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Trichoderma inoculants and straw compost improved resilience and yield in Cu-contaminated rice paddies

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Abstract. *Cuevas VC, Banaay CGB. 2022.* Trichoderma *inoculants and straw compost improved resilience and yield in Cu-contaminated rice paddies. Nusantara Bioscience 14: 1-9.* Rice paddies in Marinduque, Philippines, are copper-contaminated from tailings of two mining companies formerly operating in the province. At present, paddy-soil copper concentration ranges from 22-386 mg kg^{-1}. Crops suffer from copper toxicity and water stress due to climate-related events. The field study investigated the ability of in situ composted rice straw and *Trichoderma* microbial inoculant (TMI) to mitigate rice productivity constraints. In treated set-ups, rice straw was scattered on the paddy after harvest. Triple 14 mineral fertilizer was mixed with *Trichoderma* compost activator, broadcasted over the straws, and subsequently incorporated into the soil during land preparation. Rice seeds were TMI-coated before sowing. Rice straw composting was not done in control set-ups, and seeds were uncoated. Mineral fertilizers were applied to both set-ups. Furthermore, set-ups were categorized based on soil Cu content, such as normal, moderate, and high. Four replicates were made per season and category. Rice leaves did not show yellowing in treated paddies, indicating adequate N mineralization and plant uptake. The difference in yield was significantly higher (81%) in treated paddies compared to the control. During severe drought conditions, the mean yield in treated paddies was 1.8 t ha^{-1}, while that of control paddies was zero. The yield was significantly correlated with K inputs, mainly by compost in treated paddies. Applying rice straw compost and *Trichoderma* inoculants can be an adaptive strategy for climate change resilience and mitigation of copper toxicity in crops.

Keywords: Copper toxicity, rice, rice straw compost, *Trichoderma* activator, *Trichoderma* microbial inoculant, water stress

INTRODUCTION

Rice straw management is an essential component of rice cropping system sustainability through its conversion to compost. Rice straw is an abundant resource in all rice-growing regions of the world (Pangesti et al. 2012; Hayuningtyas et al. 2013). The national rice production average in the Philippines is around 4 tons ha^{-1} (PSA 2019). With two cropping seasons per year, a hectare of rice land can produce 5.6-8 tons ha^{-1} annually based on a straw-to-grain ratio of 0.7-1.0 (IRRI 2015b). Therefore, farmers can reduce mineral fertilizer inputs by converting rice straws to compost. Chivenge et al. (2020) mentioned that rice straws contain 80, 40, and 30% potassium (K), nitrogen (N), and phosphorus (P), respectively, which are available to the rice plant. Therefore, rice straw compost as a component fertilizer of the rice cropping system can reduce subsequent crop fertilizer requirements.

Furthermore, rice straw compost in soil reduces the impact of heavy metal contaminants on crops. Cuevas et al. (2014) reported that rice straw compost significantly improved soil pH, which decreased the availability of soil copper and significantly increased yield in rice paddies covered with mine tailings in Mankayan, Benguet. Moreover, Cuevas and Balangcod (2020) have found that the build-up of soil organic matter (OM), with a consequent decrease in available Cu, is the driving force for ecological succession in areas covered with mine tailings. In addition to heavy metal contamination in some areas, Philippine agriculture faces a difficult challenge with the current global climate change phenomenon. The Philippines is an archipelago in the tropics, with frequent typhoons yearly. Therefore, the country is vulnerable to extreme weather events (IPCC Ocean and Cryosphere 2019) and weather pattern changes. For example, the El Nino Southern Oscillation typically has a 7-year cycle; however, this duration has shortened, as shown by what the country experienced last 2018-2019, just three years after a similar event in 2015. Reduced rainfall, drought, and stronger-than-usual typhoons characterize an El Niño year. Therefore, agricultural scientists must devise different adaptive strategies to mitigate the effect of such an erratic rainfall pattern. Conversion of rice straw into compost can be one strategy since compost, through an increase in soil organic matter, raises soil water-holding capacity and reduces the impacts of water scarcity on the crop while improving soil structure that supports the crop even in adverse weather conditions (Bot and Benites 2005).

Rice farmers do not practice composting rice straw despite the benefits derived from compost. This reluctance is because the usual composting through the heap method is labor-intensive and time-consuming. They also find it difficult to gather animal manure, a nitrogen source for composting. Farmers must also apply large quantities of compost to increase yield (IRRI 2015a). Furthermore, when incorporated into the soil, incompletely decomposed

rice straw, followed by flooding of the field, can lead to nitrogen immobilization and cause yield decline. Such conditions can also lead to methane emissions that increase greenhouse gas concentrations (Devevre and Horwâth 2000). On the other hand, rice straw compost can significantly increase crop yield in mine waste-contaminated rice paddies compared to plots without compost (Cuevas et al. 2019). Likewise, the combination of compost and TMI increased yields more than the two factors applied singly. These results show that the harmful effects of using immature compost can be overcome with the proper procedures, and the farmers can derive enormous benefits from the practice. The present field study was conducted in Mogpog, Marinduque, to test whether a simple rice straw composting process and seed-coating with *Trichoderma* microbial inoculant could gain acceptance by farmers and help alleviate the harmful effects of water scarcity and copper toxicity on the crops.

MATERIALS AND METHODS

This study was conducted in Mogpog, Marinduque, Philippines, in 2018-2019, covering two dry and two wet seasons. Marinduque is a small island province located between Mindoro and Quezon provinces. Successive breaches in the tailing ponds in 1993 and 1996 and the intermittent overflow of ponds during heavy rains have led to the transport of copper to agricultural fields downstream (Figure 1). The two companies were extracting copper, and both stopped operations after the 1996 mining disaster. Since then, they have not resumed operations; however, the damage they have brought to the agricultural lands persists today.

Composite soil samples of contiguous paddies were taken from different sites identified by local people to be affected by mine tailings. The surface soil samples were taken at 0-20 cm depth. Samples were analyzed for pH, OM, P, K, and Cu contents. The soil Cu was analyzed as total Cu through the ICP-OES trace metal analysis method using HNO_3-H_2O_2 at a ratio of 7:3 (v/v) for the extraction. Soil pH and soil fertility parameters were analyzed according to Recel et al. (1988). Percentage OM was analyzed using the Walkley-Black method, available P through Bray method 1, and exchangeable K by ammonium acetate extraction (1 N ammonium acetate pH 7.0 with orbital shaking for 30 min). All analyses were done at the Agricultural Systems Institute, Division of Soil Science, College of Agriculture and Food Science, UP Los Baños.

Rice paddies were categorized as normal, moderate, or high based on soil Cu content. Information was taken from the results of soil analysis (Table 1) of samples taken before the start of the first cropping in the 2018 dry season (DS) and with guidance from existing literature (Mackie et al. 2012). The categories were as follows: normal (22-59 mg kg^{-1} Cu), moderate (110-144 mg kg^{-1} Cu), and high (290-386 mg kg^{-1} Cu).

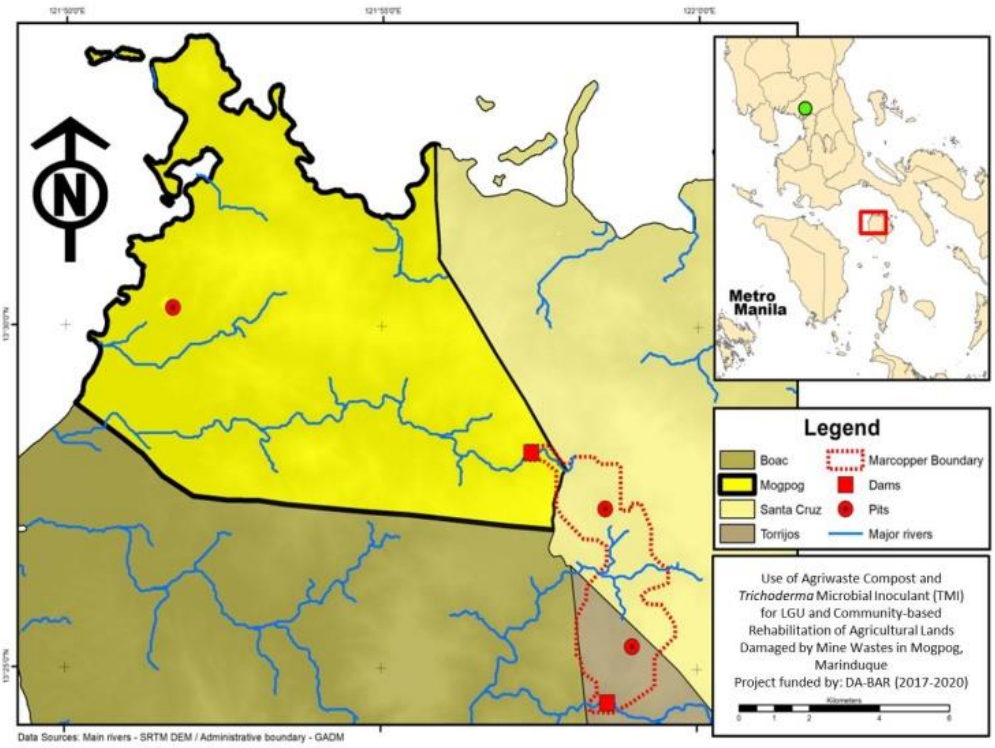

Figure 1. Map of Mogpog, Marinduque, Philippines, showing the locations of mine tailings dams and pits of two copper mining companies

Table 1. The average measurement of soil chemical properties of rice paddies at the start of the study (before DS 2018)

Soil Cu content (mg kg^{-1}) & category	No. of paddies tested	pH	OM (%)	N (%)	*P (mg kg^{-1}, Bray)	K (cmolc kg^{-1} soil)	Cu (mg kg^{-1})
22-59 (normal Cu content)	15	6.1	2.75	0.13	3.03	0.17	37
102-156 (moderate Cu content; contaminated)	11	5.09	2.42	0.14	7.88*	0.25	120
290-386 (high Cu content; severely contaminated)	15	5.32	1.94	0.11	3.13	0.29	290

Note: *Olsen method was used instead of Bray for analysis of P concentration

Trichoderma microbial inoculant (TMI) used in this study is a commercialized product developed at the University of the Philippines from strains isolated from leaf litter of Mt. Makiling in Los Baños, Laguna, Philippines. It consists of two strains of *Trichoderma ghanense* Doi (formerly identified as *T. pseudokoningii* Rifai) and one strain of UV-irradiated *T. harzianum* Rifai mixed in equal proportions. TMI was sold in a 250-g pack; 1 g contains 4.8 x 10^8 cfu g^{-1}. The *Trichoderma* compost activator used for in situ composting of rice straws is also commercially produced by BIOSPARK Corp. and consists of a local strain of *Trichoderma harzianum*. The farmer cooperators were given workshops on composting and TMI use before the start of the study. A total of 50 farmers maintained treated and the control paddies, while another 50 maintained only treated ones. In treated paddies, rice straw was scattered in paddy fields after harvest, and seeds were coated with TMI before sowing at a rate of 8.3 g kg^{-1}. The *Trichoderma* compost activator was applied to the rice straws scattered in the field after the previous season's harvest. Five kg per 0.43 ha (0.12 g m^{-2} or 12 kg ha^{-1}) triple 14 NPK mineral fertilizer was mixed with a *Trichoderma* activator and broadcasted on the scattered rice straw. The straws' initial C:N ratio was 57, then adjusted to 54 with mineral fertilizer, considering 40.7% C and 0.7% N (Nghi et al. 2020). According to package instructions, the straw was incorporated into the soil during the mechanized land preparation, and rice seeds were TMI-coated before seed sowing. Rice straw composting was not done in the control set-ups, and seeds were not coated with TMI.

Although the farmers were free to plant the inbred rice varieties of their choice, each practiced the same cultural management in treated and controlled paddies. The rice flag leaf greenness was assessed during the grain-filling stage using a Leaf Color Chart (LCC) to gauge leaf N status, reflecting N availability and uptake (Yang et al. 2003). Grain yield was measured according to the total weight of harvested grains divided by the paddy size measured in kg m^{-2} and later converted to t ha^{-1}. Nutrient inputs were computed as g m^{-2} (later converted to kg ha^{-1}) for both treated and control paddies. The N, P, and K contributions of rice straw compost in treated paddies were computed per season using the information from Dobermann and Fairhurst (2002) of 0.5%N, 0.16%P_2O_5, and 1.4%K_2O in dry straw. The amount of rice straw composted was based on the previous season's harvest. Except for DS 2018, the grain harvest in control paddies was used as a basis, assuming it was the harvest before the project started. The grain-to-straw ratio used was 1:1 (Dobermann and Fairhurst 2002). The same treatments and measurements were applied in all seasons covered by the study (DS-WS 2018 and DS-WS 2019).

In 2018, Mogpog received much-reduced rainfall compared to the previous two years (World Weather Online 2020), which persisted until August 2019 (PAGASA 2019). In Mogpog, the severity of water stress was affected by the field location and the distance from the irrigation canal. The crops suffered from water scarcity during the period of observation in addition to copper toxicity. Because these events cannot be controlled, the water-stress factor was considered in the data analysis. Water-stressed paddies were categorized into 6 groups starting with 0-no water stress from sowing to harvest. Level 1 water throughout the cropping period was present but was reduced compared to average years. Level 5 was designated when water was severely reduced throughout the cropping season. All levels between 1 and 5 depended on water availability during the different crop growth stages.

The paired t-test was used to compare rice grain yields, while Tukey's test was used to compare the mineral nutrient inputs of treated and control paddies. The multivariate analysis accounted for differences between treatments, copper levels, water stress, and interactions. In addition, regression analysis was performed to correlate yield parameters such as nutrient inputs, water stress, and soil copper level.

RESULTS AND DISCUSSION

The efficiency of rice straw decomposition

Figure 2 shows the crop stand in treated and control paddies with normal soil Cu content one month after transplant during the DS 2018. The figure shows that the observed trend is similar for all other seasons. In control paddies, leaves exhibited yellowing, indicating nitrogen deficiency, while in treated paddies, leaves had normal green color. This status was carried through the grain-filling stage, as shown in the LCC readings presented in Figure 2. Control plants had average LCC readings below three (2.33 and 2.17), while treated paddies had average LCC readings higher than three (3.5 and 3.67). LCC readings of three to four mean that the N content of the leaves was sufficient, and chlorophyll content was also enough for photosynthesis. LCC readings below three

mean that the crop is deficient in N (Yang et al. 2003). Data on nitrogen application for treated and the control paddies with normal copper concentration are presented in Table 2. In contrast, data for moderately and severely contaminated paddies are presented in Tables 3 and 4. Tukey's test showed that treated paddies had significantly higher nutrient inputs (combined fertilizers and straw compost) than the control. Tables 2-4 also show that the number of nutrient inputs received by treated paddies under little or no water stress is less than the IRRI-recommended mineral inputs to achieve the grain yield attained in treated paddies. That implies that *Trichoderma* may cause higher nutrient use efficiency since it increases yields despite lower actual mineral inputs. Previous studies have determined that *Trichoderma* spp. can improve nutrient use efficiency and support higher crop yields with fewer mineral fertilizer inputs (Fiorentino et al. 2018; Visconti et al. 2020). In addition, TMI can support the development of greener flag leaves in rice compared to untreated plants (Cuevas 2006; Banaay et al. 2012), as observed in the present study. Flag leaves are important for rice productivity because they directly deliver 50% of photosynthates to the grains (Li et al. 1998; Acevedo-Siaca et al. 2021), thus enhancing grain yield. *Trichoderma* species can increase chlorophyll and photosynthesis-related activities in host plants (Harman and Shoresh 2008a), which is consistent with the observations in this study showing increased greenness of flag leaves and higher grain yield in treated plants compared to the control plants.

The sufficient nitrogen in treated paddies (as indicated by green leaves and >3 LCC readings presented in Figure 2) may have come from the mineral fertilizers applied. The contribution of the rice straw compost since initial test results showed low soil N content (Table 1) and low soil labile amino N. This non-yellowing of leaves showed that the decomposition of rice straw by the scatter method of composting with *Trichoderma* activator plus Triple 14 NPK was sufficient. The activator jump-started the process, while the mineral fertilizer provided the ready source of nutrients for the microbial decomposers during the composting (Cuevas et al. 2019). The *Trichoderma* activator used in the study has been previously shown to effectively accelerate the decomposition process (Cuevas et al. 1988), thus allowing the rapid composting of rice straw in the field. As a result, there was no evidence of N immobilization and mineralization. Guo et al. (2018) mentioned that partially decomposed rice straw returned to the rice field, causing N deficiency and yield decline. Chivenge et al. (2020) added that partially decomposed straw had potentially adverse effects on nutrient availability and the use-efficiency of applied fertilizers for the subsequent crop. However, in this study, there was a higher yield in the treated than in the control paddies, as presented in Tables 2-4. TMI may have also aided in the mineralization of nutrients from the compost. Cuevas (2006) has shown that *Trichoderma pseudokoningii* (= *T. ghanense*, Banaay et al. 2012), a TMI component species enhances nutrient mineralization from organic matter.

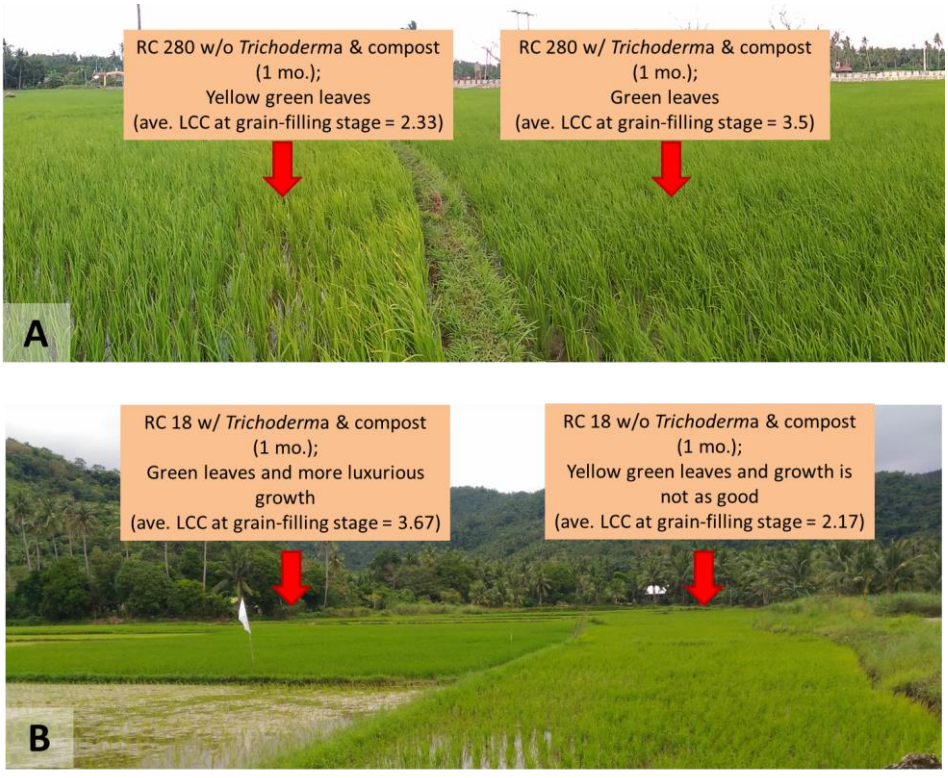

Figure 2. Comparison of standing rice crop during the 2018 dry season with the leaf color chart (LCC) readings for the same crop stand at the grain-filling stage. A. Farmer's field at Anapog-Sibucao with normal (42 mg kg^{-1}) soil Cu content and B. Farmer's field at Ino with Cu-contaminated soil (122 mg kg^{-1}); both sites are located in Mogpog, Marinduque, Philippines

Table 2. Comparison of yield, mineral fertilizers, and seasonal water stress level during the four seasons crop of 2018-2019 for paddies with normal Cu content (22-59 mg kg^{-1})

Seasons	Mean water stress level	Treated (w/ rice straw compost + TMI)				*Recommended mineral inputs for the attained yield in treated paddies (kg ha^{-1})			Control (no rice straw compost, no TMI			
		Mean yield (t ha^{-1})	Mean nutrients applied (fertilizer + compost) (kg ha^{-1})						Mean yield (t ha^{-1})	Mean mineral fertilizer applied (kg ha^{-1})		
			N	P	K	N	P	K		N	P	K
DS 2018	1	5.7	45	19	19	57	14	36	4.5	39	5.0	5.0
WS 2018	3	3.4	58	21	21	34	8.5	21	1.9	48	11	11
DS 2019	5	2.2	44	48	4.8	22	6.2	14	0	0	0	0
WS 2019	1.6	3.8	42	10	10	38	9.5	24	2.1	22	3.5	3.5
Ave		3.8	47	13.7	13.7	38	9.0	22.8	2.1	28	5.0	5.0

Note: *IRRI (2015a,b)-Steps to successful rice production. Data are the mean of 4 replicates

Table 3. Comparison of yield, mineral fertilizers, and seasonal water stress level during the four seasons crop of 2018-2019 for paddies with moderate Cu content (110-144 mg kg^{-1})

Seasons	Mean water stress level	Treated (w/ rice straw compost + TMI)				*Recommended mineral inputs for the attained yield in treated paddies (kg ha^{-1})			Control (no rice straw compost, no TMI			
		Mean yield (t ha^{-1})	Mean nutrients applied (fertilizer + compost) (kg ha^{-1})						Mean yield (t ha^{-1})	Mean mineral fertilizer applied (kg ha^{-1})		
			N	P	K	N	P	K		N	P	K
DS 2018	1.75	2.3	40	9.5	9.5	23	6.0	14	2.2	32	1.0	1.0
WS 2018	0	6.4	39	11.4	11.4	64	16	40	4.7	35.5	7.0	7.0
DS 2019	5	1.1	29	7.0	7.0	10	2.5	6.3	0	0	0	0
WS 2019	0.8	3.7	48	10	10	37	9.0	22	2.3	52	9.0	9.0
Ave		3.5	39	9.5	9.5	30	7.5	20	2.3	30	4.0	4.0

Note: *IRRI (2015a,b)-Steps to successful rice production. Data are the mean of 4 replicates

Table 4. Comparison of yield, mineral fertilizers, and seasonal water stress level during the four seasons crop of 2018-2019 for paddies with high Cu content (290-386 mg kg^{-1})

Seasons	Mean water stress level	Treated (w/ rice straw compost + TMI)				*Recommended mineral inputs for the attained yield in treated paddies (kg ha^{-1})			Control (no rice straw compost, no TMI			
		Mean yield (t ha^{-1})	Mean nutrients applied (fertilizer + compost) (kg ha^{-1})						Mean yield (t ha^{-1})	Mean mineral fertilizer applied (kg ha^{-1})		
			N	P	K	N	P	K		N	P	K
DS 2018	4.2	1.0	40	9.0	9.0	10	2.5	6.3	0.5	33	4.0	4.0
WS 2018	2.2	4.7	58	12	12	47	11.8	29.4	2.2	52	10	10
DS 2019	5	1.9	51	11.7	11.7	18	4.5	11.2	0	0	0	0
WS 2019	1.25	4.2	20	7.0	7.0	42	10	26	1.2	17.5	4.0	4.0
Ave		2.9	42.3	9.9	9.9	29.3	7.2	18.2	1.0	25.6	4.5	4.5

Note: *IRRI (2015a,b)-Steps to successful rice production. Data are the mean of 4 replicates

Furthermore, since the field was not flooded after harvest, the rice straw decomposition process was aerobic. After the wet cropping season in October/November, intermittent rain from the North-East monsoon allowed rapid decomposition in three to four weeks before the next cropping. Dobermann and Fairhurst (2000) have reported that this moist aerobic stage minimizes the adverse effects of anaerobic decomposition at the early stage of rice seedling growth. On the other hand, after the dry cropping season in April or May, the interval for the next cropping was too long (usually up to six to eight weeks) since farmers usually wait for the field to be fully saturated by rain before the start of mechanized land preparation. In this case, the activator and mineral fertilizer mixture were applied only after the first heavy rains. This practice helps ensure the proper decomposition of rice straws and avoids the harmful effects of partially decomposed straws. Figure 3 shows that, during the initial season of the study (DS 2018), in treated paddies with normal Cu content, rice straw compost added a mean of 1.9 g m^{-2} N, 0.6 g m^{-2} P, and 5.3 g m^{-2} K. In general, NPK inputs from in situ composted rice straw is higher during the wet seasons (2018 and 2019) than the dry seasons because the amount of rice straw scattered in the field before the start of wet seasons is based on dry season biomass in uncontaminated control plots (with little to no water-stress), which are usually higher than wet season biomass.

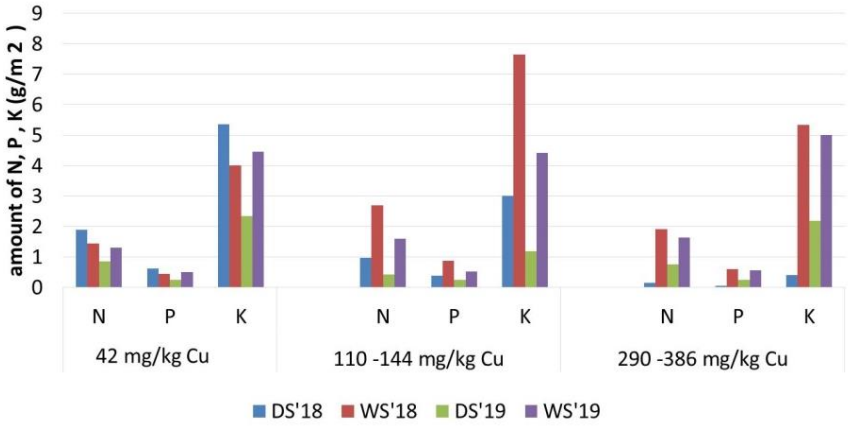

Figure 3. The amount of N, P, and K minerals contributed by the in situ composted rice straw in the compost and *Trichoderma*-treated rice paddies with different levels of soil Cu

The addition of compost to soil has both short-term and long-term benefits. Short-term benefits include the provision of NPK, enrichment of beneficial microorganisms, promotion of soil health, and provision of micronutrients like Zn, which was absent in mineral fertilizers (Bot and Benites 2005; Banaay et al. 2013; Tambone et al. 2013; Tits et al. 2014). In addition, the compost nutrients are slow-release and are less likely to be subject to leaching (IRRI 2020). Long-term benefits include improving soil physicochemical properties such as increased water holding capacity, soil aeration, and soil organic carbon (Bot and Benites 2005; Tits et al. 2014; Memoli et al. 2017). Incorporating rice straws in the paddy soil also provides ecosystem services since compost serves as a habitat and food for soil invertebrates and other soil organisms, thus increasing biodiversity (Chivenge et al. 2020). These soil organisms also participate in energy flow and nutrient cycling, providing stability to the agroecosystem (Kibblewhite et al. 2008).

Effect of nutrient inputs, water stress, and Cu level on rice yield

Multivariate tests showed highly significant differences in yield ($p<0.001$) among treatments (control vs. TMI + rice straw compost), Cu levels ($p=0.009$), and water stress levels ($p<0.001$). There were also significant interactions of Cu level × water stress ($p<0.001$) and treatment × water stress ($p=0.02$). However, the treatment × Cu level interaction was not significant ($p=0.84$). Treated paddies in all seasons and all soil Cu levels had significantly higher yields than the control. The yield was positively correlated with N, P, and K ($p= 0.01$). The overall mean yield in all treated paddies was 3.6 t ha^{-1}, while that of control was 1.9 t ha^{-1}. Thus, the overall mean increase in yield of treated paddies vs. control was 81% (Table 5). The higher concentration of nutrients from mineral fertilizers and rice straw compost resulted in better performance of treated paddies and was responsible for higher yield, especially during severe drought. The plant growth-promoting effects of TMI are also responsible for the higher yield (Cuevas 2006; Banaay et al. 2013). *Trichoderma* can increase crop yield through systemic effects on plants that include increases in the following: carbohydrate metabolism, photosynthetic rate, stomatal conductance, transpiration, internal CO_2 concentration, water use efficiency, plant height, tiller number, leaf number, panicle number, root length, root weight, and chlorophyll *a* and *b* content (Shoresh and Harman 2008a,b; Doni et al. 2014; Doni et al. 2017; Harman et al. 2019). *Trichoderma* also functions as an agent of biocontrol, natural decomposition, bioremediation, stress tolerance, and biofertilizers by helping rice plants take up nutrients from the soil (Debnath et al. 2020; Zin and Badaluddin 2020).

Table 5. Yield increase of rice in treated (with rice straw compost + *Trichoderma* microbial inoculant) and control set-ups

Set-ups	Mean rice yield (t ha^{-1}) in four seasons under different soil Cu content categories			Overall mean yield in all seasons for all soil Cu levels
	Normal Cu content (22-59 mg kg^{-1})	Moderate Cu content (110-144 mg kg^{-1})	High Cu content (290-386 mg kg^{-1})	
Treated	4.08	3.75	2.87	3.57
Control	2.31	2.43	1.08	1.96
% increase in Treated vs. the Control				81

Table 6. Differences of the mean of N, P, K inputs (in kg ha^{-1}) for treated and control paddies regardless of soil Cu concentration levels and water-stress levels, and IRRI-recommended rates for attained yield

Set-ups	N*	P*	K*	Yield* (t ha^{-1})
Treated (with Rice straw compost + TMI)	54.9 a	14.8 a	43.3 a	0.33 a
IRRI Recommendation to attain the yield observed in treated plants (for paddy fields without heavy metal contamination and water stress)	32.9 b	8.3 b	21.1 b	0.33 a
Control	25.4 b	4.5 c	4.5 c	0.18 b

Note: * In a column, means followed by a common letter are not significantly different at a 5% level of significance by Tukey's test

Tables 2-4 show data on nutrient inputs and the corresponding yields per season at different levels of soil copper. The tables also present IRRI-recommended levels of mineral fertilizers for the attained yield in treated paddies. These recommended fertilizer rates need to be added to attain a particular target yield, given that the soil type in Mogpog is clay loam (Municipality of Mogpog 2017). Close inspection of the data revealed that, in several cases, treated paddies had levels of N and P that were close to the IRRI-recommended rates for these nutrients. However, mineral K fertilizer was insufficient. For example, in treated paddies with normal Cu levels during the DS 2018 (Table 2), the K fertilizer applied was only 19 kg ha^{-1}, whereas the required K to attain the actual yield was 36 kg ha^{-1}. Similarly, the yield in treated Cu-contaminated paddies during WS 2018 was 6.4 tons ha^{-1}, but the K fertilizer applied was only 11.4 kg ha^{-1}, whereas the required K to achieve this yield was 40 kg ha^{-1}. These computations mean that the actual yield was supported by a K source other than the mineral fertilizer. Therefore, the additional K must have come from the rice straw compost (Figure 3). Tukey's test in Table 6 shows that the treated paddies had higher N, P, and K levels with combined mineral fertilizer and rice straw nutrients than the control paddies.

In the present study, the yield obtained from treated paddies was higher than in the control set-ups, indicating more significant nutrient support from inputs computed from the IRRI formula. Dobermann and Fairhurst (2002) reported that when mineral fertilizers and rice straw compost are used, nutrient reserves in the soil, such as N, P, K, and Si, are constant and may even be increased. The data and computations implied that decomposition and mineralization from the compost led to significant improvements in soil nutrient status. Chivenge et al. (2020) also supported these results and noted that rice straw incorporation could reduce mineral fertilizer inputs, supporting a greater yield. Regression analysis showed that water stress and soil Cu level had a highly significant adverse effect on rice yield (p= 0.01). However, as shown in Table 7, at zero to mild water stress level 1, the mean yield was not affected significantly in treated paddies. At the mean water stress level of 2, the average yield did not differ significantly from 0-1 and 3 levels. At water stress levels 3-5, the effect of reduced water was observed in yield reduction. However, the treated paddies performed much better than the control despite the reduction in yield. At severe drought (level 5) DS 2019, mean yields were 2.4, 1.1, and 1.9 t ha^{-1} in treated paddies with normal, moderate, and high Cu content, respectively. Meanwhile, all the corresponding control paddies had 0 yields (Tables 2-4). Such improved performance of treated paddies may be attributed to the effect of additional soil organic matter from rice straw compost, which improves soil aeration and water holding capacity (Craswell and Lefroy 2001) as well as the increase in mineral nutrients (Dobermann and Fairhurst 2002) and effects of *Trichoderma* treatment through positive influences on both nutrient-use efficiency and photosynthesis-related activities (Doni et al. 2014, 2017; Harman et al. 2019; Debnath et al. 2020).

Based on the data presented, the overall effect of soil copper on yield was low. The yield was reduced by 0.00005 and 0.00009 per unit increase in soil Cu for treated and control paddies, respectively. However, among severely contaminated paddies, there was a significant difference in yield between paddies with 290 mg kg^{-1} Cu and paddies with 386 mg kg^{-1} Cu. The former had an overall mean yield of 3.30 t ha^{-1}, while the latter had an overall mean yield of 2.6 t ha^{-1}. This difference was only observed among already severely contaminated sites, which means that Cu concentrations may already be at a level where Cu toxicity is manifested as significant yield reduction or the Cu threshold for phytotoxicity has been reached. The present study results indicate that significant yield reduction can be observed only at high Cu concentrations. Xu et al. (2006) observed that different concentrations of copper, i.e., 100 mg kg^{-1}, 300-500 mg kg^{-1}, and 1,000 mg kg^{-1} reduced rice yield by 10%, 50%, and 90%, respectively.

Table 7. Effect of water stress level on the yield of rice grown in Cu-contaminated paddy fields

Water stress level	Yield* (t ha^{-1})	
0	4.85	a
1	4.74	a
2	3.97	ab
3	2.54	bc
4	1.98	cd
5	0.88	d

*In each column, means followed by a common letter are not significantly different at the 5% level by Tukey's test

Likewise, in vegetables, significant yield reduction due to copper toxicity was observed only above 150 mg kg^{-1} soil copper concentrations (Yang et al. 2002), which is beyond the moderate category in this study. Alternatively, this low effect of Cu on yield may have been due to the inherent adaptation of the rice varieties farmers preferably use. Eduardo-Marquez et al. (2018) have observed that local rice varieties in Vietnam grown in paddy soil near open-pit coal mines contaminated with heavy metals like Cu have developed adaptations to contaminants. Rice plants have developed trace metal protection mechanisms by sequestering metals in roots or combining them with deprotonated organic acids, proteins, and polysaccharides (Eduardo-Marquez et al. 2018). It is also possible that microbial communities have adapted to heavy metal stress (Hoostal et al. 2008) and consequently confer incidental benefits for the crops.

The effect of rice straw compost and TMI use on paddies contaminated with Cu-rich mine tailings was tested by Cuevas et al. (2019) in Mankayan, Benguet. The field trial results showed that 2 kg m^{-2} application significantly improved yield and number of productive tillers. This rate was found to be optimum for rehabilitating Cu-contaminated paddies. Yield in paddy fields with TMI alone was higher when compared to the field without amendment. Yield with TMI + compost was consistently higher than paddies with TMI alone. The use of TMI in rice cropping was already suggested by Cuevas (2006), which showed that the inoculant helped make nutrients such as P and Zn more available to rice crops, increasing crop yield.

In conclusion, this two-year study showed that in situ composting by scattering the rice straws on the paddy field after harvest and inoculating with a *Trichoderma* activator mixed with triple-14 NPK mineral fertilizer is a method that farmers easily implement. Moreover, over four seasons, 100 farmers carried out the field experiment in Mogpog, Marinduque. The results showed no yellowing in treated paddy rice leaves, indicating the sufficient decomposition of straw. Furthermore, results revealed that the overall yield increase in treated paddies of 81% was significantly higher than the control. The increase in yield was due to higher mineral fertilizers that farmers applied and from nutrient contributions from rice straw compost, especially K element, and the use of TMI. This study also exhibited that using rice straw compost and TMI resulted in a modest yield even under severe water stress. Therefore, it can be an adaptive strategy for erratic rainfall patterns associated with climate change phenomena, in addition to helping alleviate the effect of Cu toxicity on crop productivity.

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Ethnobotanical study of medicinal plants used by local communities of Damot Woyde District, Wolaita Zone, Southern Ethiopia

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Abstract. *Megersa M, Woldetsadik S. 2022. Ethnobotanical study of medicinal plants used by local communities of Damot Woyde District, Wolaita Zone, Southern Ethiopia. Nusantara Bioscience 14: 10-24.* Humans have used traditional medicines, mainly of plant origins, to treat diseases. Early humans faced a tremendous challenge when searching for natural products used as medicines. This study reports on an ethnobotanical study that focused on the traditional medicinal plants used by local communities to treat human and livestock diseases. An ethnobotanical study on medicinal plants was conducted from February 2020 to October 2020. That involved semi-structured interviews, field observations, market surveys, and group discussions with informants to document information on the use and management of medicinal plants by the people of Damot Woyde District. Fifty-seven medicinal plant species belonging to 31 families have been collected in the study area inhabitants use that to treat various diseases in humans and livestock. The leading family was Asteraceae which was represented by 7 species (12.3%), followed by Rutaceae (6 species, 10.5%) and Solanaceae (5 species, 9%). Of the 57 medicinal plants collected, 36 (63.2%) were used to treat human ailments only, while 6 (10.5%) plant species were used to treat livestock ailments only, and 15 (26.31%) were used to treat both human and livestock ailments. Herbs constituted the largest number of 22 species (38.6%), followed by shrubs 18 species (31.6%), trees 15 species (26.3%), and climbers 2 species (3.51%). Leaves (31.3%) were the study area's most commonly used plant parts for preparing traditional remedies. Oral administration was the predominant mode of administration, accounting for 71%. Preference ranking analysis revealed *Allium sativum* L. was the most preferred plant species for treating the common cold. When the direct matrix ranking was analyzed, *Croton macrostachyus* Hochst. Ex. Del. was the most commonly used medicinal plant for various purposes. Our finding revealed that plant species' use plays a vital role in treating human and animal diseases in Damot Woyde District. Phytochemical and pharmacological tests are recommended mainly on frequently used medicinal plants.

Keywords: Conservation, Damot Woyde, medicinal plants, traditional medicine, Wolaita

Abbreviations: ICF**:** Informants Consensus Factor**;** JCS: Jaccard's coefficient of similarity; URTI: Upper Respiratory Tract Infection; UTI: Urinary Tract Infection

INTRODUCTION

People have used traditional medicines to treat diseases, mainly of herbal origin. Early humans faced an enormous challenge of finding natural products to use as medicines (Yuan et al. 2016). It is very likely that early humans often consumed poisonous plants in search of food but could still develop knowledge about natural medicines (Yuan et al. 2016). In several countries, considerable indigenous knowledge from the earliest times is associated with using traditional medicine (Farnsworth 2007). Many widely used products, such as herbal medicines for livestock and human health, have been developed using traditional indigenous knowledge.

Indigenous knowledge of medicinal plants has made, and can still make, a significant contribution to resolving local problems such as human and livestock diseases. Furthermore, indigenous knowledge contributes to science in many fields related to plant-based use for developing modern medicine. However, due to rapid changes in the way of life, low awareness of the importance of indigenous knowledge, lack of written documentation, the disappearance of indigenous practices, and loss of biological species, indigenous knowledge is threatened (Langton and Rhea 2005; Megersa et al. 2013).

Like everywhere in the world, local communities in Ethiopia have developed indigenous knowledge of using plants to treat diseases in humans and animals. Various studies have been conducted in different parts of Ethiopia, and many medicinal plants have been collected to treat human and livestock diseases. For example, in Kasaa et al. (2020), 266 plant species were recorded medicinal value and used by local communities in Sheka, Southern Ethiopia. Eighty-two medicinal plants were used by local communities in Kelala, Northeastern Ethiopia (Assen et al. 2021). In a similar study by Tefera and Kim (2019) and Teka et al. (2029), 105 and 244 medicinal plants were recorded to treat human and livestock diseases, respectively. The number of reported medicinal plants and their use by the indigenous people of Ethiopia suggests that the indigenous knowledge of medicinal plants and their uses is extensive. However, there is limited ethnobotanical documentation and relatively few phytochemical analyses of documented medicinal plants.

Consistent with the concentration of biological and cultural diversity, many medicinal plants are found in the south and southwest of the country (Edwards 2001; Megersa et al. 2019). That suggests a high traditional knowledge of medicinal plants in the southern part of Ethiopia, but indigenous knowledge in the region has not been systematically documented (Tefera and Kim 2019). In particular, there is no ethnobotanical study in the current study area of Damot Woyde District.

In addition, many plant species and associated indigenous knowledge are disappearing because there are no written documents on medicinal plants due to the death of knowledgeable elders without passing on traditional skills to other family members. Since knowledge of traditional medicine is passed orally from generation to generation, basic information about the use of plants and the parts used, methods of preparation, diseases treated, and other things may be lost in the process of knowledge transmission. The effects of deforestation, urbanization, and modernization are also causing rural people to abandon their natural habitat, and their knowledge, especially about herbal medicines, is slowly disappearing. Therefore, the first objective of this study was to document the medicinal plants used by the local communities of Damot Woyde District. Secondly, the study aimed to assess the threats to conservation practices and medicinal plants in the study area. The study results will benefit the development of modern medicines from plant species to treat human and livestock diseases.

MATERIALS AND METHODS

Description of the study area

Damot Woyde District is located 384 km south of Addis Ababa, 90 km from Hawassa, and 27 km from the zonal capital (Sodo). Damot Gale District borders it to the north, Humbo District to the south, Duguna Fango District to the east, and Soddo Zuria District to the west. The administrative town of the district is Beddesa. The study area lies at a longitude and latitude of 370 53'0" to 370 59'0" E and 60 55'0" to 60 57'0" N, respectively (DWDHO 2017). According to the Central Statistical Agency (CSA 2007), the population of Damot Woyde District is 91,602, with males numbering 44,861 and females 46,741. The largest ethnic group reported in Damot Woyde was the Welayta (98.6%); all other ethnic groups made up 1.4% of the population. Welaytic was the dominant first language, spoken by 99% of the inhabitants; the remaining 1% spoke all other primary languages.

The district covers an area of about 210 km^2 and has 23 kebeles. In each kebele, at least two health extension workers are responsible for providing health services to the people from house to house. The district has four health centers: Bedessa, Sake, Koyo, and Girrara. There are also twenty-nine primary and four secondary schools teaching in the district.

The ten most common diseases in the district are pneumonia, typhoid, malaria, gastritis, Urinary Tract Infections (UTI), acute febrile illness, Upper Respiratory Tract Infections (URTI), skin infections, intestinal parasites, helminths and eye diseases (DWDHO 2017). Similarly, the district has four animal health centers in Bedessa, Sake, Koyo, and Girrara. The major livestock diseases reported are anthrax, blackleg, trypanosomiasis (Gandi), flatulence, glandular swelling, cough, foot and mouth disease affecting large ruminants, including horses, and avian cholera affecting poultry.

Agriculture in the district is predominantly smallholder, mixed subsistence farming. The cropping system is mainly based on continuous cropping without fallow periods (DWDARDO 2017). In Damot Woyde District, mixed farming with livestock is widely practiced. Maize, sorghum, wheat, and barley are mainly grown in the area. Teff is also grown as an additional crop. Cultivation of Enset (*Ensete ventricosum* (Welw.) Cheesman.) is central to the cropping system on which the entire agricultural system is based, and the crop is the main source of food security and livelihoods (Olango et al. 2014). Sometimes *E. ventricosum* is grown as a mixed crop with *Coffea arabica* L. and *Persea americana* Mill. by farmers in the district. Livestock production, which includes beekeeping, poultry, small ruminants, and livestock, is another important industry in the area. The most common soil types in the study area are Eutric Nitisols associated with Humic Nitisols, which are dark reddish-brown and have deep profiles (Tesfaye 2003).

According to DWDARDO (2017), the district is divided into three major agronomic zones: (Kola 34.5%), (Woyna Dega 60.3%) and (Dega 5.2%). The rainfall distribution is bimodal, with the highest rainfall in the wet season (April to September) and the lowest rainfall in the last half of the dry season (February to March). The highest average monthly rainfall was recorded in May (179.9 mm) and the lowest in December (28.2 mm). The highest average temperature was recorded in March and April (20.5^0C) and the lowest in December (17.9^0C). In general, the mean annual temperature of the district is (19.2^0C), and total precipitation is (1271.2 mm).

Selection of the study sites

A reconnaissance survey of the study area was carried out from 10 to 25 January 2020 to obtain information on the area's agroecology, vegetation condition, and indigenous knowledge on using plants for various purposes. The study was conducted in four kebeles of Damot Woyde District, Wolaita Zone, Southern Ethiopia. The kebeles selected for the study were Dawe sake, Galcha sake, Mundeja sake, and Koyo sake (Figure 1). The four kebeles were purposively selected because of their relatively high plant diversity in the region, the practice of traditional medicine, and the recommendations of the district elders and local authorities. In addition, the three agro-climatic zones were also considered in the selection of the study sites.

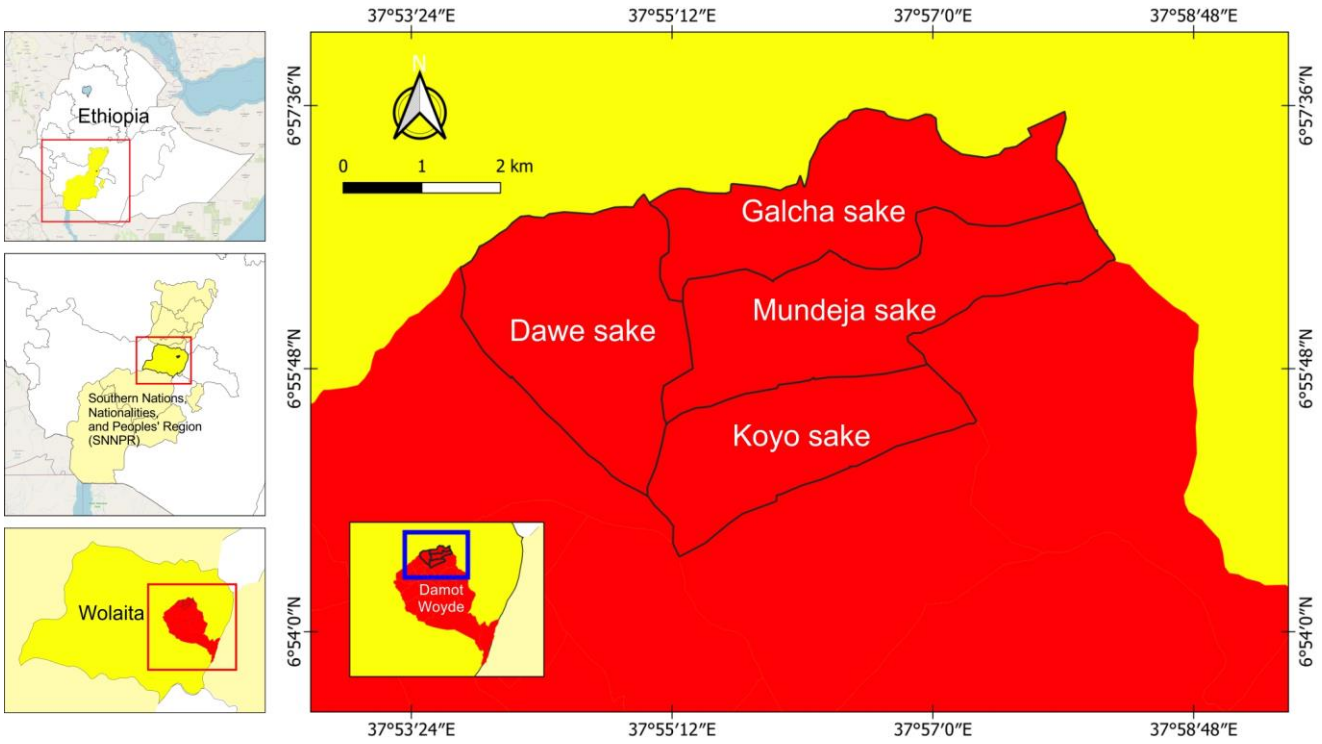

Figure 1. Map of Damot Woyde District, Ethiopia

Informant selection and sample size determination

A cross-sectional study was conducted to obtain information about the current state of the problem. With the help of random and purposive sampling, 60 (36 male and 24 female) informants aged between 34 and 73 years were selected from the four kebeles for this study. Out of the total number of informants, 36 (9 from each kebele) were randomly selected to collect valuable ethnobotanical data from the study areas, while 24 key informants (16 males and 8 females) were selected, 6 individuals from each kebele were purposively selected with the help of kebele administrative bodies and local elders. The 36 individual participants in the study were ordinary informants without formal recognition of their traditional treatment methods. On the other hand, the informants were ordinary people who had lived in the study region long and applied their knowledge of indigenous medicinal plants to their families. The quality of explanations given by particular informants during an interview was used to determine key informants. Traditional professionals who preserve indigenous knowledge of medicinal plants, such as local healers, immediately qualify as key informants.

Ethnobotanical data collection techniques

Ethnobotanical data were collected following the standard procedures of Martin (1995) and Cotton (1996) from February to October 2020. Semi-structured interviews, field observations, a market survey (two open marketplaces), and a group discussion with informants were conducted (Figure 2). The semi-structured interviews with closed and open-ended questions were conducted in English and then translated into the local language (Wolaytic). The questions in the semi-structured interview included the local names of the medicinal plants used to cure human and livestock problems, the parts of the plants used, the process of preparing the traditional medicine, and the method of administration. Field observations on the habits and habitats of the medicinal plants were carried out with the help of local guides. A market survey was conducted to assess the medicinal plants available in the market and the parts of the plants used as medicines. The informants discussed the current threats to the medicinal plants in the study area, the measures the locals took for their conservation, and the selection of multipurpose plant species.

Plant specimen collection and identification

The specimens of medicinal plants were collected from the study sites with the help of key informants (traditional healers) and coded, pressed, and dried for proper identification. Identification of the plants was done in the field at Wolayta Sodo Agricultural University and Wolayta Sodo University with the help of experts. Furthermore, additional identification of all specimens was done using authentic specimens and taxonomic keys from the flora of Ethiopia and Eritrea (Edwards et al. 1995; Edwards et al. 1997; Edwards et al. 2000). Finally, the voucher specimens were kept in the mini herbarium of Madda Walabu University.

Figure 2. The photo was taken during a group discussion on a threat to medicinal plants

Data analysis

Descriptive statistics

Descriptive statistics were used to determine the number and percentage of species, families of medicinal plants used, their growth forms, proportions of parts used, mode of preparation of the remedies, dosage, and routes of administration. The result is presented in tables, figures, and diagrams.

Informant consensus

Informants were interviewed twice on the same ideas to confirm the reliability of the information. Accordingly, the informant's information inconsistent with the previous one was rejected as irrelevant information.

Informants consensus factor (ICF)

The informant consensus factor (ICF) was considered for each group of complaints to determine the informants' agreement with the reported remedies for the group of complaints of the plant. The informant consensus factor was calculated as follows: the number of use citations in each group (*nur*) minus the number of species used (*nt*), divided by the number of use citations in each group minus one (Heinrich et al. 1998). ICF values were calculated: *ICF= nur-nt/ nur*-1. ICF values range from 0 to 1. A higher ICF value of a medicinal plant species indicates efficacy in treating a group of diseases (Trotter and Logan 1986).

Preference ranking

This method was carried out following the protocol of Martin (1995) for the six most important medicinal plants for the treatment of the common cold. Six key informants were randomly selected and participated in this exercise to identify the preferred medicinal plants for the common cold treatment. This disease was chosen because it has similar symptoms to pneumonia, most commonly reported in the region. Therefore, the researchers assume that the local population reports a common cold with similar symptoms to this disease. For each informant, six medicinal plant species were given names on paper to rank them with numbers (1-5) based on their preference or efficacy by assigning the highest value (5) to the plant species most (best) preferred to cure this disease and the lowest value (1) to the least preferred plant. Finally, the individual plant species values were summed and ranked. The plant species with the highest summed value was the most preferred.

Direct matrix ranking

Direct matrix ranking was done following Martin (1995) to compare the multipurpose use of a particular plant species based on the information collected from informants. Multipurpose plants are plants that provide various uses to local communities. The use categories include food, tools, house construction, fencing, firewood, and charcoal. Six plant species were selected from all the identified medicinal plants, and the various uses of these plants were listed. For direct matrix ranking, two focus group discussion (FGD) was conducted to determine the plants' preferences based on multipurpose criteria. Six randomly selected key informants were asked to assign a use-value to each species (5= best, 4= very good, 3= good, 2= less used, 1= least used, and 0= not used). By lottery, 6 key informants were selected from the 24 individuals. Informants were given two numbers (0 and 1), where 1 indicated that they could do the activity and 0 indicated that they could not. The average scores of each species were added together and ranked. The higher the average values of the plant species, the higher the multipurpose use.

Jaccard's coefficient of similarity (JCS)

The JCS was calculated to evaluate the composition of medicinal plant species and the degree of similarity between different areas. The similarity values were calculated between the current study area (Damot Woyde District) and ethnobotanical studies conducted in other

areas in different parts of Ethiopia. JCS is expressed as follows: JCS = c/(a + b + c), where a is the number of species in sample a, b is the number of species in sample b, and c is the number of species common to a and b (Kent and Coker 1992).

RESULTS AND DISCUSSION

Demographic characteristics

The informants (36 male and 24 female) were native to the study area. The ages of the interviewees ranged from 34 to 73 years old. However, most of the respondents (47%) were between 47-59 years of age.

Most study area informants were illiterate (55%), and 48% were farmers (Table 1).

Medicinal plants of the study area

The study area's local people collected fifty-seven species of medicinal plants to treat various diseases in humans and livestock. The families that contributed more medicinal plant species included Asteraceae with 7 species (12.3%), Rutaceae with 6 (10.5%) species and 5 species (9%) from the Solanaceae family, and other 23 families that contributed 23 (40%) species and represented by 1 species (Table 2).

Of the 57 medicinal plant species collected from the study area, 47% were from the wild, 44% were from cultivated areas, and only 9% were from both cultivated and wild habitats. Of the plant species identified, herbs accounted for the largest number, with 22 species (38.6%), followed by shrubs with 18 species (31.6%), trees with 15 species (26%), and 2 species (3.5%) were climbers (Figure 3).

Out of the 57 medicinal plant species, 36 (63.2%) were reported to treat human health problems (Table 3), 6 (10.5%) were reported to treat livestock diseases (Table 4), and 15 (26.3%) were reported for both human and livestock diseases (Table 5). The major types of human health problems identified in the study area were classified into 33 major disease categories, while 12 livestock diseases were treated locally with medicinal plants in the study area.

Medicinal plant parts used and condition of preparation

The local communities used different plant parts (leaves, fruits, seeds, shoots, roots, barks, flowers, bulbs, sap, stem, leaf and flower, leaf and seed, and rhizome) to prepare traditional medicine. However, leaves were the dominant plant parts used for the preparation of traditional medicines accounting for 21 (31.3%), followed by fruits accounting for 14 (21%) species (Figure 4).

The locals use fresh, dried, and fresh-dried medicinal plants to prepare traditional herbal medicines in the study area. As a result, most of the medicinal plants (60.6%) were used in fresh form, followed by fresh or dried form (30.3%) and dried form (9.1%), respectively.

Table 1. Demographic characteristics of informants

Demographic character	No. of informants	Percentage
Sex		
Male	36	60
Female	24	40
Age group		
34-46	21	35
47-59	28	47
Above 59	11	18
Educational background		
No formal education	33	55
Elementary	15	25
High school	6	10
College diploma	4	7
Degree	2	3
Occupation		
Farmer	29	48
Housewife	19	32
Merchant	8	13
Teacher	4	7

Table 2. Taxonomic diversity of medicinal plants

Family	Number of species	%
Asteraceae	7	12.3
Rutaceae	6	10.5
Solanaceae	5	9
Poaceae	4	7
Euphorbiaceae	3	5.3
Lamiaceae	3	5.3
Cucurbitaceae	2	3.5
Linaceae	2	3.5
Musaceae	2	3.5
Other 23 families	23	40
Total	57	100

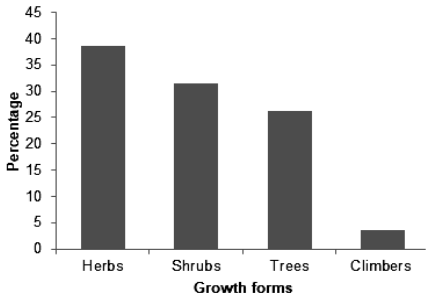

Figure 3. Growth forms of medicinal plants in the study area

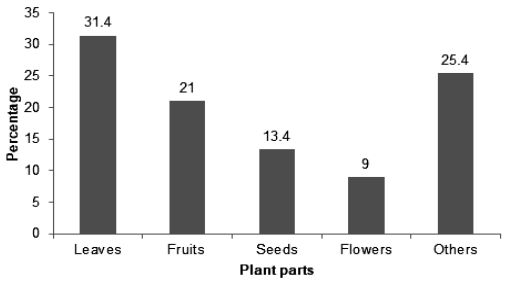

Figure 4. Plant parts used for the preparation of traditional medicine in Damot Woyde District, Ethiopia

Table 3. List of medicinal plants used for treating only human disease

Scientific name (Voucher number)	LN (No. of informants)	Family	GF	SO	PU	CO	DT	RA	Modes of preparation, application & dosage
Acmella caulirhiza Delile. (SW 15)	Idaamiya (37)	Asteraceae	H	W	Fl	F	Tonsillitis Toothache	O O	Chew & swallow a small amount of flower twice a day until recovery. Smash the flower & hold on to the infected part during pain time.
Artemisia abyssinica Sch. Bip.ex.A.Rich. (SW 16)	Ciqugniya (6)	Asteraceae	H	CU	L	F	Eye disease	Op	The leaf is smashed and rubbed or painted on the affected eye twice daily for 3 days.
Aloe debrana Christian (SW 40)	Godare uta (2)	Asphodelaceae	Sh	W	Sa	F	Allergic reaction on the skin	De	The sap of this plant is applied to the affected part once or twice a day for a week.
Catha edulis (Vahl) Endl. (SW 14)	Jimaa (4)	Celastraceae	Sh	CU&W	L	F	Common cold Abdominal pain	O	The leaf of this plant is crushed and boiled, and 2-3 coffee cups of the decoction are taken during pain time.
Capsicum annuum L. (SW 20)	Qariya (11)	Solanaceae	H	CU	Fr	F	Loss of appetite	O	The fruit of this plant is eaten daily with other food as an appetizer.
Capsicum frutescens L. (SW 21)	Mixaamixuwa (19)	Solanaceae	H	CU	Fr	F/D	Respiratory tract infection Cold Loss of appetite	O	The fruit of this plant, the bulb of *A. sativum*, is crushed together, and 2-3 spoons of the concoction per day are eaten with other food for 3 days.
Cymbopogon citratus (Dc.) Stapf (SW 39)	Gucachaa (10)	Poaceae	H	CU	L	F	Abdominal cramp	O	The leaf of this plant is crushed and boiled, and one coffee cup of the decoction is taken during pain time.
Carica papaya L. (SW 02)	Papayaa (10)	Caricaceae	T	CU	Fr	F	Gastric pain Tiredness	 O	Eat the fruit or drink the juice continuously until recovery.
Citrus x aurantium L. (SW 23)	Qomxaaxe (9)	Rutaceae	T	Cu	Fr	F	Abdominal pain	O	Eat the fruit, extract the juice, and drink one coffee cup during pain.
Citrus limon (L.) Osbeck (SW 22)	Lomiya (24)	Rutaceae	T	CU	Fr	F	Abdominal cramp Skin rash or allergic	O De	Drink one coffee cup of the juice of this plant during pain time. Crush the fruit and rub/paint on the affected skin twice daily for 3-7 days.
Citrus sinensis (L.) Osbeck (SW 24)	Birtukaaniya (2)	Rutaceae	T	CU	Fr	F	Coughing	O	Eat the fruit or drink the juice twice a day for a week.
Cucurbita pepo L. (SW 38)	Lalahiya (6)	Cucurbitaceae	Cl	CU&W	Se	F/D	Liver problem	O	The seed of this plant is crushed and boiled, and 1-2 coffee cups of decoction per day are given for one week.
Cordia africana Lam. (SW 01)	Moqota (10)	Boraginaceae	T	W	Fr	F	Abdominal pain	O	Eat the fruit 2-3 times daily during pain time.
Dovyalis abyssinica (A.Rich.) Warb. (SW 37)	Koshimiya (8)	Salicaceae	Sh	W	Fr	F	Abdominal pain/ discomfort	O	Eat a small amount of the fruit during pain time.
Echinops kebericho Mesfin (SW 25)	Boriisaa (32)	Asteraceae	H	CU&W	R	F/D	Evil eye	Na	Crush and burn a small amount of the root, then inhale its smoke during pain.
Embelia schimperi Vatke (SW 36)	Honqoquwaa (12)	Primulaceae	T	W	Se	F/D	Taeniasis Askariasis	O	Grind the seed, mix with water, and one coffee cup is given to the child (age 6-10), and two coffee cups for age >10 in morning before breakfast.
Foeniculum vulgare Mill. (SW 26)	Tatikala (5)	Apiaceae	H	CU	L	F	Kidney problem	O	The leaf of this plant is crushed and boiled, and 1-2 coffee cups of the decoction are taken when it's cooled for 3 days.

Guizotia abyssinica (L.f.) Cass. (SW 42)	Nugiyaa (6)	Asteraceae	H	CU	Se	D	Coughing	O	The roasted seed of this plant is ground, mixed with water, and boiled, and 2-3 tea cups of the filtrate are taken daily for 3 days.
Hagenia abyssinica (Bruce x Steud) J. F. Gmel. (SW 13)	Koso mitaa (12)	Rosaceae	T	W	Fl	F/D	Taeniasi or Ascariasis	O	Grind the flower, mix it with water, and one coffee cup is given to a human (age 6-10) and two coffee cups to age >10 in morning before breakfast.
Justicia schimperiana (Hochst. ex Nees) T. Anderson (SW 35)	Olomuwa (11)	Acanthaceae	Sh	W	L	F	Fibril illness	O	Grind a small amount of leaf and take one coffee cup daily for 3 days with coffee.
Linum usitatissimum L. (SW 44)	Telbaa (7)	Linaceae	H	CU	Se	D	Gastric pain	O	Boil the seed and drink the decoction 1-2 water glasses when cool during pain time.
Moringa stenopetala (Baker f.) Cufod. (SW 12)	Haalakuwaa (10)	Moringaceae	T	CU	L	F/D	Coughing	O	Crush, boil and drink the decoction (1-2 water glasses per day for 3 days.
					B	F/D	Abdominal pain	O	Grind the bark, and 1-2 spoons of the powder are mixed with one teacup of water and drink during pain.
					R	F/D	Bladder problem	O	Grind the root, then 2 spoons of the powder are mixed with one coffee cup of water and taken once a day for 3-5 days.
Musa x paradisiaca L. (SW 08)	Muziya (8)	Musaceae	Sh	CU	Fr	F	Gastritis	O	Eat the fruit during pain time.
					Sa	F	Minor bleeding	De	Cut the stem and paint/rub the sap on the cut end (bleeding skin) to assist blood clotting.
Ocimum basilicum L. (SW 28)	Kepuwa (13)	Lamiaceae	H	CU	Ag	F/D	Coughing Common cold	O	Crush the shoot of this plant, a shoot of *A. absinthium*, *A. afra*, a leaf of *E. globulus*, bulb of *A. sativum*; mix with water, and salt, boil and drink the concoction 3-6 cups of coffee daily for 3 days.
Ocimum lamiifolium Hochst. ex Benth. (SW 30)	Damaakasiya (35)	Lamiaceae	Sh	CU&W	L	F/D	Fibril illness	O	The leaf of this plant is boiled, and 2-3 tea cups of the decoction are given to humans daily for 3 days.
							Fever Eye disease	De	Smash the leaf and rub it on the skin or affected eye at bedtime.
Olea europaea subsp. *cuspidata* (Wall. ex G. Don) Cif. (SW 11)	Wagaraa (10)	Oleaceae	T	CU&W	Fr	F	Tonsillitis	O	Extract the oil and paint, or hold on to the affected part during pain.
					St	F	Toothache	O	Cut a piece of the stem of this plant, slightly heated, and hold on to the affected part of the teeth repeatedly for a few seconds during pain.
Persea americana Mill. (SW 09)	Avokaatuwa (11)	Lauraceae	T	CU	Fr	F	Dandruff	De	The fruit of this plant is peeled and painted on all parts of the affected skin or hair and waited for 8-10 hours, then washed with clean water and soap, which is repeated weekly for a month.
Phytolacca dodecandra L' Her. (SW 29)	Haanciciyaa (10)	Phytolaccaceae	Sh	W	L	F	Malaria	O	A small amount of the leaf of this plant with a bulb of *A. sativum* is crushed and mixed with water, and one cup of the concoction is taken daily for 3-5 days.
Podocarpus falcatus (Thunb.) Endl. (SW 10)	Ziga/ Zigba (8)	Podocarpaceae	T	CU&W	R/St	F/D	Evil eye	Na	Burn and inhale its smoke (dry bath) during pain time.
Rhamnus prinoides L' Her. (SW 46)	Geshuwa (10)	Rhamnaceae	Sh	CU	L	F	Tonsillitis	O	The tip of the leaf of this plant is collected, smashed, and painted on the tonsil once or twice a day for 3 days.
Ricinus communis L. (SW 47)	Qobuwa/Gulo (9)	Euphorbiaceae	Sh	CU&W	L	F	Tonsillitis	O	The leaf tip is collected, smashed, and painted on the tonsil once or twice a day for 3 days.
					Fr	F/D	Hemorrhoids	Su	Crush and paint the cream on the affected part once or twice a day until recovery.

Sida schimperiana Hochst. ex A. Rich. (SW 49)	Kindichuw (4)	Malvaceae	H	W	L	F	Skin allergic reaction	De	The leaf of this plant is crushed and applied to the affected skin daily for a week.
Solanum incanum L. (SW 48)	Buluwa (10)	Solanaceae	Sh	W	L	F	Nasal bleeding	Na	Crush the leaf and sniff the filtrate 2-3 times during bleeding.
Thymus schimperi Ronniger (SW 32)	Zinbaanuwa (3)	Lamiaceae	H	CU&W	L	F/D	Common cold	O	The leaf of this plant is boiled with water, and 2-3 tea cups of filtrate are taken daily for 3 days.
Toddalia asiatica (L.) Lam. (SW 53)	Gumaaree (9)	Rutaceae	T	W	Fl	F	Liver problem	O	The flower is boiled, and 1-2 coffee cups are taken daily for 3 days.
Vicia faba L. (SW 50)	Baa'eelaa (11)	Fabaceae	H	CU	Se	D	Swelling of the body	De	The raw seed is slightly powdered and tied to the affected part.

Note: Human (Hu), local name (LN), Growth form (GF), Sources (SO), parts used (PU), condition (CO), disease treated (DT), Rout of administration (RA), methods of preparations, application and dosage used, Wild (W), Cultivated (CU), Herb (H), Shrub (Sh), Tree (T), Climber (Cl), Bark (B), Root (R), Leaf (L), Fruit (Fr), Flower (Fl), Seed (Se), Steam (St), Sap (Sa), Bulb (Bu), Above ground (Ag), Whole Plant (Wp), Oral (O), Nasal (Na), Dermal (De), Optical (Op), Suppository (Su)

Table 4. List of medicinal plants used for treating Livestock ailments

Scientific name	LN	Family	GF	SO	PU	CO	DT	RA	Modes of preparation, Application & dosage
Ensete ventricosum (Welw.) Cheesman (SW 07)	Utaa/enset	Musaceae	Sh	CU	St	F	Retained placenta	O	The stem of this plant with a leaf of *C. arabica* is crushed and boiled, 2-3 liters of the concoction is given to a cow, and one liter to sheep and goat to expel the placenta.
Juniperus procera Hochst. Ex Endl. (SW 56)	Abeshaa xidaa	Cupressaceae	T	CU&W	L	F	Bloating	O	The leaf of *J. procera* and bulb of *A. sativum* are crushed and mixed with water half a liter of the concoction for sheep and goat, and one liter for cow and ox is given during pain time.
Leucas abyssinica (Benth.) Briq. (SW 27)	Kirkisa	Lamiaceae	Sh	W	L	F	Sudden illness Loss of appetite Diarrhea	O	The leaf is crushed and mixed with water, and half a liter is given during pain.
							Eye disease	Op	The leaf of this plant is crushed and mixed with water, and 2-3 drops of the filtrate are added to the affected eye daily for 3 days.
Lagenaria siceraria (Molina) Standl. (SW 55)	Gosiya/ kil	Cucurbitaceae	Cl	CU&W	Fr	F	Rabies	O	The fruit of this plant, the leaf of *P. dodecandra*, is crushed and mixed with salt and water, and half a liter of the concoction is given to livestock daily for a week.
Nicotiana tabacum L. (SW 45)	Tanbuwa/ Tibahu	Solanaceae	H	CU	Ag	F	Leech	Na	This plant's leaf is crushed and mixed with water, and one half coffee cup of the filtrate is nasally given to expel the leech.
							Coughing	O	The leaf of this plant is crushed and mixed with water, and one water glass is given to a cow, ox, donkey, and horse and half a water glass for sheep and goat per day for 3 days.
Withania somnifera (L.) Dunal (SW 54)	Etriwaanjiya	Solanaceae	Sh	W	L	F	Evil eye	De	The leaf of this plant, the leaf of *R. chalepensis*, is ground together and rubbed on the affected cow breast daily for a week.

Note: Livestock (Ls), local name (LN), Growth form (GF), Sources (SO), parts used (PU), condition (CO), Treatment for (TF), disease treated (DT), Rout of administration (RA), methods of preparations, application and dosage used, Herb(H), Shrub(Sh), Tree(T), Climber (Cl), Bark (B), Root (R), Leaf (L), Fruit (Fr), Flower (Fl), Seed (Se), Steam (St), Sap (Sa), Bulb (Bu), Above ground (Ag), Whole Plant (Wp), Oral (O), Nasal (Na), Dermal (De), Optical (Op)

Table 5. List of medicinal plants used for treating both Human and Livestock disease in the study area

Scientific name	LN (NI)	Family	GF	SO	PU	CO	TF	DT	RA	Mode of preparation, application & dosage
Allium sativum L. (SW 04)	Tumuwa (42)	Alliaceae	H	CU	Bu	F/D	Hu	Malaria	O	The bulb of *A. sativum* is eaten with other food once or twice a day for 3-5 days.
								Common cold	O	Crush, boil and drink the decoction (one tea cup) twice a day for 3 days.
								Coughing Asthma	O	Eat the bulb with enjera or Crush, boil and drink the decoction (1 or 2 tea cups) twice a day for 3 days.
								Respiratory tract infection	O	The bulb of *A. sativum*, the fruit of *C. frutescens* or *C. annum* are crushed together, and per day 2-3 spoons of the concoction are Eaten with other food for 3 days.
							LS	Loss of appetite (sudden illness)	O	The bulb of *A. sativum and* shoot of *A. afra* are crushed together and mixed with water, and half a water glass for sheep and goat and one water glass for cow and ox are given during pain time.
Artemisia absinthium L. (SW 18)	Naatraa (38)	Asteraceae	H	CU	Ag	F	Hu	Retained placenta	O	The leaf and stem of this plant are crushed and mixed with water one coffee cup is given to humans to expel the placenta.
								Abdominal cramp	O	Crush, mix with water, and one coffee cup is given during pain time.
							Ls	Bloating	O	The shoot of this plant and leaf of *E. globulus* is crushed together, and half a liter for sheep and goat, one liter for cow and ox, is given during pain.
Artemisia afra Jacq. ex Willd (SW 17)	Agupiya (13)	Asteraceae	H	CU	Ag	F/D	Hu	Coughing Common Cold Abdominal pain	O	The leaf of this plant, leaf of *R. chalepensis*, leaf of *C. arabica*, and bulb of *A. sativum* are crushed together, mixed with water, butter, and salt, boiled, and 3-5 coffee cups are taken per day for 3 days.
							Ls	Bloating Sudden illness	O	The leaf of this plant and a small amount of A. sativum are crushed and mixed with water, and half a liter for sheep and goats and one liter for cow and ox are given during pain time.
Coffea arabica L. (SW 03)	Tukiya (20)	Rubiaceae	Sh	CU	L	F/D	Hu	Common cold Respiratory tract infection	O	The leaf of *C. arabica*, bulb of *A. sativum*, and shoot of *A. absinthium* is crushed together, mixed with water, boiled, added salt and butter, then 3-5 coffee cup is taken daily for 3 days.
					Se	D	Hu	Wound	De	The roasted seed is ground and tied on the affected skin daily for 7 days.
					L	F	Ls	Retained placenta	O	The stem of *E. ventricosum* with a leaf of *C. arabica* is crushed, boiled, and 2-3 liter is given to cow and one liter to sheep and goat to expel the placenta.
Croton macrostachyus Hochst. Ex. Del. (SW 05)	Ankaa (46)	Euphorbiaceae	T	W	Sa	F	Hu	Wound Skin infection	De	Extract the sap or smash the leaf and apply it to the affected skin once/twice a day until recovery.
							Ls	Swelled leg	De	The leaf of this plant is slightly heated for a few seconds and covered on the affected part daily until recovery.
Cynodon dactylon (L.) Pers. (SW 41)	Suraa	Poaceae	H	W	Ag	F	Hu& Ls	Snakebite	De	The shoot of this plant, bulb of *A. sativum*, and salt are crushed together and tied on the affected part and repeated until recovery.
Eucalyptus globulus Labill. (SW 06)	Tumuwa (29)	Myrtaceae	T	CU&W	L	F/D	Hu	Common cold Coughing Fibril illness	Na	Boil the leaf, then inhale its steam (steam bath) before bed with a closed door for 3 days.
						F	Ls	Sudden illness	O	Crush the leaf with the bulb of *A. sativum*, mix it with water and give half to one liter to cow, ox, sheep, and goat during pain time.

Euphorbia tirucalli L. (SW 57)	Maxuwa/ Kinchib	Euphorbiaceae	Sh	W	Sa	F	Hu	Allergic reaction on the skin	De	The sap of this plant is applied on the affected skin once/twice a day for a week.
					Ag	F	Ls	Bloating	O	The shoot of this plant, the bulb of *A. sativum*, is crushed and mixed with water, and half a liter is given during pain time.
Hordeum vulgare L. (SW 43)	Baangaa/ Gebis	Poaceae	H	CU	Se	D	Hu	Bone break	O	The roasted seed of this plant is powdered, mixed with milk, and boiled, and 2-3 water glass is given daily until recovery.
							Ls	Bone break	O	This plant's boiled or raw seed is given to livestock (2-5) kilogram daily until recovery.
Lepidium sativum L. (SW 34)	Sifika/feto (35)	Brassicaceae	H	CU	Se/Se&L	F/D	Hu	Sudden abdominal pain	O	The seed is ground and mixed with water; one coffee cup is taken during pain. Or the seed and leaf are ground with a bulb of A. sativum and eaten 1-2 spoons with other food during pain.
								Tonsillitis Arthritis	O	The seed is ground with the bulb of *A. sativum* and eaten 1-2 spoons with other food daily for 3 days.
							Ls	Sudden illness	O	The seed is powdered and mixed with water, and half a liter is given during pain time.
Pentas schimperi (A. Rich) Vatke. (SW 19)	Daanbursaa (43)	Rubiaceae	Sh	W	L	F/D	Hu	Skeletal problem (bone break)	O	Boil the part and drink 1-2 glasses of the decoction when cool daily until recovery.
							Ls	Skeletal problem (bone break)	O	Grind the leaf, mix with cold water and give 1-2 liters daily until recovery.
Ruta chalepensis L. (SW 33)	Xalotiya/ Tenadam (40)	Rutaceae	Sh	CU	L/L& Fr	F	Hu	Evil eye	O	Crush, mix with tea/coffee, and drink 1-2 tea/coffee cups during pain time.
								Abdominal pain	O	A small amount of leaf or fruit is chewed and swallowed during pain.
							Ls	Evil eye	O	Crush, mix with water, and give one water glass to livestock during pain.
Triticum polonicum L. (SW 51)	Qanbaraa/ Aja (14)	Poaceae	H	CU	Se	D	Hu	Bone break	O	Boil the powder with milk and take 2-3 water glasses daily until recovery.
							Ls	Bone break	O	The powder is mixed with water and boiled, and 2-3 liter is given daily until recovery.
Vernonia amygdalina Del. (SW 52)	Garaa (28)	Asteraceae	Sh	CU&W	L	F	Hu	Skin rash	De	Grind the leaf and paint/rub it on the affected skin or use it as soap twice a day until recovery.
								Wound	De	Crush and paint the solution on the wound 2-3 times daily until recovery.
							Ls	Bloating Diarrhea	O	This plant's leaf is crushed and mixed with water, and one water glass is given to the cow and ox during pain.
Zingiber officinale Roscoe (SW 31)	Yenjeluwaa (27)	Zingiberaceae	H	CU	Rh	D/F	Hu	Tonsillitis	O	Chewing a small amount once/twice a day for 3 days.
								Common cold Coughing Respiratory tract infection	O	The rhizome of this plant is crushed and mixed with boiled water, and 1-2 tea cup is given for 3 days.
							Ls	Eye diseases	Op	Crushed, mixed with water, and 1-2 drops (daily) of the filtrate are added to the affected eye during pain.

Note: Human (Hu), Livestock (LS), Local name (LN), Growth form (GF), Sources (SO), Parts used (PU), Condition (CO), treatment for (TF), disease treated (DT), Rout of administration (RA), methods of preparations, application and dosage used, Herb (H), Shrub (Sh), Tree (T), Climber (Cl), Bark (B), Root (R), Leaf (L), Fruit (Fr), Flower (Fl), Seed (Se), Steam (St), Sap (Sa), Bulb (Bu), Above Ground (Ag), Whole Plant (Wp), oral(O), Nasal (Na), Dermal (De), Optical (Op), NI (Number of informants)

Preparation methods and route of administration of traditional medicine

According to the study results, people in the study area indicated they used a single plant, two, three, and more species to prepare traditional herbal medicine. In this study, there were a total of 86 herbal medicine preparations. Of these, 76.7% of the preparations were from a single plant species, 19.7% were from two species, and 3.5% of the traditional medicine preparations were from three or more species. In the study area, crushing (36%) and powdering (20%) were the most common preparation methods of traditional medicine (Table 6). Concoction: mixing/combining different ingredients to make a dish; Decoction: boiling the materials and extracting essences or active ingredients.

Local people of the study area administer traditional herbal medicines through oral, dermal, nasal, optical, and suppositories. The largest number (71%) of traditional medicine reported being administered orally (Figure 5).

Ranking of medicinal plants

In the study area, some medicinal plants were better known by informants than others. Therefore, many informants independently mentioned certain medicinal plant species for their medicinal use against diseases in humans and livestock. For example, *Croton macrostachyus* Hochst. Ex. Del., *Pentas schimperi* (A. Rich) Vatke. and *Allium sativum* L. were mentioned by 46, 43, and 42 informants, respectively (Table 3).

Importance of medicinal plants in the study area

The preference ranking of the six medicinal plant species used for treating the common cold in the study area revealed that *A. sativum* is the most effective medicinal plant for treating the common cold, followed by *Zingiber officinale* Roscoe (Table 7).

Direct matrix ranking for multipurpose medicinal plants

Seven uses and six multipurpose plant species that are not used for medicinal purposes were selected. Six randomly selected key informants were asked to assign a use-value to each species. The direct matrix ranking analysis showed that *C. macrostachyus* is the most preferred medicinal plant used by local communities of Damot Woyde District for multiple purposes, followed by *Cordia africana* Lam (Table 8).

Table 6. Method of traditional medicines preparation in the study area

Forms of preparations	Number of preparations	%
Crushing	31	36
Grinding/Powdering	17	19.7
Concoction	13	15
Decoction	11	12.8
Other forms	14	16.3
Total	86	100

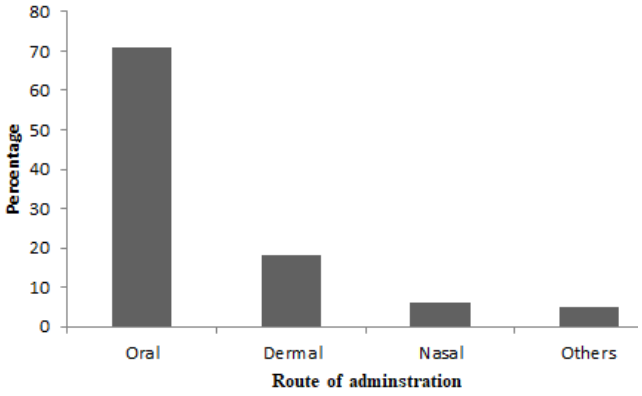

Figure 5. Route of administration of traditional medicine

Table 7. Preference ranking of six medicinal plants used to treat the common cold in the study

Types of medicinal plant species used	Informants labeled by (I_1-I_6)						Total	Rank
	I_1	I_2	I_3	I_4	I_5	I_6		
Allium sativum	5	5	5	5	5	5	30	1st
Artemisia afra	3	3	2	4	2	3	17	5th
Coffea arabica	3	1	2	3	3	2	14	6th
Eucalyptus globulus	5	4	4	5	3	4	25	3rd
Ocimum basilicum	3	2	3	4	3	4	19	4th
Zingiber officinale	5	3	5	5	5	5	28	2nd

Table 8. Direct matrix ranking of six multipurpose medicinal plants

Plant species	Use categories							Total	Rank
	Medicinal	Food	Tool	Construction	Fence	Firewood	Charcoal		
Cordia africana	3	0	5	5	4	4	4	25	2nd
Croton macrostachyus	4	0	4	4	5	4	5	26	1st
Eucalyptus globulus	4	0	3	3	3	2	3	18	5th
Olea europaea	4	0	4	5	4	3	3	23	4th
Persea americana	3	5	2	0	3	2	3	18	5th
Podocarpus falcatus	3	0	5	5	4	4	4	25	2nd

Informant consensus factor (ICF)

The frequent diseases in the study area and medicinal plants that are well-known by community members and effective in treating certain diseases have higher ICF values. For instance, the highest and lowest ICF values belong to a category of diseases (Bone problems and Snakebite, 0.95) and (Kidney problems, Liver problems, and Bladder/Urine problems, 0.4) (Table 9).

Jaccard's similarity index

The highest Jaccard coefficient of similarity in medicinal plants' composition was found between the study area (Damot Woyde District) and Berbere and Hawassa Zuria Districts (13%). In contrast, the degree of similarity was lower in the Babile district (1%) (Table 10).

Marketability of medicinal plants

Most medicinal plants were being sold in the two open markets visited. The medicinal plants encountered in the marketplaces were sold or bought for medicinal and non-medicinal uses. The other uses include; spices, food, household tools, farming tools, fencing, firewood, and constructions. In the markets, flowers of *Hagenia abyssinica* (Bruce x Steud) J. F. Gmel., fruits of *Embelia schimperi* Vatke, and the leaf of *P. schimperi* and *Nicotiana tabacum* L. (Figure 6) are more available for a low price. Usually, small plastic cups or cans are used to measure the quantity.

The threats to the medicinal plants and conservation practices in the study area

A brief group discussion was made with four traditional healers on threats to medicinal plants and the main sources of threats to medicinal plants reported by informants in the study area. Some include urbanization, agricultural expansion, over-harvesting, charcoal collection, destructive harvesting, honey cut, and habitat loss.

Even though there are many threats to medicinal plants, local people in the study area know the importance of conserving the plants. They plant in home gardens, live fences of the gardens, different worship areas, and in their plantation fields. Also, during the field observation, most informants reported that various local beliefs and cultural traditions contribute to the conservation of medicinal plants and associated knowledge in the study area includes: During the collection of parts of medicinal plants, special attention is made to save the life of the mother plant, and they took only small amount from the lateral branches without harming the main parts of the plant. Most plant remedies are only collected in the early morning before the sun rises at night or after sunset, which is believed to maintain the efficacy of the medicines. The collection of traditional medicinal plants is done only by elderly persons of the family, but the children are not allowed to collect them. This action may reduce the damage to plants by children.

Figure 6. Leaf of *N. tabacum* sold in Dawe sake market

Table 9. Informant consensus factor (ICF) of groups of ailments

Diseases categories	Number of sp	Number of use citations	ICF	Rank
Abdominal pain, Intestinal worms, diarrhea, and Gastritis	21	58	0.65	10th
Coughing and Common cold	14	67	0.8	8th
Livestock diseases (sudden illness, bloating, diarrhea, leech, rabies, and retained placenta)	12	28	0.59	11th
Respiratory tract infection, tonsillitis, and Asthma	10	85	0.89	4th
Wound, Hemorrhoids, and Body swelling	10	68	0.87	6th
Skin rash, Skin allergic, and Dandruff	8	51	0.86	7th
Eye and tooth diseases	6	83	0.94	2nd
Malaria, Fever, Tiredness, and Loss of appetite	6	44	0.88	5th
Arthritis and Fibril illness	5	66	0.94	2nd
Bone problem (injury), Snakebite	4	63	0.95	1st
Kidney problems, Liver problems, and Bladder/Urine problems	4	6	0.4	12th
Nasal bleeding, Minor bleeding, and Retained placenta	3	9	0.75	9th

Table 10. Comparison of species in the study area with those in other study areas of Ethiopia

Study areas (districts)	Species no. (a or b)	Common species (c)	Jaccard index	Similarity (%)	References
Damot Woyde	57	-	-	-	The study area
Gera district	63	12	0.09	9	(Gonfa et al. 2020)
Sheka zone	266	26	0.07	7	(Kassa et al. 2020)
Gurage	244	30	0.09	9	(Teka et al. 2019)
Yilmana Densa and Quarit	112	20	0.1	10	(Alemneh 2021)
Bench	35	4	0.04	4	(Giday et al. 2009a)
Babile	51	1	0.009	1	(Belayneh et al. 2012)
Gura Damole	30	6	0.06	6	(Assefa et al. 2021)
Hawassa Zuria	105	24	0.12	12	(Tefera and Kim 2019)
Berbere	70	19	0.13	13	(Jima and Megersa 2018)
South Omo	91	6	0.03	3	(Tolossa et al. 2013)
Meinit	51	10	0.08	8	(Giday et al. 2009b)
Ada'a	131	25	0.11	11	(Kefalew et al. 2015)

Discussion

Medicinal plants of Damot Woyde District

Of the 57 medicinal plants collected in the study area, 36 species were indicated for the treatment of human diseases, while 6 species were used for the treatment of livestock diseases and 15 species for the treatment of both human and livestock diseases. Similar results were obtained from the study of (Kassa et al. 2020; Assen et al. 2021) conducted in other parts of Ethiopia where the locals use more medicinal plants to treat human diseases than livestock diseases. That could be because people prefer human health problems over livestock health problems, as Kassa et al. (2020) justified.

Compared to previous studies conducted in Ethiopia, the present study found a low and high number of medicinal plant species. For example, Kassa et al. (2020) collected 266 plant species used by Sheka zone communities to treat human and livestock diseases. On the other hand, Teka et al. (2019) reported using 244 medicinal plants by Gurage communities. In a similar study, Alemneh (2021) collected 112 medicinal plant species in the Yilmana Densa and Quarit districts of the Amhara region. Therefore, the three authors reported more medicinal plants than the present study. Whereas, Giday et al. (2009a,b), Belayneh et al. (2012), and Assefa et al. (2021) reported 35, 51, and 30 species of medicinal plants, respectively. The difference in the number of medicinal plants could be due to the vegetation of the district, the number of informants involved in the study, the timing and duration of data collection, and the culture.

Jaccard's similarity index result on the composition of medicinal plants shows some similarities between the study area, Berbere (Jima and Megersa 2018), and Hawassa Zuria (Tefera and Kim 2019) districts. Still, less similarity was found with Babile (Belayneh et al. 2012) and South Omo (Tolossa et al. 2013) Districts. According to Megersa et al. (2013), the similarities and differences between the current study and other study areas could be due to cultural and agro-climatic conditions.

The present study revealed that the Asteraceae were represented by 7 plant species, followed by the Rutaceae. Other similar studies in Ethiopia (Teka et al. 2019; Assefa et al. 2021) and elsewhere in the world (Chaachouay et al. 2021; Khajuriaa et al. 2021) have also found that Asteraceae are the major suppliers of medicinal plants. In addition, Fabaceae (Osman et al. 2020; Alemneh 2021), Euphorbiaceae (Jima and Megersa 2018), Lamiaceae (Tuasha et al. 2018; Tamene et al. 2020), and Solanaceae (Abebe and Teferi 2021; Assen et al. 2021; Wendimu et al. 2021) were found to be dominant in the study conducted in South Wollo Zone of Amhara Region, Hulet Eju Enese District and Diguna Fango District of Wolaita Zone.

Growth form and plant parts used for traditional medicine preparation

This study shows that the most represented growth forms of medicinal plants in the study area were 22 species (38.59%). Similar studies conducted in Ethiopia reported the dominance of herbs or shrubs in the use of medicinal plants. For example, Kassa et al. (2020) reported that most medicinal plants collected in the Sheka zone belonged to herbaceous species. According to Tefera and Kim (2019), the local communities of Hawassa Zuria District collected herbaceous plants for making traditional medicine. The findings of Jima and Megersa (2018) and Demie et al. (2018) differ from the present study, as the authors reported that shrubs dominated other growths in the production of traditional medicines. The high use of herbs could be due to their relative abundance and accessibility (Kassa et al. 2020).

The current study results indicate that the local people of Damot Woyde District prepare remedies from different parts of medicinal plants. The most commonly used plant part were leaves for treating human and livestock diseases in the study area. Different research groups reported that roots or leaves are the most important plant parts for preparing traditional medicines in Ethiopia and elsewhere in the world. Among the researchers who reported leaves as the most important part in Ethiopia (Tefera and Kim 2019; Alemneh 2021; Assen et al. 2021; Assefa et al. 2021) and other countries (Tugume and Nyakoojo 2019; Hachlafi et al. 2020; Chaachouay et al. 2021), other research groups

(Tolossa et al. 2013; Kefalew et al. 2015; Jima and Megersa 2018) reported that roots were widely used plant parts for the preparation of traditional medicine. The preference for leaves over other plant parts is due to their easy availability and simplicity of drug preparation. Moreover, the storage of secondary metabolites affects the biological properties of the medicinal plant (Chaachouay et al. 2021).

The present study found that most medicinal plants (60.6%) were used in fresh form. Similarly, Tefera and Kim (2019), Kassa et al. (2020), Assen et al. (2021), and Abebe and Teferi (2021) reported the predominant use of freshly harvested plant parts for the preparation of traditional medicine. People's dependence on fresh plant parts is often due to the efficacy of fresh plant species in therapy, as the constituents are not lost before use compared to dried plant forms (Chaachouay et al. 2021). On the other hand, using fresh plant parts may endanger the plants due to frequent collection, even in dry seasons, as locals make little effort to store dried plant material for later use (Megersa et al. 2013; Tefera and Kim 2019).

Preparation methods of traditional medicine

The local people of Damot Woyde District use various methods of preparation of medicines. The preparation methods used to treat diseases in humans and livestock included crushing, pulverizing, chewing, boiling, and soaking. Crushing was the most common preparation method (36%), followed by powdering (19.7%). Similar results on the most commonly used method of preparing traditional medicine were found elsewhere in Ethiopia (Megersa et al. 2013; Jima and Megersa 2018; Demie et al. 2018; Eshete and Molla 2021). However, the results of Tefera and Kim (2019) showed that grinding was the most commonly used method by the locals in Hawassa Zuria District and elsewhere in the world (Hong et al. 2015).

Most important medicinal plants

Croton macrostachyus and *P. schimperi* were the most commonly cited medicinal plants used to treat human and livestock diseases. According to the results of the preference ranking, the most preferred medicinal plants for treating common colds in humans were *A. sativum* and *Z. officinale*. Ethnobotanical studies in Ethiopia reported different results on preferred medicinal plants to treat human and livestock diseases. Tefera and Kim (2019) showed that *Eucalyptus globulus* Labill. were the preferred medicinal plant to treat stomach pain among people in the Hawassa-Zuria district. *Stephania abyssinica* (Dillon & A. Rich.) Walp. was indicated as the preferred medicinal plant to treat stomach pain by the local communities of the Berbere district. According to Abebe and Teferi (2021), the preferred medicinal plant to treat rabies is *Phytolacca dodecandra* L' Her in the Hulet Eju Enese district. The various reports from the different research groups indicate that the local communities know effective medicinal plants to treat diseases.

According to the direct matrix ranking analysis, *C. macrostachyus* ranked first as the medicinal plant preferred by the local community for various purposes. *Cordia africana* and *Podocarpus falcatus* (Thunb.) Endl ranked second and third as the preferred medicinal plant. In similar studies in Ethiopia, *E. ventricosum* (Tefera and Kim 2019), *C. africana* (Abebe and Teferi 2021), and *Warburgia ugandensis* Sprague (Eshete and Molla 2021) were ranked first as the most used medicinal plant for various purposes by the local people in Hawassa Zuria, Hulet Eju Enese, and Suro Barguda Districts, respectively.

In conclusion, using plant species is important in treating human and animal diseases in Damot Woyde District. In the present study, 57 species of medicinal plants were recorded, and leaves were reported as the most commonly used plant parts for treating diseases. The number of medicinal plants recorded for treating human and livestock diseases indicates indigenous knowledge of traditional herbal medicine. Although medicinal plants are important in treating diseases, they are under threat. Among threats, urbanization, agricultural expansion, and over-harvesting are the major threats to the medicinal plants in the study area. Although the locals in Damot Woyde District have been using medicinal plants to treat diseases for a long time, it is useful to conduct toxicological tests in addition to pharmacological tests. The main focus should be on commonly used medicinal plants such as *C. macrostachyus* and *P. schimperi*.

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Synthesis of novel polymer quaternary ammonium salt derived from glucose as a phase transfer catalyst

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Abstract. *Alrubaie I, Salim AT, Majeed MM, Radhi AJ. 2022. Synthesis of novel polymer quaternary ammonium salt derived from glucose as a phase transfer catalyst. Nusantara Bioscience 14: 25-33.* Some new polymers containing quaternary ammonium salts based-carbohydrate starting from methyl-4,6-*O*-benzylidene-glucopyranoside have been prepared. The ammonium groups are connected to the glucose structure in various positions (2 and 3). Our synthesis used methyl-4,6-*O*-benzylidene-*α*-D-glucopyranoside through nucleophilic reagents to produce the main intermediates. The monomer glucose-quaternary ammonium salts are immobilized on the polyvinyl azide to give the final structure of the Polymer-Supported quaternary ammonium salts. In certain new kinds of phase transfer catalysts, the effectiveness of the polymer glucose-quaternary ammonium salts was tested in the Williamson etherification. Ether synthesis is a typical example of using a phase transfer catalyst. The reactions were performed in a liquid-liquid two-phase system, applying a mixture of toluene and a 50% solution of aqueous sodium hydroxide in the presence of a 20 mg/mmol catalyst (compound 9). Compared to other derivatives, the reaction of 4-nitrophenol with dibromoethylene gives the highest yields. On the other hand, compound 9 with the C16 chain gives high activity in the phase transfer catalyst.

Keywords: Carbohydrates, QAS, Quaternary ammonium salts, polymer, PTC

INTRODUCTION

Phase-transfer catalysis (PTC), a broadly utilized methodology in organic chemistry, has gained growing recognition in recent years for many reasons (Chokaouychai and Zhang 2020; Iribarren and Trujillo 2020; Pudi et al. 2020; Roagna et al. 2020; Weatherley 2020; Xu et al. 2021). For instance, phase transfer catalysis has been identified as an influential green chemistry method that decreases waste generation (Lucchese and Marzorati. 2000; Makosza, 2000; Ikunaka, 2008; Nelson and Benjamin. 2011). In the meantime, the advancement of the asymmetric variant of PTC has created considerable attention because of its possible applicability in the industrial asymmetric synthesis of pharmaceuticals (Haraguchi et al. 2010). The mechanism of PTC typically includes activation of anionic varieties by the carrier of the ion of an aqueous phase, where ions are extremely solvated and unreactive, to a nonpolar organic solvent, where more limited solvation of the ions can lead to enhanced reactivity towards a neutral substrate. Various types of PTC have been utilized, the most common ones being quaternary ammonium salts, with the tetraalkylammonium ion acting as the counter ion of the reactive anion. These quaternary ammonium salts are applied in liquid-liquid phase-transfer catalysis (Annunziata et al. 2000; Chinchílla et al. 2000; Denmark et al. 2011; Waser et al. 2012; Jia et al. 2015; Jin et al. 2018).

A general Figure 1 of the quaternary ammonium salt promoted SN2 reaction involving a (Q+X-) salt with an alkyl halide (RX) is also presented (Vander Zwan and Hartner 1978; Gianluca and Fish 2011). These versatile catalysts can catalyze many reactions, and an especially attractive application is the quaternary ammonium salts that promote nucleophilic Williamson etherification (Freedman and Bubois 1975; Vander Zwan and Hartner 1978; Annunziata et al. 2000; Chinchílla et al. 2000; Denmark et al. 2011; Gianluca and Fish 2011; Naik 2017). Most frequently, sugar-based quaternary ammonium salts are extensively utilized in various applications (Dmochowska et al. 2011; Dmochowska et al. 2016). Carbohydrate derivatives have a wide range of applications for stereochemical control as chiral auxiliaries (Kunz and Rück 1993; O'Doherty 2013), ligands (Lehnert et al. 2011; Henderson et al. 2016), organocatalysts (Phillips 2014; Sabah et al. 2020), and PTC (Bakó et al. 2012, 2015; Erfurt et al. 2020).

The building of the structure of the sugars into the chiral catalyst has many advantages: Carbohydrates utilized as starting materials are, in most states, inexpensive and readily available commercial products. In addition, carbohydrates contain functions that can create secondary connecting sites and catalytic sites (Rapi et al. 2014, 2016, 2017, 2018). Immobilizing the sugar-based quaternary

ammonium salt onto polymers allows for many advantages, such as easy handling, recyclability, and adaptation to continuous processes for this important complexant set (Leelamma and Devaky 2009; Itsuno et al. 2010).

Polymers involving ammonium salt compounds are widely used in the chemistry of polymers and have been known for a long time (Jiang et al. 2005; Liao and Ye 2018; Popescu et al. 2019). Recently, these ammonium salt compounds containing polymers have been studied intensively due to their peculiarities and self-assemblies of QAS systems (You et al. 2021). Moreover, immobilizing the QAS onto polymers allows for many advantages, such as easy handling, recyclability, and adaptation to continuous processes for this important set of compounds (Chinchílla and Nájera 2009; Haraguchi et al. 2010; Zhang et al. 2012).

In this research, we prepared poly-quaternary ammonium salt derivatives and applied them as catalysts in PTC operation in Figure 1.

MATERIALS AND METHODS

Instrumentation

FT-IR spectra were recorded using Fourier transform infrared Alpha-Broker (Germany) infrared spectrophotometer between 4000 and 600 cm^{-1}. In addition, ^{1}H and ^{13}C NMR spectra in DMSO-d_6 were obtained on Bruker spectrometer (300MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR, respectively). Mass spectra were recorded on the MS system, model 5975 quadrupole analyzer, performed at Tarbiat Modares University, Tehran, Iran. Transmittance Electron Microscopy (TEM) CM-130 microscope was installed in 2005; it is a 300 kV analytical TEM/STEM with a LaB6 source. Measurements are made at the Department of Electricity, Faculty of Engineering, Shahid Beheshti University, Iran. FESEM INSPECT S50 Measurements, Shahid Beheshti University, Iran.

Reagents and materials

1-Bromohexadecane (>99%), 1-Bromotetradecane (>99%), 1-Bromododecane (>99%), 1-Bromo decane (>99%) were purchased from Sigma Aldrich (Iran). The 3-Chloro-N, N-diethylpropan-1-amine (>99%), Methyl 4,6-O-benzylidene-α-D-glucopyranoside (>97%) were purchased from N.D.B. Co., Ltd. (China). Benzyl Alcohol (>96%),

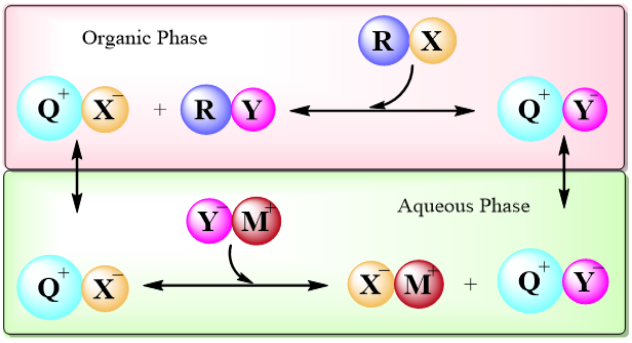


Figure 1. Mechanism of phase-transfer catalysis

Cyclohexanol (>95%), Phenol (>95%) were purchased from GCC (India). Dibromoethylene (>98%) and Benzyl Chloride (>97%) were purchased from GCC (Malaysia). Dimethyl sulfoxide (>95%), Chloroform (>95%), Sodium ascorbate (>95%), Cuprous chloride (>95%), Hexane (>96%), Ethyl acetate (>95%), Ethanol (>97%), propargyl bromide (>93%), *N,N*- Dimethylformamid (>97%), were purchased from Sigma Aldrich (US). Acetone (>99%), Diethyl ether (>98%), Tetrahydrofurane (>98%), toluene (>97%), Nitric acid (>97%), were purchased from Fisher (US). Sodium hydroxide (>94%) was purchased from BDH (England).

Synthesis of methyl-4,6-*O*-benzylidene-2,3-di-*O*-propargyl-*α*-D- glucopyranoside 2

A solution of methyl-4,6-*O*-benzylidene-*α*-D-glucopyranoside (4g, 14.16 mmol) was dissolved in 150 mL of anhydrous THF, and sodium hydride (0.68 g, 28.32 mmol) was added and cooled with an ice-salt bath. The mixture reaction was stirred for 20 mins before 2.14 mL (3.36 g, 28.32 mmol) of propargyl bromide was added slowly over 1 h. The reaction mixture is stirred for 3 days at room temperature and continues stirring until the reaction is complete. The reaction course was followed by TLC ethyl acetate: Hexane (1:1) as an eluent to afford the product. MeOH is added to the destruction of excess sodium hydride. The solvent was removed under diminished pressure, and the residue was taken up in 250 mL of CH_2Cl_2 and 150 mL of water. The organic layer was dried using $MgSO_4$, filtered, and concentrated by evaporated solvent. The crude material was purified by column chromatography and concentrated under a rotary evaporator to give desired compound 2 (70%) yellowish-white solid, m.p 76-78. FT-IR cm^{-1}: 3284 (CH- acetylenic), 3066 (CH-aromatic), 2118 (-C≡C-). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.49-7.35 (m, 5H, Ph). 5.64 (s, 1H, PhCH-), 4.90 (d, J = 3.5 Hz, 1H, H-1), 4.26 (m, 1H; H-6ax), 4.16 (m, 1H; H-6eq), 4.15-4.10 (m, 4H, -OCH_2C≡C), 3.86-3.75 (m,1H, H-2), 3.72- 3.63 (m, 1H, H-4), 3.59-3.57 (m,1H, H-5), 3.57-3.52 (m,1H, H-3), 3.42 (s,3H,-OCH_3), 3.5 (s, 2H,-C≡CH). ^{13}C NMR (75 MHz, DMSO) δ 138.00, 129.31, 128.59, 126.54 (Ph), 100.83 (PhCH-),98.32 (C-1), 81.13, 80.83, 80.73 (C-2/C-4/C-3), 78.29 (-OCH_2C≡CH), 77.94 (-OCH_2-C≡CH), 67.43 (C-6), 62.47 (C-5), 59.35 (-OCH_2C≡C), 58.06 (-OCH_2C≡C), 55.08 (-OCH_3). MS: [M+] calcd. for $C_{20}H_{22}O_6$: 358.39; found: 358,2.

Synthesis of 3-azido-*N,N*-diethylpropan-1-amine(azido-tert-amine) 4

To a solution (3.26 mL, 3 g, 22.11 mmol) of 3-chloro-*N*, *N*-diethylpropan-1-amine in (15 mL) DMSO, (1.17 gm, 18 mmol) of sodium azide was added, and the mixture was stirred for 72 hrs. The reaction course was followed by TLC ethyl acetate: Hexane (1:1) as eluent to afford the product, 50 mL of DCM was added for extraction, the organic layer was separated, and the residue was washed with DCM, the product 4 yellow liquid 3.11 mL (95%). FT-IR cm^{-1}: 2090 (azide group).

Synthesis of methyl-4,6-*O*-benzylidene-2,3-bis-*O*-[2-(4-ethyl-1H-1,2,3-triazol-1-yl)-*N,N*-diethylpropan-1-amine]-α-D-glucopyranoside 5

To a solution (3.2 g, 8.92 mmol) of compound 1 in (30 mL) of DCM, (2.54 g, 17.84 mmol) of azido-tert-amine was added. After the mixture showed homogeneity, sodium ascorbate (0.35 g, 1.78 mmol) and cuprous chloride (0.17 g, 1.78 mmol) were added, and the mixture was stirred at room temperature for 72 hrs. The reaction course was followed by TLC ethyl acetate: Hexane (1:1) as eluent. The solvent was removed under diminished pressure, and the residue was taken up in 50 mL of CH_2Cl_2 and 150 mL of water. The crude material was purified by column chromatography-ethylacetate: Hexane (1:1) and concentrated under a rotary evaporator to give desired compound 5 (76%) as a yellow solid, m.p 104-106. FT-IR cm^{-1}: 3136 (CH-triazole rings), 3066 (CH-aromatic). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.14(s, 2H,-CH-triazole ring), 8.00 (s, 2H,-CH-triazole ring), 7.55-7.35 (m, 5H, Ph), 5.67 (s, 1H, PhCH-), 4.85-4.80 (m, 1H, H-1), 4.80-4.63(m,4H, -OCH_2-), 4.42-4.30 (m,4H, -CH_2CH_2-$CH_2N(CH_2CH_3)_2$), 4.29 (m, 1H; H-6ax), 4.15 (m, 1H; H-6eq), 3.82-3.67 (m, 1H, H-4), 3.87-3.82 (m,1H, H-2), 3.60-3.51 (m, 1H, H-3), 3.66-3.62 (m,1H, H-5), 3.47 (s, 3H,-OCH_3), 2.44-2.26 (m, 8H, -$N(CH_2CH_3)_2$), 1.97-1.78 (m, 8H, -CH_2-$CH_2N(CH_2CH_3)_2$), 0.96-0.83 (t,12H, -$N(CH_2CH_3)_2$). ^{13}C NMR (75 MHz, DMSO) δ 144.90, 144.45 (-C=CH-triazole ring), 138.10, 129.27, 128.57, 126.51(Ph), 124.42(-C=CH-triazole ring), 124.05 (-C=CH-triazole ring), 100.85 (PhCH-), 98.54 (C-1), 81.15, 80.77, 80.59 (C-2/C-4/C-3), 67.48 (C-6), 65.75 (C-5), 64.07, 62.59 (-OCH_2-), 55.08 (-OCH_3), 49.31 (-CH_2-$CH_2N(CH_2CH_3)_2$), 48.09, 48.00 (-CH_2N $(CH_2CH_3)_2$), 46.63, 46.59 (-$N(CH_2$ $CH_3)_2$), 27.95, 27.89 (-CH_2-$CH_2N(CH_2CH_3)_2$), 12.06, 12.04 (-N $(CH_2$ $CH_3)_2$). MS: [M+] calcd. for $C_{34}H_{54}N_8O_6$: 670.86; found: 670.5.

Synthesis of methyl-2,3-bis-*O*-[2-(4-ethyl-1H-1,2,3-triazol-1-yl)-*N, N*-diethylpropan-1-amine]-α-D-glucopyranoside 6

To a solution (4.4 g, 6.55 mmol) of compound 3 in methanol, 100 mg of Pd-C 5% was added, then the flask was flashed with hydrogen gas to remove the O_2 atmosphere. The H_2 was filled in a balloon, attached to the flask, and stirred at RT for 72 hrs at room temperature. The reaction course was followed by TLC methanol: Chloroform (1:3) as an eluent to afford the product. Filtrating the product was concentrated. The residue was purified by column chromatography on silica gel using methanol: Chloroform (1:3) as eluent to make the product 6 (83%) yellowish semisolid. FT-IR cm^{-1}: 3399 (-OH), 3143 (-CH-triazole rings). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.14(s, 2H, -CH-triazole ring),7.99 (s, 2H, -CH-triazole ring), 5.54 (s, 1H, 4-OH), 4.83-4.79 (m, 1H, H-1), 4.42-4.32 (m, 4H, -CH_2CH_2-CH_2N $(CH_2CH_3)_2$), 4.31 (m, 1H; H-6ax), 4.16 (m, 1H; H-6eq), 3.88-3.83 (m, 1H, H-2), 3.82-3.69 (m, 1H, H-4), 3.67-3.62 (m, 1H, H-5), 3.61-3.52 (m, 1H, H-3), 3.30 (s,3H, -OCH_3), 2.48-2.30 (m, 8H, -$N(CH_2CH_3)_2$), 1.97-1.63 (m, 8H, -CH_2-CH_2N $(CH_2CH_3)_2$), 0.98-0.84 (q, 12H, -$N(CH_2CH_3)_2$), ^{13}C NMR (75 MHz, DMSO) δ 144.91, 144.45 (-C=CH-triazole ring), 124.08, 124.45 (-C=CH-triazole ring), 98.53 (C-1), 81.13, 80.66, 80.58 (C-2/C-4/C-3), 68.47(C-6), 65.72 (C-5), 64.05, 62.58 (-OCH_2-), 55.07 (-OCH_3), 49.15, 49.10 (-CH_2-CH_2-$CH_2N(CH_2$ $CH_3)_2$), 47.94 (-CH_2N $(CH_2CH_3)_2$), 46.53, 46.50 (-$N(CH_2CH_3)_2$), 27.53 (-CH_2-$CH_2N(CH_2CH_3)_2$), 11.72 (-N $(CH_2$ $CH_3)_2$). MS: [M+] calcd. for $C_{27}H_{50}N_8O_6$: 582.75; found: 582.4.

Synthesis of methy-4,6-*O*-dipropargyl-2,3-*O*-bis[2-(4-ethyl-1H-1,2,3-triazol-1-yl)-*N, N*-diethylpropan-1-amine]-α-D-glucopyranoside 7

A solution (2.8 g, 4.50 mmol) of compound 4 was dissolved in 70 mL anhydrous THF, and sodium hydride (0.21 g, 9 mmol) was added and cooled with an ice-salt bath. The mixture reaction was stirred for 20 mint before 0.68 mL (1.07 g, 9 mmol) of propargyl bromide was added slowly over 1 h. the reaction mixture is stirred for 3 days at room temperature and continues stirring until the reaction is complete. The reaction course was followed by TLC ethyl acetate: Hexane (1:1) as an eluent to afford the product. MeOH was added carefully until gas formation ceased (destruction of excess sodium hydride). The solvent was removed under diminished pressure, and the residue was taken up in 80 mL of CH_2Cl_2 and 150 mL of water. The organic layer was dried using $MgSO_4$, filtered, and concentrated by evaporated solvent. The crude material was purified by column chromatography to make the product 7 (72 %) yellowish-white solid, m.p 119-121 ℃. FT-IR cm^{-1}: 3285 ,(CH-acetylenic), 2117, (-C≡C-). ^{1}HNMR (300 MHz, DMSO-d_6) δ 8.50(s, 2H,-CH-triazole ring), -8.00 (s, 2H,-CH-triazole ring), 4.86-4.77 (m, 1H, H-1), 4.74-4.65 (m, 4H, -OCH_2-), 4.53-4.37 (m,4H, -CH_2CH_2-CH_2N $(CH_2CH_3)_2$), 4.37-4.24 (m, 4H, -OCH_2C≡C), 4.23 (m, 1H; H-6ax), 4.16 (m, 1H; H-6eq), 3.86-3.73 (m, 1H, H-2), 3.72-3.63 (m, 1H, H-4), 3.62-3.58 (m, 1H, H-5), 3.58-3.54 (m, 1H, H-3), 2.71-2.51 (m, 1H), 2.68-2.57 (m, 8H, -$N(CH_2$ $CH_3)_2$), 3.50 (s, 2H,-C≡CH), 2.07-1.90 (m, 8H, -CH_2-$CH_2N(CH_2CH_3)_2$), 1.04-0.91 (t,12H, -N $(CH_2$ $CH_3)_2$). ^{13}CNMR (75 MHz, DMSO) δ 144.90, 144.80 (-C=CH-triazole ring), 124.05 (-C=CH-triazole ring), 124.95 (-C=CH-triazole ring), 98.26 (C-1), 81.29, 80.88, 80.34 (C-2/C-4/C-3), 67.07 (C-6), 65.43 (C-5), 64.90, 62.39(-OCH_2-), 57.83, 55.57(-OCH_3), 49.75 (-CH_2-CH_2-$CH_2N(CH_2CH_3)_2$), 47.71 (-CH_2N $(CH_2$ $CH_3)_2$), 46.72 (-$N(CH_2CH_3)_2$), 25.70 (-CH_2-$CH_2N(CH_2CH_3)_2$), 11.20 (-N $(CH_2$ $CH_3)_2$). MS: [M+] calcd. for $C_{33}H_{54}N_8O_6$: 658.85; found: 658.5.

General procedure for the quaternization of amino sugars 8

Compound 7 (1.5 mmol) was dissolved in DMF. Alkyl halide: 2 equivalents were added. The reaction mixture was refluxed for 5 hrs. After the reactions were complete, followed by TLC, the mixture was filtered, and the solvent was removed under reduced pressure. The purification processes of the different ammonium salts were done by recrystallization (using petroleum ether/hexane) of the product 8 (78 %) as a brown solid, m.p 165-167ºC. FT-IR cm^{-1}: 2931, 2771, 2441, 1428, 1465, 874, 758, ^{1}H NMR

(300 MHz, DMSO-d_6) δ 8.07 (s, 2H,-CH-triazole ring), 5.95-4.86 (m, 1H, H-1), 4.78-4.56 (m, 4H, -OCH_2-), 4.39-4.33 (m,4H, -CH_2CH_2-CH_2N^+ (CH_2CH_3)$_2$), 4.23 (m, 1H; H-6ax), 4.14 (m, 1H; H-6eq), 4.14-4.08 (m, 4H, -CH_2-CH_2N^+(CH_2 CH_3)$_2$), 4.07-4.02 (m, 4H, -$OCH_2C{\equiv}C$), 3.87 - 3.76 (m, 1H, H-2), 3.76-3.67 (m, 1H, H-4), 3.64-3.57 (m, 1H, H-5), 3.56-3.52 (m, 1H, H-3), 3.49 (s, 2H,-C≡CH), 3.43 (s,3H,-OCH_3), 3.29-3.18 (m, 8H, -N^+(CH_2CH_3)$_2$), 2.41-2.24 (m, 4H,-CH_2-CH_2N^+(CH_2CH_3)$_2$), 1.89-1.78 (m, 4H,-CH_2-CH_2N^+(CH_2CH_3)$_2$), 1.53-1.25 (m, 28H, -$(CH_2)_{14}$-), 1.06-0.92 (m, 18H, -CH_3). ^{13}C NMR (75 MHz, DMSO) δ 144.92, 144.85 (-C=CH-triazole ring), 124.62(-C=CH-triazole ring), 124.30 (-C=CH-triazole ring), 98.46 (C-1), 81.47, 80.77, 80.15 (C-2/C-4/C-3),78.36,78.26 (C≡CH), 76.51, 74.28 (C≡CH), 67.23 (C-6), 58.62 (C-5), 57.86 (-OCH_2-), 57.63, 57.39, 57.14, 56.89, 55.87 (-OCH_3), 55.71, 54.65 (-CH_2-CH_2-CH_2N^+(CH_2 CH_3) $_2$), 52.66 (-CH_2N^+(CH_2 CH_3)$_2$), 50.49 (-N^+(CH_2CH_3)$_2$), 31.49-28.07 (-CH_2), 27.53, 27.02 (-CH_2-CH_2N^+(CH_2 CH_3)$_2$), 25.87, 23.46, 23.34 (-CH_2), 14.76, 14.76 (-N^+ (CH_2CH_3)$_2$), 9.24(-CH_3), MS: [M+] calcd. For $C_{53}H_{96}N_8O_6^{2+}$: 941.40; found: 941.3.

Synthesis of PVN_3

Polyvinyl chloride (PVC) (10 g) was dissolved in (30 mL) of DMSO, and then (3.5 g, 53 mmol) of sodium azide was added, and the mixture was stirred for 72 hrs. Finally, the mixture was washed extensively with water, and the product was dried to form a light-yellow solid (yield: 9.8 g).

Synthesis of polymeric QAS 9

Polyvinyl azide (PVN3) (2.4 g), compound 8 (0.00063 mol) was dissolved in (25 mL) of DCM and stirred for 15 minutes. After the mixture showed homogeneity, sodium ascorbate (0.6 g, 3 mmol) and cuprous chloride (0.3 g, 3 mmol) were added and stirred at room temperature for 72 hrs. The mixture was washed extensively with water and Ethanol. The solid product 9 was dried to form brown.

General procedure for Williamson reaction compound 10-15

A solution of alcohol (10 mmol), alkyl halides (mono-substation: 1eq, di-substation: 2 eq), and the polymeric catalyst (20 mg/mmol) was dissolved in a mixture of toluene and 50% aq. NaOH 3:2 ratio (10 mL). The mixture was stirred at a temperature of 65°C for 8 hrs. The end of the reaction was monitored by TLC (petroleum ether-ethylacetate 3:1). After filtration to recover the catalyst, 5 mL of toluene was added. The organic layer was separated by a separating funnel, washed two times with (10 mL) of water, dried over anhydrous magnesium sulfate, and filtered. The solvent was removed in a vacuum. The crude materials were purified by column chromatography.

RESULTS AND DISCUSSION

To synthesize carbohydrate derivatives with QASs, we introduced the quaternary ammonium group via a reaction between tert-amine with 1-bromo hexadecane, 1-bromotetradecane, 1-bromodecane, and 1-bromododecane. Methyl 4,6-O-benzylidene-2,3-O-bis[prop-2-yn-1-yl]-glucopyranoside (comp.2) was chosen as the beginning material, which was synthesized in our lab in one step starting with methyl 4,6-O-benzylidene-α-D-glucopyranoside (comp.1) in high yields according to literature procedure (Tankam et al. 2007). The copper-catalyzed click reaction between azides and terminal alkynes is ideal for many applications, with the balance of stability and reactivity, allowing new sugar development with a linking moiety. Furthermore, click chemistry allows the connection between alkynyl-carbohydrates (comp.2) and the azido-tert-amine (comp.4) through a heterocyclic triazole. This way, taking into account Figure 2, through 1,3-dipolar cycloaddition between a 1,3 dipole (azido-tert-amine) and a dipolarophile (alkynyl-glucose), we can obtain triazole di-substituted compound. The final step is converting compound 7 to quaternary ammonium salt compound 8.

Structures of compounds 8

The intermediate compound 7 was further converted to quaternize with 1-bromohexadecane Figure 3. In most states, reactions were terminated in 5 h. These two products were isolated from the starting materials.

The ^{1}H-NMR spectrum of compound 8a-d showed there is a new signal at the range of 1.2-1.5 ppm for the protons of the alkyl chain due to (-CH_2-) groups of the aliphatic chain; the ^{13}C-NMR spectrum of compound 6a-d showed that there was a new signal at the range of 23-32 ppm for the carbons of alkyl chains due to (-CH_2-) groups of aliphatic chain and The characteristic bands of the FT-IR measures appeared about 2826-2994 cm^{-1} (C-H of CH_2 stretch), 2400-2700 cm^{-1} (ammonium ion), 1428-1467 cm^{-1} (C-N), and 757-781 cm^{-1} (skeletal "out of plane" vibration). Furthermore, for each series of compounds, the intensity of the aliphatic C-H absorption bands within the 2826-2994 cm^{-1} region was observed to increase with the length of the *N*-alkyl chain.

Synthesis polymeric (glucose-based triazole)-supported quaternary ammonium salts (QASs) 9

The polymerization method usually needs some criteria to achieve it in a good and economic system. However, the sugar involves QAS in a protecting group approach which increases the cost of synthesis. So, the polymerization of such materials will enhance the cost, and as a result, the process will shift to a different conformation of the resulting structure. Instead, our viewpoint in synthesizing such polymeric catalysis is included in crafting this material on commercially available polymers. Among them, PVC is becoming a starting polymer to be crafted with QAS derivative 9a-d, where price, availability, and stability are crucial factors for choice. The PVC was converted to another reactive precursor, polyvinyl azide (PVN_3), by an easily achieved nucleophilic replacement reaction, in which the chloride nucleophile could replace it with the more reactive azide (Figure 4).

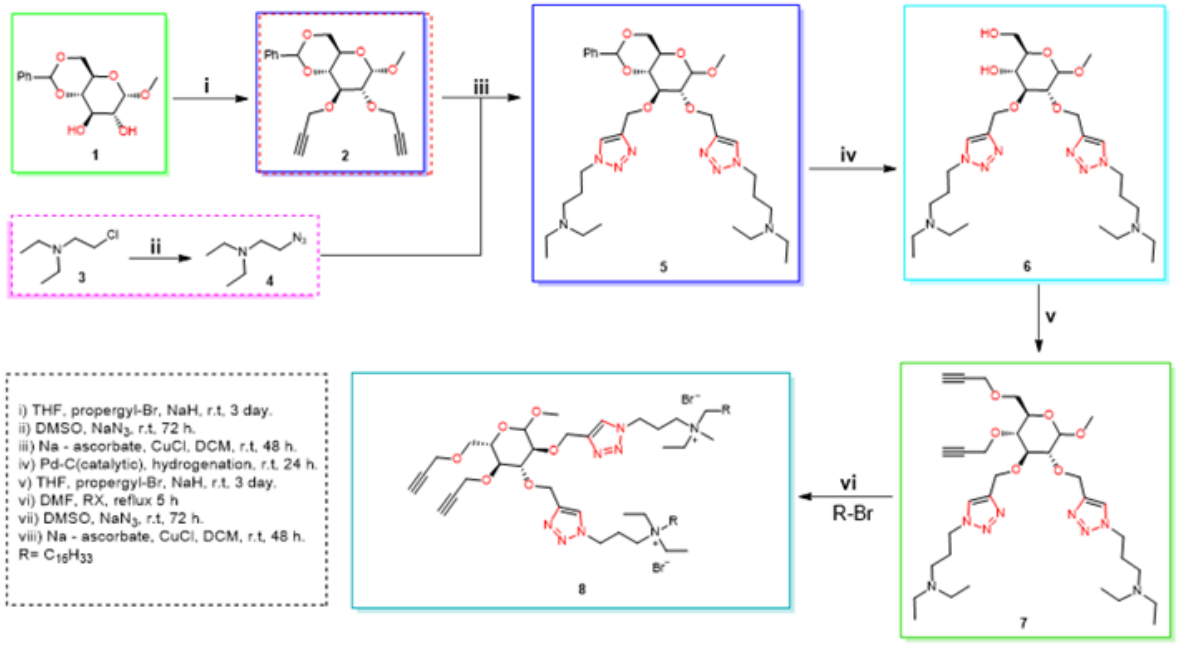
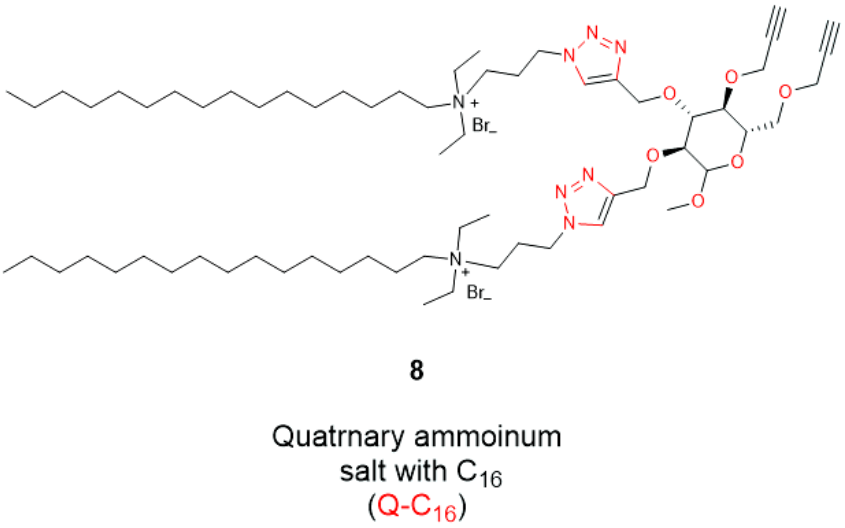


Figure 2. A synthetic strategy, generally aqueous extraction, gives ammonium salts compound 8 in reasonable yield (75%).



8

Quatrnary ammoinum salt with C_{16} ($Q\text{-}C_{16}$)

Figure 3. Quaternary ammonium salt 8

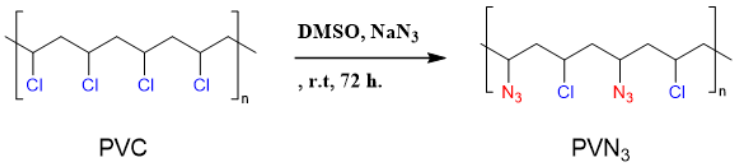


Figure 4. prepare of polyvinyl azide (PVN_3)

The FT-IR spectrum of PVN_3 showed a very strong band at 2110 cm^{-1} resulting from the starching vibration of the azide (N_3) group. Due to a review of the IR spectra of both PVC and the resulting PVN_3, the azide group was confirmed. The above reacted with propargylic glucose-QAS ether compounds 6a-d through a 1,3-dipolar Huisgen (click) cycloaddition reaction in Ethanol as the solvent, catalyzed by copper chloride and sodium ascorbate (Figure

5). The FT-IR range of the resulting polymer 9a-d revealed no alkyne CH signals at 3300 cm^{-1}. Furthermore, the peak between 2112-2118 cm^{-1} remained, indicating that there were unreacted azide groups in 9a-d due to the large volume of azide in the polymeric chain.

Field emission Scanning Electron Microscope (FESEM) analysis

The FESEM technique analyzes the materials' morphology to understand better some of the qualities that originate from the surfaces, leading to appropriate recommendations for using these materials in various applications. For example, the surface of linear polymer 9 was somewhat rougher, and some of the aggregations were seen in the morphological study of the FESEM image of linear polymer (9) (Figure 6).

Transmittance Electron Microscopy (TEM) analysis

The morphology of isolated polymer 9 (Q-C_{16}) was investigated by TEM. As was already apparent using the NTA in TEM images, a heterogeneous population of QAS groups on PVN3 was detected in Figure 7.

Application of the sugar-based quaternary salts in phase transfer catalyst Williamson ether synthesis

The catalytic activity of the new carbohydrate-based ammonium salts was tested to synthesize the ether compound. Various groups have broadly studied this type of reaction. The reactions were completed in a liquid-liquid two-phase system, applying a mixture of toluene and 50% aqueous NaOH solution in the presence of a 200 mg catalyst. The crude products were purified using preparative TLC in all cases. The results are summarized in Table 1. The ether synthesis is a typical example of using a phase transfer catalyst for the ether that is sensitive to other methods, like hydrides and alkoxides, when other side products may occur.

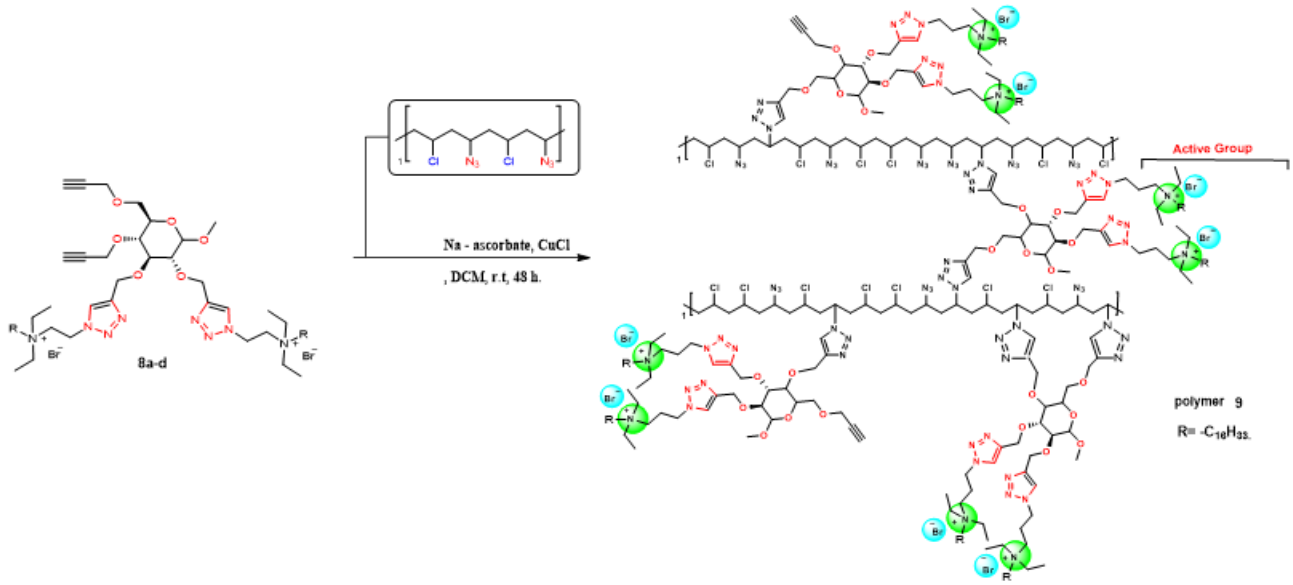
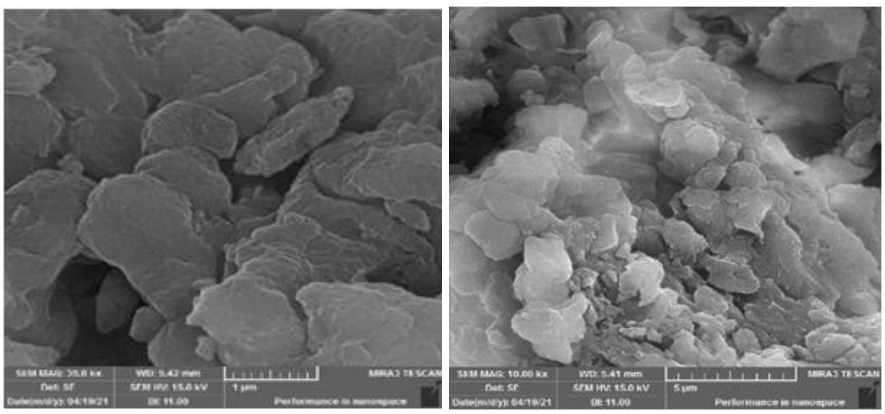


Figure 5. The steps of preparing compound 9



A **B**

Figure 6. SEM analysis of polymer 9d: A. 5 µm, B. 1 µm

However, the prevention of elimination reaction that competitive the substitution required mild conditions available under phase transfer catalytic methodology. The various starting materials, including alkyl halides, alcohols, and phenols, were investigated to use quaternary salts and effectively study the limitations. In all cases, excellent to moderated yields were obtained using the optimum condition investigated with the less reactive alkyl halides and alcohols available to this study (Figure 8). Where other side products such as hydrides and alkoxides are present, ether synthesis is a common example of utilizing a phase transfer catalyst for ethers vulnerable to other methods such as hydrides and alkoxides. However, mild conditions were needed to avoid the removal reaction during competitive substitution, which are available in phase transfer catalytic methodology.

Several starting materials, including various alkyl halides, alcohols, and phenols, were examined to determine the effectiveness of utilizing quaternary salts as well as the limitations. In all instances, excellent-to-reasonable yields were obtained under optimal conditions, which were examined using the less reactive alkyl halides and alcohols available for this analysis.

The activity of the synthesized compound 9 is studied through the interaction of Williamson with different derivatives of alkyl halides and phenols, and it is found that compound 9 gave a good yield, noting that the difference in the yield is not clear or close to some extent. As compared to Phenol, 4-nitrophenol has the highest yields of nearly all alcohols, as seen in Table 1, while hexanol has the lower yields of nearly all alcohols used in the test.

Among all alkyl halides, the benzyl chloride shows high yields with almost all alcohols compared to the ethylene dibromide, as shown in Table 1. The synthesized compounds (10-15) were identified by 1HNMR and FT-IR spectroscopy. Similarly, the hydroxyl compound structures were investigated with the same alkyl halide. The benzyl alcohol and phenols showed a high yield compared to others. The Phenol with a strongly electron-withdrawing nitro group (compounds 13) exhibited an excellent yield (95%) due to the resonance effect, which is to withdraw the electron density from the oxygen of the hydroxyl group and, as a result, weakens the oxygen-hydrogen bond. In other words, the base could easily abstract that hydrogen and form a strong phenoxy anion, which attacks the halide to produce ether. Conversely, the cyclohexanol with donating aliphatic group (compound 11) gave a lower yield (75%). With a PTC process, one can accomplish quicker reactions, greater conversions or yields, fewer byproducts, remove the requirement for expensive or hazardous solvents that dissolve all the reactants in one phase, eliminate the need for expensive raw materials, and/or reduce waste. Phase-transfer catalysts are particularly beneficial in green chemistry because they allow water to be used.

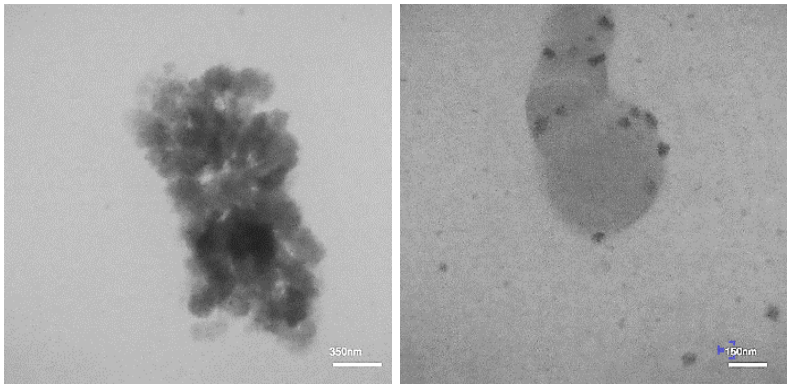


Figure 7. TEM analysis of polymer 9: A. 350 nm, B. 150 nm

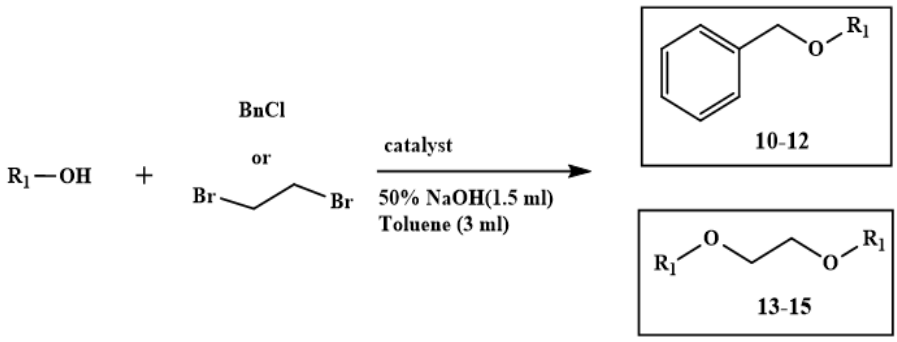
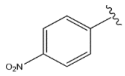
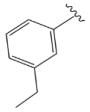


Figure 8. preparation of ether compounds

Table 1. Results of Williamson's reaction

Comp.	R_1	Temp. (C°)	Time (h)	Yield (%) 20 mg/mmol of catalyst (Compound 9)
Mono-substituted ethers				
10		65	8	91
11		65	8	80
12		65	8	75
Di-substituted ethers				
13		65	8	95
14		65	8	84
15		65	8	89

In conclusion, this paper describes the synthesis of a novel polymeric quaternary ammonium salt, PQAS, which carries a monosaccharide and is applied as a catalyst in PTC; the ammonium functionalities were put in various places on the glucose scaffold to investigate the structure-efficiency relationship of the new catalysts. When using long alkyl chains in QAS, the resultant polymer has a high enough molecular weight to be insoluble in most organic solvents. This characteristic gives stability to the polymer in various phases. In addition, they help us recover the polymer easily. The click reaction using CuCl in dichloromethane was effective enough to attach the quaternary sugar ammonium to PVN_3. The prepared cross polymer with active units of quaternary ammonium salts likely has a high affinity for PTC. By testing poly quaternary ammonium salts (9) in the Williamson reaction (as phase transfer catalysts), which includes the reaction of alkyl halide derivatives with alcohol or phenol derivatives. The reactions were performed in a liquid-liquid two-phase system. The length of the alkyl chain had a significant impact on the activity of the study phase transfer catalyst. During the reaction, Williamson: The reaction products of halides, alkyls, and alcohols differed depending on their structure; 4-nitrophenol had the highest yields of nearly all alcohols, while hexanol had the lowest yields of nearly all alcohols that were used in the test. In addition, the increase of the main units on the polymer leads to an increase in the activity of the polymer as the catalyst. Also, the chain length has an important role in the effectiveness of the quaternary salts, as we noticed during the presentation of the results.

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Agronomic diversity of several soybean putative mutant lines resulting from gamma-ray irradiation in M_6 generation

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Abstract. *Nilahayati, Nazimah, Handayani RDS, Syahputra J, Rizky M. 2022. Agronomic diversity of several soybean putative mutant lines resulting from gamma-ray irradiation in M_6 generation. Nusantara Bioscience 14: 34-39.* Soybean is one of the foremost commodities for Indonesian people. Therefore, increasing domestic production must continue to be pursued absolutely. One way to do this is to use the assembly technology of new superior varieties with better and adaptive properties. This study aims to determine the agronomic diversity and yield of soybean putative mutant lines resulting from gamma irradiation in M_6 generation. The research was conducted in Paloh Lada Village, Dewantara Subdistrict, Aceh Utara District, Indonesia, from July 2021 to October 2021. The tested genotypes consisted of 8: Kipas Putih variety (parent variety), Anjasmoro variety (comparison variety), M.1.1.3, M.5.2.1, M.5.2.3, M.1.1.8, M.1.1.9, and M.1.1.17 mutant lines. The results showed that the agronomic diversity of the tested mutants differed from the parents and comparison variety. In addition, we obtained early maturity and high yielding mutant in the M_7 generation. The mutant line with a high yield and equivalent to its parents was M.1.1.8, with a production of 4.37 tons.ha^{-1}. Mutant lines with early maturity were M.1.1.9, M.5.2.1, and M.1.1.3 lines with a harvesting age of 83 days after planting (DAP).

Keywords: Gamma-ray irradiation, genotype, M_6 generation, putative mutant lines

INTRODUCTION

Soybean is an important plant for Indonesian people (Lestari et al. 2017; Toyip et al. 2019). Increasing national soybean production is necessary to maintain food sovereignty (Harsono and Pratiwi 2017). Various efforts can be made to increase domestic soybean production by expanding the planting area, improving cultivation techniques, and using superior varieties. Assembling superior varieties can be done by plant breeding techniques to obtain genetic diversity. Various methods of plant breeding, both conventional and biotechnology, are currently available. One of the very popular breeding techniques is mutation breeding with gamma-ray irradiation.

Mutation induction with gamma rays is commonly used to obtain genetic diversity in cultivated plants, especially soybeans. Several researchers have previously used gamma-rays on various soybean genotypes, including Sohag, BARI Soybean-5, Bangladesh Soybean-4, and BAU-S/64, for genetic improvement of yield attributes (Malek et al. 2014), soybean line VX04-6828 for better agronomic performance and seeds composition (Nobre et al. 2019), Anjasari soybean for high seed yield and large seed size (Mawarni et al. 2019; Yoel and Rachmadi 2020), Korean cultivars Danbaek and Daepung for highest linolenic acid contents (Hong et al. 2019), Kipas Putih soybeans for early maturity and high yields (Nilahayati et al. 2016; Nilahayati et al. 2019), Denna 2 variety for shade-tolerant (Harsanti et al. 2020) and induction of genetic diversity of soybean genotypes PGRI-15, KY EXOTIC, NARC-, AJMERI-2, AJMER-I (Hussain et al. 2020).

It is possible to assemble local soybean varieties to obtain early maturity and high-yielding essential derivative variety using gamma-ray irradiation. A soybean mutant line from the Kipas Putih cultivar was obtained in the previous study. The selection in the M_5 generation obtained 33 putative mutant lines, including 6 early maturity mutant lines (4-14 days earlier than the parent), 3 mutant lines that harvested 8 days earlier than a parent, 19 mutant lines with high yields but not early maturity, and 7 putative mutant lines with high yields but not early maturity and have large seed weight (Nilahayati 2018). Putative mutant lines that are in early maturity are purified for several generations so that they can be continued for the release of new varieties.

The availability of early-maturity soybean varieties will overcome climate change because using short-lived varieties will reduce the risk of crop failure due to drought. Furthermore, early-maturity soybeans would be more profitable for farmers to alternate crops with rice and avoid water shortages for plants during their growth when planted after rice (Rehajeng and Adie 2013). In addition, early-maturity soybeans can provide various advantages, namely reducing pest infestations and increasing the cropping index in a year.

Therefore, the early maturity putative mutant lines obtained from the previous study need to be tested in the next generation to determine their genetic stability. The test was carried out by observing the agronomic diversity and yield of putative mutants selected by comparing their characters with the parents and the comparison varieties.

Therefore, a combination of plant breeding and agronomy is needed to improve plant characteristics and test line stability before releasing a variety.

This study used six soybean mutant lines of M_6 generation from the Kipas Putih soybean variety mutation with two checked varieties, Kipas Putih and Anjasmoro. All soybean seeds were obtained from the results of previous generations of research. These lines need to be tested for yield to determine agronomic performance with their parents.

MATERIALS AND METHODS

The research was conducted in Paloh Lada Village, Dewantara Subdistrict, Aceh Utara District, Indonesia, from July 2021 to October 2021. The plant materials used in this study were eight soybean genotypes: Kipas Putih variety (parent variety), Anjasmoro variety (comparison variety), M.1.1.3, M.5.2.1, M.5.2.3, M.1.1.8, M.1.1.9 and M.1.1.17 mutant lines. The agricultural production facilities are urea 50 kg ha^{-1}, SP-36 150 kg ha^{-1}, KCl 100 kg ha^{-1}, manure 3 tons ha^{-1}, Decis 25 EC, and Dithane-M45. A non-factorial Randomized Block Design (RBD) was utilized as the experimental design.

The planting area is cleaned of weeds that grow in the area. Then made an experimental plot with a size of 2 m x 1.5 m. A drainage ditch was made with a distance between plots and between replications of 50 cm. Seed planting is done by making planting holes in the plot with a depth of 2 cm and a spacing of 40 cm x 20 cm, then inserting 2 seeds per planting hole. Thinning was done at the age of 2 weeks after planting so that only 1 plant was left per planting hole.

Fertilization was carried out according to the recommended dosage of soybean fertilizer. The application of SP-36 and KCl fertilizers and manure was carried out 2 weeks before planting, while urea was applied at the planting time. Thinning was done at the age of 2 WAP. Weeding was done manually by removing weeds from the plot. Harvesting is done by pulling the stems of the plant by hand. The harvest criteria were that most of the leaves had turned yellow and fell, and the skin of the pods was brownish yellow in as much as 95% of the experimental plot units. In addition, observations were made on plant height, number of branches, flowering age, harvest age, number of pods per plant, seed weight per plant, seed weight per plot, and tons ha^{-1} production. Data analysis was performed by analysis of variance. If significantly different, it continues with the Least Significant Difference (LSD) Test.

RESULTS AND DISCUSSION

Plant height

The results significantly affected the character of plant height of several M_6 genotypes and their two comparison varieties. The results of further tests on the average plant height of several M_6 genotypes and their two comparison varieties can be seen in Figure 1.

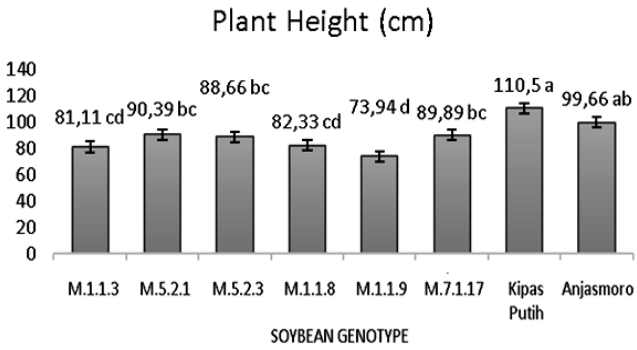

Figure 1. The effect of several soybean genotypes on plant height characters. Note: The numbers followed by the same letter in the same bar are not significantly different according to the 5% LSD test

The highest plants were found in the Kipas Putih variety, with a height at harvest reaching 110.50 cm, which was not significantly different from genotype Anjasmoro, with a height of 99.66 cm. Then followed by M.5.2.1, M.7.1.17, M. 5.2.3, M.1.1.8, and M.1.1.3 mutant lines. The shortest plant height was found in M.1.1.9 genotype with a height of only 73.94 cm, which was not significantly different from the height of M.1.1.8 and M.1.1.3 mutant lines (Figure 1). In the mutant lines of gamma-ray irradiation, the selection results in the previous generation that were tested still showed a decrease in plant height compared to Kipas Putih. The reduction of plant height due to gamma-ray irradiation treatment on plants has been found by many researchers before. Yadav et al. (2019) found that maize genotype HQGM-1 irradiated at several irradiation doses showed a reduction in plant height of 56% at an irradiation dose of 0.5 kGy. (MT et al. 2021) also reported the development of semi-dwarf, early maturing, and high-yielding mutant of rice cultivar Improved White Ponni with gamma-ray irradiation. In the M_6 generation, they found a significant reduction in plant height (up to 40% deduction). Similar results have been reported by (Mudibu et al. 2012) in soybean. In the 0.4 kGy gamma-ray treatment, plant height was shorter in the M_1 generation, with a decrease of 25%, 13%, and 38% for Kitoko, Vuangi, and TGX814-49D, respectively. In the M_2 generation, a 0.4 kGy gamma-ray irradiation dose caused a 14% decrease in plant height in the Kitoko genotype.

The plant height reduction in M_1 generation was observed for the 0.4 kGy treatment with 25%, 13%, and 38% decrease for Kitoko, Vuangi, and TGX814-49D, respectively. In M_2 generation, the 0.4 kGy irradiation significantly reduced plant height in Kitoko (14%).

Compared to planting in the previous generation (M5 generation), the plant height of the tested lines ranged from 39-59 cm, which means that it was shorter than the planting in this M_6 generation. That is due to different locations and different growing seasons. Plant height is included in the quantitative character, which is strongly influenced by the environment. Xue et al. (2019) stated that plant height is an important trait in soybean. The taller plants may give higher yields but will be more susceptible to lodging. There are many genes involved together in influencing plant

height throughout development. Genetically, plant height is a quantitative trait controlled by multiple genes.

Days of flowering, harvesting age, and number of branches

The analysis of variance showed a very significant effect on the characteristics of flowering and harvesting ages of several M_6 genotypes and their two comparison varieties. However, there was no significant effect on the character of the number of branches of several M_6 genotypes and their two comparison varieties. Further tests on the average days of flowering, the number of branches and harvesting age, and their two comparison varieties can be seen in Figures 2, 3, and 4.

The longest days of flowering were found in M.5.2.3 mutant genotype with a flowering age of 42.77 days after planting (DAP), which was not significantly different from other mutant genotypes and the parent variety. The fastest flowering age was found in Anjasmoro, which started flowering at 35.55 DAP. The research results in this generation showed an unstable appearance on the character of flowering age compared to the planting of these lines in the previous generation. Nilahayati (2018) found that in the M_5 generation, the M.1.1.3, M.5.2.1, M.5.2.3, and M.1.1.9 lines were selected based on the criteria for flowering age, which were faster than other lines with flowering age at 36-37 DAP. In contrast, MT et al. (2021) reported reduced days to flower in the M_6 generation of Improved White Ponni (IWP) paddy mutants. Reduction days to 50% flowering of WP 5-4 mutant was reduced to 13 days (11.8% reduction from 110 days in the IWP control). In the other six mutants, there was a reduction of up to 11 days in their flowering time.

The longest harvesting age was found in genotype Kipas Putih with a harvest age of 89.66 DAP, which was significantly different from the harvest age of genotypes M.1.1.17, M.5.2.3 and M.1.1.8. The earliest harvesting age was found in genotype Anjasmoro with a harvest age 82.83 DAP, which was not significantly different from the harvest age of M.1.1.3, M.5.2.1 and M.1.1.9 with harvesting ages 83.89, 83.72 and 83.05 DAP, respectively. Compared with the previous generation, the genotypes of the M.1.1.3, M.5.2.1, and M.1.1.9 mutant lines showed an early harvesting age. These mutants can be called solid mutants for harvesting age because they provide stability in the character of early harvest in several generations of planting.

The mutant genotypes, namely M.1.1.3, M.5.2.1, and M.1.1.9, had an average harvest age of 83 DAP, so they were categorized as early-maturity soybeans (78-85 days). The Previous research to obtain early maturity soybeans using gamma-ray irradiation has also been carried out by (Puspitasari et al. 2021) on Argomulyo soybean with a radiation dose of 250 Gray. As a result, they get the harvest time of the mutant line was more early (70 days) than the control (>80 days). On the other hand, the GEE-5 mutant line showed the shortest harvest time of 66.79 days. The mutant line flowering days also showed a faster flowering time (31-32 days), while the control variety was around 35 days. The highest average seed yield was found in mutant lines GBB-2 and GEE-5 compared to control varieties and other mutant lines because this mutant had a higher number of filled pods than other genotypes. However, the seed size of the mutant line was smaller, only 10-11 g, compared to the 100 seed weight of the control varieties, which was 13-16 g.

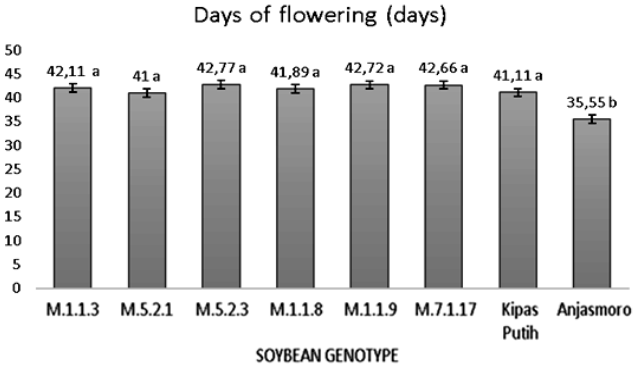

Figure 2. The effect of several soybean genotypes on the characters of days of flowering. Note: The numbers followed by the same letter in the same bar are not significantly different according to the 5% LSD test

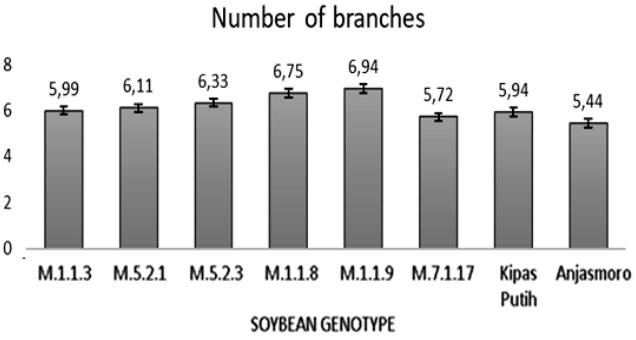

Figure 3. The effect of several soybean genotypes on the characters' number of branches. Note: The numbers followed by the same letter in the same bar are not significantly different according to the 5% LSD test

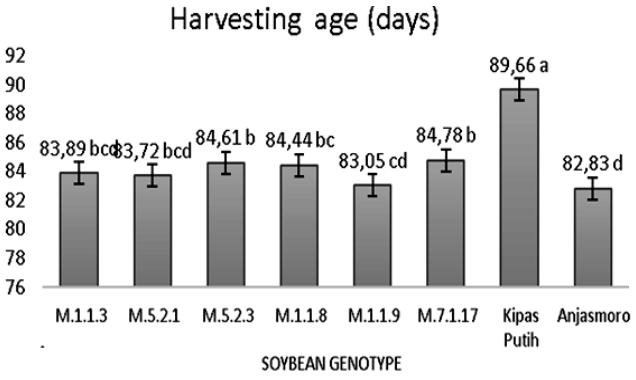

Figure 4. The effect of several soybean genotypes on the characters of harvesting age. Note: The numbers followed by the same letter in the same bar are not significantly different according to the 5% LSD test

The preference for early-maturity soybeans was higher than for late-maturity soybeans because it could increase the cropping index. It can also avoid crop failure due to drought stress that shortens pod filling mass. The length of time for pod filling is the most critical and vulnerable period in the event of a long dry season. Rahajeng and Adie (2013) said that early-maturity soybean varieties could be a solution for farmers to deal with climate change. Early maturing varieties are in great demand because they can provide various benefits, such as eliminating yield losses due to drought and pest infestations and increasing cropping in a year.

The number of the pod and 100-seed weight

The analysis of variance showed a very significant effect on the character of the number of pods and the weight of 100 seeds of several M_6 genotypes and their two comparison varieties. The results of further tests on the average number of pods and weight of 100 seeds on several M_6 genotypes and their two comparison varieties can be seen in Figures 5 dan 6.

Figure 5 shows that the highest number of pods was found in the Kipas Putih variety, which amounted to 239.89 pods, which was not significantly different from M.1.1.8 mutant line with 217.33 pods. On the other hand, the lowest number of pods was obtained in M.1.1.3 mutant line with only 97.05 pods, which was not significantly different from M.1.1.9, M.1.1.17 lines, and the comparison variety Anjasmoro.

Only one putative mutant genotype had the same number of pods as its parents, namely the M.1.1.8 genotype. There was no genotype whose number of pods exceeded the number of parent pods. Genotype M.1.1.8 in the previous generation test (M_5) had the highest number of pods compared to other genotypes, with an average of 500 pods (twice the number of parent pods). In the planting of the M_6 generation, there was a decrease in the average number of pithy pods, probably due to the different locations and planting seasons.

The largest weight of 100 seeds was found in the Anjasmoro variety at 17.34 g/100 seeds (Figure 6). The smallest 100-seed weight was found in the M.1.1.3 mutant line with 100 seed weight of 11.79 g, which was not significantly different from the Kipas Putih variety, the M.1.1.9 and M.1.1.8 mutant lines, which weighted 100 seeds, respectively 11.68 g, 11.82 g, and 12.55 g. The mutant line that increased seed size compared to its parents in the M_6 generation was the M.5.2.3 mutant line, which increased by 2 g/100 seeds. This mutant line on the previous M_5 generation planting showed a small seed size of only 10.21 g/100 seeds, which indicates that differences influence the increased seed size in the planting environment.

Soybean seed size was divided into three groups, namely small seed size (<10 g/100 seeds), medium seed size (10-14 g/100 seeds), and seed size >14 g/100 seed as large seed size (Krisnawati and Adie 2015). Based on these criteria, the putative mutant lines in this study had small seed sizes, and medium-sized seeds were not different from their parents, Kipas Putih. At the same time, the comparison variety is Anjasmoro, a large-seed soybean variety with a weight of 100 seeds 17 g, which follows the variety's description.

Seed weight/plant, seed weight/plot, and production tons.ha^{-1}

The results of the analysis of variance showed that there was a significant to a very significant effect on the character of seed weight/plant, seed weight/plot, and production of several M_6 genotypes and their two comparison varieties. Further tests on the average seed weight/plant, seed weight/plot, and production/ha on several M_6 genotypes and their two comparison varieties can be seen in Figures 7, 8, and 9.

Figure 7 dan 9 shows that the highest seed weight per plant and production per hectare was found in the Kipas Putih variety, with a seed weight of 42.25 g and production of 4.75 tons/h, which was not significantly different from the seed weight of M.1.1.8 mutant line, which was 38.84 g/plant and production of 4.37 tons.ha^{-1}. On the other hand, the lowest seed weight and production were found in M.1.1.3 mutant line with a seed weight of only 18.25 g and production of 2.05 tons.ha^{-1}, which were not significantly different from M.7.1.17 and M.1.1.9. In the M.1.1.9 mutant line, the low seed weight per plant was because many pods were contained, but the seeds produced were not good, such as the small and shriveled seeds' shape.

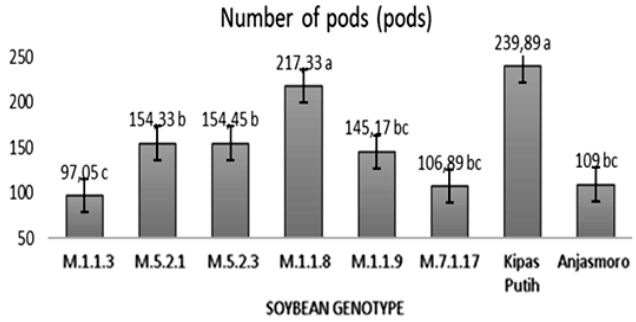

Figure 5. The effect of several soybean genotypes on the characters' number of pods and the weight of 100 seeds. Note: The numbers followed by the same letter in the same bar are not significantly different according to the 5% LSD test

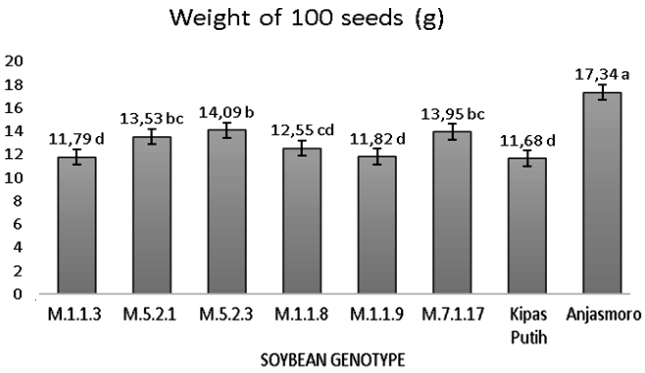

Figure 6. The effect of several soybean genotypes on the weight of 100 seeds characters. Note: The numbers followed by the same letter in the same bar are not significantly different according to the 5% LSD test

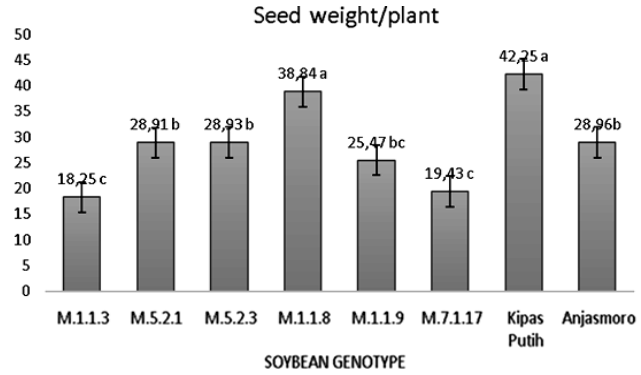

Figure 7. The effect of several soybean genotypes on seed weight/plant characters. Note: The numbers followed by the same letter in the same bar are not significantly different according to the 5% LSD test

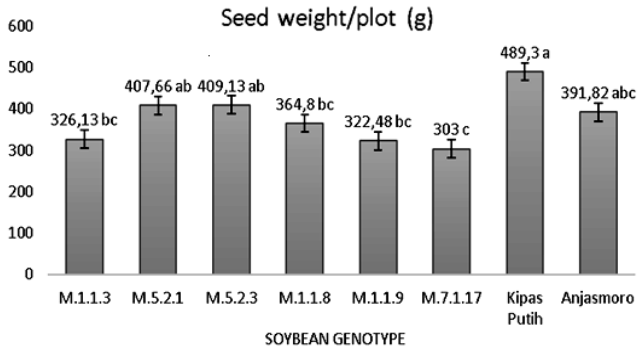

Figure 8. The effect of several soybean genotypes on seed weight/plot characters. Note: The numbers followed by the same letter in the same bar are not significantly different according to the 5% LSD test

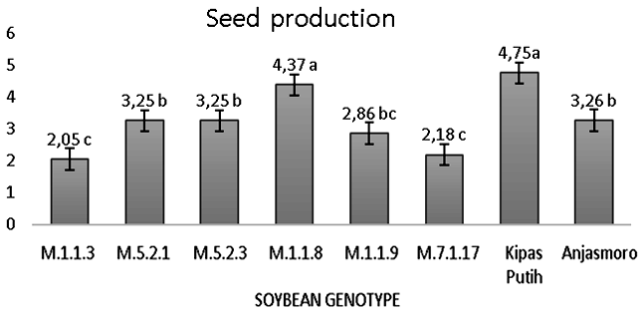

Figure 9. The effect of several soybean genotypes on production tons.ha^{-1}. Note: The numbers followed by the same letter in the same bar are not significantly different according to the 5% LSD test

Late-maturing soybeans (Kipas Putih variety) yield more seeds than early-maturing ones because their vegetative growth and reproductive periods are longer. Therefore, developing an early-maturing line without decreasing seed yield has not been easy. However, the present study showed that M.1.1.8 mutant had a significantly earlier maturing time than Kipas Putih (Figure 2), and M.1.1.8 had a seed yield similar to Kipas Putih (Figure 9). Therefore, we have successfully found an early-maturing mutant line without decreasing yield.

For measurement data of seed weight/plot (Figure 8), it can be seen that the highest seed weight/plot was found in the Kipas Putih variety, namely 489.30 g/plot, which was not significantly different from M.5.2.3, M.5.2.1 and M.1.1.8. On the other hand, the lowest seed weight per plot was found in the M.7.1.17 mutant line, which was 303 g/plot. There was a decrease in the seed weight/plots of the M.1.1.8 mutant line because the number of plants/plots that managed to grow in this line was only 50% of the total number of plants. The total 24 plants/plot managed to grow only 12 plants per plot, resulting in a decrease in seed weight/plot compared to seed/plant weight data.

These results are in line with (Asadi and Dewi 2020). They obtained soybean mutants resulting from gamma irradiation combined with crossbreeding. As a result, there were nine mutant soybean lines with better performance and 15-26% higher yield than the Panderman variety and 27-31% higher than the Anjasmoro variety. The nine selected lines will be tested for further adaptability in different locations and environments in the next generation.

Several mutant soybeans were also obtained through gamma-ray irradiation of the Panderman variety. The results on the M_5 generation of 10 tested mutants show two mutant lines, i.e., Kdl3 and Kdl8, that perform better than the other mutant lines under drought stress conditions. These mutant lines required 30.75 to 32 days to flower and 79.75 to 83.75 days to harvest. They also have relatively short plant heights of 28.25 and 23.35 cm, respectively (Yuliasti and Reflinur 2017).

In this study, sterile mutants were still found in M.1.1.3 mutant lines population. There was only one full sterility plant from the entire cultivated population, as shown in Figure 10. When entering the flowering phase, this plant shows the appearance of several flower candidates, but in 2 days they fall, so the flower candidates appear can not grow. In addition, this plant shows differences in growth when it reaches harvest age; the leaves turn yellow more quickly, and the leaves wither and fall off over time. Several other abnormal characteristics in full sterility plants were that the number of branches and leaves was less when compared to other plants.

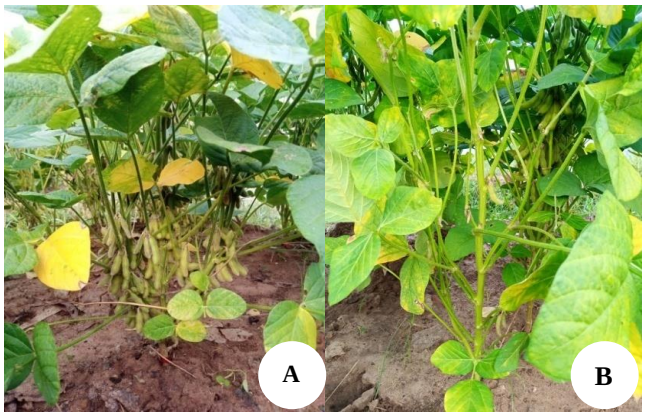

Figure 10. A. Fertile mutant lines, B. Sterile mutant lines

In a previous study, Nilahayati et al. (2016) also reported that full sterility plants showed normal vegetative growth but failed to produce flowers. Kumar and Rai (2006) investigated the study on soybean using gamma-ray irradiation and found some sterile mutants. The mutant cytology test showed the occurrence of desynapsis in the cells. In sterile plants, only slightly bivalent, while the univalent frequency is very high, which causes a very high percentage of pollen sterility. The later stages of meiosis are also severely disrupted. Plants were identified as male sterile plants because only a few pods were formed. They concluded that gamma rays may have acted on some of the genes responsible for forming synapses and chiasms at the stage of gamete cell division.

In conclusion, the genotype treatment affected all observed characters except for the number of branches and empty pods. The putative mutant line with a high yield and equivalent to its parents is the M.1.1.8 line, with a production of 4.37 tons/ha. The putative mutant lines with early maturity were M.1.1.9, M.5.2.1, and M.1.1.3 lines with a harvesting age of 83 DAP.

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Antioxidant potency of n-butanol fraction of *Ficus glumosa* leaves against oxidative stress induced by carbon tetrachloride in the kidneys of rats

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Abstract. *Abu MS, Yakubu OE, Onuche JI, Tatah SV. 2022. Antioxidant potency of n-butanol fraction on* Ficus glumosa *leaves against oxidative stress induced by carbon tetrachloride in the kidneys of rats. Nusantara Bioscience 14: 40-46.* The kidneys play several essential roles in the body, including regulating the water and ions reabsorb from glomerular filtrate in kidney tubules. It is controlled by several hormones such as antidiuretic hormone (ADH), aldosterone, and angiotensin II. This report presented the repairing effect of the n-butanol fraction of *Ficus glumosa* Delile on nephrotoxicity induced by CCl_4 in rats. Rats were divided into 7 groups with 5 animals each. Groups 1 and 2 were used as normal and vehicle controls, respectively. Group 3 was induced but treated with neither extract nor standard drug. However, Groups 3, 4, and 5 were induced by CCl_4 and administered with varying doses of the n-butanol fraction. Group 6 was induced by CCl_4 and treated with a standard antioxidant drug. The results showed that treatments with an n-butanol fraction of *F. glumosa* leave and silymarin significantly ($p<0.05$) restored the activities of SOD, GPx, and CAT comparable to normal values, i.e., 2.10±0.07 U/L, 43.8±2.49 U/L, and 34.2±2.59 U/L, respectively. In addition, the treatments reduce the MDA level in the kidney of rats treated with an n-butanol fraction comparable to 0.34±0.05 mmol/L in the normal rats. Similarly, there was a significant ($p<0.05$) reduction of urea from 5.24 mg/dL to 3.5 mg/dL (standard value) in the treated groups. Creatinine significantly ($p<0.05$) reduced from 71.2 mg/dL in the treated groups to 43.3 mg/dL in the normal group. Electrolytes, Na^+, K^+, and Cl^- levels in the CCl_4-induced rats after administration of n-butanol fraction of *F. glumosa* leaves significantly ($p<0.05$) decreased to 137.8±2.59 mmol/L, 3.98±0.54 mmol/L, and 92.8±1.92 mmol/L respectively. The significant reversal of the rats' biochemical parameters can be ascribed to the ameliorative potency of the n-butanol fraction on methanol extract of *F. glumosa* leaves on the glomerular and tubular cells, which may have improved renal function in the injured kidney.

Keywords: Antioxidant, carbon tetrachloride, *Ficus glumosa*, kidney, n-butanol fraction extract, oxidative stress

INTRODUCTION

The kidneys are organs located in the retroperitoneal space at the back of the abdominal cavity with a bean-shaped. These organs are essential to the urinary system and serve homeostatic functions such as electrolyte regulation, acid-base balance maintenance, and blood pressure regulation via salt and water balance (Vasudevan et al. 2011; Biff 2015). The kidneys also function as a natural blood filter, removing water-soluble wastes such as urea and ammonium, which are then excreted in the urine (David et al. 2015). In addition, it reabsorbs water, glucose, and amino acids into circulation and produces hormones (such as calcitriol and erythropoietin) and a key enzyme (renin) that acts in negative feedback (Le 2013). Renal failure interferes with these essential functions resulting in inefficient removal of waste products and an imbalance in osmotic pressure. It can occur quickly (acute renal failure) or gradually (acute kidney injury), often as the result of ischemia, toxins, or mechanical trauma (Setyaningsih et al. 2006; David et al. 2015).

Ficus glumosa Delile is a small to medium-sized tree with a height of 5-10 m and 2 m in diameter (Umar et al. 2013). However, it can grow into a huge tree at 2 m and 24 m in height. The branches are widely spreading, more or less horizontal, and are frequently supported by stilt roots (Alfred 2014). The bark is yellow, grey, or green-grey, smooth to somewhat rough with a few flaking pieces; the slash is reddish with white streaks; the branchlets are twiggy, finely hairy, with substantial leaf scars (Orwa et al. 2009). Bioactivities of leaf, stem bark, and gum extracts of *F. glumosa* have been previously investigated for antioxidant, anti-diabetic, anti-diarrheal, diuretic, hematological, hypolipidaemic, and toxicological effects (Mutungi et al. 2021). This study investigated the ameliorative effects of leaf extract on nephrotoxicity and oxidative stress induced by CCl_4 in albino rats.

MATERIALS AND METHODS

Fractionation of crude methanol extract of *F. glumosa* leaves

In this study were used crude methanol extract (20 g) was re-dissolved in 300 mL of distilled water. It is partitioned in a separating funnel with 400 mL of n-hexane

repeatedly with vigorous shaking. Then the mixture was allowed to stand for 30 minutes to separate into distinct hexane and aqueous layers. Next, the n-hexane fraction was collected and concentrated using a water bath. Next, to obtain the ethyl acetate fraction, the aqueous layer was repeatedly partitioned on 400 mL of ethyl acetate. Then, the aqueous layer was saturated with distilled water and repeatedly partitioned on 400 mL of n-butanol, after which the n-butanol fraction and aqueous residue were obtained. Finally, the fractions were concentrated using a water bath maintained at 45°C to obtain concentrated fractions. The concentrated fractions were kept in sealed containers and refrigerated for further use at 2- 4 °C.

Experimental design to assess the ameliorative effect of n-butanol fraction of *F. glumosa* leaves extract on CCl_4-induced kidney toxicity and oxidative stress

Animal grouping

In this study, a total of 35 albino rats were used, which were divided into 7 groups of 5 animals each:

Group 1: Normal control rats received feed and tap water only. It served as the normal control group.

Group 2: Rats were treated with 1 mL olive oil /kg body weight and served as the vehicle control group.

Group 3: In olive oil, rats were treated with 1 mL/kg body weight 50% Carbon tetrachloride (CCl4). It served as the CCl_4 control group without extract or standard drug treatment.

Group 4: Rats were treated with 1 mL/kg body weight 50% CCl_4 in olive oil + 100 mg/kg body weight/day n-butanol fraction.

Group 5: Rats were treated with 1 mL/kg body weight 50% CCl_4 in olive oil + 300 mg/kg body weight/day of the n-butanol fraction.

Group 6: Rats were treated with 1 mL/kg body weight 50% CCl_4 in olive oil + 500 mg/kg body weight/day of the n-butanol fraction.

Group 7: Rats were treated with 1 m/kg body weight 50% CCl_4 in olive oil + 100 mg/kg body weight/day of silymarin as a standard drug reference.

Ethical clearance and animal testing regulations

All animal ethical protocols were obtained and followed the animal testing regulations at Ahmadu Bello University Zaria, Zaria, Nigeria.

Induction of nephrotoxicity and oxidative stress using CCl_4 and treatment of the n-butanol fraction of F. glumosa *leaves*

On the 1st day, the experimental animals were pre-treated with 1 mL/kg body weight of 50% solution of CCl_4 in olive oil (IP). Then followed by oral administration of the CCl_4 extract intoxication after 24 hours. Then, the administration of the n-butanol fraction was continued for 21 days and challenged with 1 mL/kg body weight of 50% solution of CCl_4 once weekly. Finally, the animals were fasted for 24 hours after the last administration of the extract to be sacrificed at the end of the experiment for sample collection and analyzed subsequently (Akram et al. 2010).

Collection and preparation of animal samples

Collection and preparation of sera samples

The animals were anesthetized using chloroform and sacrificed by decapitation at the end of 21 of treatment. Then, for biochemical analysis, blood samples were collected from the throat in plain and EDTA bottles for hematological analysis. First, blood samples in plain tubes were allowed to clot; after that, then the sera were separated using Labofuge 300 centrifuge (Heraeus) at 3,000 rpm for 10 minutes. Then, the collected sera were subjected to biochemical analysis.

Collection of kidneys for homogenization

Immediately after the blood was collected, kidneys were quickly excised, then trimmed of connective tissues and rinsed with physiological saline to eliminate blood contamination, dried with filter paper by blotting, and then weighed (to calculate the relative weight) were kept on ice. Next, one gram of each kidney was randomly homogenized in 10 mL of buffer (50 mM potassium phosphate buffer, pH 7.4) using a pestle and mortar. Next, the rest of the organs for histopathological studies were placed in freshly prepared 10% formalin. Finally, the homogenate was centrifuged at 4,000 rpm (2,700 x g) for 15 minutes, and the supernatant was collected using a Pasteur pipette.

Toxicological studies on renal function of experimental animals

Determination of serum urea concentration

Serum urea concentration was assessed using the method described by (Fawcett and Scout 1960).

Principle**:** Urease breaks down urea into carbon dioxide and ammonia. Ammonia reacts with salicylate and hypochlorite to form a colored compound, dicarboxy indophenol, in an alkaline medium. The reaction is catalyzed by sodium nitroprusside. The intensity of color produced is measured spectrophotometrically at 578 nm.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{hypochlorite} + \text{salicylate} \longrightarrow \text{dicarboxyindophenols (blue compound)}$$

Procedure: Reagent (1 mL) containing sodium nitroprusside and urease was added into three clean test tubes that have been contained 0.01 mL sample, 0.01 mL standard reagent, and 0.01 mL distilled water and labeled as a test sample, standard, and reagent blank, respectively and mixed. Test tubes were incubated at room temperature (25-30°C) for 10 minutes. The absorbance of the test sample and standard were read against the reagent blank at 578 nm.

The serum urea concentration was calculated using the formula below:

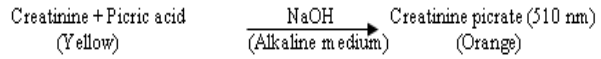
$$\text{Urea Conc.} \left(\frac{\text{mg}}{\text{dl}}\right) = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of Standard}$$

Blood Urea Nitrogen Concentration (mg/dL) = 0.467 x Urea Concentration (mg/dL)

Determination of serum creatinine concentration

The colorimetric method was used to determine serum creatinine concentration, according to Bertels and Bohmer (1973).

Principle: Creatinine in the serum reacts with alkaline picrate to form a colored complex. The rate formation of the colored complex is directly proportional to creatinine concentration. This reaction rate reaction (intensity of orange color produced) is measured colorimetrically at 510 nm and is compared to the standard.



Procedure: A working reagent (1 mL) containing picric acid and sodium hydroxide was added into two clean test tubes. The test tubes contained 0.1 mL of the test sample and 0.1 mL of standard solution and were labeled as test samples and standard and mixed thoroughly. After 20 seconds, the standard (ST1) and test sample (TS1) absorbance were read at 510 nm. Precisely 80 seconds later, absorbance for (ST2) and (TS2) of the standard and sample were read at 510 nm against distilled water (blank).

The concentration of creatinine in serum (mg/dL) was calculated using the formula below:

$$\text{Creatinine Conc.}\,(\text{mg/dl}) = \frac{TS2 - TS1}{ST2 - ST1}\ x\ \text{Concentration of Standard}$$

(ST= Standard, TS= Test Sample)

Estimation of serum sodium, potassium, and chloride ions

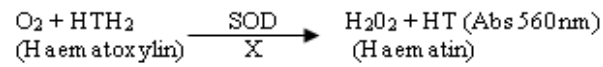
A flame photometer Model 143, equipped with an automatic diluter Model 144 (dilution ratio of 200:1) (Instrumentation Laboratory, Inc., Lexington, Mass., U.S.A.), was used. The flame photometer was calibrated using distilled water twice and a standard with a Na^+ concentration of 140 mequiv/L and a K^+ concentration of 5 mequiv/L (Instrumentation Laboratory, Inc., Lexington, Mass., U.S.A). In addition, the instrument's stability was checked with the standard solution after each sample measurement.

Determination of in vivo antioxidant status in the kidney of experimental animals

Estimation of Superoxide Dismutase (SOD) activity

Superoxide dismutase activity was measured using the method described by (Martin et al. 1987).

Principle: Auto-oxidation of hematoxylin (by increasing absorbance at 560 nm) is inhibited by SOD activity at pH 7.8. The percentage of inhibition is linearly proportional to the amount of SOD present within a specific range. SOD activity in the sample was determined by measuring the amount of haematin formed at 560 nm.



Procedure: Phosphate buffer 0.05 M, pH 7.8) (920 µL) was added to a clean test tube, followed by the addition of 40 µL of the sample (tissue homogenate), which was labeled as the test sample. A reagent test (blank/ without sample) was also prepared by adding 40 µL of assay buffer (phosphate buffer 0.05 M, pH 7.8) to another clean test tube. The mixtures were shaken and incubated for 2 minutes at room temperature. Also, 40 µL of hematoxylin was added to both sample and reagent test tubes (blank) and mixed quickly to start the auto-oxidation reaction. Following the addition of 40 µL of hematoxylin, the absorbance of the sample and reagent test (blank) was read at 560 nm every 30 seconds for 5 minutes against distilled water.

SOD activity was determined by measuring the ratios of auto-oxidation rates in the presence and absence of the sample. SOD activity in the sample was calculated as follows:

$$\text{Absorbance}_{\text{Reagent test}}\,(A_R) = \text{Absorbance}_{\text{Reagent test 2}} - \text{Absorbance}_{\text{Reagent test 1}}$$

$$\text{Absorbance}_{\text{sample test}}\,(A_S) = \text{Absorbance}_{\text{sample test 2}} - \text{Absorbance}_{\text{sample test 1}}$$

$$\%\ \text{SOD inhibition} = (1 - A_S / A_R) \times 100$$

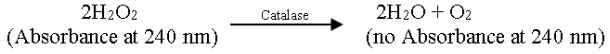
$$\text{SOD activity (U/ml)} = (1 - A_S / A_R) \times 100 \times 1.25$$

One unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the auto-oxidation of hematoxylin to haematin in 1 minute.

Estimation of Catalase (CAT) activity

Catalase activity determination follows the method described by (Aebi 1983).

Principle: Catalase scavenges hydrogen peroxide (H_2O_2) and converts it to water and molecular oxygen.



The catalase activity in the sample was determined by observing the rate of decrease in absorbance at 240 nm.

Procedure**:** Working buffer (50 mM potassium phosphate buffer, pH 7.0, 1000 µL) was added to a cuvette and used to standardize the spectrophotometer at a wavelength of 240 nm. Also, 950 µL of the mixture of working buffer (490 µL of 50 mM potassium phosphate buffer, pH 7.0) and 460 µL of 30 mM hydrogen peroxide (H_2O_2), and 50 µL of a sample (tissue homogenate) were pipetted to another clean cuvette, mixed quickly. A catalase standard was prepared by adding 50 µL of diluted catalase standard to 950 µL of working assay buffer. The decomposition rate of H_2O_2 was measured at 240 nm every 1 minute for 5 minutes. Catalase activity was determined and expressed as (U/mL) of the sample's decomposition rate given as (ΔA240nm/min).

ΔA240nm/min = Change in absorbance per minute.

$$\text{Catalase (U/ml)} = \frac{(\Delta A240\text{nm/min})}{\text{The volume of the reaction mixture}}$$

Estimation of Glutathione Peroxidase (GPx) activity

Glutathione Peroxidase Assay is a modified method of Paglia and Valentine (1967). Principle**:** Glutathione Peroxidase catalyzes the reduction of hydrogen peroxide

(H_2O_2), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH), forming $NADP^+$. As a result, it decreases absorbance (340 nm) and recycles the GSH. Since Glutathione Peroxidase is the reaction rate-limiting enzyme, the decrease in absorbance at 340 nm is directly proportional to the Glutathione Peroxidase activity.

Procedure: All reagents were prepared and used at room temperature, and samples (tissue homogenate) were placed on ice. The NADPH reagent (β-nicotinamide adenine dinucleotide phosphate and GSH reduced) was reconstituted with NADPH diluent (glutathione reductase in buffer with stabilizer and 4mM NaN_3) and was labeled as working NADPH. Next, 50 µL of the sample was added to a clean test tube, followed by 50 µL working NADPH. Also, 50 µL of working H_2O_2 (0.3 mL of 3% H_2O_2 diluted to 10 mL with assay buffer) was added to the sample test tube and equilibrated for 1 minute. Next, the blank tube was prepared by replacing the sample with 50 µL of distilled water. The mixtures in both tubes were transferred to cuvettes, and absorbance was read at 340 nm for 5 minutes with 30 seconds recording intervals against the blank sample.

Glutathione Peroxidase activity was calculated from the net rate and expressed as (U/mL).

$$GPx = \frac{2\,(mRates - mRateb)\,150}{6.22 \times 50}$$

Where;

$mRate_s$ = 1000 x ΔA_{340}/min of sample

$mRate_b$= 1000 x ΔA_{340}/min of blank

6.22 = NADPH 340 nm millimolar absorption coefficient at 1 cm path length.

150 µL = volume of the reaction mixture

50 µL = volume of sample

2= Correction factor for 2 moles GSH oxidized to 1 mole GSSG per mole NADPH oxidized.

Estimation of Thiobarbituric Acid Reactive Substance (TBARS)

A thiobarbituric acid reactive substance (TBARS) in the tissues was estimated using the method described by (Fraga et al. 1988).

Principle: Malondialdehyde formation is the basis for the TBA method used for the extent of lipid peroxidation evaluation. At low pH of 2-3 and high temperature (60^0C), Malondialdehyde (MDA) binds thiobarbituric acid (TBA) to form a pink complex (MDA-TBA) adduct, which absorbs maximally at 532 nm.

Procedure: Tissue homogenate (sample) (250 µL), 10 µL of BHT reagent (butylated hydroxytoluene in ethanol), 250 µL acid reagent (1 M phosphoric acid), and 250 µL of TBA reagent (2-thiobarbituric acid reconstituted with 10.5 ml distilled water) were added to a clean sample centrifuge tubes, mixed vigorously. A blank test was prepared by replacing the sample with 250 µL of distilled water. Then, both tubes were incubated for 60 minutes in a water bath at 60^0C. It was then cooled and centrifuged at 10,000 x g for 3 minutes. Finally, the reaction mixture in both tubes was transferred to cuvettes, and the absorbance was read at 532 nm for 5 minutes against a blank sample.

The concentration of TBARS is expressed in Malondialdehyde (MDA) equivalent (µM).

Molar extinction of MDA = 1.56 x 10^5 $M^{-1}cm^{-1}$

MDA concentration = Absorbance / 1.56 x 10^5 $M^{-1}cm^{-1}$

RESULTS AND DISCUSSIONS

Effect of n-butanol fraction of *F. glumosa* on oxidative stress and lipid peroxidation in the kidney

The effect of daily oral administration of the n-butanol fraction of *F. glumosa* for 21 days on the levels of some endogenous antioxidant enzymes (catalase, glutathione peroxidase, and superoxide dismutase) and Malondialdehyde (MDA) in the kidney of CCl_4-induced rats are represented in Table 1 showing that the Malondialdehyde (MDA) level was significantly ($p<0.05$) increased. The level of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) of the CCl_4-induced control group were significantly decreased ($p<0.05$) compared to the induced rat but treated with n-butanol fraction groups. However, compared with the normal control group, there was no significant ($p>0.05$) difference in the levels of MDA and endogenous antioxidant enzymes of the induced but treated groups.

Effect of n-butanol fraction of methanolic leaf extract of *F. glumosa* on kidney biomarkers

Figure 2 depicts the urea concentrations in the serum of normal and CCl4-induced rats after the oral administration of the n-butanol fraction of *F. glumosa* for 21 days. The result showed that urea concentration in the serum of CCl_4-induced rats but not treated with the n-butanol fraction of *F. glumosa* leaves was significantly ($p<0.05$) higher than the induced but treated with n-butanol fraction groups.

Creatinine concentrations in the serum of normal and CCl_4-induced rats after oral administration of n-butanol fraction of *F. glumosa* for 21 days are represented in Figure 3. The result showed that the creatinine concentration in the serum of CCl4-induced rats but not treated with n- butanol fraction was significantly ($p<0.05$) higher than the induced but treated with n-butanol fraction and the normal control groups.

Table 2 shows the concentrations of electrolytes (sodium ion Na^+, potassium ion K^+, and chlorine ion CL^-) in the serum of the experimental albino rats after oral administration of the n-butanol fraction of *F. glumosa* for 21 days. The result showed a significant ($p<0.05$) increase in the concentrations of these ions in the serum of the CCl_4-induced but not treated with n-butanol fraction group compared to the normal and induced but treated with n-butanol fraction groups.

Table 1. MDA, SOD, CAT, and GPx concentrations of CCl_4-induced albino rats treated orally with an n-butanol fraction of *F. glumosa* leaves

Group	MDA (mmol/L)	SOD (U/L)	CAT (U/L)	GPx (U/L)
Normal control	0.34 ± 0.05^{b}	2.10 ± 0.07^{b}	43.80 ± 2.49^{b}	34.20 ± 2.59^{b}
Olive oil only	0.36 ± 0.05^{b}	2.04 ± 0.15^{b}	42.00 ± 1.58^{b}	28.60 ± 6.22^{b}
CCl_4 only	0.84 ± 0.05^{ac}	1.68 ± 0.04^{ac}	17.00 ± 2.00^{ac}	15.20 ± 0.04^{ac}
CCl_4 + 100mg Extract	0.78 ± 0.05^{ac}	1.82 ± 0.08^{bc}	32.40 ± 2.88^{ab}	26.60 ± 4.93^{b}
CCl_4 + 300mg Extract	0.44 ± 0.08^{b}	2.02 ± 0.12^{b}	33.00 ± 0.71^{b}	27.40 ± 3.21^{b}
CCl_4 + 500mg Extract	0.36 ± 0.05^{b}	2.00 ± 0.10^{b}	40.60 ± 4.39^{b}	30.40 ± 3.21^{b}
CCl_4 + 100mg sylimarin	0.36 ± 0.06^{b}	2.10 ± 0.07^{b}	42.40 ± 3.78^{b}	27.05 ± 6.40^{b}

Note: n= 5; the values are in mean±standard deviation; the values with different superscripts down the columns are significantly different at $p<0.05$; a= significantly different from the normal control group ($p<0.05$); b= significantly different from the group treated with CCl_4 without extract or the standard drug treatment ($p<0.05$); c= significantly different from the group treated with CCl_4 and the standard drug treatment ($p<0.05$); MDA: Malondialdehyde, SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione peroxidase

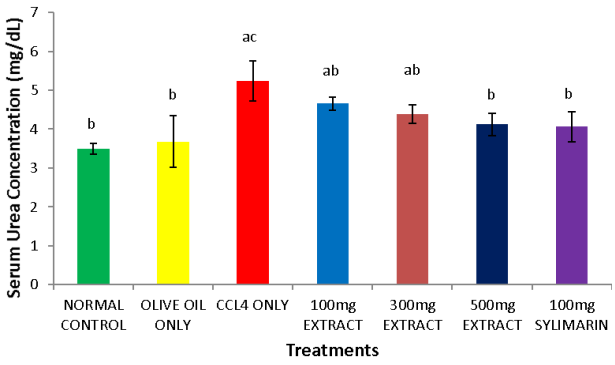


Figure 2. Serum urea levels of CCl_4-induced albino rats treated orally with an n-butanol fraction of *F. glumosa* leaves. Note: n= 5; the values are in mean±standard deviation; values with different superscripts across the bars are significantly different at $p<0.05$; a= significantly different from the normal control group ($p<0.05$); b= significantly different from the group treated with CCl_4 without extract or the standard drug treatment ($p<0.05$); c= significantly different from the group treated with CCl_4 and the standard drug treatment ($p<0.05$)

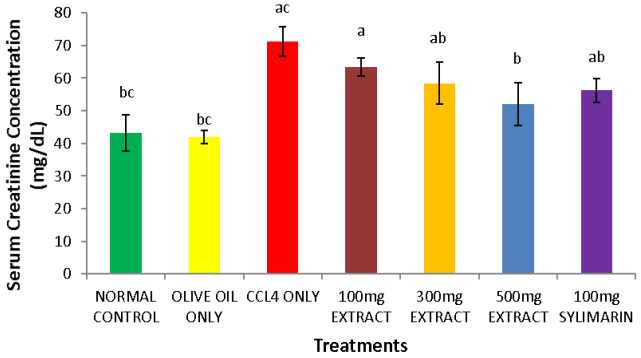


Figure 3. Serum creatinine concentration of CCl_4-induced albino rats treated orally with an n-butanol fraction of *F. glumosa* leaves. Note: n=5; the values are in mean±standard deviation; the values with different superscripts across the bars are significantly different at $p<0.05$; a= significantly different from the normal control group ($p<0.05$); b= significantly different from the group treated with CCl_4 without extract or the standard drug treatment ($p<0.05$); c= significantly different from the group treated with CCl_4 and the standard drug treatment ($p<0.05$)

Table 2. Sodium ion (Na^+), potassium ion (K^+), and chlorine (Cl^-) concentrations in the serum of CCl_4-induced albino rats treated orally with an n-butanol fraction of *F. glumosa*

Group	Na+ (mmol/L)	K+ (mmol/L)	Cl- (mmol/L)
Normal control	137.80 ± 2.59^{b}	3.98 ± 0.54^{b}	92.80 ± 1.92^{b}
Olive oil only	132.20 ± 2.88^{b}	4.24 ± 0.36^{b}	97.40 ± 2.07^{b}
CCL4 only	156.60 ± 4.51^{ac}	6.32 ± 0.41^{ac}	114.00 ± 7.91^{ac}
CCL4 + 100mg Extract	139.80 ± 2.28^{b}	4.26 ± 0.18^{b}	100.00 ± 2.92^{b}
CCL4 + 300mg Extract	132.00 ± 2.12^{b}	4.28 ± 0.18^{b}	98.60 ± 3.05^{b}
CCL4 + 500mg Extract	138.80 ± 2.77^{b}	4.30 ± 0.29^{b}	99.40 ± 2.97^{b}
CCL4 + 100mg sylimarin	137.00 ± 9.12^{b}	4.46 ± 0.23^{b}	97.80 ± 1.48^{b}

Note: n= 5; values are in mean±standard deviation; values with different superscripts down the columns are significantly different at $p<0.05$; a= significantly different from the normal control group ($p<0.05$); b= significantly different from the group treated with CCl_4 without extract or standard drug treatment ($p<0.05$); c= significantly different from the group treated with CCl_4 w and standard drug treatment ($p<0.05$)

Discussion

The levels of thiobarbituric acid reactive substances such as malondialdehyde and endogenous antioxidant enzymes are sensitive indices in free radical-induced hepatocellular damage (Meghri et al. 2019). The endogenous antioxidant enzymes are superoxide dismutase, glutathione peroxidase, and catalase. Malondialdehyde (MDA) increased significantly ($p<0.05$) in the kidney tissues of CCl_4-induced but not treated rats may be a result of increasing membrane lipid peroxidation caused by free radicals generated by CCl_4 (Shah et al. 2017). It might also be caused by the failure of antioxidant defense mechanisms to reduce free radicals' excessive formation or detrimental effects (Shaban et al. 2021). SOD, GPx, and CAT decreased significantly ($p<0.05$) in the kidney tissues of CCl_4-induced but not treated rats. It may be due to a high concentration of free radicals generated by CCl_4.

On the other hand, it may lead to inactivation (adaptive response) or inhibition of the synthetic pathways of these endogenous antioxidant enzymes. Thereby it results in low turnover (Fahima et al. 2016). However, treatments with an n-butanol fraction of *F. glumosa* leaves and silymarin could restore SOD, GPx, and CAT activity levels to almost normal and reduce the MDA level. It follows the results of Momoh et al. (2015) that the administration of *Vernonia amygdalina* Delile aqueous leaf extracts exhibited similar actions against liver damage induced by acetaminophen, equally.

The effects of the n-butanol fraction were comparable to the standard drug (Silymarin), which equally increased the activity of the endogenous antioxidant enzymes. Therefore, the result could be attributed to the free radical scavenging activity of the n-butanol fraction of *F. glumosa* leaves due to antioxidant compounds such as phenolic acids, flavonoids, and ascorbic acid (Abu et al. 2020). Therefore, it may have exerted beneficial action against pathophysiological alterations caused by superoxide, hydroxide free radicals, and hydrogen peroxide and restored the antioxidant status in the cells (Saeed et al. 2012; Letiele et al. 2020).

Furthermore, administration of CCl_4 caused nephrotoxicity as indicated by significantly elevated ($p<0.05$) urea and creatinine levels and electrolytes (Na^+ K^+ and Cl^-) concentrations in the serum of the experimental animals. These results agree with previous findings by Venkatanarayana et al. (2012) and Yacout et al. (2012). Kidney toxicity caused a rapid reduction in renal performance due to a decrease in glomerular filtration rate (GFR) and a lack of kidney ability to excrete toxic metabolites produced in the body. In addition, it results in abnormal retention of renal biomarkers like blood urea nitrogen and creatinine (Kumar et al. 2013). This study shows that significant elevation in serum urea, creatinine, and electrolyte levels can be attributed to damaged nephron structural integrity (Khan and Siddique 2012). However, the significant reduction ($p<0.05$) in urea, creatinine, and electrolytes levels in the CCl_4-induced but treated groups may be due to the repairing ability of the n-butanol fraction of *F. glumosa* leaves in the glomerular and tubular cells. Therefore, applying the n-butanol fraction of *F. glumosa* leaves may have improved renal function in kidney disorders.

In conclusion, the significant reversal of the rats' biochemical parameters can be ascribed to the ameliorative potency of the n-butanol fraction of methanol extract of F. glumosa leaves on the glomerular and tubular cells improved renal function in the injured kidney. The outcome of this research implies that the n-butanol fraction of *F. glumosa* leaves extract can be purified to manage kidney disorders.

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Short Communication: Thromboplerous hyphae of the ectomycorrhizal mushroom *Rhizopogon roseolus* with and without a host tree

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Abstract. *Putra IP, Aimi, T, Shimomura N. 2022. Short Communication: Thromboplerous hyphae of the ectomycorrhizal mushroom* Rhizopogon roseolus *with and without a host tree. Nusantara Bioscience 14: 47-52.* Thromboplerous hyphae are modified hyphae found in the basidiocarp of many agarics. However, data on their development and function, as well as on their formation in pure cultures of ectomycorrhizal mushrooms, are scarce. The aim of the present study was to characterize the cytological descriptions of thromboplerous hyphae from pure cultures of *Rhizopogon roseolus* (Corda) Th.M. Fr. with or without the host pine tree *Pinus thunbergii* Parl. Thromboplerous hyphae formed on the mycelium and mycelial cords in all experimental settings. Results revealed thromboplerous hyphae were extremely melanized, smooth, cracked on the surface, and swollen at both hyphal termini. Thromboplerous hyphae produced with a host were mainly unbranched in shape in contrast to those formed without a host that possessed additional twisted, semi-twisted, and branched forms. Some thromboplerous hyphae that grew without a host had large diameters, and few showed notable septa and clamp connections. The present study also provides evidence of the initial development of thromboplerous hyphae via vegetative and tubular hyphae. This study contributes to understanding the cytology of thromboplerous hyphae grown with and without a host tree. Further investigations on the specific functions of thromboplerous hyphae are needed to deepen the current knowledge of fungal cytology.

Keywords: Cytology, host, pure cultures, *Rhizopogon roseolus*, thromboplerous hyphae

INTRODUCTION

Hyphae are the basic structure of many fungi. They constitute the mycelium of the higher fungi and form other structures (Yafetto 2018). They can be modified in many different structures to serve various functions (Kirk et al. 2008). One of the hyphae functions, which has never been studied, is the host impact on the oil production of hyphae in many fungi. In addition, there is sparse information regarding modified hyphae, such as secretory hyphae in Basidiomycota, ranging from saprophytic to ectomycorrhizal fungi. Secretory hyphae can be observed from mycelia, rhizomorphs, mycorrhiza structure, sclerotia, and basidiomes (Clémençon 2012). One of the types of secretory hyphae is the thromboplerous hyphae, which have been cytologically less observed over decades. In addition, the terminology of thromboplerous hyphae is not uniform and inconsistently used by mycologists.

Thromboplerous (Th) hyphae, previously known as oleiferous hyphae, are hyphae whose development and function remain obscure. There was a debate regarding the term between Th hyphae and oleiferous hyphae. The term "oleiferous hyphae" originates from French literature and pertains to a lipid-composed structure (Lentz 1954). However, Clémençon (2012) rejected this term since certain hyphae do not contain lipids and suggested using the term "thromboplerous hyphae" instead. Until now, Clémençon (2005) provided the only information on Th hyphae development from the saprophytic fungi. Th hyphae are easy to distinguish from undifferentiated hyphae due to their size and structure; th hyphae are melanized, refractive, contain a solid mass of homogenously dense material, and are devoid of nuclei. Furthermore, these structures' clogged, coagulated, and gelatinous consistency represents Th hyphae's "thrombo" characteristics.

Lentz (1954) reported that the oily structure of Th hyphae is found in agarics; previous studies by various authors confirmed the presence of Th hyphae in basidiocarp tissue (Smith and Zeller 1966; Vizzini and Ercole 2012; Vizzini et al. 2012; Moreau et al. 2013). However, the only comprehensive reports available on the possible mechanisms of development and function of Th hyphae are from rhizomorphs studies by Clémençon (2002; 2003; 2005). In addition, information on this particular hypha from pure cultures is scarce (Miller et al. 1983; Clémençon 2002; 2003).

Lentz (1954) argued that Th hyphae might play an essential but unknown physiological role in fungi. Currently, the only available cytological evidence on the possible functions of Th hyphae is from a study on saprophytic fungi by Clémençon (2005); therefore, there is

a need to gather data from other species and living styles of higher fungi, especially from laboratory cultures. As thromboplera are produced on saprobic and ectomycorrhiza (ECM) forming fungi, *Rhizopogon roseolus* (Corda) Th.M. Fr., an ECM fungus that is easy to isolate into pure cultures, was used in this study. Unfortunately, only Miller et al. (1983) have recently described Th hyphae from *R. roseolus* cultures, albeit with minimal information. In addition, to date, most cytological research on ECM fungi has focused only on the plant with less attention to the fungal structure (Leyva-Morales et al. 2019). Therefore, the aim of this study was to provide cytological information on Th hyphae from pure cultures of *R. roseolus* with and without the presence of the ectomycorrhizal host.

MATERIALS AND METHODS

Fungal material

Rhizopogon roseolus (= *R. rubescens* Tul. & C. Tul.) with strain number TUFC10010 was used in this study. The fungus was collected from the Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University, Japan. The fungus was prepared on 2% malt extract agar with a pH of 5 and incubated for 21 days at 25°C before being used.

Plant material

Seeds of *Pinus thunbergii* Parl. were rinsed overnight in water and then surface-sterilized using 30% hydrogen peroxide. After being washed with sterile distilled water, the seeds germinated at 25°C for 1-2 weeks in water agar. One fine seedling with well-developed lateral roots was then transferred to half size of a five-fold dilution of modified Melin and Norkrans (1/5 MMN) medium in (90×20) mm Petri dish. The vertical plate contained 30 mL of solid medium with half-removed agar to provide space for pine shoots. The Petri dish was sealed with 3M™ transpore surgical tape and incubated in a controlled laboratory chamber with the following conditions: 25°C temperature, 50% relative humidity, and a photoperiod of 16-hour day at 5000 Lx.

Th hyphae in the presence or absence of ECM host

A one-month-old pine seedling was inoculated with a 7-mm round mycelial plug of *R. roseolus*. Four plugs were placed near the lateral roots. One mycelial plug was inoculated on a host-free 1/5 MMN medium. Petri dishes were then incubated for 12 weeks in identical conditions.

Morphological characterization of Th hyphae

A total of 50 Petri dishes of *R. roseolus* with the ectomycorrhizal host and ten plates without the host were examined. The cytology of Th hyphae was evaluated from mycelia and mycelial cords using stereo and optical microscopes. The samples were first observed using a Leica EZ4 stereo microscope before being fixed in a 3:1 solution of 99.5% ethanol and acetic acid and subsequently removed from the air. The samples were then mounted with distilled water and lactophenol cotton blue and observed using an Eclipse 80i light microscope (Nikon, Tokyo, Japan). Pictures were obtained using a DS-L2 digital camera (Nikon, Tokyo, Japan). The variables analyzed in the morphological assessment include Th hyphae position, surface ornaments, shape, length, diameter, septum, and clamp connection. The diameters of Th hyphae with and without hosts were subjected to a one-way analysis of variance. Mean values were ranked using the Student-Newman-Keuls test at $P < 0.05$.

RESULTS AND DISCUSSION

The present study showed Th hyphae's cytological characteristics and the host's impact on this structure. Previously, most studies on Th hyphae were only on the occurrence or absence of this structure (Wartchow and Cortez 2016; Assis et al. 2018; Gelardi et al. 2019), without information on its cytological aspects. The Th hyphae were found on fungal mycelia (Figure 1A-B) and the surface of mycelial cords at the center and periphery of the fungal colonies (Figure 1C-D), both with and without a host. These findings contrast with those of Clémençon (2003), who reported Th hyphae's presence in the agar medium's depths. In this study, we did not find Th hyphae inserted in the agar medium in any of the experimental sets, which differs from Clémençon (2003), who investigated Th hyphae of saprophytic fungi. In the current study, a non-obligate ECM fungus was inoculated with and without a host, yet the Th hyphae were consistently observed on the surface of the media.

The Th hyphae that grew with hosts were found near the pine roots, on the ECM mantle, and the extraradical mycelia. However, a larger number of Th hyphae were distributed near the plug of the fungal isolate (Figure 2A) compared to the center of the fungal colony (Figure 2B) or in any other places in both experimental settings. Previously, Clémençon (2005) proposed that Th hyphae function as energy reservoirs in fungi. While further investigation is needed to confirm this, it might explain why larger Th hyphae accumulated near the fungal isolate than in any other part of the colony in this study. Substrate colonization and symbiosis initiation require high energy from the saprophytic or mycorrhizal fungi (Cairney 1992; Smith and Read 2008).

The Th hyphae were easily distinguished from the common vegetative hyphae by their appearance (Figure 3A). The Th hyphae were thick-walled, grew up to 200 µm in length, had a pale to dark brown color in distilled water (Figure 3A-B), had rare septa, and were extremely melanized. This finding is consistent with a previous description by Moreau et al. (2013) of Th hyphae with brown content observed from the gleba of *Alpova komoviana* collected at the field. The Th hyphae are the type of secretory hyphae that differ from Heteroplera (Laticifera and Gloeoplera) by liquid and the occurrence of nuclei (Clémençon 2012) on the later types. In the secretory hyphae, a cytoplasm containing secreted substances is called deuteroplasm. The deuteroplasm is a modified cytoplasm filled with secondary metabolites seen

as droplets, heterogeneous, or homogeneous masses (Clémençon 2012).

In the Th hyphae of this study, the solid column containing homogeneous deuteroplasm was separated from the adjacent vegetative hyphae by the unclear septum, and no nuclei were evident. Therefore, th hyphae are devoid of nuclei and are considered dead hyphae (Clémençon 2012). The homogenous grain content of the hyphae protoplasm was also evident (Figure 3A, double arrow). The Th hyphae were generally smooth with some cracks on the surface (Figure 3A, arrow) and turned darker brown with age. In addition, the Th hyphae turned dark blue (Figure 3C) and sometimes green (Figure 3D) when stained with lactophenol cotton blue. Clémençon (2012) reported that higher fungi produce many types of secretory hyphae, which the morphology and reactivity can distinguish with stains and solutions.

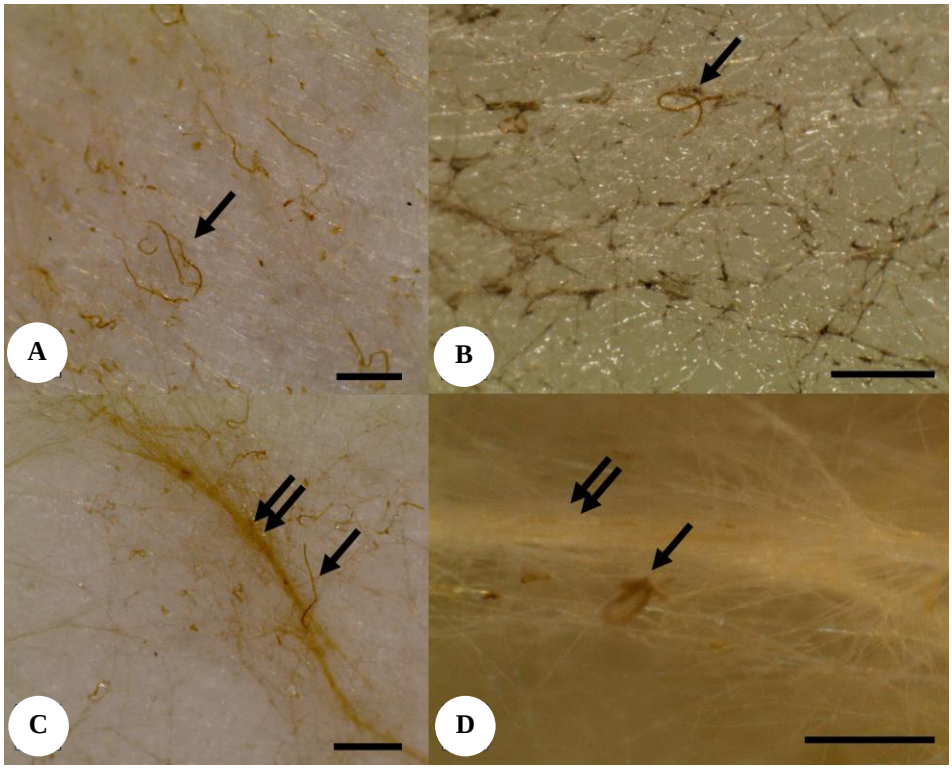

Figure 1. A-B. Thromboplerous hyphae (arrow) on the mycelia of *Rhizopogon roseolus* with and without a host, C-D. Thromboplerous hyphae (arrow) on the mycelial cords (double arrow) of *Rhizopogon roseolus* with and without a host. Bars = 200 µm

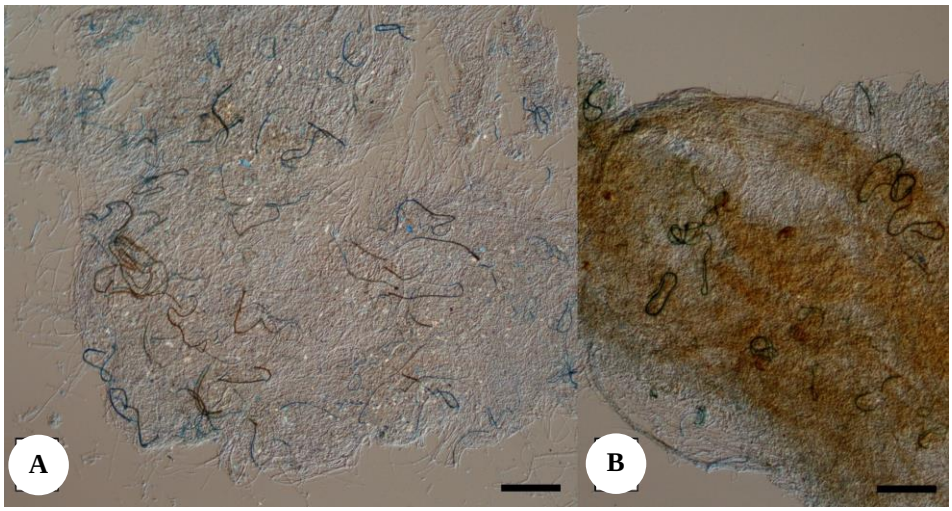

Figure 2. A larger number of *Rhizopogon roseolus* thromboplerous hyphae were found near the fungal inoculum (A) compared to the center of the fungal colony (B) with and without a host. Samples were stained with lactophenol cotton blue. Bars = 100 µm

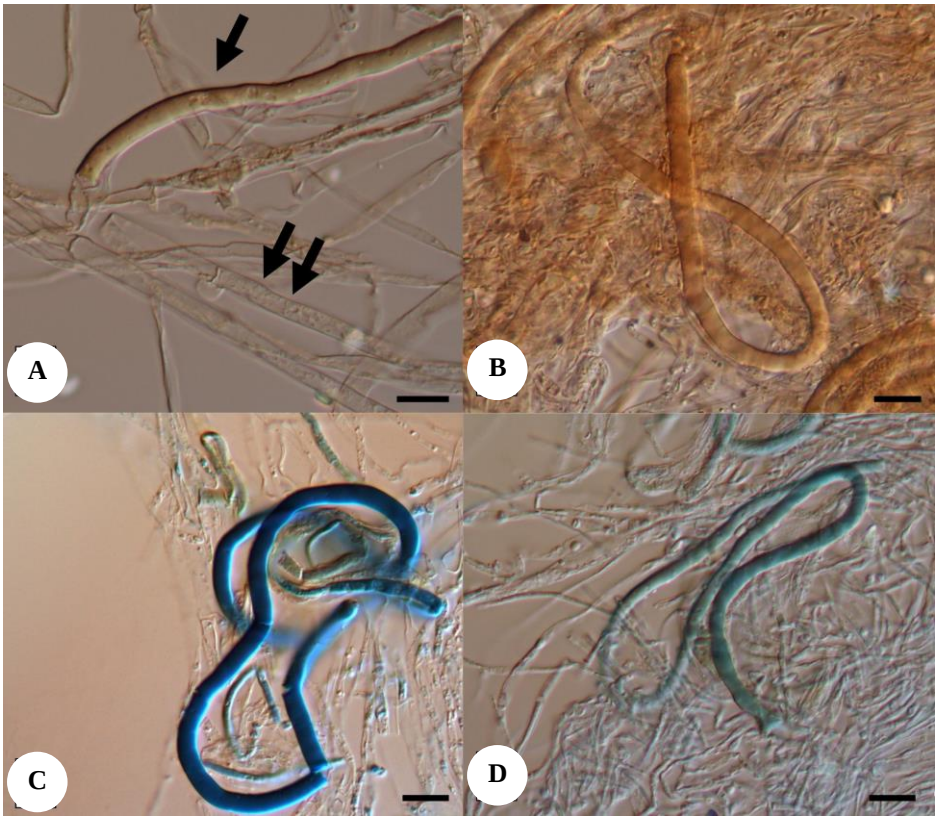

Figure 3. The common vegetative hyphae (double arrow) and melanized hyphae (arrow) with cracks on the surface (A), Thromboplerous (Th) hyphae mounted with distilled water showed brown content of Th hyphae and prominent oil drop near the hyphae (B), Th hyphae heavily stained with lactophenol cotton blue (C), Th hyphae that turned green after stained with lactophenol cotton blue (D). Bars = 10 µm

The Th hyphae were inflated at the distal or proximal ends of the hyphal terminal (Figure 4A-B). Thrombocytes of Th hyphae, usually found on old cultures (Clémençon 2002), are swollen cells at the ends of the structures. The Th hyphae of *R. roseolus* were reminiscent of oil but less refractive, a finding that contrasts with the description of Miller et al. (1983). Clémençon (2005; 2012) classified Th hyphae as secretory hyphae that never release latex. However, drops of oil were prominent near Th hyphae (Figure 3B), which changed the color of the fungal colony from white to cream. The deuteroplasm does not flow out but expands out of the damaged hyphae (Clémençon 2005; 2012).

The Th hyphae that grew with and without hosts were cytologically identical except for a few differences in shape and size. Th hyphae that grew with a host were mostly unbranched (Figure 4B), whereas those that grew without a host possessed semi-twisted to twisted (Figure 4C) and branched hyphae (Figure 4D). Clémençon (2003; 2005; 2012) reported that Th hyphae commonly grew without branches. In contrast, the present study is the first report of branched Th hyphae in fungi. Septa were evident in the middle of the Th hyphae and in the terminals where branches formed (Figure 4C-D). Th hyphae that grew without a host had slightly larger diameters than those that grew with a host (diameter 3.96 µm ± 0.7, range 3.1-5.03, n=9; diameter 4.47 µm ± 1.62, range 1.72 - 7.65, n=23, respectively). The presence of a host had no significant impact on the diameter of the Th hyphae (P> 0.05). Some of the Th hyphae that grew without a host produced exceptionally large (>9 µm) diameters (Figure 4A). Judging from the size, we suggest that large Th hyphae were derived from tubular hyphae, while vegetative hyphae initially formed the more common and smaller-sized Th hyphae.

Prior reports on the development and function of Th hyphae are scarce, and studies have been conducted exclusively on saprophytic fungi (Clémençon 2003; 2005). In this study, the cytoplasm of tubular hyphae, which had a diameter similar to that of Th hyphae, condensed into a solid homogeneous mass (Figure 5C). In addition, granules of melanizing protoplasms (Figure 5D) and empty portions of Th hyphae (Figure 5B) were also evident. Granules (Figure 5D) were always found near mature Th hyphae. The results of this study provide evidence of the initial development of Th hyphae in *R. roseolus*. Clémençon (2012) previously reported the gradual change of *Amanita citrina* protoplasm from a meromorphic (granular) to a more thrombomorphic state. In addition, Clémençon (2005) argued that Th hyphae develop from tubular hyphae based on their diameter. The present study found many smaller hyphae melanizing to Th hyphae (Figure 5B-D) than tubular hyphae. Therefore, the results suggest that Vegetative mycelia dominate th hyphae production and not the tubular hyphae.

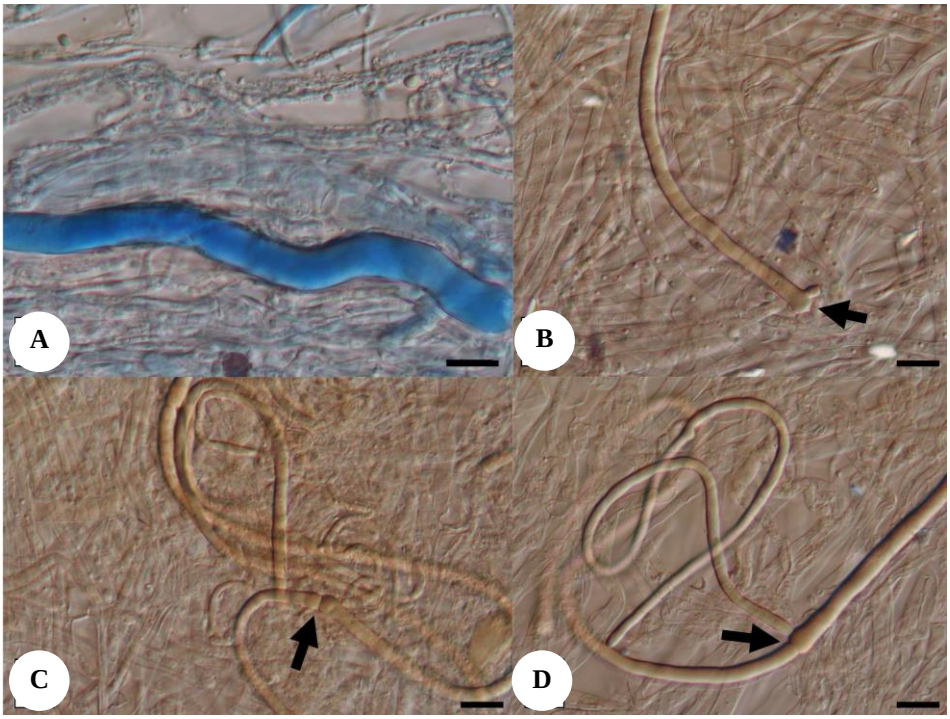

Figure 4. A. The large thromboplerous hyphae formed by specimens without a host stained with lactophenol cotton blue; B. The typical unbranched thromboplerous hyphae grown with a host, note the thrombocytes (arrow); C. The twisted shape of thromboplerous hyphae grown without a host, note the septa (arrow); D. The branched thromboplerous hyphae grown with a host, note the branch node (arrow). Bars = 10 µm

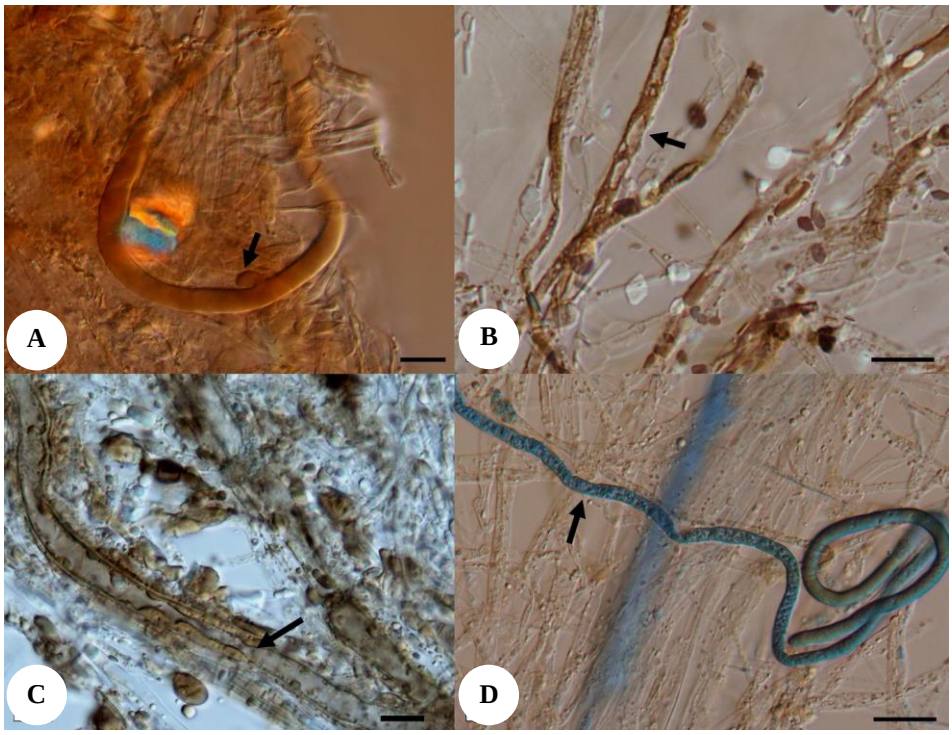

Figure 5. A. Clamp connection (arrow) of thromboplerous hyphae grown without a host, B. Melanizing hyphae showing a large vacuole (arrow), C. Tubular hyphae with condensing protoplasm (arrow), D. The homogenous deuteroplasm (arrow) of the initial formation of thromboplerous hyphae. Bars = 10 µm

Clémençon (2005) observed many clamp connections and incomplete septa in Th hyphae from the rhizomorph of *Ossicaulis lignatilis*. In this study, only a few Th hyphae that grew without a host had prominent septa (Figure 4C-D) and clamp connections (Figure 5A) compared to those that grew with a host. The clamp connection was also filled with the homogenously solid deuteroplasm. The occurrence of septa on Th hyphae in this study contrasts with the Th hyphae of *R. roseolus* described by Miller et al. (1983). Furthermore, Clémençon (2005) assumed that Th hyphae are used as reservoirs by fungi as he observed the presence of intrahyphal hyphae inside Th hyphae. While no intrahyphal hyphae were found inside Th hyphae in the present investigation. However, this study suggests that Th hyphae play an essential role in *R. roseolus* as it is produced in all stages of development of the fungal colony. However, the investigation of cytological aspects and hyphal features of *R. roseolus* was sparse (Martín and Gracia 2000; Putra et al. 2021), and more research should be done to reveal the undescribed phenomenon of the cytological characters of ECM fungi with and without a host.

The results of this study deepen our current understanding of the cytology of Th hyphae from laboratory studies. Studies on ultrastructure observations and 3D models of Th hyphae are underway, hopefully revealing more information on this aspect. In addition, further studies are needed to determine the specific roles of Th hyphae growing with or without a host in ECM fungi.

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Diversity of arbuscular mycorrhizal fungi and root colonization in *Polygonatum verticillatum*

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Abstract. *Kumar A, Tapwal A. 2022. Diversity of arbuscular mycorrhizal fungi and root colonization in* Polygonatum verticillatum. *Nusantara Bioscience 14: 53-63.* Diversity of arbuscular mycorrhizal fungi (AMF) associated with *Polygonatum verticillatum* (L.) All. was investigated in two sites of Himachal Himalaya, India. A total of 15 AMF species were isolated and identified from the rhizosphere soil of *P. verticillatum*. The spore density was 1.48 ± 1.91 and 3.99 ± 3.78 per 20 grams of rhizosphere soil at the site I and site II, respectively. Mycorrhizal colonization in the roots of *P. verticillatum* was recorded at 46.12 and 52.23 percent at the site I and site II, respectively. In addition, the mycorrhizal structures like darkly stained endophytic hyphae, coiled intracellular hyphae, Y-shaped hyphae, and 'H' connection (Arum type) were recorded.

Keywords: AM fungi, Astavarga, Mahameda, *Polygonatum verticillatum*, root colonization

INTRODUCTION

Polygonatum verticillatum (L.) All., commonly known as Whorled Solomon's Seal, belong to the family Asparagaceae. *Polygonatum* comprises 71 species (Zhao et al. 2017), mostly distributed in temperate regions. It is found in India, Pakistan, Nepal, Afghanistan, Bhutan, Korea, Russia, and in moderate climate zones of North America and Europe (Saboon et al. 2016). Its distribution is also recorded in subtropical and boreal zones of the northern hemisphere (Meng et al. 2014). In India, *P. verticillatum* distribution is reported from Kashmir to Sikkim as undergrowth in the temperate forests at an altitude range of 2000-3000 m (Pandey et al. 2006; Bisht et al. 2011).

Polygonatum verticillatum is locally known as Salam-mishri, 'Mahameda' in Ayurveda, and Tridanti, Devamani, and Vasuchhidra in Sanskrit. *Polygonatum verticillatum* has been used as a folklore remedy and is a member of the 'Astavarga' group of medicinal plants in Ayurvedic medicines. Astavarga is a group of eight astounding medicinal plants and is known for jeevaniya (vitality promoting), vaysthapan (restoring of youthful condition), body nourishment, antioxidant, and reviving properties (Buchake et al. 2010). Charak Samhita, an ancient Ayurvedic literature, has described its noteworthy role in curing cough, dyspnea, consumption, cardiac problems, and voice disorders (Baliga et al. 2013). β-sitosterol, 2-hydroxybenzoic acid (Khan et al. 2013; Sagar 2014; Saboon et al. 2016), santonin, diosgenin (Khan et al. 2015), and quinine present in *P. verticillatum* exhibit a variety of pharmaceutical properties like antioxidant activities, anti-inflammatory activity, anticancer activity (Patra et al. 2018), antimalarial activities (khan et al. 2012) antipyretic, analgesic, diuretic, sex stimulant (Wujisguleng et al. 2012; Akhtar et al. 2013; Sharma and Samant 2014; Razzaq et al. 2015) and energizer, etc. (Tariq et al. 2015; Saboon et al. 2016; Virk et al. 2016). Rhizomes of this species improve liver health and cure throat pain, headache, eye diseases, gastric troubles, epilepsy, high blood pressure, etc. (Tiwari and Chaturvedi 2016).

Plants have long been used as a source of medicinal bioactive substances to treat a variety of diseases (Gouda et al. 2016; Chalo et al. 2017; Dhakal et al. 2021). Medicinal plants accumulate an array of unique bioactive secondary metabolites that confer high specificity to the associated microorganisms in their distinctive micro-biome (Qi et al. 2012). The microorganisms present in the rhizosphere of plants play a vital role in supporting plant's health by improving plant nutrition (Jacoby et al. 2017), suppressing pathogen outbreaks (Pieterse et al. 2014), nutrient exchange and modulation abiotic stress tolerance (Baum et al. 2015; Cheng et al. 2019) such as drought, low temperature, and salinity (Sun et al. 2021). Among them, arbuscular mycorrhizal fungi (AMF) are the important functional groups that promote plant growth, provide nutrition, and improve health (Giovannini 2020). AMF facilitate nutrient uptake, mainly phosphorus, nitrogen (Campo et al. 2020), potassium, sulfur, copper, zinc, calcium, etc. (Avio et al. 2006; Prasad et al. 2017; Liu et al. 2018; Wang et al. 2018) and enhance the availability of nutrients as well as their translocation (Rouphael et al. 2015). The abiotic factors viz. light, temperature, humidity, soil fertility, and cultivation techniques influence the consistency of active ingredients in medicinal plants, as do biotic factors such as herbivory, disease-related stimuli, and reciprocal symbioses with *Rhizobium* bacteria and mycorrhizal fungi (Szakiel et al. 2011). In host plant, the AMF enhances the content of secondary metabolites (Zubek et al. 2015; Johny et al. 2021) like terpenoids,

alkaloids, and phenolics (Yadav et al. 2013; Zeng et al. 2014), cyclohexanone derivatives, apocarotenoids, phytoalexins, triterpenoids and glucosinolates (Zubek et al. 2010, 2013; Singh et al. 2013). *Polygonatum verticillatum* is little explored for the mycorrhizal association. The present study investigated mycorrhizal colonization and diversity of AMF associated with P. verticillatum in two distant sites in Himachal Himalaya, India.

MATERIALS AND METHODS

Study area

Two sites with temperate climates were selected to collect soil and root samples. The study areas include Chikkadhar, Kullu District, India (Site I) (32°12'02.13"N, 077°15'24.44" E; 2,964 m asl), and Jani, district Kinnaur (Site II) (31°29'13.1"N, 78°04'39.3"E; 2,686 m asl) in Himachal Himalaya, India. Soils up to the depth of 0-30 cm were collected in sterile polyethylene bags and carried to the laboratory.

Isolation and identification of AMF

Ten single soil samples from each sampling site were combined to form a composite sample. Isolation of AMF spores from rhizosphere soil followed the wet sieving and decanting method (Gerdemann and Nicolson 1963). Air dried soil sample (20 g) was suspended in 1000 mL water for 1 hour, and then the suspension was decanted through a series of sieves ranging from 40 µm to 700 µm arranged in descending order of pore size. The sieved material collected from sieves was observed under a stereomicroscope (Nikon SMZ 1500), and the spores were isolated using a hypodermal needle. The spore population was expressed in terms of the number of spores per 20 g of dry soil.

Taxonomic identification of AM fungal spores was done based on size, shape, color, wall structure, number of wall layers, surface ornamentation, hyphal attachments, and the presence or absence of bulbous suspensor under NIKON E-400 microscope following standard websites and taxonomic manuals (International Culture Collection of Vesicular-Arbuscular Mycorrhizal Fungi; http://invam.caf.wvu.edu), http://www.amf-phylogeny.com, http://www.zor.zut.edu.p1/Glomeromyca, Techniques in mycorrhizal research, VA Mycorrhiza and Handbook of AMF.

Assessment of root colonization by AMF

Root staining

This study used trypan blue as a stain to process roots for AMF colonization assessment. Roots were first washed thoroughly with tap water, cut into 1 cm lengths, and cleared in 10% (w/v) KOH for 1 hour at 90 °C, acidified with 1% HCl and stained with 0.05% trypan blue overnight, and then finally de-stained with lactic acid-glycerin (1:1 by volume) at room temperature (Phillips and Hayman 1970).

Assessment of root colonization-Grid-line intersect method

The gridline intersects method assessed root colonization (Giovannetti and Mosse 1980). This method is used to estimate both the proportion of infected roots and their total length. For each sample, 100 root segments were chosen at random. The roots (50 segments at a time) were evenly spread out in a Petri dish (10.2 X 10.2 cm^2) with gridlines marked on the bottom to make 0.5-inch squares. Vertical and horizontal gridlines were scanned with a stereomicroscope (Nikon SMZ 1500), and the presence or absence of colonization was noted at each location where the roots intersected a line. The root segments were spread out and examined three times. Finally, the percentage of colonization was computed by dividing the total number of colonized root segments by the total number of investigated root segments. Intersections between gridlines and roots are designated as either mycorrhizal or non-mycorrhizal. The mycorrhizal structures like hyphae, arbuscules, and vesicles were observed in root segments under a microscope. The following formula determined the percentage of root colonization:

$$\text{Root colonization (\%)} = \frac{\text{Total no. of colonized root segments}}{\text{Total no. of root segments investigated}} x\ 100$$

Diversity and data analysis

Some important ecological diversity indicators, such as spore density, relative abundance, isolation frequency, Shannon-Wiener index of diversity (H'), Simpson's index of dominance (D), Evenness (E), and Sorenson's coefficient (Cs) can be used to describe AMF community structure. According to Kavitha and Nelson (2013), spore density, to some extent, represents the biomass of AMF species. Relative abundance is defined as a percentage of the spore number of a species, which indicates the ability of distinct AMF species to sporulate. The percentage of soil samples in which a species existed, which revealed the extent of dispersion of a particular AMF species in an ecosystem, is described as isolation frequency. Finally, the Shannon-Wiener index of diversity reflected the degree of diversity.

Relative abundance (RA), isolation frequency (IF), Shannon-Wiener index of diversity (H'), Simpson's index of dominance (D), Evenness (E), and Sorenson's coefficient (Cs) were calculated as outlined by Dandan and Zhiwei (2007).

$$RA = \frac{\text{Spore numbers of a species}}{\text{Total spore number}} x\ 100$$

$$IF = \frac{\text{Number of soil samples where species occurred}}{\text{Total number of soil samples}} x\ 100$$

$$H' = -\sum Pi \ln Pi$$

Pi = ni/N,

Where, ni is the spore number of a species, and N is the total number of identified spore samples.

$$D = \sum (ni/N)^2$$

$$Evenness = \frac{H'}{H'max}$$

H'max is the maximal H' and is calculated by the following formula:

$H' = \ln S$,

Where, S is the total number of identified species per sampling site

$$\text{Sorenson's coefficient (Cs)} = \frac{2j}{(a+b)}$$

Where, a or b is the total number of species per sampling site, and j is the number of species common to both sites.

RESULTS AND DISCUSSION

Root colonization

Fungal structures like thin intraradical hyphae and coiled intracellular hyphae in cortical root cells confirm the mycorrhizal association. The fungal hyphae were Y-shaped and exhibited an arum type of mycorrhizal infection. The intraradical hyphae in cortical root cells were darkly stained, thin-walled, about 3.28 µm in width, Y-shaped septate branches, and showed 'H' connections between parallel strands of hyphae. This type of hyphal proliferation in the root cortex is known as the Arum type (Brundrett 2004). These characters can be used as diagnostic features to identify the genus of AMF in mycorrhizal roots (Morton and Bentivenga 1994). These mycorrhizal characteristics are exhibited by the *Glomus* species (Souza 2015). The roots collected from study sites showed the presence of dark septate endophytic and intracellular coiled hyphae. The mycorrhizal status of roots collected from both study sites is given in Table 1. Distinct AM vesicles and arbuscules were not recorded, but dark septate hyphae could be seen prominently in the cortical root cells (Figure 3).

The colonization by AMF in the roots of *P. verticillatum* was recorded at 46.12% and 52.23%, respectively, in Site I (Chikadhar, Kullu District) and site II (Jani, Kinnaur District). The AM fungal root colonization is influenced by soil moisture, soil texture (Herold et al. 2014; Sharma and Kothamasi 2015), humidity, temperature (Urcoviche et al. 2014; Bhardwaj and Chandra 2018), seasonal periods, and host plant (Carrenho et al. 2007; Haichar et al. 2016; Guyonnet et al. 2017), soil pH and available nutrients (N and P) (Khanam et al. 2006; Liu et al. 2016). Most medicinal plants' roots and agronomic and vegetable crops exhibit endomycorrhizal association (Gaur and Kaushik 2011). Thangavelu and Raji (2016) reported 10.38-84.55% root colonization in five pot-grown species of *Asparagus*, and the extent of root colonization by AMF varies in different plant species. Yaseen et al. (2016) recorded root range colonization in 20-80% of 20 medicinal plants from Charsadda, Khyber Pakhtunkhwa (Pakistan). At the same time, 48-100% root colonization was recorded in 46 medicinal plant species in the Western Ghats of Karnataka region (Rajkumar et al. 2012). Tapadar et al. (2017) recorded 82.98% root colonization in *Smilax perfoliata*. According to Johny et al. (2021), root colonization amounts vary between AMF and plant species. *Rhizophagus irregularis* exhibited maximum AMF root colonization (43 ± 1.00%) in Ashwagandha, followed by *Claroideoglomus claroideum* (34 ± 4.33%). Root colonization by *R. irregularis* was greatest in marigolds (73 ± 2.88%). *Claroideoglomus etunicatum* and *C. claroideum* displayed the highest affinity to plants, with root colonization rates of 73 ± 5.77 and 72 ± 2.88%, respectively, in licorice. Sinegani and Yeganeh (2016) studied the symbiosis of 48 medicinal plant species with AMF in the semi-arid regions of Iran. They reported percent root range colonization in 32.37% to 77.26%.

Diversity of AMF associated with *Polygonatum verticillatum*

15 AMF species belonging to 7 genera, viz., *Glomus*, *Funneliformis*, *Claroideoglomus*, *Acaulospora*, *Rhizophagus*, *Gigaspora*, and *Scutellospora*, were identified from the rhizosphere soil collected from site 1. Among these, *Glomus* was the dominant genus represented by 5 species, followed by *Acaulospora* (3 spp.) and *Funneliformis* (2 spp.). The species of *Glomus* are: *G. ambisporum*, *G. glomerulatum*, *G. microcarpum*, *G. macrocarpum*, *G. aggregatum*. *Acaulospora* was represented by *A. laevis*, *A. spinosa*, *A. rehmii*, and *Funneliformis* by *F. Geosporum* and *F. constrictus*. The rest of the genera were represented by one species each. Only 3 species representing two genera, i.e., *G. glomerulatum*, *F. geosporum*, and *F. constrictus*, were recorded from site II. Although much literature is available on the diversity of AMF associated with medicinal plants, *P. verticillatum* is little explored in this aspect. Gaur and Kaushik (2011) have isolated 16 AMF from three medicinal plants, i.e., *Catharanthus roseus*, *Ocimum* species, and *Asparagus racemosus* in the Uttarakhand state. Thangavelu and Raji (2016) recorded a dual association of AM and DSE in five pot-grown species of *Asparagus* (*A. aethiopicus*, *A. densiflorus*, *A. setaceus*, *A. racemosus*, and *A. umbellatus*). The spores of 15 AMF species from 10 genera were isolated by Zubek et al. (2012) from rhizosphere soils of the following medicinal plant species, i.e., lemon balm (*Melissa officinalis*), sage (*Salvia officinalis*), and lavender (*Lavandula angustifolia*). Verma et al. (2019) investigated AM diversity in seven ethnomedicinal plants from the Western Himalayas and recorded 23 AMF, where *Glomus* was dominating genus. Kumar et al. (2019) investigated 22 medicinal plants from the Hamirpur district of Himachal Pradesh for AM association, identified 43 AMF from their rhizosphere soils, and reported *Glomus* and *Acaulospora* as dominant genera. In Zhangzhou (China), 66 species of AM fungi have been found in the rhizosphere of 20 medicinal plants, with *Glomus* as the most common genus (Jiang et al. 2012). From the southern region of Fujian (China), 91 AM fungi species were isolated from medicinal plants' rhizosphere, with *Glomus* as the predominant genus (Jiang 2012).

Twenty-six species of AM fungi were isolated from the rhizosphere of *Begonia fimbriata* (Su et al. 2018), belonging to the genera *Acaulospora*, *Glomus*,

Scutellospora, and *Gigaspora*. Song et al. (2019) studied the diversity of AMF of *Sophora flavescens*, and 220 AMF species were detected, representing 8 families and 14 genera. *Glomus*, *Septoglomus*, *Rhizophagus*, *Kamienskia*, and *Sclerocystis* were the dominant AMF genera in the rhizosphere of *S. flavescens*. Koul et al. (2012) recorded 42 species of AMF in the rhizosphere of medicinal plants in India, where six AM fungal species were found in *Aloe vera*, five in *Artemisia annua*, and one in marigolds. Sundar et al. (2011) studied the association of AMF with three medicinally important plants, viz. *Eclipta prostrata*, *Indigofera aspalathoides*, and *I. tinctoria* were collected from three different localities of Kanyakumari (South India), identified 21 AMF, and recorded *Glomus* as the dominant genus. According to the classification of Krüger et al. (2012), there are 11 AMF families, 17 AMF taxa, and about 230 AMF species, where *Glomus* is the most diverse genus, exhibiting around 93 morphospecies. In our study, *Glomus* was recorded dominant genus; it conformed with studies conducted with other medicinal plants (Guadarrama and Álvarez-Sánchez 1999; Muthukumar and Udaiyan 2000; Hijri et al. 2006).

Occurrence, Relative abundance, and Isolation frequency of AMF species are given in Table. 2. A total of 15 AM fungi were identified from the site I (Chikkadhar) and 3 species from site II (Jani). Just three species were reported to be shared by both study sites. Relative abundance (RA) was found in the range of 1.51% (*A. laevis*, *A. rehmii*, *A. spinosa*, *Scutellospora gregaria*, *Scutellospora* sp. and Unknown sp.) to 34.84 % (*G. ambisporum*) in the site I and 11.11% (*F. constrictus*) to 69.44% (*G. glomerulatum*) in site II. Similarly, isolation frequency (IF) varied from 33.33% (*A. laevis*, *A. rehmii*, *A. spinosa*, *F. geosporum*, *S. gregaria*, *Scutellospora* sp. and Unknown sp.) to 100% (*C. etunicatum*, *F. constrictus*, *G. ambisporum*, *G. glomerulatum*, *G. microcarpum*, and *Rhizophagus intraradices*) from the site I and it was ranging from 0-100% (maximum 100% was found in *F. constrictus* and *G. glomerulatum*), while the majority of the AMF species were found to have zero values at site II.

The AM spore density was recorded at 1.48±1.91 and 3.99±3.78 per 20 grams in the rhizosphere soil of P. verticillatum at sites I and II, respectively (Figure 1). Low spore density may be due to the harsh environmental conditions of the study sites. Climatic and edaphic factors (Antunes et al. 2012; Sivakumar 2013; Nouri et al. 2014) significantly impact the population dynamics of AMF; rapid changes in soil nutrients can affect the AMF association and spore numbers (Khanam et al. 2006). Environmental variations, host phenology, interspecific competition, and regional spatial dynamics can influence AM fungal communities' population, distribution, and composition in various ecosystems (Öpik et al. 2006; Melo et al. 2019). Information on the AMF spore density in the rhizosphere of *P. verticillatum* is not available. Still, it was recorded in the range of 3.01 to 2860 spores by many researchers with other medicinal plants, e.g., 10 spores in *Ludwigia linifolia* and 382 spores/100 g of soil in *Leucas aspera* (Bukhari et al. 2003); 84 spores/100g in *Withania coagulans* and 147 spores in *Mitragyna parvifolia* (Panwar and Tarafdar 2006); spore density ranged from 47.53 in *Datura stramonium* to 177.4 in *Mimosa pudica* per 50 g of soil sample (Kumar et al. 2019); 27 spores/10 g of soil in the rhizosphere of *Adhatoda vasica* and 196 spores in *Zanthoxylum acanthopodium* (Singh et al. 2017); 3.01 spores density per 50 g in *Peumus boldus* and 37.30 in *Matricaria chamomilla* (Urcoviche et al. 2014); AM fungal spore density varied from 270 (*Leonurus heterophyllus*) to 2860 (*Lophatherum gracile*) per 100 g soil (Wang and Jiang 2015). Bhat et al. (2014) isolated and identified 151 spores/100g and 24 spores/100g of natural soil of *C. roseus* at two sites, and *Glomus* was found to be the predominant genus in the rhizosphere of both sites. Garampalli et al. (2012) investigated the arbuscular mycorrhizal status of 46 medicinal plant species of herbs and shrubs in the western ghats of Karnataka region, which recorded spore density ranged from 15 to 520 per 100 g of soil.

Glomus glomerulatum, *F. geosporum*, and *F. constrictus* were common to both sites, and Sorenson's Coefficient (Table 3) was found to be 0.33. The diversity indices are shown in Figure 2.

Table 1. Status of AMF association in *Polygonatum verticillatum*

Site	Root colonization (%)	AMF identified	AM structures observed		
			Arbuscules	Vesicles	Hyphae
Chikkadhar	46.12	*Glomus* (5spp.), *Acaulospora* (3 spp.), *Funneliformis* (2 spp.), *Claroideoglomus* (1 sp.), *Rhizophagus* (1 sp.), *Gigaspora* (1 sp.), *Scutellospora* (1 sp.), Unknown (1 sp.)	- (Arum type)	-	+ +
Jani	52.23	*Glomus* (1 spp.), *Funneliformis* (2 spp.)	- (Arum type) Intracellular hyphal coils present	-	++

Note: -: Absent, ++: Moderate

Table 2. Occurrence, relative abundance, and Isolation frequency of AMF species in rhizosphere soil of *Polygonatum verticillatum* (L.) All

Name of fungi	Site	Occurrence	% Freq.	D	A	RA (%)	IF (%)
Acaulospora laevis Gerd. & Trappe	I	+	7.14	0.33	1	1.51	33.33
	II	-	--	--	--	--	--
Acaulospora rehmii Sieverd. & S. Toro	I	+	7.14	0.33	1	1.51	33.33
	II	-	--	--	--	--	--
Acaulospora spinosa C. Walker & Trappe	I	+	7.14	0.33	1	1.51	33.33
	II	-	--	--	--	--	--
Claroideoglomus etunicatum (W.N. Becker & Gerd.)	I	+	21.42	1.33	1.33	6.06	100
C. Walker & A. Schüßler	II	-	--	--	--	--	--
Funneliformis constrictus (Trappe) C. Walker & A.	I	+	21.42	1.66	1.66	7.57	100
Schüßler	II	+	100	1.33	1.33	11.11	100
Funneliformis geosporum (T.H. Nicolson & Gerd.) C.	I	+	7.14	0.66	2	3.03	33.33
Walker & A. Schluessler	II	+	100	2.33	2.33	19.14	100
Glomus aggregatum N.C. Schenck & G.S. Sm.	I	+	14.28	0.66	1	3.03	66.66
	II	-	--	--	--	--	--
Glomus ambisporum G.S. Sm. & N.C. Schenck	I	+	21.42	7.66	7.66	34.84	100
	II	-	--	--	--	--	--
Glomus glomerulatum Sieverd.	I	+	21.42	3.33	3.33	15.15	100
	II	+	100	8.33	8.33	69.44	100
Glomus macrocarpum Tul. & C. Tul.	I	+	14.28	1.33	2	6.06	66.66
	II	-	---	--	--	--	--
Glomus microcarpum Tul. & C. Tul.	I	+	21.42	1.66	1.66	7.57	100
	II	-	--	--	--	--	--
Rhizophagus intraradices (N.C. Schenck&G.S. Sm.)	I	+	21.42	2	2	9.09	100
C. Walker & A. Schüßler	II	-	--	--	--	--	--
Scutellospora gregaria (N.C. Schenck & T.H.	I	+	7.14	0.33	1	1.51	33.33
Nicolson) C. Walker & F.E. Sanders	II	-	--	--	--	--	--
Scutellospora sp.	I	+	7.14	0.33	1	1.51	33.33
	II	-	--	--	--	--	--
Unknown sp.	I	+	7.14	0.33	1	1.51	33.33
	II	-	--	--	--	--	--

Note: Site I: Chikkadhar; Site II: Jani

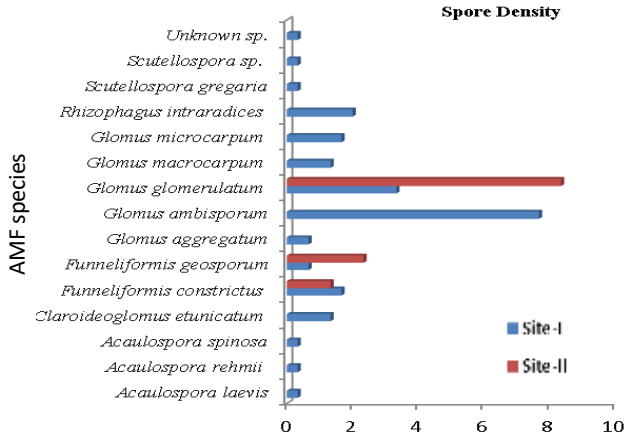

Figure 1. AMF spore density in the rhizosphere soil of *Polygonatum verticillatum*

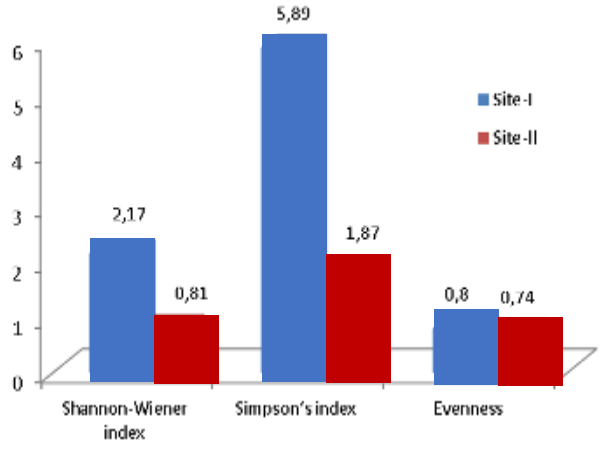

Figure 2. Diversity indices of AMF associated with *Polygonatum verticillatum*

The resources of many medicinal plants are suffering an unavoidable loss of resources due to damage to the natural habitat and long-term over-exploitation (Zhao et al. 2019; Ullah et al. 2020). *Polygonatum verticillatum* is one of many important medicinal plants, and the knowledge of its AMF association will be of immense importance. The potential AMF can be multiplied during cultivation trials for better active ingredient contents. According to the literature, plants produce significantly more secondary metabolites after AM fungal colonization (Oliveira et al. 2013; Zeng et al. 2013; Mechria et al. 2015; Pedone-Bonfim et al. 2015; Kapoor et al. 2017; Duc et al. 2021). Thus, the use of AM fungi could be employed as a strategy for crop biofortification (Antunes et al. 2011; Dutta and Neog 2016) because they influence soil fertility, plant nutrition, plant physiology, and secondary metabolism

(Wipf et al. 2014; Schweiger and Müller 2015; Cervantes-Gámez et al. 2016). Furthermore, AMF is essential for plant growth and health (Ban et al. 2017). As a result, it is critical to investigate the diversity of these fungi in the rhizosphere community structure of *P. verticillatum*. Several academics are working on identifying certain mycorrhizal fungus and their role in phytochemical production (Kumar et al. 2021).

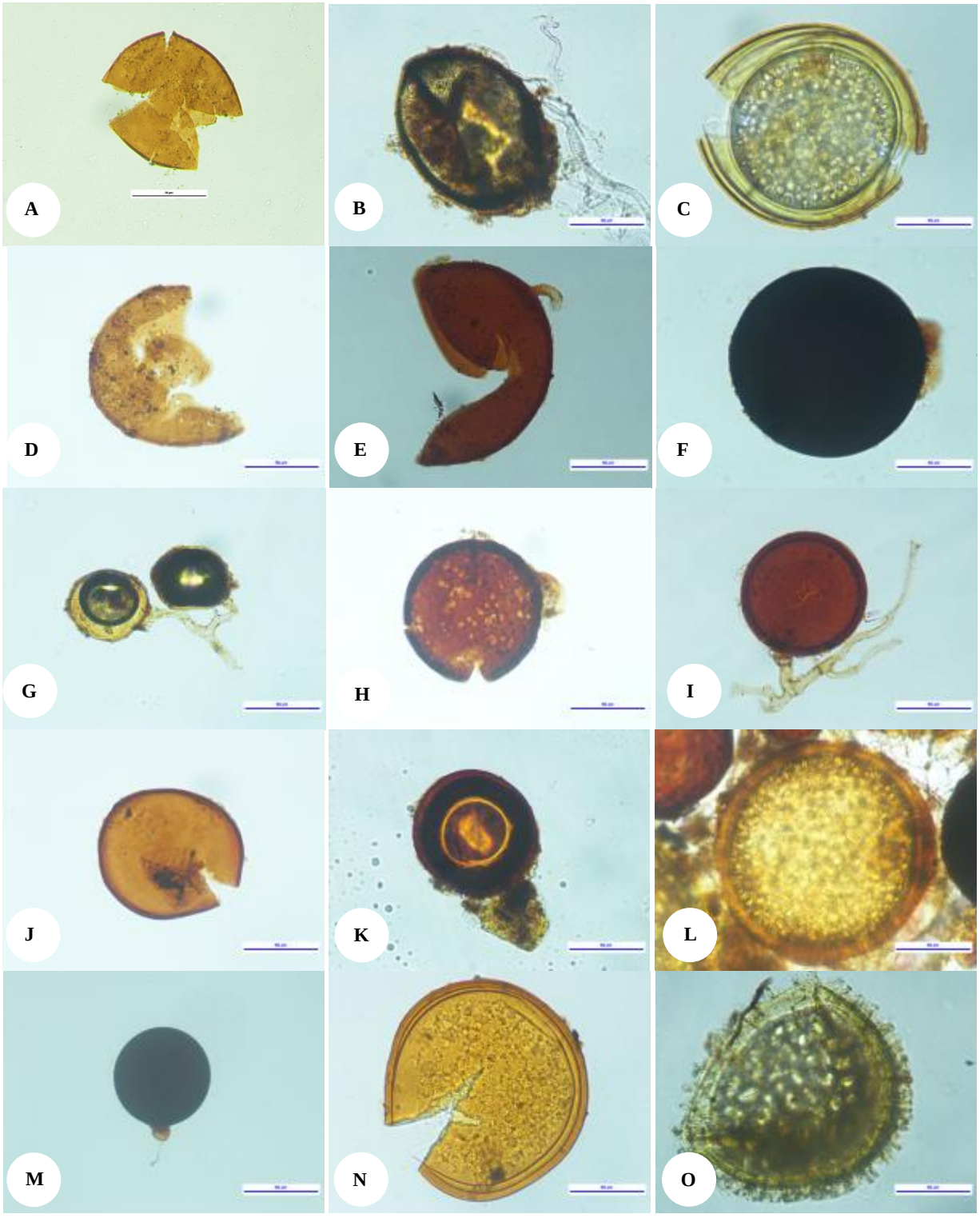

Figure 3. Diversity of AMF spores in the rhizosphere of *Polygonatum verticillatum* (L.) All. A. *Acaulospora laevis*; B. *A. rehmii*; C. *A. spinosa*; D. *Claroideoglomus etunicatum*; E. *Funneliformis constrictus*; F. *F. geosporum*; G. *Glomus aggregatum*; H. *G. ambisporum*; I. *G. glomerulatum*; J. *G. macrocarpum*; K. *G. microcarpum*; L. *Rhizophagus intraradices*; M. *Scutellospora gregariaI*; N. *Scutellospora* sp.; O. Unknown sp.

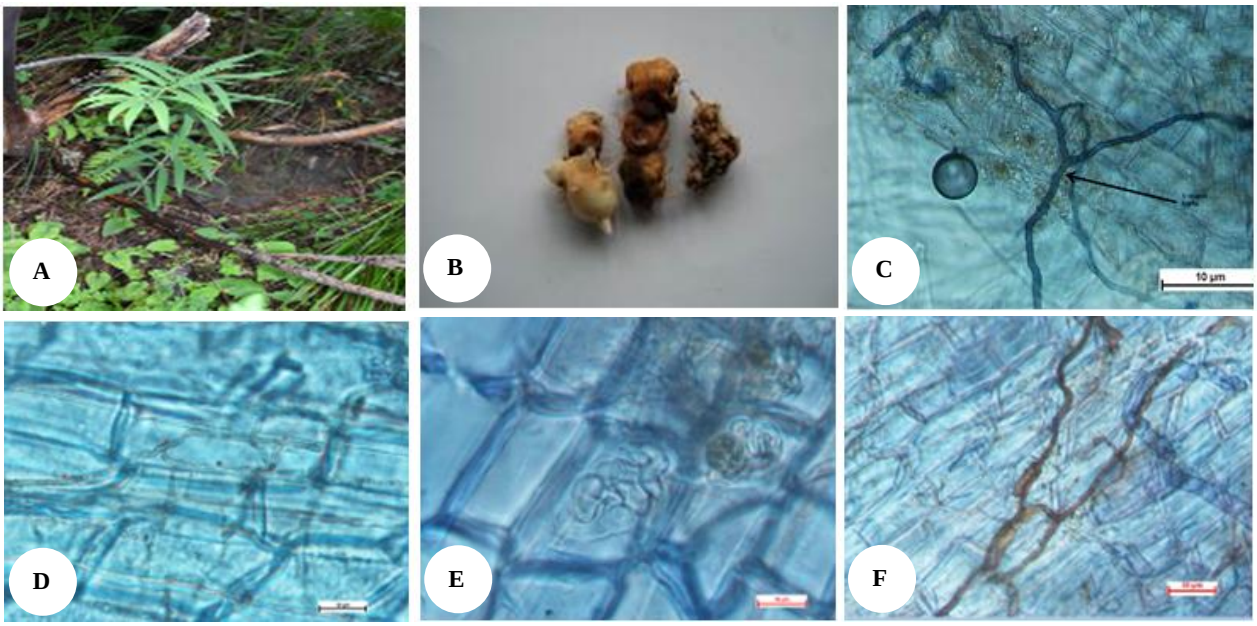

Figure 4. Status of the mycorrhizal colonization with roots of *P. verticillatum.* A. *P. verticillatum*; B. Rhizomes of *P. verticillatum*; C. Y-shapes intraradical hypha; D. H-shaped connection; E. Hyphal coils in the cortical cells; F. Dark septate hyphae

In conclusion, the present study is focused on the diversity and root colonization of AM fungi in the rhizospheric soil of a highly valuable medicinal plant, *P. verticillatum*, found in two temperate regions of the Himachal Himalaya. The plant is well-known for its pharmaceutical properties like antioxidant, anti-inflammatory, anti-cancerous, antimalarial, antipyretic, analgesic, diuretic, aphrodisiac, etc. An accurate and deep understanding of the rhizosphere microbiome is important because of its significant role in enhancing the therapeutic properties of medicinal plants. Here, we provide information on the spore density, root colonization, and diversity of AM fungi in the rhizosphere of *P. verticillatum.* The degree of colonization and spore density varied greatly between the study sites. The fungal genera *Glomus* and *Funneliformis* were the dominant genera at sites I and II, respectively. Considering the possible application of AM fungi in the future on such important medicinal plants, it appears that more attention should be paid to the dominant AM fungi in the association of medicinal plants for the process of in vitro cultivation and mycorrhizal performance so that growth and secondary metabolite production could be improved.

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In vitro thrombolytic activity of *Moringa oleifera*

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Abstract. *Kunwar B, Jain V, Verma SK. 2022. In vitro thrombolytic activity of* Moringa oleifera. *Nusantara Bioscience 14: 63-69. Moringa oleifera* Lam. (Family Moringaceae) is a medium-sized perennial tree, commonly known as drumstick tree, horse radish tree, miracle tree, *sahjan, shobhanjana, munga arak*, etc., in different languages. Leaves, flowers, and pods of the plant are edible. Various parts of the plant are utilized to treat several diseases by ethnic communities, including heart ailments. Leaves and flowers of *M. oleifera* were assessed for preliminary qualitative phytochemical analysis and in vitro thrombolytic potential. Preliminary phytochemical screening has shown the presence of flavonoids, terpenoids, cardiac glycosides, saponins, tannins, amino acids, and carbohydrates in the leaves and flowers of *M. oleifera*. Two types of methanolic extracts of both leaves (MEL-I and MEL-II) and flowers (MEF-I and MEF-II) were used to assess percent clot lysis. A significant in vitro clot lysis activity of MEL-I (36.64±1.55%), MEL-II (41.40±2.02%), and MEF-I (19.07±2.36%), and MEF-II (20.52±1.51%) was demonstrated in a concentration of one mg/mL as compared to negative control distilled water and positive control streptokinase for the first time. The edible plant's observed thrombolytic potential may be employed to prevent athero-thrombotic cardiovascular diseases. Further investigations are required to isolate the bioactive molecule responsible for the thrombolytic action.

Keywords: Clot lysis, drumstick, *Moringa oleifera*, quercetin, Streptokinase

INTRODUCTION

Thrombus is a natural physiological response of the body to prevent hemorrhage by forming a blood clot associated with circulating platelets, thrombin, and fibrin fibers. In the absence of any stimulus from the intravascular damage, significant abnormalities such as ischemia, stroke, heart attack, and deep vein thrombosis happen. The formation of a thrombus in the coronary artery leads to interference in blood circulation to the heart and results in myocardial infarction (Furie and Furie, 2008; Gaziano and Gaziano, 2018). Therefore, a Lysis of thrombus is, known as thrombolysis, urgently required during heart attack and stroke cases. For this purpose, various synthetic thrombolytic agents, such as tissue plasminogen activator (t-PA), urokinase (UK), Streptokinase (SK), alteplase, etc., are widely used to provide immediate clearing of vessels. However, these synthetic thrombolytic drugs may pose a risk of severe bleeding, anaphylactic reactions, and shock (Collen 1990). Therefore, searching for a better alternative as a thrombolytic agent regarding cost-effectiveness, safety and efficiency are required. Natural resources are generally considered safe and may provide better efficacy. In this regard, plants with fibrinolytic, thrombolytic, and antiplatelet potential can be used to reduce the risk of thrombosis. Moreover, suppose plants with such efficacy could be a part of diets. In that case, it could also help prevent the onset of such diseases as scientific studies have shown that a daily intake of five servings of plant-based foods ranging from 400-800 g/day could help in risk reduction for cardiovascular diseases (Yu et al. 2018).

The Moringa genus belongs to Family Moringaceae, a monotypic family with 13 species distributed in Asia and Africa. *Moringa oleifera* Lam. is a popular plant commonly found in North-West India, the sub-Himalayan tract, from Chenab eastward to Sarda, and cultivated all over the plains of India. It is a medium-sized (about 10 m) perennial tree, known as drumstick tree, horse radish tree, miracle tree, mother's best friend, *sahjan*, *sainjna*, *shobhanjana*, *mungna*, *shevgi*, *sehjan*, *munga arak*, etc., in various languages. Leaves are tripinnate with entire and glabrous leaflets. Flowers are odorant with five unequal yellowish-white petals. The fruit is a hanging pod with a 9-ribbed brown capsule having dark brown, globular seeds. The tree's leaves, flowers, and fruits are edible and used as vegetables (The Wealth of India 1962; Tiagi and Aery 2007; Abd Rani et al. 2018).

Moringa oleifera is a good source of vitamins, proteins, and minerals. Its plant parts are used in traditional medicine for the treatment of various human diseases, for example, anemia, arthritis, abscess, boils, blister, bone fracture, cancer, diabetes, diarrhea, coma, fever, nervous debility, spasmodic abdominal pain, eye infection, gout, heart ailment, hemorrhoids, high blood pressure, impotence, infectious diseases, influenza, irregular menses, kidney stone, night blindness, skin diseases, sprain, syphilis, throat infection, urine trouble, etc. (Bhutya 2011; Baiyeri and Akinnagbe 2013; Jain and Jain 2016).

Many pharmacological activities of *M. oleifera viz.* antimicrobial, anti-inflammatory, analgesic, antioxidant,

anticancer, anti-urolithiasis, antiasthmatic, antipyretic, anti-obesity, antihypertensive, hepatoprotective, hypoglycemic, hypolipidemic, immunomodulatory, cardiovascular, nootropic, antiulcer, etc., have been reported by several researchers (Bhattacharya et al. 2014; Amjad et al. 2015; Nfambi et al. 2015; Helmy et al. 2017; Martínez-González et al. 2017; Bhattacharya et al. 2018; Eremwanarue and Shittu 2018; Oboh et al. 2018; Islam et al. 2019; Patel and Lariya 2019; Xu et al. 2019; Kilany et al. 2020; Mabrouki et al. 2020; Padayachee and Baijnath 2020; Ali et al. 2021a; Barhoi et al. 2021; Kumolosasi et al. 2021; Palupi et al. 2021;). It is also recommended as a poultry diet due to its high nutritional and protein content (Taufek et al. 2022) and can reduce glucose in kampong chicken (Adli 2020). Because of its immense nutritional and pharmacological potential, its leaves and flowers were selected to evaluate in vitro thrombolytic potential.

MATERIALS AND METHODS

Collection, identification, and preparation of plant material

Leaves and flowers of *M. oleifera* were collected from an open land in Arvind Nagar, Sunderwas, Udaipur, Rajasthan, India. The plant was identified at the Department of Botany, Government Meera Girls College, Udaipur. A voucher specimen was preserved and further authenticated at Botanical Survey of India (BSI), Arid Zone Regional Centre, Jodhpur, Rajasthan (BSI/AZRC/I.12012/ Tech./2020-21- (Pl.Id.)/424 dated 08/02/2021, Sl. No. 4). The leaves and flowers were dried separately under shade. Dried plant parts were ground to make a fine powder for suitable plant extracts.

Preparation of plant extracts

Methanolic extracts

Methanolic extract- I (ME-I) – Five gram dried powder of leaves and flowers of *M. oleifera* were soaked in 50 mL methanol for 24 hours at room temperature with occasional stirring and filtered. This process was repeated three times with 50 mL of methanol, and then the last filtrate was evaporated in a boiling water bath at 40ºC and stored in sterile glass petri plates at 4ºC in the refrigerator. These extracts were named MEL-I (Methanolic extract of leaves-I) and MEF-I (Methanolic extract of flowers-I) and used for qualitative phytochemical analysis and preliminary evaluation of in vitro clot lysis activity.

Methanolic extract- II (ME-II) - 50 g dried powder of leaves and flowers of *M. oleifera* was soaked in 250 mL of methanol for eight days with occasional stirring and then filtered with Whatman's filter paper no. 1. The filtrates were evaporated in boiling water bath at 40ºC as described earlier (Ramjan et al. 2014). These dried extracts were stored at 4ºC in a refrigerator in sterile glass petri plates and named MEL-II (Methanolic extract of leaves-II) and MEF-II (Methanolic extract of flowers-II). MEL-II and MEF-II were used to evaluate in vitro clot lysis activity.

Aqueous extract

Four hundred milligrams of dried powder of leaves and flowers of *M. oleifera* was soaked in 20 mL distilled water, boiled for 20 minutes, and filtered with Whatman's filter paper no. 1. It was always prepared fresh for qualitative analysis of phytochemicals.

Qualitative phytochemical analysis

Suitable plant extracts (Aqueous and MEL-I and MEF-I) or dried plant powder of leaves and flowers of *M. oleifera* (wherever applicable) were screened for qualitative phytochemical analysis of amino acids, carbohydrates, terpenoids, steroids, cardiac glycosides, phlobatannins, flavonoids, polyphenols, tannins and saponins as per standard methodology (Anandjiwala et al. 2007; Jain et al. 2011; Adli 2020).

Test for amino acids

One milliliter of dilute HCL was added to two milliliters each of MEL-I and MEF-I and heated. Then, a few drops of concentrated $HNO_3$was added to the test tube. The development of yellow color indicated the presence of amino acids in the plant extracts.

Test for carbohydrates

It was performed by Fehling's test in which one milliliter each of Fehling solutions A and B was combined and added to one milliliter each of aqueous extracts of leaves and flowers in a test tube. It was then boiled in a water bath for two minutes. The appearance of a brick-red color precipitate determined the presence of carbohydrates.

Test for terpenoids

The Salkowski test was performed to detect the presence of terpenoids in the plant parts. First, in the test tube, two-milliliter chloroform was added to five-milliliter aqueous extracts of leaves and flowers each. Then, three milliliters of strong sulfuric acid were gently added along the test tube's wall to produce a layer. A reddish brown coloring of the interface indicated the presence of terpenoids.

Test for steroids

The Liebermann-Burchardt test was performed to detect the presence of steroids. First, one-milliliter chloroform was added to one milliliter each of MEL-I and MEF-I. Then, two-milliliter acetic anhydride was added, followed by the addition of two-three drops of strong sulfuric acid along the test tube's sidewalls. The development of dark green indicated the presence of steroids in the plant parts.

Test for cardiac glycosides

It was determined using the Keller-Kiliani test. First, two milliliters of glacial acetic acid were added to five milliliters of aqueous extracts of leaves and flowers each. Then one drop of ferric chloride solution was added to the test tubes. After that, one milliliter of concentrated sulfuric acid was added to the test tube's wall drop by drop. A reddish-brown ring at the intersection of two liquids indicated the presence of cardiac glycosides.

Test for phlobatannins

A few drops of one percent hydrochloric acid were added to one milliliter of aqueous extracts of both leaves and flowers and heated for two minutes. A crimson-red color precipitate indicated the presence of phlobatannins.

Test for flavonoids

It was performed using an ethyl acetate test in which 0.5 gram dried powder of leaves and flowers of *M. oleifera* each was boiled over a steam bath for three minutes after adding ten milliliters of ethyl acetate. After filtration, four milliliters of filtrates were shaken with one mL of dilute ammonia solution. The development of a yellow coloration indicated the presence of flavonoids.

Test for phenols

A few drops of neutral five percent ferric chloride solution were added to five milliliters of freshly prepared aqueous extracts of leaves and flowers. The development of dark green color indicated the presence of phenols.

Test for tannins

It was determined using Braemer's test that two milliliters of MEL-I and MEF-I were mixed in one milliliter of 10 % alcoholic ferric chloride solution. Dark blue or greenish grey color was considered for the presence of tannins.

Test for saponins

The presence of Saponin was determined by the Froth test. Five milliliters of water were added to one gram of dried powder of leaves and flowers in test tubes. The test tubes were vigorously shaken for five minutes for frothing and then kept still for ten minutes. Frothing indicated the presence of saponins in the plant materials.

Evaluation of in vitro thrombolytic activity

In vitro thrombolytic activity was evaluated as described by Prasad et al. (2006) and Prasad et al. (2007). For a preliminary assessment of in vitro thrombolytic activity, ME-I was used in the blood samples of five healthy volunteers. Then ME-II was used to assess in vitro thrombolytic activity in ten healthy volunteers. The study was conducted after institutional ethical approval (Ref.PMU/PMCH/IEC/2019, dated 26.12.2019) and obtaining informed consent. The experiment was performed following the Declaration of Helsinki 2004.

Preparation of plant extracts

A concentration of 10 mg/mL of all four extracts, MEL-I, MEF-I, MEL-II, and MEF-II, was prepared by suspending 25 mg crude methanolic extract in 2.5 mL of sterile distilled water, shaken vigorously on a vortex mixer and kept for overnight. That was filtered the following day using a syringe filter with 0.22 µ pore size to remove any microbial contamination and utilized to evaluate clot lysis.

Preparation of Streptokinase

Streptokinase, a well-known thrombolytic drug, was used as a positive control to evaluate in vitro thrombolytic activity. The commercially available lyophilized SK of 15,00,000 IU (STPase manufactured by Cadila Pharmaceuticals, Ahmedabad, India) was dissolved in 5 mL of sterile distilled water and mixed thoroughly, which served as a stock solution from which 100 µL (30,000 IU) was used for the test.

In vitro clot lysis activity

Ten mL of venous blood samples were drawn as per the study protocol in a fasting state from healthy volunteers irrespective of gender and who were not taking any medication, oral contraceptives, and anticoagulant therapy. First, 500 µL of blood was poured into previously weighed micro-centrifuge tubes and incubated at 37ºC for 45 min for clot formation. After 45 minutes, tubes were centrifuged at 2,000 rpm for 10 min to remove the serum altogether, and tubes were again weighed to determine the weight of the clot (Clot weight = weight of micro-centrifuge tube having clot – weight of micro-centrifuge tube).

100 µL of plant extracts, MEL-I, MEF-I, MEL-II, and MEF-II; 100 µL sterile distilled water (negative control) and 100 µL of Streptokinase (positive control) were added to micro-centrifuge tubes. All the tubes were incubated at 37ºC for 90 min. After that, fluid obtained after clot lysis was removed carefully from the tube using a micropipette, and tubes were re-weighed to determine the weight of the clot after lysis (weight of micro-centrifuge tube having clot – weight of micro-centrifuge tube after the clot lysis). The difference in clot weight after lysis was expressed as % clot lysis = weight of clot after lysis/weight of clot × 100. All the experiment was done in triplicate.

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM) for three replicates. Statistical comparisons are made using Student's Paired t-test using Microsoft Excel (2010).

RESULTS AND DISCUSSION

Phytochemical screening is required to determine the presence of therapeutic and physiologically important classes of bioactive compounds present in the plant material. Furthermore, this support provides a base for future quantitative phytochemical studies (Rahman et al. 2020). Preliminary qualitative phytochemical screening has shown the presence of secondary metabolites such as flavonoids, terpenoids, cardiac glycosides, saponins, and tannins besides primary metabolites, amino acids, and carbohydrates in leaves and flowers of *M. oleifera* collected from Udaipur. However, the absence of phenols and steroids in flowers and phlobatannin in the leaves of *M. oleifera* was also observed (Table 1). Flavonoids are well known for their antioxidant, anti-inflammatory, anti-diabetic, cardio-protective, antiviral, antimicrobial, and anti-proliferative potential (Wang et al. 2018).

Similarly, phenolic compounds also possess beneficial health activities such as anticancer, antioxidant, anti-

inflammatory, antimicrobial, and antithrombotic (Kumar and Goel 2019), whereas cardiac glycosides are found to be helpful in cardiac ailments and cancer (Kumavath et al. 2021). Cardiovascular benefits of terpenoids, besides hypoglycemic, anticancer, anti-inflammatory, antioxidant, and neuroprotective properties, have also been demonstrated in various scientific studies (Yang et al. 2020). Further, quantitative estimation of these prospected heart-beneficial phytochemicals in the leaves and flowers of *M. oleifera* is also required. In the present study, for the first time, both leaves and flowers of *M. oleifera* have been shown to possess significant in vitro clot lysis potential compared to distilled water as a negative control and SK as a positive control (Table 2).

Preliminary assessment of 100 µL methanolic extract of *M. oleifera* leaves (MEL-I) having a concentration of 1 mg/mL, demonstrated 36.64±1.55% in vitro clot lysis activity whereas 100 µL SK, as a positive control, demonstrated 47.98±1.34 percent clot lysis and 100 µL sterile distilled water (negative control) has shown negligible clot lysis of 3.12±0.43%. The second methanolic extract of leaves (MEL-II) demonstrated a significant ($p<0.0001$) in vitro clot lysis activity of 41.40±2.02% as compared to positive control SK, which exhibited 53.73±0.97% clot lysis and distilled water with 2.67±0.30% clot lysis activity.

As compared to leaves, flowers of *M. oleifera* have demonstrated less thrombolytic potential. MEF-1 has shown significant ($p<0.001$) clot lysis of 19.07±2.36% compared to negative control of 4.78±0.40%. MEF-II also showed 20.52±1.51% clot lysis, whereas SK exhibited 48.91±0.51% clot lysis, and distilled water demonstrated negligible 2.9±0.18% clot lysis activity. The mean differences in clot lysis percentage between positive and negative control were significant in all the observations. The results clearly indicate that water does not affect in vitro thrombolysis; hence, the contribution of water to the thrombolytic efficacy of *M. oleifera* could be considered nil. Besides, the effect of preparing methanolic extracts with two different techniques did not significantly impact the thrombolytic effectiveness of both leaves and flowers. However, this needs further detailed studies with statistical approval.

Several plant species have shown similar percent thrombolytic potential (in vitro), for example, 41.46% clot lysis activity by methanolic extract of *Cassia senna* leaves (Hossain et al. 2012), 32.58% by leaves of *Leea indica* (Sakib et al. 2021), 33.31% by leaves of *Homalomena aromatica* (Ali et al. 2021b), 34.72% by leaves of *Ficus cunia* (Hasanat et al. 2019), 21.64% by leaves of *Antidesma cuspidatum*, 20.74% by leaves of *Scaphium macropodum* and 19.90% by leaves of *Uncaria acida* (Azad et al. 2018), etc. Interestingly, the vasodilator activity of some other species of *Moringa*, such as *M. stenopetala* leaves, has also been shown in guinea pigs (Geleta et al. 2016).

Plants with antioxidant and anti-inflammatory potential are shown to possess anticoagulant effects (Lamponi 2021). Leaves of *M. oleifera* have also been shown to possess in vitro antioxidant potential (Fitriana et al. 2016) and anti-inflammatory potential (Xu et al. 2019). Similarly, flowers of *M. oleifera* have also shown in vitro anti-inflammatory activity (Alhakmani et al. 2013) and in vitro antioxidant potential (Santos et al. 2012). Furthermore, phenolic compounds and flavonoids are potent antioxidants and have anticoagulant properties (Bijak et al. 2016; Lamponi 2021). In this regard, leaves of *M. oleifera* are rich in various phytoconstituents, for example, flavonoids like quercetin, isoquercetin, quercetrin, kaemfericitin, kaempferol, isothiocyanates, hyperoside, and glycoside compounds beta-l-rhamnofuranoside, polyphenols, n-hexadecanoic acid, tetradecanoic acid, *cis*-vaccenic acid, octadecanoic acid, palmitoyl chloride, 5-*O*-acetyl-thio-octyl, gamma-sitosterol and pregna-7-diene-3-ol-20-one (Amjad et al. 2015; Bhattacharya et al. 2018; Mabrouki et al. 2020).

Table 1. Qualitative preliminary phytochemical analysis of leaves and flowers of *M. oleifera*

Phytochemical test	*M. oleifera* leaves	*M. oleifera* flowers
Saponin (*Froth test*)	+	+
Carbohydrate (*Fehling's test*)	+	+
Amino acid	+	+
Flavanoid (*Ethyl acetate test*)	+	+
Phenol (*Ferric chloride test*)	+	-
Tannin (*Braemer's test*)	+	+
Phlobatannin	-	+
Terpenoid (*Salkowski test*)	+	+
Cardiac glycoside (*Keller-kiliani test*)	+	+
Steroid (*Liebermann burchard test*)	+	-

Note: +: present, -: absent

Table 2. In vitro percent clot lysis activity was obtained for methanolic extracts of *M. oleifera* leaves and flowers (MEL-I, MEL-II, MEF-I, MEF-II), Streptokinase (30000 IU) as the positive control, and Distilled water as the negative control

Plant extract	n	Percent clot lysis (Mean±SEM)		
		Plant extract (I)	Streptokinase (II)	Distilled water (III)
MEL-I	5	36.64±1.55	47.98±1.34[a]	3.12±0.43[b,c]
MEL-II	10	41.40±2.02	53.73±0.97[a]	2.67±0.30 [b,c]
MEF-I	5	19.07±2.36	47.13±1.06 [a]	4.78±0.40 [c,d]
MEF-II	10	20.52±1.51	48.91±0.51[a]	2.9±0.18 [b,c]

Note: p-value: a. I v/s II; $p<0.0001$, b. I v/s III; $p<0.0001$, c. II v/s III; $p<0.0001$, d. I v/s III; $p<0.001$, Values are expressed as Mean ± SEM

Fresh leaves of *M. oleifera* contain 220 mg/100 g of Vitamin C, seven times more than oranges. Interestingly, vitamin C is linked with reduced risk of cardiovascular diseases (CVD) through its antioxidant and antiplatelet effects, and recent studies have shown the role of JAK-STAT, STAT, PD1, EGFR, FoxO, and chemokines signaling pathways as protection mechanisms (Gopalakrishnan et al. 2016; Zhu et al. 2021). Similarly, fresh leaves are also rich in vitamin E (448 mg/100 g), which also acts as a significant antioxidant and anti-inflammatory agent, helping with CVD (Ziegler et al. 2020). Furthermore, administration of an alkaloid, N,α-L-rhamnopyranosyl vincosamide, isolated from its leaves has shown inhibition of the ST segment elevation and heart rate and decrease of necrotic cells of cardiac muscle after seven days in cardiotoxic experimental rat models (Panda et al. 2013). Furthermore, a nitrile glycoside, niazirin, isolated from leaves has shown antioxidant activity and attenuation of the proliferation of high glucose-induced vascular smooth muscle cells (Wang et al. 2021). All such compounds might be responsible for the thrombolytic potential of *M. oleifera*, as demonstrated in the present study. However, detailed studies are required to isolate bioactive molecules responsible for clot lysis.

Abnormal thrombus formation is considered the main culprit behind cardiovascular diseases. Plants with antiplatelet, antioxidant, thrombolytic, and fibrinolytic potential can reduce the risk of cardiovascular diseases and may be utilized as a dietary intake for the prevention and early onset of such diseases. Therefore, plants can act like nutraceuticals by providing nutrition and therapeutic efficacy simultaneously and could be a better alternative than costly synthetic drugs (Aune et al. 2017; Alves et al. 2019; Albadawi et al. 2022). Plant-based diets help reduce the risk of cardiovascular diseases and prevent other mortality causes such as cancer (Aune et al. 2017). Several ethnic communities of India consume leaves, flowers, and fruits of the plant as edible (Jain and Jain 2016). Nutritionally, leaves of *M. oleifera* are rich in vitamin-A, vitamin B-choline, vitamin B1, riboflavin, nicotinic acid, ascorbic acid, E-lutein, minerals such as Ca, Mg, P, Na, K, Cu, Fe, Mn, Zn and S, amino acids like Arg, His, Lys, Trp, Phe, Thr, Leu, Met, Ile, Val, proteins and fiber content. Its flowers also contain calcium, potassium, amino acids, sucrose, alkaloids, and flavonoids, such as rhamnetin, isoquercitrin, and kaempferitrin (Gopalakrishnan et al. 2016; Bhattacharya et al. 2018; Ercan et al. 2021). Recently, enzymatically modified isoquercitrin having a 17-fold higher bioavailability than quercetin aglycone, has demonstrated significantly improved endothelial function and increased concentration of circulating quercetin metabolites in human volunteers at risk of CVD (Bondonno et al. 2020). That further implies the potential role of using Moringa as a dietary supplement to prevent CVD. High protein and fiber-rich leaves of *M. oleifera* are used for preparing various snacks and herbal tea and are also consumed to treat malnutrition and anemia (Baiyeri and Akinnagbe 2013; Alia et al. 2022). Along with the cardio-vascular beneficial uses of its chemical constituents, *M. oleifera* could be better utilized as an effective nutraceutical against thrombosis.

Leaves of *M. oleifera* have also demonstrated a reduction in cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), and malondialdehyde levels in hyperlipidemic adult male albino rats after 60 days of administration of its extract at a dose of 400 mg/kg bw (Helmy et al. 2017). The hypolipidemic potential of hydroalcoholic extract of *M. oleifera* leaves was also observed in male wistar rats. An interesting observation of this study was a significant ($p<0.001$) increase in high-density lipoprotein cholesterol (HDL-C) and a significant ($p<0.001$) decrease in the atherogenic index after 28 days of administration of *M. oleifera* extract (Rajanandh et al. 2012) both of which are considered as crucial parameters of cardiovascular diseases (Mahdy et al. 2012; Kazemi et al. 2018). Antiobesity and hypolipidemic effects were also observed after administration of a herbal extract combination comprising 60% ethanolic extract of *M. oleifera* leaves in a dose of 900 mg for 16 weeks in 66 healthy overweight adults. A significant reduction in body weight, body mass index, total body fat, waist, hip circumferences, LDL-cholesterol, and HDL cholesterol increase were observed (Dixit et al. 2018).

Moringa oleifera leaves have also shown significant hypoglycemic potential in various scientific studies. Khan et al. (2017) have demonstrated in vivo hypoglycemic effect of aqueous extract of *M. oleifera* leaves in streptozotocin and high-fat diet-induced diabetes in female wistar rats. Significant ($p<0.05$) restoration of fasting blood glucose, lipid profile, and liver marker enzyme levels was observed in both experimental models. Methanolic extract from *M. oleifera* leaves also showed a protective effect against oxidative stress in the heart of diabetic rats (Aju et al. 2019).

A recent study by Mabrouki et al. (2020) has shown significant reductions in the levels of cardiac catalase, glutathione peroxidase, and superoxide dismutase activities along with an increase in malondialdehyde in the high-fat diet-induced obese rats administered with methanolic extract of *M. oleifera* leaves for 12 weeks (200 mg/kg/bw and 400 mg/kg/bw). The study also exhibited that this effect of *M. oleifera* leaves could be attributed to its phytochemical content and antioxidant potential. Furthermore, the vasodilator effect of aqueous extract of leaves of *M. oleifera* in doses of 30 and 60 mg/kg/day was observed in L-NAME (Nω-nitro-L-arginine-methyl ester) induced hypertensive rats after three weeks. A significant reduction in blood pressure and heart rate and impairment in acetylcholine-induced mesenteric arterial relaxation was observed, along with a decrease in vascular O_{2^-} production and plasma malondialdehyde levels. This dose-dependent vasorelaxation in the endothelium of mesenteric arterial beds could be due to the release of endothelium-derived relaxing factors. In this regard, the present findings of in vitro thrombolytic action further corroborate the vasodilator potential of *M. oleifera* (Aekthammarat et al. 2019; Aekthammarat et al. 2020).

Because of the activities mentioned above and having thrombolytic potential, *M. oleifera* thus could be called a

herbal polypill by protecting against high blood pressure, high blood sugar, and higher lipid parameters in already known cases of ischemic heart disease and metabolic syndrome that have increased tendency of clot formation. Furthermore, it could also be helpful in the secondary prevention of ischemic heart disease.

The present study has shown in vitro thrombolytic potential of methanolic extracts of leaves and flowers of *M. oleifera* for the first time. However, further in vivo studies for thrombolytic potential are warranted, along with identification and characterization studies of corresponding bioactive molecules to discover safe thrombolytic agents.

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In vitro thrombolytic activity of *Moringa oleifera*

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Abstract. *Kunwar B, Jain V, Verma SK. 2022. In vitro thrombolytic activity of* Moringa oleifera. *Nusantara Bioscience 14: 63-69. Moringa oleifera* Lam. (Family Moringaceae) is a medium-sized perennial tree, commonly known as drumstick tree, horse radish tree, miracle tree, *sahjan, shobhanjana, munga arak*, etc., in different languages. Leaves, flowers, and pods of the plant are edible. Various parts of the plant are utilized to treat several diseases by ethnic communities, including heart ailments. Leaves and flowers of *M. oleifera* were assessed for preliminary qualitative phytochemical analysis and in vitro thrombolytic potential. Preliminary phytochemical screening has shown the presence of flavonoids, terpenoids, cardiac glycosides, saponins, tannins, amino acids, and carbohydrates in the leaves and flowers of *M. oleifera*. Two types of methanolic extracts of both leaves (MEL-I and MEL-II) and flowers (MEF-I and MEF-II) were used to assess percent clot lysis. A significant in vitro clot lysis activity of MEL-I (36.64±1.55%), MEL-II (41.40±2.02%), and MEF-I (19.07±2.36%), and MEF-II (20.52±1.51%) was demonstrated in a concentration of one mg/mL as compared to negative control distilled water and positive control streptokinase for the first time. The edible plant's observed thrombolytic potential may be employed to prevent athero-thrombotic cardiovascular diseases. Further investigations are required to isolate the bioactive molecule responsible for the thrombolytic action.

Keywords: Clot lysis, drumstick, *Moringa oleifera*, quercetin, Streptokinase

INTRODUCTION

Thrombus is a natural physiological response of the body to prevent hemorrhage by forming a blood clot associated with circulating platelets, thrombin, and fibrin fibers. In the absence of any stimulus from the intravascular damage, significant abnormalities such as ischemia, stroke, heart attack, and deep vein thrombosis happen. The formation of a thrombus in the coronary artery leads to interference in blood circulation to the heart and results in myocardial infarction (Furie and Furie, 2008; Gaziano and Gaziano, 2018). Therefore, a Lysis of thrombus is, known as thrombolysis, urgently required during heart attack and stroke cases. For this purpose, various synthetic thrombolytic agents, such as tissue plasminogen activator (t-PA), urokinase (UK), Streptokinase (SK), alteplase, etc., are widely used to provide immediate clearing of vessels. However, these synthetic thrombolytic drugs may pose a risk of severe bleeding, anaphylactic reactions, and shock (Collen 1990). Therefore, searching for a better alternative as a thrombolytic agent regarding cost-effectiveness, safety and efficiency are required. Natural resources are generally considered safe and may provide better efficacy. In this regard, plants with fibrinolytic, thrombolytic, and antiplatelet potential can be used to reduce the risk of thrombosis. Moreover, suppose plants with such efficacy could be a part of diets. In that case, it could also help prevent the onset of such diseases as scientific studies have shown that a daily intake of five servings of plant-based foods ranging from 400-800 g/day could help in risk reduction for cardiovascular diseases (Yu et al. 2018).

The Moringa genus belongs to Family Moringaceae, a monotypic family with 13 species distributed in Asia and Africa. *Moringa oleifera* Lam. is a popular plant commonly found in North-West India, the sub-Himalayan tract, from Chenab eastward to Sarda, and cultivated all over the plains of India. It is a medium-sized (about 10 m) perennial tree, known as drumstick tree, horse radish tree, miracle tree, mother's best friend, *sahjan*, *sainjna*, *shobhanjana*, *mungna*, *shevgi*, *sehjan*, *munga arak*, etc., in various languages. Leaves are tripinnate with entire and glabrous leaflets. Flowers are odorant with five unequal yellowish-white petals. The fruit is a hanging pod with a 9-ribbed brown capsule having dark brown, globular seeds. The tree's leaves, flowers, and fruits are edible and used as vegetables (The Wealth of India 1962; Tiagi and Aery 2007; Abd Rani et al. 2018).

Moringa oleifera is a good source of vitamins, proteins, and minerals. Its plant parts are used in traditional medicine for the treatment of various human diseases, for example, anemia, arthritis, abscess, boils, blister, bone fracture, cancer, diabetes, diarrhea, coma, fever, nervous debility, spasmodic abdominal pain, eye infection, gout, heart ailment, hemorrhoids, high blood pressure, impotence, infectious diseases, influenza, irregular menses, kidney stone, night blindness, skin diseases, sprain, syphilis, throat infection, urine trouble, etc. (Bhutya 2011; Baiyeri and Akinnagbe 2013; Jain and Jain 2016).

Many pharmacological activities of *M. oleifera viz.* antimicrobial, anti-inflammatory, analgesic, antioxidant,

anticancer, anti-urolithiasis, antiasthmatic, antipyretic, anti-obesity, antihypertensive, hepatoprotective, hypoglycemic, hypolipidemic, immunomodulatory, cardiovascular, nootropic, antiulcer, etc., have been reported by several researchers (Bhattacharya et al. 2014; Amjad et al. 2015; Nfambi et al. 2015; Helmy et al. 2017; Martínez-González et al. 2017; Bhattacharya et al. 2018; Eremwanarue and Shittu 2018; Oboh et al. 2018; Islam et al. 2019; Patel and Lariya 2019; Xu et al. 2019; Kilany et al. 2020; Mabrouki et al. 2020; Padayachee and Baijnath 2020; Ali et al. 2021a; Barhoi et al. 2021; Kumolosasi et al. 2021; Palupi et al. 2021;). It is also recommended as a poultry diet due to its high nutritional and protein content (Taufek et al. 2022) and can reduce glucose in kampong chicken (Adli 2020). Because of its immense nutritional and pharmacological potential, its leaves and flowers were selected to evaluate in vitro thrombolytic potential.

MATERIALS AND METHODS

Collection, identification, and preparation of plant material

Leaves and flowers of *M. oleifera* were collected from an open land in Arvind Nagar, Sunderwas, Udaipur, Rajasthan, India. The plant was identified at the Department of Botany, Government Meera Girls College, Udaipur. A voucher specimen was preserved and further authenticated at Botanical Survey of India (BSI), Arid Zone Regional Centre, Jodhpur, Rajasthan (BSI/AZRC/I.12012/ Tech./2020-21- (Pl.Id.)/424 dated 08/02/2021, Sl. No. 4). The leaves and flowers were dried separately under shade. Dried plant parts were ground to make a fine powder for suitable plant extracts.

Preparation of plant extracts

Methanolic extracts

Methanolic extract- I (ME-I) – Five gram dried powder of leaves and flowers of *M. oleifera* were soaked in 50 mL methanol for 24 hours at room temperature with occasional stirring and filtered. This process was repeated three times with 50 mL of methanol, and then the last filtrate was evaporated in a boiling water bath at 40ºC and stored in sterile glass petri plates at 4ºC in the refrigerator. These extracts were named MEL-I (Methanolic extract of leaves-I) and MEF-I (Methanolic extract of flowers-I) and used for qualitative phytochemical analysis and preliminary evaluation of in vitro clot lysis activity.

Methanolic extract- II (ME-II) - 50 g dried powder of leaves and flowers of *M. oleifera* was soaked in 250 mL of methanol for eight days with occasional stirring and then filtered with Whatman's filter paper no. 1. The filtrates were evaporated in boiling water bath at 40ºC as described earlier (Ramjan et al. 2014). These dried extracts were stored at 4ºC in a refrigerator in sterile glass petri plates and named MEL-II (Methanolic extract of leaves-II) and MEF-II (Methanolic extract of flowers-II). MEL-II and MEF-II were used to evaluate in vitro clot lysis activity.

Aqueous extract

Four hundred milligrams of dried powder of leaves and flowers of *M. oleifera* was soaked in 20 mL distilled water, boiled for 20 minutes, and filtered with Whatman's filter paper no. 1. It was always prepared fresh for qualitative analysis of phytochemicals.

Qualitative phytochemical analysis

Suitable plant extracts (Aqueous and MEL-I and MEF-I) or dried plant powder of leaves and flowers of *M. oleifera* (wherever applicable) were screened for qualitative phytochemical analysis of amino acids, carbohydrates, terpenoids, steroids, cardiac glycosides, phlobatannins, flavonoids, polyphenols, tannins and saponins as per standard methodology (Anandjiwala et al. 2007; Jain et al. 2011; Adli 2020).

Test for amino acids

One milliliter of dilute HCL was added to two milliliters each of MEL-I and MEF-I and heated. Then, a few drops of concentrated $HNO_3$was added to the test tube. The development of yellow color indicated the presence of amino acids in the plant extracts.

Test for carbohydrates

It was performed by Fehling's test in which one milliliter each of Fehling solutions A and B was combined and added to one milliliter each of aqueous extracts of leaves and flowers in a test tube. It was then boiled in a water bath for two minutes. The appearance of a brick-red color precipitate determined the presence of carbohydrates.

Test for terpenoids

The Salkowski test was performed to detect the presence of terpenoids in the plant parts. First, in the test tube, two-milliliter chloroform was added to five-milliliter aqueous extracts of leaves and flowers each. Then, three milliliters of strong sulfuric acid were gently added along the test tube's wall to produce a layer. A reddish brown coloring of the interface indicated the presence of terpenoids.

Test for steroids

The Liebermann-Burchardt test was performed to detect the presence of steroids. First, one-milliliter chloroform was added to one milliliter each of MEL-I and MEF-I. Then, two-milliliter acetic anhydride was added, followed by the addition of two-three drops of strong sulfuric acid along the test tube's sidewalls. The development of dark green indicated the presence of steroids in the plant parts.

Test for cardiac glycosides

It was determined using the Keller-Kiliani test. First, two milliliters of glacial acetic acid were added to five milliliters of aqueous extracts of leaves and flowers each. Then one drop of ferric chloride solution was added to the test tubes. After that, one milliliter of concentrated sulfuric acid was added to the test tube's wall drop by drop. A reddish-brown ring at the intersection of two liquids indicated the presence of cardiac glycosides.

Test for phlobatannins

A few drops of one percent hydrochloric acid were added to one milliliter of aqueous extracts of both leaves and flowers and heated for two minutes. A crimson-red color precipitate indicated the presence of phlobatannins.

Test for flavonoids

It was performed using an ethyl acetate test in which 0.5 gram dried powder of leaves and flowers of *M. oleifera* each was boiled over a steam bath for three minutes after adding ten milliliters of ethyl acetate. After filtration, four milliliters of filtrates were shaken with one mL of dilute ammonia solution. The development of a yellow coloration indicated the presence of flavonoids.

Test for phenols

A few drops of neutral five percent ferric chloride solution were added to five milliliters of freshly prepared aqueous extracts of leaves and flowers. The development of dark green color indicated the presence of phenols.

Test for tannins

It was determined using Braemer's test that two milliliters of MEL-I and MEF-I were mixed in one milliliter of 10 % alcoholic ferric chloride solution. Dark blue or greenish grey color was considered for the presence of tannins.

Test for saponins

The presence of Saponin was determined by the Froth test. Five milliliters of water were added to one gram of dried powder of leaves and flowers in test tubes. The test tubes were vigorously shaken for five minutes for frothing and then kept still for ten minutes. Frothing indicated the presence of saponins in the plant materials.

Evaluation of in vitro thrombolytic activity

In vitro thrombolytic activity was evaluated as described by Prasad et al. (2006) and Prasad et al. (2007). For a preliminary assessment of in vitro thrombolytic activity, ME-I was used in the blood samples of five healthy volunteers. Then ME-II was used to assess in vitro thrombolytic activity in ten healthy volunteers. The study was conducted after institutional ethical approval (Ref.PMU/PMCH/IEC/2019, dated 26.12.2019) and obtaining informed consent. The experiment was performed following the Declaration of Helsinki 2004.

Preparation of plant extracts

A concentration of 10 mg/mL of all four extracts, MEL-I, MEF-I, MEL-II, and MEF-II, was prepared by suspending 25 mg crude methanolic extract in 2.5 mL of sterile distilled water, shaken vigorously on a vortex mixer and kept for overnight. That was filtered the following day using a syringe filter with 0.22 µ pore size to remove any microbial contamination and utilized to evaluate clot lysis.

Preparation of Streptokinase

Streptokinase, a well-known thrombolytic drug, was used as a positive control to evaluate in vitro thrombolytic activity. The commercially available lyophilized SK of 15,00,000 IU (STPase manufactured by Cadila Pharmaceuticals, Ahmedabad, India) was dissolved in 5 mL of sterile distilled water and mixed thoroughly, which served as a stock solution from which 100 µL (30,000 IU) was used for the test.

In vitro clot lysis activity

Ten mL of venous blood samples were drawn as per the study protocol in a fasting state from healthy volunteers irrespective of gender and who were not taking any medication, oral contraceptives, and anticoagulant therapy. First, 500 µL of blood was poured into previously weighed micro-centrifuge tubes and incubated at 37ºC for 45 min for clot formation. After 45 minutes, tubes were centrifuged at 2,000 rpm for 10 min to remove the serum altogether, and tubes were again weighed to determine the weight of the clot (Clot weight = weight of micro-centrifuge tube having clot – weight of micro-centrifuge tube).

100 µL of plant extracts, MEL-I, MEF-I, MEL-II, and MEF-II; 100 µL sterile distilled water (negative control) and 100 µL of Streptokinase (positive control) were added to micro-centrifuge tubes. All the tubes were incubated at 37ºC for 90 min. After that, fluid obtained after clot lysis was removed carefully from the tube using a micropipette, and tubes were re-weighed to determine the weight of the clot after lysis (weight of micro-centrifuge tube having clot – weight of micro-centrifuge tube after the clot lysis). The difference in clot weight after lysis was expressed as % clot lysis = weight of clot after lysis/weight of clot × 100. All the experiment was done in triplicate.

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM) for three replicates. Statistical comparisons are made using Student's Paired t-test using Microsoft Excel (2010).

RESULTS AND DISCUSSION

Phytochemical screening is required to determine the presence of therapeutic and physiologically important classes of bioactive compounds present in the plant material. Furthermore, this support provides a base for future quantitative phytochemical studies (Rahman et al. 2020). Preliminary qualitative phytochemical screening has shown the presence of secondary metabolites such as flavonoids, terpenoids, cardiac glycosides, saponins, and tannins besides primary metabolites, amino acids, and carbohydrates in leaves and flowers of *M. oleifera* collected from Udaipur. However, the absence of phenols and steroids in flowers and phlobatannin in the leaves of *M. oleifera* was also observed (Table 1). Flavonoids are well known for their antioxidant, anti-inflammatory, anti-diabetic, cardio-protective, antiviral, antimicrobial, and anti-proliferative potential (Wang et al. 2018).

Similarly, phenolic compounds also possess beneficial health activities such as anticancer, antioxidant, anti-

inflammatory, antimicrobial, and antithrombotic (Kumar and Goel 2019), whereas cardiac glycosides are found to be helpful in cardiac ailments and cancer (Kumavath et al. 2021). Cardiovascular benefits of terpenoids, besides hypoglycemic, anticancer, anti-inflammatory, antioxidant, and neuroprotective properties, have also been demonstrated in various scientific studies (Yang et al. 2020). Further, quantitative estimation of these prospected heart-beneficial phytochemicals in the leaves and flowers of *M. oleifera* is also required. In the present study, for the first time, both leaves and flowers of *M. oleifera* have been shown to possess significant in vitro clot lysis potential compared to distilled water as a negative control and SK as a positive control (Table 2).

Preliminary assessment of 100 µL methanolic extract of *M. oleifera* leaves (MEL-I) having a concentration of 1 mg/mL, demonstrated 36.64±1.55% in vitro clot lysis activity whereas 100 µL SK, as a positive control, demonstrated 47.98±1.34 percent clot lysis and 100 µL sterile distilled water (negative control) has shown negligible clot lysis of 3.12±0.43%. The second methanolic extract of leaves (MEL-II) demonstrated a significant ($p<0.0001$) in vitro clot lysis activity of 41.40±2.02% as compared to positive control SK, which exhibited 53.73±0.97% clot lysis and distilled water with 2.67±0.30% clot lysis activity.

As compared to leaves, flowers of *M. oleifera* have demonstrated less thrombolytic potential. MEF-1 has shown significant ($p<0.001$) clot lysis of 19.07±2.36% compared to negative control of 4.78±0.40%. MEF-II also showed 20.52±1.51% clot lysis, whereas SK exhibited 48.91±0.51% clot lysis, and distilled water demonstrated negligible 2.9±0.18% clot lysis activity. The mean differences in clot lysis percentage between positive and negative control were significant in all the observations. The results clearly indicate that water does not affect in vitro thrombolysis; hence, the contribution of water to the thrombolytic efficacy of *M. oleifera* could be considered nil. Besides, the effect of preparing methanolic extracts with two different techniques did not significantly impact the thrombolytic effectiveness of both leaves and flowers. However, this needs further detailed studies with statistical approval.

Several plant species have shown similar percent thrombolytic potential (in vitro), for example, 41.46% clot lysis activity by methanolic extract of *Cassia senna* leaves (Hossain et al. 2012), 32.58% by leaves of *Leea indica* (Sakib et al. 2021), 33.31% by leaves of *Homalomena aromatica* (Ali et al. 2021b), 34.72% by leaves of *Ficus cunia* (Hasanat et al. 2019), 21.64% by leaves of *Antidesma cuspidatum*, 20.74% by leaves of *Scaphium macropodum* and 19.90% by leaves of *Uncaria acida* (Azad et al. 2018), etc. Interestingly, the vasodilator activity of some other species of *Moringa*, such as *M. stenopetala* leaves, has also been shown in guinea pigs (Geleta et al. 2016).

Plants with antioxidant and anti-inflammatory potential are shown to possess anticoagulant effects (Lamponi 2021). Leaves of *M. oleifera* have also been shown to possess in vitro antioxidant potential (Fitriana et al. 2016) and anti-inflammatory potential (Xu et al. 2019). Similarly, flowers of *M. oleifera* have also shown in vitro anti-inflammatory activity (Alhakmani et al. 2013) and in vitro antioxidant potential (Santos et al. 2012). Furthermore, phenolic compounds and flavonoids are potent antioxidants and have anticoagulant properties (Bijak et al. 2016; Lamponi 2021). In this regard, leaves of *M. oleifera* are rich in various phytoconstituents, for example, flavonoids like quercetin, isoquercetin, quercetrin, kaemfericitin, kaempferol, isothiocyanates, hyperoside, and glycoside compounds beta-l-rhamnofuranoside, polyphenols, n-hexadecanoic acid, tetradecanoic acid, *cis*-vaccenic acid, octadecanoic acid, palmitoyl chloride, 5-*O*-acetyl-thio-octyl, gamma-sitosterol and pregna-7-diene-3-ol-20-one (Amjad et al. 2015; Bhattacharya et al. 2018; Mabrouki et al. 2020).

Table 1. Qualitative preliminary phytochemical analysis of leaves and flowers of *M. oleifera*

Phytochemical test	*M. oleifera* leaves	*M. oleifera* flowers
Saponin (*Froth test*)	+	+
Carbohydrate (*Fehling's test*)	+	+
Amino acid	+	+
Flavanoid (*Ethyl acetate test*)	+	+
Phenol (*Ferric chloride test*)	+	-
Tannin (*Braemer's test*)	+	+
Phlobatannin	-	+
Terpenoid (*Salkowski test*)	+	+
Cardiac glycoside (*Keller-kiliani test*)	+	+
Steroid (*Liebermann burchard test*)	+	-

Note: +: present, -: absent

Table 2. In vitro percent clot lysis activity was obtained for methanolic extracts of *M. oleifera* leaves and flowers (MEL-I, MEL-II, MEF-I, MEF-II), Streptokinase (30000 IU) as the positive control, and Distilled water as the negative control

Plant extract	n	Percent clot lysis (Mean±SEM)		
		Plant extract (I)	Streptokinase (II)	Distilled water (III)
MEL-I	5	36.64±1.55	47.98±1.34[a]	3.12±0.43[b,c]
MEL-II	10	41.40±2.02	53.73±0.97[a]	2.67±0.30 [b,c]
MEF-I	5	19.07±2.36	47.13±1.06 [a]	4.78±0.40 [c,d]
MEF-II	10	20.52±1.51	48.91±0.51[a]	2.9±0.18 [b,c]

Note: p-value: a. I v/s II; $p<0.0001$, b. I v/s III; $p<0.0001$, c. II v/s III; $p<0.0001$, d. I v/s III; $p<0.001$, Values are expressed as Mean ± SEM

Fresh leaves of *M. oleifera* contain 220 mg/100 g of Vitamin C, seven times more than oranges. Interestingly, vitamin C is linked with reduced risk of cardiovascular diseases (CVD) through its antioxidant and antiplatelet effects, and recent studies have shown the role of JAK-STAT, STAT, PD1, EGFR, FoxO, and chemokines signaling pathways as protection mechanisms (Gopalakrishnan et al. 2016; Zhu et al. 2021). Similarly, fresh leaves are also rich in vitamin E (448 mg/100 g), which also acts as a significant antioxidant and anti-inflammatory agent, helping with CVD (Ziegler et al. 2020). Furthermore, administration of an alkaloid, N,α-L-rhamnopyranosyl vincosamide, isolated from its leaves has shown inhibition of the ST segment elevation and heart rate and decrease of necrotic cells of cardiac muscle after seven days in cardiotoxic experimental rat models (Panda et al. 2013). Furthermore, a nitrile glycoside, niazirin, isolated from leaves has shown antioxidant activity and attenuation of the proliferation of high glucose-induced vascular smooth muscle cells (Wang et al. 2021). All such compounds might be responsible for the thrombolytic potential of *M. oleifera*, as demonstrated in the present study. However, detailed studies are required to isolate bioactive molecules responsible for clot lysis.

Abnormal thrombus formation is considered the main culprit behind cardiovascular diseases. Plants with antiplatelet, antioxidant, thrombolytic, and fibrinolytic potential can reduce the risk of cardiovascular diseases and may be utilized as a dietary intake for the prevention and early onset of such diseases. Therefore, plants can act like nutraceuticals by providing nutrition and therapeutic efficacy simultaneously and could be a better alternative than costly synthetic drugs (Aune et al. 2017; Alves et al. 2019; Albadawi et al. 2022). Plant-based diets help reduce the risk of cardiovascular diseases and prevent other mortality causes such as cancer (Aune et al. 2017). Several ethnic communities of India consume leaves, flowers, and fruits of the plant as edible (Jain and Jain 2016). Nutritionally, leaves of *M. oleifera* are rich in vitamin-A, vitamin B-choline, vitamin B1, riboflavin, nicotinic acid, ascorbic acid, E-lutein, minerals such as Ca, Mg, P, Na, K, Cu, Fe, Mn, Zn and S, amino acids like Arg, His, Lys, Trp, Phe, Thr, Leu, Met, Ile, Val, proteins and fiber content. Its flowers also contain calcium, potassium, amino acids, sucrose, alkaloids, and flavonoids, such as rhamnetin, isoquercitrin, and kaempferitrin (Gopalakrishnan et al. 2016; Bhattacharya et al. 2018; Ercan et al. 2021). Recently, enzymatically modified isoquercitrin having a 17-fold higher bioavailability than quercetin aglycone, has demonstrated significantly improved endothelial function and increased concentration of circulating quercetin metabolites in human volunteers at risk of CVD (Bondonno et al. 2020). That further implies the potential role of using Moringa as a dietary supplement to prevent CVD. High protein and fiber-rich leaves of *M. oleifera* are used for preparing various snacks and herbal tea and are also consumed to treat malnutrition and anemia (Baiyeri and Akinnagbe 2013; Alia et al. 2022). Along with the cardio-vascular beneficial uses of its chemical constituents, *M. oleifera* could be better utilized as an effective nutraceutical against thrombosis.

Leaves of *M. oleifera* have also demonstrated a reduction in cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), and malondialdehyde levels in hyperlipidemic adult male albino rats after 60 days of administration of its extract at a dose of 400 mg/kg bw (Helmy et al. 2017). The hypolipidemic potential of hydroalcoholic extract of *M. oleifera* leaves was also observed in male wistar rats. An interesting observation of this study was a significant ($p<0.001$) increase in high-density lipoprotein cholesterol (HDL-C) and a significant ($p<0.001$) decrease in the atherogenic index after 28 days of administration of *M. oleifera* extract (Rajanandh et al. 2012) both of which are considered as crucial parameters of cardiovascular diseases (Mahdy et al. 2012; Kazemi et al. 2018). Antiobesity and hypolipidemic effects were also observed after administration of a herbal extract combination comprising 60% ethanolic extract of *M. oleifera* leaves in a dose of 900 mg for 16 weeks in 66 healthy overweight adults. A significant reduction in body weight, body mass index, total body fat, waist, hip circumferences, LDL-cholesterol, and HDL cholesterol increase were observed (Dixit et al. 2018).

Moringa oleifera leaves have also shown significant hypoglycemic potential in various scientific studies. Khan et al. (2017) have demonstrated in vivo hypoglycemic effect of aqueous extract of *M. oleifera* leaves in streptozotocin and high-fat diet-induced diabetes in female wistar rats. Significant ($p<0.05$) restoration of fasting blood glucose, lipid profile, and liver marker enzyme levels was observed in both experimental models. Methanolic extract from *M. oleifera* leaves also showed a protective effect against oxidative stress in the heart of diabetic rats (Aju et al. 2019).

A recent study by Mabrouki et al. (2020) has shown significant reductions in the levels of cardiac catalase, glutathione peroxidase, and superoxide dismutase activities along with an increase in malondialdehyde in the high-fat diet-induced obese rats administered with methanolic extract of *M. oleifera* leaves for 12 weeks (200 mg/kg/bw and 400 mg/kg/bw). The study also exhibited that this effect of *M. oleifera* leaves could be attributed to its phytochemical content and antioxidant potential. Furthermore, the vasodilator effect of aqueous extract of leaves of *M. oleifera* in doses of 30 and 60 mg/kg/day was observed in L-NAME (Nω-nitro-L-arginine-methyl ester) induced hypertensive rats after three weeks. A significant reduction in blood pressure and heart rate and impairment in acetylcholine-induced mesenteric arterial relaxation was observed, along with a decrease in vascular O_{2^-} production and plasma malondialdehyde levels. This dose-dependent vasorelaxation in the endothelium of mesenteric arterial beds could be due to the release of endothelium-derived relaxing factors. In this regard, the present findings of in vitro thrombolytic action further corroborate the vasodilator potential of *M. oleifera* (Aekthammarat et al. 2019; Aekthammarat et al. 2020).

Because of the activities mentioned above and having thrombolytic potential, *M. oleifera* thus could be called a

herbal polypill by protecting against high blood pressure, high blood sugar, and higher lipid parameters in already known cases of ischemic heart disease and metabolic syndrome that have increased tendency of clot formation. Furthermore, it could also be helpful in the secondary prevention of ischemic heart disease.

The present study has shown in vitro thrombolytic potential of methanolic extracts of leaves and flowers of *M. oleifera* for the first time. However, further in vivo studies for thrombolytic potential are warranted, along with identification and characterization studies of corresponding bioactive molecules to discover safe thrombolytic agents.

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Prevalence of β-lactamase produced in *Klebsiella pneumoniae* and *Enterobacter cloacae* isolated from gingivitis in Al-Najaf Province, Iraq

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Abstract. *Motaweq ZY. 2022. Prevalence of β-lactamase produced in* Klebsiella pneumoniae *and* Enterobacter cloacae *isolated from gingivitis in Al-Najaf Province, Iraq. Nusantara Bioscience 14: 78-83.* This study provides phenotypic and genotypic β-lactamase formation data on 26 isolates of *Klebsiella pneumoniae* and *Enterobacter cloacae* isolated from patients with gingivitis checked at Al-Kafeel clinic and private clinic in Al-Najaf Province-Iraq during the period from September 2020 to February 2021. In this study, some were detected by traditional phenotypic methods, while others were detected by phenotypic and then genotypically by using the monoplex-PCR technique. The results revealed that out of 14 β-lactam resistance *K. pneumoniae* isolates, eight isolates (57.1%) gave positive results with the direct capillary tubes method, while 12 β-lactam resistance *E. cloacae* gave 6 (50%) positive results. This result indicated that enzymatic resistance was prevalent among isolates. Furthermore, the results showed that most isolates were ESBL producers according to initial and confirmatory methods. Molecular amplification of the β-lactamase enzyme bla_{SHV} gene was detected in 8 (57.1%) and 5 (41.6%) for *K. pneumoniae* and *E. cloacae*, respectively. While all 100% of *K. pneumoniae* and *E. cloacae* isolates gave negative results for the bla_{GES} gene. This study aimed to investigate the β-lactamase formation and detection of bla_{SHV} and bla_{GES} genes in *K. pneumoniae* and *E. cloacae* isolated from gingivitis diseases.

Keywords: β-lactamase, bla_{SHV}, bla_{GES}, Enterobacteriaceae, ESBL, oral cavity disease, gingivitis

INTRODUCTION

Enterobacteriaceae are natural human gastrointestinal tract flora. Enterobacteriaceae are transiently in the mouth and are important human body pathogens. Bad hygiene, fecal-oral contamination, self-inoculation of toothbrushes, and antibiotic usage are the critical reasons for oral infection by enterobacteria in the mouth (Lafaurie et al. 2012). The mouth is an important site for research because of its anatomical and physiological features, making it a favorable site for microbial proliferation (Rocha et al. 2006). Microorganisms can be disseminated by speaking, coughing, sneezing, or breathing through aspiration via oropharyngeal secretions or transmission through saliva droplets (Fernandes et al. 2000).

The discovery and broad diffusion of novel extended-spectrum β-lactamases (ESBLs) and carbapenemases (CPEs) has resulted in a remarkable increase in antibiotic resistance among Enterobacteriaceae over the last two decades (Bush and Fisher 2011). Because carbapenems are very stable against β-lactamase hydrolysis and preserve resistance to ESBL makers, they are the ideal medicines for treating serious infections caused by ESBL-producing *Klebsiella pneumoniae* (Schroeter, 1886) Trevisan, 1887 (Colodner et al. 2004). However, carbapenem resistance in *K. pneumoniae*, mainly attributed to the formation of *K. pneumoniae* carbapenemase (KPC), has emerged, generating significant clinical difficulty and complicating treatment (Nordmann et al. 2009). KPC-1 confers moderate to high levels of carbapenem resistance, whereas KPC-2 and KPC-3 impart high levels of carbapenem resistance only with the absence of outer membrane porins (Woodford et al. 2004). KPC producers have expanded worldwide and have been found in various Gram-negative bacteria, including *Pseudomonas aeruginosa* (Schroeter, 1872) Migula, 1900 and *Escherichia coli* Mig., 1895 (Kitchel et al. 2009). This study aimed to investigate the β-lactamase formation isolates and identify bla_{SHV} and bla_{GES} genes in β-lactam-resistant isolates of *K. pneumoniae* and *E. cloacae* isolated from gingivitis diseases.

MATERIALS AND METHODS

Patients and clinical specimens

A total of 120 oral cavity specimens were collected from patients with gingivitis at the Al-Kafeel clinic and private clinic in Al-Najaf Governorate, Iraq, from September 2020 to February 2021. The patients included both sexes and the age range (7 to 65 years).

Bacterial isolates

The specimens were inoculated on different types of culture media, including blood agar and MacConkey agar, then dispersed on each plate with a sterile loop. Next, plates were incubated at 37°C for 24 hours. Following that, the plates were checked for bacterial growth. Finally, a single pure isolated colony was transferred to brain heart infusion agar for preservation and morphological

examination by Gram-staining and other biochemical tests that validated the isolates' identity (Macfaddin 2000).

Identification of bacteria

The identification of *K. pneumoniae* and *E. cloacae* were achieved according to Macfaddin (2000). Vitek-2 GN identification card was used to confirm *K. pneumoniae* and *E. cloacae* identification.

Phenotypic detection of β-lactamase

A direct capillary tube method was used for discovering β-Lactamase formation (Guido and Pascale 2005). This method includes: (i) Two milliliters of phenol red indicator solution (0.5%) were added to Penicillin G solution. (ii) Drops of NaOH solution (1 N) were added to this suspension until the color was changed to violet. (iii) One end of the capillary tube was immersed in the prepared solution until it reached the height of 1-2 cm, then immersed this end of the capillary tube was in the culture of bacterial colonies 24-h for making a bacterial plug (avoiding the formation of bubbles between the solution and the colonies). (iv) Incubated the capillary tubes vertically at 37ºC; the result was read within 15 minutes with a change in color from the top of colonies to yellow, indicating a positive result.

Initial screening for ESBL formation

By performing an initial screen test, all bacterial isolates that produced β-lactamase were found to produce ESBL. Furthermore, if the inhibition zone of ceftazidime (30 µg) disks were less than or equal to 22 mm, the isolate would be regarded as a probable ESBL producer (Koneman et al. 1997).

Confirmatory test for ESBL formation

All the β-lactamase generating isolates were further tested for confirmatory ESBL formation by three methods; these tests were included:

Disk combination test

The disk diffusion approach confirmed the phenotypic identity of probable ESBL-producing bacteria. Ceftriaxone and ceftazidime were examined separately and combined with Tazobactam and clavulanic acid. A 5 mm increase or equal in diameter in the inhibition zone for antibiotics tested in combination with Tazobactam and clavulanic acid compared to its zone when tested alone shows the presence of an ESBL-generating strain (Koneman et al. 1997).

Disk approximation test

All β-lactamase producing isolates were tested according to Batchoun et al. (2009).

Genomic methods

Extraction of DNA

Boiling was used to extract DNA from *K. pneumoniae* and *E. cloacae* isolates. In brief, young colonies of *K. pneumoniae* and *E. cloacae* isolates were suspended in 100 microliters of sterile distilled water and heated for 15 minutes in a water bath. Then, it rapidly cooled at -20ºC for one hour, centrifuged, and the supernatant was saved for amplification (Shah et al. 2017). The concentration and purity of extracted DNA can be determined by Williams et al. (2007).

Polymerase Chain Reaction (PCR) assay

To detect *K. pneumoniae* and *E. cloacae* β-lactamase genes, a monoplex PCR assay was employed to amplify various segments of the genes under study. Two genes from each type were chosen to be amplified individually and utilized in this work (Table 1).

PCR cycling conditions

The PCR mixture was built up in a total volume of 20 µL including five µL of PCR premix, 2.5 µL of each primer, and six µL of extracted DNA. The rest volume was completed to 20 µL of sterile dDW, then vortexes. The contents of PCR-reaction tubes were centrifuged quickly to mix and bring them to the bottom of the tubes, then placed in a thermal cycler PCR programmed as follows (Table 2).

Table 1. Illustrating bla_{GES} and bla_{SHV} genes

Genes	Primer sequence (5'-3')	Amplicon size (bp)	Reference
bla_{SHV}	F:GGCCGCGTAGGCATGATAGA R:CCCGGCGATTTGCTGATTTC	714	Ensor et al. (2009)
bla_{GES}	F:AGTCGGCTAGACCGG AAAG R:TTTGTCCGTGCTCAGGAT	307	Dallenne et al. (2010)

Table 2. PCR program that applies in the thermo-cycler

Gene	Temperature(°C)/Time					Cycles number
	Initial denaturation	Condition of one cycle			Final extension	
		Denaturation	Annealing	Extension		
bla_{GES}	95-7 min.	94 - 40 sec.	57 - 40 sec.	72-1 min.	72-7 min.	30
bla_{SHV}	95 -5 min.	94 - 30 sec.	55 - 60 sec.	75- 45 sec.	72-7 min.	30

Preparation of agarose gel and DNA loading

Agarose gel electrophoresis was done according to Mainiatis et al. (1982). First, Agarose gel was prepared by dissolving 0.8 g of agarose in TBE 1X (100 mL) in a glass bottle, melting to boiling. Then the solution was cooled to 50-60°C., 3µL of ethidium bromide dye was added with mixing, agarose was poured out into a gel jar after fixation the comb for making wells, then left to solidify. Next, the bubbles were carefully removed; the jar was put in the electrophoresis tank. Six microliters of the 100 bp DNA ladder were placed in the first left well of the agarose electrophoresis gel. Next, five µL of the amplified PCR product were carefully transferred to a well of the agarose electrophoresis gel. The electrophoresis tank closed with its unique lid, and the electric current was matched (70 volts for 1.5-2 h).

Detection of DNA by agarose gel electrophoresis

The gel documentation system was used to detect the electrophoresis outcome. Positive findings were identified when the DNA band base pairs samples were equivalent to the desired product size (Bartlett and Stirling 2003). Finally, the Biometra gel documentation system photographed the gel (Mishra et al. 2009).

RESULT AND DISCUSSION

Phenotypic detection of β -Lactamase producing isolates

The direct capillary tubes method was employed to identify β-lactamase synthesis in β-lactam resistance 14 *K. pneumoniae* and 12 *E. cloacae* isolates. Table 3 revealed that out of 14 β -lactam resistance *K. pneumoniae* isolates, eight (57.1%) gave positive results with the direct capillary tubes method. In contrast, 12 β-lactam resistance *E. cloacae* gave 6 (50%) positive results. This result indicated that enzymatic resistance was prevalent among isolates. However, in this procedure, 6 (42.9%) and 6 (50%) were non-β-lactamase producers in *K. pneumoniae* and *E. cloacae*, respectively.

A variety of processes can cause Non-susceptibility to β-lactam antibiotics in Gram-negative bacteria; however, the enzymatic mechanism, which includes intrinsic and acquired -lactamases, is the most common (Bush 2010; Bush and Jacoby 2010). As a result, various processes, such as porin loss, efflux pumps, lack of expression of acquired genes, and the presence of other undiscovered β-lactamases, could be linked to these discrepancies between genes detected and expressed phenotype (Davin-Regli and Pagès 2015).

The current findings are consistent with those of Al-Charrakh et al. (2011), and Gamboa et al. (2013) found that 41.2% and 58.5% of β-lactam resistant *Klebsiella* spp. isolates were able to produce β-lactamases, respectively.

The results of Table 4 indicate that 6 (42.9%) *K. pneumoniae* and 6 (50%) *E. cloacae* were negative with the capillary tube method, suggesting that these isolates may be either have no β-lactamases or production of low quantities of enzymes, making its detection more difficult (Jacoby and Bush 2009). On the other hand, the negative results could be related to the fact that β-lactamase in isolates required more time to destroy their cell wall and be released. Additionally, temperature and pH may significantly affect or reduce enzyme activity (Foley and Perret 1962).

Phenotypic detection of ESBLs Enzyme

All isolates of *K. pneumoniae* and *E. cloacae* resistant to β-lactam antibiotics were tested for ESBL production. In addition, Ceftazidime disks were used to examine how well the test isolates performed in the ESBL first screen disk test. If the inhibition zone of ceftazidime disks (30 µg) was less than 22 mm, the isolate was considered a probable ESBL producer by the CLSI (2018). The investigation discovered that 11/14 (78.5%) *K. pneumoniae* isolates tested positive for ESBL during the initial screening using a ceftazidime disk. In comparison, 9/12 (75%) *E. cloacae* isolate tested positive for ESBL, indicating that the isolate is suspected of producing ESBL.

The disk combination method was used to detect ESBL-generating isolates in the study. This approach combined ceftazidime and ceftriaxone disks with clavulanic acid and Tazobactam instead of ceftazidime and ceftriaxone disks alone. When the inhibition zone of combined disks was greater than or equal to 5 mm more significant than the inhibition zone of a single disk, the isolate was classified as an ESBL producer (Figure 1).

The results showed that 9 (64.2 %) *K. pneumoniae* and 8 (66.6 %) *E. cloacae*, out of the 24 bacterial isolates β-lactamase producers, demonstrated zone enhancement with clavulanic acid, indicating their ESBL production. Additionally, all isolates were confirmed using the disk approximation method. The enhancement of the inhibitory zone between 30 µg antibiotic disks (ceftazidime, ceftriaxone, cefotaxime, and aztreonam) and a 20/10g amoxicillin-clavulanate disk was interpreted as synergy, indicating the existence of an ESBL, in this approach.

Since the inhibitory zone of synergism has been characterized, screening test findings revealed that 7 (50%) *K. pneumoniae* isolates exhibited positive ESBL production test results versus 5 (41.6%) E. cloacae isolates (Figure 1).

Table 3. β-lactamase generating isolates by direct capillary tube method

Species	No. of Isolates	No. (%) of positive *β*-lactam producing	No. (%) of negative *β*-lactam producing
K. pneumoniae	14	8 (57.1)	6 (42.9)
E. cloacae	12	6 (50.0)	6 (50.0)
Total	26	14 (53.8)	12 (46.2)

Table 4. ESBLs produced in *E. cloacae* and *K. pneumoniae* isolates by confirmation methods

Species	No. of Isolates	No. (%) of positive	
		Disk combination	Disk approximation
K. pneumoniae	14	9 (64.2)	7 (50)
E. cloacae	12	8 (66.6)	5 (41.6)

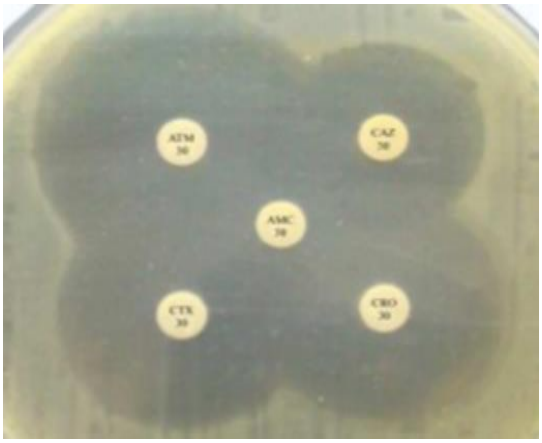


Figure 1. The positive result of ESBL formation *K. pneumoniae* on Muller Hinton agar after 24 hr of incubation at 37°C

Even though ESBLs were discovered at least three decades ago, there is still a lack of understanding of their laboratory detection and clinical importance. Failure to detect these enzymes has aided their unchecked spread and, in some cases, therapeutic failures (Yang and Zhang 2008).

The standard Kirby-Bauer disk diffusion method was used to screen for reduced sensitivity to third-generation cephalosporins and aztreonam. When the zone diameter of any of the markers matched the CLSI criteria (CLSI 2018), the isolate was termed positive for the screening test, and additional phenotypic tests were required to determine ESBL production. The discovery and broad diffusion of novel extended-spectrum β-lactamases have resulted in a remarkable increase in antibiotic resistance among Enterobacteriaceae over the last two decades (ESBLs) (Coque et al. 2008; Chong et al. 2011; Salabi et al. 2013). The most prevalent mechanism of resistance to β-lactam antibiotics among Gram-negative bacteria is the hydrolysis of the β-lactam ring by β-lactamase(s) (Bush and Jacoby 2010), however other mechanisms (e.g., changes in porin channels, efflux pumps) may contribute to or increase resistance (Bush and Fisher 2011; Canton et al. 2012).

The results partially agree with a recent study by Shakib et al. (2018) that showed that 88.6% of *K. pneumoniae* isolates produced ESBL. Moreover, Ghasemi et al. (2013) found that 60% of *K. pneumoniae* isolates ESBL producers in Shiraz, Iran. Also, agreement with Aljanaby and Alhasani (2016) pointed out that the presumptive test for ESBL production was positive for 65.5%.

The clinical microbiology laboratory faces a significant challenge in identifying ESBL producers since the affinity of ESBL-producing isolates for various substrates is varied, making detection challenging. Furthermore, in vitro, some ESBL isolates may appear susceptible to third-generation cephalosporins (Hadi 2018).

However, not all screened positive isolates were ESBL producers in the current investigation. Other mechanisms of resistance to third-generation cephalosporins and aztreonam may so exist. In organisms that produce both ESBL and AmpC, clavulanate may cause hyperproduction of the AmpC-lactamase, resulting in hydrolysis of the third-generation cephalosporin in a false-negative ESBL detection test (Thomson 2010). There are a few cases where the screening tests are positive, but the confirmatory tests are negative or inconclusive (Steward et al. 2001).

On the other hand, the coexistence of different classes of β-lactamases in a single bacterial isolate can make diagnosis difficult. The ability to detect and differentiate AmpC and ESBL-producing pathogens has epidemiological implications and may also have therapeutic implications (Al-Sehlawi 2012).

Molecular study

Molecular detection of ESBL-producing isolates

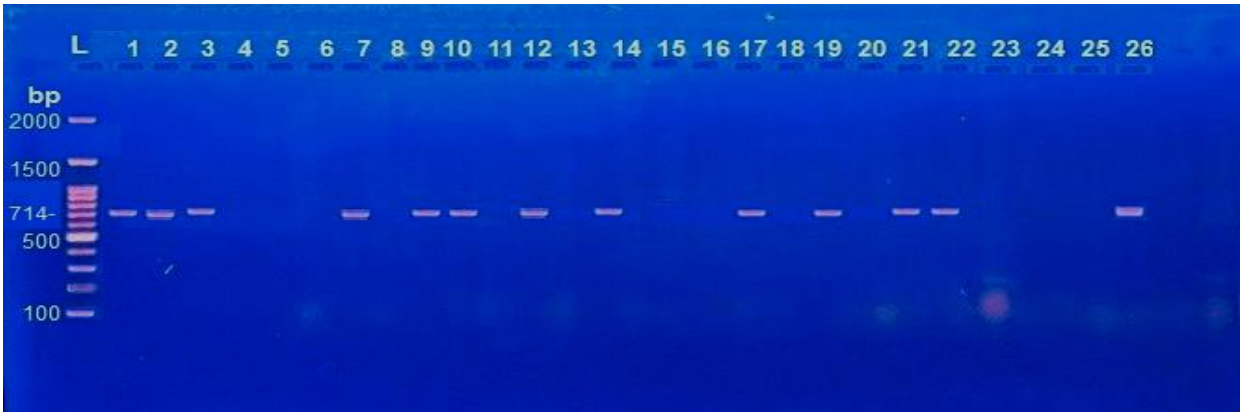
Only two genes in the families of SHV and GES were examined in the current investigation. Detection of these genes (bla_{SHV} and bla_{GES}) was performed by PCR technique. The results revealed that out of 14 *K. pneumoniae* and 12 *E. cloacae* isolates in this study were given 8 (57.1%) and 5 (41.6%) for the bla_{SHV} gene, respectively. While all 100% of *K. pneumoniae* and *E. cloacae* isolates gave negative results for the bla_{GES} gene (Figures 2A and 2B).

ESBLs have been found in *Serratia marcescens* Bizio 1823, *Enterobacter* spp., and *Citrobacter freundii* (Braak, 1928) Werkman & Gillen, 1932 isolated from many places across the world, and they are becoming increasingly common (Ferreira et al. 2010). Initially, these species' ESBLs were TEM or SHV enzymes (Dhillon and Clark 2012). However, environmental antimicrobial agent pressure could be a risk factor for ESBL gene acquisition. Furthermore, using antibiotics as feed additives in animal farming and agriculture creates selective pressure (Woodford et al. 2004).

Similar studies by Ma et al. (2005) and Szabó et al. (2005) found that 33.3% and 30.9% of *E. cloacae* isolates had the bla_{SHV} gene. In addition, Aljanaby and Alhasani (2016) showed blaSHV detected in 87.5% of K. pneumoniae isolates in Iraq.

The absence of the bla_{GES} gene in all tested isolates was discovered in this investigation, which could be attributed to the absence of the bla_{GES} gene or the presence of another subtype of a gene that could not be targeted by the primer employed in this study.

The main reason for the prevalence of β-lactamases in Iraq may be due to the extensive usage of certain third-generation cephalosporin antibiotics. ESBL-producing Enterobacteriaceae are found in high numbers in Asia. The high rate of ESBL production in impoverished countries is cause for concern; a lack of money for efficient infection control and limited access to effective antimicrobials have significant implications for reducing morbidity and death associated with these infections.



bla$_{SHV}$ gene with amplified product 714 bp

A

bla$_{GES}$ gene with amplified product 307 bp

B

Figure 2. Gel electrophoresis of PCR products from DNA of *K. pneumoniae* and *E. cloacae* isolates. A. *bla*$_{SHV}$ primers. Lanes (1, 2, 3, 7, 9, 10, 12, 14) positive results of *K. pneumoniae* isolates (17, 19, 21, 22, 26) positive results of *E. cloacae*, (L), DNA molecular size marker (100-bp ladder). B. *bla*$_{GES}$ primers. Lane, all 100% isolates show negative results (L), DNA molecular size marker (50-bp ladder).

In conclusion, Enterobacteriaceae species is the most familiar Gram-negative bacteria isolated from three types of oral cavity diseases. The *K. pneumoniae* and *E. cloacae* isolates are the commonest Enterobacteriaceae isolated from gingivitis infections. The *K. pneumoniae* and *E. cloacae* isolates appeared with *bla*$_{SHV}$ genes in β- lactam-resistant isolates, while no results for *bla*$_{GES}$ genes. ESBL phenotype was highly prevalent among *K. pneumoniae* and *E. cloacae* isolates.

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Ethnobotany of medicinal plants from Lampung Tribe around Way Kambas National Park, Indonesia

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Abstract. *Yudiyanto, Hakim N, Wakhidah AZ. 2022. Ethnobotany of medicinal plants from Lampung Tribe around Way Kambas National Park, Indonesia. Nusantara Bioscience 14: 84-94.* The local communities of the Lampung Tribe around Way Kambas National Park (TNWK), Lampung, Indonesia, utilize forest resources through local community knowledge. However, cultural modernization can potentially lead to the erosion of people's traditional knowledge. Therefore, this study is essential to be conducted. This study aimed to inventory medicinal plant species and describe local communities' knowledge of the use of medicinal plants. Semi-structured interviews and observations collected ethnobotanical data. The results were presented in a table and diagram and then analyzed qualitatively. The results showed that the local community of the Lampung Tribe around the TNWK area used as many as 69 species of medicinal plants belonging to 39 families. Zingiberaceae was the family with the highest number of species. Leaves were the plant part that was used mainly by the local community. Boiling was the most widely used mode of preparation. The Lampung Tribe uses plants to treat various diseases grouped into external and internal diseases. The local community frequently experiences external diseases.

Keywords: Ethnobotany, herbal medicine, local knowledge, Way Kambas National Park

INTRODUCTION

The plant diversity of Indonesia is very promising to be developed as raw material for traditional medicines. The local knowledge has been passed down from generation to generation (Nahdi et al. 2016). About 80% of the total plant species in Indonesia have medicinal properties for diseases that exist worldwide (Kusuma et al. 2014). Specifically, as many as 940 medicinal plants from about 20,000 plant species have been used in Indonesia (Masyhud 2010). Medicinal plants are used in traditional medicine, which is understandable as an ancient and culture-bound method of healing that humans have used to cope and deal with various diseases that have threatened their existence and survival (Abdullahi 2011). Traditional medicine is a mixture made from multiple parts of plant species that have properties to cure various diseases and have been practiced from generation to generation since ancient times (Sada and Tanjung 2010). Traditional medicinal plants are natural ingredients used as medicines based on a community experience. This kind of medicine is usually processed using a simple technology based on recipes inherited from generation to generation, following local traditions and beliefs. Even some are based on supernatural power (Nahdi et al. 2016).

The use of medicinal plants is also regulated in the Indonesian Constitution. One of the government policies is Constitution No. 23 of 1992 concerning traditional medicine, which explains an effort outside of medicine or nursing science, including methods, drugs, and treatment, which refers to knowledge, experience, and skills that are passed down from generation to generation, both original as well as those from outside Indonesia, which applied according to the prevailing norms in local society. The law also states that as part of health service efforts, traditional medicine is the responsibility of the government and the community to participate in realizing public health status.

Way Kambas National Park (TNWK) has very high biodiversity. The local communities, i.e., the Lampung tribe, around TNWK use forest resources for local community knowledge. One of the uses of forest resources is medicinal plants, which are closely related to health needs in people's daily lives. The fulfillment of these needs is carried out independently and is hereditary. This practice benefits the community to create a healthy and prosperous society.

The transformation in traditional culture and the environment often occurs due to modernization. Therefore, cultural modernization potentially leads to the erosion of people's traditional knowledge. Likewise, the practice of plant use and management as traditional medicines by the community may be lost (Giddens 2013). Moreover, the local knowledge about traditional medicinal plants is not well documented since older adults or local experts only orally transmit the information. In other words, knowledge about medicinal plants passed down orally from generation to generation is vulnerable to lose, especially with the current modernizations.

Considering such backgrounds, ethnobotanical studies of medicinal plants that the Lampung Tribe around TNWK

uses are essential to be carried out. This study aims to document local communities' knowledge and conservation of biological resources. The study aims to inventory medicinal plant species used by the Lampung Tribe around TNWK and to describe the knowledge of local communities in terms of the use of medicinal plants. Therefore, local knowledge can be well preserved and be a future legacy for the next generation.

MATERIALS AND METHODS

Study area

The study was carried out in five sub-districts around Way Kambas National Park, Lampung, Indonesia, namely Sukadana, Labuhan Ratu, Braja Slebah, Way Jepara, and Labuhan Maringgai. These sub-districts are directly adjacent to the national park area and have long been inhabited by the Lampung Tribe (Figure 1). The study was conducted from April to September 2020. The TNWK is located in East Lampung Regency, more specifically between 40°37'-50°16' South Latitude and between 105°33'-105°54' East Longitude. That is a tropical forest area sized approximately 125,631 hectares. The TNWK is a conservation area in the form of a national park established based on the Decree of the Indonesian Minister of Forestry No. 670/Kpts-II/1999 dated 26 August 1999. Geographically, TNWK is positioned at 40,37'-50,16' LS and 105,33'-105,54' BT.

The topography of TNWK is relatively flat to slightly bumpy, with a height range from 0 to 50 meters above sea level. The soil is generally podzolic, has high light content, acid soil reaction, is low in nutrients, is easy to catch water (porous), and has relatively high soil binding capacity. The TNWK has three major river sub-groups which flow into the Java Sea coast in the eastern area, i.e., Way Penet in the south area, Way Kanan and Wako in the middle area, and Pegadungan river in the northern area. A swamp forest ecosystem is mainly around the river in the eastern part of the TNWK. This area is relatively close to the ancient village where this study was conducted. In those ancient villages, the Lampung Tribe communities still preserve customary local culture, including Sukadana, Labuhan Ratu, Braja Slebah, Way Jepara, and Labuhan Maringgai still exist.

Sukadana, Labuhan Ratu, Braja Slebah, Way Jepara, and Labuhan Maringgai Sub-districts are directly adjacent to the Way Kambas National Park area. The Lampung Tribe primarily inhabits these sub-districts. The ancient villages in these sub-districts still maintain their knowledge of traditional medicine. Traditional medicine uses many plants as ingredients for an herbal drink (*jamu*), consumed as fresh vegetables or applied smeared.

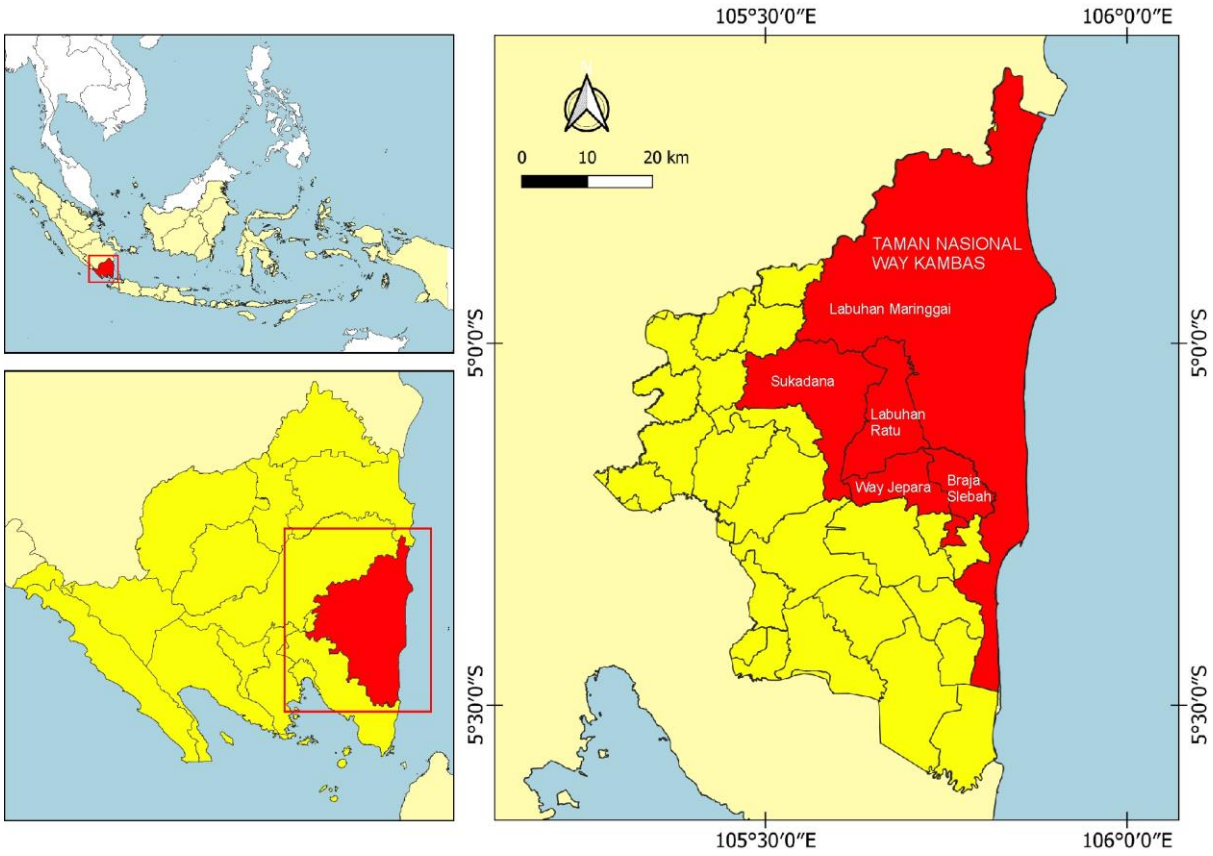

Figure 1. Map of the study area in the Way Kambas National Park, Lampung, Indonesia

Data collection

Semi-structured interviews and observations collected ethnobotanical data. Semi-structured interviews were carried out with key respondents using questionnaires prepared on a particular topic, completed with in-depth and open direct discussion. The snowball sampling technique was employed to determine key respondents (Vogl et al. 2004). The key respondents were local people who are more knowledgeable in medicinal plants than others, including village heads, traditional leaders, community leaders, or people accustomed to using medicinal plants.

Observations were made to verify plant species obtained from the interviews. The unidentified plant species were taken in a photo, made into herbarium, and identified following plant identification books such as Flora of Java (Spermatophytes only) (Backer and Van Den Brink 1963), Flora Pegunungan Jawa (Steenis 2013), Identifikasi dan Klasifikasi Tumbuhan (Ahmad 2020). The scientific names were validated using online sources, namely https://powo.science.kew.org/ and https://www.nparks.gov.sg/. In addition, observations were made by finding the presence of medicinal plants around the TNWK. Observations were carried out with respondents' assistance to directly show the plants in question and then documented.

Data analysis

The data were presented in a table and diagram and analyzed by qualitative descriptive method to describe plant species used as medicine by the community, curable diseases, plant parts used, mode of preparation, and comparison with scientific data of medicinal plants from the other area of Indonesia. We also discussed the local wisdom of the Lampung Tribe around the TNWK about their conservation action toward medicinal plants.

RESULTS AND DISCUSSION

The use of medicinal plants

This study showed that as many as 69 medicinal plants belonging to 39 families were used by the Lampung Tribe around TNWK (Table 1). This result indicates that the knowledge of the local people about medicinal plants is well maintained. This finding showed that Zingiberaceae had the highest species (9 species). The same results were also reported in other Indonesian areas, such as Wakhidah (2020), where 11 plant species of the Zingiberaceae family are used as medicinal plants by the Lampung Saibatin in the western part of Lampung Province. The Colo community in Kudus, Central Java, also uses a lot of plant species from the Zingiberaceae family (14 species) (Wahidah et al. 2021). Likewise, the Toba Batak ethnicity in North Sumatra also often uses the Zingiberaceae family as medicinal ingredients with nine species (Nasution et al. 2020). Other families that the Lampung Tribe widely uses are Poaceae (5 species), Musaceae, Piperaceae, Fabaceae, and Asteraceae (4 species each) (Table 2).

The Zingiberaceae family has been reported to have various biological activities, such as antioxidant, anti-cancer, anti-inflammatory (Tushar et al. 2010), and antibacterial (Saad et al. 2014). Plant species of the Zingiberaceae family used by the Lampung Tribe were *Alpinia galanga* (L.) Willd., *Curcuma domestica* Valeton, *Curcuma xanthorrhiza* Roxb, *Curcuma zedoaria* (Christm.) Roscoe, and *Zingiber officinale* Roscoe. These species contain essential oils that might have different bioactivity. For example, the bioactivity of the essential oil in *Z. officinale* is antiglycation (Batubara et al. 2016), while the essential oil content in *C. xanthorrhiza* acts as antihyperglycemic and anti-inflammatory (Rajkumari and Santombi 2018). Apart from the hereditary belief of the Lampung tribe, plant species of the Zingiberaceae family have been proven to contain compounds that are beneficial to health.

The second highest family used as medicine by the Lampung Tribe was Poaceae (5 species), consisting of *Imperata cylindrica* (L.) P.Beauv., *Cymbopogon citratus* (DC.) Stapf, *Zea mays* L., *Cymbopogon nardus* (L.) Rendle, and *Saccharum officinarum* cv. Badila. The leaves of *alang-alang* (*I. cylindrica*) were mixed with *Andrographis paniculata* (Burm.f.) Nees are used as herbs by drying and then brewing. The plant is believed to treat heartburn, urinary incontinence, and kidney disease. The *I. cylindrica* is also used by the Dani Tribe in Jayawijaya, Papua, as medicine for influenza and tinea versicolor (Mabel et al. 2016). The Sunda tribe in Cirebon uses the roots of *alang-alang* mixed with the leaves of avocado and *Orthosiphon aristatus* (Blume) Miq. to cure kidney disease (Hidayat and Rachmadiyanto 2017). Phytochemicals in *I. cylindrica* include alkaloids, triterpenoids, flavonoids, saponins, and tannins (Padma et al. 2013). These phytochemical compounds have various biological activities, such as analgesic abilities (Razafindrakoto et al. 2021), an antibacterial effect that can inhibit bacterial growth, such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Bacillus subtilis*, also effective against two avian intestinal worms such as *Raillietina echinobothrida* (Megnin, 1881), and *Ascaridia galli* (Schrank, 1788) (Lalthanpuii et al. 2019), and the extract can ameliorate disturbance in hematological profile due to liver damage (Dahlan et al. 2020). There is a possibility that kidney disease can be treated in line with ethnobotanical knowledge because of the analgesic activity in the roots extract of *I. cylindrica*.

Table 1. The list of medicinal plants used by the Lampung Tribe around the Way Kambas National Park, Lampung, Indonesia

Family	Scientific name	Local name	Used part	Mode of preparation	Curable disease/ efficacy
Acanthaceae	*Rhinacanthus nasutus* (L.) Kurz	Cucuk manuk, tuktuk bughung	Leaves	The leaves and flowers are cleaned, rubbed into the itchy part	Relieve itching
	Andrographis paniculata (Burm.f.) Nees	Sambiloto	Stem, leaves	The stems and leaves are washed, boiled, and boiled water is drunk	Relieve fever, aches, and pains
Amaryllidaceae	*Allium tuberosum* Rottler ex Spreng.	Kucai	Leaves, stem	Consumed as fresh vegetables. Boiled and boiled water is drunk	Healthy bones and lowering cholesterol
Annonaceae	*Annona muricata* L.	Sirsak, bulung sisrsak	Leaves	The leaves are washed, boiled, and boiled water is drunk	Cancer, diabetes, and gout
Apiaceae	*Foeniculum vulgare* Mill.	Adas poluwaras	Root	Pounded, squeezed, and brewed with warm water	Digestive medicine, flatulence, constipation, anemia, and irregular menstruation
	Coriandrum sativum L.	Ketumbar	Seed	Roasted and eaten	Eliminate body odor and female genital organ
	Apium graveolens L.	Seledri	Root, stem, leaves	Blended finely, add lemon juice, filtered, and drink every day	Gout
Apocynaceae	*Catharanthus roseus* (L.) G.Don	Tapak dara, serdadu	Root, stem, leaves	The roots and the leaves are washed, boiled, and boiled water is drunk. The leaves are washed, boiled, and boiled water is drunk	Ulcer and internal diseases, diabetes, lowering high blood pressure, tumors, and leukemia
Arecaceae	*Cocos nucifera* L.	Kelapa hijau	Coconut water	Burn the coconut and then drink the water	Lowering blood pressure and removing body toxins
Asteraceae	*Gynura procumbens* Merr.	Sambung nyawa	Leaves	As fresh vegetables or boiled leaves, strained, and drink the water	Stamina booster and malaria
	Gynura divaricata (L.) DC.	Tapak dewa, tapak limin	Leaves	Pounded leaves, then pasted to bruises.	Bruises
				The leaves are boiled, and the boiled water is drunk	Relieve kidney stones
	Sonchus arvensis L.	Tempuyung	Leaves	Fresh leaves or dried leaves are boiled, then boiled water is drunk	Fever, lowering high blood pressure, and shedding kidney stones
	Blumea balsamifera (L.) DC.	Capa, sembung, bulung capa	Leaves	Ten leaves, squeezed with a bit of water, compress all over the body, especially the soles of the feet	Febrifuge
Basellaceae	*Anredera cordifolia* (Tenore.) Steenis	Binahong	Leaves	Boiled and boiled water is drunk. The leaves are crushed and then placed on the burn	Ulcers, fever, and burn
Bignoniaceae	*Crescentia cajute* L.	Bernung, maja	Fruit, flower	Fruit is made into syrup, the leaves are boiled, and the boiled water is drunk	Relieve stomach aches, facilitate digestion, asthma
Boraginaceae	*Heliotropium indicum* L.	Sangketan, buntut tikus	Leaves	1. Boiled, and then boiled water is drunk; 2. The leaves are crushed, mixed with eucalyptus oil, and rubbed on the body	1. Shedding kidney stones, 2. Relieve fever in children

Cactaceae	*Hylocereus undatus* (Haw.) D.R.Hunt	Buah naga	Fruit	Peel and consume directly	Digestion, cardiovascular
Caricaceae	*Carica papaya* L.	Papaya	Fruit	Consumed directly or in juice	Digestion, menstrual pain, cancer
Cucurbitaceae	*Momordica charantia* L.	Pare, bulung pria	Leaves	Five leaves washed, add salt, then put into the mouth of the child/toddler with a spoon	Stimulating the appetite of children
	Cucurbita moschata (Duch.) Duch	Labu kuning, labu parang	Fruit	Fruit cut into pieces, boiled, and eaten before bed without sugar	Relieve stomach ache
Euphorbiaceae	*Ricinus communis* L.	Pohon jarak ulung	Sap	Take a leaf stalk, break it, take the sap about one tablespoon, add salt, and drink	Vomiting blood, heartburn, and ulcer
Fabaceae	*Tamarindus indica* L.	Asam jawa	Fruit	Mixed with turmeric or ginger, cleaned in a tangerine, combined with a bit of hot water, squeezed and filtered, add honey, and drink	Cough medicine, fever, rheumatism, stamina enhancer, and dysentery medicine
	Parkia timoriana (DC.) Merr.	Kedawung, kadawong	Seed	Add a little water, and paste it on the sore spot	Itching, wound infection, and stomach disorders
	Erythrina variegata L.	Dadap, khedak minyak	Leaves	Mix the leaves of the oil with hot water and then drink. Crushed leaves, add a little water, compress on the head, stomach, and chest	Fever, gout medication, launch menstruation, postpartum after giving birth
	Senna alata (L.) Roxb	Ketepeng	Flower	Leaves are boiled, filtered, then drunk	Overcoming intestinal worms, constipation, and treating sprue
Illiciaceae	*Illicium verum* Hook.f.	Kembang lawing	Flower	The flowers are washed, mashed, boiled, strained, and drunk in the water	Sciatica, fever, flu, and stamina enhancer
Lamiaceae	*Orthosiphon aristatus* (Blume) Miq.	Kumis kucing	Leaves	The leaves are washed, boiled, and drunk	Shedding kidney stones and relieving cough
Lauraceae	*Persea americana* Mill.	Alpukat	Leaf and fruit	Five leaves were washed and boiled; Fruit was eaten or juiced	Ulcer, diarrhea, and constipation
	Cinnamomum verum J.Presl	Kayu manis	Bark	Boiled with other herbal mixtures	Stamina enhancer, lowering blood sugar, rheumatic pain
Liliaceae	*Allium sativum* L.	Bawang putih	Tuber	Grated, mixed with sugar and coconut oil, and then applied to the wound	Wound medicine
	Allium cepa L.	Bawang merah	Tuber	Grated, add coconut oil, and apply to the head, stomach (center), and body	Medicine for fever/high fever in children
Malvaceae	*Ceiba pentandra* (L.) Gaertn.	Golong gandu	Leaves	The leaves are grown, add water, squeeze, drink	Constipation
Meliaceae	*Lansium domesticum* Corrêa	Duku	Bark	Bark or teak bark, cleaned, washed, ground, boiled with water from 3 cups to 1 cup, then drunk	Diabetes
Menispermaceae	*Tinospora cordifolia* (Willd.) Hook.f. & Thomson	Brotowali, bratawali	Stem	The stems are consumed directly or boiled; the water is drunk	Febrifuge antidote to toxins in the body, treating gout and preventing diabetes, cancer, and heart disease
Moringaceae	*Moringa oleifera* Lam.	Kelor	Leaves	The leaves are cooked as "sayur bening." Boil, and drink the boiled water	Maintain a healthy body, diabetes, asthma, and breast milk booster
Musaceae	*Musa x paradisiaca* L.	Pisang	Leaves	The leaves are smeared with edible oil, heated over a fire, and stick to the bruised area	Bruise
	Musa acuminata "Cavendish'	Sumbeng, jantung pisang ambon	Flower	Peel the flower, take the white part, boil it, let for a few minutes, and consume	Stomachache
Myrtaceae	*Psidium guajava* L.	Jambu biji, bulung jambew biji	Leaves	Take 20 leaves, wash, boil in 2 cups until boiling, add rice flour, stir, strain, and drink	Diarrhea
	Syzygium polyanthum (Wight) Walp.	Daun salam	Leaves	Leaves are boiled, and boiled water is drunk	Gout, gastritis, lowering cholesterol

Oxalidaceae	*Averrhoa carambola* L.	Belimbing wuluh, belimbing sayur	Fruit, flower	Cut into pieces, boiled with 3 cups of water into 1 cup, cooled, and drunk on an empty stomach before breakfast. Star fruit flowers, boiled with water from 3 cups to 1 cup, drunk when warm before going to bed or in the morning	Cholesterol medication, lowering blood pressure, and stomach acid
Pandanaceae	*Pandanus amaryllifolius* Roxb. ex. Lindl.	Bulung pandan, daun pandan	Leaves	The leaves are boiled in 3 cups of water; after 1 cup is left, add sugar and salt to taste	Nausea and stomach aches
Papilonaceae	*Erythrina subumbrans* (Hassk.) Merr.	Daun srep	Leaves	Boiled, and add honey	Lowering blood sugar reduces miscarriage, increases breast milk
Piperaceae	*Peperomia pellucida* (L.) Kunth	Tumpang air, suruhan	Leaves, stem	A handful of leaves and stems, cleaned, boiled with boiling water, then drunk	Relieves aches and pains
	Piper nigrum L.	Lada, lado	Seed	Pounded, add coconut oil, smear on the wound	Relieve pain, itching
	Piper betle L.	Sirih	Leaves	Boiled, drink the boiled water	Reduce body odor, curing red eyes
Poaceae	*Imperata cylindrica* (L.) P.Beauv.	Alang-alang	Root	As a mixture of bitter herbs, dried and brewed	Heartburn, urination expediter, and shedding kidney stone
	Cymbopogon citratus (DC.) Stapf	Sereh	Stem	Three stems crushed, put in a glass filled with drinking water, let for one night, then drink. To repelling mosquitoes, put lemongrass in the room	Menstrual pain, digestion expediter, and mosquito repellent
	Cymbopogon nardus (L.) Rendle	Sereh merah, sereh minyak	Stem	Crush stem, boiled, the boiled water is drunk	Lowering blood sugar and blood pressure
	Saccharum officinarum cv. Badila	Tebu ireng, tebu hitam	Leaves	Squeezed	Flu, coughs, and fever
	Zea mays L.	Jagung	Fruit	Take young corn, grated, applied to the smallpox wound	Smallpox
Portulacaceae	*Talinum paniculatum* Gaertn.	Ginseng jawa	Root, leaves	Swallowed or cooked for stamina booster; placed on the wound	Stamina booster; wound
	Portulaca oleracea L.	Krokot madi	Stem, leaves	Boil the stems and leaves, filter and drink the water	Cardiovascular
Rubiaceae	*Morinda citrifolia* L.	Mengkudu, bentis pace	Fruit, leaves	Fruit made in juice, strain, add sugar and honey and drink twice a day after meals. The leaves are washed, withered over the fire, squeezed until the water comes out, stick the leaves on the chest	Medicines for heart, liver and asthma, cough, preventing cancer
Rutaceae	*Citrus aurantiifolia* (Christm.) Swingle	Jeruk nipis	Fruit	Squeeze lime, add soy sauce and salt to taste, and drink three times a day	Cough
Sapotaceae	*Manilkara zapota* (L.) P.Royen	Sawo	Fruit	Young fruit peeled, grated, squeezed, and filtered	Indigestion, weight loss, fever, and inflammation
Solanaceae	*Brugmansia suaveolens* (Humb. & Bonpl. ex Willd.) Sweet	Daun kecubung	Leaves	Squeezed	Bloating
	Physalis angulata L.	Ciplukan, ketepuk	Leaves, stem, and root	The fruit can be eaten with a wrapper. Leaves, stems, and roots are boiled with water from 3 cups to 1 cup; drink regularly in the morning and evening	Lowering blood pressure
Thymelaeaceae	*Phaleria macrocarpa* (Scheff.) Boerl.	Mahkota dewa	Leaves	The leaves are crushed, affixed to the itchy skin	Itching and coughing

Verbenaceae	*Tectona grandis* L.f.	Jati	Bark	The bark is cleaned, washed, crushed, and boiled with water from 3 cups to 1 cup, ready to drink	Diabetes
Xanthorrhoeaceae	*Aloe vera* (L.) Burm.f.	Lidah buaya	Leaves	Leaves, pandan leaf, honey, and ice cubes, blend, and ready to drink	Cancer and gastritis
Zingiberaceae	*Zingiber montanum* (J.Koenig) Link ex A. Dietr.	Bangle/balai	Rhizome	Grated, put hot water, stir, and apply on the itchy body	Itchy medicine, itching allergy
	Alpinia galanga (L.) Willd.	Bulung lengkuas, lawas	Leaves	Five pieces of leaves, pounded, add 1 cup of water, squeeze and drink, also add a little salt and honey	Stimulating the appetite-, stamina enhancer, lowering cholesterol
	Etlingera elatior (Jack) R.M.Sm.	Kecombrang, tanaman honje	Stem, leaves, flower	1. The stems and leaves are crushed, then affixed to the forehead, 2. Crushed, soaked in warm water, and then drunk, 3. Young flowers and stems are cooked as vegetables	1,2. Febrifuge
	Kaempferia galanga L.	Kencur	Rhizome	Grated, squeezed, add honey and lime juice, or grated and squeezed into an appetite-enhancing herb	Cough, stimulating the appetite
	Curcuma aromatica Salisb.	Temu putih, kepoh	Rhizome	The tubers are cleaned and consumed directly as fresh vegetables	Reduce or eliminate halitosis
	Curcuma xanthorrhiza Roxb	Temulawak	Rhizome	Grated, squeezed, mixed with chicken egg yolk, one tablespoon honey, stir, and drink	Internal disease, indigestion medicine, stimulating appetite, and maintaining liver health
	Zingiber zerumbet (L.) Roscoe ex Sm.	Lepoyang, lempuyang	Rhizome	Grated, squeezed, add half a glass of water and a little salt, 2. Grated, add two tablespoons of water and a little salt, squeeze, and drink 3. Pounded, boiled with 3 cups of water to 1 cup, filtered, and drunk	1. Ulcer, 2. Cough
	Zingiber officinale Roscoe	Jahe, jahik	Rhizome	Grated, add water, and drink. Crush the red ginger, add the shallots, wrap them in banana leaves, and spread them over the fire. After the heat is evenly distributed, remove it, place it on the part of the body that is sick with rheumatism	Ulcer, flu, rheumatism
	Curcuma domestica Valeton	Kunyit	Rhizome	Grated turmeric, poured water, and drunk	Fever relief, stomachache, and ulcer

Table 2. The family of medicinal plants used by the Lampung Tribe in the villages around Way Kambas National Park, along with the number of used species

Family	Number	Family	Number
Apiaceae	3	Meliaceae	1
Acanthaceae	2	Menispermaceae	1
Amaryllidaceae	1	Moringaceae	1
Annonaceae	1	Musaceae	4
Apocynaceae	1	Myrtaceae	2
Arecaceae	1	Oxalidaceae	1
Asteraceae	4	Pandanaceae	1
Basellaceae	1	Papilonaceae	1
Bignoniaceae	1	Piperaceae	4
Boraginaceae	1	Poaceae	5
Cactaceae	1	Portulacaceae	2
Caricaceae	1	Rubiaceae	1
Cucurbitaceae	1	Rutaceae	1
Euphorbiaceae	1	Sapotaceae	1
Fabaceae	4	Solanaceae	2
Illiciaceae	1	Thymelaeaceae	1
Lamiaceae	1	Verbenaceae	1
Lauraceae	2	Xanthorrhoeaceae	1
Liliaceae	2	Zingiberaceae	7
Malvaceae	1		

In the families of Musaceae, Piperaceae, Fabaceae, and Asteraceae were only found four species each. Furthermore, plant species from Fabaceae or Asteraceae were also widely used as drugs and stamina boosters, as seen in the Tunjung Dayak community in East Kalimantan (Setyowati 2010), Lampung Saibatin (Wakhidah 2020), and Madura (Fathir et al. 2021). *Kedawung* (*Parkia timoriana* (DC.) Merr.) is an example of a species from Fabaceae whose seeds are trusted by the local people of Lampung as a remedy for itching, wound infections, and stomach disorders. The *P. timoriana* seeds have antibacterial activity (Angami et al. 2018). The activity can be used to treat itching and infection because it can inhibit the bacteria originating from wounds, according to the belief of the Lampung tribe.

Sambung nyawa (*Gynura procumbens* Merr.) is one of the species of the Asteraceae family whose leaves are used as a stamina booster and to cure malaria. The species also protects against tissue damage (Mahmood et al. 2008). In addition, antioxidant activity is also found in the extract of *G. procumbens*, especially in the roots, due to its high phenolic content (Rosidah et al. 2008). The phenolic content is likely to act as a stamina booster. Jarikasem et al. (2013) showed that *G. procumbens* could suppress the growth of *Plasmodium falciparum* 3D7 and *Plasmodium berghei* NK65. That proves that the plant can cure malaria following the knowledge of the local community of Lampung.

Plant parts used

The plant parts used by the local community of Lampung in preparation for traditional medicine included roots (7 species), tubers (9 species), stems (12 species), leaves (36 species), flowers (3 species), fruit (24 species), seeds (3 species), and sap (1 species) (Figure 2). The most used plant part was the leaf. Leaves are commonly used as the main ingredient of traditional medicine in many local communities, for example, Madurese (Purwanti et al. 2020) and the Osing Tribe of Banyuwangi (Ardiyansyah and Nurchayati 2018). The reason behind this is that the leaves can be obtained over time, not limited by season (Handayani 2015); also, the use of leaves is relatively sustainable in terms of conservation aspects (Setyowati 2010). *Golong gandu*, also called *kapok* tree (*Ceiba pentandra* (L.) Gaertn.), is a medicinal plant that uses leaves. The plant is believed to be efficacious as a remedy for constipation. The leaves are mixed with a bit of water and then squeezed. The use of the leaves of *C. pentandra* for medicine is also carried out by sub-ethnic Batak Simalungun of North Sumatra. The leaves are cleaned and boiled with water. The locals of Batak Simalungun believe that the concoction of *kapok* leaves can relieve fever (Silalahi et al. 2015).

The fruit was the most widely used plant part after leaves. This result was also reported in other regions in Indonesia, such as the Dani tribe in Jayawiyaja Regency (Mabel et al. 2016) and the Madurese in Sumenep Regency (Purwanti et al. 2020). Fruit stores products from photosynthesis and secondary metabolites of a plant. The content of phytochemicals in fruit is higher than in other plant parts (Pott et al. 2019). Therefore, it is strongly suspected that many medicinal properties are contained in the fruit that can be used as medicine. For example, the fruit of *ketepuk* (*Physalis angulata* L.) is believed to treat high blood pressure by eating the ripened fruit directly. *Kecombrang* (*Etlingera elatior* (Jack) R.M.Sm.), soldiers (*Catharanthus roseus* (L.) G. Don), and celery (*Apium graveolens* L.) are an example of some medicinal plants that all their parts can be used as medicine.

The preparation of medicinal herbs

Eight modes of preparation were commonly practiced by the Lampung Tribe (Table 3). As many as 40.5% of the preparation mode were boiling, filtering, and taking the boiled water. That was the most widely used mode of preparation, followed by pounding or grating and then squeezing it (22.7%), pounding/bruising, then adding a little water (10.8%), and engulfing it (9.4%) (Table 3). Although local people mix medicinal plants can vary, such as *lempuyang* (*Zingiber zerumbet* (L.) Roscoe ex Sm.), there are at least three methods to mix this medicinal plant. The first method was grated, squeezed, given a little extra drinking water, and ready to be consumed. The second method was to pound it, boil it until it boils and take the boiled water to drink, and the last was pounded, heated over a fire, and consumed or smeared.

Table 3 shows various modes of preparation for extraction to obtain phytochemical content that is useful as a medicine. The medical efficacy of medicinal plants depends on the extraction or preparation of the material (Odey et al. 2012). For example, extracts of medicinal plants obtained from the oven-drying method have lower phytochemical content than those obtained by freeze-drying (Mahanom et al. 1999). Thus, the various form of medicinal plant preparation by the Lampung Tribe resulted in different concentrations of phytochemicals and possible doses according to curable diseases. Boiling and filtering techniques were the highest percentages of preparation mode (40.5%). In the boiling technique, the chemical compounds of medicinal plants can be easily extracted since some phytochemical compounds can be dissolved in hot water (Syah et al. 2014). In addition, the boiling technique is reasonably practical compared to other techniques, such as pounding or grating (Mabel et al. 2016). The percentage of medicinal plants prepared by boiling was also highest in the Saibatin community of Tanggamus Regency in Lampung Province and the Tengger tribe of Ngadisari village in East java (Jadid et al. 2020; Wakhidah and Hayati 2021).

The curable disease

The local people of Lampung use plants to treat various diseases, grouped into three categories: external, internal, and internal or external diseases. The use of medicinal plants in traditional practice is mainly used to treat internal diseases (50 species), followed by external diseases (13 species), and both internal and external diseases (5 species) (Figure 3). The number of plants used to cure internal disease is directly proportional to the highest preparation mode, namely boiling. The boiling technique allows phytochemical compounds to dissolve in water, increasing their bioactivity and being absorbed quickly by the body through metabolic processes (Jadid et al. 2020). As a result, this technique is suitable for extracting important compounds in internal medicine. For example, *adas pulowaras* (*Foeniculum vulgare* Mill.) treat digestive problems, flatulence, and constipation.

For example, the use of plants for external medicine in the Tane'olen community in East Kalimantan is the highest in number (Karmilasanti and Supartini 2011). The method for treating external diseases mostly comes from a single plant by heating and then wrapping, smearing, or pounding and then smearing it. Like the Malays in Seponti District, North Kayong Regency, the external treatment method generally uses a single plant by pounding and then placing it on the affected part (Wulandara et al. 2018). External treatment is usually carried out to treat diseases that appear on the skin or are related to the skin, such as itching, fever, bloating, and scratches. One example is *the bangle* (*Zingiber montanum* (J. Koenig) Link ex A. Dietr.) used to treat itching on the skin due to fungus or allergies. The rhizomes of the plant have antioxidant activity. The concentrations of alkaloids, phenolics, flavonoids, saponins, and triterpenoids of *Z. montanum* effectively inhibit the growth of *Escherichia coli* and *Streptococcus sobrinus* (Setyani et al. 2021). Therefore, to the scientific study, the phytochemical content of *Z. montanum* is efficacious for treating itching and rashes due to bacterial activity (add references here).

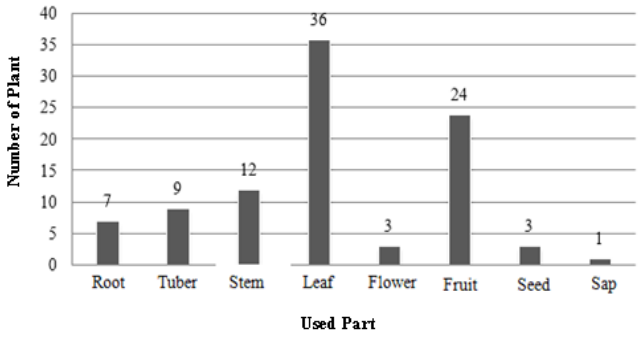

Figure 2. The used part of medicinal plants utilized by the local community of Lampung in Way Kambas Natural Park (TNWK), Lampung, Indonesia

Table 3. The mode of preparation employed by the Lampung Tribe, Indonesia, along with local citation in percent

Mode of preparation	Citation (%)
Boiled then filtered	40.5
Heated/putting on fire	5.4
Mash/grated and squeezed	22.7
Brewed with hot water	5.4
Consumed as vegetable	9.4
Pounded/bruised and add the water	10.8
Take the sap	2.7
Smeared	2.7

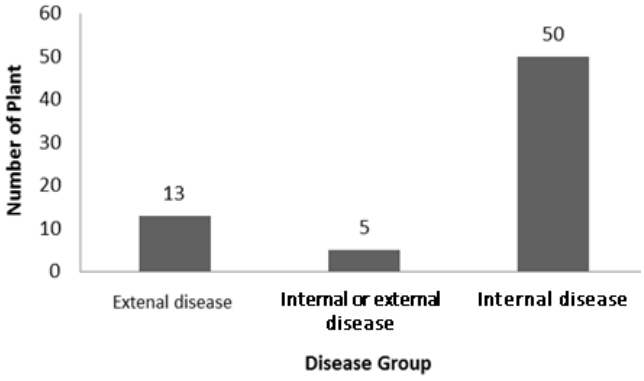

Figure 3. The number of medicinal plants classified into three groups of diseases by the Lampung Tribe in the area around TNWK

Binahong (*Anredera cordifolia* (Tenore.) Steenis) is an example of plant species used to treat internal and external diseases. The community commonly uses the plant to treat stomach ailments, i.e., stomachache, fever, and burn. The leaf extract of *A. cordifolia* has been scientifically proven to treat burns by increasing the thickness of the re-epithelialization layer in experimental mice that have been given burns (Shrivastav et al. 2018). This plant also shows antibacterial activity. The extract can inhibit the growth activities of *Staphylococcus aureus*, MRSA, *B. subtilis*, *P. aeruginosa*, and *E. coli* (Leliqia et al. 2017). Furthermore, its leaf extract has the potential to be an anti-inflammatory, so it accelerates wound healing and reduces the effects of inflammation, such as fever (Laksmitawati et al. 2017).

In conclusion, the Lampung Tribe community around the TNWK area utilizes 69 species of medicinal plants belonging to 39 families. Zingiberaceae is the highest number of used species (9 species). This result is similar to the other ethnobotanical studies in Indonesia, where Zingiberaceae is the most used plant family. The part of medicinal plants most used by the local community is leaves as many as 36 species. The leaf is the most used part of medicinal plants due to its abundance, ease of accessibility, and phytochemistry content. This finding is also reported in other regions.

Furthermore, the boiling method is the most widely used preparation mode (40.5%) in the Lampung Tribe. Some studies in Indonesia report similar findings, while others are different. It depends on the various types of curable diseases. If the highest curable disease is internal, the most preparation method may be by boiling. The local people of Lampung use plants to treat various diseases grouped into three categories: external diseases, internal diseases, and internal or external diseases. Internal diseases are the highest type of curable disease.

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Yield performances of rice varieties (*Oryza sativa*) under nano-CuO and nano-ZnO micronutrient fertilizers

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[1]Department of Botany, Faculty of Natural Sciences, Open University of Sri Lanka. Nawala, Nugegoda, Sri Lanka. Tel. +94-71-493-3922, ♥email: srwee@ou.ac.lk

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Abstract. *Sandanayake CLT, Weerakoon SR, Karthikeyan N, Somaratne S. 2022. Yield performances of rice varieties (*Oryza sativa*) under nano-CuO and nano-ZnO micronutrient fertilizers*. *Nusantara Bioscience 14: 95-103.* Rice (*Oryza sativa* L.) is a primary staple food in the world. There is an extreme need for higher rice yields to meet the ever-increasing demand with the rise in population despite the hampering impact of climatic changes. Nanotechnology plays a potential role in food security and introduces nano fertilizers as an alternative to conventional fertilizers. Therefore, the present research was carried out to determine the effects of nano micronutrient fertilizers, nano-CuO, and nano-ZnO on the yield of selected rice varieties, *Bg360*, *BW364*, *Kaluheenati*, and *Kuruluthuda*. Sol-gel and thermal decomposition methods synthesized nano-fertilizers used in the study. Nano-ZnO, nano-CuO, and nano-CuO-ZnO composite treatments were applied as a foliar spray at concentrations of 30 mg L^{-1} (T1), 60 mg L^{-1} (T2), and 120 mg L^{-1} (T3) and double deionized water served as the control (T0). The synthesized nano-fertilizers were applied during the growth stage [at 48-58 days after sowing (DAS)] and grain filling stage [100-105 DAS], while the plants were provided with appropriate levels of N, P, and K fertilizers, as recommended by Department of Agriculture. A Complete Randomized Block Design was employed with three blocks and five replicates in each block. The Yield parameters were recorded at the harvesting stage. Descriptive statistics such as mean and standard error of mean and inferential statistics were performed on the data obtained. ANOVA with interaction terms was performed to assess the significant differences between the treatments. The descriptive analyses show an increase in the yield of the rice varieties under the application of nano-fertilizers. ANOVA suggests a significant effect ($p \leq 0.05$) of nano-CuO and nano-ZnO on the yield of rice varieties used in the study. Both traditional and inbred rice varieties indicated yield response to applied nano-fertilizers.

Keywords: Micronutrients, nano-fertilizer, *Oryza sativa*, traditional and inbred rice varieties

INTRODUCTION

Rice is a semi-aquatic annual plant of the family Poaceae, the second-largest commodity produced worldwide (Hiyasmin et al. 2015). Population growth is one of the main factors leading to increased demand (Nurhasanah et al. 2016). Rice is the single most important crop and the main staple diet in Sri Lanka, according to the Department of Agriculture (2020), accounting for 34 percent (0.77 million hectares) of the total planted area. Sri Lanka's current annual rough rice production is 2.7 million tons, which meets the bulk of the country's needs. Rice demand is expected to expand at a rate of 1.1 percent per year, while rice output will need to grow at 2.9 percent per year to keep up. Increasing the cropping intensity and national average yield can achieve these production targets.

A widespread nutrient deficiency worldwide has been reported in agricultural lands, significantly decreasing crop yield. In Sri Lanka, the yields of many annual crops are much less than the potential and the world average due to an inadequate supply of mineral nutrients (Kumaragamage et al. 2011). Sri Lanka has three agroecological zones, the wet zone (>2,000 mm), the intermediate zone (1,000-2,000 mm), and the dry zone (<1,000 mm) (Bandara and Silva 2000a; 2000b). Paddy production is high in the intermediate zone and the dry zone. In dry and intermediate zones, soils are deficient in Zn and Cu (Bandara and Silva 2000a; 2000b). Moreover, poorly drained rice-growing soils of a mid-country wet zone of Sri Lanka are also deficient in zinc (Nagarajah et al. 1983).

With the green revolution, the micronutrients of agricultural lands have decreased due to the significant increase in crop yields. Hence, global crop production and food security depend highly on fertilizers input to agricultural lands. Studies have found a substantial increase in crop production due to the application of micronutrients (Zain et al. 2015). It is reported that after the green revolution era in India, nitrogen fertilizer usage in the form of urea has increased by 29% (Duhan et al. 2017). However, such large-scale use of chemical fertilizers is unsustainable in the long run (Solanki et al. 2015; Mapa 2020).

It is predicted that the world population will exceed 9 billion by 2050. Total food consumption will have to rise by 50%-70% if these people are adequately fed (Jaggard et al. 2010). Such an increase in food demand cannot be met without extensive application of fertilizers. Yet, the effectiveness of conventional fertilizers is limited by their low nutrient use efficiency (Guo et al. 2018). Nanotechnology manipulates matter on an atomic and

molecular scale (Qureshi et al. 2018). Nano fertilizers are nanostructured formulations of plant nutrients that can be applied to plants (Raliya et al. 2017). There are three ways to deliver agrochemicals into plants; seed treatments, soil amendment, and foliar sprays (Mahil and Kumar 2019). Nano fertilizers are rising as a competent substitute for conventional fertilizer application methods and can significantly supply micronutrients to plants (Sekhon 2014). Nanoparticles can be given to plants at specific times and concentrations; hence they are considered SMART (Specific, Measurable, Achievable, Relevant, and Time-oriented) delivery systems (Shaoyu Lu 2016).

According to a recent study, foliar application of Nano-ZnO fertilizers increases growth and specifically yield performances of rice varieties *Suwandal*, *Pachchaperumal*, and *Bg94-1*(Somaratne et al. 2021). Though nano fertilizers are prominent in countries such as China, India, Iran, etc., using Nanofertlizers in Sri Lankan paddy cultivation is inconspicuous. Since the yield of traditional rice remains low and there is a market for traditional rice due to its medicinal and nutritional value, this study was mainly focused on the possibility of increasing the rice yield of inbred and traditional rice varieties. This study also studies the possibility of introducing Nano fertilizers as an alternative to conventional micronutrient fertilizers. The yield performances of two traditional and two inbred rice varieties under the treatment of Nano-CuO and Nano-ZnO SMART fertilizers were assessed.

MATERIALS AND METHODS

Selection, germination, and transplant of rice varieties

Four rice varieties of the three-and-half-month category of rice varieties were chosen for the study. Inbred varieties *Bg360* (Keeri samba) and *BW364* (Red Nadu) and traditional varieties *Kalu Heenati* and *Kuruluthuda* were chosen based on the duration of cultivation, suitability for lowland paddy cultivation, and popularity among the Sri Lankan farmers, consumers, and rice distributors. Seed materials were collected from Rice Research and Development Institute (RRDI), Batalagoda, and the Regional Rice Research and Development Center (RRRDC), Bombuwela.

The seeds were germinated in plastic trays in a growth chamber at the Botany research laboratory of The Open University of Sri Lanka. After 14 days, the seedlings were transplanted in pots (22 cm x 22 cm) containing paddy soil. The recommended levels of macronutrients by the Department of Agriculture, nitrogen, phosphorous, and potassium, were added to the pot soils before seedling transplant. The pot-level experiment was conducted under greenhouse conditions in the Open University premises, Nawala. These pots consisting of five (5) rice plants were arranged in a Complete Randomized Block Design (CRBD). Five replicates of each treatment were carried out for each rice variety.

Synthesis of nano-CuO and nano-ZnO

Copper and Zinc nanoparticles were prepared using the metal acetates of the respective elements. Two different routes were followed to get the desirable surface morphologies suitable for this investigation (Saravanan et al. 2012). nano-CuO was prepared via thermal decomposition, and Nano-ZnO was prepared via a Sol-gel method.

Thermogravimetric analysis was carried out to confirm the decomposition temperatures of the precursor molecules by SDTQ600 Thermo Gravimetric Analyzer at the Sri Lanka Institute of Nano Technology under the following conditions; temperature range ambient to 800°C, Ramp 10°C/mins, Purge gas was compressed air.

Precursor chemicals Copper acetate [$Cu(CH_3COO)_2.H_2O$] and Zinc acetate [$Zn(CH_3COO)_2.2H_2O$], NaOH, and isopropyl alcohol (2-propanol) with high purity (> 99.5 %) were purchased. Analytical grade chemicals and reagents were used in this study, and deionized double distilled water was used to prepare all aqueous solutions.

Nano fertilizer characterization

Nanoparticles were characterized with XRD (X-Ray diffraction) and SEM/TEM (Scanning Electron Microscopy/ Transmission Electron Microscopy) to confirm their physical properties, viz. structure, morphology, and elemental composition. In addition, prepared Nano-CuO and Nano-ZnO particles were characterized with a Bruker D8 Focus X-ray diffractometer. Samples were compressed onto the sample holder, and the X-ray diffraction patterns were recorded under the following conditions: Cu-Kα radiation (1.54A°), a voltage of 40 kV, a current of 40 mA, 2ɵ range from 10° to 20° and a scan speed of 1 sec/step.

The average crystallite size of the nanoparticles was calculated using the Scherrer equation.

$$D = \frac{k\lambda}{\beta \cos\theta} \quad \text{Eq. 1}$$

Where; k: Scherrer's constant, λ: wavelength of X-rays, θ: Bragg's diffraction angle, β: full width at half maximum, and D: crystallite size (Scherrer 1918; Mohammadian et al. 2018).

To confirm the synthesized nanoparticles' physical properties, viz. structure, and surface morphology, Scanning Electron Microscopy was performed by Hitachi SU6600 Analytical Variable Pressure FE-SEM (Field Emission Scanning Electron Microscope) at the Sri Lanka Institute of Nanotechnology.

Nano fertilizer suspension preparation and application

Nanoparticle suspensions of different concentrations were prepared by weighing the prepared nano-CuO and nano-ZnO particles and suspending them in the double-deionized water. Nano-CuO, nano-ZnO, and the Nanocomposite of CuO and ZnO Fertilizers were used in spray applications at a concentration of 0, 30 and 60, and 120 mg L^{-1} (F0, F1, F2, and F3) and double deionized water were served as control. The treatments were carried out in two stages as a foliar spray application. During

bearing [at 48-58 Days after sowing (DAS)] and at the stage of grain filling [(100-105 DAS)]. The application was made using pump sprayers for 30 seconds per plant at a constant spraying rate. The first application was made 50 DAS on 26 July 2020 at 07.00 am. (Temperature was 24^0C, in the absence of the wind). The second application was at 100 DAS on 14 September 2020. (Temperature was 25^0C, in the absence of the wind).

Measurement of yield parameters

The yield parameters were taken during the harvest. First, the number of panicles per plant was counted and recorded (Plant Genetic Resource Center 1999). Then, after the harvesting, the length of panicles was measured from the tip of the panicle to the base. Next, the number of grains per panicle was counted and recorded for each panicle. Finally, 100- filled grains were weighed using the electrical balance and recorded.

Statistical analysis

Descriptive statistics, i.e., mean and standard error of the mean, were calculated and tabulated. The data were subjected to univariate analysis of variance (3-way ANOVA) to assess the interaction between the rice varieties, fertilizer treatment, and DAS. The significance level of the results, $p \leq 0.05$, was considered statistically significant. Non-parametric characters were subjected to the χ^2 test. Statistical analysis was carried out by using SPSS ver. 20.0.

RESULTS AND DISCUSSION

Thermogravimetric analysis (TGA)

The TGA thermogram of copper acetate monohydrate at the temperature range of 50-800°C is shown in Figure 1. The initial weight loss of 9.190% at 150°C is due to dehydration, yielding anhydrous copper acetate [$Cu(CH_3COO)_2$]. A weight loss of 52.75% resulted in the range of 250-300°C, indicating acetate decomposition and the formation of copper oxide. There was no further decomposition beyond 350°C. A nano-CuO yield of 5g was obtained from 14.06g of Copper acetate monohydrate.

The TGA thermogram of zinc acetate dihydrate at the temperature range of 50-800°C is shown in Figure 2. According to the figure, there is an initial weight loss of 16.52% at 100°C, which is due to dehydration, yielding anhydrous zinc acetate [$(Zn(CH_3COO)_2$]. A weight loss of 63.47% resulted in the range of 250-350°C, indicating acetate decomposition and the formation of zinc oxide, and there was no further decomposition beyond 350°C. A nano-ZnO yield of 5g was obtained from 22.7g of zinc acetate dihydrate.

X-ray diffraction

The XRD of the prepared CuO nanoparticles and ZnO nanoparticles are shown in Figures 3 and 4, respectively. First, the highest XRD peak was used to calculate the crystallite size, and the peak width and height were extracted with ORIGIN ver.9.5 software. Then the crystallite size was calculated using Scherrer's equation (Equation 1). The calculated crystallite size of the Nano-CuO particles was 26 nm, and that of the Nano-ZnO particles was 31 nm.

Scanning Electron Microscopic (SEM) analysis

FE-SEM (Field Emission SEM) was carried out to observe the surface morphologies of the synthesized nanoparticles. FE-SEM images of synthesized Nano-CuO and Nano-ZnO particles are shown in Figure 5 and Figure 6, respectively. Nano-CuO particles also showed agglomeration and varied particle shape from rod to spherical. The scale is one μm divided into 10; therefore, the particle size of CuO nanoparticles is confirmed to be included in the nano range. ZnO nanoparticles also showed agglomeration, which results in a greater surface-area-to-volume ratio. In addition, the shape of the nanoparticles ranges from spherical to hexagonal. The scale is 500 nm divided into 10; therefore, the size of the ZnO nanoparticles is in the nano range.

Descriptive statistical analysis of yield parameters

Standardized Mean values of the number of panicles per plant in four rice varieties were plotted against the nano fertilizer treatment and graphed (Figure 7). As per the descriptive analysis, the applied nano fertilizers affected the yield attributes. The mean number of panicles per plant in *Bg360*, *Bw364*, *Kaluheenati*, and *Kuruluthuda* was highest in CuO 60 mgL^{-1}, ZnO+CuO 60 mgL^{-1}, and ZnO60 mgL^{-1}. Comparatively, the number of panicles was higher in inbred varieties than in traditional varieties.

Standardized Mean values of the panicle length of four different rice varieties against the nano fertilizer treatment were graphed (Figure 8). The selected rice varieties showed the highest mean panicle length when treated with 60mgL^{-1} of Nano-CuO, Nano-ZnO, and the NanoCuO+Nano-ZnO. In addition, the mean panicle length was comparatively higher in the two traditional varieties than in the inbred varieties (Figure 8).

Standardized Mean values of the number of grains per panicle of four different rice varieties against the nano fertilizer treatment were graphed (Figure 9). According to the graph, the selected rice varieties showed the highest mean number of grains per panicle when treated with 60mgL^{-1} of Nano-CuO, Nano-ZnO, and NanoCuO+Nano-ZnO. The traditional variety, *Kuruluthuda*, showed the highest number of grains per panicle out of the four studied varieties.

Standardized Mean values of the weight of 100 grains of selected rice varieties were plotted against the nano fertilizer treatment (Figure 10). Results on the highest weight of 100 grains were reported in the rice plants of the selected varieties, treated with 60mgL^{-1} of Nano-CuO, Nano-ZnO, and the NanoCuO+Nano-ZnO. The mean weight of 100 grains in the two traditional varieties was more significant than in the studied inbred varieties. Between *Bg360* and *Bw364*, the latter showed a higher mean weight of 100 grains, as *Bg360* has small grains compared to the other varieties.

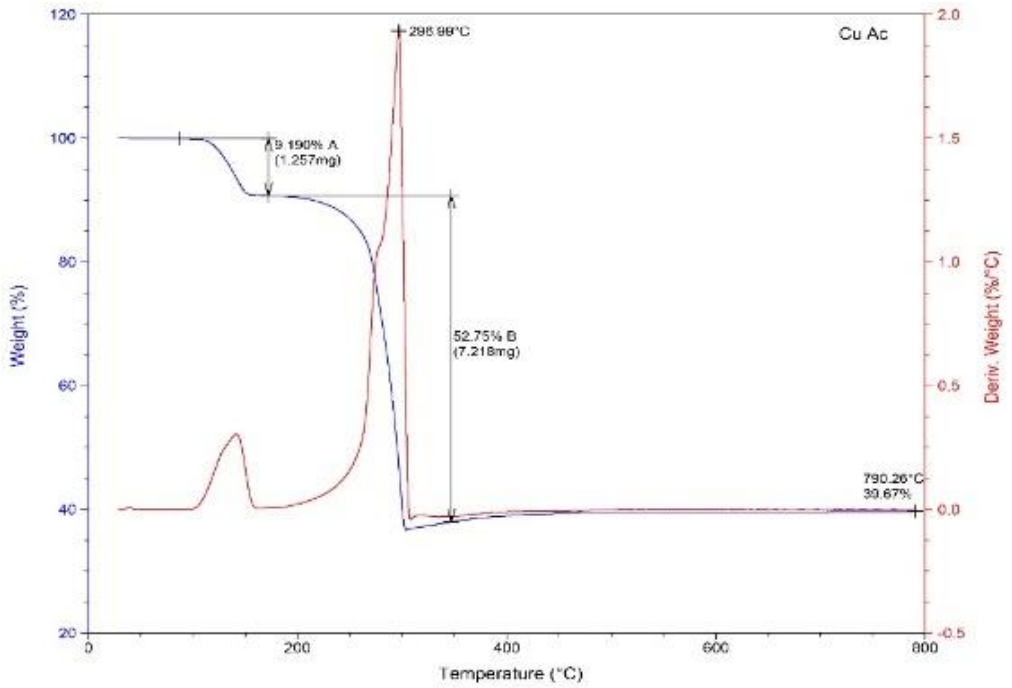

Figure 1. Two-step decomposition of Copper (II) Acetate monohydrate to form Nano-CuO

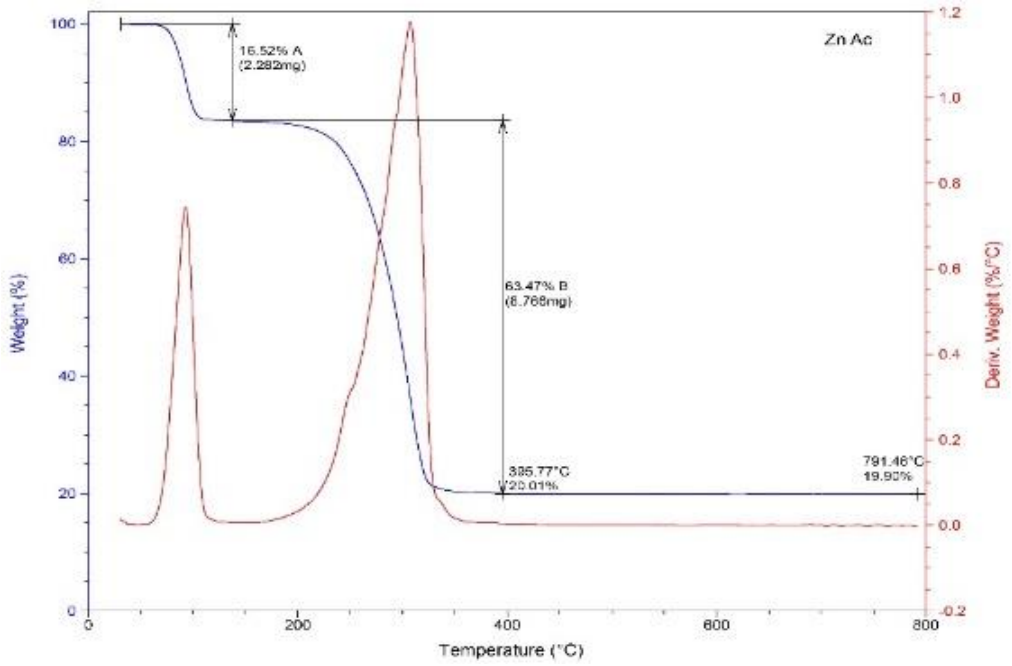

Figure 2. Two-step decomposition of Zinc (II) Acetate dihydrate to form ZnO

Inferential statistical analysis of Yield parameters

The Inferential Statistical Analysis shows the number of panicles per plant (Table 1) of the four different rice varieties subjected to univariate variance analysis. The results suggest a significant ($p \leq 0.05$) effect of the element, concentration, and variety, on the number of panicles per plant. The interactions between the subjects were insignificant in the number of panicles per plant. The panicle length of the four different rice varieties was subjected to univariate analysis of variance (Table 1). The results suggest a significant ($p \leq 0.05$) effect on the panicle length of the element, concentration, variety, and interactions between the subjects. The number of grains per panicle of the four rice types was significantly affected by the variety, element, concentration, and interactions between those factors ($p \leq 0.05$) (Table 1). The weight of 100 grains of the four distinct rice cultivars was also subjected to ANOVA (Table 1). The result suggests that the weight of 100 grains is significantly ($p \leq 0.05$) affected by the element, concentration, and variety. Also, there were substantial interactions between subjects.

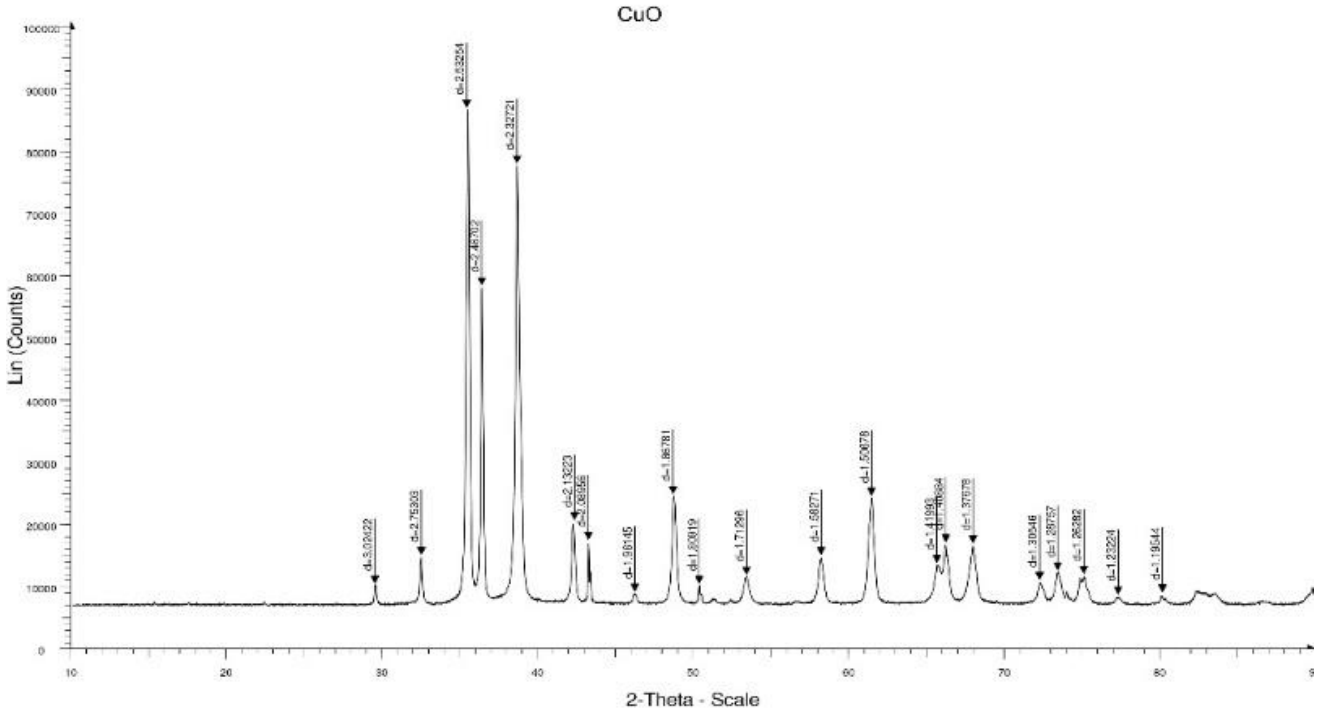

Figure 3. X-ray diffraction of CuO nanoparticles

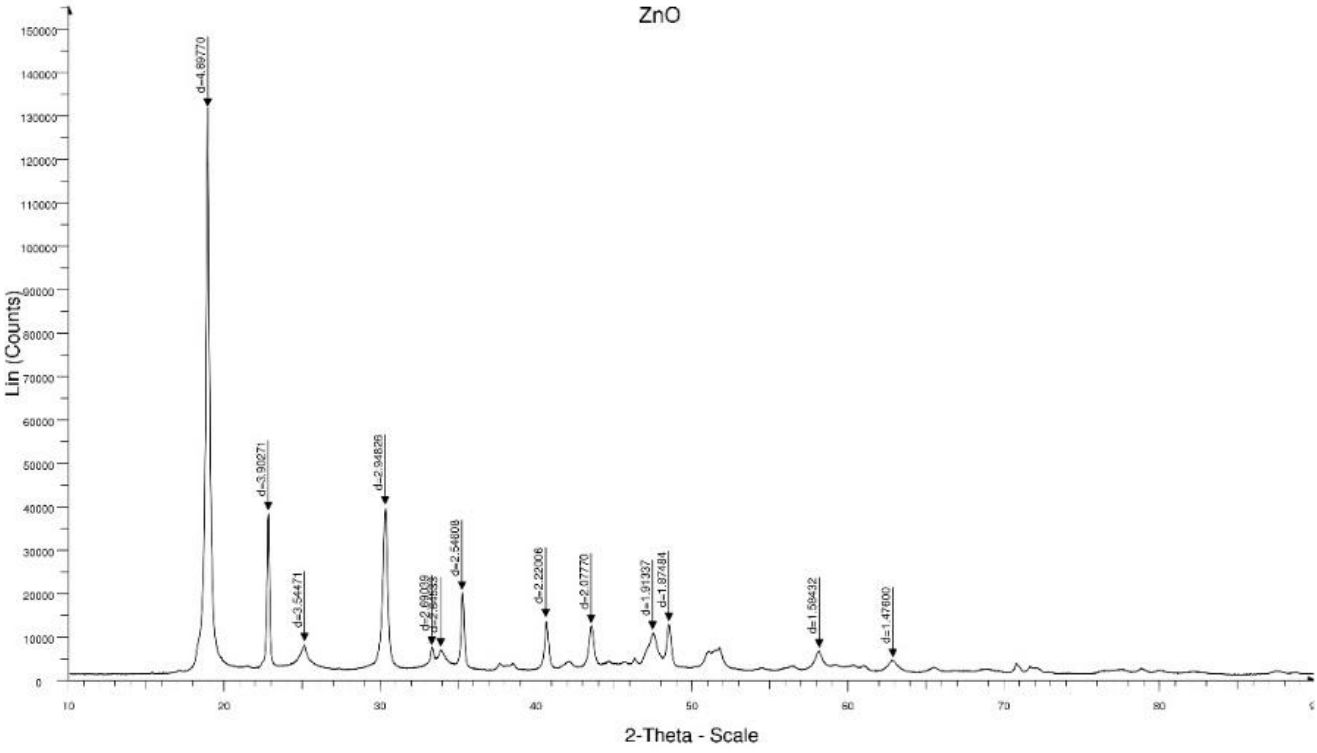

Figure 4. X-ray diffraction of ZnO nanoparticles

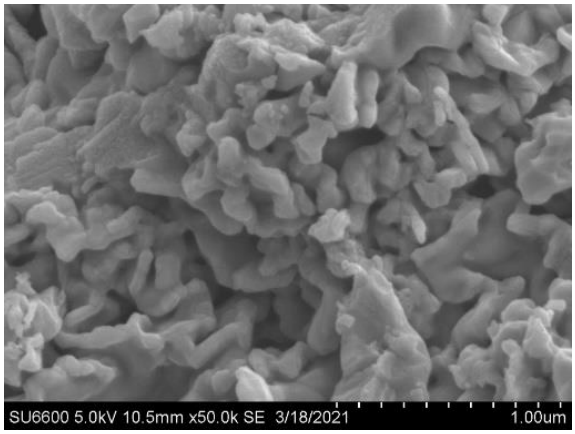

Figure 5. SEM of Synthesized CuO nanoparticles

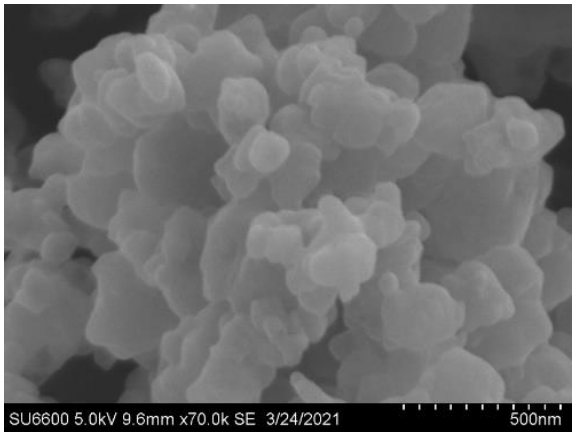

Figure 6. SEM of Synthesized ZnO nanoparticles

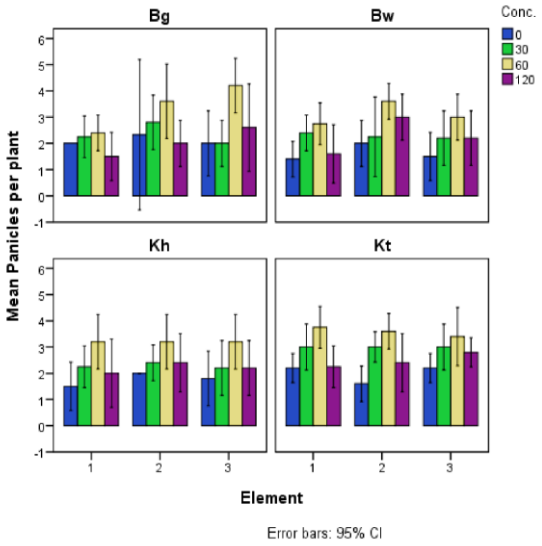

Figure 7. Mean number of panicles per plant with Different Nano fertilizer treatments in 4 different Rice varieties (1-Nano-CuO, 2-Nano-ZnO, 3-NanoCuO+Nano-ZnO)

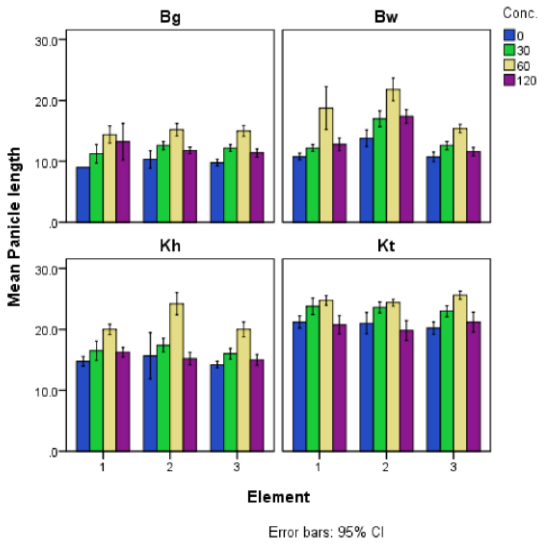

Figure 8. Mean Panicle length with Different Nano fertilizer treatments in 4 different Rice varieties (1-Nano-CuO, 2- Nano-ZnO, 3-NanoCuO+Nano-ZnO)

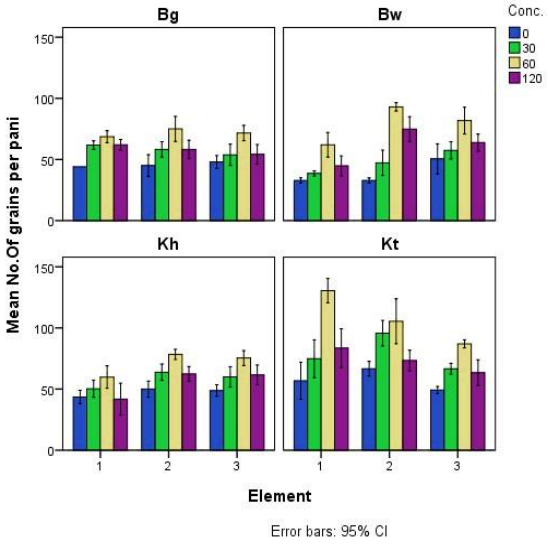

Figure 9. Mean Grains per panicle Different Nano fertilizer treatments in 4 different Rice varieties (1-Nano-CuO, 2- Nano-ZnO, 3-NanoCuO+Nano-ZnO)

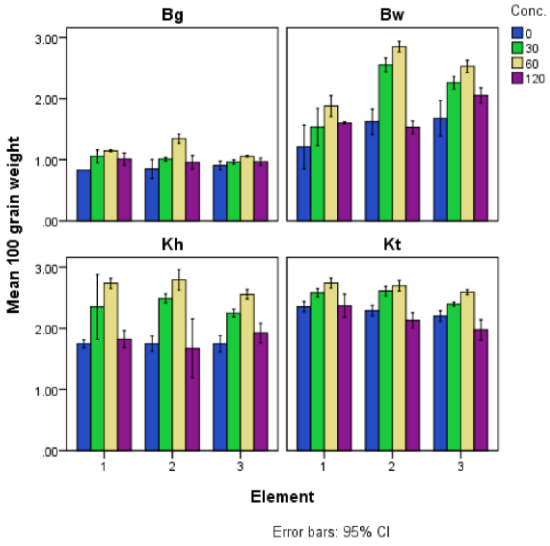

Figure 10. Mean of the weight of 100 grains with Different Nano fertilizer treatments in 4 different Rice varieties (1-Nano-CuO, 2-Nano-ZnO, 3-NanoCuO+Nano-ZnO)

Table 1. Summary of ANOVA of the yield parameters

Character	Source	df	Mean Square	Sig.
Panicles per plant	Corrected Model	46	2.011	.000
	Intercept	1	1241.169	.000
	Variety	3	2.019	.017
	Element	2	2.033	.032
	Concentration	3	17.066	.000
	Variety * Element	6	.899	.162
	Variety * Concentration	9	.373	.757
	Element * Concentration	6	.508	.511
	Variety * Element * Concentration	17	.517	.579
	Error	175	.577	
	Total	222		
	Corrected Total	221		
Panicle length	Corrected Model	46	98.102	.000
	Intercept	1	55760.442	.000
	Variety	3	989.964	.000
	Element	2	65.592	.000
	Concentration	3	295.929	.000
	Variety * Element	6	23.871	.000
	Variety * Concentration	9	9.859	.000
	Element * Concentration	6	3.999	.000
	Variety * Element * Concentration	17	3.885	.000
	Error	175	.909	
	Total	222		
	Corrected Total	221		
Number of Grains per panicle	Corrected Model	46	1538.026	.000
	Intercept	1	810479.680	.000
	Variety	3	6896.193	.000
	Element	2	1458.432	.000
	Concentration	3	10347.634	.000
	Variety * Element	6	1531.335	.000
	Variety * Concentration	9	655.769	.000
	Element * Concentration	6	161.972	.003
	Variety * Element * Concentration	17	288.872	.000
	Error	175	46.813	
	Total	222		
	Corrected Total	221		
Weight of 100 grains	Corrected Model	46	1.843	.000
	Intercept	1	716.189	.000
	Variety	3	17.338	.000
	Element	2	.361	.000
	Concentration	3	4.630	.000
	Variety * Element	6	.641	.000
	Variety * Concentration	9	.344	.000
	Element * Concentration	6	.271	.000
	Variety * Element * Concentration	17	.073	.000
	Error	175	.015	
	Total	222		
	Corrected Total	221		

Discussion

Zn, Mn, B, Fe, Cu, and Mo are the most important micronutrients for higher plants and occupy a significant portion as they are essential for increasing plant growth. Their importance increases due to their role in plant nutrition and increasing soil productivity. Certain mineral nutrients are gaseous and enter plants through leaf stomata (Solanki et al. 2015). Moreover, copper holds extreme importance in plant life. Copper is involved in cellular activities such as photosynthesis and mitochondrial respiration, carbon and nitrogen metabolism, oxidative stress protection, and cell wall synthesis. Copper act as a catalytic metal in many oxidase enzymes, such as cytochrome-c-oxidase. Copper also assists iron utilization in chlorophyll synthesis (Hänsch and Mendel 2009). Copper deficiency causes reduced pollen viability,

resulting in the formation of sterile spikelets and the development of unfilled grains (Dobermann and Fairhurst, 2000). Zinc is a crucial element in plants and is involved in Chlorophyll production, maintaining membrane activities, cytochrome, nucleotide synthesis, seed development, and stalk maturation. Under Zn deficiency, the time to reach crop maturity increases, and tillering, leaf blade area, and chlorophyll content decrease. Tillering can be halted entirely if the deficiency is severe (Dobermann and Fairhurst 2000).

Nano fertilizers can supply micronutrients to plants (Mahil and Kumar 2019). Although soil application is the most common method to supply essential nutrients to plants, higher plants can absorb mineral nutrients when applied as foliar sprays at inappropriate concentrations (Fageria et al. 2009). It has been shown that the foliar application of nano-scale nutrients increases nutrient uptake, and the aerosol spray facilitates the even distribution of the nanoparticles (Mahil and Kumar 2019). Rico et al. (2011) report that the foliar sprayed nanoparticles get transported via apoplastic, simplistic pathways, or plasmodesmata from one cell to another. Once entering the cytoplasm of a cell, these nanoparticles reach different organelles and participate in cellular metabolic processes (Solanki et al. 2015). The nanoparticle synthesis processes employed in this study, the Sol-gel method, and thermal decomposition are very effective in preparing fine nanoparticles.

The selected traditional rice varieties, *Kuruluthuda* and *Kaluheenati*, are popular among Sri Lankan farmers and consumers due to their taste and distinct medicinal properties. On the other hand, two selected inbred varieties *Bg360* and *Bw364*, are vastly cultivated in Sri Lanka due to the high yield and consumer demand. The findings of this study reveal that the foliar spray application of the Cu and Zn micronutrients in the form of nano fertilizer suspension can increase the yield of traditional and inbred rice varieties. That could be a viable option for reducing the excessive use of bulk fertilizers, which cause various environmental issues. According to the crop forecast of the Department of Agriculture in Sri Lanka, at the end of August 2020, rice cultivated was 465,191 ha, and the expected paddy production was 1.84 million metric tons. This paddy production can be increased with the application of nano fertilizers. The current study's findings support those of a previous study (Widanapathirana et al. 2018), which found that foliar application of Nano-ZnO-fertilizers improves rice's growth and yield performances varieties; *Suwandal*, *Pachchaperumal*, and Bg94-1. According to a study by Upadhyaya et al. (2015), the application of Zinc nanoparticles on rice has shown a significant physiological effect. Another study on the foliar application of Super-micro plus, an Iranian commercial nano fertilizer that contains 8% of zinc and 0.65% copper, has shown a significant effect on rice growth and yield (Jassim et al. 2019).

In conclusion, the selected rice varieties, *Bg360*, *BW364*, *Kalu Heenati*, and *Kuruluthuda*, exhibited a significant increase in the number of panicles per plant, panicle length, number and grains per panicle, and the 100-grain weight in response to the application of nano-fertilizers of CuO and ZnO. In addition, the yield increases were noticeable in both traditional and inbred rice cultivars. In preparing nano-CuO and nano-ZnO, thermal decomposition and sol-gel techniques proved fruitful. Further, studies involving the application of nanotechnology in Sri Lankan agriculture are still in their early stages, and in-depth research is required to optimize the concentration and the time of application in a varietal-specific manner.

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Ethnobotanical study on medicinal plants used by ethnic people of Gechi District, South West Oromia, Ethiopia

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Abstract. *Desalegn A, Egigu MC, Sasikumar JM. 2022. Ethnobotanical study on medicinal plants used by ethnic people of Gechi District, South West Oromia, Ethiopia. Nusantara Bioscience 14: 104-116.* This study recorded medicinal plants used by ethnic people of Gechi District, Buno Bedele zone of South West Oromia region, Ethiopia. Data were collected from 386 informants through semi-structured interviews, guided field observations, and focus group discussions. Descriptive statistics and quantitative ethnobotanical indices were used to analyze the data. Moreover, an independent t-test and one-way ANOVA were employed to investigate the effect of Sociodemographic traits on traditional medicinal knowledge. The study documented 70 medicinal plant species belonging to 61 genera and 36 families. Most plants (83.7%) were used to treat human ailments. Asteraceae (7 species) mainly represented the family. Most of the plants collected were shrubs (32.9 %), followed by herbs (25.7%). Leaves (42.3%) represented the highest part for remedy preparation. The dominant route of administration of remedies was oral (56%). Jaccard's similarity index (JI) showed a high degree of similarities (JI = 0.75-0.91) among three kebeles namely, Imboro, Koba, and Dike. The highest Informant consensus factor (ICF) value (0.73) was detected for the sensory organs category illnesses. *Juniperus procera* Hochst. Ex. Endl. was observed with the highest fidelity level (FL) index value (0.97) for the wound. The highest preference ranking (PR) was adjudged to be *Ruta chalepensis* L. for stomach ache. *Syzygium guineense* (Willd.) DC. was top-ranked as a multipurpose plant in direct matrix ranking (DR). It was observed that the Traditional Knowledge (TK) of medicinal plants was significantly ($P<0.05$) influenced by the gender, age, and educational level of the people. Therefore, our documentation of TK on medicinal plants possessed by the people of the studied area could help preserve their knowledge for extensive use.

Keywords: Ethnobotanical study, Gechi District, medicinal plants, traditional knowledge

INTRODUCTION

Indigenous people worldwide practice plant-based traditional medicine despite the massive growth of modern pharmacopeia and the advent of innumerable synthetic drugs. Traditional medicine of plant origin has endowed several current medications due to bioactive secondary metabolites. In addition, it will lead to the development of novel drugs for degenerative diseases (Cox and Balick 1994; Newman and Crag 2016). Among different regions of the world, the legacy of African people preferring traditional medicine to modern medicine for their health care is well known (Cunningham 1993; Sofowora 1996). Ethiopia is one of the cultural heritage nations in Africa, with diverse ethnic communities and a rich repository of floral diversity. Modern conventional drugs are widespread in Ethiopia, like in other countries, yet a majority of people in the country rely on traditional medicinal plants for their health care. That is mainly due to the low socio-economic status to afford modern medicine and the cultural acceptability of traditional medicine. However, studies on systematic documentation of traditional knowledge (TK) on medicinal plants are still needed in untapped territories of the country to explore the TK of diverse ethnic groups. Furthermore, the younger generation's unawareness of TK could cause the gradual depletion of biodiversity, which is a loss of TK. Also, the gradual migration of the population to urban areas of the country (Andarge et al. 2015; Jima and Megersa 2018). These factors necessitate ethnobotanical studies to explore the TK of the ethnic groups of Ethiopia.

The study area, Gechi District, Buno Bedele zone (Illu Aba Bora), also encounters the challenges mentioned above that urged the authors to tap the TK of the ethnic community dwelling in the area. The *Oromo* ethnic group inhabits the study site with strong TK on the uses of plants for curing various ailments. According to the District Health Office, for 19 *Kebele*s (*Kebele* is the lowest administration unit), only a few health facilities, viz. four health centers, three veterinary health clinics, and 17 health posts, serve the entire people and livestock. Thus, people in the study area still depend on medicinal plants to treat human and livestock diseases. Therefore, documentation of the study area's medicinal plants and associated TK is essential. The Gechi District is situated in the Southwest part of the *Oromia* regional state. Although some studies have been reported from other parts of the Southwest *Oromia* region, Ethiopia (Awas and Demissew 2009; Abera 2014; Chemeda and Mosisa 2017; Siraj et al. 2020), no ethnobotanical report was found from the Gechi District. Thus, we undertake a study to document medicinal plants and associated traditional knowledge of the ethnic

people to treat human and livestock ailments in ethnobotanically unexplored *Kebele*s of Gechi District, Buno Bedele zone, *Oromia* region, Ethiopia.

MATERIALS AND METHODS

Description of the study area

Gechi District, Buno Bedele zone of Oromia region, Ethiopia, is situated between 8°10'-8°30' N and 36°20'-36°40' E latitude and longitude, respectively (Figure 1). The district is subdivided into 19 *Kebele*s, of which three are urban, and the rest are rural. The elevation of the study area is about 1,787 meters above sea level. The district's total population was 70,478, of which 35,307 were men and 35,171 were women (CSA 2007). Islam followers (87.7%) dominate the total population, followed by Ethiopian Orthodox Christians (10.58%) and Protestants (1.66%). Most people (97.16%) speak *Affan Oromo*, and *Amharic* is spoken only by 2.09% of the population.

Selection of *Kebeles* and sampling

Before collecting data, a reconnaissance survey was performed from July 2017 to August 2017 to select *the district's Kebeles (study sites)* based on vegetation cover, traditional medicine use history, availability of medicinal plants, and traditional medicine practitioners. Accordingly, three *Kebele*s, Imboro, Koba, and Dike, were selected for the ethnobotanical survey out of 19 Kebeles. The survey was conducted between September 2017 and July 2018. A total of 386 participants (age ≥25) were interviewed for data elicitation. Of this, 366 respondents were non-traditional medicinal practitioners selected randomly. In addition, based on the information from the ordinary people of the study area, experienced and highly knowledgeable key informants (20) were selected purposively. For the interviews, the respondents were divided into three age ranges (25-40, 41-60, >60) and three levels of educational status (illiterate, elementary, and secondary schools completed (Megersa and Woldetsadik 2022).

The sample size of the selected *Kebele*s was calculated based on the total population size (11,128) of the three selected *Kebele*s by adapting the formula of Cochran (1977) used by (Eshete and Molla 2021) as follows:

$$n = \frac{N}{1 + N(e)^2}$$

Where n = sample size for a survey; *N* = the total number of populations in the three *Kebeles* (11,128); *e* = maximum variability or margin of error 5% (0.05); and 1= the probability of an event occurring.

Thus, the required sample size was:

$$\frac{11{,}128}{1+ 11{,}128(0.0025)} = 386$$

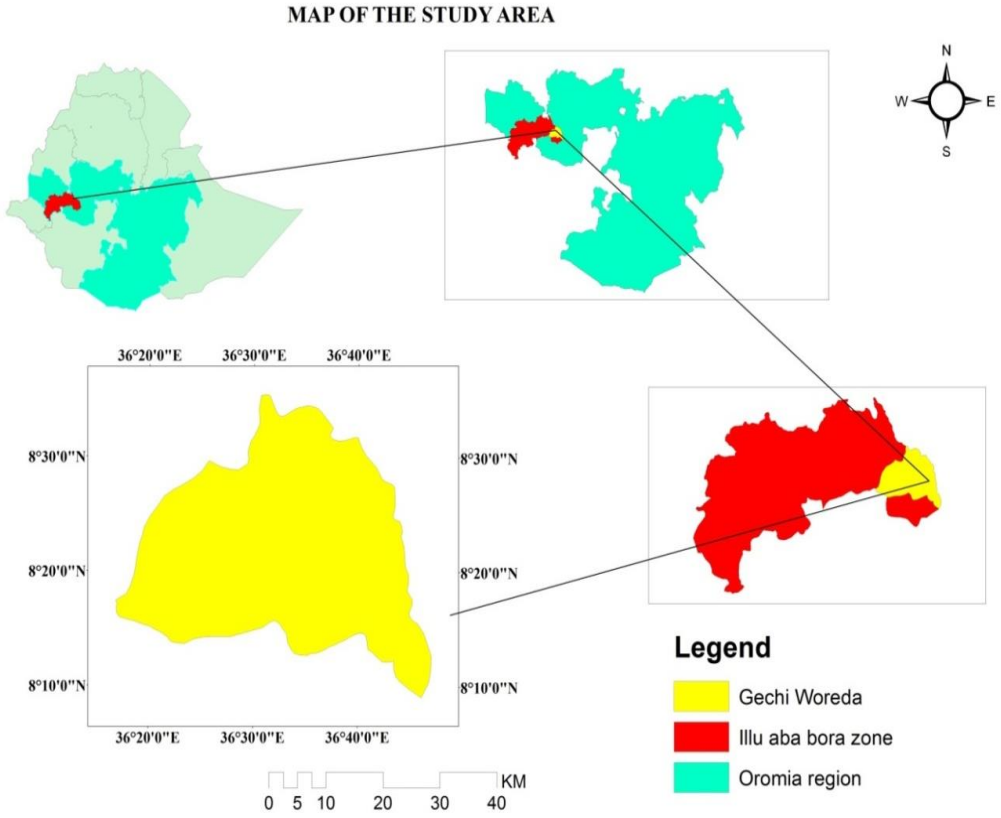

Figure 1. Location of Gechi District, Buno Bedele zone of Oromia region, Ethiopia

Field surveys for TK data collection

During the field trips, data were gathered using methodologies viz. semi-structured interviews, focus group discussion, and direct guided field walks, ethnobotanically and social-demographically. The data on ethnobotany included vernacular names of the medicinal plants, ailments treated, habits, habitat, plant part(s) used, methods of preparation of plant remedies, administration routes, dosage, etc. The socio-ethnographic data encompassed gender, age, educational level, marital status, religion, and occupation. Interviews were made in *Affan Oromo* (the language of the local ethnic group) from the questions prepared in English. Women were interviewed in their ambient vegetation after the purpose of the survey was explained to the family's elders. The ethnomedicinal plants were collected, and the voucher specimens were pressed, dried, and mounted. The identification of the plants was achieved by using Flora of Ethiopia and Eritrea (Friis 2009) and by comparing it with the authentic herbarium specimens at Haramaya University. The scientific names of the medicinal plants were verified by consulting 'the plants of the world online' (https://powo.science.kew.org/). The identified plants were deposited in Haramaya University Herbarium, Ethiopia, for future reference.

Data analysis and comparison of traditional knowledge

The data on TK gathered were statistically analyzed using descriptive statistical methods. In addition, for quantification of TK on medicinal plants, Jaccard's similarity index (JI), Informant consensus factor (ICF), fidelity level (FL), preference ranking (PR), and direct matrix ranking (DR) were computed. Furthermore, to compare TK discrepancies between the informants of different gender, age, and education status. Independent T-test and one-way ANOVA were performed in SPSS version 20. There were two categories in gender (Male vs. Female); the age category was divided into three groups (25-40, 41-60, >60), and educational status had three levels (informants with no formal education, elementary and secondary school completed). Differences were considered statistically significant at $P<0.05$.

Jaccard's similarity index (JI)

Furthermore, to compare the similarity of TK on medicinal plants among the *Kebele*s, the Jaccard's similarity index was calculated using the following formula:

$$JI = \frac{c}{a+b+c}$$

Where JI is the Jaccard similarity index, 'c' is the number of species shared by the study sites, 'a' is the number of species in study site A only, and 'b' is the number of species in study site B only. The JI values range between 0 and 1, whereby a value of 1 indicates complete similarity.

Informant consensus factor (ICF)

The ICF was employed to analyze the agreements among informants on using plant species in various ailment categories and was computed by applying the formula used earlier (Gazzaneo et al. 2005).

$$\text{ICF} = \frac{n_{ur\_}n_t}{n_{ur\_}1}$$

Where, the number of use citations for each ailment (nur) minus the number of species used (nt) for that ailment is divided by the number of use citations for each ailment minus one.

Fidelity level (FL)

The FL, the percentage of respondents claiming a specific plant for the same primary purpose, was calculated for the most frequently reported ailment category using the following equation (Teklehaymanot and Gidey 2007).

$$\text{FL}(\%) = \frac{NP}{N} x100$$

Where Np is the number of informants that claim the use of a plant species to treat a particular disease, and N is the number of informants that use the plants as a medicine to treat any given disease.

Preference ranking (PR)

The PR was calculated using the method of Martin (1995). More frequently mentioned medicinal plants (10 species) for treating stomachaches were selected for PR analysis. In the ranking analysis, randomly selected key informants (10) were enquired to rank the plant species based on their perceived efficacy in curing the disease by giving the highest value (10) for the most effective plant and the lowest value (1) for the least effective plant.

Direct matrix ranking (DR)

The DR was performed using 14 plants claimed by informants for eight use diversities following Martin's (1995) and Cotton's (1996) methods. First, ten key informants were asked to assign use-values viz., 5=best, 4=very good, 3=good, 2=less used, 1= least used, and 0=not used for each plant corresponding to each use-value. Then, the average value of use diversity given by informants for each species was taken, and the values were totaled and ranked.

RESULTS AND DISCUSSION

Traditional medicinal plants documented

The ethnobotanical survey in three *Kebele*s of Buno Bedele Zone, Gechi District, documented 70 medicinal plant species used for various ailments in this ethnic. The medicinal plant species belong to 61 genera and 36 families. About 84% of the plants were used to remedy human ailments, and 12% were utilized against livestock ailments. An insufficient number of species (4%) was used for human and livestock ailments (Table 1). The informants cited some plants for specific ailments among the medicinal plants documented. For example, *Allium sativum* was cited by 47 informants for treating malaria, the common cold, and dental disease, followed by *Datura stramonium* (38 informants) as a remedy for dandruff, wart, and toothache.

Table 1. Traditional medicinal plants documented

Scientific name (habit)	Family	Common name	Habitat	Disease treated	Treatment for	Part(s) used and mode of preparation	Route
Acacia abyssinica Hochst. ex Benth. (Tree)	Fabaceae	*Laaftoo* (O)	F	Tonsillitis	H	Root: Fresh root bark is chewed	O
				Eye disease	H	Leaf: Young leaves are squeezed, and the extract is directly applied to the infected eye	E
Allium sativum L. (Herb)	Alliaceae	*Qullubbi adii* (O)	HG	Malaria	H	Bulb: Bulb of *Allium sativum* and rhizome of *Ginger officinale* are pounded and eaten with honey	O
				Common cold	H	Bulb: Bulb is inserted into the nostrils to sniff	N
				Tooth disease	H	Bulb: Bulb is held on the tooth	O
Artemisia abyssinica Sch., Bip. ex A.Rich. (Herb)	Asteraceae	*Qoddoo* (O)	HG	Malaria	H	Leaf: Fresh leaf and *Allium sativum* bulbs are pounded and consumed	O
Asplenium monanthes L. (Epiphyte)	Aspleniaceae	*Digaluu bakkannisaa* (O)	OT	Headache	H	Leaf: Leaf is pounded with the leaf of *Cussonia ostinii* and drunk with a cup of tea	O
Bersama abyssinica Fresen. (Tree)	Melianthaceae	*Lolchiisaa* (O)	F	Plague	L	Root: Root is crushed and mixed with water to wash the cattle's body	D
				Stomach ache	H	Leaf: Leaf is smashed, mixed with water, and drunk	O
Beta vulgaris L. (Herb)	Chenopodiaceae	*Qosta* (O)	HG	Dehydration	H	Leaf: The fresh leaves are cooked with oil and salt, then after eaten	O
Bidens biternata (Lour.) Merr. & Sherff (Herb)	Asteraceae	*Cogoogitii gurraattii*	RS	Febrile illness	H	Leaf: Leaf is pounded together with the leaf of *Croton macrostachyus* and rubbed against the body	O
Bidens pilosa L. (Herb)	Asteraceae	*Maxanne* (O)	GL	Nasal bleeding	H	Leaf: Freshly squeezed leaves are sniffed	N
Calpurnia aurea (Aiton) Benth. (Shrub)	Fabaceae	*Ceekaa* (O)	AR	Wound	H	Leaf: The leaf is smashed, and the solution is added to the wounded part	D
				Scabies	L	Leaf: Leaf is pounded and mixed with water to wash the body of the animal	D
Capparis cartilaginea Decne. (Climber)	Capparidaceae	*Gooraa* (O)	RS	Toothache	H	Root: Root is chewed and held onto the teeth	O
Carissa spinarum L. (Shrub)	Apocynaceae	*Hagamsa* (O)	F	Gonorrhea and Stomachache	H	Root: Fresh root is pounded and mixed with local alcohol such as "Tella" and drunk	O
				Stomachache	H	Leaf: Pounded leaf of *Carissa spinarum* is mixed with honey and taken in a small amount every morning	O
				Headache	H	Leaf: Dry pounded leaf is smoked	N
Capparis tomentosa Lam. (Shrub)	Capparidaceae	*Harangamaa* (O)	F	Headache	H	Root: Dried root is powdered, and one spoon of the powder is mixed with alcohol and drunk	O
				Diarrhea	H	Leaf: Dried and powdered leaves are mixed with water and consumed	O
Citrus limon (L.) Burm. f. (Shrub)	Rutaceae	*Loomii* (O)	HG	Common cold	H	Fruit: Juice is drunk with tea	O
				Nasal bleeding	H	Fruit: Fruit is sniffed	N
				Tinea versicolor	H	Fruit: Juice is rubbed on the skin	D
Clausena anisata (Willd.) Hook.f. ex Benth. (Shrub)	Rutaceae	*Ulumaayii* (O)	RS	Skin infection	H	Leaf: Leaves of *Clausena anisata, Solanecio gigas,* and *Justicia schimperiana* are pounded together and creamed on the skin	D

Clematis simensis Fresen. (Climber)	Ranunculaceae	*Hidda feetii* (O)	F	Dandruff	H	Leaf: Leaves are pounded and placed on the head	D
				Tonsillitis	H	Leaf: Leaf is crushed, pressed, rolled in a clean cloth, and tied to the neck	D
				Elephantiasis	H	Leaf: Leaf of *Clematis simensis* and *Lagera aleta* are crushed, smashed, and tied to the affected part of the leg	D
Clerodendrum myricoides (Hochst.) R.Br. ex Vatke (Shrub)	Lamiaceae	*Maraasisaa* (O)	RS	Toothache	H	Leaf: Leaves are chewed directly or leaf powder mixed with butter and put on the affected tooth	O
				Toothache	H	Stem: Stem is used as a toothbrush	O
Coccinia abyssinica (Lam.) Cogn. (Climber)	Cucurbitaceae	*Ancootee* (O)	HG	Broken Bone	H	Root: The root is cooked and eaten with oat bread	O
Coffea arabica L. (Tree)	Rubiaceae	*Buna* (O)	HG	Diarrhea	H	Seed: Roasted and pounded seeds are mixed with honey and eaten	O
				Stomachache	H	Leaf: Tea made of leaves is drunk	
Croton macrostachyus Hochst. ex Delile (Tree)	Euphorbiaceae	*Bakkannisa* (O)	F	Wound	H	Sap: Sap is rubbed against the affected body part	D
				Ring Worm	H	Leaf: The leaf is crushed, and the extract is creamed on the infected area	D
Cucumis ficifolius A.Rich. (Climber)	Cucurbitaceae	*Yemidir Embuay* (A)	AR	Stomachache	H	Root: Fresh or dried root powder is mixed with water and drunk	O
				Diarrhea	B	Whole plant: Whole fresh plant is used to prepare a decoction with water and drunk by humans and cattle	O
				Ascariasis	L	Whole plant: Whole dried plant is powdered, mixed with water, and drunk	O
Cucurbita pepo L. (Herb)	Cucurbitaceae	*Buqqee* (O)	HG	Gonorrhea	H	Seed: Seed powder is mixed with water and drunk	O
Cupressus lusitanica Mill. (Tree)	Cupressaceae	*Yeferenji Tid* (A)	HG	Diarrhea (animal)	L	Leaf: Leaves are crushed, mixed with water, and given to cattle to drink	O
Cynodon dactylon (L.) Pers. Herb	Poaceae	*Coqorsa* (O)	GL	Wound	H	Above ground: Above-ground parts of *Cynodon dactylon* are crushed and rubbed on the affected skin for a week with butter	D
Datura stramonium L. (Shrub)	Solanaceae	*Asaangira* (O)	AR	Dandruff	H	Leaf: Fresh leaf is smashed, and its solution is creamed on the affected part of the skin	D
				Wart	H	Stem: Leafy stem is squeezed, and its drop is mixed with butter and creamed on the affected body part	D
Dodonaea angustifolia L.f. (Shrub)	Sapindaceae	*Kitkita* (A)	F	Wounds and eczema	H	Leaf: Fresh leaf paste is mixed with butter and applied to the affected part	D
Echinops kebericho Mesfin (Herb)	Asteraceae	*Qarabichoo* (O)	HG	Wound	H	Root: Dried root is smoked	N
Echinops macrochaetus Fresen. (Shrub)	Asteraceae	*Kusheshile* (A)	F	Toothache	H	Root: Fresh root paste is orally given with water	O
				Febrile illness	H	Root: Dried root decoction is orally given	O
Ehretia cymosa Willd. ex Roem & Schult. (Tree)	Boraginaceae	*Ulaaga* (O)	F	Stomach ache or stabbing pain	H	Leaf: The leaf is smashed, and the human takes the sap	O
Embelia schimperi Vatke. (Shrub)	Myrsinaceae	*Haanquu* (O)	RS	Taeniasis	H	Seed: Seed powder is mixed with water and drunk	O
				Hookworms	H	Seed: Seed powder is mixed with water and drunk to eliminate hookworm	O
Eucalyptus globulus Labill. (Tree)	Myrtaceae	*Bargamoo adii* (O)	HG	Stomach ache	H	Fruit: Top part of the fruit is chewed	O
				Fever	H	Leaf: Leaves are rubbed on the skin to reduce fever	D
				Common cold	H	Leaf: Leaves are boiled in water and inhaled	N

Euphorbia lathyris Georgi (Tree)	Euphorbiaceae	*Adaamii* (O)	F	Heartburn	H	Stem: Stem is chopped and boiled to fumigate ulcerated breast	D
				Rabies	P	Root: One spoon of root powder is mixed with a cup of fresh milk and given to the dog to drink	O
Ficus palmata Forssk. (Shrub)	Moraceae	*Luugoo* (O)	HG	Skin infection	H	Latex: Latex is rubbed on the skin	D
Ficus sur Forssk. (Tree)	Moraceae	*Harbuu* (O)	F	Ringworm	H	Sap: Sap is creamed on the affected skin	D
Ficus sycomorus L. (Tree)	Moraceae	*Odaa* (O)	F	Hepatitis	H	Sap: Sap is collected from the bark surface of *Ficus sycomorus* and creamed on the skin	D
Foeniculum vulgare Mill. (Herb)	Apiaceae	*Ensilal* (A)	RS	Urine retention	H	Whole plant: The whole part is pounded, mixed with water, and drunk	O
Guizotia abyssinica (L.f.) Cass. (Herb)	Asteraceae	*Nuugii* (O)	AF	Cough and Asthma	H	Seed: Seed is roasted, powdered, boiled, and drunk with honey	O
Hordeum vulgare L. (Herb)	Poaceae	*Garbuu* (O)	AF	Broken bones	H	Stem: Seed flour is made into porridge and eaten	O
Indigofera hochstetteri Bak. (Herb)	Fabaceae	*Qoricha hadha'a* (O)	AR	Tetanus	H	Leaf: Leaves are chopped, warmed on fire, and held in the affected area	D
				Tetanus	H	Root: The root of *Indigofera hochstetteri* is powdered, mixed with butter, and put on the affected area	D
Indigofera spicata Forssk. (Shrub)	Fabaceae	*Yayit Misir* (A)	F	Febrile illness	H	Leaf and stem: Fresh leaf and stem are smoked	N
Juniperus procera Hochst. Ex. Endl. (Tree)	Cupressaceae	*Yabesha Tsid* (A)	HG	Wound	H	Leaf: Fresh leaf is crushed, and the solution is creamed on the affected part	D
Justicia schimperiana (Hochst. ex Nees) T. Anderson (Shrub)	Acanthaceae	*Sensel* (A)	F	Rabies	B	Leaf: Leaf with *Salix mucronata* leaf are squeezed, and juice is given to humans and animals before food every morning	O
Kalanchoe densiflora Rolfe. (Herb)	Crassulaceae	*Endahula* (A)	RS	Wound	H	Leaf: Leaf is squeezed and creamed on the wound	D
				Elephantiasis	H	Leaf: Leaf is pounded and tied at the affected part	D
Lantana camara L. (Herb)	Verbenaceae	*Yawef qolo*(A)	F	Leishmaniasis	H	Leaf: Leaf is squeezed and sniffed	N
Lepidium sativum L. (Herb)	Brassicaceae	*Feexoo* (O)	HG	Stomach ache	L	Seed: Dried seed decoction is given to animals	O
Linum usitatissimum L. (Herb)	Linaceae	Talbaa (O)	AF	Indigestion	H	Seed: Seed powder is dissolved in water and drunk	O
Lippia adoensis Hochst. ex Walp. Var. *adoensis* (Shrub)	Verbenaceae	*Kusaayee* (O)	RS	Fungal infection	H	Leaf: Fresh leaf juice is mixed with little water and applied topically	O
				Ringworm	H	Leaf: Leaf is directly rubbed on the affected skin	D
Maesa lanceolata Forssk. (Tree)	Myrsinaceae	*Abbayii* (O)	F	Elephantiasis	H	Bark: Bark is pounded, mixed with butter, and creamed on the affected body	D
				Scabies	H	Leaf: Leaves are rubbed on the skin	D
Nicandra physalodes (L.) Gaertn. (Herb)	Solanaceae	*Haawwixii* (O)	RS	Liver problem	H	Leaf and root: Leaf and root are pounded together and mixed with cold water, and the solution is drunk	O
Nicotiana tabacum L. (Herb)	Solanaceae	*Tamboo* (O)	HG	Blotting	L	Leaf and root: Leaf and root are dried, powdered, mixed with salt, and made as bread. Slice is given to cattle for three days	O
				Gastroenteritis	L	Leaf: Fresh leaf juice is mixed with water and orally given to cattle	O

				Wound	H	Leaf: Dried leaf powder is mixed with *Coffea arabica* seed powder and applied topically	D
Ocimum gratissimum L. (Shrub)	Lamiaceae	*Daamakasee* (O)	RS	Febrile illness	H	Leaf: Leaf of *Ocimum gratissimum* is smashed, and the solution is sniffed nasally	N
				Headache, Cough	H	Leaf: Fresh or dried leaf is crushed, mixed with coffee, and drunk	O
				Allergy	H	Leaf: The leaf of *Ocimum gratissimum* is squeezed, and its drop is applied to the skin	D
Phytolacca dodecandra L'Her (Shrub)	Phytolaccaceae	*Handoode* (O)	RS	Anemia	H	Leaf: Leaf is squeezed, and juice is drunk	O
				Stomachache, Scabies, Itching, and Rabies	B	Leaf: Fresh leaf juice is mixed with water and given orally to humans and livestock	O
				Liver problem	H	Root: Root is pounded, mixed with water, and drunk	O
Podocarpus falcatus (Thunb.) Endl. (Tree)	Podocarpaceae	*Zigba* (A)	F	Stomach ache	H	Leaf: Leaf combined with the leaf of *Syzgium guineense* are pounded, mixed with water, and drunk	O
				Diarrhea	H	Leaf: Fresh leaf is smashed, and the extract is drunk	O
Premna schimperi Engl. (Tree)	Verbenaceae	*Urggeesaa* (O)	F	Eye disease	L	Leaf: Leaves are chewed and spitted on cattle eye	E
				Toothache	H	Root: Root is chewed, and the solution is held in the mouth and swallowed	O
Prunus africana (Hook.f.) (Tree)	Rosaceae	*Hoomii* (O)	F	Wound	L	Bark: Bark is powdered and added directly to wounds of donkey, mule, and horse	D
Rhynchosia elegans A.Rich. (Climber)	Fabaceae	*Tero Areg* (A)	F	Rabies	B	Leaf: Dried leaf powder is mixed with little water and drunk	O
Ricinus communis L. (Shrub)	Euphorbiaceae	*Qoobboo* (O)	HG	Amoebiasis	H	Seed: The dried seed is chewed	O
				Anthrax	H	Seed: Dried seed powder is mixed with water. and drunk	O
				Blotting	L	Root: Root is pounded with salt, mixed with cold water, and given to cattle to drink	O
Rosmarinus officinalis L. (Herb)	Lamiaceae	*Yesiga Metibesha* (A)	HG	Toothache	H	Leaf: Fresh leaf is chewed	O
Rumex abyssinicus Jacq. (Herb)	Polygonaceae	*Meqmeqo* (A)	AR	Dandruff	H	Leaf: Dried leaf powder is mixed with butter and applied to the skin	D
Ruta chalepensis L. (Herb)	Rutaceae	*Xeenaadaama* (O)	HG	Stomachache	B	Leaf: Fresh leaf is crushed with *Allium sativum* bulb and consumed	O
Rumex nepalensis Spreng. (Herb)	Polygonaceae	*Tultii* (O)	AR	Stomachache	H	Root: Roots are chewed, and juice is swallowed	O
				Skin infection	H	Leaf: Leaf is directly rubbed on the affected skin	D
				Amoebiasis	H	Root: Root is pounded and drunk with tea	O
Rumex nervosus Vahl (Shrub)	Polygonaceae	*Embacho* (A)	F	Scabies and acne	H	Stem or leaf: Fresh stem or leaf is crushed, and the solution is mixed with *lemon* juice and water to wash with	D
Salix subserrata Willd. (Shrub)	Salicaceae	*Aleltu* (O)	F	Stomachache	H	Leaf: Dried leaf powder is mixed with milk and drunk	O
Schinus molle L. (Tree)	Anacardiaceae	*Qundoo barbarree* (O)	RS	Pharyngitis	H	Fruit: Fruit is chewed for sore throat	O
				Eye disease	L	Leaf and fruit: Leaf and fruit are chewed and spitted on cattle, equines, goats, and sheep's eye	E

Solanum marginatum L.f. (Shrub)	Solanaceae	*Hiddii* (O)	RS	Plague	L	Fruit: Fresh fruit is smashed, mixed with water, and applied topically on the affected body part of livestock	D
				Skin infection	H	Fruit: Fruit is creamed on the affected skin area	D
				Stomach ache	H	Root: Root tip is chewed and swallowed	O
Syzygium guineense (Willd.) DC. (Tree)	Myrtaceae	*Baddessa* (O)	F	Hookworm	H	Bark: Bark and exudates of *Aloe pubescens* concoction are drunk	O
Triticum aestivum L. (Herb)	Poaceae	*Qamadii* (O)	AF	Skin infection	H	Seed: Seed is chewed, and the bolus is put on the swollen area	D
Verbascum sinaiticum Benth. (Herb)	Scrophulariaceae	*Gurra Harree* (O)	GL	Urinary retention	L	Root: Root is crushed, mixed with water, and given to animal	O
Vernonia amygdalina Delile (Tree)	Asteraceae	*Ebicha* (O)	F	Stomachache	H	Leaf: Fresh leaf, combined with leaves of *Rumex nervosus* and *Justicia schimperiana*, are pounded, mixed with water, and drunk	O
				Urinary retention	H	Leaf: The squeezed fresh leaf is added to water and drunk	O
				Toothache	H	Leaf: Leaves of *Vernonia amygdalina* and bulb of *Allium sativum* are chewed.	O
				Toothache	H	Stem: Stem is used as a teeth brush at the affected site	O
Vigna membranacea A.Rich. (Climber)	Fabaceae	*Hidda hantuutaa* (O)	RS	Rabies	L	Root: Root is dried, powdered, baked with *teff* flour, and given to cattle to eat	O
Zingiber officinale Roscoe *(*Herb)	Zingiberaceae	*Zingibaa*	HG	Common cold	H	Leaf: leaves are boiled in water and drunk	O

Notes: Habitat; Agricultural field (AF), Home garden (HG), Forest (F), Roadside (RS), Grazing land (GL), Around river (AR), and on the tree (OT). Treatment for; Human (H), Livestock (L), Both (B), and Pet animal (P). Route; Oral (O), Nasal (N), Dermal (D), Eye (E), Anal (A). Common Name; *Affan Oromo* (O) and *Amharic* (A)

Taxonomic diversity, habitat, and habit

Analysis of taxonomic diversity showed that Asteraceae was represented by seven species, followed by Fabaceae (6 species). Solanaceae and Lamiaceae were represented by four species each. The families such as Cucurbitaceae, Poaceae, Verbenaceae, Rutaceae, Euphorbiaceae, Moraceae, and Polygonaceae were represented by three species each. Myrtaceae, Capparidaceae, and Cupressaceae were represented by two species each, and the rest (22 families) contributed one species each. Several studies from Ethiopia (Birhanu et al. 2015; Mogosse 2016; Megersa and Woldetsadik 2022) and other countries such as Brazil and Iran (da Costa et al. 2021; Hosseini et al. 2021) reported the abundance of the Asteraceae family in their ethnobotanical records. Extensive growth and a vast number of species may be responsible for the dominance of Asteraceae. Moreover, the high recognition of this family in traditional medicine may be due to diverse bioactive molecules (Petropoulos et al. 2019).

Relating to habitat, the majority (74.28%) of the collected plants were from wild habitats, including forests, roadsides, riverbanks, agricultural fields, and grazing land, whereas 25.71% of the plants were collected from home gardens. These results agree with other reports of the country (Abera 2014). Except for those from home gardens, most medicinal plants appeared out of human management. Thus, they are deemed to be given due attention for conservation.

Concerning the life forms, the majority of the medicinal plants (32.85%) were shrubs, followed by herbs (31.42%), trees (25.71%), climbers (8.5%), and epiphytes (1.42%). Similar results from various parts of the country corroborate our findings (Lulekal et al. 2008). The broader use of shrubs and herbs could be associated with their high abundance in the area. In most other studies conducted in Ethiopia, these life forms were reported as predominant life forms used in traditional medicine (Meragiaw et al. 2016; Gonfa et al. 2020; Mukaila et al. 2021).

Plant parts used, methods of preparation, and modes of administration

For preparing plant remedies, the locals mostly use leaves followed by the root, seed, fruit, sap, stem, entire plant, bulb, and bark (Figure 2). Similar studies in different parts of Ethiopia (Bekele 2007; Ketema 2015; Weldearegay and Awas 2021) also show the leaf as a predominantly used plant in remedy preparations. Preference for the leaf over other plant parts may be due to its perceived curative potential by local people, which serves for pharmacological investigation in novel drug discovery. More dependence on leaves for remedy preparations may not endanger the survival of the mother plant (Jima and Megersa 2018).

The survey unveiled that most of the remedy preparations were in Fresh form (73%), followed by Dry shape (22%) and fresh or dried form (5%). Using a fresh form may help prevent the loss of active constituents during drying or the preparation of the drugs (Chaachouay et al. 2022). Different authors commonly reported the use of fresh plant parts in Ethiopia (Yineger et al. 2008; Abera 2014; Birhanu and Ayalew 2018; Alemneh 2021; Megersa and Woldetsadik 2022).

Furthermore, regarding route administration, most (63.41%) of the medicinal recipes were administrated orally, followed by the external dermal route of application (28.46%) for dermatological ailments, nasal (4.88 %), eye (2.44%), and anal (0.81%). Except for a few site-specific ailments, such as dermatological diseases, remedies are taken orally for most reported ailments. Our result agrees with many reports from different parts of Ethiopia (Yineger et al. 2008; Agisho et al. 2014; Kefalew et al. 2015).

Quantification of TK on medicinal plants

Jaccard's similarity index (JI)

Comparative ethnobotanical studies between people from different *Kebele*s of study sites are necessary to assess the similarity in TK. This analysis for similarity or consensus on the medicinal uses may lead to the discovery of novel plant-based drugs (Leonti 2011). In our study, JI similarity values (Table 2) suggested that people of the three *Kebele*s have more or less similar knowledge of medicinally using the same plant species. That could be the same vegetation composition, which is due to the similarity in agroecology between *Kebeles* and the high exchange of information between the people of the three *Kebeles* on the medicinal value of the reported species.

Informant Consensus Factor values

Based on the information elicited from the informants, the ailments claimed to be treated by medicinal plants were sorted into eight categories and tabulated (Table 3). The ICF values for the eight ailment categories ranged from 0.51 to 0.73. The highest ICF (0.73) was associated with illnesses of Sensory organs, followed by diseases related to the Respiratory system (0.72) and the Genitourinary system (0.62). The disease categories with minor agreement among the informants were Parasite infections (0.50) and Liver disorders (0.50).

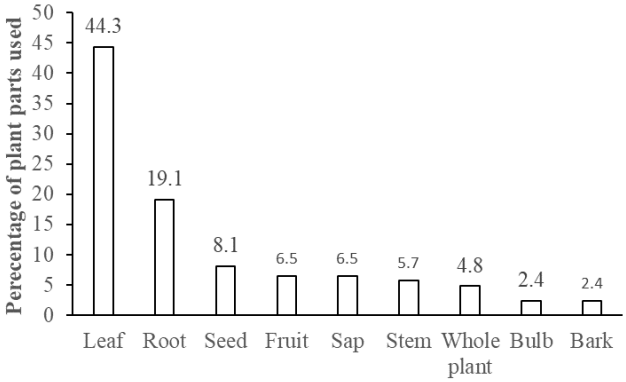

Figure 2. Plant parts used for remedy preparations

Table 2. Jaccard's similarity Index of TK between the *Kebele*s

Kebeles	Imboro	Koba	Dike
Imboro	1	0.91	0.83
Koba	0.91	1	0.75
Dike	0.83	0.75	1

The maximum ICF value for Sensory organs indicates the high information exchange and agreement on the medicinal value of the reported medicinal plants among the ethnic people of the study site. On the contrary, the least ICF for Parasite infections and Liver disorders indicates the informants' contradictory understating of this disease category or the seldom occurrence of this illness in the study area (Heinrich 2000).

Fidelity Level (FL) index

Table 4 presents the FL values for medicinal plants used for a specific ailment as informed by the respondents. Computation of FL showed that the index values varied between 0.62 - 0.97. Out of 70 medicinal plants documented, *Juniperus procera* was a highly cited plant with the highest FL index value (0.97) for wounds, followed by *Podocarpus falcatus* for treating stomachache with an FL index value of 0.90. The species with the lowest FL (0.62) was *Coccinia abyssinica* (*Anchote*) for healing a broken bone. Moreover, a previous report from eastern Ethiopia justified the claim of *J. procera* for healing wounds (Kandari et al. 2015). The maximum FL index value of *J. procera* indicated the people's high preference for this plant for the treatment of wounds. Therefore, in vivo wound healing activity in animal models and phytochemical analysis of this plant are warranted. The plants with low FL index values could also be given attention to preserving the therapeutic knowledge related to them. For instance, previous reports revealed that *C. abyssinica* is widely used in southwestern Ethiopia to patch broken bones because of its high content of calcium (Aga and Bada 1997; Ketema 2015), though its FL value appeared lower in this particular study.

Preference ranking (PR) of important medicinal plants

A simple preference ranking helps to judge the mainly used medicinal plants for treating a specific disease. The results of the PR analysis of the ten most cited plants by informants for treating stomachache are presented in Table 5. Among them, *Ruta chalepensis* ranked top, followed by *Carissa spinarum* and *Coffea arabica*. High preferences for these plants may be connected to the peoples' indigenous knowledge of their curative potential (Leonti 2011). Our result on *R. chalepensis* concord with an earlier study from southern Ethiopia (Avigdor et al. 2014). Reports from other parts of the Oromia and Amhara regions revealed that *R. chalepensis* and other medicinal plants were used to treat stomach aches (Osman et al. 2020). Hence, further pharmacological and phytochemical evaluation is essential to validate those people's traditional claim of R. chalepensis.

Table 3. Informant consensus factor (ICF) for eight ailment categories

Categories of ailments	Nt	Nur	ICF
Sensory organs (Nasal bleeding, Eye disease)	5	16	0.73
Respiratory system (Cough, Common cold, Asthma, Pharyngitis)	8	26	0.72
Genitourinary system (Gonorrhea, Retained placenta, Urinary Retention)	4	9	0.62
Common ailments (Headache, Fever, Febrile illness, Toothache)	15	36	0.6
Dermatology-related ailments (Scabies, Ring Worm, Dandruff, Wart, Wounds, Eczema, Acne, Itching, Loxoscelism)	24	56	0.58
Gastrointestinal-related ailments (Gastroenteritis, Blotting, Indigestion, Stomach ache, Diarrhea)	22	46	0.51
Parasite infections (Elephantiasis, Leishmaniasis)	4	7	0.5
Liver disorders	2	9	0.5

Notes: nur= number of use citations for each disease category; nt= number of species

Table 4. Fidelity Level (FL) index of most commonly used medicinal plants

Medicinal plants	The main ailment treated	NP	N	FL (%)
Juniperus procera	Wound	28	29	97%
Podocarpus falcatus	Stomachache	28	31	90%
Euphorbia lathryis	Heartburn	25	29	86%
Premna schimperi	Eye disease	23	27	85%
Embelia schimperi	Taeniasis	21	26	81%
Coffee arabica	Diarrhea	18	24	75%
Datura stramonium	Toothache	19	26	73%
Indigofera hochstetteri	Tetanus	18	25	72%
Croton macrostachyus	Wound	25	37	67%
Coccinia abyssinica	Broken bone	22	35	62%

Notes**:** NP= No. of informants who independently indicate the use of species; N= Total No. of informants that used the plant to treat primary ailments

Table 5. Preference ranking of medicinal plants used for treating stomachache

Plant species	Respondents' (R_1-R_{10}) Scores										Total	Rank
	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}		
Ruta chalepensis	10	10	10	10	9	9	9	9	8	6	90	1st
Carissa spinarum	10	8	9	8	5	8	7	9	9	9	82	2nd
Coffea arabica	10	10	9	7	7	9	8	8	6	4	78	3rd
Vernonia amygdalina	6	5	7	10	8	8	5	4	10	9	72	4th
Rumex nepalensis	3	5	5	7	10	4	7	9	10	8	68	5th
Podocarpus falcatus	10	10	9	7	2	3	3	7	5	5	61	6th
Solanum marginatum	10	9	2	5	4	8	9	2	3	8	60	7th
Eucalyptus globulus	1	3	6	5	6	9	10	2	4	8	54	8th
Salix subserrata	1	1	2	8	9	4	4	7	5	2	43	9th
Ehretia cymosa	3	1	1	8	1	5	2	6	1	4	32	10th

Notes: Scores given by the respondents (R_1-R_{10}) are based on the effect of the plants to cure cough, i.e., number 6 denotes the highest efficacy, and 1 denotes the lowest efficacy

Table 6. Direct matrix ranking of selected multipurpose plant species

	Use categories									
Plant species	**Charcoal**	**Construction**	**Fencing**	**Firewood**	**Food**	**Forage**	**Furniture**	**Medicine**	**Total**	**Rank**
Syzygium guineense	5	4	2	5	4	4	4	3	31	1st
Podocarpus falcatus	5	5	3	5	0	0	5	3	26	2nd
Carissa spinarum	3	0	4	4	5	4	0	5	25	3rd
Eucalyptus globulus	2	5	5	5	0	0	4	3	24	4th
Prunus africana	4	4	1	5	0	2	4	3	23	5th
Juniperus procera	3	5	3	4	0	0	4	3	22	6th
Cupressus lusitanica	3	5	2	4	0	0	4	3	21	7th
Croton macrostachyus	5	2	2	4	0	0	2	5	20	8th
Acacia abyssinica	5	2	1	4	1	1	2	3	19	9th
Ficus sur	2	3	1	3	3	3	1	3	19	9th
Maesa lanceolata	4	2	2	4	1	1	1	3	18	10th
Vernonia amygdalina	2	0	3	3	0	4	1	4	17	11th
Premna schimperi	2	2	3	3	0	1	1	4	16	12th
Bersama abyssinica	2	0	3	4	0	0	0	5	14	13th
Total	**47**	**39**	**35**	**57**	**14**	**20**	**33**	**50**	295	
Rank	**3rd**	**4th**	**5th**	**1st**	**8th**	**7th**	**6th**	**2nd**		

Direct matrix ranking for multi-utility plants

Fourteen medicinal plants were reported for seven uses other than medicinal value. Thus, direct matrix ranking was done using eight use categories. Among the use categories, the category of firewood was top-ranked with a score of 57. Therefore, other environmental-friendly alternative energy sources such as biogas should be sought to minimize the burden. Table 6 shows that *Syzygium guineense* ranked top to be used highly for the eight use categories. The fact that *S. guineense* is being highly exploited shows its possible reduction in its distribution and abundance unless care is taken. Therefore, it deserves significant attention, not lost it. Moreover, ecological investigation on its regeneration status in the study area should be investigated to understand its conservation status.

Traditional knowledge of medicinal plants

This study recorded the outcome of the analysis on the impact of socio-ethnographic factors TK on the informants. Generally, people's TK on medicinal plants is influenced by sociodemographic factors such as gender, age, and educational status (McCarter and Gavin 2015). In the present study, TK on medicinal plants significantly (P=0.002; Independent T-test) differed between male and female informants. The male informants reported more medicinal plants informed than their female counterparts, suggesting their higher traditional knowledge of medicinal plants. Our result accords with some previous reports from Ethiopia (Tefera and Kim 2019) and overseas (Pakistan) (Shaheen et al. 2017). This observed difference may be due to the oral transmission of TK on medicinal plants to sons

rather than daughters of the family (Teklehaymanot and Gidey 2007)

Our result also showed that the TK on medicinal plants varied significantly (one-way ANOVA; $P<0.001$). The informants at >60 years of age reported more (8.0) medicinal plants than those aged 41-60 and 25-40, who reported 7.0 and 5.0 medicinal plants, respectively. That suggests that older people play a critical role in transferring TK on medicinal plants to the next generation as they possess superior expertise in using medicinal plants in their vicinity for various diseases. Some previous ethnobotanical studies in Ethiopia (Giday et al. 2009; Tefera and Kim 2019; Kassa et al. 2020; Abebe 2021) and overseas (Ecuador) (Weckmüller et al. 2019) support our findings. The results also revealed that differences in educational status significantly impacted the TK on medicinal plants (one-way ANOVA; $P=0.002$). Respondents with no formal education reported more (8.0) medicinal plants than those with elementary and secondary education, which reported six plants each. The study unveiled that the higher the education among the informants, the lower the TK on medicinal plants, and the same trend was previously reported in other country areas (Giday et al. 2009; Tefera and Kim 2019; Kassa et al. 2020; Abebe 2021). On the other hand, sociodemographic traits such as occupation, marital status, and religion did not influence the TK of the informants on the use of medicinal plants in the studied area.

In conclusion, documentation of a considerable number of medicinal plants from the three *Kebele*s shows the presence of rich TK of medicinal plants by the local people and their dependence on them to treat various human and livestock ailments. Our results also implicated that gender, age, and educational status of the people influences the medicinal knowledge of the local people. Moreover, to maintain the level of medicinal plants and associated knowledge, the local people should be encouraged to conserve plants and free transfer knowledge on medicinal plants.

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Effectiveness of gel formulation of mahogany (*Swietenia macrophylla*) bark extract and its potential as an anti-inflammatory in white male rats (*Rattus norvegicus*)

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[2]Department of Forest Resources Conservation and Ecotourism, Faculty of Forestry and Environment, Institut Pertanian Bogor. Jl. Ulin Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia

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Abstract. *Nurani SG, Deluna NN, Nabila P, Falah S. 2022. Effectiveness of gel formulation of mahogany (*Swietenia macrophylla*) bark extract and its potential as an anti-inflammatory in white male rats (*Rattus norvegicus*). Nusantara Bioscience 14: 117-121.* Mahogany bark contains phytochemical compounds that have anti-inflammatory activity. This study aims to determine the anti-inflammatory activity of mahogany-bark extract gel formulation in-vivo. Gel formulations of 96% ethanol extract of mahogany bark at different concentrations were tested for anti-inflammatory activity on the paws of rats induced by 1% carrageenan. The data observed were the thickness of the foot edema of rats. Positive control was anti-inflammatory drugs (Desoximetasone), and negative control was the gel formulation without extract. Data on the thickness of rat paw edema were analyzed using the ANOVA test. The results of the anti-inflammatory test showed that gel formulation 2 with an extract concentration of 10% had a thickness reduction value of 41.83%, which was not significantly different from the positive control treatment of 44.38%. The results of the dispersion test showed that formulation 2 and formulation 3 had good dispersibility, as suggested in SNI, and all gel formulations had good adhesion. Based on these results, it was concluded that formulation 2 was the most effective in reducing rat paw edema.

Keywords: Anti-inflammatory, gel formulation, in vivo test, *Swietenia macrophylla*

INTRODUCTION

Indonesia is a country that has high biodiversity, one of which is plant diversity. About 30,000 plant species in Indonesia, and 9,600 are medicinal plants (Novaryatiin and Indah 2019). The use of medicinal plants in Indonesia has been known for a long time in some communities. For example, Indonesian people used to consume jamu as herbal medicine. Based on the traditional utilization, jamu is being developed into a therapeutic formulation using phytopharmaceuticals by herbal practitioners (Sangat and Larashati 2002; Woerdenbag and Kayser 2014; Fathir et al. 2021; Utaminingrum et al. 2022).

Mahogany (*Swietenia macrophylla* G.King, family Meliaceae) is a medicinally important plant indigenous to tropical and subtropical. It is commonly found in Indonesia. The plant parts commonly used as medicine are fruits, seeds, and leaves. Previous studies indicate various pharmacological activities of *S. macrophylla*, which exhibit antimicrobial, anti-inflammatory, antioxidant effects, antimutagenic, anticancer, antitumor, and antidiabetic activities (Telrandhe et al. 2022). In addition, various other activities include anti-nociceptive, hypolipidemic, antidiarrhoeal, and anti-infective, antiviral, antimalarial, acaricidal, antifeedant, and heavy metal phytoremediation activity (Moghadamtousi et al. 2013).

Wood processing activities produce bark as a waste that is rarely used. Bark as a waste or by-product has benefits as other plant parts. Mahogany bark extract could serve as a suitable adjuvant and promising antidiabetic phytomedicines (Falah et al. 2010). In addition, mahogany bark also has anti-inflammatory activity due to alkaloids, flavonoids, saponins, and terpenoid content (Yasotha et al. 2019).

Inflammation is the body's defense mechanism to eliminate harmful agents that damage and prepare the body for tissue recovery. Characteristics of inflammation in the body include swelling/edema, redness, heat, and pain (Naclerio et al. 2010). Inflammation that occurs in the long term and is persistent is called chronic inflammation and harms the body (Bondy et al. 2021). There are two types of anti-inflammatory drugs, namely steroid and non-steroidal anti-inflammatory drugs, which can be in the form of oral and topical drugs. Topical drugs are absorbed faster because they do not pass through the digestive tract. Topical drugs are widely used for healing inflammatory skin diseases. One of the most commonly used topical drugs is the gel form due to its good drug release and cooling effect on the skin (Garg et al. 2015).

Mahogany bark extract was formulated as a topical anti-inflammatory drug in gel formulations. Therefore, this research is expected to be a source of innovation. Therefore, this research aims to evaluate the anti-inflammatory effects of mahogany bark extract gel on white male rats induced by carrageenan.

MATERIALS AND METHODS

Sample preparation

The mahogany bark was obtained from a 38-year-old mahogany tree planted in the research forest of the Research Institute for Agroforestry Technology, Ministry of Environment and Forestry in Ciamis, West Java, Indonesia. First, mahogany bark is cut into small pieces of 2 cm x 2 cm. The small pieces are then put into the Hammer Mill to make powder and filtered to get a powder size of 40-80 mesh.

Sample extraction

In this study, using the maceration method, two hundred g of mahogany bark powder with a moisture content of 7.53% was extracted with 96% ethanol solvent in a ratio of 1:10 (%w/v). Maceration was carried out for one day in a closed container and repeated three times. The filtrate was filtered using filter paper and then evaporated using a vacuum evaporator to produce concentrated ethanol extract.

Bark extract gel formulation

Three gel formulations with various extract concentrations (F1, F2, F3) were used in this study. In addition, gel formulation without extract was used as negative control (F4) (Table 1) and formulated as the previous methods (Sugihartini and Wiradhika 2017). The gel was made in two phases, namely phase A and phase B. Phase A was composed of a gel base, namely Carbopol (6 g), dissolved in 100 mL of distilled water at 70°C. Phase B consists of methylparaben (0.18 g) dissolved in a small amount of water and added with glycerin and propylene glycol. The two phases were mixed, and 20 g of distilled water was added and stirred until homogeneous.

Anti-inflammatory activity test

The in-vivo anti-inflammatory activity was performed using carrageenan-induced male white rats (*Rattus norvegicus* Berkenhout, 1769). There were five treatment groups, namely the negative control group (F4), three gel formula treatment groups (F1, F2, F3), and the positive control (F0). The positive control used corticosteroid anti-inflammatory drugs, namely Desoximetasone®. A 1% carrageenan was made to induce rat paws to cause edema. Carrageenan (0.1 g) was put into a container and dissolved in 10 mL of 0.9% NaCl.

Table 1. Gel formulation of mahogany bark extract

Materials	F1 (g)	F2 (g)	F3 (g)	F4 (g)
Bark extract	6	12	18	0
Carbopol	6	6	6	6
Glycerin	12	12	12	12
Propylene glycol	6	6	6	6
Methyl Paraben	0.18	0.18	0.18	0.18
Aquades	120	120	120	120

Rats were injected with 0.1 mL of 1% carrageenan solution intraplantar on the sole of the rat's right foot. One hour after an injection of carrageenan solution, the thickness of the rat paws was measured using a digital caliper and recorded as initial thickness (T0). Then, each group was treated topically on the soles of the feet according to the treatment group. The thickness of the rat's right paw after administration of the gel was measured every 60 minutes for 360 minutes and at 24 and 48 hours after carrageenan induction.

Gel formulation test

The gel preparation was tested by organoleptic, pH, dispersibility, and adhesion tests. Moreover, the organoleptic test was conducted by observing the texture, color, and smell. The pH test was conducted by smearing the gel preparation on a universal pH indicator. The dispersion test was carried out by placing 0.5 g of gel on a glass lined with millimeter block paper and covered with another glass that had been weighed, then adding a load of 50, 100, and 150 g and allowed to stand for one minute. The distribution diameter was recorded and repeated three times. The adhesion test was carried out by placing 0.5 g of gel on a slide and covering it with another slide which was then given a load of 1 kg for 5 minutes. Then the load weighing 100 g is released, and the time when the two slides are released is recorded and repeated three times.

Data analysis

The data were analyzed using the Analysis of Variance (ANOVA) with a 95% confidence level to determine the significant differences in the treatments. In addition, the differences between treatments were analyzed with the Least Significance Different (LSD) test.

RESULTS AND DISCUSSION

Anti-inflammatory activity

Anti-inflammatory activity was observed based on the ability of the gel to increase or decrease the edema size on the soles of the rats' paws. That observation is made every hour. However, the paws of rats experienced inflammation after being induced by carrageenan, which was characterized by swelling due to increased blood flow and edema (tumor), as well as redness (rubor) (Figure 1).

The thickness of the rat paw edema in carrageenan-induced rats was presented in Table 2. Table 2 shows that formulation two (F2) is the closest formulation to the treatment using anti-inflammatory drugs as the positive control treatment.

The results showed that formulation 2 had a percentage reduction in edema thickness of 41.93%, that not significantly different from the percentage reduction of positive control (44.38%) (Table 3). On the other hand, formulations 1,3, and 4 had a lower percentage reduction of edema thickness which was 21.21%. 30.50%. 13.78%, respectively.

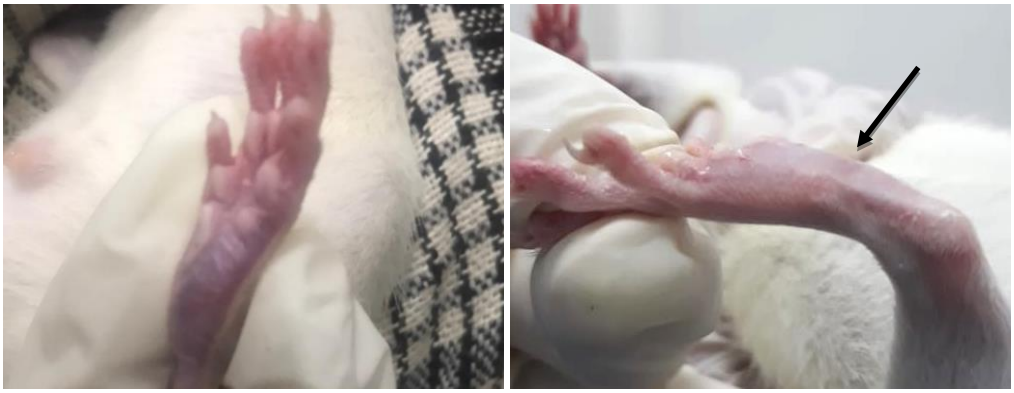

Figure 1. A. The paws of rats before carrageenan induction, B. The soles of rats' feet after carrageenan induction. Arrows indicate swelling

Table 2. The average thickness of the rat paw edema (%) in carrageenan-induced rats for two days of observation

Groups	The average thickness of edema (mm) per hour (x ± SD)								
	0	1	2	3	4	5	6	24	48
Positive control	3.28 ± 0.55	2.85 ± 0.49	2.25 ± 0.54	2.58 ± 0.39	2.78 ± 0,46	2.22 ± 0.44	2.45 ± 0.36	2.65 ± 0.64	1.80 ± 0.26
F1	2.73 ± 0.59	3.02 ± 0.66	2.68 ± 0.32	2.75 ± 0.19	3.35 ± 0.73	2.70 ± 0.51	2.45 ± 0.33	2.53 ± 0.21	2.07 ± 0.29
F2	3.40 ± 0.71	2.52 ± 0.20	2.63 ± 0.16	2.73 ± 0.42	2.13 ± 0.57	2.32 ± 0.32	2.07 ± 0.46	2.28 ± 0.23	1.85 ± 0.29
F3	2.83 ± 0.31	2.25 ± 0.24	2.58 ± 0.48	2.57 ± 0.37	2.28 ± 0.35	2.77 ± 0.37	2.27 ± 0.42	2.33 ± 0.26	1.93 ± 0.23
F4	2.73 ± 0.18	2.45 ± 0.36	2.41 ± 0.10	2.73 ± 0.87	2.82 ± 0.31	2.73 ± 0.23	2.75 ± 0.73	2.90 ± 0.29	2.35 ± 0.41

Note: Corticosteroid anti-inflammatory drug, Desoximetasone® was used as a positive control

Table 3. The percentage of the thickness of rat paw edema (%) compared to the thickness at 0-hour observation

Observation time (hour)	Groups				
	Positive control	F1	F2	F3	F4
0	100.0	100.00	100.00	100.00	100.00
1	88.90	116.30	77.46	80.36	90.14
2	70.03	100.50	81.15	92.58	88.59
3	80.65	104.44	82.25	92.61	99.68
4	86.33	127.50	62.62	81.38	103.06
5	68.99	102.34	71.89	98.72	100.01
6	77.09	91.57	62.19	81.14	100.32
24	81.24	95.57	71.64	83.48	106.07
48	55.62	78.78	58.07	69.50	86.22
∆t (0-48)	44.38^{c}	21.21^{a}	41.93^{c}	30.50^{b}	13.78^{a}

Note: Different letters indicate significant differences based on the LSD test

Table 4. The organoleptic test of the gel formulation of mahogany bark extract

Parameters	F1	F2	F3	F4
Texture	Gel	Gel	Gel	Gel
Color	Brown	Brown	Brown	Clear
Smell	Extract smell	Extract smell	Extract smell	Gel smell
pH	4	4	4	3

Gel formulation

The gel preparations were tested organoleptically by observing the texture, color, odor, and pH (Table 4). The results of the organoleptic test showed that there were differences in each gel formulation. It is due to the differences in extract concentration in gel formulations. Furthermore, increasing mahogany bark extract concentration in the gel formulation increased the gel density, darker color, a sharp odor typical of mahogany bark extract, and a higher pH. However, the pH value of the gel is still quite acidic. It is due to the acidic base of the carbopol gel. Therefore, adding other ingredients, such as triethanolamine (TEA), is necessary to increase the gel's pH.

The dispersion test resulted in the spreadability of a gel formulation of mahogany extract after loading 150 g (Figure 2). In addition, spreadability was carried out to determine the ability of the gel to spread when applied to the skin. The results of the gel dispersion in the standard range were gel formulations 2 and 3 of 6.95 cm2 and 6.60 cm2, respectively. Figure 3 shows the ability of each gel to stick in seconds in the adhesion test

Discussion

Carrageenan was chosen as an inducer of inflammation because carrageenan induction in rat paws has been widely used as a model of acute inflammation (Morris 2003). The mechanism of carrageenan-induced inflammation was as follows: the first (early) phase is about 1 hour after injection, which is associated with the release of inflammatory mediators such as histamine, bradykinin, and serotonin by mast cells in damaged tissues.

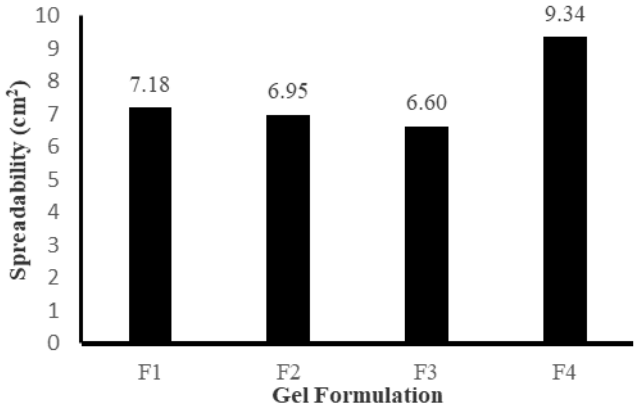

Figure 2. The spreadability of mahogany bark extract gel when added with a load of 150 g in the dispersion test

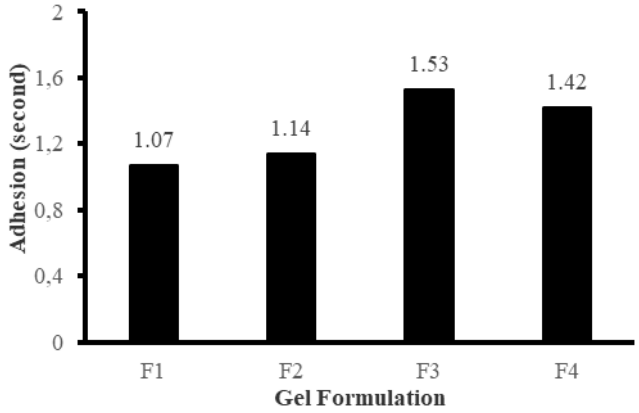

Figure 3. The adhesive properties of mahogany bark extract gel

The second phase is mainly attributed to the stage of prostaglandin production by the cyclooxygenase (COX) enzyme. This phase took about 3-6 hours after the carrageenan injection. Therefore, anti-inflammatory activity was evaluated by inhibiting the formation of these mediators, especially the product of the COX-2 enzyme. The COX-2 enzyme is an enzyme that plays a vital role in the synthesis of prostaglandin mediators as a vasodilator for edema formation (Karim et al. 2019).

Cyclooxygenase (COX) is an enzyme that regulates prostaglandins' formation from arachidonic acid, known as prostaglandin-endoperoxide synthase (PTGS). Prostaglandins are one of the mediators in the body's inflammatory response. The COX enzyme has two isoforms, namely COX-1 and COX-2. COX-1 plays a role in maintaining tissue homeostasis, synthesized essentially in various cells, and COX-2 is synthesized during inflammation induction. Therefore, COX-2 becomes the primary target inhibiting anti-inflammatory processes (Sharma et al. 2019).

COX-2 regulates the formation of prostaglandins initiated by converting arachidonic acid to prostaglandin G2 (PGG2). Followed by the peroxidase process to form prostaglandin H2 (PGH2). PGH2 is converted into several isoforms with specific functions by various specific synthase enzymes, such as TXA2, PGF2α, PGE2, PGD2, and PGI2 (Korbecki et al. 2014). Thromboxane A2 (TXA2) activates platelet aggregation and smooth muscle contraction, mainly produced from COX-1 platelets. PGI2 is synthesized by COX-1 to produce gastric mucosa and plays a role in maintaining tissue homeostasis, especially in the digestive tract. On the other hand, COX-2 is more dominant in synthesizing PGE2, leading to inflammation characterized by redness, swelling, and pain due to vasodilation of blood vessels and edema (FitzGerald and Ricciotti 2011).

Formulation 4 had the highest dispersion due to the highest extract concentration. The spreadability test showed gel formulations had a good spreadability range from 5-7 cm2. That is in the Indonesian National Standard (SNI) range number 06-2588 (Ningsih et al. 2019). Formulation 1 contains less extract than other formulations; it contains fewer phytochemical compounds that affect its anti-inflammatory activity. Formulation 3 contains more extract, but the amount of extract also affects the spreadability related to the gel's ability to deliver the drug to the skin. The increase in the extract amount decreased gel dispersion and the active substance diffusion rate through the membrane. Gels with high spreadability result in good drug delivery (Patil et al. 2019). Stickiness is also a determinant factor in gel properties (Ruis et al. 2007). The stickiness of all gel formulations is considered good because it has an average of three repetitions in one second. The requirement for good adhesion is more than one second. All gel formulations have good adhesion to the skin because they meet these requirements. Formulation 2 has good anti-inflammatory activity, pH, spreadability, and adhesion. Therefore, formulation 2 is the best gel formulation.

The anti-inflammatory activity of mahogany has been reported in seed due to the sweetening content that can downregulate the COX-2 enzyme in vitro (Mak et al. 2021). In addition, a study by Chen et al. (2015) showed that other limonoid compounds extracted from mahogany seeds inhibit nitric oxide (NO), in which the limonoid is one of the inflammatory mediators. Therefore, it can be used as an anti-inflammatory agent. Furthermore, an anti-inflammatory compound in mahogany can also be found in fruit which inhibits the superoxide anion on neutrophils (Chen et al. 2010).

Mahogany bark contains phytochemical compounds that have anti-inflammatory activity. The gel of mahogany stem bark extract at a concentration of 10% had an anti-inflammatory activity that closely resembled corticosteroid anti-inflammatory drugs. So, it can be concluded that the best gel formulation was formulation 2, with an extract concentration of 10%. Therefore, gel formulation 2 also has the potential as an anti-inflammatory herbal therapeutic drug.

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Evaluation of the effect of nicosulfuron at different times of application on the chemical component of maize (*Zea mays*)

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[1]Department of Botany, Adamawa State University. P.M.B. 25, Mubi, Nigeria. Tel.: +234-7066274008, ♥email address: taritizhe@yahoo.com
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Abstract. *Tizhe TD, Alonge SO, Iortsuun DN, Adekpe DI, Batta K. 2022. Evaluation of the effect of nicosulfuron at different times of application on the chemical component of maize (*Zea mays*). Nusantara Bioscience 14: 122-127.* This study aimed to evaluate the effect of nicosulfuron at different concentrations and times of application on the chemical compositions of maize (*Zea mays* L.) grain. A field experiment was conducted during the 2019 and 2020 cropping seasons. Furthermore, using a split-plot design, maize seed was planted; and four different concentrations of nicosulfuron (50, 100, 150, and 200 g/ha) were applied at 3, 5, and 7 Weeks After Sowing (WAS). The maize grain was analyzed using standard procedures for its proximate amino acids and mineral contents. The results showed that the different concentrations of nicosulfuron and its time of application significantly affected the proximate compositions except for the ash content. The 5 WAS application time had the significantly highest protein, crude fiber, and moisture content with 11.35, 2.60, and 12.01%, respectively, and the lowest carbohydrate (70.58%) and crude fat (2.33%). The nicosulfuron was observed to significantly affect all the amino acids and mineral (except on N) contents of the maize grain with 100 g/ha. This study recorded the significantly highest content of almost all the amino acids with 100 g/ha, and 50 g/ha was the lowest. The 5 WAS virtually had the highest amino acids, while the 3 WAS the lowest. Therefore, nicosulfuron and time of application both have a significant effect on the proximate amino acids and Mg and Ca components of maize grain. That means that using nicosulfuron at concentrations precisely above 100 g/ha and at a time other than 5 WAS negatively affects the chemical components of maize grain.

Keywords: Amino acids, the effect of time of application, herbicide, the mineral content of maize grain, nicosulfuron, proximate composition

INTRODUCTION

Maize (*Zea mays* L.) is an annual plant believed to have originated from central Mexico about 7,000 years ago from wild grass and was by the native Americans transformed into a better source of food (Ranum et al. 2014). The crop was said to have been introduced to the African continent in the 16th century. And eventually spread to all parts of the continent around the 19th century. It is a staple food crop for over 1.2 billion people in Latin America and Sub-Saharan Africa (Anon 2021). It is grown in diverse environments and consumed by people with varying socio-economic backgrounds and food preferences in Africa (Olaniyan 2015). The maize consumption rate is estimated globally to be more than 116 million tonnes, with about 30% and 21% of the consumption rates occurring globally and in Sub-Saharan Africa (SSA). All parts of the crop can be used for food and non-food products. Besides being food for human consumption, it is also used for extraction of edible oil, as feed for poultry and livestock, starch, and glucose industry (Hawaldar and Agasimani 2012; Ahmad et al. 2021). It is also used mainly in Nigeria for beer brewing, the manufacturing of fabric and adhesives, and the pharmaceutical industries (Obi and Ihedigbo 1987). Maize grain contains approximately 72% starch, 10.4% protein, 4.8% fat, 17% ash, and 2.5% fiber (Farhad et al. 2009; Ranum et al. 2014).

Miafuron 75WG is a selective systemic post-emergence herbicide used in controlling weeds in maize fields. It has nicosulfuron as its active ingredient. It controls weeds by inhibiting the plant Acetolactate Synthase (ALS) enzyme. Inhibiting the ALS enzyme system blocks the production of the amino acids, valine and isoleucine, essential building blocks of proteins and other plant components (Anon 1990). The use of herbicides in the control of weeds in crop fields was reported to significantly affect crop grains' chemical components such as crude protein, crude fiber, fat, and ash (Mehmeti et al. 2016). Shaban et al. (2016) reported a significant increase in the carbohydrate content of maize grains due to the application of some herbicides (metribuzin and acetochlor). On the other hand, significantly lower valine, leucine, and isoleucine amino acids were observed in maize seedlings treated with chlorimuron-ethyl (Alla et al. 2008). Moreover, to determine the impact of atrazine, nicosulfuron, topramezone, and mesotrione on sweet corn nutritional quality, Cutulle et al. (2018) recorded an increase in the uptake of mineral elements like phosphorus, magnesium, and manganese by 8-75%, and protein content by 4-12%. However, literature reporting the effect of nicosulfuron at different concentrations and time of application on the amino acids and other chemical components of maize grain do not abound. Therefore, it was because of this that the idea of this study was initiated.

MATERIALS AND METHODS

Description of the study area

The research was carried out during the 2019 and 2020 cropping seasons in the research farm of the Department of Crop Science, the University of Adamawa State, Mubi, Adamawa State, Nigeria. The location of the research farm falls within the North Eastern region of Nigeria between latitude $10^0$16'06" N and longitude $13^0$16'01" E. This location has an elevation of 582 m above sea level; and occupies a land of about 725.85 Km^2. The area has a tropical climate with an average annual temperature of 32^oC; and lies within the Sudan Savannah vegetation zone of Nigeria. In addition, the area has an annual rainfall of about 1056 mm and an average relative humidity ranging from 28-45% (Adebayo 2004).

Source of seed for the experiment

The maize variety used in this study, SAMMAZ 17 was obtained from the Institute for Agricultural Research (IAR), Ahmadu Bello University, Zaria, Kaduna State, Nigeria.

Treatment and experimental design

In this study, the treatments consisted of four (4) concentrations of Miafuron 75WG (at 50, 100, 150, and 200 g/ha); two controls (hoe weeding and unweeded) and herbicide application time (at 3, 5, and 7 WAS). The experiment was managed in a split-plot design and was replicated three times. The time of herbicide application was managed on the main plots, while the herbicide concentrations and controls (sub-treatments) were laid on the sub-plots. A tractor was used to plow the experimental field and harrowed to obtain a good soil tilth, and then using a cow plougher, the field was ridged with an inter-row spacing of 75 cm apart.

Seed planting

Momtaz 45 WS seed dressing chemical treated on the maize seeds to avoid seed destruction by insects in the soil. That was followed by planting three seeds per hole of about 2 inches at an inter-row spacing of 75 cm and intra-row spacing of 25 cm. Finally, the seedlings were thinned to one plant per stand after two Weeks After Sowing (WAS).

Treatment and fertilizer application

Furthermore, using a back-mounted knapsack sprayer, the herbicides were applied at three periods (3, 5, and 7 WAS) at 50, 100, 150, and 200 g/ha concentrations. As a result, NPK (15:15:15) fertilizer was applied at the rate of 400 kg/ha at 2 WAS; next, urea fertilizer was applied at 130.43 kg N/ha at 5 WAS.

Collection of maize grain samples for proximate, amino acids, and mineral compositions analysis

The maize grain samples used for the proximate, amino acids and mineral composition analysis were obtained at harvest from the field according to the treatment plot when the maize grain was fully mature and dried thoroughly. The harvested maize of each cropping season was de-husked and then shade dried, shelled, grounded into powder, and placed in a well-labeled polythene black; then taken to the laboratory for analysis.

Data collection

Determination of proximate compositions:

The maize grain proximate compositions were analyzed using the methods described by AOAC (2020).

Determination of maize grain amino acid and mineral compositions:

The maize grain amino acid and mineral compositions were determined following the methods described by AOAC (2020).

Data analysis

The data generated from this study were subjected to analysis of variance (ANOVA) by a program of Statistical Analysis Software (SAS) version 9.0, and means with significant differences were separated using Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Effect of nicosulfuron at different concentrations and times of application on the proximate compositions of maize grain

A comparison of the effect of different concentrations of nicosulfuron on maize grain proximate compositions during 2019 and 2020 trials showed that the highest carbohydrate content (72.32%) was recorded on a plot applied 50 g/ha of nicosulfuron, followed by 200 g/ha (71.93%) and the lowest (70.50%) was recorded at 100 g/ha. The lowest result was observed statistically at par with the two controls (unweeded and weeded plots) and 150 g/ha. The highest crude protein content (11.53%) was recorded at 100 g/ha but was statistically at par with those of the unweeded and weeded controls which had 11.19 and 11.37%, respectively, while the lowest protein content (10.49%) was recorded on plot applied concentration 200 g/ha which, however, was statistically similar to that of 150 and 50 g/ha. The significantly highest crude fat content (2.59%) was that of 200 g/ha, followed by that of weeded control which had 2.54%. In comparison, the lowest (2.24%) was recorded at 150 g/ha but was statistically at par with the unweeded and those of the other concentrations. The highest crude fiber (2.75%) was that of the weeded control, while the lowest (2.14%), which was only significantly different from the highest, was recorded at 200 g/ha. The moisture content (12.21%) recorded at 150 g/ha was the highest, but it was statistically similar to that of unweeded and 100 g/ha, while the lowest (11.25%) which was statistically at par with that of 50 and 200 g/ha was recorded at weeded control plot. However, the different concentrations of nicosulfuron had no significant difference in their effect on the ash content of the maize grain (Table 1).

The comparison of the effect of time of application of nicosulfuron on maize grain proximate compositions showed that the significantly highest crude protein

(11.35%), crude fiber (2.60%), and moisture (12.01%) contents were recorded on plots treated at 5 WAS; while the lowest protein (10.76%), fiber (2.18%) and moisture (11.57%) recorded at 3 and 7 WAS were statistically at par. The higher carbohydrate (71.70%) recorded at 7 WAS was significantly similar to 3 WAS, while the lowest (70.58%) was that of 5 WAS. The higher fat content (2.49%) was recorded on the plot treated at 3 WAS, but it was statistically at par with 7 WAS, while the lowest (2.33%) was recorded at 5 WAS. The 3, 5, and 7 WAS application periods had no statistically significant effect on the maize grain ash content (Table 1).

Comparing the effect of year on the proximate components showed that 2019 had the significantly highest contents of carbohydrate (71.92%) and crude fiber (2.43%), while 2020 had the highest of crude protein (11.21%) and moisture (12.33%). On the fat and ash contents, however, the 2019 and 2020 cropping seasons had no significant difference (Table 1).

The interactions between year and concentrations and year and time of application only significantly affected carbohydrate, fiber and moisture, and fiber and moisture contents, respectively. In comparison, the interactions between concentrations and time of application; and year, concentrations, and time of application did not have a significant effect only against ash content (Table 1).

Effect of nicosulfuron at different concentrations and times of application on the amino acids composition of maize grain

The effect of different concentrations of nicosulfuron on the amino acids content of maize grain comparison indicated that isoleucine (0.81 g/100 g), leucine (1.06 g/100 g), lysine (1.12 g/100 g), methionine (1.44 g/100 g), phenylalanine (1.32 g/100 g), tryptophan (1.33 g/100 g), arginine (1.39 g/100 g) and alanine (1.49 g/100 g) were statistically significantly the highest at 100 g/ha; histidine (0.74 g/100 g), threonine (1.18 g/100 g), aspartic acid (1.30 g/100 g), cysteine (1.50 g/100 g), proline (1.64 g/100 g) and tyrosine (1.89 g/100 g) recorded at 200 g/ha were significantly the highest; valine (1.41 g/100g), glutamic acid (1.77 g/100 g) and glycine (1.52 g/100 g) recorded at 150 g/ha were the highest, while the lowest of most of these amino acids were recorded at concentration 50 g/ha of nicosulfuron (Table 2).

The effect of time of application of nicosulfuron on the amino acids content of the maize grain comparison showed that 5 WAS had the statistically significantly highest content of all the amino acids while 3 WAS had the significantly lowest content of amino acids like; isoleucine (0.60 g/100 g), methionine (0.96 g/100 g), phenylalanine (0.91 g/100 g), arginine (1.07 g/100 g), glutamic acid (1.15 g/100 g) and tyrosine (1.26 g/100 g); and 7 WAS had the lowest of histidine (0.60 g/100 g), leucine (0.69 g/100 g), valine (1.15 g/100 g), aspartic acid (0.90 g/100 g), cysteine (0.95 g/100 g), proline (1.20 g/100 g) and serine (1.28 g/100 g). The lower content of lysine, threonine, tryptophan, and glycine recorded at 3 and 7 WAS were significantly similar. Comparing the effect of year on the amino acids content of the maize grain showed that 2019 had the significantly highest content of all the amino acids except cysteine, glutamic acid, glycine, serine, and tyrosine (Table 2).

Interactions between year and concentration; between year and time of application; between concentration and time of application; and between year, concentration, and time of application all had a significant effect on all the amino acid content of the maize grain except against serine (Table 2).

Table 1. Effect of nicosulfuron on the proximate compositions of maize grain obtained from the 2019 and 2020 cropping season

Treatment	Proximate compositions (%)					
	Carbohydrate	Protein	Fat	Ash	Fibre	Moisture
Concentration (g/ha) – (C)						
UW	70.89c	11.19ab	2.35bc	1.14a	2.31b	12.13a
W	70.98c	11.37ab	2.54ab	1.10a	2.75a	11.25b
50	72.32a	10.64c	2.32c	1.15a	2.20b	11.37b
100	70.50c	11.53a	2.32c	1.16a	2.29b	12.20a
150	71.27bc	10.91bc	2.24c	1.16a	2.22b	12.21a
200	71.93ab	10.49c	2.59a	1.15a	2.14b	11.70b
SE±	0.29	0.17	0.07	0.07	0.08	0.15
Time App (WAS) – (TA)						
3	71.67a	10.97b	2.49a	1.13a	2.18b	11.57b
5	70.58b	11.35a	2.33b	1.14a	2.60a	12.01a
7	71.70a	10.76b	2.36ab	1.16a	2.18b	11.85ab
SE±	0.21	0.12	0.05	0.05	0.06	0.11
Year – (Y)						
2019	71.92a	10.83b	2.39a	1.15a	2.43a	11.28b
2020	70.71b	11.21a	2.40a	1.13a	2.21b	12.33a
SE±	0.17	0.10	0.04	0.04	0.05	0.05
Interactions						
Y x C	*	NS	NS	NS	*	*
Y x TA	NS	NS	NS	NS	*	*
C x TA	*	*	*	NS	*	*
Year x C x TA	*	*	*	NS	*	*

Note: Means followed by the same alphabet within a treatment group are not statistically different at p≤0.05. Key: *: statistically significantly different; NS: No significant difference; UW: Unweeded; W: Weeded

Table 2. Effect of nicosulfuron on the amino acids compositions of maize grain obtained from the 2019 and 2020 cropping season

Treatment	Amino acids (g/100g)								
	HIS	**ISO**	**LEU**	**LYS**	**MET**	**PHA**	**THR**	**TPY**	**VAL**
Concentration (g/ha) – (C)									
UW	0.60c	0.70b	0.67e	0.71d	0.86d	0.88e	0.91d	1.11c	1.11d
W	0.52e	0.54e	0.88b	0.77c	0.94c	0.90d	0.86e	1.05d	1.14d
50	0.63b	0.64c	0.57f	0.62e	0.83e	0.84f	0.78f	0.97e	1.23c
100	0.64b	0.81a	1.06a	1.12a	1.44a	1.32a	1.07b	1.33a	1.33b
150	0.56d	0.56d	0.73d	0.79c	0.99b	0.98c	0.99c	1.05d	1.41a
200	0.74a	0.69b	0.83c	0.86b	0.99b	1.01b	1.18a	1.22b	1.22c
SE±	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
Time APP (WAS) - (TA)									
3	0.62a	0.60c	0.76b	0.76b	0.96c	0.91c	0.89b	1.03b	1.24b
5	0.63a	0.71a	0.93a	0.91a	1.06a	1.10a	1.12a	1.30a	1.34a
7	0.60b	0.66b	0.69c	0.76b	1.01b	0.95b	0.88b	1.04b	1.15c
SE±	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
Year – (Y)									
2019	0.63a	0.69a	0.85a	0.92a	1.12a	1.10a	1.04a	1.23a	1.35a
2020	0.60b	0.62b	0.73b	0.71b	0.90b	0.88b	0.89b	1.02b	1.13b
SE±	0.01	0.01	0.00	0.01	0.01	0.00	0.00	0.01	0.01
Interactions									
Y x C	*	*	*	*	*	*	*	*	*
Y x TA	*	*	*	*	*	*	*	*	*
C x TA	*	*	*	*	*	*	*	*	*
Y x C x TA	*	*	*	*	*	*	*	*	*
	ARG	**ALA**	**ASP**	**CYST**	**GLU**	**GLY**	**PRO**	**SER**	**TYR**
Concentration (g/ha) – (C)									
UW	1.32b	1.29c	0.88e	0.96d	1.09c	1.01e	1.11d	1.16a	1.19e
W	1.22c	1.35b	0.93d	0.97d	1.08c	1.10d	1.12d	1.13a	1.21e
50	1.05d	1.07e	0.88e	0.88e	1.09c	1.00e	1.16c	1.24a	1.27d
100	1.39a	1.49a	1.01c	1.40c	1.46b	1.50b	1.56b	7.70a	1.42c
150	1.22c	1.19d	1.06b	1.44b	1.77a	1.52a	1.55b	1.19a	1.75b
200	1.33b	1.37b	1.30a	1.50a	1.48b	1.45c	1.64a	1.73a	1.89a
SE±	0.01	0.01	0.01	0.01	0.01	0.01	0.01	2.58	0.01
Time App (WAS) – (TA)									
3	1.07c	1.12b	0.93b	1.12b	1.15c	1.13b	1.26b	1.41b	1.26c
5	1.45a	1.38a	1.19a	1.50a	1.64a	1.52a	1.62a	1.74a	1.77a
7	1.24b	1.38a	0.90c	0.95c	1.19b	1.13b	1.20c	1.28c	1.34b
SE±	0.01	0.01	0.01	0.01	0.01	0.01	0.01	1.83	0.01
Year – (Y)									
2019	1.29a	1.28b	1.01a	1.15b	1.29b	1.24b	1.36a	1.41b	1.39b
2020	1.22b	1.31a	1.01a	1.23a	1.36a	1.28a	1.36a	3.54a	1.52a
SE±	0.01	0.00	0.01	0.01	0.01	0.00	0.00	1.49	0.01
Interactions									
Y x C	*	*	*	*	*	*	*	NS	*
Y x TA	*	*	*	*	*	*	*	NS	*
C x TA	*	*	*	*	*	*	*	NS	*
Y x C x TA	*	*	*	*	*	*	*	NS	*

Note: Means followed by the same alphabet within a treatment group are not statistically different at $p \leq 0.05$. Key: *: statistically significantly different; WAS: Week after Sowing; UW: Unweeded; W: Weeded; HIS: Histidine; ISO: Isoleucine, LEU: Leucine; LYS: Lysine; MET: Methionine; PHA: Phenylalanine; THR: Threonine; TPY: Tryptophan; VAL: Valine; ARG: Arginine; ALA: Alanine; ASP: Aspartic acid; CYST: Cysteine; GLU: Glutamic acid; GLY: Glycine; PRO: Proline; SER: Serine; TYR: Tyrosine

Effect of nicosulfuron at different concentrations and times of application on the mineral composition of maize grain

Table 3 shows the different concentrations of nicosulfuron had no significant difference in their effect on the N and Ca content of the maize grain. However, the Mg content (0.15%) recorded in maize grain obtained from the unweeded control plot was significantly the highest, while the lowest Mg content (0.06%) at 100 and 200 g/ha was statistically at par with those of the weeded, 50 and 150 g/ha.

The effect of time of application of nicosulfuron at different concentrations on the N, Ca, and Mg contents of the maize grain comparison showed that the 3, 5, and 7 WAS time of application had no statistically significant difference in their effect on the N and Ca contents of the maize grain. Moreover, a significant difference was observed in the Mg content, with 3 WAS recording the highest Mg content (0.13%) while 7 WAS was the lowest (0.06%). However, that was statistically significantly similar to 5 WAS, which had 0.07% (Table 3).

Table 3. Effect of nicosulfuron on the N, Ca, and Mg content of maize grain obtained from the 2019 and 2020 cropping season

Treatment	Mineral composition (%)		
	N	Ca	Mg
Concentration (g/ha) – (C)			
UW	1.77b	0.18a	0.15a
W	1.81ab	0.17a	0.07b
50	1.71ab	0.18a	0.07b
100	1.82a	0.16a	0.06b
150	1.71ab	0.16a	0.10b
200	1.74ab	0.17a	0.06b
SE±	0.03	0.02	0.02
Application Time (WAS) – (AT)			
3	1.75a	0.19a	0.13a
5	1.80a	0.17a	0.07b
7	1.74a	0.16a	0.06b
SE±	0.02	0.01	0.01
Year – (Y)			
2019	1.75a	0.17a	0.06b
2020	1.77a	0.17a	0.11a
SE±	0.02	0.01	0.01
Interaction			
Y x C	NS	NS	*
Y x AT	NS	NS	*
C x AT	*	NS	*
Y x C x AT	*	NS	*

Note: Means along the column with the same letter(s) are not statistically significantly different at $p \leq 0.05$. Key: *: Statistically significantly different; NS: Not significant; UW: Unweeded; W: Weeded

A comparison of the effect of year on the three mineral content of the maize grain showed that year had no significant effect on the N and Ca content of the maize grain. Still, there was a significant effect on Mg, with 2020 recording the highest Mg content with 0.11% (Table 3).

Interactions between year and concentration, year and time of application only had significantly effected on the Mg content of the maize grain, while interactions between concentrations and time of application; and year, concentrations, and time of application only had a significant effect on the N and Mg contents of the maize grain (Table 3).

Discussion

Assessing the effect of nicosulfuron at the different concentrations on the proximate compositions of Sammaz 17 during the 2019 and 2020 trials, indicated a significant effect of the nicosulfuron concentrations on most of the proximate components of the maize variety. The highest carbohydrate, protein, crude fat, moisture, and crude fiber were recorded at concentrations 50, 100, 200, and 150 g/ha and weeded plots, respectively. The significantly higher content of most of these proximate compositions in maize grain obtained from plots treated with different concentrations of nicosulfuron and weeded control could be due to the effective weed control achieved as a result of the application of the herbicide. A similar amount of carbohydrate recorded in a maize grain that was treated with herbicide was reported by Ritter and Menbere (2001). A protein content between 4-12% was recorded in the grain of sweet maize variety treated with herbicides like nicosulfuron and atrazine (Cutulle et al. 2018). Chaudhary et al. (2010) also reported a higher percentage of protein in the grain of maize cultivated on a plot treated with herbicide than that of hand hoeing and unweeded controls. The application of herbicides which include: Sekator, Lintur 70 WG, Granstar 75 WG, and Mustang in wheat fields, was reported to bring about a significant increase in the proximate compositions like crude protein, crude fat, ash, and crude fiber of the wheat grain (Mehmeti et al. 2016). The effect of time of application of the nicosulfuron on the proximate compositions of the maize variety comparison showed that, time of herbicide application had a significant effect on most of the proximate components of the maize grain, with crude protein, crude fiber, and moisture contents being high at 5 WAS; carbohydrate at 7 WAS; and crude fat at 3 WAS. The significantly highest crude protein, crude fiber, and moisture recorded at 5 WAS time of application might be due to the effective weed control observed at that stage. The timely application of herbicides to control weeds in maize fields significantly improved the quality of maize (Karkanis et al. 2020).

The analysis of the amino acid contents of the maize variety applied nicosulfuron at different concentrations showed that the nicosulfuron had a significant difference in their effect on all the amino acids, except on serine. The significantly highest essential amino acids, which include: histidine, lysine, methionine, phenylalanine, isoleucine, leucine, threonine, tryptophan, arginine, and valine; and non-essential like alanine were recorded at the recommended concentration of the nicosulfuron, while the highest of most of the non-essential were recorded at the highest concentration of the herbicide. The determination of the effect of time of application of the nicosulfuron on the amino acids content of the maize variety indicated that the statistically significantly highest content of virtually all the amino acids, both the essential and non-essential, were recorded at the 5 WAS period of treatment application. That authenticates the significantly highest protein content of maize variety recorded at the 5 WAS, as shown in Table 1, as the increase in protein means an increase in amino acids (El-Sobki and Salem 2021). This finding might not be far from the fact that the herbicide had less injury on the plant; there was an effective weed management, especially at higher concentrations of the herbicide at that period of application. The post-emergence application of herbicides in the control of weeds in wheat and maize seedlings resulted in a significant increase in some of the amino acid content of the two crops, especially the butachlor and metribuzin-treated ones (Alla et al. 2008).

The analysis of the mineral content of the maize variety applied nicosulfuron at different concentrations showed that the nicosulfuron concentrations had no significant difference in their effect on the N and Ca content of the maize grain. That contradicts the finding of Omovbude et al. (2017), who reported a significant increase in mineral content like N, Ca, P, K, and Mg. This contradiction might be due to differences in herbicide type and application period; crops react differently to different herbicides (Soltani et al. 2007). However, comparing the mineral

content according to the time of treatment application indicated that the application period had no statistically significant difference in its effect on the N and Ca content except on Mg. This result proved right the non-significance of the effect of nicosulfuron concentrations and time of application on the ash content of the maize variety, as shown in Table 1. A similar finding was reported by Barbas and Sawicka (2020) when they discovered a nonsignificant difference in the effect of concentrations and time of application of some pre and post-emergence herbicides on the mineral content of some cereal crops.

In conclusion, nicosulfuron at concentrations of 50-200 g/ha significantly affects the proximate compositions of the Sammaz 17 maize variety. The time of nicosulfuron application also has a significant effect on most of the proximate compositions of the maize variety, with 5 WAS having the highest of most of the proximate components than 3 and 7 WAS. On the amino acid content of the maize grain, nicosulfuron at concentrations 50-200 g/ha has a significant effect, with concentration 100 g/ha (recommended concentration) recording the highest of all the essential and some of the non-essential amino acids content than other concentrations. The 3, 5, and 7 WAS time of nicosulfuron application also significantly affects the amino acids contents of the maize grain, with 5 WAS having the majority of the significantly highest amino acids content than 3 and 7 WAS. Concentrations 50-200 g/ha of nicosulfuron have no significant effect on the Ca and N of Sammaz 17 maize grain, while the 3, 5, and 7 WAS periods of application have no significant effect on the Ca and N, but on Mg content of the maize.

Therefore, nicosulfuron at different concentrations and times of application significantly affect the proximate and amino acid components of Sammaz 17 more than its Ca and N mineral content. Concentration 100 g/ha and 5 WAS time of application give the highest content of all essential amino acids content of Sammaz 17 maize grain. That means that using nicosulfuron herbicide at the recommended concentration (100 g/ha) to control weeds at 5 WAS in maize fields allows the plants to produce sufficient amino acids. Those amino acids produced, especially the essential ones could pave the way for synthesizing the necessary materials needed for increased growth and yield.

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Phenotypically and genotypically estimation of virulence factors in *Salmonella* serovar *typhi* isolated from patients with enteric fever in Al-Najaf, Iraq

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Abstract. *Zghair LS, Motaweq ZY, Lafta HC. 2022. Phenotypically and genotypically estimation of virulence factors in* Salmonella serovar typhi *isolated from patients with enteric fever in Al-Najaf, Iraq. Nusantara Bioscience 14: 128-133. Salmonella* serovar *typhi*, often known as enteric fever, causes typhoid fever and has been a major human infectious disease for centuries, surviving in poor sanitation and overcrowding. Only 64 (58.1%) Gram-negative bacteria were found from the 110 total specimens, with 46 (41.8%) Gram-positive bacteria. The 64 samples were divided into 42 (65.6%) males and 22 (34.4%) females. This work presents the isolation and identification of 64 *Salmonella typhi* isolates obtained from specimens. In addition, the flagellin gene was found in 64 isolates of probable typhoid fever patients (*fliC-d)*. In this study, phenotypic techniques were used to detect several virulence factors. The results showed that small colonies of L-form bacteria grow on the edges of a petri dish when one of the β-lactam antibiotics (a class of antibiotics that includes penicillin) is given to wild-type bacteria, showing 52 (81.3%) of isolates could produce L-form and observed in 44 (68.75%). The ability to generate CFA/I and CFA/II was found in 68.7% of isolates. The large percentage of CFA produced showed CFA/III production, 64 (100%).

Keywords: CFA, L-form, *Salmonella* serovar *typhi*, typhoid fever, virulence factors

INTRODUCTION

Salmonella is a Gram-negative bacterium belonging to the Enterobacteriaceae family, with rod-shaped bacteria. The genus *Salmonella* flagellated (flagella peritrichous-found everywhere around the cell body) is facultative-anaerobes and non-spore formation predominantly motile with cell diameters ranging from: 0.7 to 1.5 μm, lengths from 2.0 to 5.0 μm. Two genera exist, *S. enterica* and *S. bongori*, divided into six subspecies, including over 2,600 serotypes (Fàbrega and Vila 2013; Gal-Mor et al. 2014; LeLièvre et al. 2019).

As a result, increasing the chances of discovering carriers is crucial to limit the harm they bring to populations. A sensitive, specific, and quick diagnostic technique for identifying typhoid patients and carriers would be ideal. *Salmonella* species that are pathogenic attack non-phagocytic gut epithelium by delivering a specific set of effectors via finely tuned hardware involving the Type 3 secretion system (T3SS), which plays a crucial role in *Salmonella* pathogenesis (Que et al. 2013). The *S. typhi* utilizes two T3SSs: *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2). SPI-1 is a gene cluster with a 40-kb district that includes 39 genes encoding T3SS-1, its chaperones, effector proteins, and transcriptional controllers that regulate the expression of multiple destructiveness genes both inside and outside SPI-1 (Zhang et al. 2018).

Surface K antigens are the smallest common antigens discovered in *Salmonella* species and are heat-sensitive polysaccharides located on the bacterial capsule surface. Antigens of virulence (Vi) are a subclass of K antigen, Dublin, *Paratyphi* C, and *Typhi* are the only three pathogenic serovars (Wattiau et al. 2011). The capsular Vi antigen is a linear homopolymer of alpha 1-4. They are coupled to galactose aminouronic acid, which is variably acetylated at the C3 site. One of the main traits differentiating *S. typhi* from non typhoid *Salmonella* (NTS) is the production of a polysaccharide capsule named the Vi antigen. The Vi capsule reduces phagocytosis while promoting serum resistance, most likely by preventing antibodies from attacking the O-antigen (Hart et al. 2016). This work aimed to identify the phenotype of pathogenicity factors in *Salmonella* serovar *typhi* represented by capsule, L-form bacterium, and Colonization Factor Antigen type.

MATERIALS AND METHODS

The research was carried out at the Bacteriology and Molecular Laboratories, Department of Biology, Faculty of Sciences, Kufa University, Iraq.

Clinical specimens and patients

Blood samples (110) were taken from patients suffering from enteric fever at AL-Sadder Medical City and AL-Furat General Hospital/Al-Najaf-Iraq for three months, from August 2021 to November 2021. Furthermore, using sterile syringes, four milliliters of fresh venous blood were taken and separated into two halves. For the Widal test, one milliliter of blood was used, with three milliliters of blood delivered into a special screw, placed in a bact/alert 3D

apparatus incubated at 37°C for a week. If a positive sample was found, each specimen was inoculated using a direct method of inoculation on a culture of selective media, namely MacConkey, XLD, and SS agar. The inoculation cultivation dishes were incubated overnight at 37°C for 18 to 24 hours, then stored until needed (Cheesbrough 2010). Identification of *S. typhi* isolates by Microscopic Properties, Cultural Characteristics, and Biochemical Tests. GN ID cards were used to confirm *S. typhi* isolates using the automated VITEK-2 compact system. Furthermore, it was performed on each bacterial isolate to complete the final identification. The GN ID card is based on well-established biochemical (64 reactions) methodologies and newly created substrates that measure many metabolic processes (German BioMerieux Company).

Molecular diagnostic methods

Purification and extraction of DNA

The extracted *S. typhi* DNA was prepared using the boiling technique. Briefly, colonies were suspended in 100 microliters of sterile distilled water and boiled at 100°C for 15 minutes in the water bath. Then immediately frozen at -20°C for one hour, centrifugation at 14,000 x g for 10 min, and the supernatant was conserved for the amplification operation (Yang et al. 2008). The concentration and purity of DNA can be determined by Williams et al. (2007).

Polymerase Chain Reaction (PCR) assay

Thermo cycle PCR was used to re-confirm *S. typhi* diagnosis; this technique requires specific primers for the *flic-d* gene, including sequence information. The primer of flagellin gene *fliC-d*-F:5'-ACTCAGGCTTCCCGTAA CGC-3' and *fliC-d*-R: 5'-GGCTAGTATTGTCCTTATCGG-3', in the product size 763 bp (Levy et al. 2008). Five µl of master mix, five µl of template DNA mixed with 2.5µl each set of primers in a suitable PCR tube, the rest of the total volume was attained to 25 µl by sterile nuclease-free water, the mixture vortexing well. The PCR for the *flic-d* gene included a primary denaturation step, denaturation, annealing, and extension at 94°C for 4 minutes. Next, at 40 cycles at 94°C for 45 seconds, 56°C for 30 seconds, and 72°C for 45 seconds, respectively. The reaction mixture was kept at 4°C until employed after the last extension step at 72°C for roughly 10 minutes (Levy et al. 2008). Then, all PCR products were examined using 1% agarose gel electrophoresis and 3µl of ethidium bromide dye. The specific cover on the electrophoresis tank was closed, and the electric current was matched (70 volts for 1.5-2 h). Finally, the gel documentation system was used to identify the electrophoresis data.

Phenotype detection of some virulence factors

Detection of capsule (vi antigens) production

A loopful of suspected culture was mixed with a loopful of nigrosin stain on a clean and dry slide, allowing it to air dry at room temperature. Next, the slide was gently cleaned with water before being stained for 2 minutes by methylene blue stain and left to air dry at room temperature. Next, the slide was softly washed with water and viewed using an oil laboratory microscope. The nigrosin stain gives the unstained capsule a dark backdrop, while the methylene blue stain gives the cells a blue tint (Harley and Prescott 2002).

L-form detection of S. typhi

According to multiple studies, tiny colonies of L-form bacteria grow on the plate's edges when one of the β -lactam antibiotics (which includes penicillin) is administered to wild-type bacteria in a petri dish. Penicillin treatment not only selects L-forms (which are penicillin-resistant) but also stimulates L-form growth (Casadesús 2007). Penicillin discs (10µ) were used according to the Kirby-Bauer disc diffusion method. The method included the following steps : (i) A isolate of previously discovered bacteria was improved by mixing a growth from an isolated colony with 5 ml of sterile normal saline at a cell density comparable to the turbidity of McFarland tube No. (0.5), equivalent to 1.5x108 cells/ml of bacteria. Inoculums were obtained using a sterile cotton swab and streaked over Muller Hinton agar medium. With heated, sterilized forceps, the antibiotic discs were placed on the surface of the medium at evenly spaced intervals. Incubate the plate at 37°C for 18 hours (Perilla et al. 2003). (ii) According to the Domingue methods, the agar containing the bacteria was cut out from the edges of the plate as discs, then transported to the variant broth incubated at 35°C for 7-10 days. Then by using a cotton swab immersed in bacterial suspension, spread the bacteria on variant agar; after incubating for 18 hours at 35°C, the L-form or cell wall deficient bacteria were grown as fried egg colonies on variant agar. (iii) After staining with Gram stain, the bacterial colonies appear spherical or ovoid shapes and agglutinated (Domingue et al. 1979).

Haemagglutination

It was detected via the presence of clumping of erythrocytes, caused via fimbriae of *S. typhi* when D-mannose is present. The assay was carried out using the mannose-sensitive and mannose-resistant haemagglutination assays and the bacterial Haemagglutination assay-slide method. The *S. typhi* was inoculated on nutrient broth at 37°C for 48 hrs. Blood from human (O) and different animal blood types was collected under sterile conditions into Alsever's solution at a ratio of 2 volumes: 1 volume of blood and stored in the refrigerator. Each type of blood was taken, and NS was washed three times and formed up to a 3% suspension in NS. (AL-Khafagee 2018). The slide was rocked at room temperature for 5 minutes after one drop of RBC suspension was introduced to a drop of broth culture. The presence of clumping was viewed as a sign of haemagglutination. The absence of haemagglutination revealed mannose-sensitive by a comparable series of tests in which a drop of 2% W/V D-mannose and a drop of broth culture were added to the red cells. The presence of 3% haemagglutination in the presence of 2% W/V D- mannose was used to detect mannose-resistant haemagglutination. This technique was also used to identify the type of fimbria in crude oil (Vagarali et al. 2008).

RESULTS AND DISCUSSION

Salmonella typhi isolates identification

A total of 110 blood samples from typhoid patients were collected during the present study period. The first identification of *S. typhi* isolates has been based on morphological, biochemical, and microscopical studies. Gram-negative bacilli were microscopically identified as S. typhi, which are peritrichous flagellated, motile, and non-spore-producing bacteria. Although the morphological of *S. typhi* isolates utilized special media such as XLD (Xylose Lysine Deoxycholate) agar, SS (*Salmonella* Shigella) agar, and MacConkey agar, appeared on culture medium once appeared the typical characteristics at 37°C after 18-24h. *S. typhi* colonies appeared smooth, rounded, convex, non-hemolytic, and grey-white color on blood agar. However, *S. typhi* colonies on MacConkey agar looked pale yellow (non-lactose ferment), 1-3 mm in diameter, and after 18-24 hours at 37°C, as well as good development of *S. typhi* colonies on XLD agar emerged gray hue with black center colonies due to its ability to create H_2S.

The TSI, Sugars, Oxidase, Indole, Ureases, and Simmons citrate assays indicated the biochemical results of *S. typhi* isolates. In the TSI slants test, the slant and butt turned AKL/ACID red and yellow, suggesting non-fermentation of glucose on the slant and acid generation with H_2S in the bottom. Further tests of *S. typhi* isolates yielded negative results for oxidase, indole generation, urease generation, and citrate use. The identification of *S. typhi* isolates using the VITEK-2 GN ID Cards System comprised various biochemical assays. The results indicated *S. typhi* with cards IDing a wide range of good isolates (percentage from 95 to 99%). On MacConkey agar, there are 64 *S. typhi* colonies.

Typhoid fever is caused by *S. enterica* serovar *Typhi*, an acute systemic sickness that causes a significant proportion of illness and mortality, especially in impoverished nations. The *S. enterica* serovar *Typhi* infections are common among travelers returning from disease-endemic areas in Europe (Fabrizio et al. 2009).

Confirmation of *S. typhi* by PCR amplification of *FliC-d*

Salmonella typhi clinical isolates were tested using the polymerase chain reaction technique to confirm the identification of *S. typhi* with a specific gene with 763 bp. The observations are that most *S. typhi* isolates carry the *fliC-d* gene, which is typical of *S. typhi*. Furthermore, the PCR showed a total of 64 (100%) positive results from blood (Figure 1). This finding is in agreement with Khan et al. (2012), who found that out of 80 suspected typhoid fever cases, the flagellin gene (*fliC-d)* was detected by PCR in 56 (70%) cases, which matches the results of a previous study in Bangladesh, where PCR was positive in 88.7% of suspected typhoid fever cases.

The result obtained by the VITEK-2 system is the same obtained by the PCR technique. Furthermore, these results are comparable to those of Ali (2015), who discovered that the positive result from the Vitek 2 compact system and the PCR technique was 65 (32.5%).

Of 64 patients, 42 (65.6%) were male, and 22 (34.4%) were female. Therefore, males were estimated to be infected at a higher rate than females. This result is compatible with study results in the Al-Musaib District (Al-Khafaji et al. 2006) and Diyala District (Saleh 2013). That could be because most males were out-doored and could be seen as food-eating and handling or contact with other patients from this perspective (Flayyih 2017). The patients' age rate is from (1-60) years old; the patients' ages are distributed in Figure 2.

The lowest incidence was among the (31-40) and (41-50) were 4.7% and 6.3%, respectively. On the other hand, the percentage of the age group (21-30) was 7.8%, while the highest incidence was among the (0-10) 46.9 %. The disease affects age groups (51-60) and >60 at 12.5%. These findings are dissimilar to Al-Sultany's (2003) previous research, which obtained the most infective age between (16-20) at 23.5%. The results also correlated with Flayyih (2017). The age range (of 11-20) had the highest occurrence, according to the researchers (54%), while Ali (2015) found that the majority of participants in his study were aged (51-60) years. These results also compare with Prince (2002), who found the ages (35-10) the most infective, as in Table 1.

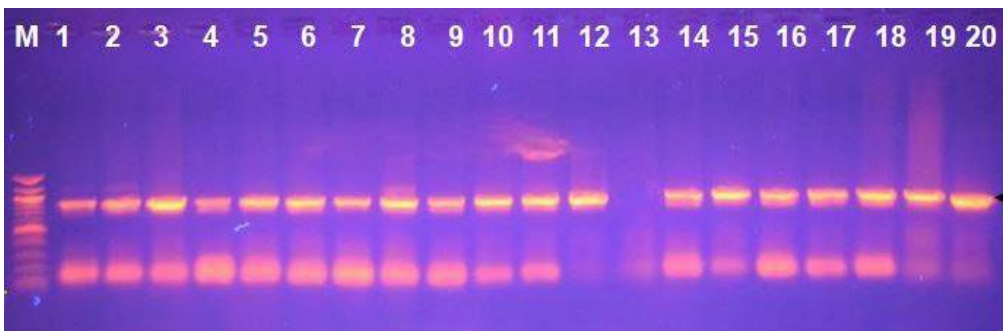


Figure 1. PCR product of *fliC-d* gene primers with product 763 bp gel electrophoresis Lane (M): DNA molecular size marker (100-bp ladder), Lanes (1-20): positive *fliC-d* gene results

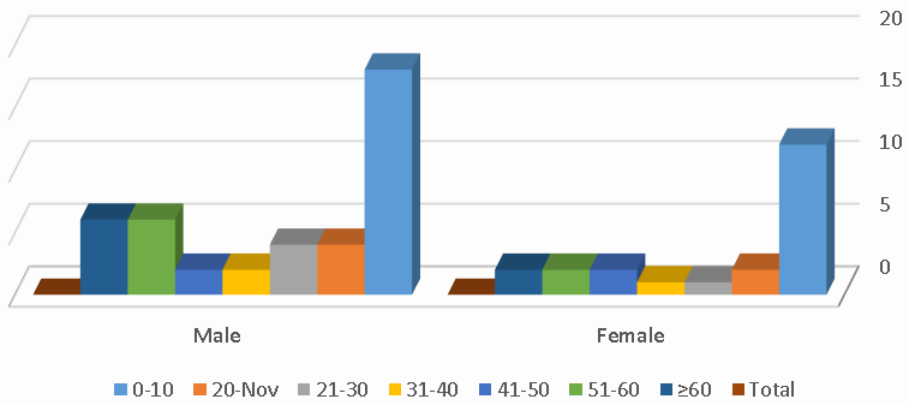


Figure 2. Age rate from female and male *S. typhi* patients

Table 1. Prevalence of *Salmonella* serovar *typhi* according to the age groups

Age group	Total no. (%)	Female	Male
0-10	30 (46.9)	12	18
11-20	6 (9.4)	2	4
21-30	5 (7.8)	1	4
31-40	3 (4.7)	1	2
41-50	4 (6.3)	2	2
51-60	8 (12.5)	2	6
≥60	8 (12.5)	2	6
Total	64 (100)	22 (34.4)	42 (65.6)

Salmonella typhi virulence factors detection

S. Typhi's ability to create a large number of all virulence factors.

Capsule detection

The encapsulated isolates of *S. typhi* were detected using the nigrosin stain. The findings revealed that 56 isolates (87.5%) were encapsulated isolates with tiny polysaccharide capsules**.**

The *S. typhi*, unlike most other *S. enterica* serovars, may produce a carbohydrate capsule known as Vi-CPS antigen. The production of this antigen, which is influenced by environmental inputs, is critical for extracellular survival and protection against neutrophil oxidative bursts. TNF-αresponse in human macrophages is likewise reduced following absorption. According to current thinking, Vi-CPS is implicated in immune evasion during infection in the human host, hence critical during infection (Eed et al. 2011).

Hemolysin production

The results of the *S. typhi* isolates' virulence factors revealed that none of the *S. typhi* isolates produced hemolysin in a blood agar medium**.** These results agree with the findings of Flayyih (2017).

Colonization Factor Antigen (CFA):

Figure 3 shows that 44 (68.75%) isolates have CFA/I and CFA/II. Because of the significant production of CFA, 64 (100%) of *S. typhi* isolates were found to produce this component. CFA/I and CFA/II were also identified in *S. typhi* isolates, albeit in lower percentages than CFA/III. These results disagree with the findings of Ali (2015). According to the findings, 31% of isolates could produce CFA/III, 15% could produce CFA/II, and 92% of *S. typhi* isolates could produce CFA I.

Fimbriae are thought to play an essential function in epithelial cell adhesion. Fimbriae mediate bacterial colonization and host-cell communication by binding to specific host receptors. Fimbrial adhesins control the bacterial pathogen's fate in the host as well as the progression of the disease process. Type-1 fimbriae are also significant in determining the organism's pathogenicity. Jaroni's (2014) experiments revealed that a mannose-resistant haemagglutinin was required for *Salmonella* to attach to target cells. Fimbriae 1 is thought to play a crucial function in adhesion to epithelial cell surfaces (which facilitates bacterial colonization) and determining *S. typhi* pathogenicity.

The adherence of bacteria in mucous surfaces or epithelial cells of the gastrointestinal tract revealed a link between mannose-sensitive hemagglutinin (MSHA) or type 1 fimbria and bacterial pathogenicity. Among the isolates, CFA/II exhibited the lowest prevalence. This factor produces agglutination in chicken blood and helps bacteria bind to unique and complex carbohydrate receptors on small intestinal epithelial cells (Hamid and Jain, 2008).

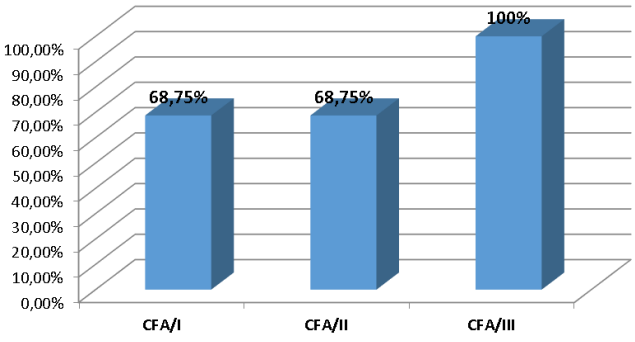
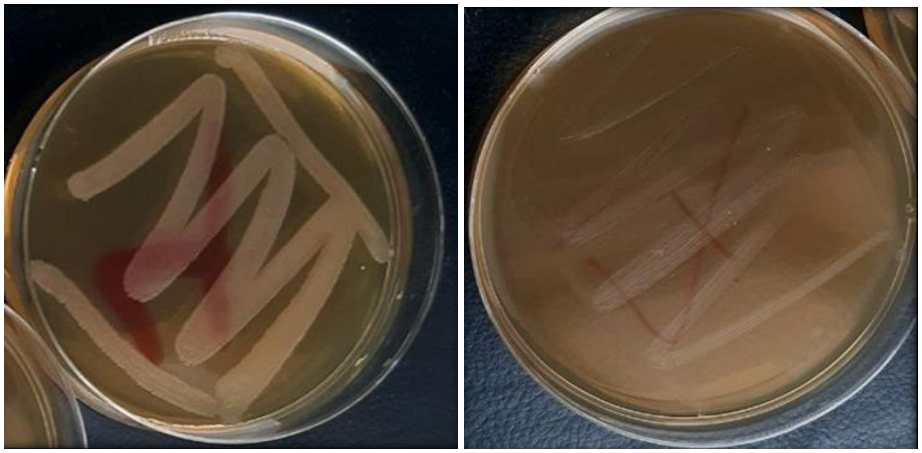


Figure 3. Types of colonization factor antigen in *Salmonella* serovar *typhi*



A **B**

Figure 4. L. form of *Salmonella typhi* isolates after incubation at 37°C for 24 hrs. A. L. form cell on variant agar B. control cell on variant agar

Table 2. Showed isolates bacteria to produce L. form

Num	L-form	Total
1-64	positive	52
	negative	12
total		64

L. form detection

According to multiple studies, tiny colonies of L-form bacteria grow on the plate's edges when one of the β - lactam antibiotics is administered to wild-type bacteria in a petri dish. Likewise, penicillin treatment not only selects for L-forms (which are penicillin-resistant) but also causes L-form growth (Casadesús 2007).

Table 2 showed that 52 (81.3%) isolates could produce L-form. This result agrees with Al-Sultany's (2003) findings, which found that 82.3% of isolates could lose their cell wall and produce L-form after culturing on special media prepared for this target. After staining with Gram stain and examining with a light microscope, the bacterial colonies appear as spherical or ovoid shapes and agglutinated (Kalaivani et al. 2014), as in Figure 4.

In conclusion, this study found that about 100% of *S.* serovars *Typhi* isolated from the blood of enteric fever patients had many virulence factors by phenotypic tests.

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