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Zingiber officinale flower photo by S.K. Siddhartthan

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Smith J, Jones M Jr, Houghton L et al. 1999. Future of health insurance. N Engl J Med 965: 325-329. DOI: 10.10007/s002149800025.

Book:

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Chapter in the book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds.). Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.). Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from the internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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Antioxidant potential of ginger extract on metals (lead, cadmium, and boron) induced oxidative stress in maize plant

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Abstract. *Ezeonu CS, Tatah SV, Imo C, Yakubu OE, Garba QH, Arowora K, Umaru IJ, Abah MA, Abu MS, Yohanna ER, Timothy M. 2022. Antioxidant potential of ginger extract on metals (lead, cadmium, and boron) induced oxidative stress in maize plant. Asian J Trop Biotechnol 19: 45-51.* Plants have a high potential to accumulate heavy metals, which may have a toxic effect on them. These heavy metals can induce the generation of reactive oxygen species in plants which may affect their physiological activities. This research aimed to examine ginger extract's antioxidant potential in treating metals (lead, cadmium, and boron) in contaminated soil in which maize was cultured. Maize seedlings were grown in pots containing soil (A-H) induced with lead acetate, cadmium, and boron (1 g each) and treated with 1 g of ginger extract, simulated for 40 days. The uptake and distribution of the heavy metals (lead, cadmium, boron) with possible induction of oxidative stress alteration in the activity of the antioxidant defense system of the maize plants were determined. The inhibitory effect of ginger against lead, cadmium, boron induced oxidative stress in maize seedlings was also determined, and the percentage inhibition showed an increase in extract concentration of ginger. From the result, maize seedlings grown from 10-40 days with 1 g of lead, 1 g of cadmium, and 1 g of boron showed significant ($p<0.05$) increased lipid peroxidation in the whole maize plant compared to the control. However, ginger extract caused a significant ($p<0.05$) decrease in the accumulation of lipid peroxide concentration in the seedlings. In addition, there was a marked increase in antioxidant enzyme activities in the lead, cadmium, and boron-contaminated soil. The result also showed that lead, cadmium, and boron-induced oxidative stress in maize seedlings could be ameliorated by ginger extract and antioxidant enzymes, which are the biomarkers for metal-induced oxidative stress in maize plants. The result obtained showed a significant ($P<0.05$) increase in SOD activity in maize seedlings grown in pots B and G (15.95±1.34 nmol/mg and 15.85±0.49 nmol/mg, respectively); B and C (10.70±1.14 nmol/mg and 10.95±0.07 nmol/mg, respectively) and A and H on the soils contaminated with 1 g lead, cadmium, and boron, respectively. SOD activity was observed to be higher in pots E (28.47±1.65 nmol/mg) and H (21.29±1.12 nmol/mg) (boron contaminated soil) when compared with lead and cadmium contaminated soils. The study has clearly shown that lead, cadmium, and boron toxicity induces oxidative stress in maize plants which the ginger antioxidant effect could reduce.

Keywords: Antioxidant enzymes, ginger extract, maize plant, metals, oxidative stress

INTRODUCTION

Naturally, heavy metals are contaminants and pollutants to plants, humans, and the environment because of their persistence, high toxicity, and easy transmission through the food chain. These metals are widely spread in the air, water, and soil and easily enter living organisms (Kim et al. 2015; Irawati et al. 2017; Tatah et al. 2017). As a result, heavy metals often coexist in soil and plant systems. The buildup of heavy metals in soils can restrict soil function, result in toxicity to plants, and contaminate the food chain by affecting food quality and safety (Van Bussel et al. 2014). Because most plant roots are located in the soil, they play important roles in the absorption of heavy metals (Topolska et al. 2014). In a soil environment, they inhibit seed germination, stunt seedling growth, and threaten plant metabolic reactions for proper growth and development, resulting in low yields (Kasfori and Petrovic 2008). High levels of heavy metals in soils normally result in oxidative damage to plants either directly or indirectly by triggering an increased level of reactive oxygen species generation that generally causes damage to biological molecules such as proteins, membrane lipids, chloroplast pigments, enzymes, nucleic acids, etc. (Malecka et al. 2001). These free radicals include superoxide radical (O_2^{*-}), hydroxyl radical (OH^{*-}), and hydrogen peroxide (H_2O_2) that are produced as by-products during membrane-linked electron transport reactions and by associated metabolic pathways (Khaki et al. 2009). Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species and the biological system's ability to readily detoxify the reactive intermediates produced or failure to easily repair the damages caused (Halliwell 1994; Ojochenemi et al. 2019).

Maize (*Zea mays* L.) is an edible flowering plant in the Gramineae family and is a warm-season crop that is easily grown during the spring and summer. Additionally, maize serves as the main food source for humans and animals around the world. It is considered one of the main cereal crops of West Africa. It is the fourth most consumed cereal

in Nigeria in the past two decades, after sorghum, millet, and rice. Maize is the world's highest supplier of calories, with a caloric supply of about 19.5%. It provides more calories than rice (16.5%) and wheat (15.0%) (Badu-Apraku et al. 2021). It is one of the most important staple foods in the world today. Maize is also the most important staple food in Nigeria, and it has grown to be the local 'cash crop' most especially in the south-western part of Nigeria, where at least 30% of the cropland has been devoted to small-scale maize production under various cropping systems (Badu-Apraku et al. 2021). Maize can also be used in animal feed as a feedstock source (Zhema et al. 2022). To meet the ever-increasing demand for maize for both domestic consumption and the export market, farmers are now employing different methods of farming, such as the application of fertilizers, pesticides, compost manure, and irrigation to improve and protect the maize (Abah et al. 2021). However, agricultural practices such as applying phosphatic fertilizers, pesticides, and refuse-derived composts contribute to heavy metals in the soil (Wang and Sun 2013; Okoli et al. 2021). Because the roots of maize plants are located in the soil, they can absorb heavy metals, which accumulate in the plant's tissues over time, resulting in oxidative damage to plants either directly or indirectly (Hechmi et al. 2015). However, Ginger (*Zingiber officinale* Roscoe) which has been reported to be a strong antioxidant substance, can mitigate or prevent a generation of free radicals. It is considered a safe herbal medicine with few and insignificant side effects (Ali et al. 2008). Ginger and its constituents are stated to have antiemetic, antithrombotic, anti-hepatotoxic, anti-inflammatory, stimulant, cholagogue, androgenic and antioxidant properties (Khaki et al. 2009). This research is aimed at assessing the absorption and distribution of heavy metals (lead, cadmium, and boron) in the root and shoot of maize plants which induced oxidative stress and to determine the inhibitory potential of ginger as well as a possible alteration in the activity of some defensive enzymes of maize plants.

MATERIALS AND METHODS

Collection of soil sample

Soil samples were collected randomly from different locations within Federal University Wukari, Taraba State, Nigeria, to be induced with metal ions.

Collection of ginger rhizomes

Ginger rhizomes were collected from the Wukari market region, Taraba State, Nigeria, before being transported to the Biochemistry Laboratory, Federal University Wukari, Taraba State, Nigeria.

Preparation of ginger extracts

Ginger rhizomes were reduced into smaller pieces, and the rhizomes were dried at a certain temperature in an air oven for 7 days. Then, the rhizomes were ground and sieved through a mesh of fine size to obtain a powdered sample of whole ginger. The powdered ginger sample (500 g) was extracted via maceration in ethanol for a period of 48 hrs using the method described by Aguawa and Mittal (1981), modified by Tugbobo et al. (2018).

Experimental design

Maize seeds were surface sterilized with hypochloric acid solution (0.1%) for 10 mins and then rinsed with distilled water before planting. After 24 hrs of inhibition of seeds in water (seed priming), the seedlings were raised in pots. The pots were grouped in which some were treated with metals and ginger extract, while others served as control before planting the seeds. Optimum relative humidity for 12 hrs photoperiod was maintained. The pots were formed into (8) groups: A, B, C, D, E, F, G, and H. Three plants were used in each treatment as biological replicates. (i) Pot A: 2 kg of soil microcosm treated with 1 g ginger extract as a positive control. (ii) Pot B: 2 kg of soil microcosm only served as normal control. (iii) Pot C: 2 kg of soil microcosm contaminated with 1 g of lead acetate. (iv) Pot D: 2 kg of soil microcosm contaminated with 1 g of cadmium. (v) Pot E: 2 kg of soil microcosm contaminated with 1 g of boron. (vi) Pot F: 2 kg of soil microcosm contaminated with 1 g of lead and treated with 1 g of ginger extract. (vii) Pot G: 2 kg of soil microcosm contaminated with 1 g of cadmium and treated with 1 g of ginger extract. (viii) Pot H: 2 kg of soil microcosm contaminated with 1 g of boron and treated with 1 g of ginger extract. The seeds were then sown and allowed to germinate in pots (A-H) for 40 days. Germinated seeds were then observed in a secured environment.

Determination of metallic contents (lead, boron, and cadmium) in maize seedlings

Metallic contents in maize seedlings were determined using the atomic absorption spectrometry (AAS) method as described by Tugbobo et al. (2018). A fresh whole plant sample was collected and surface sterilized with 1ml of HCl acid and then with 1 mM Na2EDTA for the surface-bound metal ions under study and then dried in an oven at 50ºC for 3-days. Dried samples were mashed into a fine powder using a blender and then mixed with concentrated H_2SO_4. Mixed samples were dissolved in de-ionized distilled water, and the metal content (lead, boron, and cadmium) was determined.

Determination of anti-oxidant potentials of ginger

The antioxidant activity of ginger in maize was determined using the method described by Ohkawa et al. (1979) and modified by Tugbobo et al. (2018). The whole plant was pounded in cold saline (1/10 w/v) with 10 up-and-down strokes in mortar and pestle. The homogenate was centrifuged for 10 min at 10,000 x g to obtain the supernatant and incubated with the different metal ions under study (boron, cadmium, and lead) and ginger extract at varied concentrations together with de-ionized water at a total volume of 300 µL at 37ºC for 1 hr. The color reaction was monitored by adding 200 µL, 250 µL, and 500 µL each of 8.1% Sodium Dodecyl Sulfate (SDS) and acetic acid at pH 3.4 and 0.6% TBA, respectively. The solution was incubated at 97ºC for 1 hr, and absorbance was read at 532 nm.

Oxidative stress assay

The level of lipid peroxidation products was determined using the Heath and Packer (1968) method. Fresh root and shoot samples were ground in 0.25% thiobarbituric acid (TBA) in 10% TCA using a mortar and pestle. The mixture was heated at 95ºC for 30 minutes, cooled in an ice bath, and centrifuged at 10,000 x g for 10 minutes. The absorbance of the supernatant was read at 532 nm, while a total of 0.25% TBA in 10% TCA served as the blank. The concentration of lipid peroxides and the oxidative-modified proteins of plants were quantified and expressed as total TBARS as mol/g^{-1} fresh weight using an extinction coefficient of 155 mM^{-1} $cm^{-1.}$

Preparation of plant extract for antioxidant enzyme assays

The fresh leaves (500 mg) from the stressed plants were ground into a fine powder with liquid nitrogen. The powdered samples were homogenized with 1.2 mL of 0.2 M potassium phosphate buffer (pH 7.8), followed by centrifugation at 14,000 g for 20 minutes at 4ºC. Then, the supernatant was removed, and the pellet was re-extracted again. The collected supernatants were stored at 4°C for various antioxidant enzyme assays.

Superoxide dismutase assay

Superoxide dismutases are metalloproteins that occur in different isoforms with different metal co-factors. The activity of Superoxide Dismutase (SOD) was determined by calculating the reduction in the absorbance of formazone formed by the reaction between the superoxide and dye (Nitrobluetetrazolium (NBT)) by the enzymes (Dhinda et al. 1981). Fifty (50) nM phosphate (pH 7.8), 2 nM ethylenediaminetetraacetic acid (EDTA), 9.9 nm L-Methionine, 55 nm NBT and 0.025% triton-X were freshly prepared before the estimation. The extract was filtered and centrifuged at 22,000 x g for 10 mins at 4ºC. Test tubes were arranged, and 1 mL of phosphate buffer, 20 µL of EDTA, 100 µL of L-Methionine, 10 µL Triton X-100, 10 µL Riboflavin, 20 µL of plant extract, and lastly, 1.2 mL of NBT have added accordingly, and mixture without plant extract served as the control. The absorbance of samples was recorded at 475 nm using the UV-visible spectrophotometer. The enzyme activities were expressed as units/ mg of protein.

Catalase (CAT) assay

Catalase is mostly present in the cytosol and peroxisomes that catalyze H_2O_2 and O_2. Therefore, the catalase activity was examined by the Beers and Sizer method (1982). First, the homogenate was centrifuged at 22,000 x g for 10 mins at 4ºC, after which the supernatant was used for the enzyme assay. The assay mixtures include the plant extract, 100 nM potassium buffer (7.0), and 75 nM hydrogen peroxide. Then the absorbance was measured at 240 nm using a UV-Visible spectrophotometer. Finally, the catalase activity was assayed using an extinction coefficient of 39.4 mM^{-1} cm^{-1}.

Glutathione reductase assay

Glutathione reductase is an NADPH enzyme, and it catalyzes oxidized glutathione (GSSG) into reduced form (GSH). The co-factor for GR is flavin adenine dinucleotide (FAD) and cysteine, which are present in chloroplast, cytosol, and mitochondrial. Glutathione reductase was assayed by Schaedle and Bassham's (1997) method. The assay mixture was freshly prepared, consisting of 0.75 mM DTNB, 0.1 mM NADPH, 1 mM GSSG, and 10 µL crude plant extract. GSSG was finally added to the assay mixture to increase the adsorption at 340 nm. The activity was calculated using the extinction coefficient of 6.2 mM^{-1} Cm^{-1}.

Statistical analysis

The data were analyzed using statistical package for social science (SPSS) software version 23, and the group means were compared for significance at $p \leq 0.05$. Data were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Metals uptake by growing maize seedlings in soil microcosm for 40 days (mg/kg)

Figures 1, 2, and 3 show the uptake of metals by soil microcosm for 40-days by the growing maize seedlings. The highest level of maize seedling absorption of lead (86. 32 mg/kg) in pot C, cadmium (74.42 mg/kg) in pot G, and boron (85.45 mg/kg) in pot H was observed on day 40, and the least level of lead, cadmium and boron absorption (20.20 mg/kg) were observed on day 10 in pot A.

Level of lipid peroxidation

Figures 4, 5, and 6 show the total oxidative stress in the growth of the maize plant for 40 days. The results obtained in these tables showed an increase in the production of lipid peroxides in maize plants with their maximum and minimum levels on days 10 and 40, respectively.

Inhibitory potential of ginger extract

Figure 7 shows the inhibitory potential of ginger extract in metal-induced oxidative stress on maize plants. 160 mg/mL of the ginger extract showed the highest inhibitory potential on lead (84%) and the lowest inhibitory potential on boron (52%). Ten (10) mg/mL of the ginger extract showed the highest inhibitory potential on lead (16%) and the lowest inhibitory potential on cadmium (14%).

SOD enzyme activity in maize grown in metals-contaminated soil with and without ginger treatments

Figures 8, 9, and 10 show the effect of heavy metal (lead, cadmium, and boron) uptake on superoxide dismutase activity in pre and post-ginger extract-treated soil in maize seedlings. The result obtained in Figures 8, 9, and 10 showed a significant ($P<0.05$) increase in SOD activity in maize seedlings grown in pots B and G (15.95±1.34 nmol/mg and 15.85±0.49 nmol/mg, respectively); B and C (10.70±1.14 nmol/mg and 10.95±0.07 nmol/mg, respectively) and A and H on the soils contaminated with 1 g of lead, cadmium, and boron, respectively.

Catalase enzyme activity in maize grown in metal-contaminated soil with and without ginger treatments

Figures 8, 9, and 10 also shows the effect of heavy metals (lead, cadmium, and boron) uptake on catalase activity in pre and post-ginger extract treatment in maize plant. For catalase (CAT) activity, it was observed that maize seedlings grown on boron-contaminated soil pots H (0.99±0.02 nmol/mg) were significantly (P<0.05) lower than the normal control (B) that was grown on lead (1.08±0.08 nmol/mg), cadmium (1.98±0.12 nmol/mg) and boron (1.24±0.06 nmol/mg). However, in pots F (3.99±0.01 nmol/mg) and G (2.96±0.93 nmol/mg) treated with the sample extract, CAT activity was appreciably higher in maize plant tissues when compared to control.

Glutathione reductase enzyme activity in maize grown in metals-contaminated soil with and without ginger treatments

Figures 8, 9, and 10 show the effect of heavy metals (lead, cadmium, and boron) uptake on glutathione reductase activity in pre and post-ginger extract treatment in maize plants. Glutathione Reductase (GR) activity was observed to be higher in pots treated with lead acetate (C), cadmium (D), and boron (E) when compared with normal control (B). Similarly, GR activity was significantly (P<0.05) higher in pot A treated with extract on maize seedlings growing in pots F2.70±0.42 nmol/mg), G (1.98±0.28 nmol/mg), and H (1.89±0.14 nmol/mg).

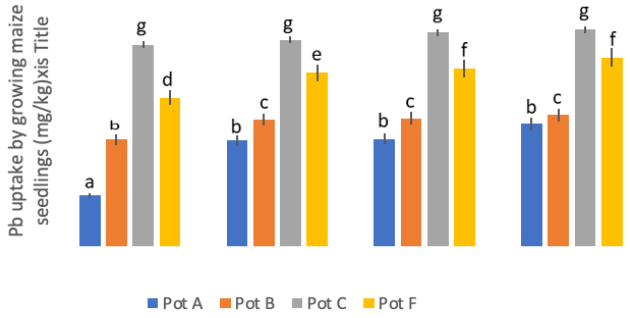

Figure 1. Lead uptake by growing maize seedlings (mg/kg)

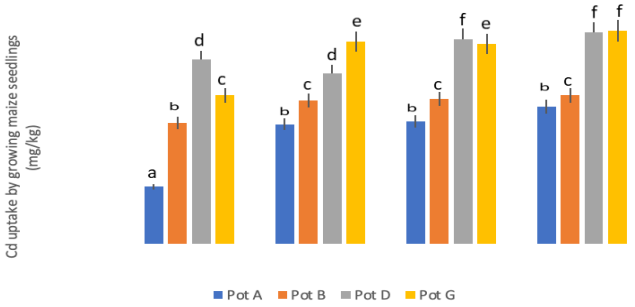

Figure 2. Cadmuim uptake by the growing maize seedlings (mg/kg)

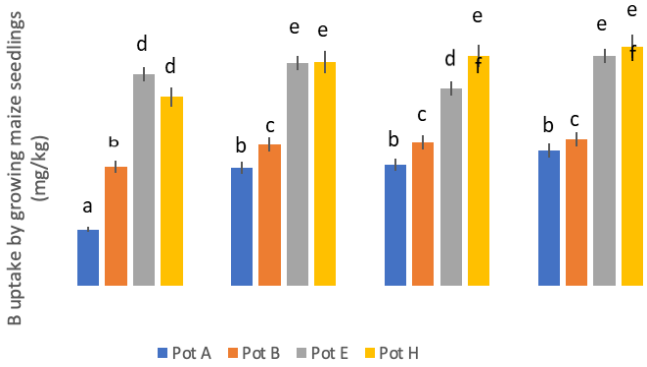

Figure 3. Boron uptake by growing maize seedlings (mg/kg)

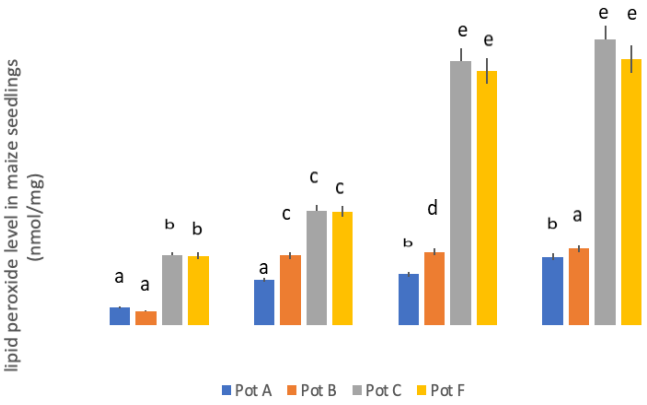

Figure 4. Level of lipid peroxide in maize seedlings with and without lead contamination (nmol/mg). Note: n=2, results are shown as mean values ± SD. Values with different superscripts are significantly different (p<0.05)

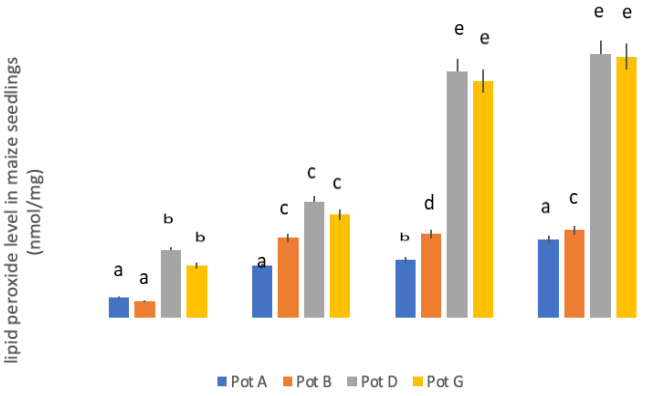

Figure 5. Level of lipid peroxide in maize seedlings with and without cadmium contamination (nmol/mg). Note: n= 2, results are shown as mean values ± SD. Values with different superscripts are significantly different (p<0.05)

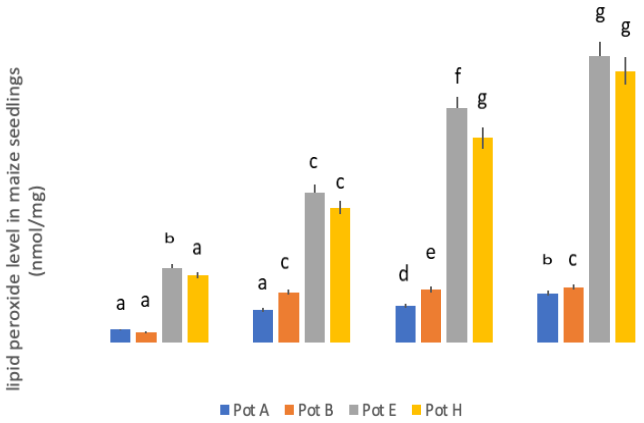

Figure 6. Level of lipid peroxide in maize seedlings with and without boron contamination (nmol/mg). Note: n= 2, results are shown as mean values ± SD. Values with different superscripts are significantly different ($p<0.05$)

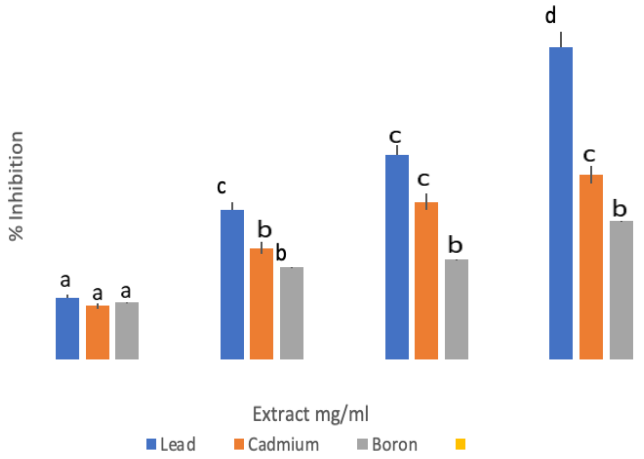

Figure 7. Inhibitory potential of ginger extract on metals (lead, cadmium, boron) induced oxidative stress in maize grown on 2 kg of soil (group B)

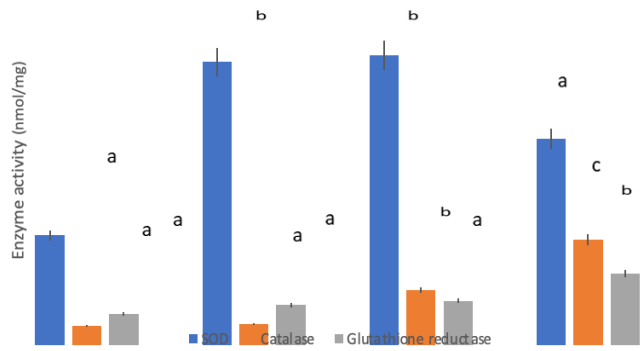

Figure 8. SOD, Catalase and Glutathione enzyme activity in maize grown in lead acetate contaminated soil with and without ginger treatments (nmol/mg). Note: n= 2, results are shown as mean values± SD. Values with different superscripts are significantly different ($p<0.05$)

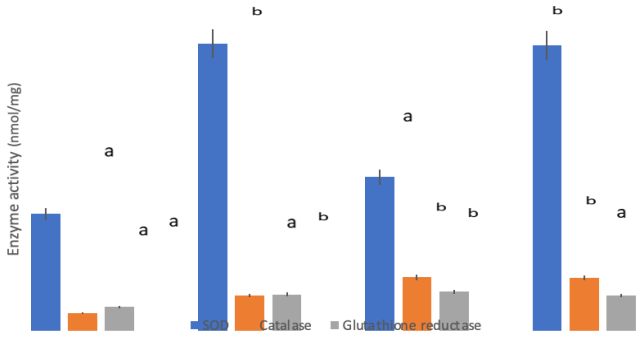

Figure 9. SOD, Catalase and Glutathione enzyme activity in maize grown in cadmium contaminated soil with and without ginger treatments (nmol/mg). Note: n= 2, results are shown as mean values± SD. Values with different superscripts are significantly different ($p<0.05$)

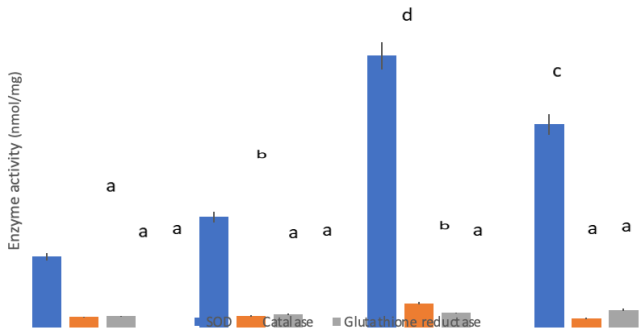

Figure 10. SOD, Catalase and Glutathione enzyme activity in maize grown in boron contaminated soil with and without ginger treatments (nmol/mg). Note: n= 2, results are shown as mean values ± SD. Values with different superscripts are significantly different ($p<0.05$)

Discussion

The contamination of ecosystems and exposure to toxic metals is a significant worldwide burden. For this reason, bio-monitoring techniques are getting more relevant because they may help recognize contaminated areas or crops, distribute metals in the ecosystem, and control potential environmental hazards caused by heavy metals pollution (Ugulu 2015).

Figures 1, 2, and 3 show the uptake of metals by soil microcosm for 40-days by the growing maize seedlings. The highest level of maize seedling absorption of lead (86. 32 mg/kg) in pot C, cadmium (74.42 mg/kg) in pot G, boron (85.45 mg/kg) in pot H was observed on day 40, and the least level of lead, cadmium and boron absorption (20.20 mg/kg) were observed on day 10 in pot A. The increase of these heavy metals in maize plants based on their age could result from the constant interaction between the metal and the root of the plant (maize) through their concentration in the soil (Rosselli et al. 2006). However, comparing pots with contamination of heavy metals and

when 1 g of ginger extract was used in treating soils contaminated with metals, the reducing absorption of metal by maize plants was possible except for cadmium and boron, perhaps due to the chelating ability of the ginger extract on soil metals thus increasing the antioxidant potential in the maize plants by reducing the metals available for absorption or absorption of the ginger extract by maize seedling thereby increasing the antioxidant potentials in maize plants as explained by Tanvir et al. (2017).

Figures 4, 5, and 6 show the total oxidative stress in the growth of the maize plant for 40 days. The results obtained in these tables showed an increase in the production of lipid peroxides in maize plants with their maximum and minimum levels on days 10 and 40, respectively. The accumulation in lipid peroxides levels could be due to an increase in the retention of heavy metals by the roots of the maize plants. However, pots that were treated with 1 g of ginger extract per 2 kg of soil microcosm (pots F, G, and H) showed a decrease in the level of lipid peroxides when compared with pots contaminated with 1 g of metals per 2 kg of soil microcosm only (pots C, D, and E). The increase in the level of lipid peroxides in pots C, D, and F could be attributed to the ability of the heavy metals to catalyze one electron (e-) transfer reaction that generates reactive oxygen species, thereby generating more lipid peroxides (Lozano et al. 1996). Therefore, the decrease in the level of lipid peroxides in pots F, G, and H could be attributed to the preventive effect demonstrated by ginger due to the presence of ginger constituents, which enhances its antioxidant activity, as reported by Tanvir et al. (2017).

Antioxidants can decrease oxidative damage directly by reacting with free radicals or indirectly by inhibiting the activity or expression of free radical generating enzymes or enhancing the activity or expression of intracellular antioxidant enzymes (Tanaka et al. 1985). From the result presented in Figure 7, the inhibitory potential of ginger extract in metal-induced oxidative stress on maize plants could be possible due to the hydrogen donating ability of ginger extract. The percentage inhibition shows an increase with an increase in extract concentration of ginger.

Plants generally possess an essential antioxidant defense system used naturally to combat oxidative damage. Figures 8, 9, and 10 show the effect of heavy metal (lead, cadmium, and boron) uptake on superoxide dismutase activity, catalase activity, and glutathione reductase activity in pre and post ginger extract treated soil in maize seedlings. The result obtained in Figures 8, 9, and 10 showed a significant ($P<0.05$) increase in SOD activity in maize seedlings grown in pots B and G (15.95±1.34 nmol/mg and 15.85±0.49 nmol/mg, respectively); B and C (10.70±1.14 nmol/mg and 10.95±0.07 nmol/mg, respectively) and A and H on the soils contaminated with 1 g of lead, cadmium, and boron respectively. Therefore, SOD activity was observed to be higher in pots E (28.47±1.65 nmol/mg) and H (21.29±1.12 nmol/mg) (boron contaminated soil) when compared with lead and cadmium contaminated soils. This possibly may be because boron is absorbed more by maize plants than lead and cadmium. Hence, excessive boron reduces the defensive potential of SOD in maize plants. SOD activity has been reported to increase under heavy metal toxicity, and this increase in response to stress could be due to de novo synthesis of the enzyme (Lozano et al. 1996).

Figures 8, 9, and 10 also shows the effect of heavy metals (lead, cadmium, and boron) uptake on catalase activity in pre and post-ginger extract treatment in maize plant. For catalase (CAT) activity, it was observed that maize seedlings grown on boron-contaminated soil pots H (0.99±0.02 nmol/mg) were significantly ($P<0.05$) lower than the normal control (B) that was grown on lead (1.08±0.08 nmol/mg), cadmium (1.98±0.12 nmol/mg) and boron (1.24±0.06 nmol/mg). However, in pots F (3.99±0.01 nmol/mg) and G (2.96±0.93 nmol/mg) treated with the sample extract, CAT activity was appreciably higher in maize plant tissues when compared to control. Catalase activity is an antioxidant enzyme responsible for the degradation of hydrogen peroxide. Therefore, the decline in CAT activity in pots D, F, and G could be attributed to lead, cadmium, and boron toxicity, respectively which could delay the removal of hydrogen peroxide and peroxides mediated by catalase which in turn enhances free radical-mediated lipid peroxidation in maize plant.

Figures 8, 9, and 10 show the effect of heavy metals (lead, cadmium, and boron) uptake on glutathione reductase activity in pre and post-ginger extract treatment in maize plants. Glutathione Reductase (GR) activity was observed to be higher in pots treated with lead acetate (C), cadmium (D), and boron (E) when compared with normal control (B). Similarly, GR activity was significantly ($P<0.05$) higher in pot A treated with extract on maize seedlings growing in pots F2.70±0.42 nmol/mg), G (1.98±0.28 nmlo/mg), and H (1.89±0.14 nmol/mg). The increased GR activity suggests a possible involvement of GR in regenerating Glutathione (GSH) from Glutathione (GSSG) under lead toxicity to increase the GSH/GSSG ratio and thus, increase the total glutathione pool (Noctor and Foyer 1998).

In conclusion, the study has clearly shown that lead, cadmium, and boron toxicity induces oxidative stress in maize plants which the ginger antioxidant effect could reduce. In contrast, antioxidant enzymes play a pivotal role in combating plant oxidative stress.

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The deleterious effects of chitosan application on red chili pepper growth and capsaicin biosynthesis under drought stress

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Abstract. *Aziz MA, Esyanti RR, Meitha K, Dwivany FM. 2022. The deleterious effects of chitosan application on red chili pepper growth and capsaicin biosynthesis under drought stress. Asian J Trop Biotechnol 19: 52-61.* The production of red chili pepper is often impeded by drought. Chitosan is considered as a promising alternative natural fertilizer and defense elicitor. This study aimed to investigate the effect of chitosan on red chili plant growth and capsaicin biosynthesis under drought stress. At the onset of the generative phase, the plant was subjected to 1 mg mL^{-1} chitosan, 50% drought, or chitosan-drought treatment. Observations were made on several growth parameters, yield, PAL expression, PAL activity, and capsaicin content. The results showed that chitosan-drought treatment significantly decreased plant growth and yield. The PAL gene expression was up-regulated around 129-fold higher than control, followed by increased PAL activity and capsaicin content about 1.22 and 1.39-fold higher than control, respectively. PAL activity and capsaicin content on chitosan-drought treated plants were lower than on individual chitosan applications. Therefore, we suggested that the double treatment of chitosan-drought might decrease red chili pepper production and capsaicin content. Interestingly, the individual chitosan treatment significantly increased PAL expression level, PAL activity, and capsaicin content compared to other treatments. Furthermore, it was suggested that chitosan might play a role in the red chili plant defense system involving capsaicin biosynthesis through the phenylpropanoid pathway.

Keywords: Capsaicin, *Capsicum annuum*, chitosan, drought, growth, PAL expression

INTRODUCTION

Red chili pepper (*Capsicum annuum* L.) is one of the most favored agricultural commodities with a high economic value. The popularity of the commodity is because of its various nutritional content such as protein, fat, fiber, minerals, vitamins A, B, and C, as well as capsaicin, a secondary metabolite known to provide heat sensation, increase body temperature and burn calories (The Indonesian Ministry of Agriculture 2015; Aziz et al. 2021). However, the fluctuating production of chili pepper causes a damaging impact on scarcity and market price escalation. In addition to pathogen infections, drought is major stress causing low productivity. According to the statistical data (The Indonesian Ministry of Agriculture 2016), it is indicated that a significant decrease in the chili harvested area due to the prolonged dry season, around 35.98% and 63.00%, occurred in 1987 and 1990, respectively.

In recent years, global climate change has been the most frequent abiotic stress (Khan et al. 2014). In brief, plants respond to water stress by regulating proline accumulation (Solichatun et al. 2022) and stomatal behavior mediated by abscisic acid (ABA) as the defense regulator. It plays a role in maintaining a plant's water capacity. However, this causes a reduction in photosynthesis, hampered plant growth and productivity (Iriti et al. 2009; Phimchan et al. 2012). Drought stress in chili plants might cause flowers and immature fruits to fall easily. In addition, the fruit tends to contain a higher level of capsaicin (Sung et al. 2005). Mahmood et al. (2021) added that flowering is one of the most sensitive stages of the chili plant affected by drought.

Capsaicin is one of the specific secondary metabolites of the phenol group in the genus of Capsicum, which is well known to cause pungency. Capsaicin is synthesized through the phenylpropanoid and fatty acid pathways. In addition to being responsible for pungency, capsaicin has many benefits in pharmaceutical properties, including anti-inflammatory, antioxidant, analgesic, and anti-carcinogenic (Gonzalez-Zamora et al. 2013). The biosynthesis pathway of capsaicin involves four main enzymes, including phenylalanine-ammonia-lyase (PAL), cinnamic acid-4-hydroxylase (C4H), p-coumaric acid-3-hydroxylase (C3H), and caffeic acid-o-methyltransferase (CAOMT). PAL regulates capsaicin production using phenylalanine as the main precursor. Capsaicinoid is synthesized from vanillylamine and isocapryl-CoA through capsaicinoid synthetase (CS) activity, then accumulated in the placenta (Sung et al. 2005). Various environmental conditions influence capsaicin biosynthesis. The higher level of capsaicin is one of the plant's biological responses in increasing resistance against pathogenic attacks. Veloso et al. (2013) proved that capsaicin could inhibit the growth

of *Verticillium dahliae* Kleb. by 33% and *Phytophthora capsici* Leonian by 63.7%. On the other hand, Solichatun et al. (2022) stated that the resistance increment of paprika (*C. annum*) against drought stress could be performed using the KCl seed priming technique.

Increasing plant growth and resistance using environmentally friendly technologies is continuously developed. This is by public awareness regarding the use of synthetic compounds causing any hazardous impacts on living things' sustainability. Chitosan is a natural compound of acetylated chitin derivatives that are easily decomposed (Mondal et al. 2013; Pichyangkura and Chadchawan 2015). The role of chitosan as a plant biostimulant is suggested through several mechanisms such as supplying the nitrogen (El-Tanahy et al. 2012), increasing macronutrient and micronutrient absorption (Chookhongkha et al. 2012; Jang et al. 2012), increasing the activity of many enzymes related to protein metabolisms and increasing nitrogen transport to leaves (Mondal et al. 2013). In addition, it is suggested that chitosan promotes plant resistance by increasing the secondary metabolism of phenolic compounds such as capsaicin (Khan et al. 2003; Aziz et al. 2021). However, the role of chitosan on plant growth and capsaicin accumulation under drought stress has not been thoroughly studied. Therefore, this study aimed to investigate the effect of chitosan on red chili plant growth and capsaicin biosynthesis under drought stress.

MATERIALS AND METHODS

Plant materials and treatments

This research was conducted at the greenhouse of the Institut Teknologi Bandung, Indonesia. This experiment was performed for ± 112 days, with the average photoperiod being 12 hours. This study uses the commercial red chili pepper (*C. annuum*) Laba cultivar from East-West Seed Indonesia Ltd. The cultivar is widely cultivated due to its superior defense system against pest and microbial infection and the ability to produce 18-20 tons of Ha^{-1}. The individual red chili pepper was grown in a plastic polybag with a 3 kg planting medium containing soil, husks, and organic fertilizer with the corresponding ratio (4:3:2, v:v:v). The plantlets were irrigated up to 100% field water capacity for the vegetative stages (7 weeks). At the onset of the generative phase, indicated by the emergence of flower buds, the plantlets were subjected to the treatments of chitosan (Chi), chitosan and drought (Chi-D), drought (D), and control (C) up to sixteen weeks of planting (n = 3 replications). Several growth parameters, including plant height and the number of leaves, followed by the number of flowers and fruits, were observed during the treatment (weeks 8 to 16). These were made to show the effect of chitosan on plant growth and yield under drought conditions. In addition, several environmental microclimate conditions, including pH, light intensity, humidity, and temperature, were also recorded.

A 1 mg mL^{-1} Chitosan solution, prepared as described in Esyanti et al. (2019) and Aziz et al. (2020), was sprayed once a week by foliar feeding technique to Chi and Chi-D treatments. Esyanti et al. (2019) stated that 1 mg mL^{-1} chitosan solution maintained the growth rate of the red chili plant cultivar. Drought treatment was applied to reach 50% of field water capacity by applying 500 mL of water (in two days) subjected to D and Chi-D treatments. Dorji et al. (2005) suggested that a 50% of water deficit is a feasible irrigation strategy to maintain plant growth performance. In addition, the control group (C) was irrigated with 100% field water capacity but not sprayed with any solutions. Fresh leaves and fruits were collected 55-60 days after flowering (harvesting period) (Sung et al. 2005). The fresh leaves from three plants were pooled into one sample and then used for RNA extraction to analyze PAL gene expression and PAL enzyme activity, while the fresh fruits were used for capsaicin content measurement.

RNA extraction and qPCR analysis

The total RNA was extracted from the leaves sample (50 mg) using the PureLinkTM RNA mini kit (Invitrogen). RNA quality was visualized in a 1.5% (w/v) agarose gel with gel-red as a coloring agent by using an electrophoresis instrument. Meanwhile, the quantity of RNA was measured using a nanodrop spectrophotometer (Eppendorf, biospectro-meter) to evaluate the purity of RNA and quantify RNA concentration. DNAse treatment was performed using DNAseI (Thermo Scientific), while cDNA synthesis was carried out using iScript cDNA Synthesis Kit (Bio-Rad) containing reverse transcriptase enzyme. In addition, the quality of cDNA was determined in a 2% agarose using CaUbi3 as the housekeeping gene by using the specific primer (Table 1). The Polymerase Chain Reaction (thermal cycler) protocol was performed as follows: 95°C 3 min, 95°C 30 s, 58°C 30 s, 72°C 1 min, 72°C 5 min (35 cycles).

Table 1. Primer for PAL and CaUbi3 genes

Primer	Accession number	Nucleotide (5' to 3')	Size (bp)
PAL	NM_001324603		283
Forward		TGGGCTTAATCTCATCAAGG	
Reverse		TAGGTTGAGCTGCAGGTATC	
CaUbi3	AY486137.1		201
Forward		TCCATCTGCTCTCTGTTGACG	
Reverse		CCCCAAGCACAATAAGACATTGT	

The PAL gene's amplification was performed using specific primers (Table 1). After being evaluated in a 2% agarose electrophoresis, sequencing was performed through Macrogen service, Korea. Further, the quantitative PCR was performed using instruments and software from MyGo Pro with the dye from Toyobo (Thunderbirds SybrqPCR Mix QPS-201). The quantification cycle (Cq) score was then used to quantify the expression level through a relative method using the corresponding formula (Livak and Schmittgen 2001).

PAL enzyme activity assay

Sung et al. (2005) adopted the PAL enzyme activity assay method with a few modifications. In brief, 0.5 gr of chili leaves were crushed using liquid nitrogen and added by a 3 mL protein extraction buffer. The samples were homogenized at high speed for 1 minute and then centrifuged at 7000 rpm for 40 minutes. The supernatant was then centrifuged at 10000 rpm for 10 minutes, then transferred to the vial bottle followed by adding 5 mL of 50 mM Tris-HCl buffer pH 8. The total protein was quantified by using the Bradford method. The absorbance was measured by using UV-Vis spectrophotometer at λ595 nm. Bovine Serine Albumin (BSA) was used as the standard solution at concentrations of 0, 1, 2.5, 5, 10, and 15 ppm.

PAL activity assay was carried out by mixing 1 mL of 100 mM Tris-HCl, 0.5 mL of 10 mM L-phenylalanine, 0.4 mL of sterile deionized water, and 0.1 mL protein extract. The mixture was incubated at room temperature for 1 hour. The reaction was ended by adding 0.5 mL of 6 M HCl. In addition, 7.5 mL of diethyl ether was added to bind the polar compound, and then the mixture was stored in a -20 freezer. After 2 phases were formed, diethyl ether was discarded. The frozen extract was dried using a freeze dryer, and the residue was dissolved using 3 mL of 50 mM NaOH. The absorbance was measured at λ290 nm. PAL activity was quantified by using cinnamic acid as a standard solution at concentrations of 0, 0.3125, 0.625, 1.25, and 2.5 ppm (Sung et al. 2005).

Capsaicin content assay

Capsaicin was extracted from the chili placenta by using the maceration method. The quantification method of capsaicin level was adopted from Sung et al. (2005) with a few modifications using HPLC (C-R7A Plus Chromatopac, Shimadzu). The placenta was immediately sampled from harvested chili fruits and dried at 60°C for 30 hours, then crushed and macerating tissue in 5 mL of 96% ethanol for 24 hours. Macerated tissue was then filtered in a 50 mL beaker glass and incubated at room temperature to evaporate ethanol. Finally, the dried filtrate was dissolved using 1 mL of 100% methanol and re-filtered using oil-free cotton into a 2 mL microtube for immediate assay or storage at 4°C.

A 2.5 µL of the extract was injected into the HPLC system. A mix of acetonitrile and aqua bides at a ratio of 6:4 (v:v) with a rated flow of 0.7 mL min^{-1} was used as the mobile phase. It runs for 12 min $sample^{-1}$ by using absorbance at λ280 nm. Before running, acetonitrile solution was filtered using a Polytetrafluoroethylene (PTFE) filter, while aqua bides used nitrocellulose membrane. Capsaicin standard (SIGMA, Lot #BCBJ2271V) solution at the concentration of 0, 125, 250, and 500 ppm was prepared by diluting from capsaicin powder.

Statistical analysis

The effect of chitosan, drought, and the interaction of both treatments towards the increase in height, PAL genes expression level, PAL enzyme activity, and the capsaicin content were analyzed by Two-way ANOVA performed using IBM SPSS statistics 22 software. In addition, the significant differences in the treatments were determined using the Tukey HSD test at $P < 0.05$ using the R studio 3.5.2 (32/64 bit) application.

RESULTS AND DISCUSSION

Chitosan application under drought conditions inhibited plant growth and productivity

The microclimate condition, pH, humidity, and temperature of the media and light intensity, humidity, and temperature of the environment, are shown in Table 2. There was no significant difference between the pH and temperature of the media in all treatments. However, the drought-treated Chi-D and D plants showed a lower media humidity at 43.44% and 46.56%, respectively. In addition, the average relative humidity was recorded at 78.96%, a light intensity of 10240 lux, and temperature ± 27.78°C.

To determine the effect of chitosan-drought (Chi-D) application on the red chili pepper plant, it is important to investigate the plant growth performance parameters such as height increment and the number of leaves described in Figures 1A and 1B. The similar performance in the vegetative phase determined the plant's growth variability. The identical growth parameters indicate it at week 8 for the number of leaves and flowers and at week 10 for the number of fruit (Figure 1). According to the difference between the plant height in weeks 8 and 16, it was found that the double treatment of chitosan and drought (Chi-D) caused the lowest increase in height significantly, which is 52.00 cm. in addition, the highest increase in height was recorded in the C group, 64.67cm, although was not significant to Chi and D treated plant which is 62.50 cm and 60.33 cm, respectively. Furthermore, we also documented an average decrease of ± 5 cm in drought-treated plants compared to the control.

The effect of chitosan application during drought stress conditions was also determined based on the number of leaves of each treated plant from weeks 8 to 16. All treatment groups showed an increment in the number of leaves from week 8 until 12, which tends to be constant until week 16. Overall, Chi-D treated plants possessed the least number of leaves from week 11, totaling 114.33 in week 16. Chi-D and D-treated plants showed a similar trend until week 16. The individual chitosan group (Chi) showed the highest number of leaves recorded in weeks 8 to13, then possessed the equal number with C groups until week 16. Chi-D and D treatments suppressed the number of leaves since week 11 compared to Chi and C groups.

Table 2. The microclimate condition recorded during the treatment

	Media			Environment		
	pH	**Humidity (%)**	**Temperature (°C)**	**Light Intensity (Lux)**	**Humidity (%)**	**Temperature (°C)**
Chi	6.66	63.61	27.44	10240	78.96	27.78
Chi-D	6.64	43.44	27.53			
D	6.63	46.56	27.59			
C	6.63	77.11	27.41			

Note: Chi: 1 mg mL^{-1} chitosan; Chi-D: 1 mg mL^{-1} chitosan and 50% drought; D: 50% drought; C: control

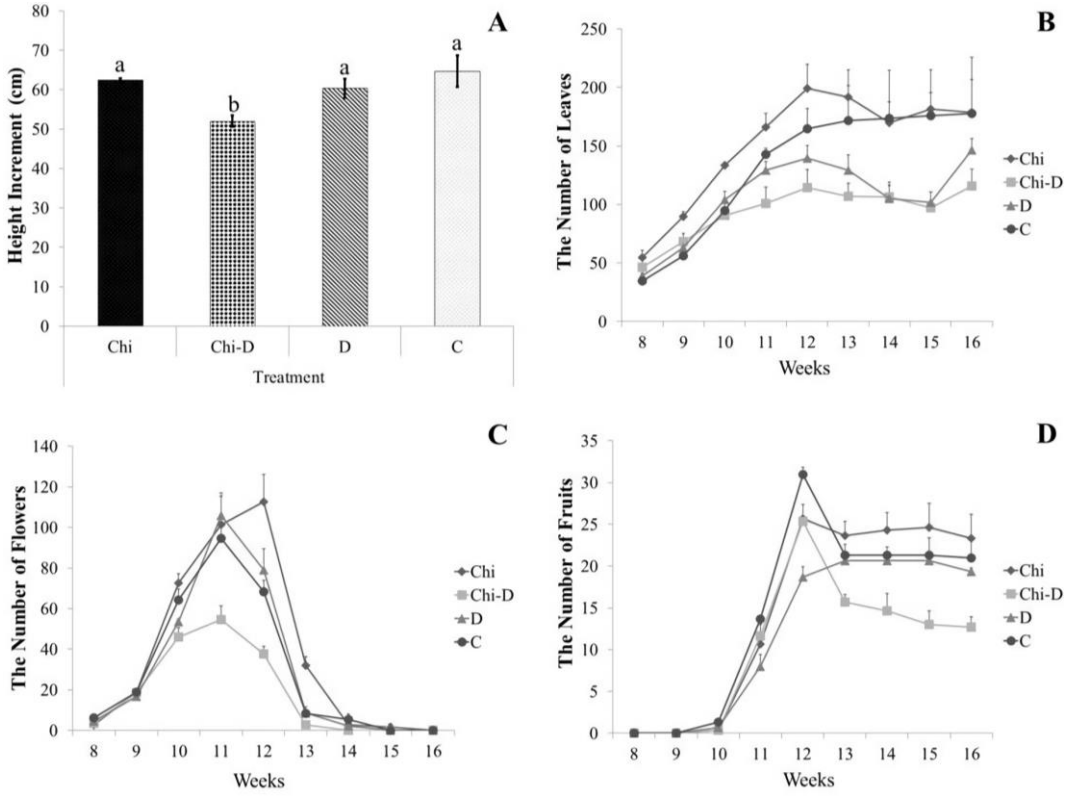

Figure 1. The growth performance of red chili pepper plants. The height increment was calculated from the difference at the start (week 8) and end (week 16). The number of leaves, flowers, and fruits was measured from the start (week 8) until the end (week 16) of treatment. The bars represent the mean of each growth parameter with the corresponding standard error (n = 3). A significant value is indicated by the letter on top of each bar, $p < 0.05$. Chi: 1 mg mL^{-1} chitosan; Chi-D: 1 mg mL^{-1} chitosan and 50% drought; D: 50% drought; C: control

All treatments were given before the commencement of the generative phase. Throughout this phase, the number of flowers followed by the number of fruits was also recorded. The results showed that chitosan application during drought conditions (Chi-D) led to the least total of flowers. The number of flowers peaked in week 12 when treated with chitosan (Chi). A steep decrease was recorded starting in week 11 in almost all treatments, which was thought to be due to abscission and fruit development. An exponential fruit emergence was recorded in weeks 11-12. However, the immature fruits did not entirely reach the mature stage, with fruit abscised as early as week 12. Chi-treated plants were relatively able to maintain fruit numbers with the highest yield at the harvesting period in week 16, followed by C and D groups. Chi-D-treated plants could only produce the least mature fruits, although the total number of fruits in week 12 coincided with Chi-treated plants.

According to the results, all growth parameters showed a massive change in chitosan treatment under drought conditions. The significantly lowest height increment and the small number of flowers, leaves, and fruits were recorded in the double treatment of chitosan-drought (Chi-D). This follows the suggestion of Iriti et al. (2009) that combining chitosan and drought treatment in plants can cause a synergistic response in increasing plant defense response but result in stunted growth. Malerba and Cerena (2016) added that the effect of chitosan application on plant biological responses, such as resistance to stress and increased productivity depends on the chemical composition, application period, and application intensity.

Therefore, it is suggested that the application of 1 mg mL^{-1} chitosan in the generative phase of the chili plant has no significant effect on balancing the harmful effects of stress. Meanwhile, Bistgani et al. (2017) proved that the application of chitosan in 50% drought conditions caused a decrease in the fresh weight of thymus plants up to 29.98% and 5.60% along with the two times cultivation. However, the results contrast with the previous studies performed in *Ocimum basilicum* L. (Malekpoor et al. 2016) and *Ricinus communis* L. (Karimi et al. 2012) that chitosan application could enhance the physiological aspects under drought treatment.

The individual chitosan application could increase the number of leaves, flowers, and fruit. This is supported by Salachna and Zawadzinska (2014) showed that the application of chitosan significantly increases the plant height of the freesia plant by about 10 cm compared to control, accompanied by an increase in chlorophyll level. Furthermore, according to Dzung et al. (2011), chitosan could improve the function of the nitrogen source in forming leaf organs and the nitrogen can contribute to plant growth, such as forming new cells and cell elongation. In addition, applying chitosan can accelerate the flowering period and increase the number of flowers. Dzung et al. (2011) also stated that chitosan application could reduce the abscission rate of coffee up to 15.25% compared to control. This was by an increase in chlorophyll level, mineral accumulation, and plant growth. In addition, Malerba and Cerena (2016) showed that the application of chitosan could increase the productivity of various plants such as tomatoes, grapes, strawberries, and coffee.

A 50% drought stress could decrease plant growth and productivity. Him and Radhauane (2015) observed that a 50% drought caused a decrease in plant height up to 13-15%. Dorji et al. (2005) and Sung et al. (2005) stated that a 50% drought caused a reduction in the chili pepper fresh weight up to 34.7%, the number of fruits by 20%, and the fresh weight of fruit up to 22% compared to control. Zamljen et al. (2020) stated that 50% drought treatment on two different chilies species significantly decreases yield quantity caused by the abscised flower. Mahmood et al. (2021) added that drought treatment subjected to hot and sweet pepper at flowering and pod formation stages significantly decreases the flower survival percentage, the number of fruits per plant, and the fruit weight. Drought can stimulate the synthesis of jasmonic acid (JA) and suppress the action of gibberellic acid (GA), thus affecting impeded plant growth. In addition, plants respond to drought by activating abscisic acid (ABA), which can induce stomatal closure and reduce transpiration (Iriti et al. 2009). However, this also impacts CO_2 absorption, so the photosynthetic activity decreases, resulting in a degradation of plant growth and survival (Anjum et al. 2011).

The combination treatment of 1 mg mL^{-1} chitosan application and a 50% drought (Chi-D) caused a decrease in plant physiological characteristics followed by the yield compared to control. This is supported by the microclimate data indicating the lower humidity recorded at 46.56 and 43.44% for D and Chi-D, respectively. It was in line with the lack of water capacity due to the treatment. In addition, it was presumed that the chitosan-drought treatment caused plants to experience excessive stress, which can trigger the more closed stomata induced by reactive oxygen species (ROS) production (Pichyangkura and Chadchawan 2015). This assumption is in line with the investigation of Aziz et al. (2020) that the double treatment of chitosan and drought caused the highest stomatal closure compared to the drought and chitosan treatments separately as well as control. This phenomenon caused a decrease in photosynthetic efficiency due to the limited absorption of CO_2 and induced the abscission of main plant organs. Maser et al. (2018) explained that plants induced by either chitosan application or drought could promote higher Ca_{2+} accumulation in guard cells and the continuous release of K^+. This causes a decrease in the turgor pressure of the guard cells so that the stomata are closed.

Chitosan application during drought conditions increased PAL gene expression

To quantify the relative gene expression, the validation in every single step needs to be performed. According to the total RNA quality and quantity assay, there are 2 bands observed under UV light visualization with the sizes of 28s and 18s (Figure 2), by means that the total RNA was successfully extracted. The quantitative assay using a nanodrop spectrophotometer showed that the purity of RNA (ratios λ260 and λ280) ranged from 2.14 to 2.15, while the quantity ranged from 327.7 to 664.4 µg mL^{-1}. Based on the sequencing result for ± 260 bp of the PCR product, after being analyzed using the BLAST technique, a 97% identity value was confirmed with the CaPAL gene (accession number: NM_001324603) (Table 3). PAL gene was selected to analyze the influence of chitosan on plant defense response under drought stress. The gene was selected from the previous study that Phenylalanine ammonia-lyase (PAL) is considered one of the key enzymes on capsaicin biosynthesis coded by the PAL gene (Sung et al. 2005; Khan et al. 2014).

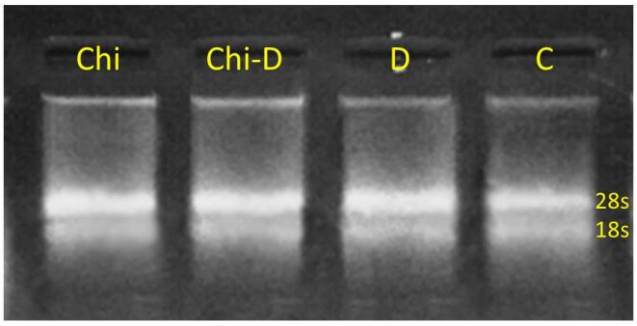

Figure 2. The total RNA quality and quantity assay

Table 3. BLAST result of PAL & CaUbi3 genes

PAL gene

Capsicum annuum phenylalanine ammonia-lyase-like (LOC107843092), mRNA
Sequence ID: **NM_001324603.1** Length: 2318 Number of Matches: 1
Range 1: 1564 to 1822

Score	Expect	Identities	Gaps	Strand	Frame
444 bits(240)	8e-121()	257/264(97%)	5/264(1%)	Plus/Minus	

Features:

```
Query  1     GCAGGTATCATCAGTCATAGGTCAAACAAGTATTCCCTGATCCACGACTCGAAGCAACTC  60
             ||||||||||||||| |||||| ||||||||||||||||||| ||||||||||||||||||||
Sbjct  1822  GCAGGTATCATCAG-CATAGG-CAAACAAGTATTCCCTG-TCCACGACTCGAAGCAACTC  1766

Query  61    CTTTTCCGCAGAATCCTTGCTGGATGAAGTTCACCGTTAGCGCCCATTGTCAAAGTTCTC  120
             |||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1765  CTTTTC-GCAGAAT-CTTGCTGGATGAAGTTCACCGTTAGCGCCCATTGTCAAAGTTCTC  1708

Query  121   TTAGCTACTTGGCTGACTGTGTTCTTGACTGCATTCTTCAAGTTTTCTTCCAAGTGCCTC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1707  TTAGCTACTTGGCTGACTGTGTTCTTGACTGCATTCTTCAAGTTTTCTTCCAAGTGCCTC  1648

Query  181   AAGTCTATAGCTTGGCAAAGAGCCACGAGATAGGTTGATGACATCAGCTTCAAGATGTCA  240
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  1647  AAGTCTATAGCTTGGCAAAGCGCCACGAGATAGGTTGATGACATCAGCTTCAAGATGTCA  1588

Query  241   ACCGCCTCGGCTGTTTTCCTTGAT  264
             || ||||||||||||||||||||||
Sbjct  1587  ACTGCCTCGGCTGTTTTCCTTGAT  1564
```

CaUbi3 reference gene

Capsicum annuum ubiquitin-conjugating protein mRNA, complete cds
Sequence ID: **AY486137.1** Length: 578 Number of Matches: 1
Range 1: 276 to 444

Score	Expect	Identities	Gaps	Strand	Frame
287 bits(155)	8e-74()	165/169(98%)	3/169(1%)	Plus/Plus	

Features:

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Query  1     CCAATGAT-CGCTCGTA-CAG-AATTGCTCACATGTACAAGACTGACAGGGCCAAATACG  57
             || |||| |||||||| ||| ||||||||||||||||||||||||||||||||||||||
Sbjct  276   CCGATGATCCGCTCGTACCAGAAATTGCTCACATGTACAAGACTGACAGGGCCAAATACG  335

Query  58    AGACCACTGCTCGTAGCTGGACTCAGAAATATGCTATGGGATAATGGACAAAATCGTCTC  117
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336   AGACCACTGCTCGTAGCTGGACTCAGAAATATGCTATGGGATAATGGACAAAATCGTCTC  395

Query  118   CAGGCATGTCTGGGACTTTTGCAACAACAATGTCTTATTGTGCTTGGGG  166
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  396   CAGGCATGTCTGGGACTTTTGCAACAACAATGTCTTATTGTGCTTGGGG  444
```

The target gene, PAL, was amplified using the specific primer (Table 1). As shown in Figure 3A, the particular bands of PAL and the housekeeping CaUBi3 gene described that both genes were successfully amplified. Since the specificity of primers with the size ± 300 bp for PAL and ± 200 bp for CaUbi3 was confirmed, the quantitative PCR was then performed. According to the investigation, we found that the expression level of the PAL gene was up-regulated in all treatments (Figure 3B). The relative expression level of the double treatment of chitosan and drought (Chi-D) was recorded as the highest at 129-fold higher than control, followed by chitosan (Chi) and drought (D) treatment at 73 and 4-fold higher than control, respectively. Based on the statistical analysis, the PAL expression level in each treatment was significantly different. Thus, we suggested that chitosan application (Chi) might increase the expression level of the PAL gene, and it was more pronounced when applied under drought conditions.

Besides being involved in plant growth, PAL is the important gene in synthesizing secondary metabolites, capsaicin, which acts as a plant defense system (Gonzalez-Zamora et al. 2013; Kim and Hwang 2014). The previous studies supported the up-regulation of PAL gene in all treatment groups and demonstrated the increment of PAL gene expression level 12-fold higher than control after 1 mg mL^{-1} chitosan application (Mejia-Teniente et al. 2013) and 3.61-fold higher after drought stress treatment (Khan et al. 2014). Drought could induce ABA and JA synthesis (Golldack et al. 2014). Meanwhile, the application of chitosan also induced similar signaling pathways involving H_2O_2, ABA, and JA in activating defense responses such as stomatal closure and several genes expression related to abiotic stress (Pichayangkura and Chadchawan 2015). Interestingly, both chitosan and drought activate a similar signaling pathway, so that chitosan and drought applied together could modulate the excessive defense responses, including the expression of resistance genes such as PAL.

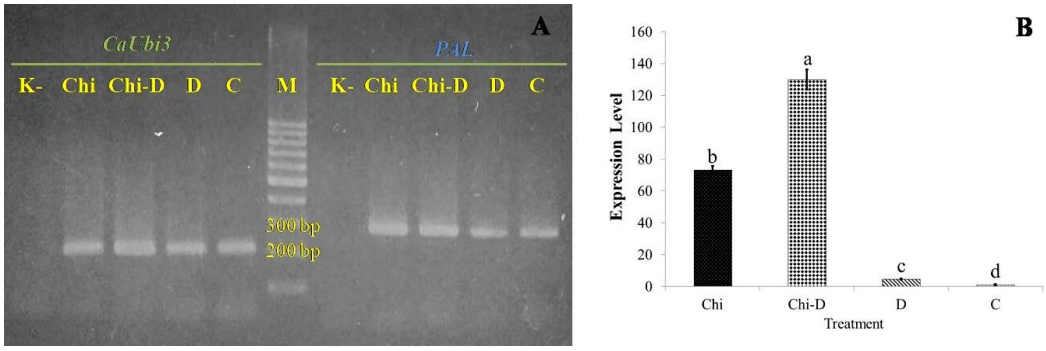

Figure 3. The visualization of PAL gene amplification coupled with CaUbi3 housekeeping gene (A) and the PAL gene expression level of red chili plant treated by chitosan application under drought stress condition (B). The bars represent the mean of PAL gene expression with the corresponding standard error (n = 3). A significant value is indicated by the letter on top of each bar, p <0.05. 35 PCR cycles. Chi: 1 mg mL^{-1} chitosan; Chi-D: 1 mg mL^{-1} chitosan and 50% drought; D: 50% drought; C: control; K-: negative control; M: marker

Therefore, it is known that both drought and chitosan could enhance the expression level of the PAL gene, and the combination was suggested to increase the higher gene expression level than those treatments separately. This is in line with the finding of Ali et al. (2021) that demonstrated the up-regulation of strictosidine synthase (STR), deacetylvindoline-4-O-acetyltransferase (DAT), peroxidase 1 (PRX1) and geissoschizine synthase (GS) genes up to 5.6 folds on *Catharanthus roseus* (L.) G.Don under drought stress. However, the addition of 1% of chitosan nanoparticle found a higher expression level.

Chitosan application under drought conditions inhibited PAL enzyme activity

After being expressed, the PAL gene will be translated into the PAL enzyme (Sung et al. 2005). According to Figure 4, it was indicated that either chitosan application (Chi) or drought (D) treatment is suggested to increase PAL activity. Interestingly, although not significantly different, the double treatment of chitosan and drought (Chi-D) showed lower PAL activity than the Chi group. Chitosan and chitosan-drought treatment significantly increased the PAL activity at 1.35 and 1.22-fold higher than control, respectively, followed by the drought treatment group recorded at 1.03-fold higher than control. Thus, we suggested that chitosan application (Chi) could promote PAL activity, but the activity slightly decreased when applied under drought conditions (Chi-D).

Drought stress conditions could affect the activity of PAL as the first enzyme activated in the synthesis of capsaicin through the phenylpropanoid pathway (Sung et al. 2005). In Figure 4, it is indicated that 1 mg mL^{-1} chitosan (Chi) application could significantly increase PAL activity compared to the control. This is supported by Khan et al. (2003) that applying a chitosan oligomer could increase the activity of PAL in soybean leaves. It was suggested as an initial response to stress, either due to elicitation or pathogen infection. According to the observation, both Chi-D and D treatments could potentially enhance PAL activity compared to control. However, although experiencing the highest PAL expression level, PAL activity in the Chi-D treatment group tends to be lower than in the Chi treatment group. This is suggested as the consequence of the dramatic increase in ROS levels caused by the excessive stress experienced by the plant (Pichyangkura and Chadchawan 2015). This would lead to the oxidative damage of proteins, DNA, and lipids (Anjum et al. 2011). However, Ali et al. (2021) demonstrated that the application of 1% of chitosan nanoparticles under drought conditions could mitigate the reduced growth performance accompanied by the increment of proline accumulation and antioxidant activity such as catalase (CAT) and ascorbate peroxidase (APX) so that it reduced H2O2 and malondialdehyde (MDA) accumulation. In addition, PAL activity might fluctuate depending on the level of stress experienced by the plants. Khan et al. (2003) stated that PAL activity increased from the 24th to 36th hour after the chitosan treatment, then decreased when entering the 48th hour. Sung et al. (2005) added that a 50% drought caused PAL activity in the pericarp to increase at 1.18-fold higher than control at 40 days after flowering (DAF). Still, they decreased 0.9-fold lower at 50 DAF than the control, while the opposite response occurs in the placenta.

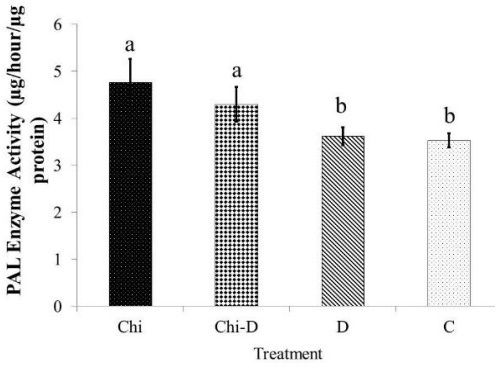

Figure 4. The PAL activity of red chili plant treated by chitosan application under drought stress condition. The bars represent the mean of PAL activity with the corresponding standard error (n = 3). A significant value is indicated by the letter on top of each bar, p < 0.05. Chi: 1 mg mL^{-1} chitosan; Chi-D: 1 mg mL^{-1} chitosan and 50% drought; D: 50% drought; C: control

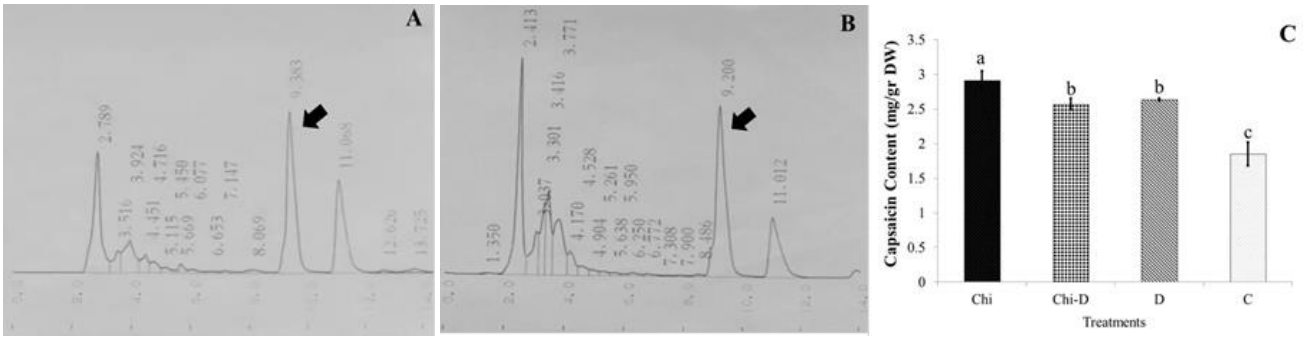

Figure 5. The chromatograms of capsaicin measurement using HPLC instrument in the 500 ppm capsaicin standard (A) and sample (B). The capsaicin content (C) of red chili plant treated by chitosan application under drought stress conditions. The bars represent the mean of capsaicin content with the corresponding standard error (n = 3). A significant value is indicated by the letter on top of each bar, $p < 0.05$. Chi: 1 mg mL^{-1} chitosan; Chi-D: 1 mg mL^{-1} chitosan and 50% drought; D: 50% drought; C: control

Chitosan application under drought conditions decreases capsaicin accumulation

Capsaicin biosynthesis of chili pepper plants was the critical plant defense mechanism against pathogenic attack, so the regulation after chitosan application was essential to evaluate either under normal or drought conditions. Based on the chromatograms of capsaicin standard (Figure 5A) and sample (Figure 5B) obtained from the HPLC instrument, it was shown that the capsaicin compound peak was detected at the 9th minute. Regarding this study (Figure 5C), we suggested that either chitosan application (Chi) or drought treatment (D) could enhance capsaicin content significantly. Chitosan application showed significant capsaicin content enhancement up to 1.57-fold higher than control, followed by the drought treatment at 1.42-fold higher than control. Surprisingly, the capsaicin content at chitosan-drought (Chi-D) treatment increased only 1.39-fold higher than control, although not significant compared to D treatment. In addition, we suggested that chitosan application under drought conditions slightly decreases the capsaicin level compared to individual chitosan or drought treatment.

The level of PAL expression and PAL activity plays an important role in the biosynthesis regulation of secondary phenolic metabolites, capsaicin (Gonzalez-Zamora et al. 2013). Based on Figure 5, it is shown that capsaicin levels in Chi, Chi-D, and D treatment groups are significantly higher than control. In line with PAL activity, the individual chitosan application (Chi) was recorded to increase capsaicin levels compared to other treatment groups significantly. Khan et al. (2003) stated that the application of chitosan oligomer could increase soybean's total phenol content, which is estimated to be in accordance with the increase in PAL activity. Meanwhile, Sung et al. (2005) stated that a 50% drought could increase the level of capsaicin in the placenta up to 2.56-fold higher than control. Zamljen et al. (2020) stated that 50% drought treatment on *C. annum* caused higher capsaicin and dihydrocapsaicin content, while the higher concentration of ascorbic acid and total phenolic content were recorded at optimal irrigation. Ali et al. (2021) added that drought stress promotes the higher accumulation of alkaloid content, and a further increase was observed with the addition of 1% chitosan nanoparticle treatment.

However, the highest PAL expression in the Chi-D treatment group was not in line with the slight decrease in PAL activity, and the capsaicin level recorded was not significantly different from the D treatment group. This was suggested to support our previous assumption that plants experienced the excessive stress caused by the double treatments, which triggers the higher ROS level (Pichyangkura and Chadchawan 2015), as stated by Anjum et al. (2011) that a higher level of ROS might cause damage to plant cells through increased lipid peroxidation, protein degradation, and DNA degradation. It was suggested to contribute to the capsaicin degradation in the Chi-D group resulted in a lower amount of capsaicin than Chi and D groups. Jeeatid et al. (2017) stated that the appropriate water stress could significantly increase capsaicinoid content in some hot pepper species depending on each genetic variability. It was supported by Mahmood et al. (2021) that drought stress decreased reproductive growth parameters and pungency of pepper fruit as most of the plant energy was consumed in defense antioxidant molecules. However, several enzymes related to capsaicin biosynthesis, either from phenylpropanoid or fatty acid pathways, are also suggested to affect capsaicin accumulation. Therefore, this study needs to be continued to gain a more profound understanding of the responses of those enzymes after Chi-D treatment.

In conclusion, double treatment of chitosan-drought red chili plants significantly decreased the growth performance recorded in plant height increment, followed by the small number of leaves, flowers, and fruit. Therefore, chitosan application was suggested that could not alleviate plant growth under drought stress. The PAL expression level after chitosan-drought treatment was recorded up-regulated 129-fold higher than control. However, PAL activity and capsaicin content were decreased. In addition, the individual chitosan application significantly increases the PAL expression level, PAL activity, and capsaicin level compared to the control. Thus, we suggested that chitosan application might play a role in the defense system of red

chili pepper plants involving capsaicin biosynthesis through the phenylpropanoid pathway.

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Phytochemical composition of *Dichrocephala integrifolia* crude extracts, antiviral activity and toxicity

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Abstract. *Hadun AH, Mbaria JM, Aboge GO. 2022. Phytochemical composition of* Dichrocephala integrifolia *crude extracts, antiviral activity and toxicity. Asian J Trop Biotechnol 19: 72-81.* Human herpes simplex viruses are among the world's most ubiquitous human infections. Generally, there are two types of Human Herpex simplex viruses (HSV), HSV-1 and HSV-2, infecting over 90% of people with either one or both. HSV-1 is a viral disease to cause genital and oral lesions. As a major antiviral drug to treat HSV infections, Acyclovir has proven unsatisfactory as resistance, and severe side effects in pregnant mothers and infants have been frequently reported. The high prevalence of HSV, lack of vaccines, and limited treatment options warrant an urgent need for more effective anti-HSV agents. The aim of this study was to investigate phytochemical composition, in vitro anti-HSV, and in vivo and in vitro toxicity of *Dichrocephala integrifolia* (L.fil.) Kuntze crude extracts. Leaves, roots, flowers, and stems of *D. integrifolia* were collected from Mabariri Village in Nyamira County, Kenya. The identification by a botanist in the school of biological sciences, University of Nairobi, Kenya. The materials were extracted with methanol and water, and Qualitative tests were conducted to determine alkaloids, flavonoids, saponins, tannins, glycosides, and terpenoids presences. The MTT assay investigated in vitro cytotoxic activity using Vero cell lines from the center for viral research Kenya Medical Research Institute (KEMRI). Antiviral activity has assessed the ability to protect normal cells (Vero cell lines) from HSV attack. In vivo toxic effects in female Swiss albino mice using oral acute toxicity protocols by OECD. The qualitative phytochemical showed the extracts contained tannins, flavonoids, alkaloids, terpenoids, phenols, glycosides, and saponins. It was not cytotoxic to Vero cells except the flower's methanolic extract, which had a CC_{50} value of 71.31± 2.65 µg/mL. The extracts interference adsorption step of HSV-1 blocks the virus's epitopes on the cell's membrane. Methanolic extracts of the stem, leaves and aqueous extracts of leaves inhibited HSV-1 virus from causing a cytopathic effect, with IC_{50} values of 63.95±5.36 µg/mL, 54.45±3.45 µg/mL, 86.20±7.56 µg/mL. Methanolic flower, aqueous root, and methanolic leaves extract show virucidal with IC_{50} values of 45.27±2.41 µg/mL, 0.333±1.23 µg/mL, and 30.53±4.51 µg/mL. Oral administration to mice at 300 mg/kg and 2,000 mg/kg did not result in any toxic effects or mortality. In all the groups, no major behavioral or appearance changes were observed. Pharmacologically phytochemicals such as flavones, phenols, terpenoids, and tannins in antimicrobial action support this plant's pathologies management. The cytotoxicity, efficacy, and acute oral show no major toxicity; preparing antiviral herbal remedies may be safe for patients. Further research into plant mechanisms and isolating the bioactive agents are needed; studying toxic effects is also recommended to formulate pharmacological products.

Keywords: Antiviral, cytotoxicity, *Dichrocephala integrifolia*, Herpes Virus Simplex, in vivo safety, phytochemical

INTRODUCTION

Traditional medicine has been practiced since immemorial times (Dery et al. 1999). It is still a vital component in health care systems, especially in low-income communities in developing nations. It is used against diseases like Acquired Immune Deficiency Syndrome (AIDS), Herpes Simplex Viruses (HSV), Ebola, influenza, malaria, cancer, diabetes, tuberculosis (TB), and Human Immunodeficiency Virus (HIV), among others (Balick and Cox 1995a). Many people, especially those in developing countries, mostly rely on drugs of natural origin for their healthcare requirements (Cunningham 1993; Balick and Cox 1995b; Mworia 2000). The World Health Organization (WHO) approximates that nearly 6 billion people use plant-derived products for their primary healthcare needs (Choudhry et al. 2004). For example, approximately 84% of Peru's population prefers traditional medicine to conventional drugs for their primary healthcare. They believe that herbal products are less toxic, easily accessible, and well-tolerated; thus, traditional medicines are considered safe and combine conventional drugs (Bussmann et al. 2007). In Africa, many communities use medicinal plants to treat many human diseases, mainly due to unstable economies (Fakung et al. 2011). In Ethiopia, up 80% of the people relies on natural products for prophylaxis and treatments of different human ailments. In Kenya, almost 90% of the population uses complementary and alternative medicine from natural sources at least once for various health conditions (Chirchir et al. 2006), and various plants or parts harbor active principles responsible for their medicinal properties.

Despite the wide scope of clinically active synthetic and semi-synthetic antibiotics, the search for new efficacious anti-infective drugs remains paramount due to resistance and the emergence of new parasite strains or new therapeutic targets (Fair and Tor 2014). Currently, the need for substances with antiviral activity is high since the drugs used to treat viral infections are not readily available. A

major problem has been the emergence of mutant viral strains not responding to the available antiviral drugs. The greatest drawback in the fight against human simplex virus infections is the rapidly evolving drug resistance, increasing the cost of treatment. Virus resistance to Acyclovir which has been allied to mutations at the TK gene has been documented (Morfin and Thouvenot 2003). Herpes simplex virus strains resistant to Acyclovir have been isolated from normal hosts and often in patients with recurrent infections. Immune-compromised people, such as HIV-AIDS patients, are at great risk of attack by these strains (Morfin and Thouvenot 2003). These drawbacks call for a multifaceted approach to curb the disease.

Plants have been used since ancient times to fight against various diseases, including viral ones (Kinghorn et al. 2011). A natural product-based approach could help discover safe new leads with diverse targets to the virus, reducing chances of resistance development. The ethnomedical studies and bioprospecting of medicinal plants may therefore facilitate the extraction of vital compounds that could be potent and cost-effective with fewer side effects. Diseases of viral origin have been treated with natural products from plants for decades (Newman and Cragg 2007; Kinghorn et al. 2011). However, few studies have been conducted to evaluate the plants with antiviral activities, and the number of active compounds has been isolated from higher plants (Farnsworth and Kaas 1981; Kingstone 2011; David et al. 2015). These studies suggested that selecting plant materials based on ethnomedical use gives a higher lead generation than screening programs for search from general synthetic products (Kingstone 2011). However, most of the plants used for medicinal purposes by different communities have not been investigated thoroughly using scientific techniques. In this study, *Dichrocephala integrifolia* (L.fil.) Kuntze was selected for scientific validation based on its ethnomedical uses.

Furthermore, a qualitative screening of the phytochemicals in plant crude extracts was done using standard procedures (Evans 2009). In vitro antiviral (against HSV) properties and cytotoxicity of the extracts were performed to ascertain their potency and safety in managing HSV. The study aimed to screen for the phytochemical compounds present in the crude extracts of *D. integrifolia*; to determine the in vitro antiviral activity of extracts of *D. integrifolia* against HSV; to determine the in vitro cytotoxic effects of *D. integrifolia* extract against Vero cell lines, and to determine the in vivo mammalian toxicity of the methanolic and aqueous extracts of *D. integrifolia* using Swiss albino mice.

MATERIALS AND METHODS

Study design

The study was carried out using laboratory based *in vitro* and in vivo models to ascertain the antiviral potential and safety of the plant extracts. Vero cells were used as the in vitro cell model, while Swiss albino mice were used as the in vivo animal model. In addition, qualitative phytochemical analysis was carried out using standard chemical tests to determine the presence or absence of different active ingredients such as alkaloids, saponin, tannins, flavonoids, glycosides, and terpenoids.

Study site

The study activity was done at the University of Nairobi Campus of Agriculture and Veterinary Science in Nairobi, Kenya. Plant materials were collected from Mabariri (S 00^0 31. 367', E 034^0 56. 426', Nyamira County, Kenya). Laboratories were well equipped with good facilities and a clean bench. In vivo work was done in the Animal House Laboratory.

Medicinal plant collection

The parts of plant *D. integrifolia*, namely the leaves, roots, flowers, and stems, were collected from its natural habitation in Mabariri S 00^0 31. 367', E 034^0 56. 426', Nyamira County, Kenya (Figure 1). The plants were collected from a single location due to their availability. The whole plant was uprooted, weighing approximately 4,000 g of wet matter. They were placed in a khaki envelope and immediately transferred to the University of Nairobi laboratories, where they were stored under shade in the room (25°C). Mr. Antony Mutiso, a botanist at the school of biological sciences at the University of Nairobi, did taxonomic identification and authentication. The Voucher specimen number AHH2015/01 was then deposited for future reference at the University of Nairobi herbarium on the Chiromo campus.

Preparation of plant parts

The plant materials were immediately washed separately and allowed to dry at room temperature in a clean, well-ventilated room at the departments of public health pharmacology and toxicology. The dried parts were then ground to a fine powder using Gibbons electric grinding machine (Wood-Rolfe Road Tollesbury, Essex, UK). Finally, the samples were packed in translucent paper bags and stored at room temperature until use.

Reagents, chemicals, and assay kits

Dimethyl sulfoxide (DMSO), ethanol, fetal bovine serum/ heat-inactivated newborn calf serum, Acyclovir, Eagle minimum essential medium (EMEM), trypsin, penicillin, streptomycin, trypan blue and 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyl tetrazolium bromide (MTT) were procured from Sigma (USA) according to the Nairobi University procurement policies and stored in standard condition as stated on their labels during the study period. Briefly, DMSO was stored in a well-ventilated and cool place (25°C) (Ashe 2016), MTT at 4°C in the dark (American Type Culture Collection, 2011), and EMEM at 4°C in the dark when not in use (ATCC 2016).

Figure 1. Map of Nyamira County, Kenya. Source: Kenya Mpya 2012 (http://www.kenyampya.com/index.php?county=Nyamira)

Extraction of plant materials

Aqueous extraction

Leaves, flowers, stems, and roots prepared crude extracts. Extraction was performed based on a modification to the method previously described by Awoyinka et al. 2007. The ground plant materials were weighed on an analytical balance (Mettler PM 4600) (200 g) and extractedby maceration using 1,000mL water. The samples were submerged in the water, and extraction was allowed to proceed for 48 hours with manual shaking. The samples were then filtered using the filter on a funnel. Finally, the water extracts were freeze-dried using Edwards freeze dryer, Modulyo, to obtain a dry powder that was transferred to clean sample bottles, weighed, andstored in a freezer at -20 °C until use.

Methanol extraction

Three hundred and fifty grams (350 g) of the grounded roots, stem, leaves, and flowers were extracted by maceration using methanol as solvent (Parekh et al. 2005). First, the samples were submerged into 800 mL of methanol in a flat-bottom flask plugged with cotton gauze, and extraction was allowed for 48 hours with frequent shaking. The samples were then filtered using filter paper on a funnel. Finally, the extracts were concentrated under a vacuum using a rotary evaporator B-480 (Búchi-technik IK AG, Switzerland) at 40°C concentrate samples. The extracts were then transferred to clean sample bottles, weighed, labeled, and stored in a freezer at -20°C until use.

Evaluation of phytochemical constituents of *Dichrocephala integrifolia*

Different qualitative chemical tests were conducted to determine the presence or absence of different phytochemicals, including flavonoids, tannins, saponins, alkaloids, glycosides, phenols, and terpenoids in crude extracts of *D. integrifolia.* The results were evaluated by visual inspection as a change in color or precipitation. Qualitative chemical tests for the detection of bioactive compounds proceeded as follows:

Test for tannins

Approximately 0.8g of the dried methanolic and aqueous extracts were dissolved in 15 mL of distilled water and boiled, then later filtered. Next, a few drops of ferric chloride were added to the resultant filtrate. A bluish-Green precipitate indicates the presence of tannins (Evans 2009; Segelman et al. 1969).

Test for saponins

The presence of saponins was determined by dissolving approximately one gram (1 g) of the plant extracts in boiling water for 5 minutes and allowed to stand for 15 minutes. The formation of a stable froth of more than 2 cm and persisting for at least 40 minutes indicated saponins (Kapoor et al. 1969; Evans 2009).

Test for alkaloids

The presence of alkaloid was confirmed by dissolving approximately 0.5 g of the extract(s) with about 10 mL of 1% hydrochloric acid. The mixture was boiled for 5 minutes then, followed by filtering. The filtrate was put in two test tubes of 2 mL each. Mayer's reagent was added to the first test tube with 2 mL of the filtrate, and the appearance of a cream-colored precipitate was a positive confirmation of the alkaloids' presence. Several drops of Dragendorff's reagent were added to the second test tube with 2 mL of the filtrate, and the Reddish-brown precipitate confirmed the presence of alkaloids (Salehi-Surmaghi et al. 1992; Evans 2009).

Test for glycosides

Cardiac glycosides-Keller-killiani test. Keller-killiani test was used to confirm the presence of cardiac glycosides in the extract. First, one hundred and fifty milligrams (150 mg) of each extract was mixed with 1.5 mL of glacial acetic acid containing some element of ferric chloride ($FeCl_3$) solution. Next, this solution added 0.5 mL of concentrated sulphuric acid to the side of the test tube. As a result, the appearance of a brown ring at the interface of the two layers, with the lower acidic layer turning blue-green, is a positive presence of cardiac glycosides (Ajaiyeobu 2002).

Modified Borntrager's test. One gram (1 g) of crude plant extract was boiled in 3 mL of 10% hydrochloric acid in a test tube for 4 minutes. Next, it was filtered while still

hot, cooled, and shaken with 3 mL of chloroform. Then the upper layer of chloroform was removed and shaken with half of its volume with dilute ammonia. A rose pink to red color produced in the ammonia layer indicates the presence of glycosides (Evans 2009).

Keddie test. One gram (1 g) of the crude extract was dissolved in chloroform and evaporated to dryness, then 2 drops of concentrated alcohol and 3 drops of benzoic acid. The purple color indicates the presence of glycosides whose aglycone moiety has an unsaturated lactone ring (Evans 2009).

Tests for flavonoids

One gram of the crude plant extracts was dissolved in 10 mL of distilled water and then filtered using a Whatman filter. Then, 0.5 mL of the filtrate was mixed with 6mg of magnesium turnings, followed by adding 0.05 mL of concentrated sulphuric acid. The presence of magenta red observed after five minutes confirmed the presence of flavonoids (Brain and Turner 1995).

Test of phenols

Approximately one gram (1 g) of grounded crude extracts was dissolved in two milliliters of 2% iron (111) chloride, and the appearance blue-green precipitate indicates the presence of phenols (Evans 2009).

Test for terpenoids

Four milliliters of the crude extracts were mixed with 2 mL of chloroform solution and then evaporated to dryness in a water bath. Then, a few drops of concentrated H_2SO_4 were added slowly to the test tube wall. The formation of reddish brown coloration and green color in the test tube's upper layer indicates the presence of terpenoids (Evans 2009).

In vitro assay

Cell line and culture media

Vero cell E6 obtained from CTMDR/KEMRI was used in this study. The cells were cultured and maintained using Minimum Essential Medium (MEM) with 2% of fetal bovine serum, two antibiotics (streptomycin100 µg/mL and penicillin 100 units/mL), and retained in 5% CO_2 incubator (Thermo Fisher Scientific, Toll-Free, USA) at 37°C. The media was removed after 24hrs, the cells were washed with phosphate buffer saline (PBS), and a new medium was added. The cells were then incubated to attain confluence, and upon formation of 100% confluence, the supernatant was harvested and stored at -85°C (Lamorde et al. 2010).

Cytotoxicity determination on Vero cell lines

Vero cell E6 was seeded at a concentration of 50,000 cells/well (in 100 µL of maintenance media) into a flat bottom microtiter cell culture enabled 96-well plates (Sigma, USA) and incubated in a 5% CO_2 incubator at 37°C. Crude methanol and aqueous extracts of *D. Integrifolia* in the concentration range of 0.45-100 µg/mL were exposed to the Vero cell lines and incubated in a 5% CO_2 incubator of 37°C in humidified air for 48 hrs. The cell was then washed with phosphate buffer saline (PBS), and ten micrograms (10 µL) of tetrazolium dye (5 mg/mL) was then added and incubated in 5% CO_2 incubator of 37 °C in humidified air for 2 hrs. Mitochondrial dehydrogenase, a biomarker of a living cell, interacts with MTT dye, reducing it to insoluble formazan. The formazan formed corresponds to the number of live cells. According to Tolo et al. (2007), the trypan blue exclusion method was used for cell viability. Formazan formation was confirmed using an inverted light microscope and then solubilized with 50 µL of 100% DMSO, and optical density (OD) was read at 562 nm in a 96-well microtiter plate multiplex reader.

HSV-1 isolate culture

A clinical isolate of HSV 1 virus was obtained from the center for viral research (KEMRI) was propagated in Vero cells in a T75 flask and allowed to grow in a 5% CO_2 incubator at 37°C until the complete cytopathic effect was seen. Then, the virus was harvested through the freeze-thaw technique, suspended in phosphate buffer saline (PBS), and centrifugation was done at 3,000 rpm for 15 minutes. Finally, the virus stock (supernatant) was stored at 35% sorbitol at -80°C until use (Lamorde et al. 2010).

Determination of the antiherpetic activity of Dichrocephala integrifolia *in Vero cells*

The study was carried out using the method previously described by Alem et al. (2016). Different experimental approaches were employed to characterize the effect of the extracts on various stages of virus replication and probable mechanisms of action. The targeted replication cycles important to virus growth were: attachment, a fusion of virus envelope to the plasma membrane of cells, and replication of viral proteins (Alem et al. 2016). In addition, the following procedures were followed: post-treatment studies, pre-treatment studies, and virucidal effects.

Treatment after virus infection (Post-infection treatment)

Virus suspension in serum-free media at 10^6 $TCID_{50}$ was incubated with cells in a 5% CO_2 incubator at a temperature of 37°C for I hour. Then the cells were washed with PBS, incubated with the serially diluted extracts, and grown in a 5% CO_2 incubator at 37°C for 24 hours. The extract was removed, and then all cells were washed with PBS. Fresh media was then added. Cytopathic effect was observed daily. After 48 h, the cells' ability to reduce MTT dye to formazan, as earlier described, was determined. The percentage protection of the extract to the cells was calculated as [(A-B)/(C-B) x 100], where A, B, and C (A- untreated cells, B- blank, C- treated cells) indicate the optical densities (OD) measured in a spectrophotometer at 562 nm with a reference filter of 690 nm of the tested extract with virus-infected cells, virus and cell controls. The 50% half maximum inhibitory concentration (IC_{50}) is the extract concentration that protects 50% of treated infected cells to compare with cell control using regression analysis. The extract's therapeutic index (TI) for the antiviral activity was determined by calculating the ratio CC_{50} divided by IC_{50} (Alem et al. 2016). The same procedure was done for Acyclovir (positive control).

Treatment before virus infection (Pre-infection treatment)

Fifty (50) µL of the serially diluted extract was incubated in a 5% CO_2 incubator with Vero cells for 24 hours. After washing with PBS, the cells were incubated with 50 µL of 10^6 $TCID_{50}$ virus suspensions in serum-free MEM for 1 h, washed with phosphate buffer saline (PBS), and grown with fresh media. Cytopathic effect was observed, and the same protocol for cell viability followed as mentioned in the post-treatment experiment. The same procedure was done for Acyclovir (positive control).

Investigation of virucidal activities

Different non-toxic concentrations of crude extract were tested for antiviral properties by virucidal assay. Fifty µL of 10^6 $TCID_{50}$ of HSV-1 virus suspensions were incubated with various concentrations of the crude extracts at 37°C in a 5% CO_2 incubator for 1 hour (crude extracts + virus suspension). Solvents (used to dissolve crude extracts) and virus suspension were kept blank, and cells alone as the control. After 1 h, 100 µL of each mixture (crude extracts + virus suspension) were added to monolayer cultures grown in 96 well plates and incubated for 48 hrs. The cytopathic effect was observed under a light microscope. The effect of the extracts of the cells was evaluated using the MTT assay method. The cell protection/inhibition percentage was calculated using the MTT assay formula. The same procedure was done for Acyclovir (positive control) (Figure 2).

In-vivo assay

Handling of the animals during experimentation and personal protective equipment

The female Swiss mice were handled per guidelines and protocols established for Laboratory Animals by the organization for economic development and co-operations (OECD/OCD 2001). The study was carried out at Pharmacology and Toxicology Laboratories on the agriculture and veterinary sciences campus, University of Nairobi. The experimental laboratories were suitable for animal biosafety level two (ABSL/BSL) with a strict aseptic technique using NaOCL at 10% and alcohol at 70%. The mice were restrained using universal mice restrainer for ease of identification, weighing, and drug administration. The laboratory was well-ventilated to avoid breathing dust, fumes, gas, mist, vapors, and spray. The principal investigator and personal assistant always used latex gloves, lab coats, and face masks. At the same time, anti-tetanus and anti-rabies vaccines were made available in the refrigerator in case of injuries. Animals were disposed of through incineration after anesthesia.

Animal model

Fifty-seven adult female Swiss albino mice aged 8 weeks and weighing 20-25 g were used to investigate the acute toxicity of active crude extract(s). The animals were obtained from the University of Nairobi Kabete animal facility. Ethical approval was obtained from the faculty of veterinary medicine Biosafety, Animal Care and Use Committee (BACUC), University of Nairobi Reference BACUC/J56/74093/2014.

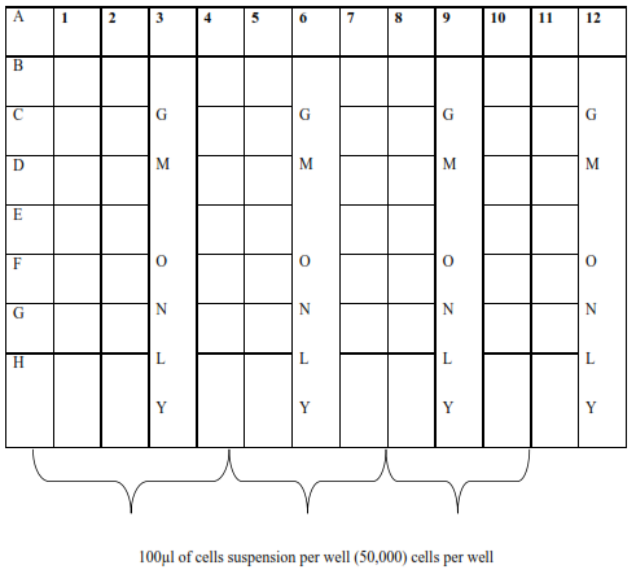

Figure 2. 96 well-plate cell suspension setting

Housing and feeding conditions

The mice were housed in a group of three in polycarbonate cages of 35 cm in length × 25 cm width × 18cm height fitted with wire mesh tops. They were kept in cages for ten days before dosing to allow adjusting to the laboratory conditions. Cages were cleaned once a week, and the bedding husk was replaced two times a week. The Temperature of the study room was maintained at 23 - 27˚C, with a relative humidity of between 50-60%. A cycle of 12-hour light and 12-hour dark was maintained. Standard mice pellet diet (Unga Feeds) was given plenty, and water was also provided with automatic water dispensers ad libitum during acclimatization and the experiment period.

Determination of in-vivo toxicity of the extract of D. integrifolia *using Swiss albino mice*

This experiment was carried out according to OECD/OCDE guideline 423 (2001) with adaptation. After acclimatization, the mice were identified by marking the tail with a permanent marker for easy identification. The identified mice were weighed using a weighing balance (mettle PM 4600), and their weight was recorded and released back to their cages. The extracts were prepared in double distilled water to the desired concentration. Before dosing, animals fasted for four hours and weighed before oral administration of a single dose initiated at 300 mg/kg of the test subsistence was selected. The food was further withheld for another 3 hours after administration of test subsistence. Based on the result, a subsequent higher dose of 2,000 mg/kg body of the test substance was administered to each mouse with an oral gavage needle. Physiological saline was administered to the control group. Individual observations of toxicity wellness parameters such as change of fur, lacrimation, mucous membrane inflammation, excessive salivation, drowsiness, convulsions, tremors, body weight, morbidity, and mortality were recorded. The weight was recorded on days

0, 7, and 14. The data obtained were presented in tables, and the LD_{50} values were determined statistically (OECD /OCDE 2001).

Data analysis

Data obtained from the study was put as a mean ± standard error of the mean (SEM) of the three independent experiments. Data was transferred onto a graph pad prism version 7, and paired t-test was used to compare the change of weights pre and post-treatment. Selectivity indices were determined as the ratio of CC_{50} to IC_{50} (CC_{50}/IC_{50}). A *p-value* less than 0.05 was considered statistically significant. The concentration that inhibited 50 % viability of the cells and cytotoxic concentration 50 were evaluated by linear regression curve. The dosage required to kill 50% of the animals (LD_{50}) was calculated using Acute Oral toxicity guidelines (OECD/OCD 2001). Briefly, the sequential design was used to determine the needed doses concerning the body weight, as shown in Table 1.

Disposal of cells and experimental animals and Ethical considerations

All the used cell lines were disposed of following the protocols that the University of Nairobi Ethical Committees set. Briefly, the liquid waste containing cells was autoclaved and disposed of in the sanitary drain, followed by water. Animal remains shall be tagged with prominent poison tags in outer bags or containers. After confirming the death, the mice carcasses were placed in transparent and sealable polyethylene bags. Finally, they were disposed of by incineration per the University of Nairobi Faculty of Veterinary Sciences disposal protocol (AVMA 2013).

The study was carried out at the University of Nairobi at the department of public health pharmacology and toxicology. Permission to carry out the study and ethical clearance was granted by the biosafety, animal care, and use committee (BACUC) at the University of Nairobi at the Faculty of Veterinary Medicine reference BACUC/J56/74093/2014. The research was conducted according to the University of Nairobi guidelines on laboratory animal use and care.

RESULTS AND DISCUSSION

Phytochemical composition of different extracts of *Dichrocephala integrifolia* plant

The plant was screened for secondary metabolites using standard procedures and contained various pharmacologically important compounds, including phenols, flavonoids, tannins, glycosides, terpenoids, alkaloids, and saponins. Flavonoids were absent in the methanolic root extract, and glycosides were absent in the aqueous extract of flowers' root and methanolic extract. The result is shown in Tables 2 and 3.

***In vitro* cytotoxicity of extract of *Dichrocephala integrifolia* against Vero cell lines**

All extracts were not toxic to Vero cells except the flower's methanolic extract, which showed slight toxicity with a CC_{50} value of 71.31 ± 2.65. According to the national cancer Institute, CC_{50} values greater than 100µg/mL are deemed safe. The result is shown in Table 4.

***In vitro* antiviral activity of extract of *Dichrocephala integrifolia* against Herpes simplex virus**

Pre-treatment of cells results shows that *D. integrifolia* methanolic extracts of the stem, leaves and aqueous extracts of leaves inhibited the ability of HSV-1 virus to cause cytopathic effects Vero cells with IC_{50} values of 63.95±5.36, 54.45±3.45, 86.20±7.56 respectively. Post-treatment of cells, only the methanolic extract of the flower and the aqueous extract of the leaves protected the cell from cytopathic effects caused by the virus at IC_{50} values 86.20±7.56 and 82.44±7.92, respectively. The other extracts had IC_{50} values greater than 100 µg/mL (Table 5).

In vitro virucidal activity of extract of *Dichrocephala integrifolia* against Herpes Simplex Virus

The methanolic extract of the flower, aqueous extract of the root, and the methanolic extract of leaves showed direct inactivation of the virus when the extracts were incubated with the virus before incubating with the cells at the cells IC_{50} values of 45.27±2.41, 0.333±1.23 and 30.53±4.51 respectively. In addition, the plant extracts selectively inhibited the growth of the virus. That is shown by the selectivity indices obtained in Table 6.

In vivo toxicity of the extract of *Dichrocephala integrifolia* using Swiss albino mice

Tables 7 and 8 show the control and treated animals' body weights and mice's general behavior at both 300 and 2,000 mg/kg body weight. The results showed no mortality or gross changes in animal behavior and appearance at 300 and 2,000 mg/kg. All animals depicted normal increments in weight and no significant differences between the control and test groups, which indicates that the extract does not affect the growth of the mice. No mortality was observed at 300 mg and 2000 mg/kg body weight doses of different extracts from different parts of the plant, indicating an LD_{50} of >2000 mg/kg body weight.

Table 1. Working estimate of LD_{50} for use in Stage 2 of the sequential design derived from mortality in a limit dose test at 2000 mg/kg-bwt

Mortality (%)	10	20	30	40	50	60	70	80	90
Working Estimate of LD_{50}	3606	2944	2541	2244	2000	1782	1574	1358	1109

Source: OECD /OCD (2001)

Table 2. Phytochemical composition of methanolic extracts of *Dichrocephala integrifolia* plant

Phytochemical	*D. integrifolia*			
	Leaves	Root	Flowers	Leaves
Saponins	+	+	+	+
Alkaloids	+	+	+	+
Phenols	+	+	+	+
Flavonoids	+	-	+	+
Glycosides	+	+	+	+
Terpenoids	+	+	+	+
Tannins	+	+	+	+

Note: +: Present, -: Absent

Table 3. Phytochemical composition of aqueous extracts of *Dichrocephala integrifolia* plant

Phytochemical	*D. integrifolia*			
	Leaves	Stem	Flowers	Root
Saponins	+	+	+	+
Alkaloids	+	+	+	+
Phenols	+	+	+	+
Flavonoids	+	+	+	+
Glycosides	+	+	+	-
Terpenoids	+	+	+	+
Tannins	+	+	+	+

Note: +: Present, -: Absent

Table 4. Cytotoxic effect of *Dichrocephala integrifolia* extracts on Vero cells (normal)

Study extract	Solvent	CC_{50} values (µg/mL)
D. integrifolia stem	Water	>100
D. integrifolia stem	Methanol	>100
D. integrifolia flowers	Methanol	71.31 ± 2.65
D. integrifolia flowers	Water	>100
D. integrifolia roots	Water	>100
D. integrifolia roots	Methanol	>100
D. integrifolia leaf	Methanol	>100
D. integrifolia leaf	Water	>100
Acyclovir	N/A	>100

Note: >100: Depicts that the CC_{50} value of the sample tested was above 100µg/mL; therefore, it could not be obtained within the concentrations exposed to the cells

Table 5. The IC_{50} values of *Dichrocephala integrifolia* extract from pre-treated and post-treated Vero cells

Plant parts	Extracts	Pre-treatment IC_{50} (µg/mL)	Post-treatment IC_{50} (µg/mL)
D. integrifolia stem	Water	>100	>100
D. integrifolia stem	Methanol	63.95±5.36	>100
D. integrifolia flowers	Methanol	>100	45.270±4.31
D. integrifolia flowers	Water	>100	>100
D. integrifolia roots	Water	>100	>100
D. integrifolia roots	Methanol	>100	>100
D. integrifolia leaf	Methanol	54.45±3.45	>100
D. integrifolia leaf	Water	86.20±7.56	82.44±7.92
Acyclovir	Water	4.772±7.81	>100

Note: IC_{50}: Inhibitory Concentration 50 (IC_{50})

Table 6. The potential of various plant parts extracts and Acyclovir (positive control) to prevent cell damage by HSV virus

Plant parts	Extracts	CC^{a}_{50} (µg/mL)	IC^{b} (µg/mL) 50	Selectivity Index (SI^{c})
D. integrifolia stem	Water	>100	>100	N/A
D. integrifolia stem	Methanol	>100	>100	N/A
D. integrifolia flowers	Methanol	71.31± 2.65	45.27±2.41	1.58
D. integrifolia flowers	Water	>100	>100	N/A
D. integrifolia roots	Water	>100	0.333±1.23	>300.3
D. integrifolia roots	Methanol	>100	>100	N/A
D. integrifolia leaf	Methanol	>100	30.53±4.51	>3.28
D. integrifolia leaf	Water	>100	>100	NA
Acyclovir	N/A	>100	24.51 ±3.57	>4.080

Note: a: Cytotoxic concentration 50 (CC_{50}), b: Inhibitory Concentration 50 (IC_{50}), c: Selective index = CC_{50}/IC_{50}, NA – Not applicable - the data presented spectacle means (± standard error) of three independent experiments performed

Table 7. Changes in body weight of Swiss albino mice following administration of crude extracts of *Dichrocephala integrifolia* at 300 mg/kg

Study extract	Day 0	Day 7	Day 14	*P* value
Leave extract (aq)	21.53±2.12	23.91±2.15	25.35±2.00	0.693
Flower extract (aq)	20.11±1.45	23.65±2.45	25.00±2.13	0.554
Root extract (aq)	25.10±3.64	25.31±3.73	27.85±4.45	0.156
Root extract (me)	24.50±1.73	24.57±2.01	25.74±1.69	0.213
Stem extract (aq)	23.27±2.96	23.86±1.89	25.00±2.52	0.076
Flower extract(me)	21.53±0.67	22.06±2.43	24.04±0.78	0.092
Leave extract (me)	26.30±4.77	26.16±2.11	27.72±0.15	0.108
Stem extract (me)	21.73±0.68	22.06±0.53	22.27±2.00	0.074
Negative control	22.49±0.48	22.00±2.00	22.65±0.35	0.726

Note: All mice groups (n = 3) were administered with the crude extracts at 300 mg/kg, aq: Water extract, Me: Methanol extract

Table 8. Changes in body weight of swiss albino mice following administration of crude extracts of *Dichrocephala integrifolia* at 2000 mg/kg

Study extract	Day 0	Day 7	Day 14	*P* value
Leave extract (aq)	26.12±0.12	26.71±2.00	26.20±0.37	0.866
Flower extract (aq)	23.19±0.38	23.51±2.11	23.07±2.05	0.594
Root extract (aq)	25.48±0.39	25.95±0.61	25.24±0.45	0.646
Root extract (me)	24.61±0.33	24.97±2.13	24.65±0.33	0.918
Stem extract (aq)	23.32±0.19	23.50±2.22	23.89±0.40	0.080
Flower extract(me)	22.51±1.90	22.68±1.57	22.95±0.45	0.671
Leave extract (me)	25.84±0.19	25.79±2.11	25.64±0.22	0.336
Stem extract (me)	28.44±0.43	28.15±2.15	28.18±0.17	0.423
Negative control	25.16±0.32	25.00±2.57	25.10±0.20	0.857

Note: All mice groups (n = 3) were administered with the crude extracts at 2000mg/kg, aq: Water extract, Me: Methanol extract

Discussion

Phytochemical compounds from plants have been used to treat different diseases, including diseases of viral origin (Abad et al. 1999; Li et al. 2009; Kohn et al. 2015). The following phytochemical compounds from plants have been reported to have antiviral activity in different previous studies alkaloids (Martin 1987; McMahon et al. 1995), flavonoids (Pengsuparp et al. 1995; Lin et al. 1999), saponins (Sindambiwe et al. 1998), terpenes (Bourne et al. 1999), tannins (Ferrea et al. 1993). In this study, the phytochemical component of aqueous and methanol crude extracts of a *D. integrifolia* screened using qualitative analysis have tested positive for the presence of alkaloids, flavonoids, phenols, saponins, tannins, and terpenoids except for root methanol and root aqueous that lacked flavonoids and glycoside respectively. This result is consistent with previous studies (Mohammed and Teshale 2012). Therefore, the current antiviral activity could be associated with these important pharmacological compounds present in *D. integrifolia* crude extracts, especially the glycosides, flavonoids, phenolics, terpenoids, and tannins, which can be further investigated by isolating the bioactive compounds.

The study reports on the cytotoxicity of different extracts from different parts of the plant on normal monkey kidney cells (Vero). Methanol extract from the flower exhibited moderate toxicity, while other parts of the plant were safe. Decoctions from plants have traditionally been widely used in treating various diseases without scientific justification for their safety (Okumu et al. 2016). Most reports on the toxicity of plants based crude drugs are often associated with liver toxicity (Agbor et al. 2010). The broad traditional use of *D. integrifolia* warrants evaluation for its toxicity properties considering public health protection to the plant extracts, which could cause undesirable consumer effects. Cytotoxicity activities of alkaloids and flavonoids have also been reported (Özçelik et al. 2011); therefore, flower cytotoxicity effects might be due to phytochemicals such as flavonoids present in the extracts. Further study must be done to identify the exerted bioactive compounds causing cytotoxicity in leaves.

In the current study plants, in vitro antiviral activity of four-part *D. integrifolia* crude extracts evaluated against herpes simplex virus revealed that all the investigated parts had antiviral against herpes simplex virus. Previous studies have reported antiviral activity in different medicinal plants. Despite reports on the antiviral and general antimicrobial activity of plants in the Asteraceae family, studies on *D. integrifolia*'s antiviral activity are lacking. When the virus was exposed to the cell before treatment with the extracts, different parts of the plant exhibited moderate inhibitory effects could be due to the blocking of cellular receptor cellular receptors preventing virus entry in the cell. Plant extracts with antiviral activity may inhibit the virus through several mechanisms, such as blocking specific proteins for viral entry or working directly on the

virus itself (Yang et al. 2013). The methanol of the flower and aqueous extract of leaves exhibited a moderate inhibitory effect when incubated with infected cells; this could be due to its interference at some stage of virus replication inside the cells. Aqueous extracts of root and methanol extracts of leaves that exhibited the highest antiviral activity were incubated with the virus before incubation with the cells, which is for the first time the antiviral activity of this plant is reported.

All animals experienced a normal increment in weight, and no drastic differences between the control and test groups were observed. Therefore, the absence of these toxicity indicators means that the extracts were safe at these doses and that their lethal doses are much higher. Oral acute toxicity has been used widely in evaluating herbal remedies' safety (Rang et al. 2001). For example, the administration of *D. integrifolia* extract to ethanol-administered mice brought transaminases toward normal values after a significant increase in alcohol dosage. In addition, the aqueous extract of the plant prevented the development of hepatic tissue abnormalities and improved hepatic function in ethanol-induced hepatic damage (Florence et al. 2017). Additionally, Franco et al. (2015) found *Chresta martii* (DC.) H.Rob., a plant in the Asteraceae family, to have no acute toxicity and no mortalities recorded. Still, debates rumors on the rationale of extrapolating animal model results to humans. However, previous studies have proved that the mice model is a better predictor for human lethal dosage than rats (Walum et al. 1995).

The results show that different roots and flower extracts of *D. integrifolia* could be good candidates in searching for new anti-HSV leads. The safety observed in mice is recommendable, given the wide use of the plant in ethnobotanical medicine. The current study lays the basis for further research on the isolation of bioactive compounds and further evaluation of the mechanisms of action of the plants bioactive at the molecular level. This study provides a partial scientific justification for using *D. integrifolia* in HSV infections. Given the high cost, unbearable side effects, and unavailability of the current antiviral drugs targeted towards HSV to most people in rural areas, the current study provides hope that new cheap antiviral drugs could be obtained from this plant.

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Identifying bacteria associated with diseased *Oreochromis niloticus* in Lake Kariba, Zambia

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Abstract. *Sakala T, Mdegela RH, Hangombe BM. 2022. Identifying bacteria associated with diseased* Oreochromis niloticus *in Lake Kariba, Zambia. Bioteknologi 19: 62-70.* The recent intensified aquaculture projects in Lake Kariba, Zambia, have brought about disease outbreaks in cultured *Oreochromis niloticus* (Linnaeus, 1758). This study aimed to identify bacteria associated with diseased *O. niloticus* and establish their antibacterial resistance patterns. Caged fish were identified as diseased based on behavioral and physical abnormalities, including swimming in circles, swimming in lateral or dorsal recumbency, ocular opacity, hyperpigmentation, fin erosions, and ulcerations. A total of 25 sick and 4 healthy fish were sampled. Samples from the liver, spleen, brain, abdomen, kidney, blood, and ulcers were inoculated on 10% sheep-blood agar and nutrient agar. Then the isolates were classified by genera using biochemical tests and standard culture. Furthermore, the bacterial isolates were tested for resistance to commonly used antibacterial compounds in aquaculture using the disc technique; the spleen, eyes, and liver had the highest number of pathologies in descending order. A total of 15 bacteria genera were identified, where *Lactococcus/Streptococcus* genera had the highest prevalence with 46.2%, then *Aeromonas* at 11.5%. All isolates have been observed to have multiple drug resistance, with two isolates, each of *Lactococcus Streptococcus* and *Aeromonas* exhibiting complete resistance to most antibiotics tested. The results suggest that increased biomass in diseased cages may be the main risk factor for the disease, with the immune and regulatory organs being first to succumb. The lake environment is a mixing vessel of various microorganisms that show multiple antibacterial resistance. Therefore, risk factors surrounding the existence of these bacteria genera must be accessed, and a more comprehensive, comparative study in antibiotic resistance on farmed, in contrast to wild species, in Lake Kariba, Zambia.

Keywords: *Aeromonas*, bacteria, Kariba, *Oreochromis niloticus*, *Streptococcus*

INTRODUCTION

Fish are a crucial food source worldwide, providing energy, protein, and a range of essential nutrients as they are eaten by over 2.9 billion people (FAO 2014). As people are consuming more fish, the dependence on the fisheries and aquaculture sector has increased (FAO 2016). Aquaculture has intensified in many parts of the world, including Chile, Brazil, China, India, Norway, Bangladesh, Morocco, Nigeria, Uganda, Ghana, and Egypt (FAO 2014; FAO 2016). This intensive production has brought with it the development of disease outbreaks. The host (fish), pathogen, and environment triad are generally balanced-relationship, regulated primarily by the immune system. However, some aquaculture-related practices and consequences, such as high densities, can cause stress leading to lowered immunity and, ultimately, disruption of the balanced triad relationship (Huicab-Pech et al. 2016). That can result in bacterial disease outbreaks in which microflora found within the natural aquatic environment may be implicated (Austin 2006; Helmy and Atallah 2015). Antibacterial agents have been used in small sub-therapeutic doses in feed as growth promoters or to prevent disease in fish. For example, they have been used practically to treat an entire population to protect healthy individuals until the sick fish die, and the infection subsides. This form of oral treatment leads to sub-therapeutic doses, enabling selection for resistance in bacteria (Thorsen 2014).

Currently, the most common species farmed in Zambian aquaculture are from the Cichlid family, namely, *Oreochromis andersonii* (Castelnau, 1861) (64%), *Tilapia rendalli* (Boulenger, 1897) (20%), *O. niloticus* (Linnaeus, 1758) (5.2%), and *O. macrochir* (Boulenger, 1912) (5%). Commercial *O. niloticus*, due to its hardy nature and economical production (Popma and Masser 1999), has been a profitable source of income on Lake Kariba, Zambia. However, with reports of resistant bacteria recently have been investigated and confirmed in *O. niloticus* and catfish in aquaculture within Africa (Ekundayo et al. 2014; Tiamiyu et al. 2015), this study aims to lay a foundation for resistance to antibacterials and the prevalence of bacteria associated with sick fish in cages in Lake Kariba.

The growing cage culture of Nile tilapia (*O. niloticus*) on Lake Kariba, Zambia, has risen because of the many advantageous qualities of the species. However, there has been an outbreak of the bacterial disease in cultured *O. niloticus*, with reported cases from 2015 to the present. On-going anthropogenic activities on Lake Kariba and recently established aquaculture projects may have affected the aquatic environment. These may have changed the environmental setup, leading to a situation where bacteria

populations increase as the host, *O. niloticus*, is concentrated in one location. Therefore, there is a need to investigate the disease in fish cultured commercially, the sensitivity to antibacterial agents of the cultured fish, and the establishment of baseline information. Furthermore, there is no information on public health issues surrounding fish found in this lake.

This study aimed to investigate the most prevalent bacterial isolates present on Lake Kariba and their sensitivity towards commonly used antimicrobials in aquaculture or present within the lake environment.

MATERIALS AND METHODS

Study area

The study area for this research was a commercial cage farm within the Siavonga town in the Southern Province of Zambia (Figure 1). The site is located at latitude 16°S 28.318", longitude 28°E 38.52", on Lake Kariba. Lake Kariba is one of the world's largest artificial lakes and reservoirs by volume, covering an area of 5,580 square kilometers, which lies 1.300 kilometers upstream from the Indian Ocean, along the border between Zimbabwe and Zambia.

Lake Kariba is home to a variety of freshwater fish species, including Tigerfish (*Hydrocynus vittatus* Castelnau, 1861), Catfish (*Clarias gariepinus* Burchell, 1822), Barbel (*Barbus barbus* Linnaeus, 1758), Labeo (*Labeo rohita* Hamilton, 1822), Jack (*Caranx lugubris* Poey, 1860), Vundu (*Heterobranchus longifilis* Valenciennes, 1840) and Bream/Tilapia (*O. niloticus*). These were introduced and reared under cage aquaculture facilities (Mudenda 1994).

Sample size

Sampling was such an outbreak investigation. In line with this, to enable detection at a 95% confidence level, infected animals must have a case of clinical infection which requires sampling 10 diseased fish; combined to form pools of a maximum of 5 fish each. These include detecting asymptomatic carriers that involve samples combined in pools of no more than 5 fish per pool (OIE 2003). According to Midlyng et al. (2000), the recommended minimum number of adult fish (>150g) to be sampled for outbreak investigation is a sample size of 5.

Sampling technique

A total of 29 fish were captured during feeding time using a disinfected scoop net from four different cages on Lake Kariba. The subtotal of 25 'sick' fish and four 'healthy' fish were selected purposively from four of the nine cages experiencing disease and increasing mortalities. Fish were initially thoroughly examined for external lesions, measured (total length and circumference) using a standard measuring tape, and weighed using an electronic balance.

Swabs were collected from the abdomen, spleen, liver, brain, eye, gonads, blood, and skin lesions on each fish. The swabs were then inoculated onto freshly prepared, appropriately labeled Nutrient agar and 10% sheep blood agar (HiMedia Laboratory Pvt, Mumbai, India) plates by streaking using sterile disposable loops on-site. Individual organ samples were then collected and stored in 10% buffered formalin, and blood smears were prepared. Culture plates and samples were stored at room temperature for 24 hrs and then transported to the laboratory under icepacks at -4°C. The organisms were initially grown on Nutrient and Blood Agar media (HiMedia Laboratory Pvt, Mumbai, India) for primary isolation. Next, culturing was done on-site by streaking with a sterile inoculating loop.

Isolation and classification of bacteria

Culture of bacteria

Samples were processed at the School of Veterinary Medicine, Department of Paraclinical Studies, University of Zambia (Woodland 2004). Isolation involved three stages; firstly, the different primary bacteria isolates were individually sub-cultured on Nutrient and Blood agar (HiMedia Laboratory Pvt, Mumbai, India). Then, to ensure that possible contaminants were absent, they were incubated at room temperature (25°C) for 24 hrs. Finally, based on colony morphological appearance, all different bacteria colonies from all organs of each sampled fish were each noted and labeled clearly.

Gram stain and morphology characteristics

The pure colonies produced were then Gram-Stained to determine Gram-Positive or Gram-Negative nature and microscopic morphological appearance. Each pure colony was first emulsified in sterile normal saline on a well-labeled, clean, dry glass slide. That was then air-dried and fixed under a Bunsen burner. After that, the slides were stained with Crystal Violet solution for 45 seconds and washed under gently running water. The slides were then flooded with iodine solution for 45 seconds and washed under gently running water again. The slides were then decolorized with 70% alcohol solution, followed by gentle washing under running water. Finally, the slides were counter-stained with safranin solution for 45 seconds, followed by gentle washing under running water. They were then air-dried and viewed at X100 magnification under oil immersion. The microscopic morphology characteristics and Gram stain were then viewed and noted (Buller 2014). Finally, the following biochemical tests were performed to confirm the suspected isolates: Simmons Citrate, SIM (Sulphur, Indole, Motility) test, Triple Sugar Iron test, Urease, and carbohydrate 'sugar' utilization tests (Esculin, Galactose, Raffinose, Salicin, Maltose monohydrate, Xylose, Mannitol, Trehalose, Insulin, Sorbitol, Lactose monohydrate, and Glucose).

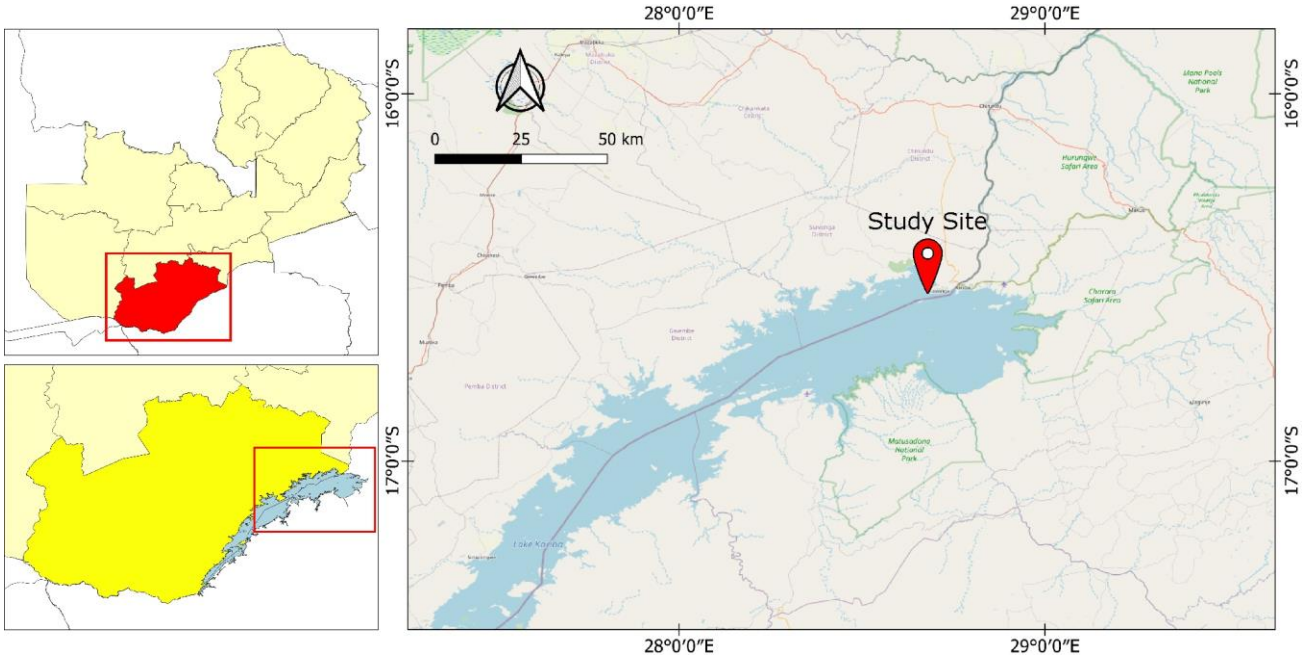


Figure 1. Study area- commercial cage farm on Lake Kariba, Zambia

Phenotypic identification of bacterial isolates

Sulphur indole motility test. The SIM media was prepared according to the manufacturer's protocol (HiMedia Laboratories, India). It was distributed in short tubes and autoclaved at 121°C for 15 minutes. The tubes were allowed to solidify for 24 hrs, producing a clear, light-yellow colored media. The pure isolates grown on nutrient agar were then collected using a sterile straight-wire loop and inoculated into the media by stabbing once under a Bunsen burner to maintain sterility. That was followed by incubation for 24 hrs at 37°C. After 24 hrs, the media was viewed for motility (cloudy appearance) and production of Sulphur gas (blackening). That was followed by adding two to three drops of Kovac's reagent to the suspension using a dropper and waiting briefly for 5-10 seconds for the reaction. The formation of a pink-colored ring that rises to the surface indicated a positive Indole result, whereas the presence of no pink-colored ring meant a negative Indole result.

Simmons citrate test. The Simmons citrate agar was prepared according to the manufacturer's protocol (HiMedia Laboratories, India). The agar was prepared according to the manufacturer's protocol. It was distributed in long tubes and autoclaved at 121°C for 15 minutes. The tubes were then allowed to solidify for 24 hrs, producing a clear, crystal-green colored media. Pure isolates of the organisms were then collected from nutrient agar and inoculated into the agar using a sterile straight-wire loop by gently streaking the media slant under the Bunsen burner to maintain sterility. The media was then incubated for 24 hrs at 37°C. The Citrate agar was green in color before inoculation. However, a positive result was obtained when the color changed to blue, meaning that the citrate was utilized, whereas in a negative result, there was no color change, and the media remained green.

Triple sugar iron test. The TSI agar was prepared according to the manufacturer's protocol (HiMedia Laboratories, India). First, it was distributed in long tubes and autoclaved at 121°C for 15 minutes. Next, the tubes were allowed to solidify for 24 hrs, producing a light-orange colored media. Next, the pure isolated colony was picked with a sterile, straight-wire loop, followed by the first stabbing of the agar and then gently streaking the slant under the Bunsen burner to maintain the surface of the slant sterility. It was then incubated at 37°C for 24 hrs. The results were read and interpreted according to Table 2.

Urease test. The urease media was prepared according to the manufacturer's protocol (HiMedia Laboratories, India). First, it was distributed in short tubes and autoclaved at 121°C for 15 minutes. Next, the tubes were allowed to solidify for 24 hrs, producing a yellowish-orange clear media. The pure isolates grown on nutrient agar were then collected using a sterile, straight-wire loop and inoculated into the media by stabbing once under a Bunsen burner to maintain sterility. That was followed by incubation for 24 hrs at 37°C. After 24 hrs, the media was observed for color change; a brick-red color meant a positive result, while a yellowish-orange color meant a negative result, meaning that the bacteria did not utilize the media.

Identification using sugars

The different strains were tested for the biochemical reaction using 12 sugars and alcohol; disaccharides (maltose monohydrate, trehalose), hexoses (glucose, mannose, and galactose), pentose (xylose), polyhydric alcohols (mannitol, sorbitol, inulin, and salicin), trisaccharides (raffinose) and Esculin (HiMedia Laboratories, Mumbai, India), were prepared according to manufacturer's protocol. That was performed in a tube of phenol red broth containing either one percent sugar or

alcohol, followed by inoculation with a single bacterial isolate using a sterile straight wire. The broth tubes were incubated at 30±0.5°C for 48 hrs, and the results were recorded as positive if the production of acid condition induced a change in the red phenol indicator from pink to yellow.

Antibacterial susceptibility test of bacterial isolates

The Kirby-Bauer disc diffusion antibacterial sensitivity test method was used to test the antibacterial resistance of the bacterial isolates. A sum of 9 antibacterial agents belonging to 5 antibacterial classes (Beta-lactam Penicillins, macrolides, tetracyclines, aminoglycosides, and sulphonamides) were used to determine the antibiograms of the isolates. Ten antimicrobial drugs were used, among them Penicillin-G (P 10 µg), Amoxiclav (AMC 30 µg), and Amoxicillin (AMX 10 µg) (HiMedia Laboratory Pvt, Mumbai, India), selected for being readily available and efface against Gram-Positive bacteria. In addition, cefotaxime (CTX 30 µg), Ciprofloxacin (CIP 5 µg), and Norfloxacin (NX 10 µg) (HiMedia Laboratory Pvt, Mumbai, India) have known efficacy against Gram-Negative bacteria. In contrast, Tetracycline (TE 30 µg), Erythromycin (E 5 µg), and Co-trimoxazole (COT 25 µg) (HiMedia Laboratory Pvt, Mumbai, India) are broad in their effect (Thompson MICROMEDEX 2003).

Mueller-Hinton agar was prepared according to the manufacturer's protocol (HiMedia Laboratory Pvt, Mumbai, India). First, the organisms were purified on nutrient agar. Next, a loop full of bacterial colonies was collected using a sterile, round-wire inoculating loop, which was then streaked onto the Muller Hinton agar surface plate until their surface was thoroughly covered under Bunsen flame to ensure a sterile environment. Next, using a pair of sterile forceps, the antibiotic discs were removed from the dispensers and then gently placed on the agar while ensuring each disc was fixed on the agar surface. Next, the discs were placed equidistant from each other, with only five antibiotic discs placed per plate to ensure clarity of results. The plates were then placed upside down and incubated for 24 hrs at room temperature (NCCLS 2000). The sensitivity of each isolate was then read by measuring the clear, circular diameter around each disc. These results were recorded in millimeters and later classified as susceptible, intermediate, and resistant.

Data analysis

Microsoft Excel 2010 was used for data storage and computation of prevalence. Chi-square tested the relationship between weight and disease severity at a 95% confidence interval.

RESULTS AND DISCUSSION

Field observations of sampled fish

Based on appearance and behavior, observations indicating the presence of disease included abnormal swimming, positioned in dorsal or lateral recumbency, and swimming in circles. The skin was abnormal, with a highly pigmented or black external appearance. The ulcers were present around the mouth and body with eroded fins. The most affected fins were the dorsal and tail fins. The eyes were blind (appearing white) and exophthalmos or protruding eyes.

Bacterial isolates

Primary isolation was performed on-site. Out of the initial 462 plates inoculated, 98 plates did not grow, and 364 plates had growth; 179 isolates were on Blood Agar, and 185 isolates were on Nutrient Agar. These were sub-cultured in the laboratory on Nutrient and Blood agar giving pure colonies (Figure 2). Gross morphology on both media was then recorded (Table 3).

Chi-square test

χ^2calculated value = 0.529

χ^2 expected value = 3.841

P (α) = 0.05; χ^2 Calculated value < 3.841 reflects no statistical significance H_O has been accepted

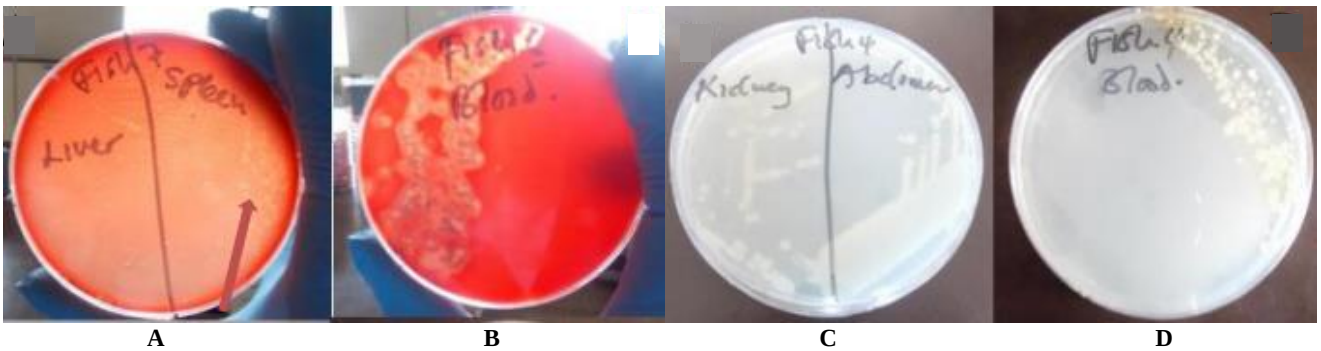
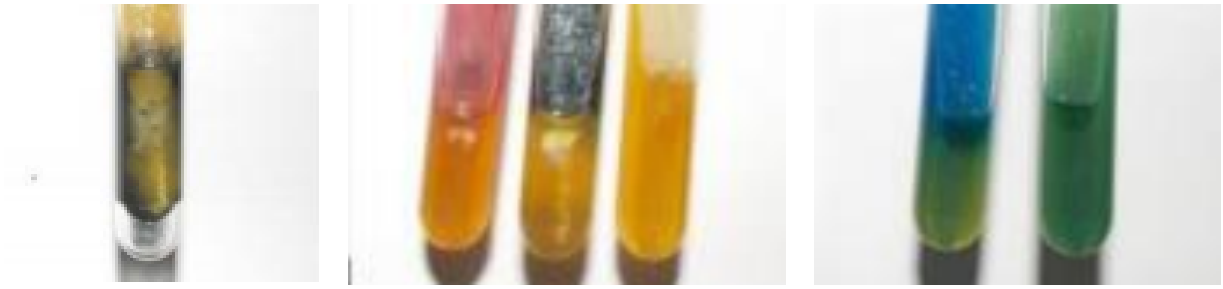


Figure 2. Labeled bacterial isolates: A. Liver and spleen isolates of fish 7 showing hemolysis on blood agar, B. Blood isolates of fish 6 showing hemolysis on blood agar, C. Kidney and abdominal isolates of Fish 4 on nutrient agar, D. Blood isolate of fish 4 on nutrient agar



A B C

Figure 3. Biochemical test results of labeled bacterial isolates: A. TSI illustration of acid throughout medium, hydrogen sulfide production (blackening) and gas production, B. TSI result, from left; acid butt and alkaline slant, middle; acid throughout medium and hydrogen sulfide production (blackening), right; acid throughout medium (Table 1), C. Simmons citrate result, left; blue slant positive test result, right; no color change negative test result

Gram-staining and morphology

The sub-cultured (purified) colonies were then Gram-Stained and observed under oil emersion at X100 magnification. Bacteria were classified based on gram-staining and morphology characteristics. Based on gross colony description and Gram-Stain, 78 bacterial isolates were selected and segregated as representative of all different isolates present from each fish. Of these, 56 were Gram-Positive, and 22 were Gram-Negative.

Biochemical tests

A sum of 16 biochemical tests was performed on each fish's representative bacterial colonies (Figure 3). The biochemical test results were compared to those stated in the literature to identify the bacterial colonies (Buller 2004).

Table 1. Gross pathologies observed in the organs of sick fish during post-mortem

Sites sampled	Frequency of observed pathologies (n=31)	Percentage (%)
Brain	3	9.7
Eye	9	29.0
Abdominal cavity	4	12.9
Spleen	7	22.6
Liver	6	19.4
Gonads	0	0
Kidney	2	6.5
Blood	0	0

Note: n: Total number

Table 2. Interpretation of triple sugar iron agar reaction slants

Appearance	Reactions
Acid butt: yellow, alkaline; slant: red	Glucose fermented
Acid throughout medium: butt and slant yellow	Glucose, sucrose and/or lactose fermented
Gas bubbles in butt and medium frequently split	Gas production
Butt shows blackening	Hydrogen sulfide produced
Unchanged or alkaline butt and slant: medium red throughout	None of the three sugars fermented

Source: Carter (1984)

Antibiograms

Table 6 summarizes the resistance profiles of bacteria isolates to the selected antibacterials.

Table 3. Prevalence of bacterial isolates at genus level based on morphological characteristics, Gram-Staining, and biochemical test screening

Identified bacterial isolates (genus)	No. of isolates (n=78)	Prevalence %
Aeromonas spp.	9	11.5
Aequorivita spp.	1	1.3
Enterococcus spp.	2	2.6
Serratia spp.	1	1.3
Lactococcus/Streptococcus spp.	36	46.2
Citrobacter spp.	1	1.3
Corynebacterium spp.	6	7.7
Edwardsiella spp.	2	2.6
Acinetobacter spp.	1	1.3
Bacillus spp.	2	2.6
Klebsiella spp.	1	1.3
Staphylococcus spp.	3	3.9
Norcardia spp.	1	1.3
Carnobacterium spp.	1	8.0
Rhodococcus spp.	1	1.3
Unidentified Bacteria	5	6.4

Note: n: Total number, %: percentage

Table 4. Frequency of bacteria identified at genus level in fish sampled

Bacteria identified (genus)	No. of fish associated with the bacteria
Aeromonas spp.	8
Aequorivita spp.	1
Enterococcus spp.	2
Serratia spp.	1
Lactococcus/Streptococcus spp.	25
Citrobacter spp.	1
Corynebacterium spp.	3
Edwardsiella spp.	1
Acinetobacter spp.	1
Bacillus spp.	2
Klebsiella spp.	1
Staphylococcus spp.	3
Norcardia spp.	1
Carnobacterium spp.	6
Rhodococcus	1

Table 5. Frequency of bacteria isolated from the internal organs of diseased *Oreochromis niloticus* at the genus level

Identified bacterial isolates	Brain	Eye	Abdominal cavity	Spleen	Liver	Gonads	Kidney	Blood
Aeromonas	-	3	1	1	1	-	2	1
Aequorivita	-	-	-	-	-	1	-	-
Enterococcus	-	1	-	1	-	-	-	-
Serratia	1	-	-	-	-	-	-	-
Lactococcus/Streptococcus	4	4	3	6	4	3	4	4
Citrobacter	-	-	1	-	-	-	-	-
Corynebacterium	2	-	-	-	-	-	3	1
Edwardsiella	-	-	1	-	1	-	-	-
Acinetobacter	-	-	-	1	-	-	-	-
Bacillus	1	-	-	-	-	-	-	1
Klebsiella	-	-	-	-	-	-	-	1
Staphylococcus	-	1	1	-	-	1	-	-
Norcardia	-	-	-	-	-	1	-	-
Carnobacterium	-	-	2	1	-	1	-	2
Rhodococcus	-	1	-	-	-	-	-	-
Total	8	10	9	10	6	7	9	10

Table 6. Antibacterial resistance profiles of bacteria isolates

	Bacteria% resistance														
A/B	**Aer**	**Aeq**	**Et**	**Ser**	**Lt/Stp**	**Cit**	**Cor**	**Ed**	**Acn**	**Bac**	**Kle**	**Stph**	**Nr**	**Carn**	**Rhd**
P 10µg	88.9*	-	100	100*	61.1	-	83.3	100*	-	100	100*	100	-	66.7	100
AMC 30 µg	100*	100*	100	100*	36.1	-	50.0	50*	-	100	50*	33.3	-	33.3	100
AMX 10 µg	100*	-	100	100*	72.2	-	83.3	50*	100*	100	50*	100.0	-	66.7	100
CTX 30 µg	44.4	-	100*	-	61.1*	-	50.0*	50	-	50*	100	66.7*	-	50.0*	-
TE 30 µg	55.6	-	50.0	-	13.9	-	33.3	50	-	100	50	-	-	33.3	-
E 5 µg	100	-	50.0	-	30.6	-	66.7	100	-	100	50	100.0	-	50.0	-
CIP 5 µg	55.6	-	100*	-	41.7*	-	33.3*	-	-	50*	50	33.3*	-	50.0*	-
COT 25 µg	33.3	-	100	-	75.0	100	50.0	50	-	50	100	33.3	-	66.7	100
NX 10 µg	44.4	-	100	-	69.4	100	16.7	-	-	50	100	33.3	-	66.7	-

Note: P: Penicillin, AMC: Amoxiclav, AMX: Amoxicillin, CTX: Cefotaxime, TE: Tetracycline, E: Erythromycin, CIP: Ciprofloxacin, COT: Co-trimoxazole, NX: Norfloxacin, A/B: Antibiotic, Aer: *Aeromonas*, Aeq: *Aequorivita*, Et: *Enterococcus*, Ser: *Serratia*, Lt/Stp or *Lacto/Strep.*: *Lactococcus/Streptococcus*, Cit: *Citrobacter*, Cor: *Corynebacteria*, Ed: *Edwardsiella*, Acn: *Acinetobacter*, Bac: *Bacillus*, Kle: *Klebsiella*, Stph: *Staphylococcus*, Nr: *Norcardia*, Carn: *Carnobacteria*, Rhd: *Rhodococcus*, *Probable Natural resistance

Discussion

The importance and significance of this study have been driven mainly by reports of disease outbreaks in commercially cultured *O. niloticus* on Lake Kariba, Zambia. These outbreaks have been recorded from as far back as 2014, thus the need to study bacterial organisms that may be associated with these outbreaks.

The fish was first observed for abnormal features and behavior in their natural habitat. Upon careful observation, 'sick' fish were singled-out. That was based on common clinical signs, including abnormal swimming, positioned in dorsal or lateral recumbency, swimming in circles, skin that was highly pigmented, giving an almost black external appearance, eroded fins, dorsal and tail fins visible, ulceration around the mouth and body, blind eyes (appearing white) and exophthalmos or protruding eyes (Austin and Austin 2007; Noga 2010; Parker 2012).

The clinical signs that were observed in the cultured *O. niloticus* have been associated with infection by bacteria, namely; *Aeromonas* (Belém-Costa et al. 2006), *Pseudomonas* (Amutha and Kokila 2016), *Edwardsiella* (Amal and Zamri-Saad 2011; Dong et al. 2015), *Flavobacterium* (Al-harbi et al. 2005; Huicab-Pech et al. 2016) and *Streptococcus* (Iregui et al. 2004; Musa et al. 2009; Anshary et al. 2014; Pretto-Giordano et al. 2015) species.

It has been said that waters with a high organic load, which favors the multiplication of bacteria, rapidly changing temperatures, overcrowding, trauma, and transportation are the most encountered environmental stress factors which predispose to clinical disease in fish. Intensive fish culture systems, such as Lake Kariba, are particularly likely to give rise to these factors (Roberts 2012; Huicab-Pech et al. 2016); as fish grow within the cage, their body size increases. The farm management reported that sick fish originating from cages delayed harvest by 2-3 months. A market size of 700 g is attained in about 4 months, according to Anonymous (2016), though market traders, restaurants, and the public generally require varying weights regularly. In Zambia, the ideal harvest size of fish is 400-500 g. Therefore, it could be concluded that the sick fish's delay in harvest resulted in increased biomass beyond that which the caged environment could support. These resulted in a stressful condition that opened the opportunity for infection by opportunistic organisms.

A Chi-square test was performed to determine any correlation between the sick fish's weights and infection severity. Mild infection was characterized by petechial hemorrhage and fin erosion. Severe infection was characterized by clinical signs, including blindness, open wounds, ulcers, and abscesses, among those presented with mild infection. Results revealed no significant relationship between the parameters; the weight of the sick fish was not related to the severity of outward clinical signs observed.

The organs with the most abnormalities included the eye, spleen, and liver, with 29.0%, 22.6%, and 19.4%, respectively (Table 5). The eye is one of the most sensitive organs, with the retina having among the highest oxygen demands of any tissue in the body (Helfman et al. 2009). With any homeostatic imbalances, the eye is often one of the first to show signs of underlying disease. That was clearly observed during the examination, with signs of blindness, opacity, and protrusion (exophthalmos) in most sick fish. The spleen is one of the organs primarily responsible for immunity, with the liver involved in blood chemistry maintenance. Perhaps for these reasons, they were severely affected by the disease.

Based on bacterial culture, morphology, Gram-Staining characteristics, and a series of biochemical evaluations and classification, an overwhelming majority, at 46.2%of these isolates, were identified as *Lactococcus* or *Streptococcus* species. Furthermore, 11.5% were identified as being *Aeromonas* species. Among the other 15 bacterial genera identified, *Corynebacterium* was 7.7%, and *Staphylococcus* was 3.9%. The rest was belonged to other bacteria. Therefore, based on the study, the pathogens most likely present in diseased *O. niloticus* on Lake Kariba include *Streptococcus*/*Lactococcus*, *Aeromonas*, *Corynebacterium*, *Carnobacterium*, and *Staphylococcus* species. *Streptococcus*/*Lactococcus* and *Aeromonas* species have been some of the most widely implicated bacteria in disease outbreaks, mainly due to their opportunistic nature (Belém-Costa et al. 2006; Austin and Austin 2007; Musa et al. 2009; Amal and Zamri- Saad 2011; Abdelsalam et al. 2013; Ahmed and El-Refaey 2013; Haenen et al. 2013; Anshary et al. 2014; Pretto-Giordano et al. 2015; Amutha and Kokila 2016; Noga 2010; Roberts 2012; Huicab-Pech et al. 2016). They can survive in the natural environment in a dormant state and invade host tissues once there is destabilization in the environment and/or host. *Streptococcus*/*Lactococcus* was the most isolated across all eight organ sampling sites. The fish observed as being 'sick' had manifested full-blown disease due to stressful cage conditions, which was proven by the clinical signs and post-mortem lesions documented in sampled fishes 1, 8, and 14. These lesions included fin, nasal and buccal erosion and ulceration, ascites, and under-belly and petechial body hemorrhages. These are lesions characteristic of Aeromonas infection (Austin and Austin 2007; Ibrahim et al. 2008; Woo 2011; Roberts 2012).

Bacteria from the family Streptococcae are found within the natural aquatic environment and are known to be naturally opportunistic. Disease caused by these bacteria has been associated with poor husbandry and excessive stocking densities (Roberts 2012). Specific causative agents of disease outbreaks worldwide include *Lactococcus garvieae* (Woo 2011; Roberts 2012; Helmy and Atallah 2015), *Streptococcus iniae* (Mcnulty et al. 2003; Baiano and Barnes 2009; Pretto-Giordano et al. 2015) and *Streptococcus agalactiae* (Iregui et al. 2004; Jafar et al. 2008). Disease outbreaks usually occur when fish have been exposed to stress, including increased water temperature, suboptimal water oxygen levels, or overcrowding for a long time. *Streptococcosis*, theoretically, affects all fish sizes; however, bigger fish (from 100 g to market size) are usually most susceptible to the disease (MSD Animal Health 2006). *Lactococcus*/ *Streptococcus* was isolated in sick fish, with an average weight of 547.8 g. These fish exhibited clinical signs of abnormal swimming, positioned in dorsal or lateral recumbency, spiral swimming, and lesions, including numerous hemorrhages all over the body, wounds, ulcers, and hyperpigmentation. That is documented in the literature (Al-harbi et al. 2005; Musa et al. 2009; Noga 2010; Roberts 2012; Ahmed and El-Refaey 2013; Huicab-Pech et al. 2016) as being characteristic of *Lactococcosis*/ *Streptococcosis*.

The eye is also a major point of bacterial infiltration, being one of the most sensitive organs of the fish (Helfman et al. 2009). In particular, *Streptococcus agalactiae* is known to cause unilateral and bilateral ocular lesions and has a tropism for the central nervous system (CNS) (Iregui et al. 2004; MSD Animal Health 2006; Jafar et al. 2008; Roberts 2012). The study revealed that nine (9) out of the twenty-five (25) fish from which *Lactococcus*/*Streptococcus* was isolated had either ocular lesions or both ocular and brain lesions. These findings show to the possible causative agent is *Streptococcus agalactiae*. *Lactococcosis*/*Streptococcosis* generally leads to inflammation and necrosis of the liver, spleen, kidney, eye, and brain, and septicemia as a hematogenous infection (MSD Animal Health 2006; Roberts 2012). That has been evidenced by the organs in which bacteria were isolated from the various fish.

The sick fish were overdue for harvest by 2 months with maintained regular feeding. Caged fish have a relatively small surface area to volume ratio compared to ponds and raceways. That was coupled with a more restricted food supply (dispersing feed over a comparatively small area of the cage) that resulted in greater competition and more pronounced disparity in food acquisition among individuals (Beveridge 2004). In this case, stocking the 900 m3 cages was on the upper limit, thus more contact between individuals. Competitive and defensive feeding behavior such as high speed, jaw protrusion, and spreading of the fins (Helfman et al. 2009), coupled with biomass beyond that which the cage is meant to support, would result in a high incidence of injury, leading to open wounds. Diseases such as Streptococcosis are transmitted horizontally from fish to fish (via cannibalism and skin injuries) and from the aquatic environment to fish (MSD Animal Health 2006).

Among the bacteria isolated in the fish included *Bacillus* (Table 4), which has been shown to have probiotic properties as lactic acid-producing bacteria. The same as

Carnobacterium and *Rhodococcus*, which have also been discovered to have the same probiotic properties (Takyi et al. 2012). Bacteria, including *Norcardia* and *Citrobacter*, are known to be commensals in the aquatic environment and surrounding soil (Roberts 2012; Takyi et al. 2012). *Staphylococcus*, *Serratia*, and *Klebsiella* are opportunistic bacteria and may manifest in heavily stressed fish, evidenced by the study. These bacteria may pose public health risks associated with human pollution, as with some Enterococci species (Marcel and Sabri 2013). *Aequorivita* is a genus under the Flavobacteria family, mostly associated with living and dead phytoplankton in the natural environment (Bowman and Nichols 2017). *Edwardsiella septicemias* and ulcerative conditions have been documented in various fish species worldwide (Austin and Austin 2007; Roberts 2012; Huicab-Pech et al. 2016). Bacterial species under the *Corynebacteria* genera have been implicated in widespread disease conditions affecting the kidney in fish species other than *Oreochromis* (Woo 2011; Buller 2014). *Acinetobacter* species have been labeled as emerging fish pathogens in other fish species. These strains have been commonly known as microorganisms transmitting antibiotic resistance genes, which may greatly impact the resistance transfer in aquaculture (Kozińska et al. 2014).

With disease outbreaks in Zambia in *O. niloticus* cage aquaculture, the active surveillance of disease pathogens has been a priority. Disease-causing pathogens isolated and confirmed at the molecular level include *Streptococcus iniae*, *Lactococcus garvicae*, and *Aeromonas hydrophila* (Hangombe and Ndashe 2015). The bacteria isolated were tested for sensitivity against some commonly used antibacterial agents in aquaculture. Some documented antibacterials include ROMET 30® or ROMET TC® (sulphadimethoxine and Ormetoprim), Aquaflor® (Florfenicol), and Terramycin® in feed formulations (Sekkin and Kum 2011; Kelly 2013). Intensification and the advent of disease outbreaks have occasion for developing resistant bacterial strains. The study looked at nine (9) commonly used antibacterial compounds, which were meant to give an overview of the profiles of antibacterial classes on the market as treatment options in fish health. According to the research findings, the thirty-six (36) *Lactococcus/Streptococcus* expressed varying levels of antibacterial resistance, with one isolate having total resistance to all the antibacterials tested. The results also showed a set of two having the same resistance patterns and another three having the same pattern, which could indicate them being the same strain. The most effective antibacterial compound was Tetracycline, with the bacterial isolates showing the lowest resistance of 13.9%. Co-trimoxazole was the most effective against the *Aeromonas* spp. (33.3%). Two isolates from different fish had the same resistance profile, indicating that these could be one in the same strain. All six (6) isolates of the Corynebacteria expressed different levels of resistance, with Norfloxacin being the most effective against it (16.7% resistance), Carnobacteria being the most sensitive to Amoxicillin (33.3% resistance), and *Staphylococcus* was most sensitive to Co-trimoxazole, Amoxicillin, and Norfloxacin (all 33.3% resistant).

Multiple resistances have been expressed towards antibacterials commonly used worldwide in aquaculture practices. These results otherwise suggest the possibility of undocumented and/or unregulated use of antibiotics by aquaculture communities on Lake Kariba. There currently have been no records of antibacterial use by fish farmers on Lake Kariba (Pers. comm. 2015). On the contrary, the antibiogram profiling of the isolates revealed multiple resistance to most of the antibacterial agents. Potential sources of this resistance include antibiotics flushed into the lake from surrounding human settlements and animal husbandry practices. In addition, bacterial populations within the water and the fish may have gained resistance through mutation upon exposure to these antibacterials. Alternatively, the bacteria within the ecosystem may have an innate resistance to the selected antibacterials. These phenomena may only be answered by further in-depth antibacterial screening and testing of the lake and surrounding environment.

This study isolated fifteen (15) bacterial genera from diseased *O. niloticus* on commercial cage fish farms on Lake Kariba. It reflected the vast number of ubiquitous, opportunistic bacterial organisms in the aquatic environment. This profile is relevant to the future of aquaculture establishments on Lake Kariba as intensification practices advance. Furthermore, the bacterial isolates all expressed varying levels of resistance to commonly used and available antibacterials, which ought to be revered in the different lakes' practices and could also be a potential concern to public health.

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Pectinase production from a local isolate of *Aspergillus niger* using orange bagasse as a carbon source

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Abstract. *Timothy M, Mayel MH, Yohanna ER, Adondua MA, Chinekwu UK, Binunga BB, Janet T. 2022. Pectinase production from a local isolate of* Aspergillus niger *using orange bagasse as a carbon source. Asian J Nat Prod Biochem 19: 81-86.* Pectinases are a group of enzymes that catalyze the breakdown of pectin substances. The largest industrial applications of pectinases are in fruit juice extraction and clarification. However, it is in short supply, but due to the gradual increase in global population, fruit juice industries will need to increase the production of fruit juices, hence the need for mass production of pectinase. Little or no work has been done on producing pectinase from the locally sourced fungus *Aspergillus niger* in Wukari using orange bagasse as a carbon source. The present investigation was carried out to produce pectinase from a locally sourced fungus, *A. niger*, in Wukari, Taraba State, Nigeria. *A. niger* was identified morphologically and screened for pectinase production. Submerged fermentation was carried out, and the crude pectinase was harvested, with maximum enzyme production at 72 hrs. The effect of pH on pectinase activity was assessed, and different temperatures were used to test for pectinase activity. The crude enzyme's kinetic constants (K_m and V_{max}) were also determined. The optimal pH of pectinase was alkaline, at pH 8.0. The temperature at the crude enzyme expressed the highest activity was 50ºC. The crude enzyme activity was tested with the effect of substrate concentration; the optimum substrate concentration was discovered to be 2.0%. At the end of this research, the results showed that pectinase could be mass-produced to serve the needs of industries in demand to make good products from fruits available, considering the population increase.

Keywords: *Aspergillus niger*, catalytic activity, concentration, pectinase, substrates

INTRODUCTION

Pectin is a type of structural fiber found in the primary cell wall and an intracellular layer of plant cells, mainly in fruits, such as apples, oranges, and lemons, among others, where they contribute to the firmness and structure of plant tissues (Sathyanarayana and Panda 2003; Shaibu et al. 2022). They are high molecular weight acid polysaccharides primarily made up of α (1→4) linked D-galacturonic acid residues (Kashyap et al. 2001). For example, citrus fruit contains 0.5%-3.5% pectin, which is largely present in the peel portion of the fruit (Sathyanarayana and Panda 2003).

Pectinases are a group of enzymes that catalyze the breakdown of pectins. They are classified according to their way of an attack on the galacturonan part of the pectin molecule. They can be distinguished between pectin methylesterases (EC 3.1.11.1) that deesterify pectins to low methoxyl pectins or pectic acid, and pectin depolymerases, which split the glycosidic linkages betweengalacturonosyl (methyl ester) residues. Polygalacturonases (PGs) split glycosidic linkage next to free carboxyl groups by hydrolysis, while pectate lyase (Pl) split glycosidic linkages next to free carboxyl groups by β-elimination. Both endo types of PGs and PALs (EC 3.2.1.15 and EC 4.2.2.2, respectively) are known by splitting the pectin chain randomly. ExoPGs (EC 3.2.1.67) release monomers or dimmers from the non-reducing end of the chain, whereas exo-PALs (Pectate disaccharide-lyase) (EC 4.2.2.9) release unsaturated dimmers from the reducing end. Highly methylated pectins are degraded by endo-pectin lyases (PL; EC 4.2.2.10) and also by a combination of PE (Pectinesterase) with PG or PAL (Sarkanen 1991). Pectinases have various sources, including bacteria, fungi, plants, insects, protozoans, and nematodes. Even though many fungi and bacterial species are known to be producers of pectinase, the fungi *Aspergillus niger* is the most commonly used in the industrial production of pectinase because they have a wide range of carbon sources and can easily be handled (De Vries and Visser 2001).

Considering the rising population globally, there is a concurrent rapid growth in food demand. The fruit juice industry constitutes a major part of the food industry, and pectinase is a key ingredient in producing food materials like wines and juices of fruit origin. According to Abdel-Moshen et al. (2016), pectinases take 25% of the ever-increasing global demand for enzymes in the food industry. Pectinase can be produced using submerged or solid-state fermentation (El Enshasy et al. 2018). They are known to have a wide range of applications in the biotechnology industry, from vegetable oil and fruit juice extraction and clarification to cotton scouring, wastewater treatment, and degumming of plant fibers (Abdel-Moshen et al. 2016). In tea fermentation, pectinase acts as an accelerator in breaking down the pectin present in the cell walls of tea leaves and also destroys the foam-forming

component of instant tea powders by hydrolyzing the pectins. The change in color of tea during the fermentation also results in the development of a characteristic aroma (Garg et al. 2016). Another use of pectinase is in biorefineries for hydrolyzing pectin present in pectin-rich agro-industrial wastes (Biz et al. 2014). These agro-industrial wastes are processed into simple sugars to be converted into bioethanol or used as fermentable sugars (Collares et al. 2012).

The value of pectinases in the food industry has made them an overly expensive commodity that is not affordable by many, despite their importance. Should pectinases be made more available at a less expensive rate, the fruit juice and wine industries will surely do better in producing more juice with no clouds or haze. However, the reverse is the case, in that there are fewer producers of the enzyme, and it is less available than expected, especially in Wukari, Taraba State, or even Nigeria as a whole. To meet the high pectinase demand, discovering new microbial sources capable of producing higher yields is imperative. Unfortunately, little or no work has been done on producing pectinase from locally sourced fungus, *A. niger,* in Wukari using orange bagasse as a carbon source. The present investigation was carried out to produce pectinase from a locally sourced fungus, *A. niger,* in Wukari, Taraba State, Nigeria.

MATERIALS AND METHODS

Study area and collection of orange bagasse samples

The present study was conducted at Federal University Wukari Central Research Laboratory, Wukari, Taraba state, Nigeria. Samples of orange bagasse were collected from Wukari new market, along Wukari-Jalingo Road, and brought to Central Research Laboratory in Federal University Wukari, Taraba state. The collected samples were washed with distilled water, minced into small particles, and sun-dried (approximately 30°C) for 5 days.

Sample preparation

Samples of orange bagasse were dampened and allowed for 5 days to decay. One gram of the decaying sample was weighed aseptically into 9 mL of sterile distilled water separately and shaken thoroughly. The dilutions were subsequently made up to 10^{-4} and poured plating of 10^{-2} and 10^{-4} was done in sterilized potato dextrose agar (PDA). After sterilization, streptomycin (100 mg/L) was added to the PDA to prevent bacterial growth. Plates were incubated in an inverted position at room temperature for 7 days. After incubation, the plates were observed for fungal growth, as Yu and Xu (2018) reported.

Morphological identification

The plates with colonies of fungal growth were identified by observing their microscopic characteristics, such as color, texture, appearance, and diameter of colonies compared to the atlas, and suspected colonies of *Aspergillus* species were subcultured and allowed to grow for 4 days.

Screening of fungal isolates

The screening method of Fahmy et al. (2008) was used. Five milliliters of iodine solution was flooded on one agar plate containing selected fungal colonies and incubated at room temperature for two days. The zone of hydrolysis around the colonies indicated the pectinolytic activity of *A. niger* and the colonies were preserved for further study. The primary screened fungal isolates were inoculated on pectinase screening agar medium (PSAM) and incubated at room temperature for 42 hrs. The PSAM contained as follows (in g/100 mL): $(NH_4)_2HPO_4$, 0.3; KH_2PO_4, 0.2; K_2HPO_4, 0.3; $MgSO_4$, 0.01, pectin, 1.0; and agar, 2.5.

Fermentation for pectinase production

Submerged fermentation (SmF) was carried out using the method of Tobechukwu et al. (2014). Four 250 mL Erlenmeyer flasks containing 50 mL of sterile cultivation medium made up of 0.1% NH_4NO_3, 0.1% $MgSO_4.7H_2O$, 0.1% $NH_4H_2PO_4$ and 1% ground orange bagasse were autoclaved at 121ºC for 15 min. To every sterile flask, one disc of actively growing *Aspergillus* species from a 4 days agar medium was added using a cork borer of diameter 10 mm and then plugged properly. The culture was incubated for 4 days at room temperature. Then, a flask was selected at 24 hrs intervals, and the mycelia biomass was separated by filtration using a muslin cloth. The filtrates were analyzed daily for pectinase activity by taking the absorbance using a spectrophotometer at 540 nm.

Polygalacturinase assay

One (1) mL of 1.0% sodium acetate buffer (pH 5.5) was added to each of the three test tubes labeled Tube 1, Tube 2, and Blank. Afterward, 1 mL of the enzyme (Pectinase) was added to Tube 1 and 2. The mixtures in the three test tubes were incubated for 5 min at 50ºC so that the enzymes in the mixtures could acclimate under this condition. To each of the three test tubes, 1 mL 1% pectin, which served as the substrate, was added and incubated for 30 min at 50ºC. 1.5 mL 3, 5 dintrosalicyclic acid reagent was added to each test tube. Pectinase activity was ascertained by taking the absorbance of the mixtures in the test tubes using a spectrophotometer at 540 nm.

Effect of pH on pectinase activity

The effect of pH on enzyme activity was determined using the method of Yu and Xu (2018). **S**odium acetate buffer (1M; pH range, 4.5-5.0), sodium phosphate buffer (1M; pH range, 6.0-7.0) and Tris-HCl buffer (1M; pH range, 8.0-9.0) were used. Tubes containing 0.5 mL of the respective buffers were mixed with 0.5 mL of the enzyme. Then, 1 mL of 0.5% (w/υ) pectin solution was added and all the tubes were then incubated at 30ºC for 10 min, after which the residual activity of the enzyme was assayed to obtain the optimum pH.

Effect of temperature on pectinase activity

The method of Tobechukwu et al. (2014) was used to assay for pectinase activity. Different temperatures range from 25-50ºC at 5ºC intervals, taking 1 mL of the crude enzyme and 1 mL of pectin into 12 test tubes (each

temperature with a test tube and an enzyme-blank test tube) in a water bath and the temperature at which the enzyme expressed maximum activity was taken to be its optimum temperature.

Effect of substrate concentration on pectinase activity

The method of Tobechukwu et al. (2014) was used to assay for the effect of substrate concentration on pectinase activity. The substrate (pectin) was prepared in different concentrations ranging from 0.5-2.0% at intervals of 0.5% in 8 test tubes to evaluate substrate concentration on pectinase activity. One millimeter of enzyme suspended in acetate buffer (0.1M, pH 5.4) was mixed with 1 mL of the various substrate concentrations, and the absorbance was taken using a spectrophotometer at 540 nm. The substrate concentration that showed the highest activity of the enzyme was taken to be the optimum concentration for the enzyme activity.

Kinetic parameters of pectinase

The method reported by Tobechukwu et al. (2014) was used in the determination of the kinetic constant and maximum velocity (K_m and V_{max}) of pectinase.

Statistical analysis

The results observed are the mean of three independent experimental replicates (n=3), and values are represented as the mean ± standard error.

RESULTS AND DISCUSSION

Screening of fungal isolates

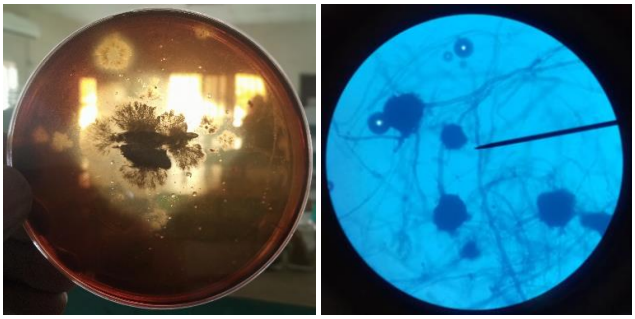
The ability of some fungal isolates to produce pectinase was tested by propagating on Pectinase Screening Agar Medium (PSAM). The result shows that the primary screened fungal isolates upon inoculation on PSAM and incubation at room temperature for 42 hrs showed the appearance of a clear zone which is an indication of pectinases production (Figure 1A). In addition, the morphological appearance of fungal isolates was identified by observing their macroscopic characteristics, such as color, texture, and appearance, under a microscope. The result revealed the following morphological characteristics: Shape-filamentous; Surface-suede-like surface; Opacity-opaque; color-blue-green (Figure 1B).

Fermentation for pectinase production

The selected isolates from the primary screening method were subjected to solid-state fermentation in a suitable medium and observed. The isolates were found to be able to consume pectin and produce pectinase. Pectinase production increased gradually over 24 hours of incubation until optimum pectinase production was achieved. After the optimum incubation period, pectinase production began to decrease. The result also revealed that pectinase production after 4-day fermentation gave maximum production on day 3 (Figure 2).

Effect of pH on pectinase activity

This study investigated the effect of various pH values on pectinase activity. At pH 2.0, pectinase activity was observed to be 2 µ/L. At pH 4.0, pectinase activity was 4 µ/L. At pH 4.5, pectinase activity was seen to be 6.9 µ/L. A decrease in pectinase activity was observed at pH 7.0 and 9.0, recording 5.0 µ/L and 5.1 µ/L, respectively. Maximum pectinase activity (7 µ/L) was recorded at pH 8.0. Varying the pH level affected pectinase activity (Figure 3).



A **B**

Figure 1. A. Screened fungal plate showing the appearance of a clear zone. B. Morphological appearance of fungal growth under the microscope

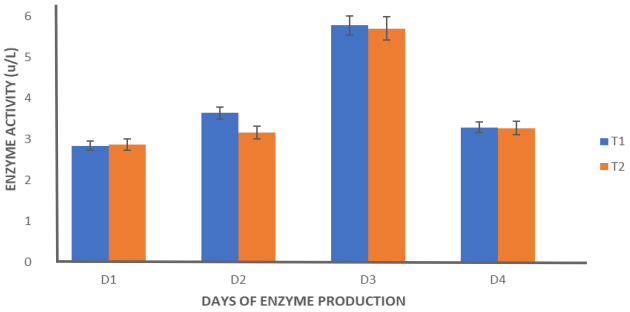


Figure 2. Pectinase production from submerged fermentation. The data represents mean ± standard error of replicates (n = 3). *T1- Tube one; T2- Tube two; D1-day one; D2- day two; D3- day three; D4-day four

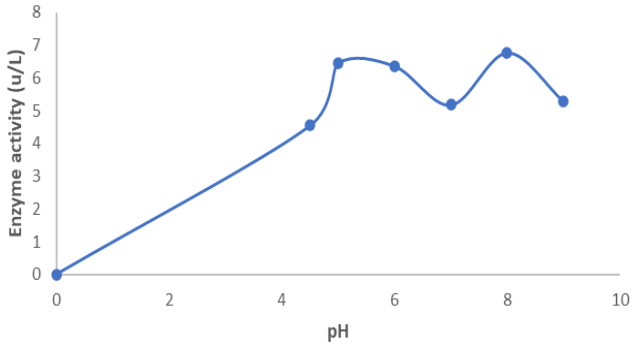


Figure 3. Effect of pH on pectinase activity

Effect of temperature on pectinase activity

The present study revealed that temperature change showed an effect on pectinase activity. At 10°C, pectinase activity was observed to be 1.8 µ/L. At 30°C, pectinase activity was observed to be 3.2 µ/L. A sharp decrease in pectinase activity was seen at 40-45°C, after which a sharp increase in pectinase activity was observed as the temperature kept increasing. The maximum temperature for pectinase activity observed was 50°C (Figure 4).

Effect of substrate concentration on pectinase activity

The result revealed that enzyme activity changed as substrate concentration varied. An increase in pectinase activity was observed as substrate concentration increased. Pectinase activity was quite stable from 0.5 substrate concentration to 1.0 substrate concentration. After which, an increase in pectinase activity was observed. At 0.5 substrate concentration, pectinase activity was observed to be 2.5 µ/L. At 1.0 substrate concentration, pectinase activity was observed to be 2.5 µ/L. At 1.5 substrate concentration, pectinase activity was observed to be 3.0 µ/L. Pectinase activity was revealed to be highest at 2.0 substrate concentration. At this substrate concentration, pectinase activity was 3.3 µ/L (Figure 5).

Kinetic parameters of pectinase

In this study, the kinetic parameters of pectinase were investigated. At 0.5 substrate concentration, the rate of reaction was found to be 0.18. At 1.0 substrate concentration, the rate of reaction was 0.32. At 1.5 substrate concentration, the rate of reaction was found to be 0.47. At 2.0 substrate concentration, the reaction rate was 0.59. A progressive increase in the reaction rate was observed as substrate concentration for the reaction increased. The K_m and V_{max} values for pectinase were found to be 5.93 and 2.22, respectively. The kinetic data and reciprocal plot (Lineweaver-Burk plot) for pectinase is presented below (Table 1; Figure 6).

Discussion

Pectinases have various screening assays that can be used to ascertain their presence in a pectin-containing medium. In this study, the screened fungal plate showed the appearance of a clear zone, connoting pectinase activity. Sudeep et al. (2020) reported that PSAM was used to grow the microorganism for 48 hrs. Iodine was used to flood the plates, and the appearance of a clearance zone affirmed pectinase activity. Fahmy et al. (2008) also reported using iodine to flood plates to observe the clearance zone on a plate.

The pectin extracted from orange bagasse was used to induce pectinase production in *A. niger* which had more pectinase activity under submerged fermentation. The entire fermentation process was carried out at a temperature of 50°C. The incubation period that gave peak production was 72 hrs, an average of 96 hrs, as reported by Tobechukwu et al. (2014) and 48 hrs reported by Sudeep et al. (2020). Chowdhury et al. (2017) reported maximum polygalacturonase activity on the 5th day of fermentation with *Penicillium chrysogenum*. Various factors can influence the production of pectinases. These include; concentration of nutrients, pH, temperature, moisture content, and influence of extraction parameters on the recovery of pectinases.

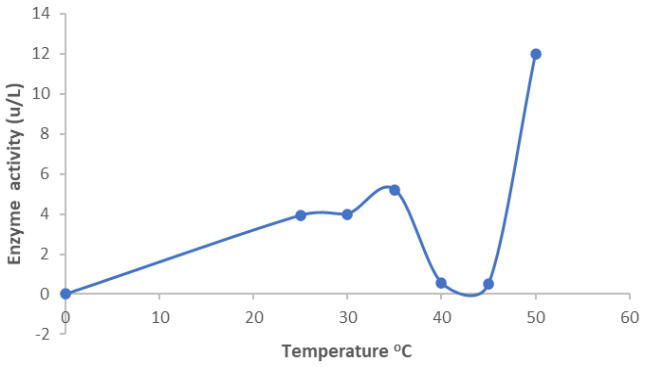


Figure 4. Effect of temperature on pectinase activity

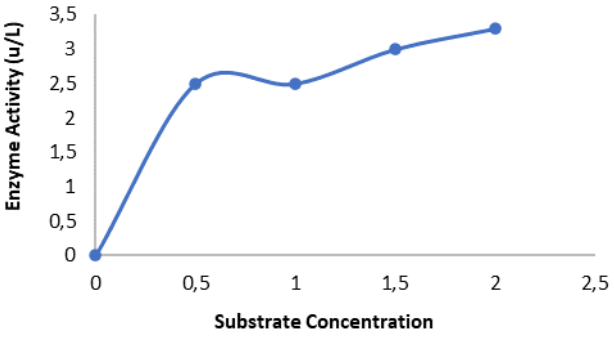


Figure 5. Effect of substrate concentration on pectinase activity

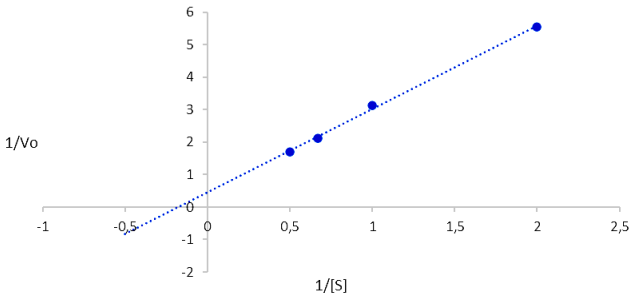


Figure 6. Lineweaver-Burk plot used for determination of Enzyme K_m and V_{max}. Intercept = 0.4499; V_{max} = 2.22

Table 1. Kinetic data for pectinase

[S]	V_o	1/[S]	$1/V_o$
0.5	0.18	2	5.56
1	0.32	1	3.13
1.5	0.47	0.67	2.13
2	0.59	0.5	1.7

One of the key factors that affect enzyme activity is pH. Some enzymes are more active in an acidic medium, while others prefer the neutral range and alkaline medium. The effect of pH on pectinase activity was determined at a pH range of 4.5-9.0 at intervals of 1.0 from pH 5.0 to check whether the enzyme activity is best expressed in an acidic, neutral, or alkaline medium. However, the pH that showed maximum enzyme activity was 8.0 (Figure 3), showing that the enzyme is more active in an alkaline medium. This is contrary to the report by Yu and Xu (2018), which showed maximum enzyme activity at pH 5.0, and De Vries and Visser (2001), which reported maximum activity at pH 6.5, both acidic media. Sudeep et al. (2020) reported that an acidic pH of 4.0-4.5 supports high pectinase activity. The pH optima for 30 fungal pectinases reported by Niture and Pant (2004) ranged from 2.5 to 6.0. According to Jayani et al. (2005), most microbial pectinases have an optimal pH of 3.5-5.5. However, Shet et al. (2018) reported that certain pectinolytic enzymes are more active in an alkaline medium; hence, this crude enzyme can be classified under such alkaline pH pectinases. According to Debing et al. (2006), pectinases are denatured at pH 8-9.5.

Temperature also greatly affects pectinase activity as it may reduce, give stable activity or express the maximum activity of the enzyme. The effect of temperature on pectinase activity in this study was assayed by subjecting the enzyme to different temperature values ranging from 25ºC-50ºC. The temperature at which the crude enzyme was most active was 50ºC (Figure 4). Khatri et al. (2015) and Sudeep et al. (2020) reported maximum pectinase activity at a temperature range of 30-50ºC. Jubayer et al. (2017) also reported that a temperature range of 40-45°C supports high pectinase activity. According to Jayani et al. (2005), most microbial pectinases have an optimal temperature range of 30-50ºC. Hence, the optimal temperature for pectinase activity obtained in this work agrees with the values obtained in the mentioned literature. These reports show that the enzyme can act optimally at temperatures between 30-50ºC.

Different substrate concentrations from 0.5-2.0% substrate (pectin) were used to test for the effect of substrate concentration (at intervals of 0.5) on the enzyme activity. As a result, the concentration of the crude enzyme was observed to express the highest activity at 2.0% pectin (Figure 5). On the other hand, Jubayer et al. (2017) reported maximum pectinase activity at 1.0% pectin concentration.

The kinetic parameters (K_m and V_{max}) of the enzyme were calculated by taking the double reciprocal of the plot. K_m and V_{max} of pectinase obtained were 5.93 and 2.22, respectively (Figure 6). Pectinases share a high degree of sequence homology, but their rate of pectin hydrolysis and hence, their kinetic constants can differ (Jubayer et al. 2017). Yu and Xu (2018) reported a K_m of 1.0 mg/mL and a V_{max} of 85 U/mg protein for pectinase isolated from *P. chrysogenum*. Siddiqui et al. (2012) reported a K_m value of 0.22 mg/mL for polygalacturonase from *Rhizomucor pusillus* isolated from decomposing orange peels. K_m values less than 0.15 and up to 5.0 mg/mL (<0.15-5.0 mg/mL) and specific activities 8.8-7000 U/mg were reported for some fungal pectinases by Sharma and Giridhar (2011).

In conclusion, the mass production of crude pectinase from a local isolate of *A. niger* in Wukari, using orange bagasse as a carbon source, is achievable under the following conditions; an incubation period of 72 hrs, pH of 8.0, a temperature of 50ºC, and substrate concentration of 2.0%. Depending on conditions that affect the enzyme activity per location, the aforementioned parameters can be adjusted to favor the mass production of pectinase for industrial use. The results obtained in this research can guide producers of pectinase on what parameters to vary for mass production of this enzyme.

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