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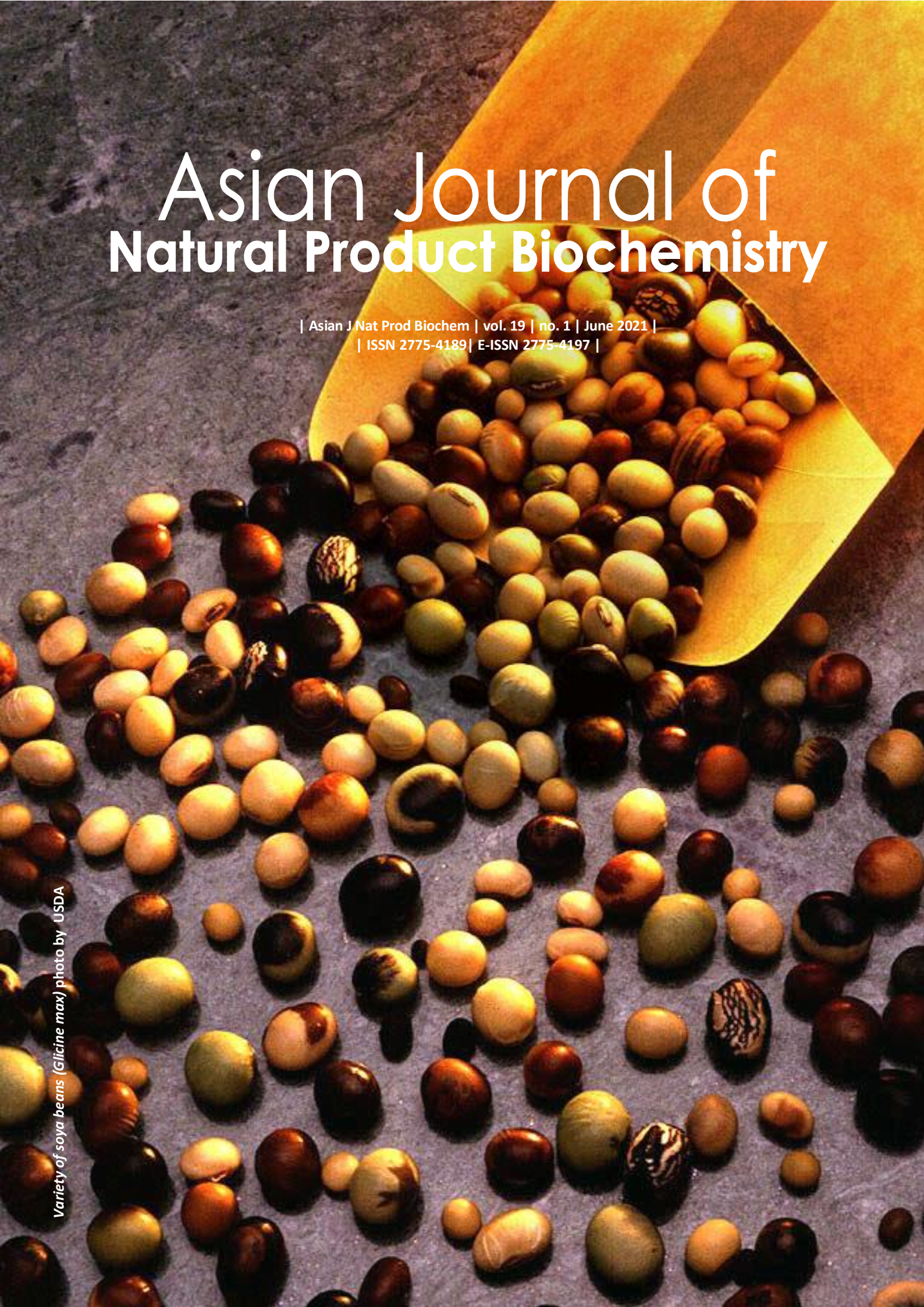

Variety of soya beans (Glicine max) photo by USDA

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Introduction is about 600 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for a manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgements** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

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The usage of "et al." in long author lists will also be accepted:

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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Review: Microbial induced mineralization of calcium carbonate for self-healing concrete

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Abstract. *Elkhateeb WA, Elnahas MO, Daba GM. 2021. Review: Microbial induced mineralization of calcium carbonate for self-Healing concrete. Asian J Nat Prod Biochem 19: 1-9.* Low-cost solutions achieving concrete self-healing are attracting researchers' attention. Generally, a concrete self-healing mechanism has been accomplished so far by three approaches: autogenous healing, encapsulation of polymeric material, and microbial-induced mineralization of calcium carbonate. The microbial approach seems an attractive, potent, and relatively cheap way to achieve concrete self-healing. Hence, this review elucidates the microbial concrete self-healing mechanisms and compares the roles of fungal and bacterial mediated self-healing concrete.

Keywords: Bacteria, biotechnology, calcium carbonate, fungi, self-healing concrete

INTRODUCTION

Concrete structures are the primary elements in any infrastructure system (Ghali et al., 2018; Hassoun and Al-Manaseer, 2020). Concrete infrastructure suffers from cracks that are formed due to many factors, such as freeze-thaw cycles, drying shrinkage, delayed ettringite (hydrous calcium aluminum sulfate mineral) formation, creep and fatigue, and reinforcement corrosion (Roodman et al. 1995; Vinoth et al. 2016; Menon et al. 2019). The problem with cracks is that they significantly weaken the durability of concrete structures because they channel in water, gases like oxygen, and carbon dioxide, which could potentially corrode the reinforcement of steel (Nama et al., 2015; Bossio et al., 2017). Furthermore, cracking may promote severe degradation of the non-mechanical properties of concrete (Menon et al., 2019). However, the implementation of continuous maintenance often requires extensive work and investment. Hence, low-cost maintenance and repair of harmful cracks without onerous work have attracted researchers' attention. A significant amount of research has been conducted during the past decade to understand how these harmful cracks could heal themselves without human intervention or interference. Three approaches have so far achieved the self-healing mechanism in concrete: autogenous healing (Edvardsen 1999), encapsulation of polymeric material (Dry 1994), and biologically induced mineralization of calcium carbonate (Decho 2010; Sisomphon et al. 2012; Reddy 2013; Krajewska 2018; Žáková et al. 2019). In this field, the microbial approach has attracted serious attention (Zhu and Dittrich, 2016; Joshi et al., 2017; Chaurasia et al., 2019). The history of using microbes to solve the concrete cracks problem started when a scientist (Hendrik Marius Jonkers) was inspired by the ability of the human body to heal minor damages on its own (self-healing). From this point, the word (Bioconcrete) has seen the light to describe a type of concrete capable of healing itself naturally. Using bioconcrete in the construction and maintenance of old structures will contribute to preserving the structure of the concrete and reducing overall costs (Harshali et al., 2016; Alves et al., 2019; Castro-Alonso et al., 2019). Additionally, microbe's contributions in this field are added to the list of biotechnological applications that emphasize microbes' ability to continuously serve humanity (Daba et al., 2018; Elkhateeb and Daba, 2019; Waghmode et al., 2019; Algaifi et al., 2020). The term "microbe" refers to many different types of organisms, but research work on self-healing concrete has been so far restricted to bacteria (Achal et al., 2011; Krishnapriya and Babu, 2015; Jin et al., 2018; Ruan et al., 2019). Bacteria-mediated self-healing concrete based on biologically induced mineralization processes has been extensively studied during the past decade (Dick et al. 2006; De Muynck et al. 2008; 2010; Jonkers et al. 2010; Nosouhian et al. 2016; Seifan et al. 2018). Many studies have demonstrated that bacteria are capable of precipitating calcium carbonate, which possesses high compatibility with concrete compositions, and this precipitation step is conducted via various biologically induced mineralization processes (Stanaszek-Tomal and Kozak, 2017; Wang et al., 2012; Ersan et al. 2015; Khaliq and Ehsan 2016; Seifan et al. 2016; Zhang et al. 2017; Gautam 2018).

The major technique employed to make bacteria-mediated self-healing concrete, spores of bacteria spores and their nutrients, are supplemented to the concrete matrix during the concrete mixing process. Later, cracks appear in concrete due to many reasons, and water comes in contact

with the dormant bacterial spores existing in the concrete mixture from the beginning. As a result, the bacterial spores grow and precipitate calcium carbonate to treat concrete cracks in situ. Then after full self-healing of cracks and consumption of all water inside, bacteria return to dormancy again. For old structures where bacterial spores were not mixed from the beginning with a concrete mixture, injection or spraying bacterial spores and their nutrients into the cracks are the techniques used to deal with that case (Pal et al. 2020). On the other hand, fungi appear as the potent perfect candidate for this mission (Martuscelli et al. 2020). Interestingly, many studies have also reported the microbial approach's ability to improve the physicomechanical characteristics of concrete, cement, and mortar (Abo-El-Enein et al., 2013; Sharaky et al., 2018; Girometta et al., 2019).

Microbial $CaCO_3$ has the advantage of being environmentally friendly; also, it is an economical material that could find promising and potential applications in the engineering field. It could protect the concrete and stone surfaces. Also, it could repair the developed flaws and defects and reduce the consolidation of loose particles (Cheng and Cord-Ruwisch 2012; De Muynck et al. 2008; Van Tittelboom et al. 2010; Wang et al. 2012). This review described microbial concrete self-healing by comparing fungal and bacterial mediated self-healing concrete roles.

Bacterial concrete healing

It is known that the lifetime of the concrete structures is negatively affected by several factors that lead to the formation of micro-cracks followed by the entrance of different materials, such as carbon dioxide, water, chlorine ions, etc., into these cracks. Finally, the results are corrosion and degradation of the concrete, which will need regular and expensive repairs as well as many maintenance works. Moreover, it is difficult to avoid further cracking in reinforced concrete due to many factors, including early-age shrinkage, thermal effects, mechanical loading, or it could be a combination of more than one factor (De Rooij et al. 2013; Isaacs et al., 2013).

Nowadays, several studies aim to find an economic bacterial-originated self-healing concrete in order to reduce the needed repair and maintenance of concrete structures. Autogenous concrete self-healing depends mainly on the concrete composition, and the substantial damage could be protected by the hydration reaction of the cementitious materials within the concrete structure. About 15-25% of the cement is found in an un-hydrated form in the concrete matrix, so once cracking occurs, these exposed un-hydrated cement grains will start to absorb water, and the hydration process will start, which in turn fills up and heals the developed cracks. This self-healing process is known as autogenous healing. This process could be efficient but only for very narrow cracks (Ter Heide 2005; Ter Heide and Schlangen 2007).

$$CaO + H_2O \rightarrow Ca(OH)_2 \quad (1)$$

$$Ca(OH)_2 + CO_2 \rightarrow CaCO_3 + H_2O \quad (2)$$

Bacterial spores have gained great importance in self-healing due to their ability to precipitate calcium carbonate. However, these spores show some drawbacks, including a short lifetime due to the presence of an alkaline medium as well as shrinkage of spores (Jonkers et al. 2010; Jonkers 2007). Cracks with widths up to 0.5 mm could be healed with these spores (Rao et al., 2013). The bacterial spores have the ability to be capsulated and incorporated into the concrete, which induces the concrete healing ability (Jonkers et al. 2010). When the weather gets humid, the bacteria become activated and start to reproduce, and then they will germinate in the calcium lactate. After that, the bacteria combine with the carbonate ions, and finally, insoluble calcium carbonate is formed, which is responsible for filling the cracks (Ramachandran et al. 2001). This process is known as Microbial Induced Calcium Carbonate Precipitation (MICCP), also known as Bio-mineralization (Kavia and Hema 2015).

After the bacterial activation process, many metabolic processes occur, including photosynthesis, sulfate reduction, and urea hydrolysis, which cause the formation of by-products, including calcium carbonate. *Bacillus* species represent the mineralized microorganism that is widely used in bio concrete. Under unfavorable conditions, *Bacillus* species form dormant spores that can resist several bactericidal factors such as heat, alkali, dryness, and organic solvents (Setlow 1994).

Various bacteria play an important role in calcium carbonate precipitation. Among these bacteria, we can mention *Bacillus cohnii, Bacillus halodurans, Bacillus pasteurii, Bacillus pseudofirmus Bacillus sphaericus, Bacillus subtilis*, etc. (Figure 1) (Vijay et al. 2017). Many factors affect the precipitation process, such as calcium concentration, the concentration of dissolved inorganic carbon, pH as well as nucleation sites (Jonkers 2011). Another important factor is the ability of the bacteria to utilize the organic nutrients and convert them to inorganic calcite crystals (insoluble), which in turn fill the concrete cracks (De Rooij et al., 2013).

The spray drying method was employed to obtain a huge amount of bacterial powder needed for concrete production. The produced-enriched bacterial solution was added to the spray dryer, where the bacterial solution was produced as a liquid droplet by atomization. Then rapidly, under high temperatures, it dries inside the spray dryer to finally form a powdery material. These steps cause the bacterial vegetative cells to be transformed into spores due to the presence of unfavorable conditions (high temperature and dryness), and the final obtained product is microbial spore powder (Zhang and Qian 2020).

Immobilization of the bacterial strain on polyurethane and spore formation techniques are gaining great attention in bonding the formed cracks in the concrete. Where the bacterial carbonate precipitation is produced due to the ureolytic activity as well as the bio-mineralization of the bacteria. In the presence of a calcium source, the bacterial strains are able to precipitate calcite through producing urea. The bacterial strains are added to the concrete along with calcium sources, phosphorous, and nitrogen

components. These ingredients may stay in the concrete for up to 200 years.

Effect of bacteria on concrete criteria

It was reported that the bacterial metabolic activity occurring in concrete improves concrete performance overall, including compressive strength (Saifee et al., 2015). Other studies found that the compressive strength increased significantly by 42% upon applying 105 cells/mL concentration and increased the tensile strength by about 63% after 28 days of treatment (Saifee et al., 2015). It was also found that concrete mass loss due to acid exposure was greatly reduced upon the microbial treatment.

Concrete water absorption ability was also evaluated, and the results showed a smaller mass increase for bacterial concrete compared to the control untreated concrete. Assuming that the bacterial concrete shows less porosity (Stanaszek-Tomal 2020). Moreover, introducing bacteria to the concrete also decreases the mass loss upon exposure to chloride and improves its compressive strength (Ravindranatha et al., 2014).

Bacillus pasteurii was added to concrete, and the results showed an increase in the initial strength of concrete. Moreover, the *Bacillus subtilis* strain was used in biological concrete since this strain is able to survive in extreme temperatures (from 30 ◦C up to 700 ◦C) (Manikandan and Padmavathi 2015), and also, an obvious increase in the concrete compressive strength was detected (Jagadeesha Kumar et al. 2013). *Bacillus flexus*, a bacterial strain that is not reported as calcite precipitating, also shows high compressive strength (Jagadeesha Kumar et al., 2013).

The bacteria can be introduced into the concrete using vascular systems or capsules. Some industrial by-products, including fly ash and silica fume, could replace the cement in concrete in order to reduce the porosity in concrete. The silica fume has the advantage of being porous and very fine and thus exhibits high bonding strength, which makes concrete denser and reduces the concrete permeability (Morsali et al. 2019). It was reported that replacing cement with other recycled or natural products helps in reducing the amount of cement content used in concrete, which helps decrease Co emission in the cement manufacturing process.

Various inorganic porous materials have been used by researchers recently (Alazhari et al. 2018), such as materials are graphite nano-platelets (Khaliq and Ehsan 2016), ceramsite (Chen et al. 2016), lightweight aggregates (Chen et al. 2019; Zhang et al. 2017), hydrogel (Wang et al. 2014), zeolite (Bhaskar et al. 2017), polyurethane, glass tubes (Wang et al. 2010), as well as expanded clay particles or expanded perlite (Jiang et al. 2020). These porous materials are employed as carriers protecting the bacteria from the concrete unfavorable alkaline environment. A suitable environment is created inside the pores that help bacteria grow safely. A study was reported where the sugar coating was used in order to immobilize both nutrients and bacteria (Jiang et al. 2020).

Some researchers applied *Bacillus pasteurii* bacteria with rice husk ash (15%) as well as micro-silica (10%) into the concrete. The results showed an increase in bacterial strength by 21% comparing to the control sample (at 10^5 cells/mL and after 28 days) (Ameri et al., 2019). Since the nanomaterials are well established in many studies, some researchers use nanoparticles or microparticles of iron oxide or bentonite, which aid in immobilizing the bacteria (Shaheen et al., 2019). It was found that bacteria immobilization with iron oxide-based media was able to heal concrete cracks with widths up to 1.2 mm, and the compressive strength markedly increased to 85% of that of the control samples. However, bentonite immobilization resulted in cracks healing with widths up to 0.15 mm and 0.45 mm, where the compressive strength increased by 45% and 65%, respectively c,ompared to the control sample.

The microbial self-healing concrete is applied mainly at the sluice chamber bottom plate as well as at the junction of the side walls. The cementitious materials produce a huge amount of hydration heat during the hydration process, where the concrete internal temperature increases sharply (Ataie 2019). On the other hand, the outer surface of the structure loses heat so fast, creating an obvious temperature gradient through the sidewall (Yang et al. 2016). The difference in temperature between internal layers and the surface of the concrete resulted in shrinkage and, finally, damage to the concrete surface due to the formation of cracks (Yang et al. 2019). Besides, the bottom plate was placed before the sidewall, leading to a restriction of the free contraction of the newly poured concrete (of the sidewall). That's why microbial self-healing concrete is added to this part.

These bio-concrete exhibit many advantages. Applying bio-concrete significantly increases the strength of concrete. It also provides effective resistance to freeze-thaw conditions and reduces the corrosion and permeability properties compared to conventional concrete. Besides the economic advantage as it reduces the repairing and maintenance costs. On the other hand, the bio-concrete may show some drawbacks, where some of the used bacteria that grow in concrete may be harmful for atmosphere and human health. So its usage is limited to some structures. Also, the cost of this kind of concrete is high comparing to the conventional concrete (about 7-28% more than conventional concrete) (Kumari 2015). The durability of bio-concrete depends on many factors. The bacterial strain itself is considered the most important factor that affects the strength of the concrete. The studies showed that *Bacillus subtilis* increases the concrete strength by 6.42% and 9.16% after being used for 7 days and 28 days respectively, however, *Bacillus pasteurii* was found to increase the strength by 29.99% and 29.97% after 7 days and 28 days, (Bashir et al. 2016) respectively.

Bacillus sphaericus showed the highest concrete strength where the strength of the concrete increased by about 65.93% and 52.42% for 7 days and 28 days respectively. Other studies reported the effect of *Bacillus cereus* and *Bacillus pasteurii* on cement mortar compressive strength and it was observed 38% and 28% strength increment respectively (Maheswaran et al. 2014). Moreover, *Sporosarcina pasteurii* shows compressive strength of 38.2 MPa and 44 MPa for 28 days and 91 days

respectively (Chahal et al. 2012) however incorporating *Bacillus pasteurii* exhibit no negative effect on concrete hardened properties (Gavimath et al. 2012). Applying *Shewanella* in bio-concrete shows positive potential and compressive strength was increased by 17% and 25% after 7 and 28 days respectively. Nevertheless, *Escherichia coli* shows no effective change in the compressive strength upon incorporation in bio-concrete (Ghosh et al. 2005). In conclusion, selection of microorganisms is considered a critical factor to alter the concrete properties (Ghosh et al. 2005).

Moreover, comparing to the control concrete, the chloride penetration and water permeability decrease significantly (Li et al. 2012), after the concrete bio-remediation by applying *Bacillus pasteurii* and *Bacillus cereus* (Maheswaran et al. 2014). It was reported that incorporating *Bacillus halodurans* Strain KG1 in the concrete mix resulted in a decrease in the porosity and water absorption 12.4% and 20% at 91 days respectively (Kunal et al. 2016), plus an obvious increase against water penetration and other hazardous materials was reported (Senthilkumar et al. 2014). The studies also showed that *Bacillus sphaericus* addition to the concrete mix improves its strength against permeability as a result of bio deposition (Basheer and Cleland 2011; Basheer et al. 1997). Another advantage of bio-remediated concrete is its capacity to resist chloride penetration which in turn decrease corrosion rate of reinforcement (Dai et al. 2010; Ibrahim et al. 1997; Medeiros and Helene 2009). it was also reported in another article that *Bacillus pasteurii* reduces chloride permeability, porosity, and water absorption, and interestingly the incorporation of bacteria in the concrete mix reduces the capillary absorption capacity by about 20% compared to control mix (Giannaros et al. 2016).

Cement-based concrete together with added and GGBFS (ground granulated blast furnace slag) and silica fume was found to increase the compressive strength where the mixture containing about 35% GGBFS exhibits a compressive strength of 56 N/mm^2 (Depaa and Kala 2018). And the mixture reached its maximum strength (37 N/mm^2) with an addition of 12.5% of silica fume resulted in increasing the strength (37 N/mm^2). The compressive strength of concrete was greatly enhanced to reach the maximum value at a cell concentration of 10^5 /mL (Reddy et al. 2015). Other studies showed that 5% of bacterial additives together with calcium lactate were employed in microbial concrete and resulted in improving the concrete compressive strength to be 49.5 MPa at 28 days (Babu and Siddiraju 2016).

Bacillus pasteurii was also cultured on a medium modified with the addition of calcium chloride and urea (Ramakrishnan et al. 1999). The compressive strength of cement mortar was reported to be 65 MPa for 28 days where that of the control mortars was 55 MPa, without the addition of any bacterial cells. The authors observed an improvement in compressive strength in mortars as it increased by 17% for 7 days, and by 25% for 28 days (Ghosh et al. 2005).

Cost for bacterial concrete production

Some studies focus on the cost of applying microbial concrete comparing to the utilization of conventional concrete (Vekariya and Pitroda 2013; Reddy et al. 2015; Dinesh et al. 2017). It was reported that the cost of microbial concrete is about 2.3 to 3.9 times higher than that of conventional concrete. The reason for this high price is that the cost of bacteria and the required nutrients consumed to develop the material account for about 80% of raw materials cost.

Some nutrient ingredients such as cheap industrial wastes (lactose mother liquor produced from starch industry or liquid corn) that contain high amount of protein content have been used in order to reduce the cost of bacterial concrete production (Jonkers 2007). Using these materials significantly reduces the price of the production. Moreover, the self-repair capability of the bacterial concrete that results in extending the building life and hence reducing the total cost is not recognized by the investors.

The majority of the contractors provide buildings warranty for nearly 10 years and during this period of time, few cracks were observed. Thus, the advantages and benefits got from using bacterial concrete might not be touched for many years that could be more than ten years. This reason results in discouraging the contractors to use this bacterial concrete. However, the benefits of using self-healing concrete are definitely beyond any economic advantages (Silva et al. 2015). More studies are required to reduce the cost of culturing bacteria that will lower the initial cost, and this will attract the attention of the contractors.

Why fungi are the perfect candidates used for self-healing concrete

One of the major obstacles facing the use of bacteria in calcium mineral precipitation is the limited ability of the majority of bacteria to survive and resist the extreme environment in concrete such as high alkalinity, varied temperature, and dry condition of concrete (Seifan et al. 2016). So far there has been little success with respect to the long-term healing efficacy and in-depth consolidation, mainly due to the limited survivability and carcinogenic ability of the bacteria, and the recorded viability of bacterial spores embedded in concrete did not exceed six months (Jonkers 2011). Due to the above-mentioned, further screening for other types of microorganisms having the ability to catalyze calcium mineral precipitation becomes of great potential importance (Luo et al. 2018).

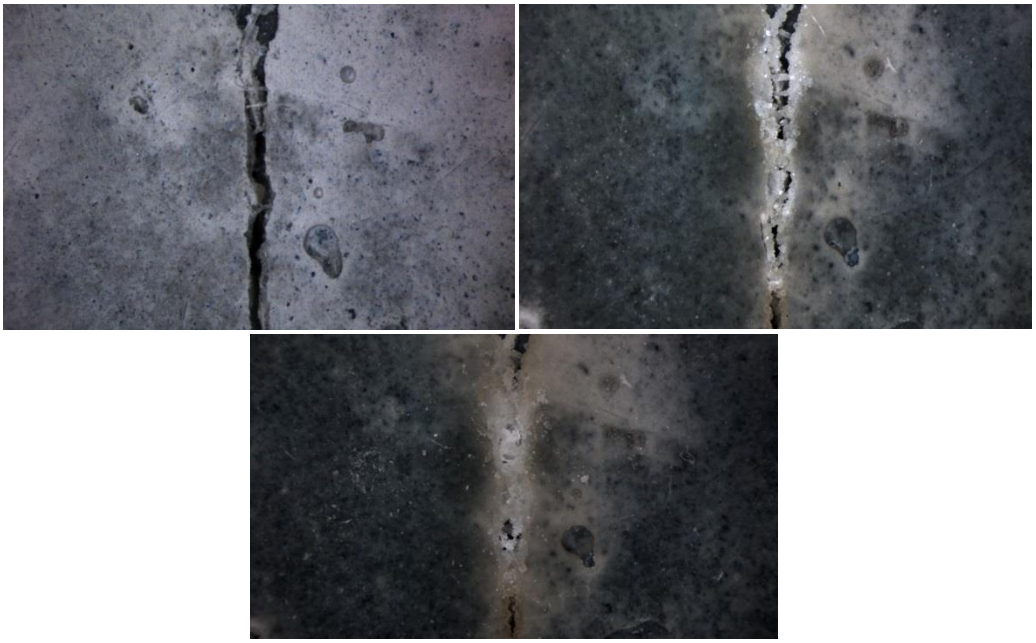

Figure 1. Bacterial self-healing concrete at different stages (hosted by https://www.thestructuralengineer.info)

Fungi could be one of the best candidates to be used as self-healing agent due to many reasons. First of all, filamentous fungi grow as threadlike structures called hyphae which grow to create an intertwined 3D network called mycelium which acts as biological fiber that improves the physicomechanical characteristics of bioconcrete (Martuscelli et al. 2020). Also, filamentous fungi have higher surface-to-volume ratios, and hence possess a larger fraction of organic substrates available for mineral precipitation (Menon et al. 2019). Moreover, fungi can survive in metal-polluted environment (Dias et al. 2002). The fungal mediated self-healing concrete possesses long term self-healing capacity that heals wider cracks within shorter periods of time. Beside, fungal spores and nutrients, could lead to no negative consequences on concrete compressive strength (Jin et al. 2018; Menon et al. 2019). Fungi can also promote large amount of calcium carbonate precipitates within short periods of time (Rautaray et al. 2003; 2004; Ahmad et al. 2004; Luo et al. 2018).

Generally, fungal mineral precipitation is conducted through induced biomineralization and organomineralization processes (Menon et al. 2019). There are two critical factors influencing the amount of produced Calcium carbonate which are Ca^{++} concentration, and carbonate alkalinity (Bindschedler et al. 2016). Many fungal metabolic activities can increase carbonate alkalinities such as physicochemical degassing of fungal produced CO_2, water consumption, oxidation of organic acids, nitrate assimilation, and urea mineralization (Menon et al. 2019). On the other hand, fungal metabolic activities can also affect the concentration of calcium ions. Regulation of calcium ions concentration within fungal cells takes place by keeping Ca^{++} concentrations sufficiently low in the cytoplasm by pumping it out of the cell or by binding it onto cytoplasmic proteins (Bindschedler et al. 2016).

Mechanism of calcium ions precipitation is a way to protect fungal cells from the calcium-rich environment characterizing concrete, which represents stress for fungal cells due to cell toxicity by high concentrations of calcium ions and subsequent osmotic pressure. To decrease the internal calcium ions concentrations, fungal cells produce oxalic acid which converts calcium ions to calcium oxalates (Neville 1996; Verrecchia 2000). Precipitation of Calcium carbonate may be due to a similar passive mechanism to immobilize excessive calcium ions. Similarly, high alkalinity represents stress on fungal cells that are faced by precipitating Calcium carbonate to protect the fungus intracellularly (Figure 2). The cell walls of fungi contain chitin which can bind calcium ions. Interestingly, both living and dead fungal biomass can bind ions onto their cell walls, then bound calcium ions can interact with the dissolved carbonate, resulting in precipitation of calcium carbonate on the fungal hyphae which means that fungi could be used as self-healing agents. Besides being an eco-friendly process, the application of microbial precipitation of $CaCO_3$ by urea hydrolysis fills cracks in concrete as a surface solution to cracks problem, or as an integrated curative agent. This type of treatment will ensure no need for future repair or inspection, which promotes increased durability of the structure, and decrease overall cost of maintenance (Mamo and Mattiasson 2019; Martuscelli et al. 2020).

Preliminary laboratory investigations to evaluate fungal ability to precipitate calcium carbonate is focussing usually on placing the fungus under investigation onto cured concrete plate then observe change in pH (increase) resulting from the ability of the fungus to dissolve Ca $(OH)_2$ from concrete. Analyses such as X-ray diffraction

(XRD) and scanning electron microscope (SEM) are usually conducted to visualize mycelial growth and confirm that the crystals precipitated on the fungal hyphae were composed of calcite (Luo et al. 2018; Sheir et al. 2020). Some of the important criteria that control choosing fungi in concrete self-healing to make sure that the chosen alkaliphilic fungus can be easily cultured and could survive in the extreme environment of concrete, also to be not pathogenic to human or animals health (Qian et al. 2019; Sidiq et al. 2019). Also, it is better to choose fungus showing high ureolytic activity (Kumari et al. 2016; Bhina et al. 2019). Many fungal species have been reported as promising bio-based self-healing concrete such as *Aspergillus niger* (Sayer et al. 1997); *Serpula himantoides* (Gharieb et al. 1998); *Paecillomyces lilacinus*, and *Chrysosporium* spp. (Magan 2007); *Neurospora crassa* (Li et al. 2014); *Pestalotiopsis* sp. and *Myrothecium gramineum* (Li et al. 2015); *Trichoderma reesei* (Luo et al. 2018); *Aspergillus nidulans* (Menon et al. 2019). Besides the previously mentioned optical analyses, Many techniques are used in order to evaluate concrete self-healing efficiency including measuring concrete stiffness, strength, and durability (Kang and Zhongxian 2012). Water and gas permeability, and absorption tests are also used for this purpose (Huang and Ye 2015). However, it is not easy to know the appropriate strength of concrete because it depends on the material structure as well as the mechanical reactions in concrete composites.

Interestingly, the reported role of fungi in the biomineralization process extends also to calcite, metals, and sand (Bindschedler et al. 2016; Oggerin et al. 2016; Povedano-Priego et al. 2017; Fang et al. 2018; Pasquale et al. 2019).

Future suggestions

The production of self-healing concrete is an interesting point that attracts the attention of many scientists. This review aims to refer to the previous researches that show the production methods of the self-healing concrete as well as dealing with some of the problems that face the production methods.

It was mentioned previously that the self-healing ability is based on placing some materials inside the concrete structure during its production process and which resists further damage. The key in this repairing process is the activation of the added repair materials and this activation initiates under stress when the concrete is subjected to stress. The self -repair could be active or passive. The active self-repair occurs as a result of an external activation for the used repair material, such as activation due to heating effect. On the other hand, the passive self-repair process is due to an automatic reaction to an external agent and it takes place without any human intervention (Łukowski and Adamczewski 2013).

Many studies show that there is great progress in producing these materials. Bacterial and other additive modification leads to very promising results. This was proved in concrete with nano-TiO_2. And it was found that it is sufficient to apply bacterial concrete only as topping plaster or a coating. Nowadays, many studies are conducted using bacterial concrete in repair concrete or mortar spraying (Sisomphon et al. 2012).

Although microbial concrete greatly improves the building materials quality, it is not found to be used on an industrial scale. This return to the difficulty in predicting future technology for these new materials. Many problems that are facing the bacterial concrete have been discussed by Saifee et al. (2015).

Many of the problems have been solved by many scientists who are almost reaching to promising solutions for these problems. Nevertheless, the problem of dealing with harmful bacteria is still needs more effort to find more safe strains that could be harmless to human health. Plus, there is not enough research about the durability of these materials nor biological cement environment effects that could occur upon biological corrosion. So future studies are needed to answer many questions such as does the produced calcium carbonate protects the used materials, and to what extent it would affect the growth of other microorganism's spores that are dispersed in air?

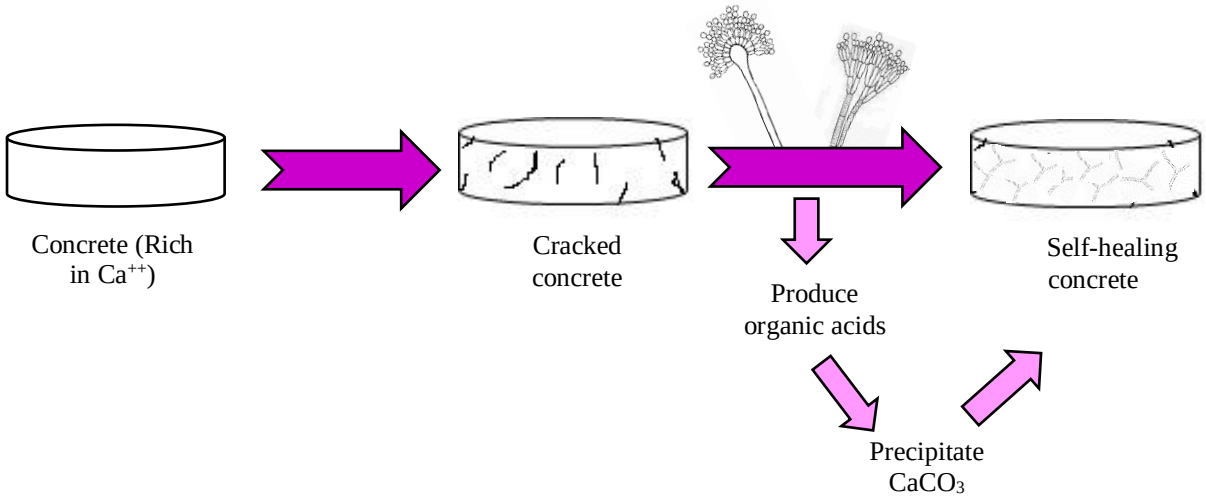

Figure 2. Schematic representation showing fungal self-healing concrete

CONCLUDING REMARK

Bacterial and fungal-mediated self-healing concrete based on biologically induced mineralization processes has been extensively studied during the past decade, but it still has important limitations. Fungi, as one of the best candidates to be used as self-healing agents due to their superior ability to adjust to the deleterious environment of concrete and extraordinary capability to promote calcium mineralization, should no longer be deserted. Also, investigation on fungi-mediated self-healing concrete is urgently needed. On the other hand, genetic engineering techniques should be employed to improve properties of promising strains capable of participating in microbial self-healing concrete.

REFERENCES

Abo-El-Enein SA, Ali AH, Talkhan FN, Abdel-Gawwad HA. 2013. Application of microbial biocementation to improve the physicomechanical properties of cement mortar. HBRC J 9 (1): 36-40.

Achal V, Mukherjee A, Reddy M. 2011. Effect of calcifying bacteria on permeation properties of concrete structures. J Ind Microbiol Biotechnol 38 (9): 1229-1234.

Ahmad A, Rautaray D, Sastry M. 2004. Biogenic calcium carbonate: calcite crystals of variable morphology by the reaction of aqueous Ca2+ ions with fungi. Adv Funct Mater 14: 1075-1080.

Alazhari M, Sharma T, Heath A, Cooper R, Paine K. 2018. Application of expanded perlite encapsulated bacteria and growth media for self-healing concrete. Construction Building Mater 160: 610-619.

Algaifi HA, Bakar SA, Sam AR, Ismail M, Abidin AR, Shahir S, Altowayti WA. 2020. Insight into the role of microbial calcium carbonate and the factors involved in self-healing concrete. Construction Building Mater 254: 119258.

Alves L, Alves L, Mello M, Barros SD. 2019. Characterization of Bioconcrete and the Properties for Self-Healing. In: Multidisciplinary Digital Publishing Institute Proceedings 38 (1): 4-12.

Ameri F, Shoaei P, Bahrami N, Vaezi M, Ozbakkaloglu T. 2019. Optimum rice husk ash content and bacterial concentration in self-compacting concrete. Construction Building Mater 222: 796-813.

Ataie FF. 2019. Influence of cementitious system composition on the retarding effects of borax and zinc oxide. Materials 12 (2019): 2340.

Babu NG, Siddiraju S. 2016. An experimental study on strength and fracture properties of self-healing concrete. Int J Civil Eng Technol 7: 398-406.

Basheer L, Cleland DJ. 2011. Durability and water absorption properties of surface-treated concretes. Mater Struct 44: 957-967.

Basheer P, Basheer L, Cleland D, Long A. 1997. Surface treatments for concrete: assessment methods and reported performance. Construction Building Mater 11: 413-429.

Bashir J, Kathwari I, Tiwary A, Singh K. 2016. Bio concrete-the self-healing concrete. Indian J Sci Technol 9: 1-5.

Bhaskar S, Hossain KM, Lachemi M, Wolfaardt G, Kroukamp M. 2017. Effect of self-healing on strength and durability of zeolite-immobilized bacterial cementitious mortar composites. Cement Concrete Composites 82: 23-33.

Bhina MR, Salim M, Masroor MS. 2019. An overview on fungi as self-healing agent in biomineralization of calcite. Int Res J Eng Technol 6: 2980-2989.

Bindschedler S, Cailleau G, Verrecchia E. 2016. Role of fungi in the biomineralization of calcite. Minerals 6 (2): 41-59.

Bossio A, Lignola GP, Fabbrocino F, Monetta T, Prota A, Bellucci F, Manfredi G. 2017. Nondestructive assessment of corrosion of reinforcing bars through surface concrete cracks. Struct Concrete 18 (1): 104-117.

Castro-Alonso MJ, Montañez-Hernandez LE, Sanchez-Muñoz MA, Macias Franco MR, Narayanasamy R, Balagurusamy N. 2019. Microbially Induced Calcium carbonate Precipitation (MICP) and its potential in Bioconcrete: Microbiological and molecular concepts. Front Mater 6: 126. DOI: https://doi.org/10.3389/fmats.2019.00126.

Chahal R. Siddique, A. Rajor. 2012. Influence of bacteria on the compressive strength, water absorption and rapid chloride permeability of fly ash concrete. Construction Building Mater 28: 351-356.

Chaurasia L, Bisht V, Singh LP, Gupta S. 2019. A novel approach of biomineralization for improving micro and macro-properties of concrete. Construction Building Mater 195: 340-351.

Chen H, Qian C, Huang H. 2016. Self-healing cementitious materials based on bacteria and nutrients immobilized respectively. Construction Building Mater 126: 297-303.

Chen H-J, Peng C-F, Tang C-W, Chen Y-T. 2019. Self-healing concrete by biological substrate. Materials 12: 4099.

Cheng L, Cord-Ruwisch R. 2012. In situ soil cementation with ureolytic bacteria by surface percolation. Ecol Eng 42: 64-72.

Daba GM, Elkhateeb WA, Thomas PW. 2018. This era of biotechnological tools: an insight into endophytic mycobiota. Egypt Pharm J 17 (3): 121.

Dai J-G, Akira Y, Wittmann F, Yokota H, Zhang P. 2010. Water repellent surface impregnation for extension of service life of reinforced concrete structures in marine environments: the role of cracks. Cement Concrete Composites 32: 101-109.

De Muynck W, Cox K, De Belie N, Verstraete W. 2008. Bacterial carbonate precipitation as an alternative surface treatment for concrete. Construction Building Mater 22: 875-885.

De Muynck W, De Belie N, Verstraete W. 2010. Microbial carbonate precipitation improves the durability of cementitious materials: a review. Ecol Eng 36: 118-36.

De Muynck W, Debrouwer D, De Belie N, Verstraete W. 2008. Bacterial carbonate precipitation improves the durability of cementitious materials. Cement Concrete Res 38: 1005-1014.

De Rooij M, Van Tittelboom K, De Belie N, Schlangen E. 2013. Self-healing phenomena in cement-Based materials: state-of-the-art report of RILEM technical committee 221-SHC: self-Healing phenomena in cement-Based materials. Springer 2013.

Decho AW. 2010. Overview of biopolymer-induced mineralization: what goes on in biofilms?. Ecol Eng 36 (2): 137-144.

Depaa R, Kala TF. 2018. An experimental study on fly ash as self-healing material. Int J Appl Eng Res 13: 5920-5925.

Dias MA, Lacerda IC, Pimentel PF, De Castro HF, Rosa CA. 2002. Removal of heavy metals by an *Aspergillus terreus* strain immobilized in a polyurethane matrix. Lett Appl Microbiol 34 (1): 46-50.

Dick J, De Windt W, De Graef B, Saveyn H, Van der Meeren P, De Belie N. 2006. Bio-deposition of a calcium carbonate layer on degraded limestone by *Bacillus* species. Biodegradation 17: 357-367.

Dinesh S, Shanmugapriyan R, Namitha S. 2017. A Review on Bacteria-Based Self-Healing Concrete. Imperial J Interdiscip Res 3 (1): 2454-1362.

Dry C. 1994. Matrix cracking repair and filling using active and passive modes for smart timed release of chemicals from fibers into cement matrices. Smart Mater Struct 3: 118-123.

Edvardsen C. 1999. Water permeability and autogenous healing of cracks in concrete. ACI Mater J 96: 448-454.

Elkhateeb WA, Daba GM. 2019. Myrothecium as promising model for biotechnological applications, potentials and challenges. Biomed J Sci Tech Res 16 (3): 12126-12131.

Ersan YC, De Belie N, Boon N. 2015. Microbially induced CaCO3 precipitation through denitrification: an optimization study in minimal nutrient environment. Biochem Eng J 101: 108-118.

Fang C, Kumari D, Zhu X, Achal V. 2018. Role of fungal-mediated mineralization in biocementation of sand and its improved compressive strength. Int Biodeterior Biodegrad 133: 216-220.

Gautam BR. 2018. Bacteria based self-healing concrete-a bacterial approach. Int J Eng Sci 57-61.

Gavimath C, Mali B, Hooli V, Mallpur J, Patil A, Gaddi D, Ternikar C, Ravishankera B. 2012. Potential application of bacteria to improve the strength of cement concrete. Int J Adv Biotechnol Res 3: 541-544.

Ghali A, Favre R, Elbadry M. 2018. Concrete structures: Stresses and deformations: Analysis and design for serviceability. CRC Press.

Gharieb MM, Sayer JA, Gadd GM. 1998. Solubilization of natural gypsum (CaSO42H2O) and formation of calcium oxalate by *Aspergillus niger* and *Serpula himantioides*. Mycol Res 102: 825-830.

Ghosh P, Mandal S, Chattopadhyay B, Pal S. 2005. Use of microorganisms to improve the strength of cement mortar. Cement Concrete Res 35: 1980-1983.

Giannaros P, Kanellopoulos A, Al-Tabbaa A. 2016. Damage recovery in self-healing concrete. In: Proceedings of HealCon Conference, Delft, the Netherlands, 28-29 November 2016.

Girometta C, Picco A, Baiguera R, Dondi D, Babbini S, Cartabia M, Pellegrini M, Savino E. 2019. Physico-mechanical and thermodynamic properties of mycelium-based biocomposites: a view. Sustainability 11 (1): 281.

Harshali J, Mitali S, Neha A, Pragati B. 2016. Bio concrete and bacteria-based self-healing concrete. Int J Res Eng Technol 5: 95-99.

Hassoun MN, Al-Manaseer A. 2020. Structural concrete: theory and design. John Wiley & Sons, New York.

Huang H, Ye G. 2015. Self-healing of cracks in cement paste affected by additional Ca^{2+} ions in the healing agent. J Intelligent Mater Syst Struct 26 (3): 309-320.

Ibrahim M, Al-Gahtani A, Maslehuddin M, Almusallam A. 1997. Effectiveness of concrete surface treatment materials in reducing chloride-induced reinforcement corrosion. Construction Building Mater 11: 443-451.

Isaacs B, Lark R, Jefferson T, Davies R, Dunn S. 2013. Crack healing of cementitious materials using shrinkable polymer tendons. Struct Concrete 14: 138-147.

Jagadeesha Kumar B, Prabhakara R, Pushpa H. 2013. Effect of bacterial calcite precipitation on compressive strength of mortar cubes. Int J Eng Adv Technol 2: 486-491.

Jiang L, Jia G, Jiang C, Li Z. 2020. Sugar-coated expanded perlite as a bacterial carrier for crack-healing concrete applications. Construction Building Mater 232: 117222.

Jin C, Yu R, Shui, Z. 2018. Fungi: A neglected candidate for the application of self-healing concrete. Front Built Environ 4 (62): 1-8. DOI: https://doi.org/10.3389/fbuil.2018.00062.

Jonkers H. 2007. Self healing concrete: a biological approach. In Self-healing materials. Springer 195-204.

Jonkers HM. 2011. Bacteria-based self-healing concrete. Heron 56: 1-122.

Jonkers HM, Thijssen A, Muyzer G, Copuroglu O, Schlangen E. 2010. Application of bacteria as self-healing agent for the development of sustainable concrete. Ecol Eng 36: 230-235.

Joshi S, Goyal S, Mukherjee A, Reddy MS. 2017. Microbial healing of cracks in concrete: a review. J Ind Microbiol Biotechnol 44 (11): 1511-1525.

Kang C, Zhongxian J. 2012. Evaluation of autogenous healing ability of cementitious composites [Dissertation] Nagoya University (2012): 1-7.

Kavia K, Hema C. 2015. Dressing of structural cracks using Bio Concrete. Indian J Sci Technol 8: 1-4.

Khaliq W, Ehsan MB. 2016. Crack healing in concrete using various bio influenced self-healing techniques. Construction Building Mater 102: 349-357.

Krajewska B. 2018. Urease-aided calcium carbonate mineralization for engineering applications: A review. J Adv Res 13: 59-67.

Krishnapriya S, Babu DV. 2015. Isolation and identification of bacteria to improve the strength of concrete. Microbiol Res 174: 48-55.

Kumari B. 2015. Microbial concrete: A multi-purpose building material-An overview. Int J Adv Eng Technol 7: 1608-1619.

Kumari D, Qian XY, Pan X, Achal V, Li Q, Gadd GM. 2016. Microbially-induced carbonate precipitation for immobilization of toxic metals. In: Advances in applied microbiology. Acad Press 94: 79-108.

Kunal Siddique R, Rajor A, Singh M. 2016. Influence of bacterial-treated cement kiln dust on strength and permeability of concrete. J Mater Civil Eng 28: 04016088.

Li PH, Wang K, Wang ZJ. 2012. Remediation and improvement of concrete by bacterial mediated carbonate deposition. Adv Mater Res 3373-3376.

Li Q, Csetenyi L, Gadd GM. 2014. Biomineralization of metal carbonates by *Neurospora crassa*. Environ Sci Technol 48 (24): 14409-14416.

Li Q, Csetenyi L, Paton GI, Gadd GM. 2015. $CaCO_3$ and $SrCO_3$ bioprecipitation by fungi isolated from calcareous soil. Environ Microbiol 17 (8): 3082-3097.

Łukowski P, Adamczewski G. 2013. Self-repairing of polymer-cement concrete. Bull Polish Acad Sci. Tech Sci 61 (1): 195-200.

Luo J, Chen X, Crump J, Zhou H, Davies D, Zhou G, Jin C. 2018. Interactions of fungi with concrete: significant importance for bio-based self-healing concrete. Construction Building Materials 164: 275-285.

Magan N. 2007. Fungi in extreme environments. The Mycota 4: 85-103.

Maheswaran S, Dasuru S, Murthy AR, Bhuvaneshwari B, Kumar V, Palani G, Iyer NR, Krishnamoorthy S, Sandhya S. 2014. Strength improvement studies using new type wild strain *Bacillus cereus* on cement mortar. Curr Sci 50-57.

Mamo G, Mattiasson B. 2019. Alkaliphiles: The emerging biological tools enhancing concrete durability. Springer 172: 293-342.

Manikandan A, Padmavathi A. 2015. An experimental investigation on improvement of concrete serviceability by using bacterial mineral precipitation. Int J Res Sci Innov 2: 48.

Martuscelli C, Soares C, Camões A, Lima N. 2020. Potential of fungi for concrete repair. Proc Manufacturing 46: 180-185.

Medeiros M, Helene P. 2009. Surface treatment of reinforced concrete in marine environment: Influence on chloride diffusion coefficient and capillary water absorption. Construction Building Mater 23: 1476-1484.

Menon RR, Luo J, Chen X, Zhou H, Liu Z, Zhou G, Zhang N, Jin C. 2019. Screening of fungi for potential application of self-healing concrete. Sci Rep 9(1): 1-12.

Morsali S. Isildar G, Tahni A. 2019. The application of bacteria as a main factor in self-healing concrete technology. J Building Pathol Rehabil 4 (2019): 7.

Nama P, Jain A, Srivastava R, Bhatia Y. 2015. Study on causes of cracks & its preventive measures in concrete structures. Int J Eng Res Appl 5 (5): 119-123.

Neville AM. 1996. Properties of Concrete. Pearson Higher Education, New Jersey.

Nosouhian F, Mostofinejad D, Hasheminejad H. 2016. Concrete durability improvement in a sulfate environment using bacteria. J Mater Civil Eng 28 (1): 04015064.

Oggerin M, Tornos F, Rodriguez N, Pascual L, Amils R. 2016. Fungal iron biomineralization in Rio Tinto. Minerals 6 (2): 37-48.

Pal S, Mohanty I, Panda I. 2020. Self-healing conventional concrete using bacteria. In: Recent Developments in Sustainable Infrastructure. Springer, Singapore.

Pasquale V, Fiore S, Hlayem D, Lettino A, Huertas FJ, Chianese E, Dumontet S. 2019. Biomineralization of carbonates induced by the fungi *Paecilomyces inflatus* and *Plectosphaerella cucumerina*. Int Biodeterior Biodegrad 140: 57-66.

Povedano-Priego C, Martín-Sánchez I, Jroundi F, Sánchez-Castro I, Merroun ML. 2017. Fungal biomineralization of lead phosphates on the surface of lead metal. Minerals Eng 106: 46-54.

Qian C, Zhou H, Wang K. 2019. Factors affecting morphology of microbially induced calcium carbonate. J Microbiol Exp 7 (2): 101-14.

Ramachandran SK, Ramakrishnan V, Bang S. 2001. Remediation of concrete using micro-organisms, ACI Mater J Am Concrete Inst 98: 3-9.

Ramakrishnan V, Deo K, Duke E, Bang S. 1999. SEM investigation of microbial calcite precipitation in cement. In: Proceedings of the International Conference on Cement Microscopy. Int Cement Microsc Assoc 406-414.

Rao M, Reddy S, Hafsa M, Veena P, Anusha P. 2013. Bioengineered concrete-a sustainable self-healing construction material. Res J Eng Sci 2278 (2013): 9472.

Rautaray D, Ahmad A, Sastry M. 2003. Biosynthesis of CaCO3 crystals of complex morphology using a fungus and an actinomycete. J Am Chem Soc 125: 14656-14657.

Rautaray D, Ahmad A, Sastry M. 2004. Biological synthesis of metal carbonate minerals using fungi and actinomycetes. J Mater Chem 14: 2333-2340.

Ravindranatha, Kannan N, Likhit ML. 2014. Self-healing material bacterial concrete. Int J Res Eng Technol 3n(3): 656-659.

Reddy V, Seshagiri Rao M, Sushma S. 2015. Feasibility Study on Bacterial Concrete as an innovative self crack healing system. Int J Mod Trends Eng Res 2: 642-647.

Reddy MS. 2013. Biomineralization of calcium carbonates and their engineered applications: a review. Front Microbiol 4: 314. DOI: https://doi.org/10.3389/fmicb.2013.00314.

Roodman D, Lenssen N, Peterson J. 1995. A building revolution: how ecology and health concerns are transforming construction. Worldwatch Institute, Washington, DC.

Ruan S, Qiu J, Weng Y, Yang Y, Yang E, Chu J, Unluer C. 2019. The use of microbial induced carbonate precipitation in healing cracks within reactive magnesia cement-based blends. Cement Concrete Res 115: 176-188.

Saifee S, Lad D, Juremalani J. 2015. Critical appraisal on Bacterial Concrete. IJRDO-J Mechanical Civil Eng 2456-1479.

Sayer JA, Kierans M, Gadd GM. 1997. Solubilisation of some naturally occurring metal-bearing minerals, limescale and lead phosphate by *Aspergillus niger*. FEMS Microbiol Lett 154: 29-35.

Seifan M, Samani A, Berenjian A. 2016. Bioconcrete: next generation of self-healing concrete. Appl Microbiol Biotechnol 100: 2591-2602.

Seifan M, Sarmah AK, Samani AK, Ebrahiminezhad A, Ghasemi Y, Berenjian A. 2018. Mechanical properties of bio self-healing concrete containing immobilized bacteria with iron oxide nanoparticles. Appl Microbiol Biotechnol 102 (10): 4489-4498.

Senthilkumar V, Palanisamy T, Vijayakumar V. 2014. Fortification of compressive strength in Enterococcus microorganisms incorporated microbial cement mortar. Int J Chem Tech Res 6: 636-644.

Setlow P. 1994. Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. J Appl Bacteriol 76: 49S-60S.

Shaheen N, Khushnood R, Khaliq W, Murtaza H, Iqbal R, Khan M. 2019. Synthesis and characterization of bio-immobilized nano/micro inert and reactive additives for feasibility investigation in self-healing concrete. Construction Building Mater 226: 492-506.

Sharaky AM, Mohamed NS, Elmashad ME, Shredah N. 2018. Application of microbial biocementation to improve the physicomechanical properties of sandy soil. Construction Building Mater 190: 861-869.

Sheir DH, Amer O, Elkhateeb WA, Daba GM. 2020. Elucidation of myco-deterioration affecting calcareous construction materials of the Egyptian architectural heritage. Boreal Environ Res 25: 27-56.

Sidiq A, Gravina R, Giustozzi F. 2019. Is concrete healing really efficient? A review. Construction Building Mater 205: 257-273.

Silva F, Boon N, De Belie N, Verstraete W. 2015. Industrial application of biological self-healing concrete: challenges and economical feasibility. J Commercial Biotechnol 21 (1): 31-38.

Sisomphon K, Copuroglu O, Koenders E. 2012. Self-healing of surface cracks in mortars with expansive additive and crystalline additive. Cement Concrete Composites 34: 566-574.

Stanaszek-Tomal E, Kozak A. 2017. Mineral and organic coatings modified nano-TiO2 addition as elements of sustainable building. Sustainability 1-10.

Stanaszek-Tomal E. 2020. Bacterial concrete as a sustainable building material? Sustainability 12: 696-670.

Ter Heide N, Schlangen E. 2007. Self-healing of early age cracks in concrete. In: First international conference on self-healing materials 1-12.

Ter Heide N. 2005. Crack healing in hydrating concrete. Delft University of Technology 12-82.

Van Tittelboom K, De Belie N, De Muynck W, Verstraete W. 2010. Use of bacteria to repair cracks in concrete. Cement Concrete Res 40: 157-166.

Vekariya MS, Pitroda J. 2013. Bacterial concrete: new era for construction industry. Int J Eng Trends Technol 4 (9): 4128-4137.

Verrecchia EP. 2000. Fungi and sediments. In: Riding RE, Awramik SM (eds) Microbial Sediments. Springer-Verlag, Berlin.

Vijay K, Murmu M. Deo S. 2017. Bacteria based self-healing concrete–A review. Construction Building Mater 152: 1008-1014.

Vinoth T, Rahimunissa A, Manikandeswari S, Valavan E, Vadivel P, Thangam D. 2016. Causes and remedies on concrete cracks-A review. Int J Eng Manag Res 6 (5): 550-551.

Waghmode MS, Gunjal AB, Bhujbal NN, Patil NN, Nawani NN. 2019. Eco-Friendly Construction. In: Reusable and Sustainable Building Materials in Modern Architecture. IGI Global, America.

Wang J, Dewanckele J, Cnudde V, Van Vlierberghe S, Verstraete W, De Belie N. 2014. X-ray computed tomography proof of bacterial-based self-healing in concrete. Cement Concrete Composites 53: 289-304.

Wang J, Van Tittelboom K, De Belie N, Verstraete W. 2010. Potential of applying bacteria to heal cracks in concrete. In: Proceedings of the second international conference on sustainable construction materials and technologies: 1807-1818.

Wang JY, De Belie N, Verstraete W. 2012. Diatomaceous earth as a protective vehicle for bacteria applied for self-healing concrete. J Ind Microbiol Biotechnol 39: 567–577.

Yang Y, Ma L, Huang J, Gu C, Xu Z, Liu J, Ni T. 2019. Evaluation of the thermal and shrinkage stresses in restrained high-performance concrete. Materials 12 (2019): 3680.

Yang G, Gu C, Bao T, Cui Z, Kan K. 2016. Research on early-warning index of the spatial temperature field in concrete dams. SpringerPlus 5 (2016): 1968.

Žáková H, Pazderka J, Rácová Z, Ryparová P. 2019. Effect of bacteria bacillus pseudofirmus and fungus *Trichoderma reesei* on self-healing ability of concrete. Acta Polytechnica CTU Proc 21: 45-45.

Zhang J, Liu Y, Feng T, Zhou M, Zhao L, Zhou A. 2017. Immobilizing bacteria in expanded perlite for the crack self-healing in concrete. Construction Building Mater 148: 610-617.

Zhang X. Qian C. 2020. Engineering application of microbial self-healing concrete in lock channel wall. Case Stud Construction Mater (2020): e00398.

Zhu T, Dittrich M. 2016. Carbonate precipitation through microbial activities in natural environment, and their potential in biotechnology: a review. Front Bioeng Biotechnol 4: 4-25. DOI: https://doi.org/10.3389/fbioe.2016.00004.

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Hematological, serum biochemical and histopathological effects of selected herbs and combinations on *Trypanosoma brucei* infected West African dwarf sheep

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Abstract. *Olatunde OA, Jegede HO, Ameen SA. 2021. Hematological, serum biochemical and histopathological effects of selected herbs and combinations on* Trypanosoma brucei *infected West African dwarf sheep. Asian J Nat Prod Biochem 19: 10-16.* The present work was carried out to study the hematological, serum biochemical and histopathological effects of selected herbal diets on trypanosome-infected West African dwarf sheep. Five incorporated herbal diets were tested where A is the control diet, B is *Citrus aurantifolia*, C is *Ocimum gratissimum*, D is *Vernonia amygdalina + Citrus aurantifolia*, and E is *Ocimum gratissimum + Vernonia amygdalina*. Sheep on Diets E, A, C, B, and D consumed 856.4g/d, 830g/d, 750.0g/d. 734.0g/d and 687.0g/d, respectively, showing more acceptability to diet E. Additionally, sheep on Diet E was highest in RBC, WBC, Hb, lymphocyte, PCV, and basophils. The least hematological indices were recorded for the animals on the Control diet (A), showing that all herbal diets improved blood indices of the infected sheep. The ALP concentration was significantly different (17.000i.u/l) in Diet B compared to other Diets. At the same time, the least ALP was noted for sheep on Diets A and B. Herbs and combinations used in this study significantly improved both hematological and pathological lesions seen in experimental trypanosomosis caused by *Trypanosoma brucei* infection in sheep. The best results were obtained from Diet E combinations.

Keywords: Hematology, herbal diets, pathology, production, trypanosomosis

INTRODUCTION

Herbal phytochemical constituents in diet or administered to animals by any routes undergo biotransformation by the principal metabolic organs like the liver, kidney, and lungs (Aliu 2007). The detoxification of any substance in the animal body involves several enzymes, cytochrome oxidases, and microsomal enzymes (Aliu 2007). The metabolism of xenobiotics or any foreign substance in the animal's body could affect the hepatic enzymes principally and various biochemical parameters: bilirubinbin and conjugated bilirubin concentration, electrolytes, creatinine, urea, protein, and cholesterol. Yakubu et al. (2005) evaluated the effects of plants by assessing their biochemical parameters, which give insight into the effect of phytochemical substances on biochemical parameters to note the detrimental effect or beneficial effects of the substances. Some factors could influence the positive or negative effect of plants on animal health amongst which include species of animal, age, the concentration of the substance, lethality of substance, the health status of the animal, sex, nutritional status of the animal, geographical zone from which plant is obtained and route of administration (Awoyomi et al. 2013).

Trypanosomosis is a protozoan disease affecting various animals, including sheep, and causing severe hematologic problems, the most prominent being anemia (Joshua et al., 1996), where the severity of the disease and tolerance of the parasite is affected by other factors, such especially the stress (Itard 1989). Most of the herbs thereby selected in this study have been known for their antioxidant effects.

This study, therefore, aimed to evaluate the safety and efficacy of some of these herbs, where hematological, biochemical, and histopathological parameters of sheep infected with *Trypanosoma brucei* and treated with selected spices and herbs.

MATERIALS AND METHODS

Study area

The study was conducted at the Teaching and Research Farm of the Faculty of Agriculture, University of Ilorin. The study lasted for three (3) weeks.

Procedures

Experimental animals and management

Fifteen (15) West African dwarf (WAD) adult sheep (2-4 years) used for this study were randomly assigned to five (5) treatments consisting of three (3) animals per treatment group, in a Completely Randomized Design. Ten (10) ml of blood samples were collected using an 18-gauge needle via the jugular vein of the sheep, and 5ml each was

distributed into bottles coated with ethylene diamine tetraacetate (EDTA) and plain bottles for hematology and serology, respectively.

Inoculation Procedure

The strain of *Trypanosoma brucei* was obtained from the Nigerian Institute of Trypanosomiasis Research (NITR), Vom, Jos. The parasites were inoculated into two albino rats. The rats were later bled during the first wave of parasitemia into an EDTA container (Ethylene diamine tetraacetic acid). The phase-contrast buffy coat technique (Biobaku et al. 2008) was used to detect and quantify trypanosomes in the blood samples using Standard methods (Murray et al. 1983). WAD sheep previously screened for trypanosome were inoculated intramuscularly with 3mls of Phosphate Buffered Saline containing 1.5×10^6 *Trypanosoma brucei*. The trypanosome species was confirmed in the infected WAD sheep following a laboratory blood test (Murray et al. 1983) on the 6th day post-infection.

Preparation of plant spices

Some plant samples (leaves from *Citrus aurantifolia* with voucher no: UILH/003/983; *Ocimum gratissimum* with voucher no: UILH/002/1984; *Vernonia amygdalina* with voucher no: UILH/001/1023) were collected within the University of Ilorin, Nigeria. The samples were identified at the Department of Plant Science, University of Ilorin, Nigeria. The collected samples were washed in a running tap to remove soil and dust particles. It was later air-dried on the laboratory bench for five days. The dried samples were milled with pestle and mortar into a powdery form. The powdered samples were stored in a dry, clean container with a lid. These were mixed with prepared diets at a 5% inclusion rate, as shown in Table 1. In combinations, e.g., (VA+CA), herbs were mixed in equal ratios i.e., 1:1. The diets were fed to the sheep for three weeks based on the group they belonged to.

Experimental Diets and Feeding

Throughout the experiment, animals were fed experimental diets (Table 1) based on 3% of their weight twice per day, and water was given to them ad libitum. They were on these diets until the experiment was terminated at the end of three weeks.

Hematological analysis

The blood samples were taken at 1 week and 2 weeks post-treatment and analyzed for Packed Cell Volume (PCV), Hemoglobin concentration (Hb), Red Blood Cell (RBC) counts, and differential leucocytes by standard hematological techniques (Jain 1986).

Serum biochemical analysis:

Five (5 mL) blood sample was collected into plain (anticoagulant free) plastic tube and allowed to clot at room temperature within 3 hours of collection. The serum samples were separated and stored at -20°C for biochemical analysis. The serum concentration of total protein, albumin, aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were determined by the colorimetric method as described by Reitman and Frankel (1975). Blood glucose was determined by the standard method described by Kaneko and Howard (1989).

Histopathological examination:

Post-study, one animal per group (A-E) was sacrificed in order to obtain fresh samples for examination. According to Drury and Wallington, samples of the spleen, kidney, and liver were collected in formalinized saline solution for histopathology and were processed and routinely stained with hematoxylin and eosin (H&E) according to Drury and Wallington (1967). The lesions were examined under a microscope (x100).

Data analysis

Data resulting from our study were expressed in Means and standard error of the mean (SEM). Monitored physiological variables were analyzed using analyses of variance (ANOVA) for repeated measures, and $P<0.05$ was accepted as significant.

Table 1. Composition of the experimental diets for West African Dwarf (WAD) Sheep

Ingredient %	**A (Control)**	**B (CA)**	**C (OG)**	**D (VA+CA)**	**E (OG+VA)**
Cassava waste	60.00	55.00	55.00	55.00	55.00
Plant extract	-	5.00	5.00	5.00	5.00
Soyabean meal	16.00	16.00	16.00	16.00	16.00
Rice husk	22.00	22.00	22.00	22.00	22.00
Vitamin-Minerals					
Premix	1.00	1.00	1.00	1.00	1.00
Salt	1.00	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00

Note: B. *Citrus aurantifolia*, C. *Occicum gatissimum*, D. *Vernonia amygdalina* + *Citrus aurantifolia*, E. *Occicum gatissimum* + *Vernonia amygdalina*

RESULTS AND DISCUSSION

Hematological observations

Sheep on Diets E, A, C, B, and D consumed an average of 856.4g/d, 830g/d, 750.0g/d. 734.0g/d and 687.0g/d, respectively. It was observed from Tables 2 and 3 that RBC and PCV were significantly different ($p<0.05$) across the various dietary treatments, and likewise are % eosinophils and % monocytes concentration ($p<0.05$). WAD sheep in the control group and Diet B (CA) had a significantly ($p<0.05$) lower WBC concentration (4.56- 4.9 $\times10^9$/L), respectively, when compared to other dietary treatments. However, sheep on Diet D (VA+CA) had numerically higher (7.23×10^9/L) WBC levels than other dietary treatments. Hemoglobin concentration difference was not significant ($p>0.05$) across treatments though it was numerically higher in Diet E (OG+VA). WAD sheep placed on Diet D (VA+CA) and Diet E (OG+VA) showed higher lymphocytes of 66% but lower neutrophils of 28-29%. PCV was significantly lower ($p<0.05$) in Diet E (OG+VA) (27.66%) compared with control A (9.0%); however, Diet E (OG+VA) and Diet D (VA+CA) were similar. WBC followed the trend, with control Diet A lowest (4.1×10^9/L, and Diet E (OG+VA) being the highest (8.4×10^9/L). Animals on Diet D (VA+CA) also showed higher (6.5×10^9/L) WBC count than those on Diet B (CA) and Diet C (OG). The same trend was observed in RBC and Hb count, as Diet E (OG+VA) had higher counts (2.4×10^{12}/L) and (6.4g/dl), respectively, when compared across other dietary treatments. Lymphocytes (%) were significantly higher ($p<0.05$) in the herbal mixtures (66.00-67.00%) than in the single herb diet, but in terms of % neutrophils, in herbal mixtures was lower (22-26%) than in other (33-39%), with diet B (CA) having the highest (44%).

Serum biochemical observations

From Table 4, ALP was significantly higher ($p<0.05$) in Diet B (CA) (17.0 IU/L) when compared with the control (12.667 IU/L) and Diet D (VA+CA) (12.0 IU/L), however, observable significant ($p>0.05$) differences were recorded for Diet C (OG), Diet E (OG+VA) and the control. AST concentration showed that diet mixtures, Diet D (VA+CA) and Diet E (OG+VA), were significantly higher ($p<0.05$) at 44.67 IU/L and 46.00 IU/L, respectively, compared to other dietary treatments. Glucose level was lowest ($p>0.05$) in Diet E (OG+VA) treatment (2.96 mmol/l) but highest in control A (4.23 mmol/l). This trend continued in the ALT as the control group was significantly lower (17.00 IU/L, $p<0.05$) than those observed in other treatments. Serum protein also followed the trend, with the control at 23.9 mmol/l ($p<0.05$). However, Diet B (CA) had the highest ($p<0.05$) serum protein level (32.1 mmol/l).

Tables 4 and 5 show that AST and Albumin levels were significantly lower ($P<0.05$) in Diet E (OG+VA) (13.0 IU/L) and (13.9 mmol/L), respectively, compared across dietary treatments, AST was higher (44.0 IU/L, $p<0.05$) in Diet D (VA+CA) than Diet B (CA) (37.0 IU/L), B(OG) (33.0 IU/L) and the control A (33.0 IU/L). ALT in Diet E (OG+VA) was significantly higher ($P<0.05$) at 50.00 IU/L than that observed in other treatments, diet B (CA) treatment also was higher (29.0 IU/L, $p<0.05$) than the control A (16.0 IU/L) and to both Diet C (OG) and Diet D (VA+CA) which were comparable in ALT concentration (24-25 IU/L, $p<0.05$). In terms of serum glucose level, no significant difference ($P>0.05$) was observed across all dietary treatments.

Histopathology observations

The spleen demonstrated various degrees of severity of hyperplasia of the lymphoid follicles. The cortical and medullary regions of the lymph nodes showed congestion of the sinuses, proliferation of mononuclear cells, erythrophagocytosis, and hemosiderosis. The kidneys similarly demonstrated various degrees of congestion, perivascular and interstitial mononuclear cell infiltration, thickening of glomerular capsules, desquamation of tubular cells, and protein casts in the tubules.

Table 2. Hematological Indices of WAD Sheep one-week post-infection placed on various experimental (herbal-based) diets

Parameters	A Control	B CA	C OG	D VA+CA	E OG+VA
Red Blood Cell (x 10^{12}/L)	1.33±0.15	1.30±0.11	1.20±0.20^{c}	1.57±0.65	1.67±0.39
White Blood cell (x 10^9/L)	4.57±0.09^{a}	4.90±0.43^{a}	6.50±0.29^{b}	7.23±1.23^{b}	6.80±0.99^{b}
Haemoglobin (g/dl)	3.60±0.12^{b}	4.10±0.22^{ab}	4.30±0.13^{ab}	5.00±0.33^{ab}	5.20±0.44^{a}
Lymphocytes (%)	54.000±0.45^{a}	58.00±1.06^{b}	55.00±0.96^{a}	66.00±0.66^{c}	66.00±0.62^{c}
Neutrophils (%)	39.00±1.11^{a}	39.00±1.77^{a}	32.00±1.22^{b}	28.00±1.07^{b}	29.00±1.09^{b}
Eosinophils (%)	3.00±0.32	3.00±0.28	5.00±0.45	5.00±0.42	1.33±0.25
Packed Cell Volume (%)	15.00±0.09	15.00±0.13	13.00±0.20	18.00±0.23	20.00±1.92
Basophils (%)	0.67±0.02^{ab}	0.67±0.03^{ab}	0.01±0.01^{a}	0.67±0.08^{ab}	3.00±0.12^{a}
Monocytes (%)	0.67±0.02	0.67±0.04	0.67±0.02	0.01±0.00	1.00±0.04

Note: Values are Mean ±S.E.M. Means with different superscripts along the same row are significantly different ($P<0.05$), CA: *Citrus aurantifolia*; OG: *Occimum gratissimum;* VA+CA: *Vernonia amygdalina + Citrus aurantifolia;* OG+VA: *Occimum gratissimum + Vernonia amygdalina*

Table 3. Hematological Indices of WAD Sheep two weeks post-infection placed on various experimental (herbal-based) Diets

Parameters	A Control	B CA	C OG	D VA+CA	E OG+VA
Red Blood Cell (x 10^{12}/L)	1.00±0.09^{a}	1.30±0.13^{b}	1.50±0.15^{c}	1.60±0.22^{c}	2.40±0.32^{d}
White Blood Cell (x 10^{9}/L)	4.10±0.48^{a}	5.20±0.56^{b}	5.50±0.65^{c}	6.50±0.88^{d}	8.40±0.89^{e}
Haemoglobin (g/dl)	2.90±0.09^{a}	4.00±0.34^{c}	3.20±0.31^{ab}	4.60±0.39^{d}	6.40±0.26^{e}
Lymphocytes (%)	53.00±0.61^{a}	55.00±0.63^{b}	60.00±0.67^{c}	67.00±0.76^{d}	67.00±0.60^{d}
Neutrophils (%)	39.00±0.45^{d}	44.00±0.46^{e}	33.00±0.40^{c}	22.00±0.74^{a}	26.00±0.62^{b}
Eosinophils(%)	4.00±0.12^{c}	1.00±0.03^{a}	5.00±0.11^{d}	10.00±1.12^{e}	2.00±0.44^{b}
Packed Cell Volume (%)	9.00±0.45^{a}	14.00±0.34^{c}	13.00±0.76^{b}	17.00±0.88^{d}	27.67±0.82^{e}
Basophils (%)	1.33±0.05^{b}	0.00±0.00^{a}	0.01±0.01^{a}	0.01±0.01^{a}	3.00±0.10^{c}
Monocytes (%)	3.00±0.14^{e}	0.01±0.01^{a}	1.33±0.11^{bc}	1.00±0.04^{ab}	2.33±0.46^{d}

Note: Values are Mean ±S.E.M. Mean with different superscripts along the same row are significantly different (P<0.05), CA: *Citrus aurantifolia*; OG: *Ocimum gratissimum;* VA+CA: *Vernonia amygdalina* + *Citrus aurantifolia;* OG+VA: *Ocimum gratissimum* + *Vernonia amygdalina*

Table 4. Serum Biochemical Indices of WAD Sheep one-week post-infection placed on various experimental (herbal-based) diets

Serum parameters	A Control	B CA	C OG	D VA+CA	E OG+ VA
ALP (IU/L)	12.67±0.89^{ab}	17.00±1.92^{e}	16.00±1.96^{d}	12.00±2.69^{a}	13.67±0.88^{c}
AST (IU/L)	29.00±1.34^{a}	35.00±1.03^{c}	31.00±1.92^{b}	44.67±1.62^{d}	46.00±0.98^{e}
GLUC (mmol/L)	4.23±0.45^{e}	3.23±0.54^{b}	4.00±0.21^{d}	3.80±0.27^{c}	2.97±0.31^{a}
ALT (IU/L)	17.00±0.93^{a}	27.00±1.12^{e}	24.00±1.02^{b}	26.67±1.00^{d}	24.67±1.40^{c}
Total Protein (mmol/L)	23.90±0.62^{a}	32.10±0.70^{d}	25.43±0.69^{ab}	27.40±0.81^{bc}	30.00±0.97^{cd}
Albumin (mmol/L)	15.53±0.09^{c}	15.80±0.12^{d}	16.00±0.23^{e}	14.80±0.11^{a}	14.85±0.11^{ab}

Note: Values are Mean ±S.E.M. Means with different superscripts along the same row are significantly different (P<0.05), CA: *Citrus aurantifolia*; OG: *Occimum gratissimum;* VA+CA: *Vernonia amygdalina* + *Citrus aurantifolia;* OG+VA: *Occimum gratissimum* + *Vernonia* amygdalina, ALP: Alanine Phosphatase; AST: Aspartate aminotransferase transaminase; GLUC: Glucose; ALT: Alanine aminotransferase transaminase; Albu: Albumin.

In the study, the livers showed various degrees of vascular congestion, perivascular cuffing of mononuclear cells, hepatocellular degeneration, and erythrophagocytosis in group A while in groups (B-E), the lesions are milder. The spleen of trypanosome-infected animals in group A showed depopulated red pulp, the proliferation of macrophages characterized by erythrophagocytosis and haemosiderosis, as well as the proliferation of plasma cells. The lesions are milder in various groups treated with spices (B-E).

Discussion

Studies have shown a progressive decrease in PCV and RBC in sheep (Mackenzie et al. 1978; Okaiyeto et al. 2010) infected with trypanosomes. The normal PCV values for healthy sheep ranged from 27-45% (Pugh 2002); therefore, the increase in PCV of sheep from 20-27% that was observed in Diet E (OG+VA) two weeks post-infection with *T. brucei* suggested effective trypanosomatid or trypanotolerant effect of certain herbs or just an increased repair mechanism resulting from the combined antioxidant effects of the plants. Marcotty et al. (2008) observed that mean PCV was a good indicator of the health status of animals in an endemic area. In this study, the upturn of PCV in group E is indicative of a recovery from the effects of the trypanosome infection. The level of anemia or the PCV usually gives a reliable insight into an infected animal's disease status and productive performance (Van den Bossche et al. 2001). With improved PCV in blood, enhanced nutrient transport is achieved, leading to efficient feed utilization and absorption, implying better body weight gain (Katunguka-Rwakishaya et al. 1998). Furthermore, the increase observed in the level of RBC and hemoglobin in Diet E (OG+ VA) between weeks one and 2 and the significant difference between the control and other diets duly buttresses the point that it proffers a better immunosuppressive potential against the trypanosome. It has been shown that the onset of anemia, characteristics of trypanosomiasis, occurs within a few days of inoculation. There are conspicuous indications that the onset of anemia in African trypanosomiasis may be markedly related to interference with the erythrocyte membrane caused directly by parasite attack on red blood cells (Orhue and Nwanze 2009; Biobaku et al. 2009). It has also been suggested that products secreted by the protozoan play a significant role in the disruption of the red cell membrane (Li et al. 2007; Biobaku et al. 2008; Awoyomi et al. 2013), e.g., reduction in red cell membrane sialoglycoprotein secondary to increased activity of plasma sialidases promoting the rapid destruction of red blood cells (Awoyomi et al. 2013). The observable faster recovery rate from anemia (i.e., increased RBC count) in Diet E (OG+ VA) showed that Diet E (OG+ VA) herbal treatment exhibited remarkable potency in protecting against the hematological problems accompanying *T. brucei* infection in sheep. On the one hand, it is possible that the herbs

may possess the ability to maintain the structural and functional capacity of the erythrocytic membrane or erythropoietic tissues to varying levels in the face of a hemiparasitic infection, having anti-anemic properties (Ikpeazu et al. 2019). On the other, since *T. brucei* infection is associated with a considerable generation of free radicals (Igbokwe et al. 1994), the antioxidant activity inherent in these plants is used (Awah 2010; Ho et al. 2012) may contribute significantly to the overall effects observed in this study. Furthermore, the herbs may possess a measure of trypanocidal activity or immuno-stimulating properties that helps put the parasite at bay and thus also control the injurious effects of uncontrolled hemiparasite proliferation.

It is worthy of note that compared with the control, the normal WBC level of sheep, which is 4-12x10^9/L in the herbal mixtures' treatment, increased in response to the pathogenic invasion. Diet E (OG+VA) recorded a higher WBC count and subsequently a higher % lymphocyte count. This implies that the immune system is fighting back to suggest host tolerance to the pathogen. In two separate studies, Oduye and Okunaiya (1971) and Murray et al. (1978) showed that the trypanotolerant N'dama cattle breed significantly had a higher level of WBC, particularly eosinophils, after being exposed to the pathogen. The increased WBC observed in all herbal based diets (B, C, D, and E) compared with control diet A could also be inferred that several phytochemicals interact in the herbal mix, especially flavonoids, to assist the body in resisting diseases (Hoet et al. 2007), by boosting their capacity to ward off infection (WBC activation) even in the presence of an overwhelming infection like *T. brucei* in this study. Medicinal plants commonly have several chemicals working together catalytically and synergistically to produce a combined effect that surpasses the total activity of the individual constituents. The combined action of these substances increases the activity of the main medicinal constituent by speeding up or slowing down its assimilation into the body (Mahomoodally et al., 2005; 2010). Secondary substances from plant origins might increase the stability of the active compound(s) or phytochemicals, minimize the rate of undesired side effects, and have an additive, potentiating, or antagonistic effect (Mahomoodally et al. 2005; 2010). Alternatively, differences in levels of cellular response and degree of immunosuppression might just reflect differences in levels of parasitemia observed in the infected animals (Morrison et al. 1987).

Hypoalbuminemia observed on animals on diets B, C, D, and E are characteristic of an infection mode in an animal body system. This is evident in the uptake of albumin-bound fatty acids, lipoprotein, and hemomodulation (Mbaya et al., 2012). Elevation in globulin due to enhanced antibody secretion in response to infection would undoubtedly have contributed immensely to the observed hyperproteinemia.

The increase in serum total protein (Table 5) may have been due to the increased release of tissue-specific enzymes and other intracellular proteins secondary to parasite-induced cell membrane disruption. Although within the normal range, the increased transaminase was observed in all herbal treated diets (B, C, D, and E). This result supported an earlier study that reported a marked elevation in the serum levels of alkaline phosphatase (ALP), aspartate aminotransaminase (AST), and alanine amino transaminase (ALT) in *Trypanosoma brucei* infected rabbits (Dina et al. 2002). As observed in the various treatments (Tables 4 and 5), elevations in these enzymes are usually secondary to tissue damage. This is because such damage results in the leakage of these enzymes from their intracellular stores into plasma. The significant increase in the transaminases commonly accompanies such liver diseases as toxic hepatitis, acute liver necrosis, or hepatic cirrhosis. Increased AST is often observed in hemolytic anemia, myocardial infarction, and cholestatic diseases of the liver (Felix and Mello 2005). OG is a known hypoglycemic agent (Aguiyi et al. 2000), which agrees with our findings in this study. However, this hypoglycemic effect is shown to be amplified in combination with VA in diet E. Many hemoprotozoans, such as trypanosomes, depend on the host glucose for aerobic glycolysis (Riou and Bernard 1980). This will prevent glucose utilization by the parasites.

Table 5. Serum biochemical indices of WAD Sheep two weeks post-infection placed on various experimental (herbal-based) Diets

Serum parameters	A (Control)	B (CA)	C (OG)	D (VA+CA)	E (OG+ VA)
AST (IU/L)	30.00±1.34^b	37.00±1.23^d	33.00±0.34^c	44.00±2.34^e	13.00±0.76^a
ALT (IU/L)	16.00±1.58^a	29.00±2.39^d	24.00±1.34^b	25.00±1.40^c	50.00±1.95^e
GLUC (mmol/L)	4.10±0.92^e	3.30±0.86^b	4.00±0.96^d	3.90±0.71^c	3.20±0.63^a
ALP (IU/L)	12.00±1.34^b	17.00±1.14^c	15.00±1.12^d	11.00±0.88^a	28.00±1.46^e
Total Protein (mmol/L)	20.70±0.23^a	30.30±0.49^c	29.60±0.45^b	32.40±0.57^e	32.00±0.56^d
Albumin (mmol/L)	14.60±0.23^c	15.50±0.18^d	13.90±0.04^a	14.20±0.05^b	13.90±0.19^a

Note: Values are Mean ±S.E.M. Means with different superscripts along the same row are significantly different ($p<0.05$), CA: *Citrus aurantifolia*; OG: *Ocimum gratissimum;* VA+CA: *Vernonia amygdalina + Citrus aurantifolia;* OG+VA: *Ocimum gratissimum + Vernonia amygdalina,* ALP: Alanine Phosphatase; AST: Aspartate aminotransferase transaminase; GLUC: Glucose; ALT: Alanine aminotransferase transaminase; Albu: Albumin.

In this study, the presence of increased hemosiderosis indicates the major role the spleen plays in the destruction of red blood cells during trypanosomiasis in the control group A. This conforms to observations of Taylor and Authie (2004). The presence of grander lesions, such as erythrophagocytosis in *T. brucei* illnesses, especially those in the acute phase of infection, could have led to the rapid development of anemia regardless of the increased erythroid hyperplasia usually present in this phase of the disease (Anosa et al. 1992). The mild enlargement of the lymphoid nodules of the spleen in the *T. brucei* infections could indicate the declining role of humoral immunity in chronic trypanosomosis (Moulton 1986). These observations are milder in other groups (B-E) fed various spices, and this may be due to modulating effects of spices on the pathogenesis of ovine trypanosomiasis.

In this study, vascular congestion, disorganization of the hepatic cords, perivascular mononuclear cell infiltration, hepatocyte degeneration, hyperplasia of Kupffer cells, and erythrophagocytosis in the liver of infected animals. These are common histological lesions in ovine trypanosomosis, but milder in other groups fed various spices. The observed lesions in group A might be because the diet in group A had no herbs as compared with other diets (B-E), which are known antioxidants aiding in tissue repair and aiding in successful recovery from the lesions observed (Taylor and Authie 2004). There are differences in the extent of these reactions depending on the parasite species and species in the stages of infection.

In conclusion, herbs and combinations used in this study have significantly improved the lesions (both hematologic and pathologic) seen in experimental trypanosomosis caused by *T. brucei* infection in West African dwarf sheep. The best results were obtained from Diet E OG+VA combinations.

REFERENCES

Aguiyi JC, Obi CI, Gang SS, Igweh AC. 2000. Hypoglycaemic activity of *Ocimum gratissimum* in rats. Fitoterapia 71: 444-446.

Aliu YO. 2007. Veterinary Pharmacology. 1st eds. Tamaza publishing, Zaria.

Anosa VO, Logan-henfrey LL, Shaw MK. 1992. A light and electron microscopic study of changes in blood and bone marrow in acute haemorrhagic *T. vivax* infection in calves. Vet Pathol 29: 33-45.

Awah FM. 2010. Antioxidant activity, nitric oxide scavenging activity and phenolic contents of *Ocimum gratissimum* leaf extract. J Med Plant Res 4: 2479-2487.

Awoyomi OJ, Biobaku KT, Kehinde OO, Adebowale OO, Oyewusi JA. 2013. A survey of ethnoveterinary botanicals in Ogun State and their public health implication. Niger J Anim Prod 40: 198-208.

Biobaku KT, Abodunrin CO, Ajibola ES, Adenubi OT, Olukunle OJ, Thomas FC. 2009. Trypanocidal effect of aqueous extracts of *Khaya senegalensis* and *Allium sativum* with natural magnesium supplement in rat. Nig J Parasitol 30: 90-95.

Biobaku KT, Ajagbonna OP, Ajayi OL, Adebowale OO. 2008. Effect of magnesium chloride and diminazene aceturate in *Trypanosoma brucei* inoculated wistar albino rat. J Univ Agric Abeokuta B7: 64-70.

Dina OA, Saba AB, Adedapo AA, Akinyemi OA, Omobowale TO. 2002. Comparative efficacy study of Homidium bromide, diminazene aceturate and their combination on New Zealand White Rabbit, experimentally infected with *Trypanosoma congolense*. Trop Vet 20: 153-159.

Drury RAB, Wallington EA. 1967. Carleton's histological techniques. 4th eds. Oxford University Press, New York.

Felix JP, Mello D. 2005. Farm Animal Metabolism and Nutrition. CABI, United Kingdom.

Li SQ, Fung MC, Reid S, Inoue N, Lun ZR. 2007. Immunization with recombinant beta-tubulin from *T. evansi* induced protection against *T. evansi*, *T. equiperdum* and *T. brucei* infection in mice. Parasite Immunol 29:191-199.

Ho WY, Liang WS, Yeap SK, Beh BK, Yousr AHN, Alitheen NB. 2012. In vitro and in vivo antioxidant activity of *Vernonia amygdalina* water extract. Afr J Biotechnol 11: 4090-4094.

Hoet S, Pieters L, Muccioli GG, Habib-Jiwan JL, Opperdoes FR, Quetin Leclercq J. 2007. Antitrypanosomal activity of triterpenoids and sterols from the leaves of *Strychnos spinosa* and related compounds. J Nat Prod 70: 1360-1363.

Igbokwe IO. 1994. Mechanisms of cellular injury in African trypanosomiasis. Vet Bull 64: 611-620.

Ikpeazu VO, Emmanuel O, Ekweogu CN, Akara EU, Ugbogu EA. 2019. A comparative nutritional assessment of leaf extracts of *Ocimum gratissimum* and *Solanum aethiopicum*. Am J Biomed Res 7: 27-31.

Itard J. 1989. African animal trypanosomiasis. Manual of Tropical Veterinary Parasitology. C.A.B International, Wallingford.

Jain NC. 1986. Schalm Veterinary Haematology. 4th ed. Lea and Febiger, Philadelphia.

Joshua RA, Neilson JS, Oladosu LA. 1996. Heterophile antibodies to chicken erythrocytes in sheep infected with *Trypanosoma congolense*. Onderstepoort J Vet Res 63: 253-258.

Kaneko JJ, Howard CF Jr. 1989. Carbohydrate metabolism. In: Loeb WF, Quinby FW (eds) Clinical Chemistry of Laboratory Animals. Pergamon Press, New York.

Katunguka-Rwakishaya E, Murray M, Holmes PH. 1998. The influence of supplementation with cotton seed cake on the resistance of goats to primary and secondary challenges with *Trypanosoma congolense* and on their response to treatment. Vet Parasitol 70: 67-76.

Mackenzie PK, Boyt WP, Nesham VW, Pirie E. 1978. The aetiology and significance of the phagocytosis of erythrocytes and leucocytes in sheep infected with *Trypanosoma congolense* (Broden, 1904). Res Vet Sci 24: 4-7.

Mahomoodally MF, Gurib-Fakim A, Subratty AH. 2005. Antimicrobial activities and phytochemical profiles of endemic medicinal plants of Mauritius. Pharm Biol 43: 237-242.

Mahomoodally MF, Gurib-Fakim A, Subratty AH. 2010. Screening for alternative antibiotics: an investigation into the antimicrobial activities of medicinal food plants of Mauritius. J Food Sci 75: M173-M177.

Marcotty T, Simukoko H, Berkvens D, Vercruysse J, Praet N, Van den Bossche P. 2008. Evaluating the use of packed cell volume as an indicator of trypanosomal infections in cattle in eastern Zambia. Prev Vet Med 87: 288-300.

Mbaya A, Kumshe H, Nwosu CO. 2012. The mechanisms of anaemia in trypanosomosis: a review. In: Silverberg D, (eds) *Anaemia*. INTECH Open Access Publisher, Croatia.

Morrison WI, Goddeeris BM, Teale AJ, Groocock CM, Kemp SJ, Stagg DA. 1987. Cytotoxic T-cells elicited in cattle challenged with *Theileria parva* (Muguga): evidence for restriction by class I MHC determinants and parasite strain specificity. Parasite Immunol 9: 563-578.

Moulton JE. 1986. Structural and functional changes in the lymphoid organs of cattle infected with *T. congolense*. Trop Vet 4: 97-106.

Murray M, Morrison WI, Murray PK, Clifford DJ, Trail JCM. 1978. Trypanotolerance: a review. World Anim Rev 31: 2-12.

Murray M, Trail JCM, Turner DA, Wissocq Y. 1983. Livestock productivity and trypanotolerance. Network Train Manual, ILCA.

Oduye OO, Okunaiya OA. 1971. Haematological studies on the White Fulani and N'Dama breeds of cattle. Bull Epizoot Dis Afr 19: 213-218.

Okaiyeto SO, Ajanusi OJ, Sackey AK, Tekdek LB. 2010. Changes in some haematological values associated with mixed *Trypanosoma congolense* and *Haemonchus contortus* infection in Yankassa sheep. Vet Res 3: 9-13.

Orhue NEJ, Nwanze EAC. 2009. Anti anaemic properties of *Scoparia dulcis* in *Trypanosoma brucei* infected rabbits. Afr J Biochem Res 3: 245-249.

Pugh DG. 2002. Sheep and Goat Medicine, 1st eds. WB Saunders, Philadelphia.

Reitman S, Frankel S. 1975. A calorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. Am J Clin Pathol 28: 56-63.

Riou GE, Bernard J. 1980. Berenil induces the complete loss of kinetoplast DNA sequences. Biochem Biophys Res Commun 9: 350-354.

Taylor K, Authie ML. 2004. Pathogenesis of animal trypanosomiasis. In: Maudlin I, Holmes PH, Miles MA (eds) *The trypanosomoses*. CABI Publishing, Oxfordshire.

Van den Bossche PRGJ, Rowlands GJ. 2001. The relationship between the parasitological prevalence of trypanosomal infections in cattle and herd average packed cell volume. Acta Trop 78: 163-170.

Yakubu MT, Adebayo OJ, Egwim EC, Owoyele VB. 2005. Increased liver alkaline phosphate and aminotransferase activities following administration of ethanolic extract of *Khaya senegalensis* stem bark to rats. Biokemistri 17: 27-32.

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Short Communication: Detection of *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* ATCC 7644 in processed meat products using Real-Time PCR Multiplex Method

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Abstract. *Sophian A, Purwaningsih R, Igirisa RPJ, Amirullah ML, Lukita BL, Fitri RA. 2021. Short Communication: Detection of* Salmonella typhimurium *ATCC 14028 and* Listeria monocytogenes *ATCC 7644 in processed meat products using Real-Time PCR Multiplex Method. Asian J Nad Prod Biochem 19: 17-20.* The detection of *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* ATCC 7644 in processed meat products was carried out using Multiplex Real-Time PCR (qPCR) in the Microbiology and Molecular Biology Laboratory at the Indonesian Food and Drug Authority in Gorontalo. The purpose of this study was to provide alternative testing methods for food products circulating in the market. The sample consisted of 25 samples of processed meat products spike with *Salmonella typhimurium* ATCC 14028 phase 2 and *Listeria monocytogenes* ATCC 7644 phase 2. The method used in the study was qPCR analysis using the SYBR Green method, while DNA isolation used the direct PCR method. Data analysis was carried out based on Cycle threshold and Melting temperature based on two main criteria. Cycle threshold (Ct) analysis determines the sample's Ct value and compares it with the control. Melting temperature (Tm) analysis determines the temperature at which 50% of double-stranded DNA changed to a single standard and compares it with the melting temperature of positive control. The results showed *Salmonella typhimurium* ATCC 14028 in the processed meat was detected at an average Ct value of 10.34 and a Tm value of 85.70. The presence of *Listeria monocytogenes* ATCC 7644 in the samples was recognized at an average Ct value of 14.04 and an average Tm value of 80.07. It can be concluded that the real-time multiplex PCR method can be used to detect *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* ATCC 7644 by using the melting curve (Tm) analysis.

Keywords: *Listeria monocytogenes*, Multiplex PCR, qPCR, *Salmonella typhimurium*

Abbreviations: TSA: Tryptic Soy Agar, TSB: Tryptic Soy Broth

INTRODUCTION

Indonesia has a variety of foods derived from processed meat, including shredded meatballs, rendang, beef jerky, sausages, nuggets, etc. The biggest challenge in serving healthy and hygienic foods is food preparations free from bacterial contamination. The primary source of bacterial contamination of processed meat is the cleanliness of raw materials. A study by Sugiyoto et al. (2015) showed that the origins of microbial contamination in traditional markets originated from water used to clean hands or butcher knives.

Contamination of *Salmonella typhimurium* and *Listeria monocytogenes* in food can result in "foodborne disease," i.e., diseases caused by consuming contaminated food or drink. Therefore, it is necessary to obtain the method for pathogenic bacteria using molecular techniques. Among the molecular methods often used to detect pathogenic bacteria is polymerase change reaction (PCR), i.e., Real-time PCR analysis (qPCR) (Oliveira et al. 2018). Molecular analysis for the detection of pathogenic bacteria using real-time PCR has advantages compared to conventional methods. The average time required for bacterial identification was 50-52 hours by the direct PCR method (24 hours for sample enrichment, 24 hours for selective enrichment, and 1.5 hours for real-time PCR analysis).

One of the real-time PCR methods is the Multiplex PCR technique. The first use of Multiplex PCR was in 1988 to detect the deletions in the dystrophin gene (Chamberlain et al. 1988). In 2008, multiplex PCR was used to analyze microsatellites and SNPs (Hayden et al. 2008). The procedures and components in a multiplex PCR reaction are the same as a regular PCR. However, the amplification process is carried out simultaneously by reading several gene targets in a single analysis. Multiplex PCR contains various sets of primers with a mixture of single PCR reagents to produce amplicons of varying sizes specific to different DNA sequences (Chamberlain et al. 1988). Research on the application of the multiplex PCR technique to bacteria was carried out by Gosiewski et al.

(2012), who applied seven different target genes from the *B. streptococci* strain. It was also carried out on several bacteria that cause foodborne diseases, such as *E. coli* and *Coliform* (Molina et al. 2015).

Regulation of The FDA (BPOM) of the Republic of Indonesia No. 13 of 2019 concerning the Requirements for Maximum Limits of Microbiological Contamination. It regulates the contamination limits of pathogenic bacteria in food products. The category of meat and meat products (referred to as hunted animals) does not allow to contain pathogenic *Salmonella*. Therefore, product monitoring is crucial to ensure that the circulating products are free from pathogenic bacterial contaminants.

This study was conducted to develop alternative methods for detecting *Salmonella typhimurium* and *Listeria monocytogenes* in processed meat products. This study's results might be used as an improved method to detect *Salmonella typhimurium* and *Listeria monocytogenes* in processed meat products based on the molecular test.

MATERIALS AND METHODS

Materials

Twenty-five samples of processed meat (meatball, jerky, sausage, beef burger, shredded), Tryptic Soy Broth (TSB) enrichment media, Tryptic Soy Agar (TSA) / Nutrient Agar (NA), QuantiNova SYBR Green PCR kit (Qiagen).

Sample setup

Twenty-five samples of processed meat products were spiked with positive control of *Salmonella typhimurium* ATCC 14028 phase 2 and *Listeria monocytogenes* ATCC 7644 phase 2.

Isolation from enriching media

Weigh 10 grams of processed meat, then add 90 mL of Tryptic Soy Broth (TSB). Incubate at 35-37° for 18-24 hours. After incubation, scratch one loopful on the TSA or NA agar medium, then incubate at 35-37° for 18-24 hours.

DNA isolation

DNA isolation was carried out by Direct PCR (without the extraction process). Bacterial colonies grown on TSA or NA medium were dried in physiological NaCl by taking 1 ose of bacterial colonies and slowly clouding it in a NaCl solution until the resulting turbidity is equivalent to 1 MacFarland standard. This solution was used as a DNA template.

qPCR analysis

Cycling and melt curve analysis was carried out using qPCR (QIAGEN 5 Plex) with the 2-step cycling method: Denaturation 95ºC for 45 seconds and Annealing / Extention 60ºC for 45 seconds. The primer for *Salmonella typhimurium* detection was InvA Forward primer (5'-ATC AGT ACC AGT CTT CTT ATC TTG AT-3 '), reverse (5'-TCT GTT TAC CGG GCA TAC CAT-3'). The primer for *Listeria monocytogenes* detection was the Forward primer (5 'CTA AAG CGC GAA TCT CCC TT 3'), reverse (5 'CCA TTG TCT TGC GCG TTA AT 3').

Master mix solution

Ten µL of master mix solution contained five µL Sybr green master mix, one µL forward primer, one µL reverse primer, one µL water-free RNase, and two µL DNA templates (Sophian et al. 2020).

Positive Control

Salmonella typhimurium ATCC 14028 phase 2, enriched in TSA or NA and scratched on the agar slant media, was used as a positive control. One ose of *Salmonella typhimurium* ATCC 14028 was taken and slowly clouded in physiological NaCl and equalized to 1 MacFarland standard.

Negative control

NTC (No Template Control) was used as a negative control that contained a master mix combined with primers and free nucleic acids water. The total negative control volume is ten µL consisting of 5 µL master mix SYBR Green, one µL forward primer, one µL reverse primer, and three µL RNase free water (Sophian *et al*. 2020).

Data analysis

Data analysis was carried out based on two main criteria: Cycle threshold (Ct) and melting point (Tm). Cycle threshold (Ct) analysis determines the sample's Ct value and compares it with the control. Melting temperature (Tm) analysis determines the temperature at which 50% of double-stranded DNA changed to a single standard and compares the melting temperature to the melting temperature of positive control (Sophian *et al.*, 2020).

RESULTS AND DISCUSSION

Real-time PCR analysis

Twenty-five processed meat samples were spiked with positive phase 2 bacterial standards control. The contaminated processed meat was analyzed by multiplex PCR to detect two bacterial targets in a single analysis. The results obtained as presented in (Table 1).

The analysis of 25 processed meat samples showed that all samples were positively contaminated with *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* ATCC 7644. It can be seen by comparing with the positive controls. The qPCR analysis results showed that positive control *Listeria monocytogenes* ATCC 7644 was detected at Ct 15.08, and *Salmonella typhimurium* ATCC 14028 was detected at Ct 18.50. The Ct values of processed meat samples were detected at 10.34 - 14.04. The negative control did not show any Ct value because the negative control was not amplified in the qPCR amplification process.

Table 1. The results of the qPCR analysis

qPCR analysis	Value	
	Ct	Tm
Negative control	-	-
Positive control *Salmonella typhimurium* ATCC 14028	18.50	85.20
Positive control *Listeria monocytogenes* ATCC 7644	15.08	80.20
Samples detected *Salmonella typhimurium* ATCC 14028	10.34	85.70
Samples detected *Listeria monocytogenes* ATCC 7644	14.04	80.07

Note: Ct and Tm values are the average value of 25 data replications

Cycling analysis (Ct)

The Ct analysis results showed differences in Ct values of samples detected by multiplex with two bacteria as positive controls. The Ct value of processed meat samples suspected to be contaminated with *Listeria monocytogenes* ATCC 7644 was 14.04, while the Ct value of samples contaminated with *Salmonella typhimurium* ATCC 14028 was 10.34 (Figure 1).

The Ct curve results can not indicate that processed meat samples are contaminated with pathogenic bacteria *(Salmonella typhimurium or Listeria monocytogenes).* It is because the Ct values are different, but it does not have a significantly different pattern (Figure 1). The amplification curve of the Ct value of positive control of bacteria with that of samples does not separate to form a pattern. The Ct value cannot differentiate species in real-time PCR analysis using multiplex techniques because the Ct value was influenced by DNA template concentration and purity. The same sample with different concentrations was detected at different Ct.

Melting temperature curve (Tm) analysis

The results showed that processed meat samples suspected of contamination with *Listeria monocytogenes* ATCC 7644 have a Tm value of 80.7. Samples suspected to be contaminated with *Salmonella typhimurium* ATCC 14028 have a Tm value of 85.7 (Figure 2).

The Tm value in the qPCR analysis is influenced by the composition and size of the nucleotides. In the multiplex techniques, differences in composition and size of nucleotides are requirements for designing the primers. In the melt curve, fluorescence signals provide information on when double-stranded DNA bands begin to separate after annealing. The melt curve produces a specific single peak of each band. The presence of multiple peaks caused by differences in melt points can be used to differentiate bacterial species.

Figure 2 shows two patterns with two different shapes. The difference indicated differences in the melt curve pattern caused by the difference in the melting temperature (Tm) of different primers. This difference is the key to the success of melt curve analysis in multiplex detection.

Positive control *Listeria monocytogenes* ATCC 7644 (red curve) is formed at the melting point 80.7. In contrast, positive control *Salmonella typhimurium* ATCC 14028 (yellow curve) is formed at the melting point 85.7. These two melt points can be used as a reference in the sample to detect and differentiate contaminated samples with *Salmonella typhimurium* or *Listeria monocytogenes.*

Discussion

Detection of *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* ATCC 7644 in processed meat products using Multiplex Real-Time PCR (qPCR) was carried out by quantitative methods using a Quantinova SYBR Green (Qiagen) kit. Twenty-five processed meat samples were spiked with *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* ATCC 7644.

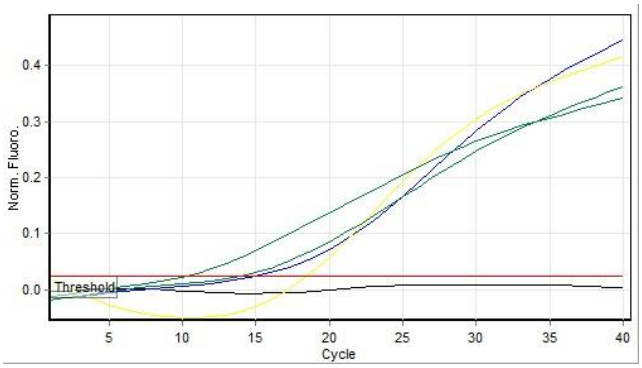

Figure 1: Curve of Ct amplification qPCR analysis. (blue: *Listeria monocytogenes* ATCC 7644), (yellow: *Salmonella typhimurium* ATCC 14028, (green: Sample)

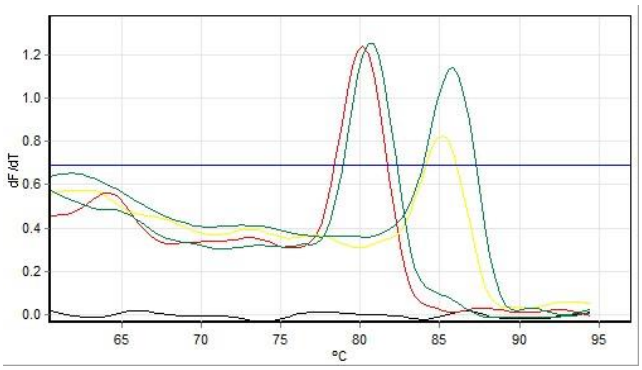

Figure 2: The melting curve of *L. monocytogenes* ATCC 7644, *S. typhimurium* ATCC 14028, and processed meat sample using the multiplex qPCR. (black: negative control; red: *Listeria monocytogenes* ATCC 7644; yellow: *Salmonella typhimurium* ATCC 14028; green: sample).

This study used the PCR direct technique is, a molecular detection technique without the DNA extraction process. Samples originating from NA are then clouded and equalized to standard 1 MacFarland, used as DNA templates. The disadvantage of this technique is the presence of inhibitors so that their purity and concentration cannot be ascertained. However, this method has advantages in terms of time and cost because it does not require extraction kits and shortens the analysis.

McLauchlin et al. (2000), in their research on cream and cheese, revealed that type of matrix influences PCR readings. However, the results of this study showed that the matrix did not significantly affect DNA amplification. All the samples were enriched on TSA or NA growth media and then scratched on the selective media so that the growing colonies would be separated from the matrix.

It can be concluded that the real-time multiplex PCR method can be used to detect *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* ATCC 7644 using melt curve analysis (Tm). However, the Ct analysis cannot specifically reveal samples contaminated with *Listeria monocytogenes* ATCC 7644 or *Salmonella typhimurium* ATCC 14028, while the Tm value can be used to distinguish them. Further study needs to be done to determine the level of primary specificity mixed in one master mix formula.

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REFERENCES

BPOM. 2019. Regulation of the Food and Drug Supervisory Agency Number 13 of 2019. Concerning the Limits of Contamination of the Food and Drug Supervisory Agency of the Republic of Indonesia. Jakarta. [Indonesian].

Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT. 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acid Res 16 (23): 11141-11156.

Gosiewski T, Monika B, Heczko PB. 2012. The application of multiplex PCR to detect seven different DNA targets in group B streptococci. Folia Microbiol (2012) 57 (3): 163-167.

Hayden, MJ, Nguyen, TM, Waterman, A, Chalmers, KJ. 2008. Multiplex-ready PCR: a new method for multiplexed SSR and SNP Genotyping. BMC Genomics. 9: 80. DOI: 10.1186/1471-2164-9-80

McLauchlin J, Narayanan GL, Mithani V, Neill GO. 2000. The detection of enterotoxin and toxic shock syndrome toxin genes in *Staphylococcus aureus* by polymerase chain reaction. J Food Prot 63: 479-488.

Molina F, Acedo E.L. Rafael T, Isidro R, Antonia G, Jose R. 2015. Improved detection of *Escherichia coli* and *coliform* bacteria by multiplex PCR. BMC Biotechnol 15: 48. DOI: 10.1186/s12896-015-0168-2.

Oliveira ACS, Matheus C Rosa, Jéssica LB, Yasmine AM, Milena MAF, Gabrielle Virgínia FC, Andréia SS, Roberta SS, Josyane BS, Fábio FL, Talita BR, Carina M. 2018. Validating the Efficiency of a Simplex PCR and Quantitative SYBR Green qPCR for the Identification of *Salmonella spp*. DNA. J Food Microbiol Saf Hyg 3: 1. DOI: 10.4172/2476-2059.1000130.

Sophian A. Purwaningsih R, Lukita B L And Cahyaningsih E. 2020. Detection of *Salmonella typhimurium* ATCC 14028 in supplement health product liquid preparation using Real-Time PCR (qPCR). Biofarmasi J Nat Prod Biochem 18: 61-65.

Sugiyoto, Kusuma A, Veronica W. 2015. Microbial content in beef from several traditional markets in Bandar Lampung. Integr Anim Husbandry Sci J 3 (2): 27-30 [Indonesian].

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Short Communication: Analysis of purity and concentration of extracted DNA on salted fish processed food products

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Abstract. *Sophian A. 2021. Short Communication: Analysis of purity and concentration of extracted DNA on salted fish processed food products. Asian J Nat Prod Biochem 19: 21-24.* Analysis of purity and concentration of extracted DNA on salted fish processed food products was carried out in the microbiology and molecular biology testing laboratory of the Food and Drug Administration in Gorontalo. The purpose of this study was to analyze the extracted DNA on salted fish processed food products based on the concentration and purity values in the A260 / A230 and A260 / A280 wavelengths. The method used for purity and concentration analysis was the absorbance method using a nanophotometer. The samples used were 10 types of salted fish processed food products sampled from 5 (five) traditional markets in Gorontalo City. The sample was extracted using the spin column method with the Dneasy Mericon Food Kit (50) paint kit. 69514 (Qiagen). The research data showed that extracted sample concentration was in the range of 24,600 - 27,150 with an average of 25,745, while the purity value measured at A260 / A280 wavelength was obtained with a purity range between 1,668 - 1,768 with an average of 1,729. Based on the results of this study, it can be concluded that the results of DNA extraction carried out on salted fish and processed food products show a value that is in the category of good DNA extraction results.

Keywords: Concentration, DNA, fish, purity, salty

INTRODUCTION

Analysis of extracted DNA's concentration and purity is the first step and is an important part that must be done in carrying out DNA-based molecular testing. The success rate of extraction can be determined by the purity and concentration value produced. At this stage, extraction is carried out by separating DNA from other components such as fat and protein using chemical and enzymatic processes. This study corresponded to Corkill and Rapley (2008) that the extraction stage is the initial key to the molecular analysis process's success. WheAt this stage, the sample will pass through three main processes, namely the process of lysis, or cell wall destruction, separation of DNA from other components such as protein and fat, and purification processes.

According to Holme and Peck (1998), the lysis process in DNA extraction removes the genetic material in the cell membrane to be used in molecular analysis. This solution can be done with the help of chemical and enzymatic processes. The chemicals and remaining enzyme residues resulting from the lysis process will be purified in the extraction process to produce inhibitor-free DNA. It is necessary to analyze the purity and concentration of the extracted DNA. The extraction method used is a spin column extraction method or column centrifuge with the Dneasy Mericon Food Kit kit combined with a robotic extraction system. This extraction tool works automatically and reduces the role of humans in DNA extraction. The conventional extraction method takes several steps to add chemicals and requires an extended processing time (± 10 hours) (Andreas et al. 2000). Therefore, to save time and control the error rate in researchers, modifications are made to combine these two types of methods. The advantage of this system is that it has a more stable extraction yield compared to conventional methods. On the other hand, humans are critical factors contributing to errors in the DNA extraction process.

The purpose of this research is to provide preliminary information regarding DNA extraction techniques in processed salted fish samples. It can be used as preliminary research to develop authentication and identification tests of processed fish species traded in the market. It can also monitor the types of species used as raw materials for processed food based on salted fish by looking at whether the species used are categorized as protected or free species that can be traded.

MATERIALS AND METHODS

Materials

This study's materials were processed food products of salted fish, RNA-free water, and a Dneasy Mericon Food Kit (50) paint extraction kit. 69514 (Qiagen).

Sample preparation

The sample was weighed as much as 1 g, added 1 mL of Food Lysis Buffer, and 25 µl of proteinase K were

homogenized by vortex for 15 seconds. The sample was then incubated at 65°C for 60 minutes while in a shaker with a speed of 1100 rpm. The stage was continued by lowering the sample temperature by leaving it at room temperature for 30 seconds, then putting it in an ice block/freezer for 10 minutes. After cooling, the samples were centrifuged at a speed of 2500xg for 10 minutes. Samples undergoing the centrifuge process will then form 2 phases, carefully pipette 500 µl of chloroform into a new 2 mL tube and remove 700 µl of the clear layer without touching the precipitation at the bottom of the tube. And put it in a tube containing 700 µl of chloroform and vortex for 15 seconds, then centrifuge at 14000xg for 15 minutes. Take 350 µl of the clear layer. Put it in Qiacube, and use the standard method with 60 ul of EB buffer elution. The eluted DNA can be used directly for real-time PCR processing or stored at -20ºC or - 80ºC for long storage.

Qiacube setup

The initial stage started with inputting the Qiacube protocol for the Dneasy Mericon Food Kit kit. All systems are carried out automatically using a robotic system so that people's involvement is only during the initial lysis stage. The protocol used is to extract total DNA from raw or processed food material with standard methods. The sample was piped 350 µl into a 2 mL tube and then placed into the Qiacube. After that, proceed with arranging the kit, which will be used according to the protocol map. After all the results are appropriate, then the tool is run.

Purity and concentration analysis

Purity and concentration were analyzed using a nano photometer NP80 (IMPLEN). Method setting; Nucleic acid, dsDNA type, nano volume mode, 2 µL sample volume, nucleic acid factor 50.00, background correction 320 nm, air bubble recognition off, manual dilution factor 1.000.

DNA yield

After knowing the concentration and purity values, the next step is calculating the yield. DNA yield is the final DNA product calculated using the formula:

DNA yield (µg) = DNA concentration × total sample volume (mL)

Data analysis

Data analysis was carried out by comparing the purity and concentration values against DNA standards, where the purity at wavelength A260 / A280 was in the range of 1.7–2.1, while the concentration was greater than 20 ng / mL (Leninger 1975; Matlock 2015) or 2.0-2.5 (Eppedorf 2016).

RESULTS AND DISCUSSION

Data from concentration and purity analysis

The analysis of concentration and purity was carried out using a nano photometer, and the results were obtained (Table 1). It can be seen that the value of extracted sample concentration is in the range of 24.600 - 27.150 with an average of 25.745. The purity value was measured at A260 / A230 wavelength, and the results were obtained with a purity range between 0.814 - 0.874 with an average of 0.834. while purity values were measured at the wavelength A260 / A280, the results were obtained with a purity range between 1.668 - 1.768, with an average of 1.729.

According to Eppendorf (2016), the optimum purity value of DNA at the wavelength A260/A280 is in the range 1.8-1.9, while for RNA, it is in the range 1.9-2.0. This is different from the opinion expressed by Kirby (1990); Sambrook (1989) stated that the results of DNA extraction are good if the purity value is in the range of 1.8-2, the concentration is greater than 20 (ng / µl). When viewed from Table 1 above, the purity value that reads the wavelength A260/A280 shows a value below 2.00. Therefore, it can be concluded that the extracted DNA does not fall into a good DNA range. To conclude that a sample extracted can be tested using real-time PCR, the purity and concentration values that are the benchmark for isolation are good because of developments. Real-time PCR technology allows amplification to occur at even low concentrations depending on the sensitivity of a PCR device.

DNA yield

The research conducted obtained an average of 1357.50 with the lowest yield value of 1230.00 and the highest yield value of 1287.30. Other results are presented in Table 2.

The absorbance method is one method to perform yield analysis using a spectrophotometric instrument. The absorbance readings were carried out at a wavelength of 260 nm or (A260). This is because DNA at this wavelength absorbs light so that the resulting turbidity can be used to estimate the amount of DNA detected. Readings are carried out in the instrument's linear range (0.1 - 1.0).

Table 1. Extracted nano photometer data

Sample	Concentration ng/µL	Purity (A260/A280)
Sample 1	24.600	1.668
Sample 2	24.650	1.724
Sample 3	24.650	1.761
Sample 4	25.150	1.729
Sample 5	25.250	1.766
Sample 6	26.700	1.768
Sample 7	26.450	1.740
Sample 8	27.150	1.729
Sample 9	26.450	1.690
Sample 10	26.400	1.720
Average	25.745	1.729

Table 2. DNA yield

Sample	Concentration ng/µL	Total Dilution Volume (µl)	Yield
Sample 1	24.600	50	1230.00
Sample 2	24.650	50	1232.50
Sample 3	24.650	50	1232.50
Sample 4	25.150	50	1257.50
Sample 5	25.250	50	1262.50
Sample 6	26.700	50	1335.50
Sample 7	26.450	50	1322.50
Sample 8	27.150	50	1357.50
Sample 9	26.450	50	1322.50
Sample 10	26.400	50	1320.00
Average	25.745	50	1287.30

Discussion

In general, the DNA extraction process using the Phenol-Chloroform extraction system consists of three processes: cell lysis, purification, and precipitation. The lysis stage was carried out with proteinase K and Sodium Dodecyl Sulfate (SDS) enzymes. At this stage, SDS will lyse fats and proteins in the cell membrane so that the contents in the cell membrane come out. This process is carried out by heating at a temperature of 70°C while being shaken. This heating activates the proteinase K enzyme to carry out lysis (Renshaw 2015) actively. The Phenol-Chloroform extraction system uses phenol to bind proteins, fats, and carbohydrates, separated from other macromolecules. Phenol and Chloroform Isoamyl Alcohol-bounds proteins and polysaccharides will settle to the tube's bottom. DNA and water are in the top layer (Kado et al. 1981). To separate and purify the DNA is using a centrifuge column, where at the end of the washing process using alcohol, the remaining salt and phenol contained in the sample will come out and leave the DNA pellets. Sterile distilled water or nucleotide-free water can be used to pull the DNA pellets from the spin column.

This study's extraction technique combines conventional methods using a centrifuge and robotic techniques using Qiacube. The advantage of combining these methods is to produce a more stable DNA extraction. Table 1 shows the data from the DNA nanodrop reading results extracted. The DNA extraction system using a robotic system is the same as conventional extraction. The difference is in the use of robots to perform pipetting and centrifuge.

Analysis of purity and concentration is read using a nanophotometer by measuring the absorbance value at a wavelength of A260/A280. The A260/A280 wavelength is a standard method for detecting DNA concentration and purity value. In the salted fish samples analyzed, the sample matrix composed of fish meat and salt seeping into the meat also requires caution in carrying out DNA extraction to produce good DNA isolates for analysis at the next step, namely the amplification process.

Leninger (1975) stated that of the 5 nucleotide compositions that make up DNA or RNA, when reading the absorbance at wavelength A260/A280, they would show varying values, namely: guanine (1.15), adenine (4.50), cytosine (1.51), uracil (4.00) and thymine (1.47). The purity analysis results obtained from the absorbance readings are the average of these four or five nucleic acids' absorbance values. It is the basis for determining the purity value in general for DNA analysis in the range (of 1.8-2.0). For RNA, the range value will be greater than this value because one of the constituent components is uracil, which, when compared with DNA composed of thymine, uracil has a higher value, namely (4.00), so that if averaged, the purity value will be higher when paired with DNA.

Apart from all analyzes of the extracted DNA, the final stage of DNA analysis, of course, lies in the PCR itself, whether in further research it can detect the extracted DNA or not because the purity and concentration values shown are initial analyzes to assess the success of the DNA extraction process.

The results of DNA yield showed that the amount of DNA was quite large, namely around 1278.30. This value is considered very good compared to the DNA yield value required for DNA amplification using real-time PCR. Each PCR has different detection limit detection capabilities ranging from 0.01-2 p / µL (Perandin et al. 2004; Cnops et al. 2011; Kamau et al. 2013; Xu et al. 2015; Srisutham et al. 2017). Suggestions for further research should be continued until the confirmation test stage to prove that the results of DNA extraction are not good enough; the results can still be used.

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REFERENCES

Andreas E, Sumantri C, Nuraini H, Farajallah A, Anggraeni A. 2010. Identification of GH|AluI and GHR|AluI genes polymorphisms in Indonesian buffalo. J Indon Trop Anim Agric 35: 215-221. [Indonesian]

Cnops L, Jacobs J, Van Esbroeck M. 2011. Validation of a four-primer real-time PCR as a diagnostic tool for single and mixed *Plasmodium* infections. Clin Microbiol Infect 17 (7): 1101-1107.

Corkill G, Rapley R. 2008. The Manipulation of Nucleic Acid: Basic Tools & Techniques in Molecular Biomethods Handbook. 2nd ed. Humana Press, New York.

Eppendorf. 2016. Nucleic Acid Photometry. Check of critical parameters. Eppendorf AG, Hamburg, Germany. eppendorf@eppendorf.com, www.eppendorf.com

Holme DJ, Peck H. 1998. Analytical Biochemistry. 3rd ed. Pearson Education Limited, Harlow, UK.

Kado CI, Liu ST. 1981. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145 (3): 1365-1373.

Kamau E, Alemayehu S, Feghali KC, Saunders D, Ockenhouse CF. 2013. Multiplex qPCR for detection and absolute quantification of Malaria. PLoS One 8 (8): e71539. DOI: 10.1371/journal.pone.0071539.

Kirby LT. 1990. DNA Fingerprinting: An Introduction. M. Stockton Press, New York.

Leninger AL. 1975. Biochemistry, 2nd ed. Worth Publishers, New York.

Matlock B. 2015. Assessment of Nucleic Acid Purity. Technical Note 52646. Thermo Fisher Scientific, Wilmington, MA, USA.

Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L, Medici MC, Arcangeletti MC, Snounou G, Dettori G, Chezzi C. 2004. Development of a real-time PCR assay for detection of Plasmodium

falciparum, *Plasmodium vivax* and *Plasmodium ovale* for routine clinical diagnosis. J Clin Microbiol 42 (3): 1214.

Renshaw. 2015. The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. J Mol Ecol Resour 15 (1): 168-176.

Sambrook J, Fritsch F, Miniatis T. 1989. Molecular Cloning Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Pr, New York.

Srisutham S, Saralamba N, Malleret B, Rénia L, Dondorp AM, Imwong M. 2017. Four human Plasmodium species quantification using droplet digital PCR. PLoS One 12 (4): e0175771. DOI: 10.1371/journal.pone.0175771.

Xu W, Morris U, Aydin-Schmidt B, Msellem MI, Shakely D, Petzold M. 2015. SYBR green real-time PCR-RFLP assay targeting the Plasmodium cytochrome B gene - A highly sensitive molecular tool for malaria parasite detection and species determination. PLoS One 10 (3): e0120210. DOI: 10.1371/journal.pone.0120210.

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Antifungal activities of methanol extracts of some medicinal plants against germination and growth of *Colletotrichum destructivum* O'Gara in culture

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Abstract. *Enyiukwu DN, Amadioha AC, Ononuju CC. 2021. Antifungal activities of methanol extracts of some medicinal plants against germination and growth of Colletotrichum destructivum O'Gara in culture. Asian J Nat Prod Biochem 19: 25-29.* Cowpea is an important grain and leafy vegetable in many tropical locations. Anthracnose caused by *Colletotrichum destructivum* O'Gara is one of the several factors constraining the economical production of the crop in warm, humid areas. The use of resistant varieties and synthetic pesticides to control the disease in the crop are disadvantaged for the variability of the pathogen, leading to resistant failure of cultivars in many cowpea growing areas; and pesticide residues in the treated crops, which ultimately engender mammalian toxicity, respectively. This study evaluated the effects of *Alchornea cordifolia*, *Tabernaemontana pachysiphon*, and *Lantana camara* as eco-friendly fungicides against the pathogen in culture. The results showed that the extracts inhibited the fungus in a dose-wise manner. At 75 and100 % concentrations, *L. camara* strongly inhibited the spore germination and radial growth of the fungus better than all concentrations of *T. pachysiphon* and *A. cordifolia*, whereas *A. cordifolia* extracts exhibited the lowest inhibition at all concentrations. Generally, the fungitoxicity of benomyl was found higher, inhibiting 90.15 % spore germination and 96.32 % radial growth of the pathogen. However, at 75%, 100% *L. camara* and 100% *T. pachysiphon*, which significantly ($P \leq 0.05$) inhibited (80.02%, 84.21 %, and 80.91%) spore germination and (87.33%, 90.87 % and 85. %) radial growth of the fungus respectively compared well with inhibition effects recorded from benomyl. Therefore, these plants can be used as viable protectants of cowpea against anthracnose (*C. destructivum*) in smallholder farming systems of the humid tropics; to enhance its production and farm economy.

Keywords: Anthracnose, *Colletotrichum destructivum*, cowpea, fungicides, plant extracts

INTRODUCTION

Cowpea is an integral part of several cuisines consumed in many countries of the tropics and sub-tropics, including South Africa, Nigeria, and some parts of Asia. The leaves, pods, and grains rich in protein and phosphorus are cherished organs of the crop, widely eaten as vegetables or pulses amongst many natives (Nielsen 1997; Enyiukwu et al. 2018a).

Anthracnose, a seed or thrash-borne disease caused by *Colletotrichum destructivum* O'Gara, is a major biotic challenge to the economical production of the crop in many cowpea growing locations (Adegbite and Amusa 2008; Enyiukwu et al. 2018b). The disease is pan-tropical, reducing grain yield by up to 50 % in susceptible cowpea cultivars. In very severe outbreaks, anthracnose can cause total crop failure (Begum et al. 2007: 2013; Amadioha and Enyiukwu 2019a). Besides, reducing the quality of grain and vegetables due to loss of vital biochemicals also results from attacks of the pathogen on the crop. Recently, we reported a mean loss of major nutrients (protein, lipids, carbohydrate) of 38.29 %, 28. 95 %, 22.55 %, and 18.14 % for the leaves, seeds, husks, and stem, respectively, of infected cowpea 8 weeks after planting; while loss of 11.86 % Ca and 13.63 % P was recorded in the crop grains in storage about the same period (Amadioha and Enyiukwu 2019a, b).

Though good agronomic practices and the use of resistant varieties are invaluable strategies for curbing the disease (Awurum et al. 2001; Enyiukwu and Awurum 2013); however, the use of resistant varieties has the disadvantage of being short-lived due largely to the variability of the anthracnose fungus which could lead to resistance failure in the cultivars (Podila 1993; Enyiukwu et al. 2014). Generally, chemical treatments are effective in arresting the development and spread of the pathogen. Still, such interventions have been trailed by several forms of mammalian toxicities, congenital disabilities, allergies, male sterility, and even death owing to residues of the pesticides in crops and the environment (BMC 1992; Awurum and Enyiukwu 2013). These, amongst other factors, ignited the search for alternatives or at least complements to the synthetic pesticides by scientists (Asawalam and Emosairue 2006; Nwaogu and Wokocha 2018).

Several biological activities have been reported about many families of tropical higher plants against a wide array of microorganisms, such as viruses, bacteria, and fungi, which are pathogenic to humans and plants (Enyiukwu et al. 2014; Mgbeahuruike et al. 2017, 2018; Enyiukwu 2019). Interestingly *Alchornea cordifolia*, *Tabernaemontana pachysiphon*, and *Lantana camara* have long been used in

ethnobotany and phytotherapy of many cultures. Recent antimicrobial evaluations of these plants on storage pathogens showed that they inhibited *Botrydiplodia fumigatus*, *Aspergillus fumigatus*, and *Penicillium notatum* (Amienyo and Ataga, 2007; Naz and Bato, 2013; Duru et al. 2015). However, the antifungal activities of extracts or compounds derived from these medicinal plants against *Colletotrichum destructivum* are scarce. Therefore, the purpose of this study is to evaluate the inhibitory effects of methanol extracts of *Alchornea cordifolia*, *Tabernaemontana pachysiphon*, and *Lantana camara* in culture against *C. destructivum* O'Gara and causing anthracnose of cowpea in Nigeria.

MATERIALS AND METHODS

Collection and preparation of plant materials

Leaves of the plant materials [*Alchornea cordifolia* (Schumach. and Thonn.) Mull. Arg., *Lantana camara* Linn. *and Tabernaemontana pachysiphon* Stapf] were obtained from the University community and Umudike in Abia State. The leaves were washed thoroughly in tap water, rinsed with sterile distilled water, and air-dried on the laboratory bench for 20 days. After that, they were milled separately into a fine powder using a hand milling machine (Model Corona Lavesh 250) to obtain 300 g of each sample which was stored separately in air-tight bottles. Each powder was weighed out separately in 25, 50, 75, and 100 g in different 250 mL conical flasks, to which 100 mL of 30 % methanol was added, and the flask was closed with foiled stoppers. They were allowed to stand for 2 h. They were then strained separately through 4-folds of sterile cheesecloth into different 200 mL beakers to obtain the respective methanol filtrates of 25, 50, 75, and 100 % concentrations of the test samples (Amadioha 2003).

Preparation of culture medium (PDA)

About 39.5 grams of potato dextrose agar (PDA) (Oxoid™ ThermoScientific Product, England, UK) was dissolved in 1000 mL of sterile distilled water in a 1000 mL conical flask to which 4 drops of lactic acid were added and then stirred vigorously before closing the flask with foiled cotton wool and autoclaving at 15 pounds per square inch (Psi) (152 cmHg, 120^{o}C) pressure for 15 minutes.

Isolation and identification of the causal organism

Bits of cowpea (*Vigna unguiculata* L. Walp.) (Var. IAR 48) pods with typical anthracnose symptoms (12 mm) were sterilized in 70% ethanol for 1 min and then washed several times with 50 mL of sterile distilled water. The tissues were plated in blotter paper and incubated for 7 days at 27^{0}C. The mycelial growth from the plated cowpea tissues was subcultured on PDA slants.

The color and colony characteristics of the isolate were observed under the microscope and recorded. Slides were prepared, fixed, mounted, and examined under a low/high power Olympus digital compound microscope fitted with the software Scopevision version 9.0. The morphological characteristics of the conidia and structures of the fungus captured in photographs were used to compare and confirm its identity concerning the illustrated species by Shen et al. (2001), Damm et al. (2014), and monographs of the International Mycological Institute (IMI 1995).

Pathogenicity test: A spore suspension of the fungal isolate (1 x 10^5 spores/mL of distilled water) was sprayed with a sterile syringe on healthy (uninfected) 7-day-old cowpea seedlings. The seedlings inoculated with the isolate showed severe infection of anthracnose disease with water-soaked lesions covered with black acervuli all over the hypocotyls after 3 days of inoculation. Severely affected seedlings became weak, deteriorated, and eventually died. Control seedlings were sprayed with sterile distilled water. They grew well without black or tan-colored, water-soaked lesions. The isolate caused typical symptoms in the cowpea plant, similar to the original isolate, which confirmed it as a pathogen. The test fungus was re-isolated from the diseased seedlings, re-examined, and confirmed identical with the previously isolated and inoculated isolate.

In vitro experiment

Preparation of spore suspension

The spores of *Colletotrichum destructivum* O'Gara were collected from 10-day-old culture-agar stock in Petri dishes by lifting 60 cm^2 pieces into a beaker containing 200 mL of sterile distilled water. This was sieved through 4-folds of sterile cheesecloth to remove agar and mycelia fragments, and the filtrate was centrifuged for 10 minutes. The spore suspension was then adjusted to 10^5spores/mL of sterile distilled water using a hemocytometer counting slide.

Effect of plant extracts on spore germination of Colletotrichum destructivum

The method of Amadioha (2003) was adopted to evaluate the effect of the extracts of the test plants on the germination of the spores of *C. destructivum.* A disc (3mm) of the fungus was placed in 3mL of the different concentrations (25, 50, 75, and 100%) of the crude methanol extracts of the different plants or benomyl (as a placebo) contained in different test tubes. The test tubes and their contents were then centrifuged for 10 minutes. After that, the resulting supernatants were then filtered through 4-folds of cheesecloth. A drop (0.05 mL) of the different concentrations was placed separately on 3 sterile slides and incubated for spore germination at 27^{o}C for 24 h in a humid chamber. The controls were maintained similarly but consisted of sterile water or benomyl. Further spore germination was stopped by adding one drop of lactophenol in cotton blue to each slide preparation. The effect of the tissue extracts from the plant materials on the germination of the test fungus spores was determined by examining 100 randomly selected pathogen spores under a microscope. Records of the number of germinated spores for each treatment/replicate were taken and then used to determine the percentage inhibition of spore germination of the pathogen compared to the controls using the formula by Amadioha (2003) as:

$$\% \text{ Inhibition of spore germination} = \frac{gc-gt}{gc} \times 100$$

Where:

gc = average number of germinated spores of the test fungus with control

gt = average number of germinated spores of the test fungus with treatment

Effect of the extracts on radial growth of Colletotrichum destructivum

One (1) mL of different concentrations (25, 50, 75, and 100%) of the crude methanol extracts of the plant materials were smeared separately on the surface of solidified PDA by gentle swirling motion (Amadioha 2003). A disc (3 mm) of the 10-day-old culture of the pathogenic fungus was transferred to the Petri dishes center, which had been marked underneath with two perpendicular lines intersecting at the center. The dishes were covered and incubated at 27^0C for 7 days. The controls were maintained similarly but with sterile distilled water or benomyl mixed with the PDA in the dishes. The radial growth of the pathogen was measured along the perpendicular lines with a meter rule 7 days after incubation.

The fungitoxicity of the extract was determined as a percentage of mycelial growth inhibited and calculated by the formula as adopted by Amadioha (2003; Amadioha et al. 2019):

$$\% \text{ Growth inhibition} = \frac{dc-dt}{dc} \times 100$$

Where:

dc = average diameter of the fungal colony with control

dt = average diameter of the fungal colony with treatment.

Data analysis

The experiments were conducted in Completely Randomized Design (CRD), consisting of 14 treatments replicated 3 times. The whole experiment was repeated twice. Data collected from the study were analyzed by analysis of variance (ANOVA) using the general linear model procedure in Genstat Release (Windows/PC Vista, version 12.10) at a significant level of 5 %. Means were separated and compared using Fisher's LSD at the probability of 0.05.

RESULTS AND DISCUSSION

Results

The result of *in vitro* effect of the crude methanol extracts of the test plant materials and benomyl on the spore germination and radial growth of the fungus is presented in Table 1. The result showed that both the crude methanol extracts and benomyl significantly ($P \leq 0.05$) inhibited the spore germination and radial growth of the fungus in the culture. It also revealed that crude extracts of the test plants varied in their inhibitory activities against the fungus. Among the three plants, *L. camara* at 100 % concentration was the most toxic to the fungus and exhibited 84.21% and 90.87% inhibition of spore germination and radial growth of the pathogen. *T. pachysiphon* at 100 % concentration exhibited 80.91% and 85.14% inhibition for spore germination and radial growth of the pathogen, respectively, while *A. cordifolia* was the lowest inhibition effect on the test parameters of the fungus at all concentrations.

Similarly, at 75% concentration, *L. camara* inhibited the germination of spores of the pathogen by 80.02% and impeded radial growth of the fungus by 89.33%. In general, the sensitivity profile of the pathogen to the toxicants at 75% concentration was *L. camara*>*T. pachysiphon*>*A. cordifolia*. However, the fungi toxic activities of the plant extracts were not as effective as benomyl which inhibited spore germination and radial growth of the fungus by 90.15% and 96.32%, respectively. However, results of the inhibition effects obtained for *L. camara* at 75% and 100% dosages and *T. pachysiphon* at 100% concentration of incorporation in the media (Table 1) compared statistically ($P \leq 0.05$) well with those of benomyl for the same parameters respectively.

Discussion

Incorporation of methanol extracts of *L. camara* into the culture medium in this study strongly inhibited spore germination and significantly ($P \leq 0.05$) retarded radial growth of *C. destructivum* better than extracts of *T. pachysiphon* and *A. cordifolia* (Table 1).

Table 1: Fungioxic effect of methanol extracts of test plants and benomyl on the spore germination and radial growth of *Colletotrichum destructivum*

Treatment	Percentage growth inhibition (%)	
	Spore germination*	Radial growth**
Alchornea cordifolia		
100%	51.23	59.08
75%	47.08	51.12
50%	41.20	48.07
25%	41.09	45.55
Tabernaemontana pachysiphon		
100%	80.91	85.14
75%	63.45	69.01
50%	58.33	64.33
25%	50.13	60.04
Lantana camara		
100%	84.21	90.87
75%	80.02	87.33
50%	77.46	84.50
25%	70.10	79.89
Fungicide (Benomyl)	90.15.	96.32
Control (Methanol)	0.00	0.00
LSD (0.05)	**9.315**	**11.018**

Note: Data are means of values from 2 separate experiments replicated 3 times

Several *Colletotrichum* species. caused anthracnose disease in cowpea, which has been effectively controlled by using fungicides of plant origin. *Xylopia aethiopica, Azadirachta indica, Cymbopogon citratus, and Piper nigrum* were reported to inhibit the growth of *Colletotrichum lindemuthianum in vitro* and *in vivo* (Amadioha and Obi 1998; Amadioha 2003). In a trial, extracts of *Piper guineense* substantially inhibited spore germination of *C. destructivum* (Enyiukwu and Awurum 2011).

Similarly, *Monodora myristica, Argemone mexicana, Tephrosia purpurea, Diodia scandens, and Cyathula prostrata* were reported to retard the development of *C. destructivum* in culture (Akinbode and Ikotun 2008; Obi 2011; Ogu and Owoeye 2013; Obi and Barriusa-Vargas 2014). In all these evaluations, the botanicals acted by inhibiting spore germination and retarding the pathogenic organisms' radial growth and mycelial biomass (Amadioha, 2003; Mukherjee et al. 2011; Ogu and Owoeye, 2013; Obi and Barriusa-Vargas 2014). Across concentrations in this study, extracts of *A. cordifolia, T. pachysiphon,* and *L. camara* showed moderate to strong inhibition of both spore germination and radial growth of *C. destructivum*, thereby corroborating the reports of findings of the other workers above.

However, in this study, the antifungal activity of the test plants against the spore germination and radial growth of *C. destructivum* were inferior to benomyl and did not confirm the report of Costa da Silva et al. (2012; 2014), where the extract of *Hyptis marrubioides* was significantly superior to carbendazim in inhibiting the spore germination and radial growth of *C. truncatum* and *Phakopsora pachyrhizi* causing anthracnose disease and rust of soybean respectively. The pesticide activity of botanicals is based on the type of active chemical principle and the amounts of functional groups involved (Enyiukwu and Awurum 2012). Therefore, the fewer fungi toxic efficacy of test plant extracts in this study may indicate that their active ingredients differ widely in structure, type, and amount of substituent functional chemical groups from benomyl.

In general, it was noted that the fungi toxic activity of the tissue extracts increased with the concentration of test extracts (Table 1). This also agrees with the reports of other investigators (Suleiman and Emua 2009). The fungitoxicity profile of the concentrations of plant extracts on the test fungus in this study was 100%>75%>50%>25%. The antifungal activity of botanicals was influenced by the concentration or amount of the active chemical compounds in the medium, which in turn is dependent on the compound's solubility in the extraction solvent (Amadioha 2003). Therefore, the differential toxicity of the botanical extracts against the pathogen may have been influenced by the solubility of the active principles in methanol. The chemical compounds of *L. camara* were perhaps more soluble than *T. pachysiphon* and *A. cordifolia*.

Enyiukwu (2017) noted the presence of Dodecanoic acid (commonly called lauric acid) in *L. camara* (42.57 %)), *T. pachysiphon* (29.69 %), and *A. cordifolia* (15.87 %). Dodecanoic acid (1,2,3-propanetryl ester) is reported to possess stronger antimicrobial activities than the other fatty metabolites (Nakatsuji et al. 1995; Rajeswari et al. 2012; Omotosho et al. 2014; Axe 2017; COC 2017). Hence, a higher amount of Dodecanoic acid (1,2,3-propanetryl ester) found in *L. camara* and *T. pachysiphon* may be the reason for high fungi toxic activities against *C. destructivum* over *A. cordifolia*.

Therefore, these plant extracts can be used as possible fungicides in smallholder farming systems to protect cowpeas from anthracnose caused by *C. destructivum* O'Gara; and hence improve the productivity of the crop and food security in the sub-region

REFERENCES

Adegbite AA, Amusa AN. 2008. The major economic field diseases of cowpea in the humid agro-ecologies of South-Western Nigeria. Afr J Biotech 7 (25): 4705-4712.

Akinbode AO, Ikotun T. 2008b. Efficacy of certain plant extracts against seed-borne infection of *Colletotrichum destructivum* on cowpea (*Vigna unguiculata*). Afr J Biotechnol 7 (20): 3683-3685.

Amadioha AC, Enyiukwu DN. 2019. Biochemical composition of seed and husk of cowpea (*Vigna unguiculata* L. Walp.) infected by *Colletotrichum destructivum* O'Gara in storage. Ann Res Rev Biol 31 (1): DOI: 10.9734/arrb/2019/v31i130034.

Amadioha AC, Enyiukwu DN. 2019. Alterations of biochemical composition of leaf and stem of cowpea (Vigna unguiculata L. Walp.) by *Colletotrichum destructivum* O'Gara in Nigeria. J Exp Agric Intl 33 (2): DOI: 10.109734/IJEAI/2019/v33i230138.

Amadioha AC, Kenkwo PC, Markson AA. 2019. Fungitoxic potentials of extracts of plant origin against fungal rot pathogens of cassava (*Manihot esculenta* Crantz) in storage. Ann Res Rev Biol 31 (1): DOI: 10.9734/arrb/2019/v31i130036.

Amadioha AC. 2003. Evaluation of some plant leaf extracts against *Colletotrichum lindemuthianum* in cowpea. Acta Phytopathologica et Entomologica Hungarica 38: 259-265.

Amienyo CA, Ataga AE. 2007. Use of indigenous plant extracts for the protection of mechanically injured sweet potato (*Ipomea batatas* (L.) tubers. Sci Res Essays 2 (5): 167-170.

Asawalam EF, Emosairue SO. 2006. Comparative efficacy of *Piper guineense* (Schumm. Thonn.) and primophos-methyl as poisons against *Sitophilis zeamais* (Motsch). E-J Environ Agric Food Chem 5 (5): 1536-1545.

Awurum AN, Enyiukwu DN. 2013. Evaluation of the seed-dressing potentials of phytochemicals from *Carica papaya* and *Piper guineense* on the germination of cowpea (*Vigna unguiculata* L. Walp.) seeds and incidence of the seed-borne fungi. Continental J Agric Sci 7 (1): 29-37.

Awurum AN, Amadioha AC, Emechebe AM. 2001. Effect of cropping systems on disease development in cowpea in Umudike, Southeast Nigeria. J Sustain Agric Environ 3 (1): 31-38.

Axe Anonymous. 2017. Fight acne and infections with lauric (dodecanoic) acid. www.draxe.com [April 2, 2017].

Begum JMAJ, Venudevan B, Jayanthi M. 2013. Storage fungi in groundnut and the associated seed quality deterioration-A review. Plant Pathol J 12 (3):127-134.

Begum MM, Saviah M, Puteh AB, Abidim MAZ. 2007. Detection of seed-borne fungi and site of infection by *Colletotrichum truncatum* in naturally infected soybean. J Agric Res 2 (9): 812-819.

British Medical Association (BMA) 1992. Guide to Pesticide Chemical and Health. Edward Press Inc., London, UK.

Campos-Vegas R, Loarca-Pina G, Oomah BD. 2010. Minor components of pulses and their potential impacts on human health. Food Res Intl 43: 461-482.

Chikwendu JN, Igbatim AC, Obizoba IC. 2014. Chemical composition of processed cowpea tender leaves and husks. Intl J Sci Res Publ 4 (5): 1-5.

COC (Coconut Oil Central) Anonymous 2017. Lauric (dodecanoic) acid the mightiest antibacterial fatty acid known to man. www.coconut-oil-central.com, [April 2, 2017].

Costa da Silva, A, Estevao de Souza P, Amaral DC, Zeviani WM, Pinto J.EBP. 2014. Essential oils from *Hyptis marrubioides*, *Aloysia*

gratissima and *Cordia verbenacea* reduce the progress of Asian soybean rust. Acta Scientarum Agronomica 36 (2): 150-166.

Costa da Silva A, Estevao de Souza, P, Da Cruz S, Machada, J, Da Silva BM, Pinto JE.BP. 2012. Effectiveness of essential oils in the treatment of *Colletotrichum truncatum*-infected soybean seed. Trop Plant Pathol 37 (5): 305-313.

Damm U, O'Cornell RJ, Groenevald JR, Crous PW. 2014. *Colletotrichum* species complex, hemibiotrophic pathogens of forage and field crops. Stud Mycol 79: 31-84.

Duru CM, Anyadoh-Nwadike SO, Okechukwu RI. 2015. Antimicrobial activity and phytochemical analysis of aqueous and ethanolic extracts of the bark of *Tabernaemontana pachysiphon* Stapf. Sci J Public Health 3 (1-5): 8-13.

Enyiukwu DN. 2017. Effects of extracts of some medicinal plants on *Colletotrichum destructivum* O'Gara causing anthracnose disease of cowpea (*Vigna unguiculata* L. Walp.) in Nigeria. [Dissertation] Michael Okpara University Agriculture Umudike, Nigeria.

Enyiukwu DN, Amadioha AC, Ononuju CC. 2018. Biochemical composition, potential food and feed values of aerial parts of cowpea (*Vigna unguiculata* L. Walp.) Greener Trends Food Sci Nutr 1 (1): 11-18.

Enyiukwu DN, Amadioha AC, Ononuju CC. 2018. Significance of cowpea leaves for human consumption. Greener Trends Food Sci Nutr 1 (1): 1-10.

Enyiukwu DN, Awurum AN, Ononuju CC, Nwaneri JA. 2014. Significance of characterization of secondary metabolites from higher plants in plant disease management. Intl J Advance Agric Res 2 (2): 30-46

Enyiukwu DN, Awurum AN. 2013. Fungitoxic effects of *Carica papaya* and *Piper guineense* extracts on *Colletotrichum destructivum* in the glasshouse. Continental J Agric Sci 7 (1): 23-28.

Enyiukwu DN, Awurum AN. 2012. Comparative fungitoxicity of benomyl and extracts of *Carica papaya* roots and seeds and *Piper guineense* seeds on *Colletotrichum destructivum* O'Gara. Continental J Biol Sci 5 (1): 26-31.

Enyiukwu DN. 2019. Nature's Pharmacy Vol. 1: A Focus on *Carica papaya* L. Greener Scholarly Publications (GSP), Lagos Nigeria.

Enyiukwu DN, Awurum AN. 2011. Effects of phytochemicals from *Carica papaya* roots and seeds and Piper guineense seeds on germination of spores of *Colletotrichum destructivum* O'Gara. Continental J Biol Sci 4 (2): 55-59.

Igbatim CA, Chikwendu JN, Obizoba IC. 2014. Chemical and organoleptic potentials of soups based on cowpea leaves and husks. IOSR J Environ Sci Toxicol Food Technol 8 (11): 11-22.

IMI. 1995. Description of Pathogenic fungi and Bacteria Nos. 316-317. International Mycological Institute), Kew, UK.

Mgbeahuruike EE, Yrjonen T, Vourela H, Holm Y. 2017. Bioactive compounds from medicinal plants: focus on Piper species. South Afr J Bot 112: 54-59.

Mgbeahuruike EE, Holm Y. Vourela H, Amandikwa C, Fyrquist P. 2018. An ethnobotanical survey and antifungal activity of *Piper guineense* used for the treatment of fungal infections in west African traditional medicine. J Ethnopharmacol 229: 157-166.

Mukherjee SK, Islam MR, Shahid SB. 2011. Efficacy of some plant extracts on the mycelia growth of *Colletotrichum gloeosporioides*. J Bangladesh Agric Univ 9 (1): 43-47.

Nakatsuji T, Kao MC, Fang JY, Zouboulis CC, Zhang L, Gallo RL, Chung-Mung H. 2009. Antimicrobial property of lauric acid against *Propionibacterium acnes*: its therapeutic potentials against inflammatory *Acne vulgaris*. J Invest Dermatol 129 (10): 2480-2488.

Naz R, Bano A. 2013. Phytochemical screening, antioxidants and antimicrobial potential of Lantana camara in different solvents. Asian J Tropical Med 1808 (13): 60104-60108.

Nielsen SS, Ohler TA, Mitchell M.C. 1997. Cowpea leaves for human consumption: production, utilization and nutrient composition. In: Singh BB (eds.). Advances in Cowpea Research, Ibadan.

Nwaogu AG Wokocha RC (2018). Effects of extracts of some botanicals against three species of fungi isolated from cocoa (*Theobroma cacao*) pods. Greener J Med Plants 1 (1): 001-007.

Ogu GE, Owoeye AE. 2013. In vitro control of anthracnose disease of cowpea (*Vigna unguiculata* L. Walp.) caused by *Colletotrichum destructivum* O'Gara with *Cyathula prostrata* L. and *Diodia scandens* SW leaf extracts. J Intl EthnoPharm 2 (1): 29-36.

Omotosho AE, Oluwafemi EO Maikailu S. 2014. Phytochemical analysis of *Cnidoscolus aconitifolius* (Euphobeaceae) leaf with spectrometric techniques. Nigerian J Pharm Appl Res 3 (1): 009-014.

Podila GK, Rogers LM, kolattukudy P. 1993. Chemical signals from avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. Plant Physiol 103 (1): 267-272. DOI: 10.1104/pp.103.1.267.

Rajeswari G, Murugan M, Moghan VR. 2012. GC-MS analysis of bioactive components of *Hugonia mystax* L. (Linaceae). Res J Pharm Biol Clin Sci 3 (4): 301-308.

Shen S, Goodwin P, Hsiang T. 2001. Hemibiotrophic infection and identity of the fungus *Colletotrichum destructivum* causing anthracnose of tobacco. Mycol Res 105 (11): 1340-1347.

Suleiman MN Emua SA. 2009. Efficacy of four plant extracts in the control of root rot disease of cowpea. Afr J Biotechnol 8 (16): 3806-3808.

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Insights into low biological activity of wax apple (*Syzygium samarangense*) juice by in vitro phytochemical investigation with special reference to metabolomics

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Abstract. *Majumder S, Acharyya S, Ghosh A, Chakraborty S, Sarkar S, Saha S, Bhattacharya M. 2021. Insights into low biological activity of wax apple (*Syzygium samarangense*) juice by in vitro phytochemical investigation with special reference to metabolomics. Asian J Nat Prod Biochem 19: 30-38.* Wax-apple (*Syzygium samarangense*) is an Asian waxy and juicy fruit that is reported as antidiarrheal and useful in diseases like dysentery. Moreover, no stronger medicinal evidence has been found associated with this fruit and its juice to ensure more biological activities and composition. Being edible, waxy and juicy, this fruit is quite popular in coastal areas and islands, but the absence of stronger medicinal and biochemical evidence associated with this fruit or its juice has created a worldwide underutilizing status. In this research, we intended to investigate phytochemical characteristics and composition of this fruit juice through detailed biochemical tests and metabolomics. Qualitative detection tests for bioactive groups of molecules (tannin, coumarin, cardiac glycosides, terpenoids, flavonoids, phenol, etc.), antioxidant assay, and antibacterial test simply showed its low in vitro biological activity. A GC-MS based metabolomics was performed where presence of wax components like long-chain hydrocarbons has been revealed. Based on the GC-MS based metabolomics a proposed pathway of wax apple's wax biosynthesis has been established. The overall study strongly clarified the absence of bioactive components in this fruit and proved that the fruit, named wax apple, is actually a source of natural waxes that can be responsible for its claimed antidiarrheal property.

Keywords: GC-MS analysis, hexadecane, Java apple, metabolomics, *Syzygium samarangense*, wax

Abbreviations: WAJ: Wax apple juice; GC-MS: Gas Chromatography-Mass Spectrometry, DPPH: 2,2-diphenyl-1-picrylhydrazyl, DBP: Dibutyl phthalate

INTRODUCTION

Wax apple is an underutilized waxy tropical fruit commonly called Java apple, samarang rose apple, jambu semarang (Indonesia) and jamrul in Bengal (India), also known as, jambu ayer rhio (Malaya), pini jambu (Ceylon), chom pu kao, or chom pu kio (Thailand), makopa (Philippines), cashu di Surinam, or Curacaose appel (Curacao), wax jambu and water apple (Morton 1987; Lim 2012). The fruit is usually white or light-red, sometimes greenish-white or cream-colored, pear-shaped, narrow at the base and flattened with fleshy calyx lobes at the apex. The skin of the fruit is very thin; the flesh is white, spongy and dry to juicy, slightly acidic and flavorless. The fruit is usually eaten fresh but is also used in salads. In Thailand, it is most popular in addition to spicy shrimp salad (Fruitsinfo 2021). In Malaya, the greenish ones are eaten raw with salt or may be cooked as a sauce or stewed with true apples (Morton 1987). The wax apple tree is an evergreen tree (5-15 m tall) growing only at the lower altitudes up to 1,220 m in India (Morton 1987). According to WCSP (2021) (Royal Botanic Gardens, Kew) wax jambu is identified as *Syzygium samarangense* (Blume) Merr. & L.M.Perry, J. Arnold Arbor. 19: 115 (1938) belonging to the family Myrtaceae. The tree is indigenous in Asia from Malaya to the Andaman and Nicobar Islands. It is widely found in the Philippines islands, Thailand, Cambodia, Laos, Vietnam, Taiwan, India and Bangladesh. Now it is also very popular in the places like Jamaica, Suriname and the islands of Curacao, Aruba and Bonaire, etc. Khandaker and Boyce (2016) studied the Botany of wax apple in-depth in a review article where three edible cultivars of wax apple were described viz. 'Masam manis pink', 'Jambu madu red' and 'Giant green' cultivar from south Asia. Giant green cultivar is the greenish-white wax apple (creamy white when ripen), also known as white wax apple or 'Jamrul' (mainly grown in sub-Himalayan plains of West Bengal, India) was chosen for this study. The complete distribution of these cultivars (both native and exotic ranges) has been reported in that article where regions of Southeastern Asia (sub-mountain plains of eastern Himalayas) have been stated. Jambu madu red (red wax apple) and Masam manis pink (pink wax apple) are reported to be native to Malay region (Khandaker and Boyce 2016).

This tropical plant and its fruit are reported to be useful in diseases like dysentery, amenorrhea, diabetes, cough, headaches, and fever. A compound named vescalagin, which was isolated from fruits of *Syzygium samarangense*, showed hypoglycaemic activity (Shen et al. 2013). According to

Ghayur et al. (2006), Reynertson et al. (2008) and Shen et al. (2013) the fruits can exhibit antidiarrheal activities. Not only the fruits but other parts of jamrul plant as well have reported health benefits. The leaf extract revealed immunomodulatory, analgesic and anti-inflammatory and anti-hyperglycemic effects as well as analgesic, anti-inflammatory and CNS activities (Kuo et al. 2004; Amor et al. 2004; Shahreen et al. 2011; Resurreccion-Magno et al. 2005) whereas the astringent bark is used as mouthwash, abortifacient as well as in diarrhea, helminthiasis, etc. (Morton 1987). The ethanolic extract of bark of this tropical showed dose dependent anthelmintic activities (Gayen et al. 2016). Interestingly, flowers of this plant are astringent and used in Taiwan to treat fever and halt diarrhea (Morton 1987; Mollika et al. 2014). Bioactive compounds like triterpenoids and chalcone were also reported in aerial parts of *Syzygium samarangense* (Srivastava et al. 1995).

Though there are several reports on ethnomedicinal use of java apple, but investigations and identification of compounds in its fruit juice (main edible part of the plant) by chromatography technology and metabolomics have not yet been conducted. Even, to date, biochemistry didn't reveal the reason why the word "wax" is used with the name of this fruit. So, to meet up these lacunae, our research was designed to study the phytochemical profile of this fruit juice with in vitro biochemical experiments.

MATERIALS AND METHODS

Collection of sample and its preparation

Matured creamy-white colored wax apple fruits ("Jamrul" in West Bengal, India and Bangladesh) were harvested from a matured plant in Jayantika Tea Estate, Darjeeling (26°32'06.0"N, 88°16'18.0"E) and were brought fresh to laboratory for downstream experiments (Figure 1). A herbarium specimen was also prepared and deposited in Kalimpong College Herbarium (Voucher no. KPGC/MB/90) where a proper identification of the fruit (White wax apple or *Syzygium samarangense* (Blume) Merr. & L.M. Perry) was done by botanists of Kalimpong College (West Bengal). After collection fresh fruits were washed under running cold water to remove dust and were rubbed with blotting paper to remove water from its surface. Selected healthy fruits were crushed and squeezed properly to separate the juice (Figure 1) completely from the pulp. Wax apple juice (WAJ) was then filtered and used in further study.

Qualitative chemical tests

Qualitative detection tests for bioactive groups of molecules like tannin, coumarin, cardiac glycosides, steroid, terpenoids, flavonoids, phenol, proteins, starch and reducing sugar in WAJ were done using the protocols of Ghosh et al. (2020) and Das et al. (2020) (Table 1).

Antioxidant activity (DPPH assay)

Antioxidant or free radical scavenging activity by DPPH assay was conducted following the protocol of Bhattacharya et al. (2009) with slight modifications. To 2800 µL of the methanol solution of 0.2 mM DPPH (SRL, India), 200 µL of WAJ was added. The mixture was vortexed vigorously and incubated at room temperature for 30 minutes in dark. Absorbance was measured at 517 nm by UV-Vis spectrophotometer. DPPH scavenging activity was measured as µg AAE/mL (ascorbic acid equivalent) because ascorbic acid was used to prepare the standard curve to quantify the antioxidant value. Result is given as means of total of five replications.

Antibacterial test

Antibacterial activity of WAJ was tested by well diffusion method following Ghosh et al. (2020) and Das et al. (2020). Overnight grown broth culture of two gram positive (*Staphylococcus aureus* and *Bacillus cereus*) and two gram negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria were used for the present study to assess the antibacterial activities of synthesized samples. Mueller-Hinton (MH) agar media (HiMedia) was used for this test. The media was autoclaved and plated under sterile conditions in laminar air flow cabinet. 100 µL of bacterial strains were added separately to each plate containing media. Circular wells were dug out by sterilized steel cork-borer. 100 µL of each WAJ was poured into the well by using sterilized pipettes. The plates were incubated overnight at 37 ºC for 24 hours.

GC-MS based metabolomics

Fresh jamrul juice or WAJ (1 mL) was dissolved in 1 mL methanol (Sigma Aldrich, Germany) prior for GC-MS analysis following the protocol of Majumder et al. (2020) and Chakraborty et al. (2021). Being widely used ideal solvent in phytochemistry compared to another organic solvent, methanol was chosen for extraction. Moreover, polarity of methanol as an organic solvent shows closeness with that of water (primary solvent of any fruit juice). GCMS-QP2010 Plus (Shimadzu Co., Japan) was used in this analysis where DB-5 fused-silica capillary column (0.25 µm film thickness, 0.25 mm internal diameter and 30 m of length). Analysis was performed by injecting 1 µL each sample with a split ratio of 20:1. Injection temperature was 260°C and interface temperature was set to 270°C. Ion Source temperature was adjusted to 230°C. Helium gas (99.9%) was used as carrier gas. Total flow rate and column flow rate were 16.3 mL/min and 1.21 mL/min respectively. Mass spectra were recorded at 5 scan/sec with a scanning rate of 40-650 m/z. The compounds were identified after comparing the spectral configurations obtained with that of available mass spectral database. The compounds were detected using library databases like NIST08s.LIB and WILEY8.LIB. The chromatogram (TIC or Total Ion Chromatogram) is based on the intensity of fragments produced by the ionization. Quantification of the amount (area %) of each compound was done on the basis of peak areas.

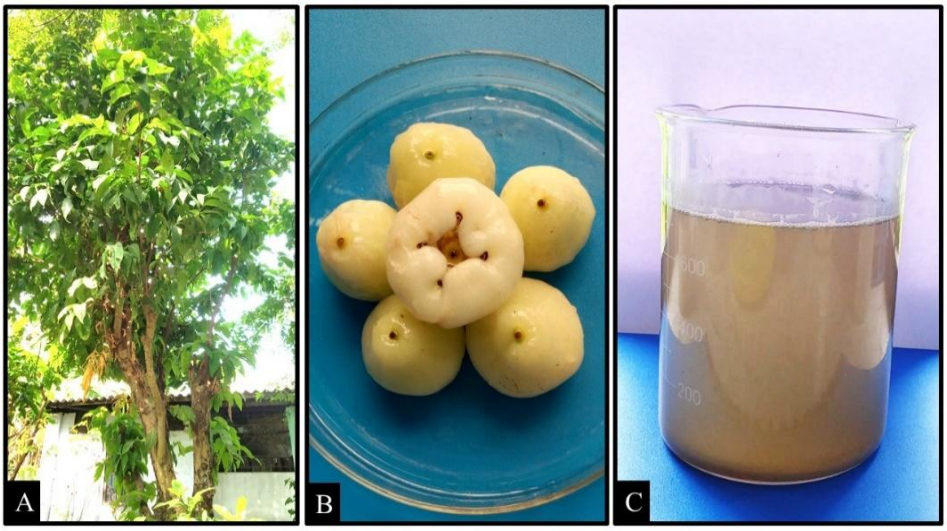

Figure 1. A. Wax apple or *Syzygium samarangense* tree, B. Fresh *Syzygium samarangense* fruits or wax apples (white wax apple or jamrul), C. Handmade wax apple juice from freshly harvested fruits

Table 1. Methods and results of qualitative chemical tests

Phytochemicals	Qualitative detection method
Flavonoids	Few drops of 10% $FeCl_3$ solution was added to 1 mL of sample. A green or blue color would indicate the presence of flavonoids.
Tannin	To 0.5 mL sample and few drops of HNO_3 was added. The reddish to yellow color of the solution would indicate the presence of tannins.
Cardiac glycosides	0.5 mL of sample were evaporated and dissolved in 1 mL glacial acetic acid. 1 drop of 10% FeCl3 solution followed by 1 mL of Conc. H_2SO_4 was added by the side of test tube. Appearance of brown color rings at the interface would indicate the presence of cardiac glycosides.
Protein	1 mL of 4% NaOH solution and a few drops of 1% CuSO4 solution were added to 3 mL of sample solution. A violet or pink color would indicate presence of protein.
Coumarin	Few drops of NaOH solution was added to 1 mL of sample. Yellow coloration would indicate the presence of coumarin
Terpenoid	50 µL sample was evaporated. The remaining was dissolved in chloroform and concentrated H2SO4 was added from the sidewall of test tubes. Formation of red to reddish-brown coloration at the base would confirm the presence of terpenoids.
Steroid	For test of 0.5 mL samples were evaporated and dissolved in 2 mL chloroform. 2 mL of Conc. H_2SO_4 was introduced carefully by the sidewall of the test tube. Formation of red color ring would confirm the presence of steroid.
Phenol	5 mg $FeCl_3$ was added to 1 mL of sample, followed by vigorous shaking. Green coloration would indicate the presence of Phenol
Starch	Iodine solution wax added drop by drop into the sample. Dark blue coloration would indicate the presence of starch.
Reducing sugar	Benedict's reagent was added to the sample, heated and it turns yellowish-orange. The final color would confirm the presence of reducing sugar.

Data analysis

The data obtained from GCMS analysis were further analyzed by studying available literature. Biosynthesis pathways of metabolites have been discussed and a probable GC-MS based pathway of WAJ metabolites has been proposed after studying literature and KEGG PATHWAY database (Majumder et al. 2020).

Spectrophotometric investigation

UV-visible absorbance spectrum analysis is a very preliminary spectrum-based compound determination analysis unlike GC-MS (which is scientifically more profound), but here, this spectrophotometric absorbance spectrum was taken into consideration as a cross-check strategy to determine presence of the only major compound of WAJ i.e. hexadecane. Protocols described by Pektaş et al. (2009) and Smith et al. (1967) were followed in this experiment with a slight modification. For comparison marketed hexadecane (Sigma Aldrich, Germany) was used. Samples were prepared following the same concentration where hexadecane (pure) being finely soluble in diethyl ether was dissolved in the targeted solvent and WAJ was

also extracted with diethyl ether (fresh juice was not used for being a waterbased sample). After zeroing with diethyl ether, absorbance spectrum of the samples was taken (200-800 nm wavelength) using Cary 60 UV-Vis Spectrometer (Agilent) and graphs were obtained thereafter.

RESULTS AND DISCUSSION

Qualitative chemical tests

Qualitative tests can provide an idea about presence of valuable groups of organic molecules in test sample. Where none of the organic groups like tannin, coumarin, cardiac glycosides, steroid, terpenoids, flavonoids, phenol, proteins, and starch were detected in fresh juice of WAJ except reducing sugar. Juice of any tropical fruit juices usually contains flavonoids or phenolic acids that confer free radical scavenging activity to a great extent, so, it was quite surprising that fruit juice of wax apple tested completely negative for the presence of those components.

Antioxidant activity (DPPH assay)

DPPH free radical scavenging activity of fresh WAJ resulted in 55.59±0.819 µg AAE/mL (Ascorbic acid equivalent where IC50 value is 43.11). Previous studies on fruit juices are considered (described in the discussion section) then our experiment on WAJ definitely showed very low level of inhibition which actually has supported the low antioxidant activity of the fruit juice.

Antibacterial test

No visible inhibition zone was found in the plates after three repeated tests which confirmed that WAJ has no significant antibacterial property against these bacterial samples.

Gas chromatography-mass spectrometry analysis and metabolomics

Gas chromatography-mass spectrometry analysis of WAJ dissolved in methanol revealed presence of six different compounds. The chromatogram of GC-MS showing peaks is depicted in Figure 2 and Table 2.

Out of the six compounds detected by GC-MS, only three compounds (hexadecane; dibutyl phthalate or DBP; and elaidic acid, methyl ester) are found to have potential bioactive properties. Detected compounds trans-1-hexanoyl-2-(1-(phenylseleno)-1-(trimethylsilyl) methyl-cyclopropane and cyclododecasiloxane, tetracosamethyl are reported contaminations (GC-MS contaminants) from the column bleeding and siloxane is a column matrix component along with silyl, silane, siloxyl- are derivatives. So, hexadecane, a long chain alkane or wax is the only abundant compound with 77.26% peak area here (mass spectrum is given in Figure 3). Elaidic acid, methyl ester is a bioactive unsaturated omega-9 fatty acid ester and trans-isomer of oleic acid which is a food component also (National Center for Biotechnology Information 2021). Elaidic acid, methyl ester also consists of long-chain and these types of long-chain fatty acid esters are also considered as waxy components (Whitaker et al. 2001). 5-Tetradecyne was also detected with a minor area in GC-MS analysis 5-tetradecyne is a long chain alkyne hydrocarbon which is also a wax. Biosynthesis pathways of WAJ waxes (hexadecane and elaidate) have been studied and a pathway has been proposed which is based on KEGG pathway database and other reviewed literature and general rules of phytochemical synthesis and has been described in the section 'Discussion' section.

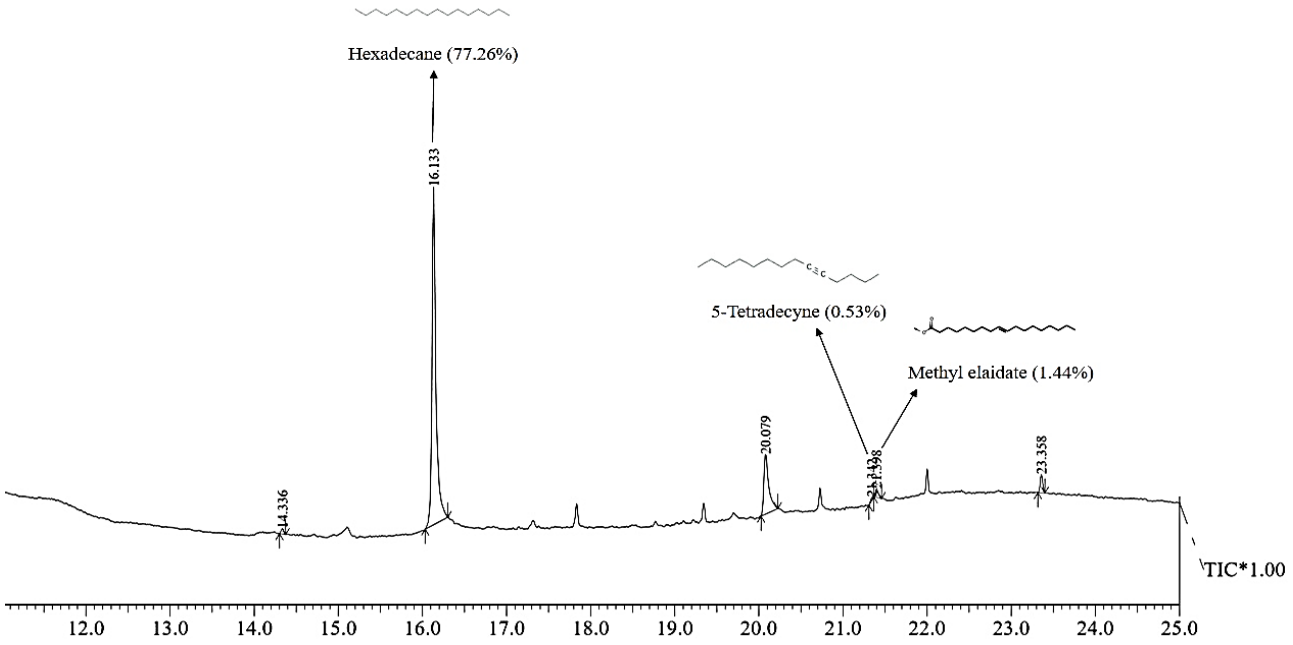

Figure 2. GC-MS chromatogram of WAJ showing peaks of waxes like hexadecane, 5-tetradecyne and methyl elaidate

Table 2. GC-MS derived compounds in WAJ

Peak no.	R. Time	Area	Area%	Name
1	14.336	27387	0.75	Trans-1-hexanoyl-2-(1-(phenylseleno)-1-(trimethylsilyl)methyl)cyclopropane
2	16.133	2820658	77.26	Hexadecane
3	20.079	632605	17.33	Dibutyl phthalate or DBP
4	21.342	19472	0.53	5-Tetradecyne
5	21.398	52550	1.44	Elaidic acid, methyl ester
6	23.358	98260	2.69	Cyclododecasiloxane, tetracosamethyl
		3650932	100	

Spectrophotometric determination of hexadecane

Both images of the absorbance spectrums obtained after the analysis are given in Figure 4 which, being finely and parallel overlapped, has clearly indicated that both WAJ and hexadecane have very much similarities. A slight dissimilarity is visible because raw WAJ contains not only hexadecane but also other hydrocarbons or fatty acids (detected in GC-MS). But, the level of accuracy with the graph of hexadecane (pure) shown by raw WAJ definitely proves the majority of hexadecane in it.

Discussion

Antioxidants have diverse role to play in the life of organisms by maintaining a balance between pro-oxidation and anti-oxidation (Bhattacharya and Chakraborty 2015). After assessing and observing all the preliminary phytochemical experiments like qualitative test, anti-oxidant assay, and antibacterial test, it was confirmed that this fruit juice has no such significant bioactivity. Previously, many scientists (Rekha et al. 2012; Zou et al. 2016) have done profound research on fruit juices and their antioxidant properties which has helped in this research to evaluate the antioxidant property of WAJ in a comparative way and according to those previous studies, DPPH scavenging value of 5.5 mg AAE by 100 mL of a juice is very much low. Moreover, absence of important bioactive groups in WAJ such as, flavonoids, phenols, cardiac glycosides and coumarins, is also proportional to its antioxidant and antibacterial activity. So, finally, a GC-MS-based metabolomic study became needful to know about the phytochemicals or the metabolites present in this fruit juice which might help to investigate the low bioactivity of WAJ at a molecular level. In previous research, Khandaker et al. (2015) conducted antioxidant, antimicrobial and other biochemical tests for three cultivars of wax apple, where red and pink wax apple showed significantly higher bioactivity compared to green giant cultivar (white wax apple). This research conforms to our findings.

GC-MS analysis solved our confusion regarding low in vitro bioactivity was resolved. All the results are parallel to the results for the preliminary tests where no significant antioxidant or antimicrobial activity was detected. But, what was found, was only some huge peaks of compounds biosynthesized through wax biosynthesis pathway. Actually, in nature, long chains of alkanes, alkynes, fatty acids, esters (mainly hydrocarbons with long aliphatic alkyl chains), etc. are considered waxes. So, components like hexadecane, elaidate and 5-tetradecyne detected in WAJ are the waxes of wax apple juice. Being the major component, it is justified that hexadecane is the main wax of wax apple and it should be the reason behind mentioning the word wax before this fruit's name. Hexadecane is a liquid wax, viscous in nature. Alkanes are saturated hydrocarbons and chemically very inert or less reactive as they are apolar molecules (Labinger and Bercaw 2002; Singh et al. 2012) but, they can be broken down into potential bioactive forms (mainly fatty acids) under the influence of microorganism (Singh et al. 2012). It is reported that microbial biodegradation of hexadecane (Volke-Sepulveda et al. 2003) is the only probable way where bio-active components like hexadecanol, hexadecanal, hexadecanoic acid, etc. are synthesized from the compound. Other waxes like methyl elaidate (long-chain fatty acid ester) and 5-tetradecyne (long-chain alkyne) were also found but detected in a minute quantity in this research. Interestingly, fatty acid esters and long-chain hydrocarbons have a close physicochemical affinity with natural waxes in nature as fatty acid esters like oleates, elaidates and palmitates are often found in beeswax (Buchwald et al. 2009; Tulloch 1980) along with alkanes (WAJ compound hexadecane) and alkynes (WAJ compound 5-tetradecyne). Other than beeswax, there are several examples regarding plants or vegetable wax where cuticular or epicuticular wax, seed oil extracts, fruit coatings, etc. are reported to contain several long-chain hydrocarbons along with fatty acids esters (Seigler 1998; Majumder et al. 2020a). So, wax apple is not going to be the only example here. Furthermore, none of the detected natural compounds of WAJ has any flavor which also justifies the flavorlessness of wax apple.

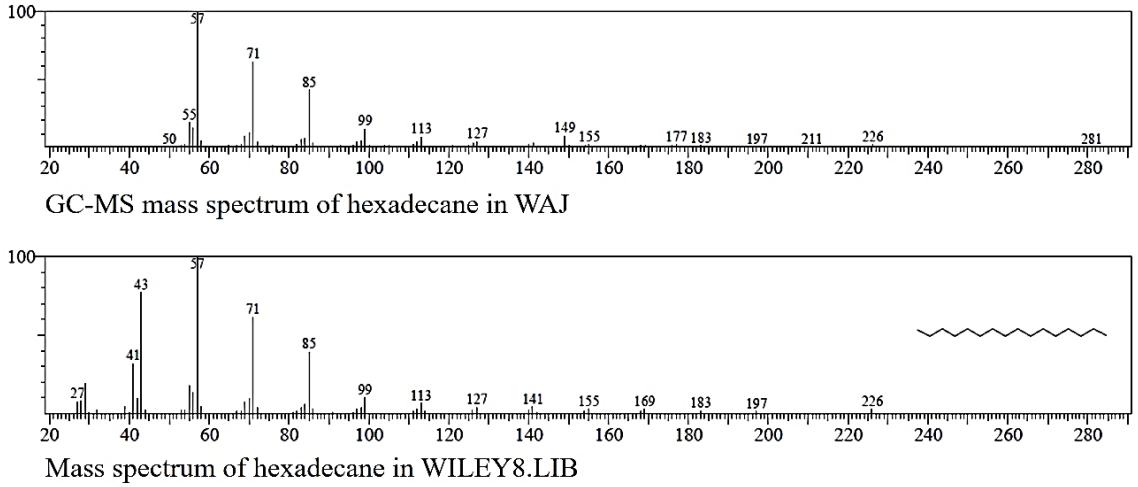

Figure 3. GC-MS mass spectrum of major WAJ compound hexadecane

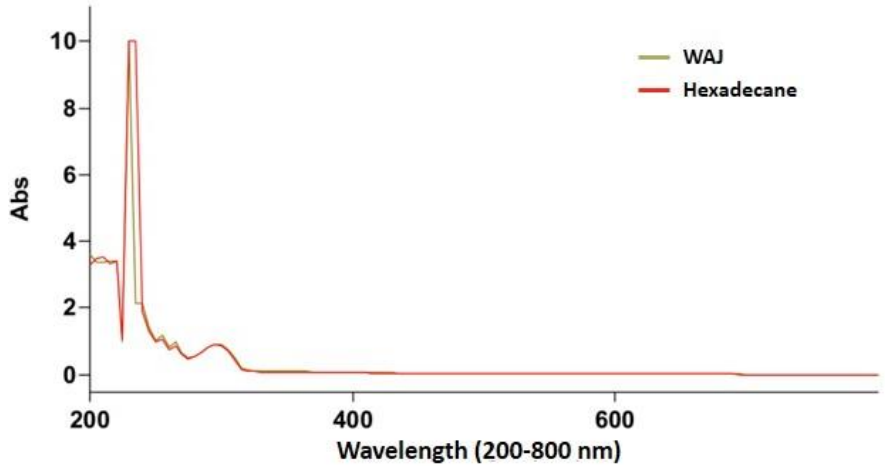

Figure 4. UV-visible spectrum of hexadecane and WAJ

Biosynthesis pathway of wax like long-chain alkanes, alkynes, fatty acids, their esters, etc. are established in KEGG (Kyoto Encyclopedia of Genes and Genomes) database (KEGG 2021) where unsaturated fatty acids after biosynthesis and elongation produce metabolites such as elaidates (C18) or other long-chain fatty acids, wax esters and hexadecane (long-chain alkanes), etc. Wax biosynthesis generally begins with unsaturated fatty acid biosynthesis in the plastid. Then after fatty acid elongation (FAE), long-chain fatty acids like C16 (palmitic acid), C18 (oleic acid or elaidic acid), C22 (docosanoic acid) or very long chains (C24-C34) are synthesized, and parallely elongated products like several alkanes, secondary alcohols, ketones, primary alcohols and wax esters are produced (KEGG 2021; Kunst and Samuels 2003). Interestingly, two wax components of WAJ, hexadecane and elaidic acid, methyl ester are intermediates of the same pathway which has no report to date but follows the general wax biosynthesis pathway and theoretically possible if nature of reactions is concerned. In between synthesis of elongated long-chain fatty acid through and synthesis of fatty acyl-CoA, fatty acid oxidation occurs on the outer mitochondrial membrane by acyl-CoA synthetase where one molecule of coenzyme A and one molecule of adenosine triphosphate (ATP) are used (Oxidation of Fatty Acids 2020). Then, oxidation of the fatty acyl-CoA occurs in the mitochondrial matrix via a sequence of reactions known as β-oxidation. Moreover, production of heptadecanal (a probable precursor of hexadecane) from acyl Co-A of an octadecanoic acid is reported in living organisms (Foulon et al. 2005) while synthesis of odd chain fatty acid (C-17, a probable precursor of hexadecane) from even chain (C-18) is also established (Jenkins et al. 2015), but in bacteria. However, in plant wax biosynthesis pathway the fatty acyl Co-A is converted into long-chain aldehydes, alkanes, secondary alcohols and ketones respectively through a series of decarbonylation reactions which involves loss of CO, thus, the number of carbon seems to be decreased one by one from the chain. But, there is no reported pathway to define formation of hexadecane from elaidic acid, so, Figure 5 is proposed. Previously reported decarbonylation reactions like, formation of heptadecane from octadecanal (Cheesbrough and Kolattukudy 1984) in plants or formation

of hexadecane from heptadecanal (Foo et al. 2017) in micro-organisms can also support this GC-MS metabolomics based probable pathway describing possibilities of formation of hexadecane from elaidic acid (an octadecanoic acid) through enzymatic reaction. Scientist Kunst and Samuels (2003) have done a profound study on this pathway, mainly cuticular wax biosynthesis pathway. According to their research, from fatty acid synthesis to formation of C18 (octadecanoic acids) takes place in plastid while fatty acid elongation to very long-chain fatty acid (VLCFA) formation occurs in endoplasmic reticulum (ER). Furthermore, acyl reduction and decarbonylation reactions occur on domains of the ER (associated with plasma membrane) and finally deposited in the cell wall as cuticular waxes. Generally, regarding aliphatic natural wax components, their class and site of synthesis depend on the chain length like n-alkanes, secondary alcohols, ketones, fatty alcohols, long-chain fatty acids, VLCFA, aldehydes and wax esters where WAJ component elaidic acid (C18) is a long-chain fatty acid (not VLCFA) and hexadecane (C16) is an alkane (not VLC alkane). According to previous reports, it is highly likely that enzyme decarbonylase is located in the cell wall matrix (Cheesbrough and Kolattukudy 1984; Kunst and Samuels 2003) and this proposed pathway also supports the activity of decarbonylase as a C18 fatty acid is detected which is suggested to be the precursor of the major product i.e., a C16 alkane. Moreover, the presence of hexadecane and the majority of this liquid wax in wax apple also suggest that no more fatty acid elongation (VLCFA synthesis) occurred after synthesizing the only detected C18 fatty acid. So, decarbonylation occurred in wax apple soon after C18 fatty acid synthesis in plastid which blocked synthesis of farther VLCFAs or wax esters. However, according to Yeats and Rose (2013), the enzymatic basis of alkane synthesis is still a long unresolved question, mainly for long-chain alkanes (non-VLC) while microbial biosynthesis of alkanes from C18 fatty acids is more established. Brown et al. (2019) have done an extensive study on microbial biosynthesis of alkane where C25-C33 range has been categorized as VLC alkanes and shorter alkanes (like C16 or hexadecane of WAJ) as mid-chain alkanes. According to their study, biosynthesis of alkanes in eukaryotic cells occur directly from C12, C14, C16, C18, and C20 saturated fatty acids through enzymatic decarboxylation by OleT or fatty acid decarboxylase (Belcher et al. 2014; Brown et al. 2019). But, some recent research articles (Sorigué et al. 2017; Huijbers et al. 2018) have described enzyme fatty acid decarboxylase activity in eukaryotes (in photosynthetic algae) has increased the accuracy of this proposed alkane biosynthesis pathway from C18 fatty acids. It is reported that in the chloroplast membrane of microalgae *Chlorella* and *Chlamydomonas*, fatty acid photo decarboxylase (FAP) converts exogenous fatty acids to alkanes (Sorigué et al. 2017). The proposed biosynthetic route of WAJ wax is very simple and has been adapted from KEGG pathway database and other literature described earlier (given in Figure 5).

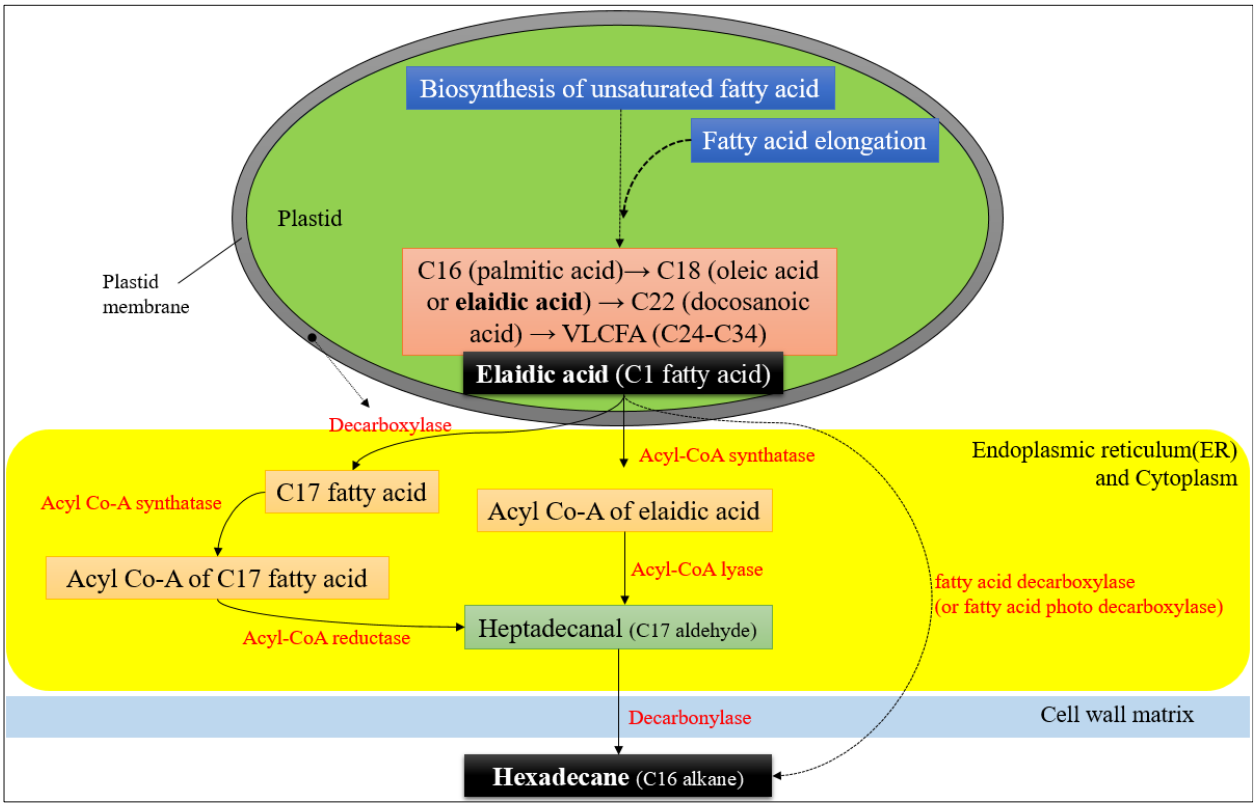

Figure 5. Proposed biosynthesis pathway of the WAJ metabolome (Wax)

Source of DBP (another major compound of WAJ) in wax apple juice is confusing because it has been already reported as contamination from lab wares (Majumder et al. 2020b) and the probable reason for presence of phthalate in WAJ can be its exposure to laboratory materials like Eppendorf tubes, beaker, GC-MS machinery, etc. However, DBP is reported as highly bioactive (antioxidant, antibacterial) glucose-derived component (Zhang et al. 2018) from shikimic acid pathway. While, results and observations of qualitative chemical tests antioxidant and antibacterial tests, GC-MS metabolomics-based pathway studies and spectrophotometric evaluation, etc. oppose the occurrence of DBP in WAJ. Although, presence of reducing sugar suggests chemical reactions for synthesis of dibutyl phthalate from sugars (glucose) of fresh WAJ as none of any sugar-derived compound was detected in GC-MS, but due to lacking strong reports it cannot be considered.

Reportedly, wax apple is useful in diseases like dysentery or diarrhea and, interestingly, revealed metabolites of WAJ also support this health claim. After assessing GC-MS analysis and spectrophotometric evaluation, presence of huge amount of hexadecane in this fruit juice has been proven which, being viscid or gelatinous liquid in nature, may definitely be the reason behind antidiarrheal activity. Moreover, elaidate is known to increase plasma cholesteryl ester transfer protein (CETP) activity which lowers HDL cholesterol (Abbey and Nestel 1994). But, presence of the compound in very minute amount, showing negative results in all of the above in vitro phytochemical tests and absence of major phytochemical groups surely demote the medicinal value of WAJ by giving the "low bioactive" tag on it. Absence of bioactive groups of molecules, low in vitro antioxidant activity, absence of inhibition zones in antimicrobial assays, abundance of wax compound hexadecane, and design of unexplored biosynthesis pathway of wax apple's wax biosynthesis through metabolomics; are the key findings of this study. The outcome of this research and evidence of microbial biodegradation of hexadecane may increase the utility of wax apple by using this low bioactive fruit in different research areas like fermentation biotechnology where biodegradation can convert this low bioactive substrate into various bioactive forms. Moreover, this research suggests that bioactive potentiality of any plant cannot be depended only on results of biochemical assays, metabolomics can play the role of an important parameter to judge acceptability of any underutilized product. Both Masam manis pink and Jambu madu red are reported as better in quality regarding yield, flavor and functional uses unlike the giant green cultivar (greenish-white wax apple) which was chosen for this study because of its availability and significance as a native cultivar in our study area (sub-Himalayan plains of West Bengal, India). Research on genetics and ecology of this plant are imperative to resolve the dilemma. Large quantity of hexadecane found in plant can be explored for beneficial purposes.

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REFERENCES

Abbey M, Nestel PJ. 1994. Plasma cholesteryl ester transfer protein activity is increased when trans-elaidic acid is substituted for cis-oleic acid in the diet. Atherosclerosis 106 (1): 99-107. DOI: 10.1016/0021-9150(94)90086-8.

Amor EC, Villasenor IM, Yasin A, Choudhary MI. 2004. Prolyl endopeptidase inhibitors from *Syzygium samarangense* (Blume) Merr. & L. M. Perry. Z. Naturforsch 59c: 86-92. DOI: 10.1515/znc-2004-1-218.

Belcher J, McLean KJ, Matthews S, Woodward LS, Fisher K, Rigby SE, Nelson DR, Potts D, Baynham MT, Parker DA, Leys D. 2014. Structure and biochemical properties of the alkene producing cytochrome P450 OleTJE (CYP152L1) from the *Jeotgalicoccus* sp. 8456 bacterium. J Biol Chem 289 (10): 6535-50. DOI: 10.1074/jbc.M113.527325.

Bhattacharya M, Chakraborty S. 2015. Free radicals and naturally occurring antioxidants. J Pharm Phytochem 3: 1-7.

Bhattacharya M, Mandal P, Sen A. 2009. In vitro detection of antioxidants in different solvent fractions of Ginger (*Zingiber officinale* Rosc.). Indian J Plant Physiol 14 (1): 23-27.

Brown S, Loh J, Aves S, Howard T. 2019. Alkane Biosynthesis in Bacteria. Springer. DOI: 10.1007/978-3-319-78108-2_7.

Buchwald R, Breed MD, Bjostad L, Hibbard BE, Greenberg AR. 2009. The role of fatty acids in the mechanical properties of beeswax. Apidologie 40 (5): 585-594. DOI: 10.1051/apido/2009035.

Chakraborty S, Majumder S, Ghosh A, Saha S, Bhattacharya M. 2021. Metabolomics of potential contenders conferring antioxidant property to varied polar and non-polar solvent extracts of *Edgaria darjeelingensis* CB Clarke. Bull Natl Res Centre 45 (1): 1-12. DOI: 10.1186/s42269-021-00503-3.

Cheesbrough TM, Kolattukudy PE. 1984. Alkane biosynthesis by decarbonylation of aldehydes catalyzed by a particulate preparation from *Pisum sativum*. Proc Natl Acad Sci 81 (21): 6613-6617. DOI: 10.1073/pnas.81.21.6613.

Das AK, Ghosh A, Majumder S, Saha S, Acharyya S, Sarkar S, Chakraborty S, Mukherjee M, Bhattacharya M. 2020. Characterization of tea and tea infusion: A study of marketed black tea samples from some tea-growing regions of India. J Pharm Phytochem 9 (5): 1532-1540. DOI: 10.22271/phyto.2020.v9.i5v.12553.

Foo JL, Susanto AV, Keasling JD, Leong SSJ, Chang MW. 2017. Whole-cell biocatalytic and de novo production of alkanes from free fatty acids in *Saccharomyces cerevisiae*. Biotechnol Bioeng 114 (1): 232-237. DOI: 10.1002/bit.25920.

Foulon V, Sniekers M, Huysmans E, Asselberghs S, Mahieu V, Mannaerts GP, Van Veldhoven PP, Casteels M. 2005. Breakdown of 2-hydroxylated straight chain fatty acids via peroxisomal 2-hydroxyphytanoyl-CoA lyase: a revised pathway for the α-oxidation of straight chain fatty acids. J Biol Chem 280 (11): 9802-9812. DOI: 10.1074/jbc.M413362200.

Fruitsinfo, 2004-2021. https://www.fruitsinfo.com/Wax-jambu-Exotic-fruits.php. Accessed on 2 April 2021.

Gayen PR, Al Hossain AM, Saifuzzaman M, Faroque ABM. 2016. Anthelmintic activity of ethanolic extract of *Syzygium samarangense* (Blume) Merril & Perry. Dhaka Univ J Pharm Sci 15(1): 109-111. DOI: 10.3329/dujps.v15i1.29204.

Ghayur MN, Gilani AH, Khan A, Amor EC, Villasenor IM, Choudhary MI. 2006. Presence of calcium antagonist activity explains the use of *Syzygium samarangense* in diarrhoea. Phytother Res 20: 49-52. DOI: 10.1002/ptr.1801.

Ghosh A, Majumder S, Saha S, Bhattacharya M. 2020. Characterization of nonpolar and polar solvent extracts from some tea plantation shade tree leaves with special reference to antioxidant and antibacterial activities. Res Plant Biol 10: 15-21. DOI: 10.25081/ripb.2020.v10.6235.

Huijbers MM, Zhang W, Tonin F, Hollmann F. 2018. Light-driven enzymatic decarboxylation of fatty acids. Angewandte Chemie Int Edition 57 (41): 13648-13651. DOI: 10.1002/anie.201807119.

Jenkins B, West JA, Koulman A. 2015. A review of odd-chain fatty acid metabolism and the role of pentadecanoic acid (C15: 0) and

heptadecanoic acid (C17: 0) in health and disease. Molecules 20 (2): 2425-2444. DOI: 10.3390/molecules20022425.

KEGG. 2021. KEGG: Kyoto Encyclopedia of Genes and Genomes. Accessed April 2, 2021. https://www.genome.jp/kegg/pathway.html

Khandaker MM, Boyce AN. 2016. Growth, distribution and physiochemical properties of wax apple (*'Syzygium samarangense'*): A review. Aust J Crop Sci 11 (12): 1640. DOI: 10.21475/ajcs.2016.10.12.PNE306.

Khandaker MM, Sarwar MJ, Mat N Boyce AN. 2016. Bioactive constituents, antioxidant and antimicrobial activities of three cultivars of wax apple (*Syzygium samarangense* L.) fruits. Res J Biotechnol 10 (1): 7-16.

Kunst L, Samuels AL. 2003. Biosynthesis and secretion of plant cuticular wax. Prog Lipid Res 42 (1): 51-80. DOI: 10.1016/S0163-7827(02)00045-0.

Kuo YC, Yang LM, Lin LC. 2004. Isolation and immunomodulatory effect of flavonoids from *Syzygium samarangense*. Planta Med 70: 1237-39. DOI: 10.1055/s-2004-835859.

Labinger JA, Bercaw JE. 2002. Understanding and exploiting C–H bond activation. Nature 417 (6888): 507-514. DOI: 10.1038/417507a.

Lim TK. 2012. Syzygium samarangense. In Edible Medicinal and Non Medicinal Plants. Dordrecht, Springer. DOI: 10.1007/978-94-007-2534-8_105.

Majumder S, Ghosh A, Bhattacharya M. 2020. Natural anti-inflammatory terpenoids in *Camellia japonica* leaf and probable biosynthesis pathways of the metabolome. Bull Natl Res Centre 44 (141): 1-14. DOI: 10.1186/s42269-020-00397-7.

Majumder S, Ghosh A, Chakraborty S, Bhattacharya M. 2020a. GC-MS analysis reveals *Dendrobium candidum* is a mosquito-attractant orchid with mosquitocidal compounds. Int J Mosq Res 7 (6): 09-12. DOI: 10.22271/23487941.2020.v7.i6a.483.

Majumder S, Sarkar S, Ghosh A, Acharyya S, Saha S, Chakraborty S, Bhattacharya M. 2020b. Photosynthetic organs of wild Indian tea tree are rich in patchouli components: a GC-MS based metabolomics. Nat Prod Res 1-5. DOI: 10.1080/14786419.2020.1851222.

Mollika S, Islam N, Parvin N, Kabir A, Sayem MW, Luthfunnesa SR. 2014. Evaluation of analgesic, anti-inflammatory and CNS activities of the methanolic extract of *Syzygium samarangense* leave. Glob J Pharmacol 8 (1): 39-46.

Morton JF. 1987. Fruits of Warm Climates. JF Morton, Miami, Florida.

National Center for Biotechnology Information. 2021. PubChem Compound Summary for CID 637517, Elaidic acid. https://pubchem.ncbi.nlm.nih.gov/compound/Elaidic-acid. Accessed April 2, 2021.

Oxidation of Fatty Acids. 2020. https://chem.libretexts.org/@go/page/234043. Accessed on April 2, 2021.

Pektaş G, Dinç E, Baleanu D. 2009. Combined application of continuous wavelet transform-zero crossing technique in the simultaneous spectrophotometric determination of perindopril and indapamid in tablets. Química Nova 32 (6): 1416-1421.

Rekha C, Poornima G, Manasa M, Abhipsa V, Devi JP, Kumar HTV, Kekuda TRP. 2012. Ascorbic acid, total phenol content and antioxidant activity of fresh juices of four ripe and unripe citrus fruits. Chem Sci Trans 1 (2): 303-310. DOI: 10.7598/cst2012.182.

Resurreccion-Magno MH, Villasenor IM, Harada N, Monde K. 2005. Antihyperglycaemic flavonoids from *Syzygium samarangense* (Blume) Merr. and Perry. Phytother Res 19: 246-251. DOI: 10.1002/ptr.1658.

Reynertson KA, Yang H, Jiang B, Basile MJ, Kennelly EJ. 2008. Quantitative analysis of antiradical phenolic constituents from *Syzygium samarangense* (Myrtaceae) fruits. Food Chem 109: 883-890. DOI: 10.1016/j.foodchem.2008.01.021.

Seigler DS. 1998. Plant Waxes. In Plant Secondary Metabolism. Springer, Boston, MA. pp. 51-55. DOI: 10.1007/978-1-4615-4913-0_4.

Shahreen S, Banik J, Hafiz A, Rahman S, Zaman AT, Shoyeb MA, Chowdhury MH, Rahmatullah M. 2011. Antihyperglycemic activities of leaves of *Syzygium samarangense* of Bangladesh. Afr J Tradit Complement Altern Med 9: 287-291. DOI: 10.4314/ajtcam.v9i2.16.

Shen SC, Chang WC, Chang CL. 2013. An extract from wax apple (*Syzygium samarangense* (Blume) Merrill and Perry) effects glycogenesis and glycolysis pathways in tumor necrosis factor-α-treated FL83B mouse hepatocytes. Nutrients 5: 455-467. DOI: 10.3390/nu5020455.

Singh SN, Kumari B, Mishra S. 2012. Microbial degradation of alkanes. In Microbial degradation of xenobiotics. Springer, Berlin. DOI: 10.1007/978-3-642-23789-8_17.

Smith JWG, De Grey GE, Patel VJ. 1967. The spectrophotometric determination of ampicillin. Analyst 92 (1093): 247-252. DOI: 10.1039/an9679200247.

Sorigué D, Légeret B, Cuiné S, Blangy S, Moulin S, Billon E, Richaud P, Brugière S, Couté Y, Nurizzo D, Müller P. 2017. An algal photoenzyme converts fatty acids to hydrocarbons. Science 357 (6354): 903-907. DOI: 10.1126/science.aan6349.

Srivastava R, Shaw AK, Kulshreshtha DK. 1995. Triterpenoids and chalcone from *Syzygium samarangense*. Phytochemistry 38 (3): 687-689. DOI: 10.1016/0031-9422(94)00739-G.

Tulloch AP. 1980. Beeswax—composition and analysis. Bee World 61 (2): 47-62. DOI: 10.1080/0005772X.1980.11097776.

Volke-Sepulveda TL, Gutiérrez-Rojas M, Favela-Torres E. 2003. Biodegradation of hexadecane in liquid and solid-state fermentations by *Aspergillus niger*. Bioresour Technol 87 (1): 81-86. DOI: 10.1016/S0960-8524(02)00207-9.

WCSP. 2021. World Checklist of Selected Plant Families. Facilitated by the Royal Botanic Gardens, Kew. http://wcsp.science.kew.org/Accessed on 2 April 2021.

Whitaker BD, Schmidt WF, Kirk MC, Barnes S. 2001. Novel fatty acid esters of p-coumaryl alcohol in epicuticular wax of apple fruit. J Agric Food Chem 49 (8): 3787-3792. DOI: 10.1021/jf010409n.

Yeats TH, Rose JK. 2013. The formation and function of plant cuticles. Plant Physiol 163 (1): 5-20. DOI: 10.1104/pp.113.222737.

Zhang H, Hua Y, Chen J, Li X, Bai X, Wang H. 2018. Organism-derived phthalate derivatives as bioactive natural products. J Environ Sci Health: Part C 36 (3): 125-144. DOI: 10.1080/10590501.2018.1490512

Zou Z, Xi W, Hu Y, Nie C, Zhou Z. 2016. Antioxidant activity of Citrus fruits. Food Chem 196: 885-896.