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Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

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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]

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Comparative study of crude and refined edible oils of sunflower and peanut

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Abstract. *Mohammed EI, Ali EA. 2017. Comparative study of crude and refined edible oils of sunflower and peanut. Biofarmasi J Nat Prod Biochem 15: 1-4.* The objective of this study was to compare crude and refined edible oils of sunflower and peanut from their physical properties like color, moisture, density and refractive index, and chemical properties like peroxide value, zero fatty acid, acid value, saponifiable and non-saponifiable value. For example, after the refining process, the moisture content of sunflower oil was reduced from 0.07% to 0.02%, and from 0.13% to 0.02% on peanut oil, and the peroxide value of sunflower oil was decreased from 13.94 meq/kg to 2.77 meq/kg and from 3.137 meq/kg to 0.2 meq/kg on peanut oil. Gas chromatographic applied on sunflower oil showed that stearic acid area percentage was decreased from 0. 1.8805 to 0.3510 after refining, and for peanut oil, it decreased from 11.1643 to 1.0281 after refining. This study showed marked differences in the physicochemical properties of sunflowers and those of peanuts. These properties of each crude oil were significantly changed when it was subjected to the refining process. GC determined fatty acid components of both crude and refined. The study showed striking differences in the crude and refined oils from sunflower and peanut types.

Keywords: Crude, edible, oils, peanut, refined, sunflower

INTRODUCTION

Oil and fats are water-insoluble substances of plants or animals, predominantly glyceryl esters of fatty acids or triglycerides. The word fat is generally used to refer to solid or, more precisely, semi-solid triglycerides at ordinary temperatures. In contrast, the word oil is used for liquid triglycerides under the same condition. No clear-cut distinction can be made between the two words, and both are used interchangeably except for important differences in solids and liquids (Bailey 1979).

The major sources of vegetable oils and fats are several hundred plants and animals that produce enough fats and oils to ensure processing into vegetable oils; however, few sources are numerically significant. Bovine, pig, and poultry are the main sources of commercial animal fat (Woodgate and van der Veen 2014). At the same time, the main source of vegetable oils is palm and palm kernel, soybean, rapeseed, sunflower seed, peanut, cottonseed, coconut, and olive (USDA 2009).

Sunflower (*Helianthus annuus*) oilseed comes from Argentina, where there are five major oilseed crops. The main fields of oilseed production are Eastern Europe, including Ukraine, Russia, Romania, Cina, and Argentine. It is also the major oilseed grown in southern France, Spain, and Turkey (FAOSTAT 2015). As its name suggests, the sunflower is well known for its heliotropism. The exact composition of sunflower seeds varies according to their origin. The un-decorated seed may contain 42-48% oil (Gunstone 2002; Phillips et al. 2005).

Groundnuts or peanuts (*Arachis hypogaea*) are annual herbaceous plants of leguminous branches originating from tropical America. The most important countries producing groundnut are China, India, Nigeria, the United States, and Sudan (FAOSTAT 2014). Groundnuts contain 40-55% oil (Ozcanand Seven 2003; Phillips et al. 2005).

The objective of this study was to compare crude and refined vegetable oils of each sunflower and peanut for the physical properties such as color, moisture, density and refractive index, and chemical properties such as peroxide values, free fatty acids, acid values, saponifiable and non-saponifiable number.

MATERIALS AND METHODS

Materials

The plant samples were collected from the Arab Sudanese Vegetable Oil Company (ASVOC), North Khartoum, Sudan, according to the standard sampling method to ensure that they are representative samples.

Methods

The methods are referred to by Kirk et al. (1991), D'Arcy and Hawes (2003), Ozcan and Seven (2003), Kenndler (2004), Shahidi (2005), Abitogun et al. (2008), AOCS (2009), and Hamm et al. (2013).

Color. This test was done by Digital Lovibond Tintometer, cell of 4 inches for crude oil, and Digital Lovibond PFX995 Tintometer, cell of 5.25 inches for refined oil. The glass cell was cleaned, dried, filled with the oil, and placed in the tintometer. The oil color was matched with the standard slides, i.e., red, yellow, and blue colors. The results were recorded.

Moisture. Digital Mettle Toledo did this test, and 4 g of the sample was taken.

Density. The density of oil was determined by a pycnometer (50 mL). The pycnometer was first dried and weighed empty, and after being filled with sample and water, it was weighed to determine the weight of the sample and water. The volume of the sample to calculate the density of the oil sample, and at the end, calculated the density by the equation below:

D=W (sample)/V (sample)

Where,
D=Density of oil
W (sample) =weight of sample. g.
V (sample) =volume of sample.

Refractive index. The refractive index was determined using a refractometer. A drop of oil was placed on the surface of the lower prism. The prisms were closed, and the mirror and light were adjusted until a dark borderline was visible on the cross wire. The refractive index was determined.

Peroxide value. 5 g of the sample were weighed in a flask, to which 25 cm^3 of glacial acetic acid and chloroform (3:2) and 0.5 cm^3 of potassium iodide were added. The flask was closed, shaken, and left to stand in the dark for 1 minute, preferably at 15-25 C°. About 30 cm^3 of water was added and titrated against 0.01 M sod thiosulphate with vigorous stirring using the starch indicator to detect the endpoint, namely when the color of the solution changed from blue to colorless.

Free fatty acid. 7 g of the sample were weighed, to which 50 cm^3 of neutral alcohol (ethanol 95%) and 2 cm^3 of phenolphthalein were added. The solution was titrated against 0.1M sodium hydroxide until its color changed to pink to indicate the endpoint.

Acid value. 25 cm^3 diethyl ether was mixed with 25 cm^3 alcohol and 1 mL of phenolphthalein solution (1%) and carefully neutralized with 0.1 M alkali. 1-10 g of the oil was dissolved and titrated against aqueous 0.1 M sodium hydroxide while it was constantly shaken until a pink color which persisted for 15 sec, was obtained.

Saponifiable number. 2 g of the oil was weighed and poured into a conical flask, and 25 cm^3 of the alcoholic potassium hydroxide solution was added. At the same time, it was being shaken frequently, and a reflux condenser was attached to the flask, which was heated in boiling water for 1 hr. 1 mL of phenolphthalein (1%) solution was added. The hot excess of alkali was titrated with 0.5 M hydrochloric acid (titration = an mL). A blank was carried out. Utmost care was taken during the titration. The titrated liquid in the sample flask was retained to determine the unsaponifiable matter.

Determination of non-saponifiable matter. After titrating the saponifiable number, the neutralized liquid was made alkaline again with 1 cm^3 of aqueous 3 M potassium hydroxide solution, transferred to a separator, and washed with water (50 mL less the volume of 0.5 N hydrochloric acid used). The solution was extracted while still warm 3 times with 50 cm^3 quantities of diethyl ether. Each ether was extracted into another separator containing 20 cm^3 of water. After the third extract had been added, the combined ether extracts were shaken with the first 20 cm^3 of washing water and then vigorously with the next two 20 cm^3 quantities. The ether extract was washed twice with 20 cm^3 of aqueous 0.5 M potassium hydroxide solution and at least twice with 20 cm^3 quantities of water until the washing water was no longer alkaline to phenolphthalein. The ether extract was poured into a weighed flask; the solvent was evaporated off, and the residue was dried at no more than 80 ◦C and weighed to constant weight.

Fatty acid analysis: [Injection port SPL1]. Injection Mode: Split. Temperature: 250°C. Carrier Gas: N2/Air. Flow Control mode: Pressure. Pressure: 94.2kpa. Total Flow: 16.2 mL/min. Column Flow: 1.00 mL/min. Linear Velocity: 28.2cm/sec. Purge Flow: 3.0 mL/min. *[Column Oven]* Initial Temperature: 100°C. Equilibration Time: 0.0min. *[Column Information]* Column Name: DB-1. Serial Number: US6554753H. Film Thickness: 0.25um. Column Length: 30.0m. Inner Diameter: 0.25mm ID. Column Max Temperature: 350°C. Description: non polar hydrocarbon/poly nuclear/aromatic/steroid. *[Detector channel 1 FID1]* Temperature: 300°C Signal Acquire: Yes. Sampling Rate: 40 msec. Stop Time: 60.0min. Delay Time:0.00min. Makeup Gas: N2/Air. Makeup Flow:30.0 mL/min. H_2 Flow:40.0 mL/min. Air Flow:400.0 mL/min.

RESULTS AND DISCUSSION

Physical tests

The physical properties of crude and refined sunflower and edible peanut oils are in Table 1, including color, moisture, density, and refractive index.

Chemical tests

The chemical properties of crude and refined sunflower and edible peanut oils are in Table 2, including peroxide value, free fatty acid, acid value, saponifiable number, and non-saponifiable matter.

Table 1. the Lovibond tintometer color of crude and refined oils of sunflower and peanut

The oil	Color			Moisture	Density	Reactive index
	Red	Blue	Yellow			
Crude sunflower	4.5	0.9	25	0.07%	0.9169 g.cm-3	1.468
Refined sunflower	1.0	0.0	5.0	0.02%	0.9177 $g.cm^{-3}$	1.467
Crude peanut	3.6	0.8	25	0.13%	0.9094 $g.cm^{-3}$	1.468
Refined peanut	1.0	0.0	4.2	0.02%	0.9209 $g.cm^{-3}$	1.466

Table 2. Peroxide free fatty acid values, acid value, saponifiable number, and non-saponifiable matter of crude and refined oils of sunflower and peanut

The oil	Peroxide value	Free Fatty acid	Acid value	Saponifiable number	Non-saponifiable matter
Crude sunflower	13.94 meq/kg	1.27%	2.4713 g/mol	192.1425	8
Refined sunflower	2.77 meq/kg	0.04%	0.2054 g/mol	186.5325	1.5
Crude peanut	3.137 meq/kg	0.3615%	1.8963 g/mol	190.74	4.5
Refined peanut	0.2 meq/kg	0.08%	0.1996 g/mol	189.3375	1

Table 3. Fatty acid composition of crude and refined oils of sunflower and peanut

Peak #	Compound name	Area%
Crude sunflower oil		
1	Undecanoicacid	0.3963
2	Tridecanoicacid	2.7035
3	Myristicacid	0.4355
4	Cis-10-Pentadecenoic Acid	20.6702
5	PalmitoleicAcid	67.8046
6	Linolelaidic Acid	0.3610
7	Elaidic Acid	0.9562
8	Stearic Acid	1.8805
9	Arachidonic Acid	3.5917
10	ErucicAcid	1.0078
11	Tricosanoic acid	0.1927
Total		100.0000
Refined sunflower oil		
1	Undecanoic acid ME	0.0779
2	Tri decanoic acid M	0.5825
3	Myristic acid M.E	0.1396
4	Cis-10-pentadeceno	7.9634
5	Palmitoleic acid M.E	0.1993
6	Palmitic acid M.E	89.6607
7	Heptadecanioc acid	0.2822
8	Oleic acid M.E	0.0087
9	Stearic acid M.E	0.3510
10	Cis-8,11,14-Eicosath	0.918
11	Arachidonic acid M	0.5398
12	Erucic acid M.E	0.1030
Total		100.0000
Crude peanut oil		
1	Undecanoic acid ME	0.2243
2	Tri decanoic acid M	0.8616
3	Cis-10-pentadeceno	59.3641
4	Palmitoleic acid M.E	0.7531
5	Linolelaidic acid M	1.1275
6	Elaidic acid M.E	2.3556
7	Stearic acid M.E	11.1643
8	Cis-8,11,14-Eicosath	0.2619
9	Cis-11-Eicosath	16.6382
10	Heptadecanioc acid	0.1342
11	Erucic acid M.E	6.1132
12	Tricosanoic acid M.E.	1.0020
Total		100.0000
Refined peanut oil		
1	Tricosanoic acid M.E.	0.5157
2	Myristic acid M.E	0.1617
3	Cis-10-pentadecenoic acid M.E	12.3999
4	Palmitoleic acid M.E	0.4664
5	Palmitic acid M.E	83.0663
6	Linolelaidic acid M.E	0.5223
7	Stearic acid M.E	1.0281
8	Cis-11-EicosnoicM.E. Cis-11,14,1-	1.4202
9	Erucic acid M.E	0.4194
Total		100.0000

The fatty acid composition of crude and refined oils of sunflower and peanut can be seen in Table 3.

Discussion

The physicochemical properties of crude and refined oils of sunflower and peanut were studied. The physical property of crude and refined sunflower oil is as follows: Bleaching the color of the sunflower oil was clearly noticed when the crude product was refined. The Lovibond reading was decreased from 4.5 to 1.0 for red, from 0.9 to 0.0 for blue, and from 25 to 5 for yellow. Similarly, the moisture content was reduced from 0.07% to 0.02%. Likewise, the refractive index decreased from 1.468 to 1.467. The density, however, was slightly increased from 0.9169 $g.cm^{-3}$ to 0.9177 $g.cm^{-3}$.

The physical properties of crude and refined peanut oil are as follows: bleaching of the color of the oil peanut was clearly noticed when the crude product was refined. The Lovibond reading was decreased from 3.6 to 1.0 for red, from 0.8 to 0.0 for blue, and from 25 to 4.2 for yellow. Similarly, the moisture content was reduced from 0.13% to 0.02%. Likewise, the refractive index decreased from 1.468 to 1.466. The density, however, was high increased from 0.909 $g.cm^{-3}$ to 0.920 $g.cm^{-3}$.

The chemical properties of crude and refined sunflower oil: The peroxide value decreased from 13.94 meq/kg to 2.77 meq/kg. Similarly, the free fatty acid was decreased from 1.27% to 0.04%. The acid value decreased from 2.4713 to 0.2054. The saponifiable number decreased from 192.1425 to 186.5325, and the non-saponifiable matter decreased from 8 to 1.5.

The chemical properties of crude and refined peanut oil: The peroxide value was decreased from 3.137 meq/kg to 0.2meq/kg. Similarly, the free fatty acid was decreased from 0.3615% to 0.08%. The acid value decreased from 1.8963 to 0.1996. The saponifiable number decreased from 190.74 to 189.3375, and the non-saponifiable matter decreased from 4.5 to 1.

The fatty acid of crude sunflower oil detected were palmitoleic acid, cis-10-pentadecenoic acid, arachidonic acid, tridecanoic acid, stearic acid, erucic acid, elaidic acid, myristic acid, undecanoic acid, linolelaidic acid and tricosanoic acid and their area%. The area % were 67.8046%, 20.6702%, 3.5917%, 2.7035%, 1.8805%, 1.0078%, 0.9562%, 0.4355%, 0.3963%, 0.3610%, and 0.1927% respectively.

The fatty acid of refined sunflower oil which were detected were palmitoleic acid, cis-10-pentadecenoic acid, arachidonic acid, tridecanoic acid, stearic acid, erucic acid,

myristic acid, undecanoic acid, palmitic acid, heptadecanoic acid, oleic acid and cis-8, 11, 14-eicosatic acid and their area %. The area % were 0.4775%, 20.1220%, 1.6902%, 2.0329%, 0.7481%, 0.3366%, 0.3158%, 0.2397%, 72.1860%, 1.29886%, 0.2613% and 0.2914% respectively.

In crude sunflower oil, linoleic, linolenic, and oleic acid were not found, and in refined one, linoleic and linolenic acid was not found. In crude sunflower oil, the contribution of palmitoleic acid, cis-10-pentadecenoic acid, arachidonic acid, tridecanoic acid, stearic acid, erucic acid, elaidic acid, myristic acid, undecanoic acid, linolelaidic acid, and tricosanoic acid was found to be high in comparison with refined one.

The fatty acid of crude peanut oil which were detected were palmitolic acid, cis- 10-pentadecenoic acid, tridecanoic acid, stearic acid, erucic acid, elaidic acid, undecanoic acid, linolelaidic acid, tricosanoic acid, cis-8, 11, 14-eicosatic acid, cis- 11-eicosenoic acid and heneicosanoic acid. The area % were 1.6999%, 50.0597%, 2.3096%, 11.3800%, 8.9181%, 2.5748%, 0.4202%, 1.0842%, 1.1639%, 0.4105%, 19.7005% and 0.2784% respectively.

The fatty acid of refined peanut oil which were detected were palmitoleic acid, cis- 10-pentadecenoic acid, tridecanoic acid, stearic acid, erucic acid, linoleic acid, cis- 11- eicosenoic acid, myristic acid, and palmitic acid. The area % were 0.4664%, 12.3999%, 0.5157%, 1.0281%, 0.4194%, 0.5223%, 1.4202%, 0.1617% and 83.o663% respectively.

In crude peanut oil, myristic, palmitic, oleic, linoleic, linolenic, arachidic, gadoleic, and behenic acids were not found; in refined one, oleic, linolenic, arachidic, gadoleic, and behenic acids were not found. In crude peanut oil, the contribution of tridecanoic, cis-10-pentadecenoic, palmitoleic, stearic, cis-11-eicosenoic, and erucic acid was found to be high in comparison with refined ones. The sample may be forged as long as the crude and refined peanut sample shows no arachidic acid, and peanut oil is famous for its arachidic acid component.

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Investigating the degrading properties of three different strains of fungi on commonly used pesticides in Guyana

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Abstract. *Fareed R, Ansari A, Seecharran D, Munroe L. 2017. Investigating the degrading properties of three different strains of fungi on commonly used pesticides in Guyana. Biofarmasi J Nat Prod Biochem 15: 5-14.* Bioremediation is the use of microbes to remove various contaminants from the environment. The present research work was carried out from 2014 to 15. *Aspergillus niger, Aspergillus flavus,* and *Penicillium* spp. were employed for the biodegradation of pesticides commonly used in Guyana. Initially, the fungi were screened for ligninolytic potential by observing the decolorization/degradation of a synthetic dye (Remazol Brilliant blue) in PDA agar. The degradation of dyes was noted by the change in the original color of the dye and the visual disappearance of color from the fungus-treated Petri plates. In addition, the accumulation of the dye by the fungal mycelium was also noticed. The fungi were then tested for their tolerance to the pesticides Diuron, Malathion, and Diazinon, respectively. Using PDA agar with amoxicillin on solid media to prevent bacterial growth. The pesticides were introduced to the fungi by way of disc diffusion. Discs of a known diameter were infused with the respective pesticides at different concentrations and placed at strategic points around the inoculated fungal mycelium. The extent of inhibition was measured by comparing the growth diameter in the sample versus the growth in the control. The pesticide diazinon was found to have inhibited the fungi more when compared to Malathion and Diuron. Finally, the fungi were used to degrade the pesticides in a liquid culture-Peptone Broth. The pesticide Diuron was selected because the fungi thrived best in all pesticide concentrations. The pesticide Diuron concentration was placed in flasks containing the peptone broth with mycelial discs of the respective fungal strain. The final concentration of the pesticide was determined by using High-Performance Liquid Chromatography (HPLC). The concentration of the pesticide in each strain of fungi was decreased. *Aspergillus niger* was found to have the highest rate of pesticide degradation, followed by *Aspergillus flavus* and *Penicillium*.

Keywords: *Aspergillus flavus*; *Aspergillus niger*, degradation, bioremediation, fungi, HPLC, ligninolytic potential, microbes, pesticides, *Penicillium*

INTRODUCTION

Environmental pollution from human activities is a major challenge faced in today's society. The industrialization development over the last five decades has resulted in an exponential increase in the production and consumption of chemicals (Coelho- Moreira et al., 2013). Consequently, improper management and disposal of these chemicals contaminate soils, groundwater, and surface waters. In addition, these chemicals have high persistence; unknown environmental pathways and their potential to bioaccumulate often result in food loss, and mutagenic and carcinogenic effects on man and other organisms within an ecosystem (Fetzner 2002; Sinha et al. 2011). Ultimately, using indigenous or introduced microorganisms to decontaminate waste sites allows for an environmentally friendly and economical solution to many hazardous pollution problems. This results in biological degradation becoming an increasingly popular alternative for treating hazardous wastes (Christian et al. 2005).

The degradation process involves converting molecules to smaller compounds that may be toxic or non-toxic or removing pesticide molecules through simple absorption or adsorption mechanism (Reece et al. 2014). Sinha et al. (2011) defined xenobiotics as compounds that are alien to a living individual and tend to accumulate in the environment. He further explained that both natural and anthropogenic activities result in the accumulation of wide ranges of toxic xenobiotic compounds in the environment. Without being metabolized by soil organisms, many xenobiotics would reach toxic concentrations. Most xenobiotics are cleared through multiple enzymes and pathways.

Fungi belonging to the genera *Aspergillus* and *Penicillium* are diverse and are classified in the family Trichocomaceae. These species are commonly found in soil, decaying organic materials, and animal feed. Trichocomaceae are saprobes and possess diverse physiological properties. Some species are opportunistic pathogens, while others are exploited in biotechnology to produce enzymes, antibiotics, and other products (Leite et al., 2012).

Organophosphates are some of the most common and toxic insecticides used today, adversely affecting humans and other organisms at low levels of exposure (Pesticide Action Network 2002). Malathion, also referred to as carbophos, maldison, and mercaptothion is a non-systemic, wide-spectrum organophosphorus insecticide used to control the household and agricultural pests (Ramadevi et al., 2012). When present in the soil, it undergoes various transformations that produce a complex pattern of metabolites. The fate of Malathion in the soil is controlled

by the chemical, biological and physical dynamics of soil transformation activity (Ramadevi et al., 2012). Diazinon is a non-systemic organo-phosphate insecticide used on home gardens and farms to control many sucking and leaf-eating insects. It also has veterinary uses against fleas and ticks (Nyakundi et al., 2011).

Bioremediation or utilizing microorganisms to degrade toxic organo-pollutants is an efficient, economical approach that has been successful in laboratory studies. The conventional mechanisms for removing pesticides in the soil, such as chemical treatment, volatilization, and incineration, have met public opposition. This is because of the large volumes of acids and alkalis produced, the potentially toxic emissions, and the elevated economic costs (Nyakundi et al., 2011; Kumar-Praveen and Bhat, 2012). Overall, most physical and chemical cleaning technologies are expensive and rather inefficient. These clean-up methods do not suit large farms since only small soil samples are required. They are done in laboratories and hence require a lot of resources because the contaminated soil has to be excavated at a site and moved to a storage area where it can be processed. Due to environmental concerns associated with the accumulation of pesticides in food products and water supplies, there is a great need to develop safe, convenient, and economically feasible methods for pesticide remediation. For this reason, several biological techniques involving the biodegradation of organic compounds by microorganisms like bacteria and fungi have been developed (Nayakundi et al., 2011).

Coelho-Moreira et al. (2013) highlighted that diuron could be transformed abiotically via hydrolysis and photo-degradation reactions, but these reactions occur at very low rates under natural conditions. Due to this, diuron is known as a potential water contaminant. Degradation products of diazinon include diazoxon and oxypyrimidine. Oxypyrimidine is the main soil and water degradation product of diazinon. Diazinon can be converted to diazoxon in the atmosphere via ultraviolet light. Diazinon released to surface waters or soil is subject to volatilization, photolysis, hydrolysis, and biodegradation (Nayakundi et al. 2011).

The transformation of diuron by *P. chrysosporium* in liquid cultures has already been documented in both stationary and shaken conditions (Coelho- Moreira et al., 2013). Singh and Singh (2010) used *Aspergillus flavus* as the experimental organism to degrade two textile dyes, namely Bromophenol blue and Congo red. This fungus has shown garnered positive results for dye degradation and decolorization.

This study was executed to examine microbial degradation in three strains of fungi, namely, *Aspergillus niger, Penicillium* spp., and *Aspergillus flavus*. The degradation of the dye Remazol Brilliant Blue R was observed in a solid medium, and pesticide degradation was done in a liquid medium. The aim of this research was to investigate the degrading properties of the three different strains of fungi on commonly used pesticides in Guyana. It focused on the following objectives: (i) To compare the ligninolytic potential of each fungal strain using Remazol Brilliant Blue dye. (ii) To compare the tolerance of each fungal strain to different concentrations of the pesticides Malathion, Diuron, and Diazinon. (iii) To determine the ability of fungi to degrade the pesticides in liquid cultures. (iv) To determine the biomass production of each fungus in liquid cultures.

MATERIALS AND METHODS

The present research work was carried out from 2014 to 15 to study the degrading properties of three fungal strains on commonly used pesticides in Guyana. The fungal cultures were obtained from the University of Guyana's laboratory. There were sub-cultured and maintained for three weeks before the commencement of the experiment. This ensured that the spores were active.

The pesticides were selected based on their availability and popularity to local farmers in Guyana. The pesticides Malathion (insecticide), Diuron (herbicide), and Diazinon (insecticide) were selected. They were then diluted to the desired concentrations required for the experiment. The Dye Remazol Brilliant blue was used, a class of anthraquinone derivative dye used in the textile industry.

Preparation of agar

The agar used was Potato Dextrose agar. This was prepared by dissolving the medium ingredients (Potato infusion, dextrose, and agar) over a heat source for approximately 15 minutes and was subsequently autoclaved for 20 minutes. The antibiotic amoxicillin was added to inhibit the growth of bacterial colonies. The dye Remazol Brilliant Blue R was added to the agar prepared to assess the ligninolytic potential of the fungi; this was a 1.0 % (w/v) dye solution prepared using the potato infusion.

Monitoring decolorization

Decolorizing dyes from the Petri plates was assessed by change in original color (as compared to control) and by the visual disappearance of color from the Petri plates. The radial growth of fungal mycelium and change in color was measured after a fixed interval for four days. The culture plates containing dyes were examined for the visual disappearance of color from the media of the Petri plates when compared to their respective controls.

Figure 1 shows Petri plates infused with the Remazol Brilliant Blue R dye and a plug of the respective fungi placed in the middle. As the fungi grow outwards, the decolorization growth diameter can be monitored. The extent of decolorization will determine the ligninolytic potential of each fungus.

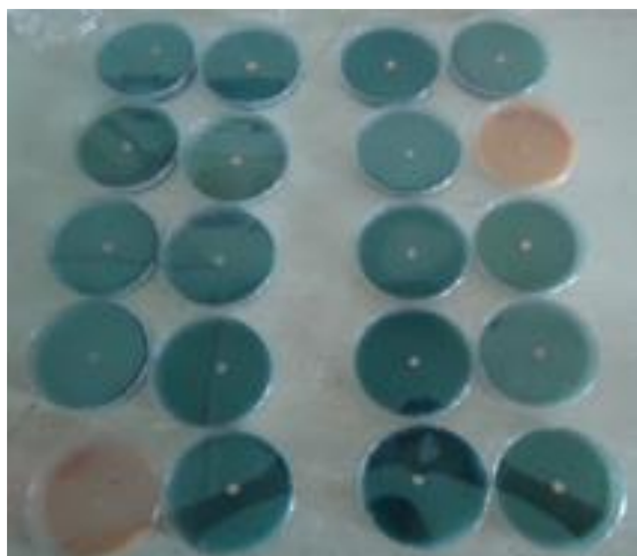


Figure 1. Infusion of Petri plates with the dye Remazol Brilliant Blue R and plugging of the respective fungi

Figure 2. inhibition zones and decolorization zones

Step 1- Assessing the ligninolytic potential of each strain of fungi

Approximately 30 ml (± 1) of the medium was poured into each previously sterilized Petri plate, which was then allowed to cool. The Petri plates were inoculated with a plug of the respective strain and maintained at room temperature for 10 days. The decolorized zone formation was observed daily and was calculated using the formula

I = C-T/C x 100

Where, I = Percentage of inhibition in fungal growth, C = Growth in terms of colony diameter in control, and T = Growth in terms of colony diameter in the sample.

The plates were done in triplicates, and two types of (2) control were prepared, first with fungi only and second with dye only. The first control was used to compare the fungal growth with and without dyes. The second was used to compare the visual disappearance of color from the inoculated plates.

Step 2 -Tolerance test of fungi to different concentrations of each pesticide

The plates were inoculated with the respective fungal strains, and discs infused with the different concentrations of the pesticides were placed around the fungal inoculum. It was maintained at room temperature, and inhibition zones and zones of decolorization were observed (Figure 2). For each strain of the fungus, there were three plates with different concentrations of pesticides. This was done in triplicates for each strain. Three types of control were prepared, first with fungi only, second with dye only, and finally with pesticide (3) only. The first control was used to compare the fungal growth with and without dyes. The second was used to compare the visual disappearance of color from the inoculated plates, and the third was used as a reference.

Step 3- Ability of the fungi to degrade pesticides in liquid cultures.

Agar plugs of 10 mm diameter were removed from an agar plate with the respective culture growing for 2 weeks. This was inoculated into 500 ml Erlenmeyer flasks containing 60 ml stock solution (peptone broth). A known amount/pesticide concentration was inoculated with the respective fungal strain. Each fungal strain was tested against a known concentration of the pesticide diuron. This was maintained at room temperature for seven (7) days. Each solution was then subjected to an HPLC analysis to determine the final concentration of pesticide. The fungal Biomass was collected by filtration, and the filter paper was dried for 24 hours and weighed.

RESULTS AND DISCUSSION

The rapid increase in industrialization and agricultural practices have resulted in the accumulation and contamination of many harmful chemicals in the environment. The frequency and ubiquitous use of artificial "xenobiotics" have led to immense effort to implement new technologies to purge these contaminants from the environment (Ramadevi et al. 2012). For instance, approximately thirty percent (30%) of agricultural produce is lost to pests, thus rendering pesticides an indispensable weapon in agriculture. However, the indiscriminate use of pesticides has inflicted serious repercussions on biodiversity and human health. Bioremediation is a promising alternative that exploits microbes' natural ability to remove soil contaminants. This method is environmentally friendly, minimally hazardous, and versatile (Ramadevi et al., 2012).

The degradation of dyes serves as an indicator of the extent of degradation that would occur when the microbes are subjected to pesticides (Ramya et al., 2007). According to Couto et al. (2001), there is a good correlation between the biodegradation of pollutants and the decolorization of polymeric dyes by ligninolytic fungi. Therefore, the decolorization of such dyes is a simple method to assess the degrading ability of the extracellular enzymes secreted by these fungi. The degrading ability of the extracellular enzymes is referred to as its ligninolytic potential. Singh and Singh (2010) highlighted that the production of extracellular enzymes such as laccase is produced by *Aspergillus* spp. In addition, the breakdown of most organo-pollutants by fungi is closely linked with ligninolytic metabolism. (Coelho- Moreira et al. 2013).

Aspergillus niger, Penicillium spp., and *Aspergillus flavus* yielded positive results for dye degradation/decolorization. This was indicated by a change and slow disappearance of the color of the dyes from the dye-containing media of the Petri plates. Additionally, a zone of different colors which is usually lighter around the fungal colony was also observed. Figure 3 shows decolorization zones observed in all strains of fungi.

This color change can be attributed to the production of extracellular enzymes by the applied fungi, which occurs during the biodegradation of the dye. Extracellular oxidases such as laccases, lignin peroxidase (Lip), and manganese peroxidase (MnP), which are involved in lignin degradation, have also been reported to decolorize dyes (Ramya et al. 2007).

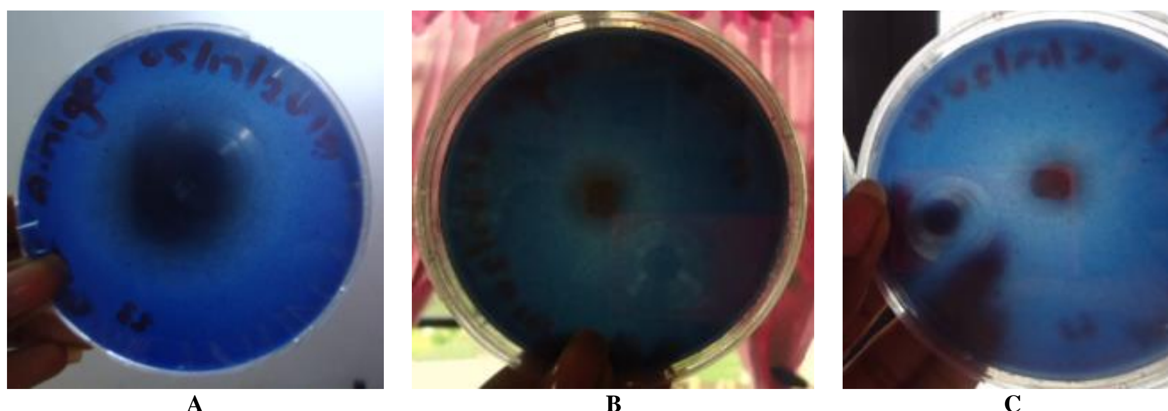
In addition to the production of extracellular enzymes, absorption of the dye by the mycelium of the fungi was also observed as the mycelium of the fungi appeared blue as it grew on the surface of the dye (Knapp and Reece, 1995; Ramamurthy and Umamaheswari 2013) highlighted that absorption of dyes to the microbial cell surface is the primary mechanism of dye decolorization. This accumulation of chemicals with microbial biomass can take place on living or dead biomass. Fungal biomass is an efficient adsorbent because it contains natural polysaccharide chitin and its derivative chitosan in the cell walls. Chitosan possesses a unique molecular structure with a high affinity for many classes of dyes.

Table 1 represents the decolorization diameter of the Fungi *Aspergillus niger*, *Penicillium*, and *Aspergillus flavus,* respectively. The values in each table compare the growth of fungi in agar treated with the dye Remazol brilliant blue and a biotic control (without dye). For all the strains of fungus, the control exhibited higher growth rates when compared to the samples treated with the dye. This shows that the dye had some inhibitory effect on the growth of the fungi. Figure 4 shows the growth rate of each fungus in the presence of the dye Remazol Brilliant Blue compared to the growth in the control.

Comparison of the growth rate in the agar infused with the dye, *Penicillium* spp. recorded the highest growth rate followed by *A. niger* and then *A. flavus*. For days two through four, *A. niger* had the highest growth rate, followed by *A. flavus* and finally *Penicillium* spp.

Table 1. Decolorization diameter values and % decolorization (Mean ± SD) for *Aspergillus niger, Aspergillus flavus,* and *Penicillium*

Day	Sample treated with dye (mm)	Control (mm)	Rate of decolorization (%)
	Aspergillus flavus		
1	14.00 + 1.73	14.33+ 1.53	2.33
2	39.00 + 1.00	39.33 + 0.58	0.85
3	55.33 + 2.52	59.67 + 1.53	7.26
4	68.00 + 2.00	72.33 + 1.53	5.99
	Aspergillus niger		
1	14.67 + 2.52	16.00±1.00	8.33
2	39.67 + 0.58	40.33±0.58	1.65
3	65.33 + 2.52	70.00±5.00	6.67
4	74.67 + 3.06	76.33±1.53	2.18
	Penicillium		
1	16.67 +1.53	17.00 + 1.00	1.96
2	37.33 + 2.52	40.33 + 0.58	7.44
3	52.67 + 9.45	58.00 + 2.65	9.20
4	68.67 + 1.53	76.00 + 1.00	9.65



A B C

Figure 3. A. *Aspergillus niger*, B. *Penicillium* spp., C. *Aspergillus flavus*

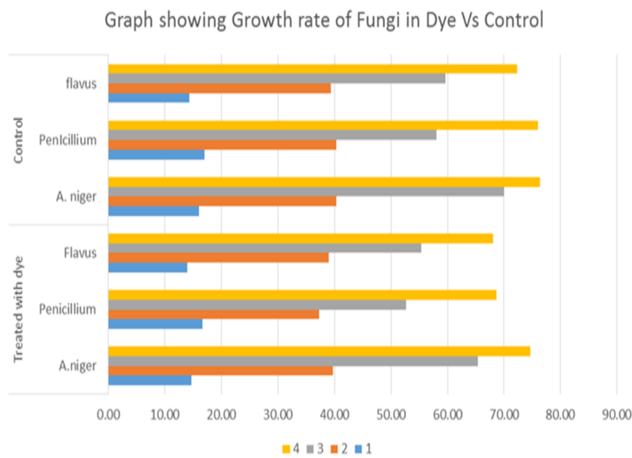


Figure 4. The growth rate of Fungi in dye vs. control

Similar patterns were observed in control with regard to day one. On day two, *Penicillium* spp. and *A. niger* recorded the same growth rate, followed by *A. flavus*, Day three, shows *A. niger* with the highest rate, followed by *A. flavus. Finally*, day four shows *A. niger* with the highest growth rate, followed by *Penicillium* and finally *A. flavus.*

To summarize*, A. niger* and *Penicillium* spp. recorded the highest growth rates for days one, two, and four in media treated with the dye.

When comparing the decolorization rates (%) (Table 1), all strains of fungi exhibited varying decolorization rates. *Aspergillus niger* recorded the highest decolorization rate on day one, followed by *Aspergillus flavus* and finally *Penicillium* spp. For the fungi, *A.niger,* and *A. flavus*, a decrease on the second day was observed, followed by an increase in the rate on day three and, finally, a drop in the rate on the final day. However, *Penicillium* spp. recorded a steady increase in the rate of decolorization. Figure 5 shows average values of dye decolorization (%) for four days to determine which strain of fungi had the highest overall ligninolytic potential. *Penicillium* spp. recorded the highest ligninolytic potential, followed by *A.niger* and finally *A. flavus.*

Initially, *Aspergillus niger* recorded the highest decolorization rate; however, as time progressed, the rate of decolorization decreased linearly. This can be attributed to the production of toxins that hindered the growth of the fungi and, consequently, the decolorization rate. As it rapidly decolorized the dye in the initial stages, the toxins produced during metabolism accumulated and hindered the production of extracellular enzymes, ultimately reducing the rate of decolorization.

Penicillium spp. recorded the highest decolorization rate, the rate increased linearly as time progressed. There was no drop in the rate of decolorization because the toxins were produced in insufficient quantities to hinder the growth of the fungi. In addition, the fungus did not decolorize the dye rapidly in its initial stages, thus limiting the effects of the amount of toxins produced. *Aspergillus flavus* recorded the second-highest decolorization rate; the same pattern was noticed with *Penicillium*, with the initial decolorization rate being low and a linear increase as time progressed.

As with *Aspergillus niger, Penicillium* and *Aspergillus flavus* also recorded a drop in decolorization rate as time progressed. However, the growth of the fungi was rapid and covered the entire Petri plate before any more observations could be recorded.

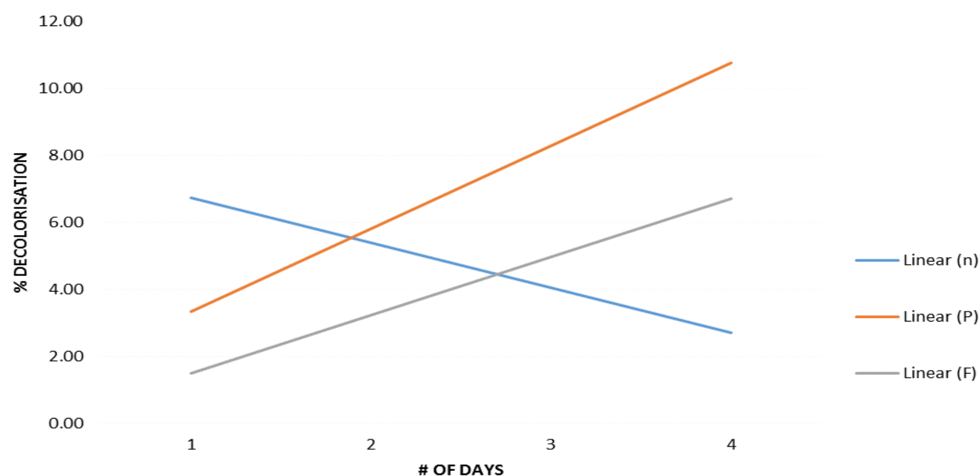


Figure 5. Rate of decolorization of each fungus (n = *Aspergillus niger*; P = *Penicillium* and F = *Aspergillus flavus*)

It was seen that decolorization occurred by two mechanisms, mainly biosorption and enzymatic degradation. However, these reactions did not occur under optimal conditions. For instance, each fungal strain did not regulate physicochemical parameters such as media composition, pH, and temperature. Some strains of fungi thrive best in media with glucose as the carbon source (Ramadevi et al., 2012). Additionally, nitrogen demands for growth and especially enzyme production differ markedly among fungal species. According to Coelho-Moreira et al. (2013), the production of ligninolytic enzymes with *P. chrysosporium* is much more effective under the conditions of nitrogen limitation. On the other hand, *B. adusta* produces more LiP and MnP in nitrogen-sufficient media (Pavko. 2011). Regarding pH (Knapp and Reece, 1995), the optimum pH depends on the medium, fungus, enzyme system, and decolorization under consideration. The majority of researchers suggest that the optimum pH values are likely to be in the range of 4–4.5. The optimal decolorization temperature for a particular process has to be selected from case to case according to the mentioned parameters (Singh and Singh 2010).

Hussaini et al. (2013) defined pesticides as any substance or a mixture of substances that are used to control destructive pests such as insects, plant disease organisms, and weeds, including many other living organisms such as nematodes, arthropods other than insects, and vertebrates that endanger our food supply, health, or comfort. The term pesticide can also refer to chemical substances that alter the biological processes of living organisms (Timchalk 2001).

The application of pesticides may adversely affect the different life forms within ecosystems. Coelho-Moreira et al. (2013) elucidated that approximately 90% of agricultural pesticide applications never reach their target organisms; instead, they are dispersed through the air, soil, and water.

Ramadevi et al. (2012) highlighted that Aspergillus spp. is an effective biodegrading organism and can be effectively used to create bioremediation systems in malathion-contaminated soil. It was noted that due to their high biodegradation activity, this fungal strain has the potential to be used as biological agents for the remediation of soil, water, or crops contaminated with pesticide malathion (Ramadevi et al. 2012). Leitao (2009) explained that *penicillium* spp. has demonstrated their ability to degrade different xenobiotic compounds with low co-substrate requirements and could be potentially interesting for developing economically feasible processes for pollutant transformation. Furthermore, *Penicillium* strains can live in saline environments, an advantage of these microorganisms over the others in the bioremediation field (Leitao. 2009).

The three strains of fungi were placed in solid media with different concentrations of the pesticides Diuron, Malathion, and Diazinon. The fungi growth diameter and decolorization were measured and observed during growth. *Aspergillus niger* recorded the highest average growth rate in all concentrations for each pesticide except for 10% Diazinon and the recommended dosage (Table 2). *Penicillium* spp. was second, followed by *Aspergillus flavus*. However, the percent inhibition values (Table 3) show that *Aspergillus niger* had the highest inhibition percent in Diuron at 1%, Malathion at 1%, and for all concentrations of Diazinon.

The percent inhibition values take the growth of the fungi in the sample and express it as a percent of the growth in the control. Hence the extent of inhibition of each pesticide and concentration can be calculated. Figures 6-7 are observed inhibition zones and each fungus's growth diameter. These were formed initially as the fungal mycelia grew outwards, occupying the plate. However, these inhibition zones disappeared after three days, most likely because the fungi acclimatized to the physiochemical changes in the media due to the pesticides.

Table 2. Average growth diameter (mm) for each fungus in different concentrations of each pesticide for 4 days.

Fungal strain	Diuron			Malathion			Diazinon		
	1%	10%	R.D	1%	10%	R.D	1%	10%	R.D
A. flavus	28.14	35.42	28.06	38.625	35.08	34.67	35.83	35.33	37.67
A. niger	43	44.67	43.92	44.67	46.33	43.67	38.50	25.67	33.58
Penicillium spp.	38.92	36.63	37.75	40.625	37.25	34.96	36.58	25.50	32.17

Table 3. Percent (%) inhibition of each fungus in different concentrations of each pesticide for four days

Fungal strain	Diuron			Malathion			Diazinon		
	1%	10%	R.D	1%	10%	R.D	1%	10%	R.D
A. flavus	10.23	13.97	8.02	4.06	12.84	20.05	13.39	12.97	9.98
A. niger	12.81	8.70	9.04	10.85	5.55	11.37	30.92	46.48	40.54
Penicillium spp.	8.80	13.96	12.58	3.84	13.23	20.02	16.93	41.48	26.52

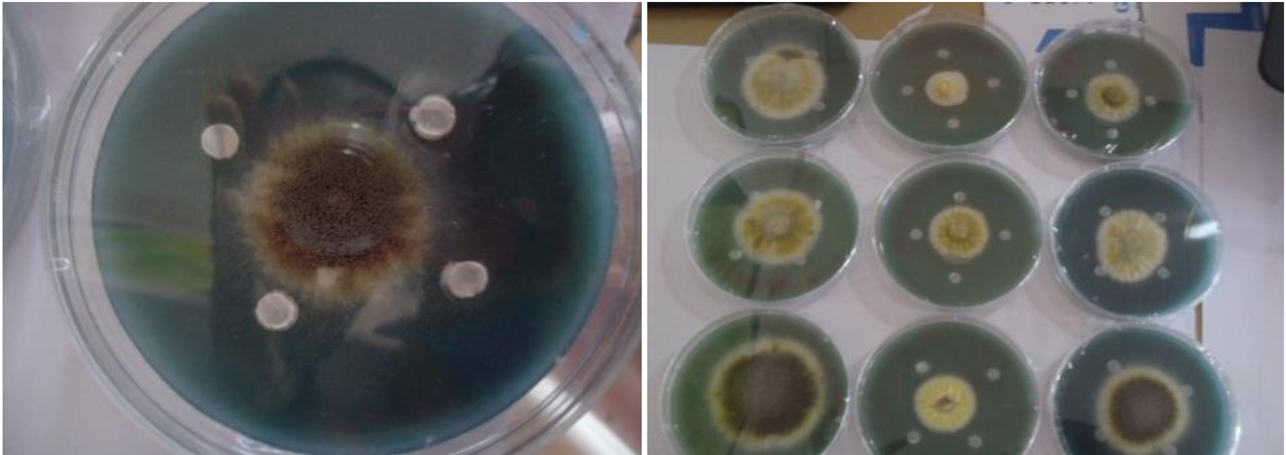


Figure 6. Inhibition zones

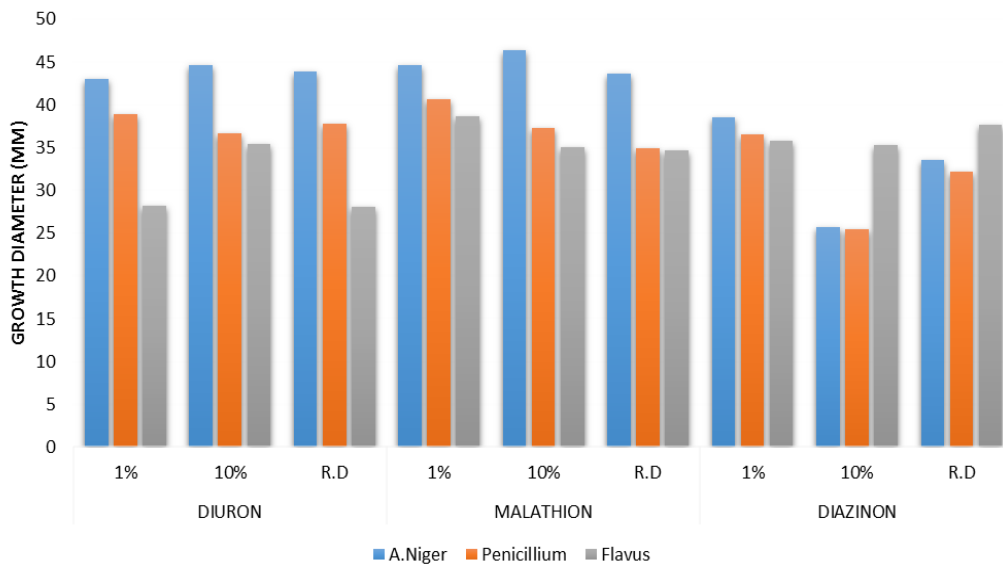


Figure 7. Growth diameter of each fungus in the different concentrations of the pesticides Diuron, Malathion, and Diazinon

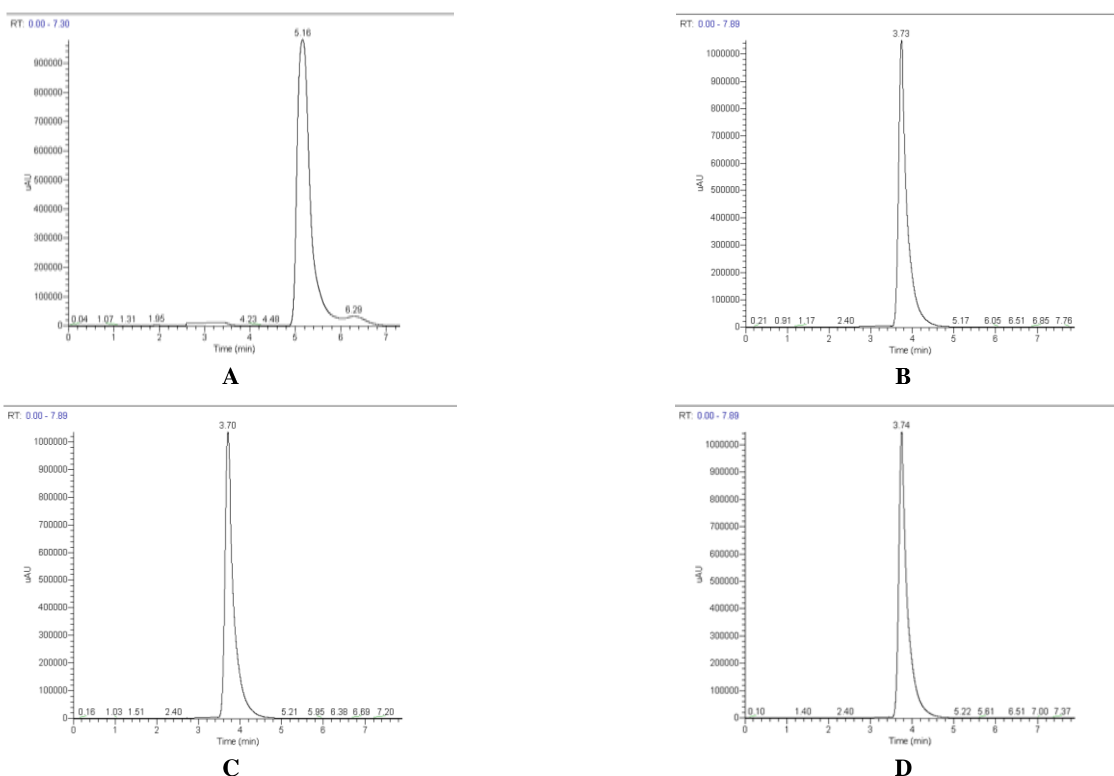
When comparing the fungal growth (Table 2), the fungi thrived best in Diuron and Malathion's pesticides. However, decolorization was observed in all plates treated with the three pesticides.

Aspergillus niger grew best in all concentrations of pesticides except for 10% diazinon, followed by *Penicillium* in all concentrations except 10% diazinon. *Aspergillus flavus* had the lowest growth rate in all pesticides except Diazinon 10% and the recommended dosage. Even though inhibition was present in the plates treated with pesticides, decolorization was observed in all the plates. They grew outwards and over the discs infused with various concentrations of pesticides. This shows that the pesticides did not affect the fungal strains' production and secretion of extracellular enzymes.

The ANOVA tests revealed significant differences in the inhibition rate in Diuron 1% and for Malathion and Diazinon recommended dosages. No significant differences were noticed between fungi except in the pesticide Diazinon (not shown).

After the initial screening process, which involved assessing the ligninolytic potential and pesticide tolerance, the fungal strains were allowed to grow in liquid cultures. The only carbon source available was from the nutrient broth and a known concentration of Diuron (Coelho-Moreira et al., 2013). Diuron is a phenyl urea herbicide applied to various crops, especially sugar cane. The compound acts in photosynthetic organisms by blocking electron transport. The initial concentration was 30 ppm after the High-Pressure liquid chromatography (HPLC) analysis was conducted.

The respective fungal strain was inoculated with the pesticide and nutrient broth. After the fungal biomass was collected, the filtrate was analyzed using HPLC, and the final concentration of the pesticide subjected to each fungal strain was determined. The final concentration of Diuron was determined by using HPLC analysis (Figure 8).



Figures 8. A. Retention time of the initial (pure) pesticide sample before it was treated with each fungus. B. After *A. flavus*, C. After *A. niger*, D. After *Penicillium* spp.

The retention time is the amount of time it takes for the compound (Diuron) to travel through the column during separation to the detector. The retention time is assigned to a corresponding peak, indicating that the interest compound is being detected. By comparing retention times, the difference in concentration can be seen by noting the decrease in retention times and the area under the peak. The retention time peaks were done in duplicates and were observed at 5.16 and 5.15 minutes, respectively. This correlates with retention times of Diuron reported in the literature, which is usually around 5-6 minutes.

Figure 9 depicts the retention time peaks of the pesticide after being treated with each of the respective fungi. Compared to the control sample's retention time peak, the time recorded after being treated with the fungi was less. The retention time peaks for the sample treated with the fungi all peaked around three minutes. In addition, the area under the curve was larger for the control sample compared to the samples treated with fungi. *Aspergillus niger* recorded the fastest retention time, followed by *Aspergillus flavus* and then *Penicillium* spp. The graphs produced from the HPLC analysis provided insight into Pesticide degradation, as the decreased retention times and reduced area under the curve suggest the pesticide concentration decreased. By using data garnered from these graphs, values of the concentration of Diuron in Parts per million (PPM) were determined (Table 4).

It was evident that degradation occurred because the concentration decreased in all the samples treated with fungi. The concentration of the control was still found to be the same as initially recorded.

The fungus *Penicillium* spp. recorded the highest pesticide concentration of 26.77 ppm, followed by *Aspergillus flavus* with 26.63 Ppm and finally *Aspergillus niger* with 26.60 ppm. It can be seen that *Aspergillus niger* was most effective in degrading the pesticide Diuron as it recorded the lowest concentration of pesticide. Concerning biomass production in the pesticide Diuron, *Aspergillus niger* had the highest production, followed by *Aspergillus flavus* and, finally, *Penicillium* (Table 5).

Table 4. Percentage degradation (MEAN $\pm$ SD) of each strain of fungus on the pesticide Diuron

Fungal strain	Initial concentration of pesticide (ppm)	Final concentration of pesticide (ppm)	% Degradation
A. flavus	30.709±0.077	26.630±0.081	86.718
A. niger	30.709±0.077	26.601±0.057	86.625
Penicillium	30.709±0.077	26.770±0.043	87.176

Table 5. Biomass production (MEAN + SD) of each strain of fungi in the pesticide Diuron

Fungal strain	Sample treated with pesticide (g)	Control (g)
A. flavus	0.38±0.01	0.38±0.01
A. niger	0.40±0.01	0.41±0
Penicillium	0.38±0.01	0.39±0.01

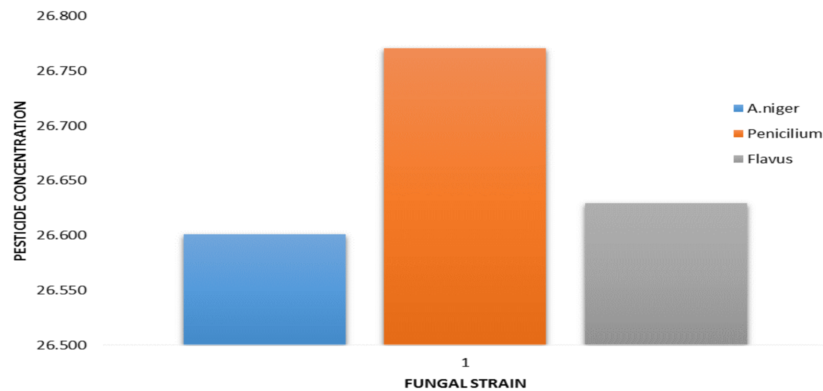


Figure 9. The final concentration of pesticide after being treated with each fungus

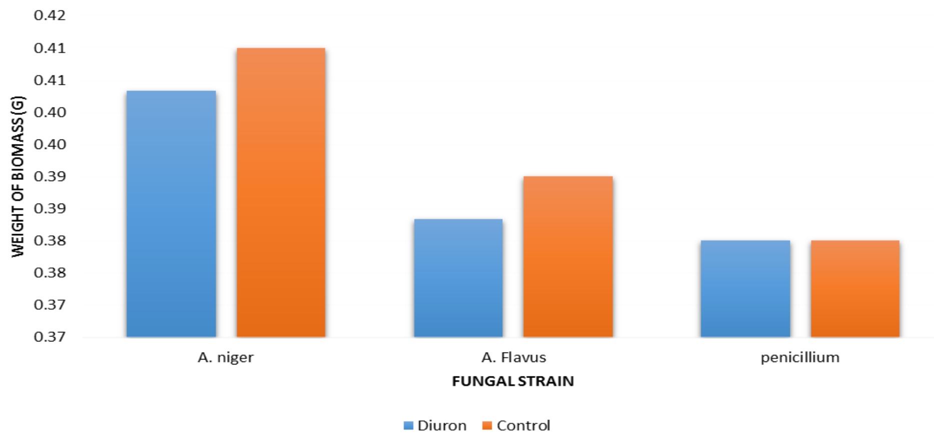


Figure 10. Biomass production in pesticide vs. control

However, biomass production in the controls for *Aspergillus niger* and *Aspergillus flavus* were higher when compared to the production in the pesticide-treated sample. This shows that the pesticide affected/inhibited the biomass production of the fungi. However, this was not observed in *Penicillium* species which shows that biomass production is the same in control and pesticide. This might be due to increased tolerance of the *Penicillium* spp. to the pesticide. Additionally, the fungi *Aspergillus niger* and *Aspergillus flavus* thrived better in the liquid media with the pesticide and in control than *Penicillium* spp. Figure 10 represents these observations.

Additionally, it was observed that there is a relationship between biomass production and pesticide degradation. For instance, *Aspergillus niger* recorded the highest degradation and highest biomass production. Similar trends were noticed for *Aspergillus flavus* and *Penicillium* spp. However, this is expected since more growth/spore production would utilize more of the pesticide as a carbon source, reducing the pesticide concentration.

In conclusion, mycoremediation is an environmentally friendly alternative that can be used to clean up toxins in the environment. This study found that the fungal strains; *Aspergillus niger, Penicillium* spp., and *Aspergillus Flavus* all exhibited ligninolytic potential as they all decolorized the textile dye remazol brilliant blue. The strain *Penicillium* was shown to have the highest decolorization rate, followed by Aspergillus flavus and *Aspergillus niger*. The three strains of fungi were more tolerant to the pesticides Malathion and Diuron as it was observed that Diazinon had the highest inhibition percent in all strains of the fungi tested. Concerning pesticide degrading properties, *Aspergillus niger* recorded the highest rate, followed by *Aspergillus flavus* and *Penicillium* spp.

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Isolation and characterization of mannanase, xylanase, and cellulase from marine bacteria *Bacillus* sp.

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Abstract. *Yopi, Djohan AC, Rahmani N, Jannah AM. 2017. Isolation and characterization of mannanase, xylanase, and cellulase from marine bacteria* Bacillus sp. *Biofarmasi J Nat Prod Biochem 15: 15-20.* Isolation, identification, and characterization of mannanase, xylanase, and cellulase-producing indigenous marine bacteria have been conducted from a total of 20 isolates. Based on 16S rDNA sequence analysis, three potential isolates are obtained and identified *Bacillus subtilis* (M8), *Bacillus tequilensis* (X4), and *Bacillus cereus* (C9). The potential strains M8, X4 and C9 can produce mannanase, xylanase and cellulase activities such as 9.5 U/mL; 0.36U/mL;0.56U/mL with optimum pH and temperature 6.0;50ºC, 5.5;70ºC and 8;50ºC, respectively. Based on the TLC analysis, mannanase from M8 and xylanase from X4 has the potential to hydrolyze mannan and xylan for producing oligosaccharides with the size around tri-hexasaccharide as the main product.

Keywords: *Bacillus* sp., cellulase, hemicellulase, marine bacteria, oligosaccharide.

INTRODUCTION

The utilization of cellulose by microorganisms involves a substantial set of fundamental phenomena beyond those associated with enzymatic hydrolysis of cellulose (Lynd et al. 2002). Marine biodiversity microorganism has high potency for biotechnology and the enzymatic environment in Indonesia. There is a deep interest in searching for new enzymes source of cellulase and hemicellulase for industrial application (Sukumaran et al. 2005).

Marine microorganisms have been regarded as a reservoir, not only for novel natural products but also for valuable genes and enzymes (Guo et al. 2013). Several xylanases produced by marine microorganisms from special habitats, such as the deep-sea hydrothermal field (Wu et al. 2006), the Antarctic marine soil (Collins et al. 2002), and marine sediment (Guo et al. 2009), were shown to have special properties, such as hyperthermostability (Wu et al. 2006), cold adaptation (Collins et al. 2002; Guo et al. 2009) and salt-tolerance (Guo et al. 2009; Liu et al. 2012).

The utilization of marine bacteria to produce cellulase and hemicellulase has not been used widely in Indonesia. There is a report related to isolated marine bacteria from Pari island, which have the ability to produce mannanase (Djohan 2014). We have already collected marine microbes from Bali Island and already identified them using 16S rRNA as cellulase and hemicellulase marine microbe belonging to *Bacillus* sp.

This research intends to isolate and characterize cellulose and hemicellulose deriving from marine microbes for new enzyme applications in biotechnology fields, especially to convert abundant cellulose and hemicellulose biomass into monosaccharides that can be converted into bioethanol through fermentation yeast.

MATERIALS AND METHODS

Isolation mannanase, xylanase, and cellulase producing marine bacteria

Mannanase, xylanase, and cellulase-producing bacteria were isolated from seawaters and sediment collected on Bali Island. The Locust Bean Gum (LBG), CMC, and Xylan are mediums for screening and purifying bacteria and contain 0.5% of substrate (LBG, CMC, and Xylan), 0.075% of peptone, 0.05% of extracted yeast which were then diluted in Artificial Sea Water (ASW) at pH 6.0 (Mandels and Sternberg 1976). The purified colonies were preserved at 4ºC for further study.

Screening of mannanase, xylanase, and cellulase producing marine bacteria

Pure cultures of bacteria isolates were transferred individually in CMC, xylan, and LBG agar plates. After incubation for one day, these agar plates were flooded with 0.2% red congo and allowed to stand for 30 min at room temperature for counterstaining the plates, NaCl 1 M solution was thoroughly used. The bacteria colonies with the largest clear zone were selected to identify and produce cellulase, xylanase, and mannanase in submerged fermentation.

Identification and phylogenetic analysis 16S rDNA sequence

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) technique using a pair of primer 9F: 5'-AGRGTTTGATCMTGGCTCAG-3' and 1492R:

5'TACGGYTACCTTGTTAYGACTT-3' (Burggraft et al. 1992). The obtained bands were stained in Bio and were visualized by a UV transilluminator.

Production of mannanase, xylanase, and cellulase

For fermentation, Mannanase, xylanase, and cellulase were produced by three potential isolates (M8, X4, and C9), precultured in a 100 mL flask containing 10 mL of ASW cultivated at 30ºC for one day. The preculture medium was seeded into a 300 mL flask containing 30 mL of ASW medium with adjusted cell 0.02 (OD λ=660) and incubated at 30ºC for six days.

Mannanase, xylanase, and cellulase activity assay

Mannanase, xylanase, and cellulase activity were assayed by measuring the reducing sugars using the dinitro salicylic acid (DNS) method (Miller 1959). Cellulase activity was measured using substrate 0.5% of CMC (Sigma) and dissolved in 50 mM sodium phosphate buffer, pH 7.0. An appropriately diluted enzyme solution (250 µL) was incubated with 250 µL of the substrate solution at 60ºC for 15 min. The amount of reducing sugars released in the enzyme reaction was assayed by mixing 750 µL of DNS solution, heating it at 100ºC for 10 min, then cooling it on ice for 10 min, and measuring the absorbance at 540 nm. One unit of cellulase activity is defined as the amount of enzyme that releases 1 µmol of D-glucose per minute under the experimental condition given.

Xylanase activity was measured using 250 µL of the substrate solution of 0.5% xylan (Sigma). An appropriately diluted enzyme solution (250 µL) dissolved in 50 mM sodium phosphate buffer with pH 7.0 was incubated at 60ºC for 15 min. After incubation, 750 µL DNS solution was added to end the reaction. The amount of reducing sugars released in the mixture was determined, with xylose as the standard using the DNS method (Miller 1959). One unit of xylanase activity is defined as the amount of enzyme capability to release 1 µmol of xylose per minute under the assay condition.

Mannanase activity was measured using a substrate of 0.5% LBG (sigma) which was dissolved in 50 mM of sodium phosphate buffer, pH 7.0. An appropriately diluted enzyme solution (250 µL) was incubated with 250 µL of the substrate solution at 60ºC for 15 min. The amount of reducing sugars released in the enzyme reaction was assayed by mixing 750 µL of DNS solution, heating it at 100ºC for 10 min, cooling it on ice for 10 min, and measuring the absorbance at 540 nm. One unit of mannanase activity is defined as the amount of enzyme that liberates 1 µmol of D-mannose per minute under the experimental condition given.

Characterization of optimum pH and temperature

The optimum enzyme activity pH was examined at pH 3.0-10.0 under standard assay conditions. Fifty mM of various buffers were used: sodium citrate (pH 3.0-5.0), sodium phosphate (pH 6.0-8.0), and glycine NaOH (pH 9.0-10.0). The enzyme reaction was incubated at 50ºC for 15 min in the presences of 0.5% (w/v) LBG for mannanase, 0.5% (w/v) xylan for xylanase and 0.5% (w/v) CMC for cellulose. The effect of temperature on enzyme activity was performed at a temperature ranging from 30-90ºC in 50 mM acetate buffer at optimum pH for 15 min.

Cellulosa and hemicellulose hydrolysis analysis by using thin-layer chromatography

Hydrolysis on mannan substrates (LBG, Konjac glucomannan, Ivory nut, porang), xylan (xylan), and cellulose (CMC) was carried out at 50ºC in 50 mM of sodium phosphate buffer, pH 6, containing 0.5% for each substrate. The enzyme-substrate ratio (v/v) was 1: 1and the reaction time was from 0, 0.5, 1, 2, 4, 8, 12 and 24 hours. The reaction was carried out in 1.5 mL of Eppendorf containing 100 µL of reaction mixtures in the dry block. Thin Layer chromatography (TLC) of oligosaccharides was carried out on silica gel $60F_{254}$ plates (Merck Art 20x20 cm) and eluent with a solvent mixture of n-Butanol/Acetic acid/Water (12: 6: 6, v/v/v). The spots were visualized by TLC dye reagent, i.e., DAP (0.5 g α-diphenylamine, 25 mL acetone, 2.5 mL phosphate acid, 0.5 ml aniline) and subsequently heated at 120ºC for 15 min. The samples were applied in equal quantities (4µL). Glucose, mannose (Sigma-Aldrich, U.S.A.), mannobiose (M2), mannotriose (M3), mannotetraose (M4), and mannopentaose (M5) and mannohexaose (all from Megazyme, Ireland) were used as a standard.

RESULTS AND DISCUSSION

Isolation of cellulose and hemicellulose from marine bacteria

The research collected 20 isolates from a screening medium of artificial seawater and bacteria that could produce several types of enzymes, such as mannanase, xylanase, and cellulase enzyme. Then they were screened further for their abilities to excrete enzymes in some substrates, such as the locust bean galactomannan substrate, which is a medium for getting the mannanase, xylene for getting the xylanase, and CMC for getting the cellulase potential marine microbe. Isolation on cellulose and hemicellulose of marine bacteria using some substrate (locust bean gum, xylan, CMC) has been done using congo red analysis (Figure 1). For the mannolytic microbe, we obtain 11 isolates that produce mannanase. Still, only the M8 isolate produces the highest mannanase activity; for xylanase, we obtain one isolate X4, and for cellulase, we obtain one isolate C9.

Identification of the purified isolates based on the 16S rDNA sequence

Based on the sequence analysis of the 16S rDNA gene against the GenBank database, there is an indication that 3 selected isolates were closely related to the members of the genus Bacillus. The phylogenetic analysis showed about 99% similarity between isolate M8 and *Bacillus subtilis,* isolate X4 and *Bacillus tequilensis,* and isolate C9 and *Bacillus cereus* (Table 1).

Production of mannanase, xylanase, and cellulase

The crude enzyme production was done from marine bacteria (M8 isolates for mannanase, X4 for xylanase, and C9 for cellulase) for 144 hours of culture (Figure 2), which were grown in ASW medium with shaking (180 rpm) at 30ºC.

The members of the genus *Bacillus* produce large varieties of extracellular cellulase and hemicellulase, such as cellulase, mannanase, and xylanase, which are significantly important enzymes for industrial demand. These quantitative enzyme tests have been conducted to determine the ability of each isolate to produce an enzyme using commercial substrates as a carbon source. The isolates produce enzymes using commercials substrate as a carbon source with maximal activity at 72 hours of culture, such as xylanase 0.36 U/mL, mannanase 9.5 U/mL, and cellulase 0.56 U/mL.

Characterization of optimum pH and temperature

The effect of pH on the activity of Mannanase M8, xylanase X4, and cellulase C9 was carried out in the range 3-10, as shown in Figure 3. The crude mannanase M8 was active at a wide pH range from 5.0-10.0, xylanase X4 from 3.0 to 10.0, and cellulase C9 from 3.0-9.0. The highest mannanase, xylanase, and cellulase activity were observed at pH 6.0, 5.5, and 8.0, respectively (Figure 3.A, 3.C, 3.E). The increase or decrease of pH depends on enzyme activity. The effect of temperature was studied on each enzyme activity by varying the value from 30ºC to 100ºC. The effects of temperature on the activity of Mannanase M8 were shown in Figure 5.B with the optimum temperature at 70ºC and high enzyme activity of 9.5 U/mL. The report of xylanase X4 was shown in Figure 5.D, in which the optimum temperature is 70ºC, with the activity of 2.2 U/mL. The report of cellulase C9 is shown in Figure 5.F, which optimum temperature is 50ºC with an activity of 0.26 U/mL. The enzyme activity increased when the temperature increased from 40ºC, but the activity started to decline as the temperature increased above 80ºC and became completely denatured at 100ºC. Similar findings were also reported by some species, i.e., *Bacillus* sp and *B.subtilis* YJ1, which have an optimum temperature of 50ºC (Kim et al. 2011).

Table 1. The phylogenetic analysis showed about 99% similarity between the isolated M8 and *Bacillus subtilis,* isolate X4 and *Bacillus tequilensis,* and isolate C9 and *Bacillus cereus*

Code of strain	Isolated from	Identified as*
M8	Sea water	*Bacillus subtilis*
X4	Sea water	*Bacillus tequilensis*
C9	Sea water	*Bacillus cereus*

Note: *by 16S rRNA gene sequencing

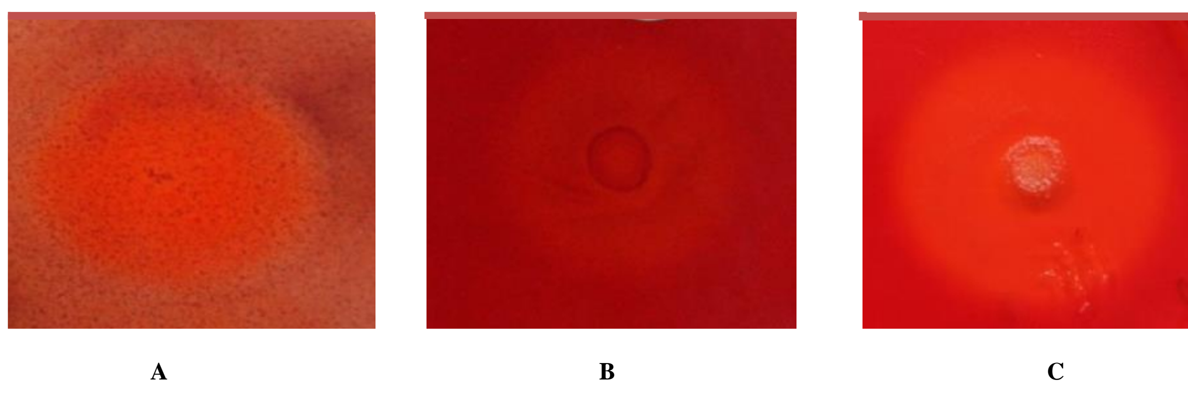

Figure 1. Congo red analysis from (A) mannolytic M8, (B) cellulolytic C9, and (C) xylanolytic X4 in a medium that uses a specific substrate (LBG, xylan, and CMC) for 24 hours of incubation

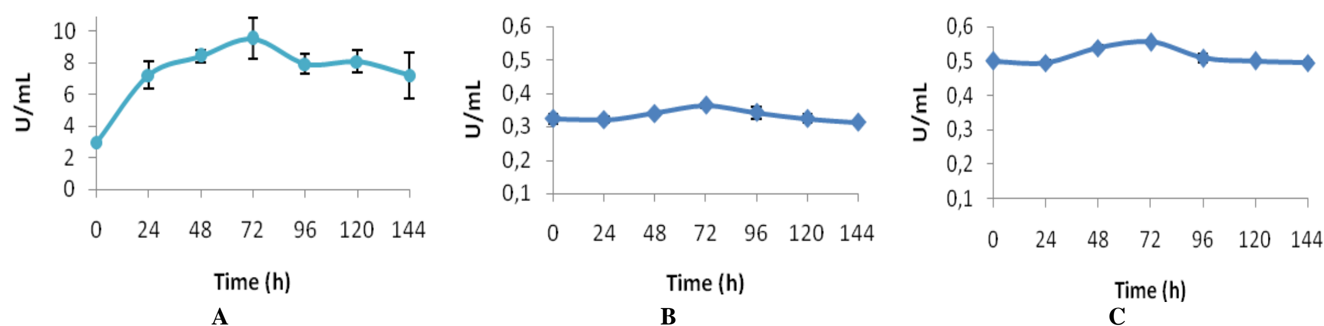

Figure 2. Crude enzyme production from potential marine bacteria with 24 hours retention times for initial optimation, such as mannanase M8 (A), xylanase X4 (B), and cellulase C9 (C).

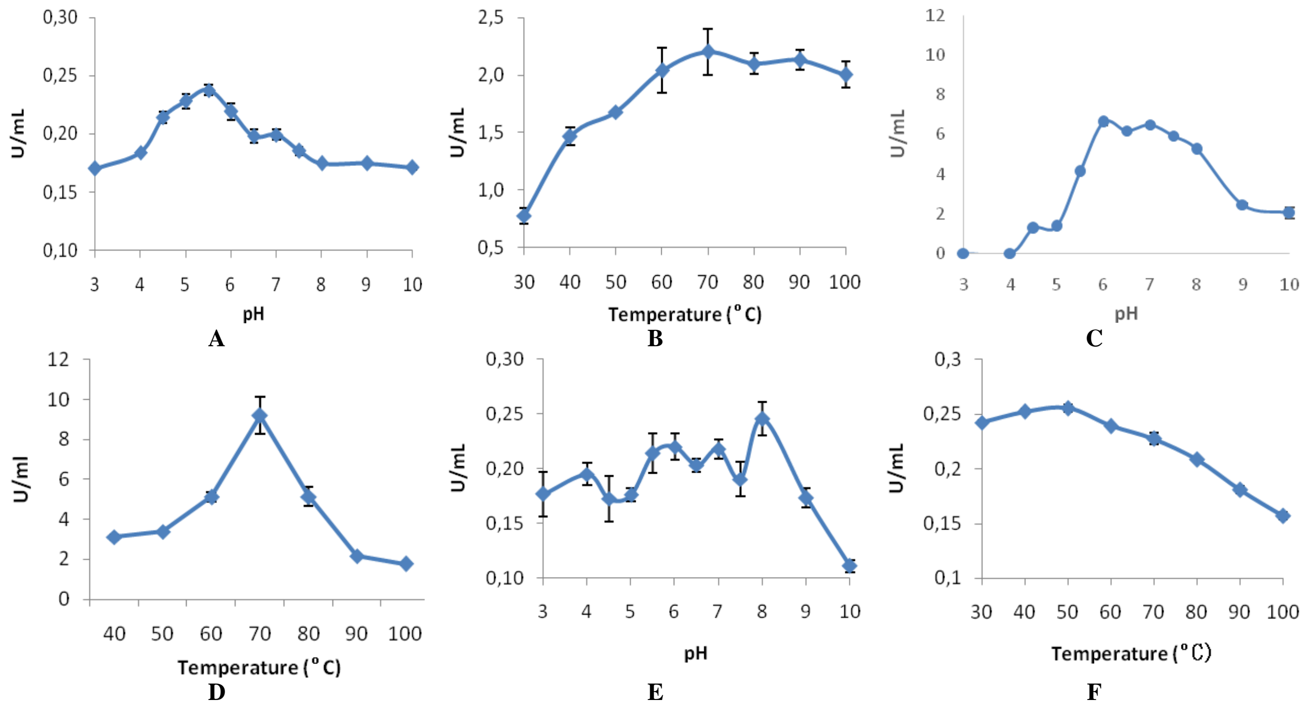

Figure 3. Characterization of pH and temperature xylanase from isolate X4 (A, B), mannanase from isolate M8 (C, D), and cellulase from isolate C9 (E, F)

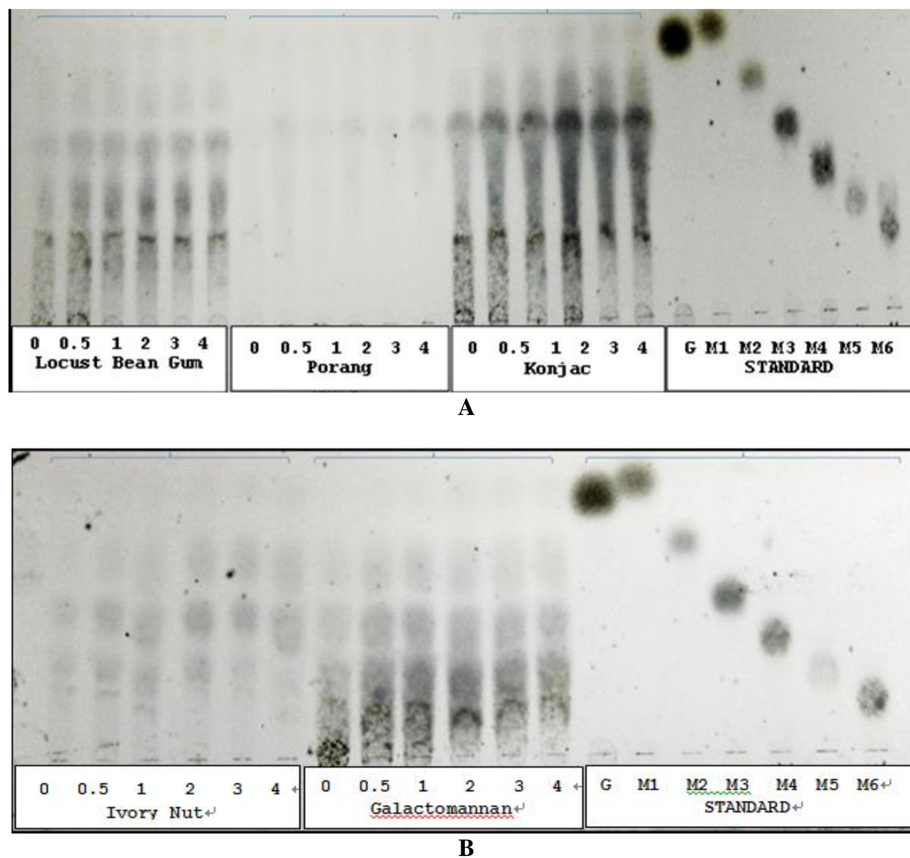

Figure 4. Thin-layer chromatography analysis of hydrolysis products mannanase M8 using various mannan as substrates, namely, LBG, porang, konjac glucomannan (A) and galactomannan, and ivory nut (B). Standards (STD): glucose (G), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5), and mannohexaose (M6). Substrate: enzyme (v/v) = 1: 10 and 1: 5, Substrate concentration 0.5% in 50 mM sodium phosphate buffer (pH 6.0), reaction time (hours): 0, 0.5, 1, 2, 3 and 4 at 30°C condition.

Product analysis by thin-layer chromatography

The hydrolysis of mannan sources such as locust bean gum, porang, konjac, ivory nut, and galactomannan by marine bacteria M8 was shown in Figures 4.A and 4.B. The product was analyzed using the TLC method and resulted in several types of oligosaccharides, such as mannose, mannobiose, mannotriose, and mannotetraose, as the main product.

The oligosaccharide production from porang substrate showed the thin spot result because porang was a raw material that was more difficult to hydrolyze than a commercial substrate that was already pure. The experiment showed that the hydrolysis process by M8 continuously produced various oligosaccharide products in different reaction times and continued to increase until it completely degraded in short times.

The hydrolysis of cellulose sources such as bagasse, porang, and konjac glucomannan by Cellulase C9 is shown in Figure 5. The product was analyzed using the TLC method and resulted in a thin chromatogram or almost spotless. It is because baggase and porang were still raw materials. On konjac glucomannan, it resulted from thin spots of cellulose compound.

The hydrolysis of xylan sources such as xylan birchwood, EFB, and baggase by xylanase X4 is shown in Figure 6. The products were identified as xylotetraose, xylopetaose, and xylohexaose, which were the main products of the hydrolysis process.

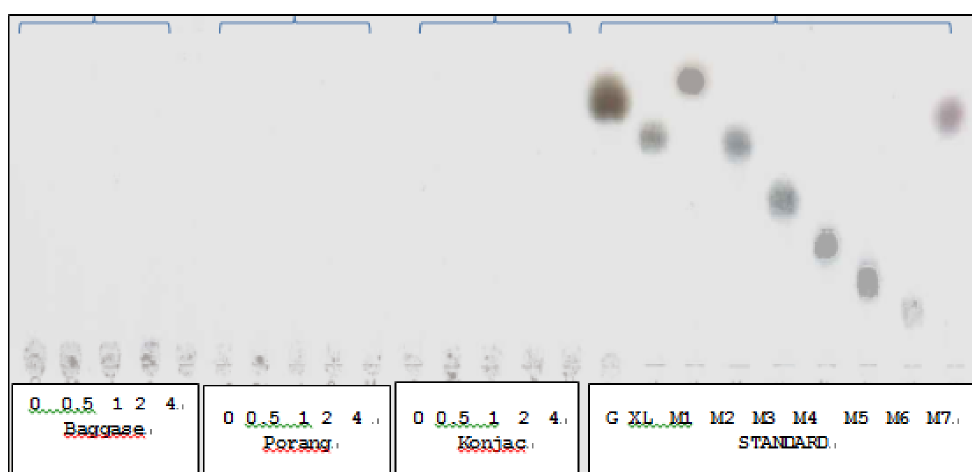

Figure 5. Thin layer chromatography analysis of hydrolysis products of cellulase C9 using Carboxymetylcellulose, Baggase, EFB as a substrates. Standards (STD): glucose (G), xylose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5) and mannohexaose (M6). Substrate: enzyme (v/v) = 1: 10 and 1: 5, Substrate concentration 0.5% in 50 mM sodium phosphate buffer (pH 6.0), reaction time (h): 0, 0.5, 1, 2, 3 and 4 at 30ºC condition

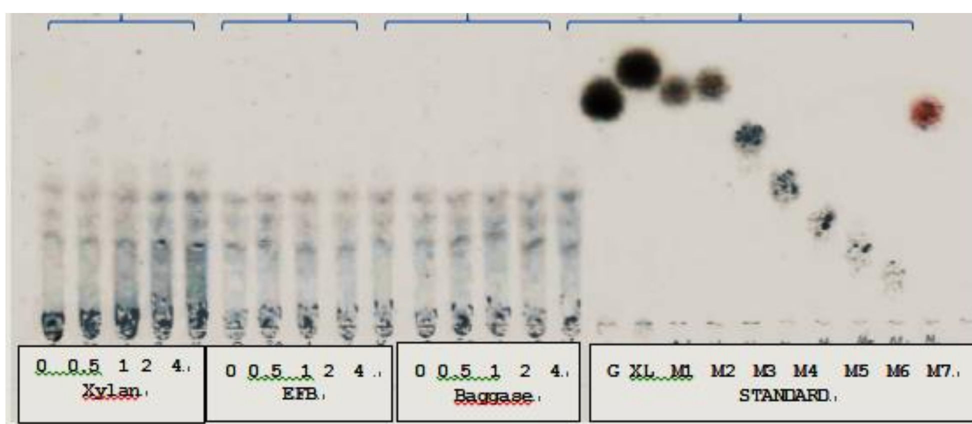

Figure 6. Thin layer chromatography analysis of hydrolysis products of xylanase X4 using xylan, EFB, Bagasse as a substrates. Standards (STD): glucose (G), xylose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5) and mannohexaose (M6). Substrate: enzyme (v/v) = 1: 10 and 1: 5, Substrate concentration 0.5% in 50 mM sodium phosphate buffer (pH 6.0), reaction time (hours): 0, 0.50, 1, 2, 3 and 4 at 30ºC

Discussion

The screening method uses congo red staining application by adding various substrates, i.e., CMC for cellulose, locust bean galactomannan substrate medium for mannanase, and xylan for xylanase, into ASW medium abilities to excrete enzyme in substrates are shown. The clear zone area that appears at the medium surface shows the positive activity of converting substrate (polymer) into specific derivate sugar by an enzyme from potential isolate for each substrate. The oligosaccharide production from locust bean gum as mannan source results in mannose, mannobiose, mannotriose, and mannotetraose. It means that mannanase from Bacillus subtilis has two enzyme types, exoenzyme, and endoenzyme, for polymer hydrolysis. Hydrolysis of xylan sources such as xylan birchwood, EFB, and baggase by Bacillus *tequilensis* results in xylotetraose, xylopetaose, and xylohexaose as the main product of the hydrolysis process, and it means Bacillus *tequilensis* bacteria belongs to exoenzyme type. Cellulase production from Bacillus cereus shows weak activity in raw biomasses.

In conclusion, the isolation of xylanolytic, mannolytic, and cellulolytic marine bacteria has been conducted from 20 isolates. It has been able to identify three potential isolates based on 16S rDNA, and this process resulted in M8 (*Bacillus subtilis*), X4 (*Bacillus tequilensis*), and C9 (*Bacillus cereus*). Based on the TLC analysis, Mannanase M8 and Xylanase X4 had the potential to produce oligosaccharides with the size around tri-hexasaccharide as the main product.

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The analysis of nutrient and fiber content of banana (*Musa paradisiaca*) sold in Pontianak, Indonesia

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Abstract. *Hasanah R, Daningsih E, Titin. 2017. The analysis of nutrient and fiber content of banana (*Musa paradisiaca*) sold in Pontianak, Indonesia. Biofarmasi J Nat Prod Biochem 15: 21-25.* This study aimed to determine the effect of varieties of bananas and marketplaces on the nutrients and fiber of bananas sold in Pontianak. Completely Randomized Design (CRD) Factorial model with main factors of varieties of banana (barangan, masak hijau, singapura), market places, namely traditional market, fruit stores, and side road kiosk, and the combination of varieties and market places of banana. The variable tests were carbohydrate, glucose, fructose, sucrose, protein, lipid, vitamin C, crude fiber, water, and ash content. The result was processed with SAS application 6.12 version using ANOVA CRD Factorial and significances followed by LSD α=0.05. Results found that banana varieties significantly affected total carbohydrate, glucose, fructose, sucrose, vitamin C, lipid, and water but did not significantly affect crude fiber and ash. The marketplaces did not significantly affect total carbohydrate, glucose, fructose, sucrose, protein, vitamin C, crude fiber, and water but significantly affected ash content. The combination between varieties and marketplaces significantly affected specific nutrient content. Barangan was good on total carbohydrates, vitamin C, and ash, while masak hijau was highest on glucose, fructose, sucrose, and crude fiber. In addition, singapura was highest in protein, lipid, and water.

Keywords: *Musa paradisiaca,* nutrient content, Pontianak

INTRODUCTION

One of the local berries which are spreading a lot in Pontianak, West Kalimantan, is banana. Banana is easily obtainable and scattered in various places of sale fruit from traditional markets and fruit shops to the side road kiosk. Based on an observation, the species and varieties which were sold in Pontianak are barangan, masak hijau, kepok, ambon kuning, mas, raja, tanduk and nipah. As a local fruit sold at lower and reachable prices, bananas have various health benefits, such as helping to expedite our digestive system. Bananas also contain starch as the main source of energy-producing carbohydrates, vitamins, and minerals. A sweet taste fruit is also high in minerals such as potassium, magnesium, phosphorus, iron, and calcium. Bananas also contain vitamins, namely C, B complex, B6, and active serotonin as a neurotransmitter in the smooth functioning of the brain (Triyono 2010).

Local fruits such as bananas (personal communication) are distributed to various marketplaces in fresher conditions and require less time than imported fruits that need more time for distribution. Local bananas should be fresher and nutritionally better because they are taken directly from the harvesting place. It affects the freshness of the fruit and metabolic transportation processes that can affect the nutritional content of the fruit. However, nutritional information about bananas traded in Pontianak is still unknown. Bananas scatter in various places of sale fruit with different distribution conditions, which also cause the difference in the nutrient content of each fruit. Traditional markets, fruit shops, and roadside kiosks have different circumstances. The traditional market and the side road kiosk have the more open condition than fruit stores. The traditional market and roadside kiosks' temperature is higher than in the fruit store. Another thing that also affects the nutrient content of the fruit is the storage area. A constant low temperatures (cold) storage can extend the physical quality (color and appearance/freshness, texture, and flavor) and nutritional value, especially vitamin C of imported fruit. A room temperature storage will lead to a quicker decrease in physical quality-organoleptic quality, and nutritional value, followed by the decay process (Tawali et al. 2004).

In fact, the consumption of fruits in Pontianak city is not as much as in other cities. BPS Pontianak (2014) data showed that fruit consumption was as much as 1.09% in Pontianak city in 2013. Lack of fruit consumption can be influenced by the level of the average income of the community (BPS Kalbar 2014). Based on the level of education, 27.44% of the people of Pontianak had elementary education, 4.90% of them had junior high school education, and 24.64% of them had senior high school education. In comparison, 11.69% of them had an SMK education, and 21.33% of these people had a university education. The profession level indicates the level of welfare. Rahardjo (2012) revealed that high education would be able to sustain a more feasible life. Although bananas can be purchased at affordable prices, climacteric they are perishable and easy to be rotten. The durability of the fruit is also a consideration for people to

buy a banana. This study aimed to know the nutritional content of bananas sold in Pontianak City, West Kalimantan, Indonesia.

MATERIALS AND METHODS

Materials

The materials used in this study were 168 grams of various flesh of bananas (masak hijau, barangan, and Singapura) from various marketplaces in Pontianak City, West Kalimantan, Indonesia.

Research method

The research was conducted from March to April 2016 in the Laboratory of Biological Education, Faculty of Teacher Training and Education (FKIP) and Laboratory of Technology of Agriculture Product, Faculty of Agriculture, Tanjungpura University (Untan), Pontianak, Indonesia, using a completely randomized design (CRD) factorial. Three varieties and three marketplaces were used as main variables. Fruit observation was done by observing the morphology and anatomy of bananas which display the outer (physical) texture in the form of fruit rind; fruit skin color; freshness; and the shape of the fruit, while appearances observation was conducted with consideration of fruit ripeness including aroma (smell), taste and texture of the fruit flesh. The study began with preparing materials; preparing 168 grams of bananas meat which were used in the observation such as total carbohydrates observation by direct calculation (Plummer 1971), namely, by calculating the number of total carbohydrates by reduction of 100% - grams of fat, protein, water, and ash. Benedict's quantitative test included glucose, fructose, and sucrose tests (Sudarmadji et al. 1997). The protein test was done using the spectrophotometric method. A fat test was done using soxhlet (SNI 2006a). The Vitamin C test was done using the iodine titration method; the level of Vitamin C was calculated by the total numbers of Iodine in ML 0:01 N) x (0.88 mg ascorbic acid) (Sudarmadji et al. 1997). The water content test was done using the gravimetric method (SNI 2006b). The crude fiber test used the gravimetric method (Sudarmadji et al. 1997). The Ash test used the done principle of direct incineration of ash content (Sudarmadji et al. 1997).

Data analysis

Data were analyzed using SAS version 6.12 applications in 1996 with RAL Factorial models using ANOVA. If the results showed a significant effect, it was continued using *Least Square Different* (LSD) at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Result

Masak hijau bananas were the sweetest when they were compared to barangan and singapura. It also has a little bit of softer banana flesh. Nonetheless, all fruit had good aromatic (Table 1). All of the fruits were unbruished.

Morphology and appearances of banana varieties sold in Pontianak were shown in Table 1, while nutrients and fiber contents were shown in Table 2.

Discussion

Physical characteristics

The observation of characteristics of bananas (Table 1) consisted of observations of skin texture, color, freshness, shape, taste, aroma, and texture of the fruit flesh adjusted to the standard of consumable banana by Kerbel (2003). Masak Hijau has a softer flesh texture than those Barangan and Singapura. This statement refers to Jafarizadeh-Malmiri et al. (2011) that banana fruit is climacteric and ripens rapidly after harvest. As reported by Marriott (1980), the ripening of banana is marked by the peel color change from green to yellow, the hydrolysis of starch to simple sugars, the softening of the pulp, and the development of aroma. It could also indicate a different maturity (Kerbel 2003).

Table 1. Morphology and appearances of banana varieties which were sold in Pontianak City, West Kalimantan, Indonesia

Fruit varieties	Morphology	Appearances
Barangan (*Musa paradisiac* var. barangan)	The peel texture is smooth, with black spots at some parts; the peel color is bright yellow, and fresh; the shape of the fruit is not bruised and rotten.	Sweet taste, aromatic, soft.
Masak Hijau (*Musa paradisiaca* var.sapientum)	The peel's texture is smooth, does not have a scratch, there are black spots at some parts, the peel is dark green, fruit does not wither, with a proportional shape.	Very sweet taste, aromatic, and a little bit softer.
Singapura (*Musa paradisiaca* var.singapura)	The peel's texture is smooth, the fruit peel is bright yellow, fruit does not wither; fresh, solid, and in proportional shape.	Sweet taste, aromatic, soft.

Table 2. Results of ANOVA analysis of the nutrients and fiber per 100 grams of barangan, masak hijau and singapura which were sold in Pontianak City, West Kalimantan, Indonesia

Variables	Total carbo-hydrates	Glucose	Fructose	Sucrose	Fat	Protein	Water	Crude fiber	Vit. C	Ash
Unit	**g**	**mg**	**mg**	**mg**	**g**	**g**	**g**	**g**	**mg**	**g**
Varieties										
Barangan	26.68 a ***	4639.5 b ***	4886.9 c ***	4546.7 b ***	0.33 ns	0.96 b **	70.98 c ***	1.28 ns	8.71 a**	1.05 ns
Masak hijau	23.6 b	5219.7 a	5532.9 a	4801.9 b	0.33	0.90 b	74.39 b	1.51	8.64 a	0.75
Singapura	20.26 c	4899.9 b	5194.0 b	5115.3 a	0.34	1:03 a	77.54 a	1.45	6:26 b	0.82
Market places										
Traditional markets	24.17 ns	4830.0 ns	5088.8 ns	4733.4 ns	0:33 ns	0.96 ns	74.46 ns	1.61 a **	8.63 ns	0.85 ns
Fruit stores	23.40	4988.9	5288.2	4889.1	0:33	0.96	73.63	1.27 b	7.54	0.90
On the side road	23.00	4940.3	5236.8	4841.5	0:34	0.96	74.83	1.36 ab	7.43	0.87
Combination										
Barangan and traditional markets	25.16 ns	4513.35 bc *	4691.13 b **	4423.08 bc *	0.35 ns	1.08 a **	72.27 ns	1.09 c *	9.97 ns	1.13 ns
Barangan and fruit shop	27.87	4445.91 c	4712.66 b	3182.91 c	0.30	0.93 b	69.9	1.13 bc	8.53	1.03
Barangan and on the side road	27.01	4959.35 ab	5256.91 a	4860.16 abc	0.35	0.85 b	70.80	1.62 abc	7.63	0.98
Masak hijau and traditional markets	24.22	5130.46 a	5438.28 a	5027.84 a	0.32	0.91 b	73.98	2.03 a	8.31	0.58
Masak hijau and fruit stores	23.83	5218.18 a	5531.27 a	5113.81 a	0.35	0.88 b	74.09	1.35 bc	8.80	0.85
Masak hijau and on the side road	22.84	5310.58 a	5629.21 a	5204.36 a	0.33	0.90 b	75.10	1.15 bc	8.80	0.81
Pisang singapura and traditional markets	20.82	4846.12 abc	5137.03 ab	4749.33 abc	0.35	0.90 b	77.12	1.68 ab	7.63	0.83
Pisang singapura and fruit shop	20.81	5302.47 a	5620.62 a	5196.42 a	0.33	1.08 a	76.94	1.36 bc	5.28	0.82
Pisang singapoura and on the side road	19.15	4551 bc	4824.33 b	4460.00 bc	0.33	1.11 a	78.60	1.30 bc	5.87	0.82

Note: ns = *non significant* * = *significant* at α = 0.05 ** = highly significant at α = 0.01 *** = very highly significant at α = 0.001. The letters on the back of the mean value which are not the same showed significant difference when tested by LSD α = 0.05.

Total carbohydrate content in bananas

There were differences in the total carbohydrate content of banana varieties, but there was no significant difference in the marketplaces and a combination of both (Table 2). The highest total carbohydrates are contained in barangan (26.68 g) and the lowest in singapura (20.26 g). Based on USDA (2016) standard, the standard of total carbohydrates in 100 grams of material is 22.84 grams. In combination, the highest total carbohydrate content was barangan sold in the fruit shop, and the lowest was singapura sold on the roadside kiosk. Still, statistically, it made no significant difference. The ripening level of bananas influences the nutritional content of bananas. Carbohydrates, glucose, fructose, and sucrose are directly proportional to the level of maturity of the fruit. Fitriningrum et al. (2013) stated that during fruit ripening, there are changes in various aspects, such as structure, texture, color, flavor, and biochemical processes. In the morphological observation of fruit (Table 1), masak hijau has a softer texture than the other bananas. It could indicate a faster ripening process than the other bananas. The age of bananas was unknown. Otherwise, the fruit's mature level and storage time affected the nutrient and fiber content. Fruit storage in a close and covered place can cause a decrease in O_2 and an increase in CO_2 and this situation can delay the time of ripening process. Delayed ripening process can reduce respiration and ethylene production. Storage of fruit in an open and uncovered place can increase O_2 and decrease CO_2 that will accelerate respiration product and increase ethylene production (Praeger et al. 2013).

Glucose, fructose and sucrose contents in bananas

There were differences in content of glucose, fructose and sucrose of bananas on fruit varieties and combinations, but there was no significant difference in bananas sold in the place of sales (Table 2). The highest glucose content was in masak hijau (5219.7 mg) and lowest was in the barangan banana (4639.5 mg). The highest glucose was masak hijau sold in the side road kiosk, and the lowest was barangan sold in the fruit shop. The highest fructose content was in masak hijau (5532.9 mg) and the lowest was in the barangan (4886.9 mg. The highest sucrose content was singapura banana (5115.3 mg) and the lowest was in barangan (4546.7 mg). For the combination of varieties and those sold in market place, there were significant differences. The content of sucrose of masak hijau banana was highest regardless to the place of sale followed by singapura sold in fruit store. Levels of glucose, fructose and sucrose in fruit become higher when the fruit has ripened. Adao and Gloria (2005) said that fructose was

formed at a faster rate which is consistent with the higher fructose levels accumulated in the ripened fruit. The fruit will be sweeter after the organic acid or starch is converted into sugar molecules that can reach a concentration of 20% in ripen fruit (Campbell et al. 1999). During the process of fruit ripening, via the enzymatic reaction, the starch will be broken down into simple sugars such as glucose, fructose and sucrose so that the fruit will become sweet (Fitriningrum et al. 2013). High sugar level of banana was characterized by observations when it was being sampled (Table 1), and was associated with post-harvest of fruit climacteric period. This is supported by Adeniji and Barimalaya's (2008) statement that after it is ripen, the skin turned from bright green to yellow because the chlorophyll of fruit was destroyed, and the color change occurred. The flesh became softer and sweeter due to the breakdown of sugar and the increase in its concentration.

The fat content in bananas

There are no differences in the fat content on banana varieties, bananas sold in market places and both combination (Table 2). Banana which has the highest fat content was singapura, namely, 0.34 grams which is not significantly different from masak hijau and barangan. For the market places, the highest fat content was on barangan sold in the side road kiosk, namely, 0.34 grams, followed by barangan sold in fruit shop and traditional markets which was 0.33 gram, According to USDA (2016), the three bananas have similar fat content with bananas in common.

The protein content of bananas

There were significant differences on the protein content of fruit varieties and on the combination of varieties and the ones sold in market places, but there was no significant difference on the market places. Singapura has a higher protein content (1.03 g) than masak hijau (0.96 g) and barangan (0.90 g). Climateric fruit such as bananas have high protein content when they are mature. Morphology on the skin colour has been observed (Table 1). According to Fabi et al. (2010), the increase of ethylene was characterized by discoloration of the fruit skin. Dominguez-Puigjaner et al. (1992) also states that the banana is climacteric fruits such as pears, apples, peaches, tomatoes and avocado which will have high levels of synthetic ethylene when they are mature. It is believed that ethylene regulates the expression of genes involved in the maturation, due to increased levels of protein in the banana pulp tissue which is in line with the maturity of the fruit. The high ethylene is indicated by the fragrant aroma of bananas.

The water content of bananas

There were significant differences of water content in the varieties of bananas but there was no difference in the market place and the combination (Table 2). The highest water content was 77.54 grams in Singapura and the lowest was in barangan (70.98 g). Siahaan et al. (2012) reported the water content was in line directly with the level of maturity of the fruit. The more mature the fruit, the higher the water content. the highest water content was in masak hijau, but the lowest water content was in Singapura bananas.

Crude fiber content of bananas

There were no significant differences of crude fiber content amongst the banana varieties, but there were highly significant difference in banana sold in the market place and significant difference in the combination of both (Table 2). The content of crude fiber is inversely proportional to the content of carbohydrates and sugar. Changes in crude fiber content during the maturation process occur because of polysaccharide degradation (Fitriningrum et al. 2013).

The vitamin C content of bananas

There was a significant difference on the vitamin C content of the fruit varieties, but not on the fruit sold in the market place and the combination (Table 2). The highest vitamin C was in Barangan (8.71 mg), which similar with that in masak hijau banana (8.64 mg) however vitamin C content of singanpore was different significantly (6.26 mg). The content of vitamin C in bananas decreases when ripe fruit was becoming ripen (Wall et al. 2006).

The ash content of bananas

There were no differences of ash content in the varieties, in bananas sold in market place, and the combination (Table 2). The ash content of fruit is inversely proportional to the level of maturity of the fruit. The more mature the fruit is, the lower the ash content is. Susanto (2009) stated that the higher the water content, organic matter content is getting higher; then ash content will be lower.

General discussion

Bananas are climacteric fruit influenced by the level of maturity, age, post-harvest, and the characteristics of the fruit in climacteric phases. According to Kader (1999) the maturity is the most important part in climacteric fruit. Maturity determines the length of life of the fruit until the post harvest period. Ripeness stage of fruits was characterized and classified by color, firmness and taste as unripe (physiological maturity stage), half-ripe and full-ripe (consumption stage) (Hernandez et al. 2006). Based on the results of analysis, it is showed that there were significant difference of fruit varieties to the carbohydrate, glucose, fructose, and sucrose, vitamin C, protein and water, but it did not affect the content of ash, crude fiber and fat. Overall for bananas sold in the market places, the nutrients in the fruit store was higher than in the traditional markets and side road kiosk. A test has been done to answer the question on the hypothesis, that there were significant differences on the content of crude fiber, but has no effect on carbohydrate, glucose, fructose, sucrose, vitamin C, fat, protein, water and ash, due to market place of bananas, so that there was the influence of a combination of banana varieties with the sale of bananas in the city of Pontianak.

Differences in the contents of the fruit on the sale were affected by storage temperature and air temperature.

According to Yulianti (2014) hot temperature can cause physical damage. According to the observation (Table 1), bananas were fresh. Therefore, the difference could come from other factor such as maturity at the time the bananas were sold. However, the sample age was not known. OECD (2010) said that good macronutrients such as potassium, nitrogen, phosphor, calcium, magnesium, and sulphur were needed in large amount for plant growth. Soil with lower pH and content of mangan and aluminium in it may be toxic and affect the plant growth, then it will decrease fruit quality. In addition, water in growing time causes fruit ripening.

Based on this research, we can choose a banana with the nutrients and fiber suited our needs respectively. Masak hijau were suitable to be processed for consumption or to be consumed directly, since they had high sugar content, and also fiber content that can help the digestive system. Banana fruit can be consumed as food reserves because they contain a high carbohydrate, and it also had high level of vitamin C and high content of ash which was better to be consumed directly without being processed first. Singapura has content of moisture, fat, and protein that can also be consumed directly without being processed.

We can buy Barangan, Masak Hijau and Singapura in three places, namely, traditional Market, fruit store and the road side kiosk because, generally, bananas in these three places did not have significant differences.

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Short Communication: Spermicidal properties of *Durio zibethinus* in the Mandiangin Forests, South Kalimantan, Indonesia

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Abstract. *Nurliani A, Kartinah N. 2017. Short Communication: Spermicidal properties of* Durio zibethinus *in the Mandiangin Forests, South Kalimantan, Indonesia. Biofarmasi J Nat Prod Biochem 15: 26-28.* Side effects caused by a condom with synthetic spermicide encourage researchers to find alternative spermicides from plants with fewer side effects. The bark of durian extract is a potential candidate for herbal spermicide because it could decrease the percentage of human spermatozoa quality in vitro at the concentrations of 2%. This study evaluated its spermicidal activity in vitro on human spermatozoa's motility, movement velocity, viability, and morphology. The gel formulation with 2% of the bark of durian extract was developed using hydroxypropyl methylcellulose as a gelling agent with 3 different concentrations, namely, 1.5, 2, and 2.5 %. Evaluation of gel preparations, including physical appearance, viscosity, spreadability, and pH, was done to obtain the best formula. Based on the evaluation, the best performance of gel was achieved by adding 2% of HPMC. Furthermore, the spermicidal activity of the gel with the bark of durian extract was tested and compared to the bark of durian extract without gel, gel without bark of durian extract, and the fresh sperm as control. Formulation of gel with the bark of durian extract significantly decreased all parameters of spermatozoa quality. Thus, a formula containing 2% of the bark of durian extract with HPMC 2% possesses appreciable spermicidal potential.

Keywords: Durian, gel, human sperm, Kalimantan, spermicide

INTRODUCTION

Since the 19th century, the condom has become one of the most popular contraceptive methods globally and used for at least 400 years. To increase its effectiveness, some condoms are lubricated with spermicide chemicals such as nonoxynol-9 (Kestelman and Trussell 1991). However, recent studies indicate that nonoxynol-9 may cause irritation and increase the risk of HIV (Asif 2013).

To resolve this problem, efforts to develop safe and effective contraceptives must be made. Herbal contraceptives are in popular demand because they have fewer side effects. The previous investigation revealed that the barks of durian extract have spermicidal activity on human spermatozoa in vitro. The extract from the bark of durian could decrease the percentage of quality of human spermatozoa in vitro at the concentrations of 2% (Nurliani and Santoso 2010).

Gels are often used pharmaceutically as lubricants and as carriers for spermicidal agents (Esposito et al. 1996). Therefore, this study was designed to formulate and evaluate spermicidal gel containing bark of durian extract for application in humans as an herbal spermicide in condoms.

MATERIALS AND METHODS

Plant materials

The bark of durian was collected from the forest region of Mandiangin, South Kalimantan, Indonesia.

Chemicals

Ethanol (Merck Ltd), Hydroxypropyl Methyl Cellulose (HPMC), propylene glycol, glycerin, methylparaben, propylparaben, Hank's Balanced Salt Solution (HBSS) Gibco®, eosin-Y, nigrosin, Giemsa, and methanol.

Sample

Semen samples were donated by 6 healthy fertile men (25-30 years old). Semen samples were collected by masturbation in sterile glass cups after at least 3 days of sexual abstinence.

Preparation of bark powder

The bark was dried under shade and then powdered coarsely with a mechanical grinder. The powder was passed through sieve No. 40 and stored in an airtight container for further use.

Preparation of extracts

Every 50 g of dried powder of bark was subjected to soxhlet apparatus. It was exhaustively extracted with 250

mL of ethanol solvent in a soxhlet apparatus. The temperature was maintained at (60-70ºC). The solvents were removed by distillation under reduced pressure, and the resulting semisolid mass was vacuum-dried using a rotary flash evaporator to obtain the extract.

Preparation of gel formulation

The spermicidal gel was formed from a mixture of HPMC, propylene glycol, glycerin, methylparaben, propylparaben, and 2 % of the bark of durian extract. There are three formulas with various concentration of HPMC, i.e., 1.5% (F1); 2% (F2); and 2.5% (F3). HPMC was dispersed in distilled water with continuous stirring. Distilled water was taken, and the required quantity of methylparaben and propylparaben was dissolved by heating in a water bath. The solution was cooled, added glycerin, and mixed with the first solution. Further required quantity of durian bark extract was mixed with the above mixture, and the volume was made up to 100 mL by adding the remaining distilled water. Finally, full ingredients were mixed properly into the HPMC gel with continuous stirring, and propylene glycol was added dropwise to the formulation for consistency (Sudipta et al., 2011).

Evaluation of gel formulation

Physical evaluation. The color and odor of the prepared gels were checked.

Measurement of pH. The pH of the gel was measured using a pH meter.

Spreadability. The spreadability of the gel formulations was determined by measuring the spreading diameter of 1 g of gel between two horizontal plates (20 cm x 20 cm) after one minute. The standard weight applied on the upper plate was 50 g.

Viscosity. The viscosity of the gel was measured using a Brookfield viscometer with the spindle. The reading was taken at 1000 rpm using spindle no. 4.

Sample preparation

Semen samples were donated by 6 healthy fertile men (25-30 years old). Semen samples were collected by masturbation in sterile glass cups after at least 3 days of sexual abstinence. Spermatozoa free of seminal plasma were obtained by centrifugation at 1,900 rpm for 20 minutes and adjusted in Hank's Balanced Salt Solution (HBSS) media. Then it was made into a pellet by microcentrifugation at 1000 rpm for 10 minutes (Purwaningsih 2000).

Experimental procedure

Semen samples were added to the gel of bark extract preparation and then assessed immediately for these parameters below.

Sperm motility. A drop of the evenly mixed sample was immediately placed on a clean and dry glass slide covered with a coverslip. This slide was then examined under the binocular microscope (Olympus, Japan) at magnifications of x10 and x40. At least five fields were rapidly examined, and 100 spermatozoa were counted (Hyacinth et al., 2012).

Movement velocity of sperm. The velocity of spermatozoa was measured by calculating the time (seconds) needed by motile spermatozoa to reach 1 box microhaemocytometer. The velocity of spermatozoa is defined with a micrometer per the second unit.

Sperm viability. One drop of above-treated sperm mixed with 2 drops of 1% EosinY. After 30 s, 3 drops of 10% Nigrosin solution were added. A drop of treated sperm-Eosin-Nigrosin mixture is placed on a clear microscope slide, allowed to dry, and observed under a microscope. Live spermatozoa had whiteheads, and dead spermatozoa had heads stained red or dark pink. A total of 100 spermatozoa were evaluated manually on each slide at right-field optics at 400 x magnification (Eliasson 1977).

Sperm morphology. The sperm morphology of treated sperm was studied under the microscope using the EosinY and Nigrosin staining methods described above. A drop of sperm-eosiny-nigrosin mixture treated with gel of bark extract was examined separately at 400X under a phase-contrast microscope to record any change in the morphology of the sperm (Jayendran et al. 1994).

Statistical analysis

Data were analyzed with the SPSS system and presented as mean±standard deviation (SD). Statistical significance was evaluated with a Nonparametric test (Kruskal-Wallis), and the difference was considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

The result of the physical evaluation of gel formulation can be seen in Table 1. The physical evaluation showed that all the formulations show similar organoleptic properties with brownish coloration due to the plant extract and a specific smell. The pH of the gel formulations was in the range of 4.61-4.96, which lies in the normal pH range of the skin and would not produce any skin irritation. The ideal pH value for the gel is 4.5-7 (Wasiaatmadja 1997). The spreadability of gel was evaluated to test the ease of applicability of gels on skin. The spreadability of formulated gels was decreased as the concentration of polymer increased. Spreadabilities of formulated gels (F1, F2, and F3) were 5.26, 5.00, and 7.93 cm, respectively. The ideal value of spreadability for the gel is 5-7 cm (Garg et al. 2002). Hence, the spreadability of the F2 and F3 formulation was better than F1formulation. Viscosity is an important parameter for characterizing the gels, affecting the spreadability. The viscosity of gels increased with the increase in polymer content, which may be due to the increase in the formation of the three-dimensional cross-linking gel structure, as expected. The ideal viscosity value for the gel is 2,000-50,000 Cps (SNI 1996). By comparing the viscosity values, the F3 formulation has an over the viscosity value. The viscosity of the F1 and F2 formulations were more ideal than the F3 formulation. According to the evaluation result, the F2 formulation with a 2% proportion of HPMC resulted in the best gel formulation.

Tabel 1. Evaluation of gel formulation

Formulations	Color and odor	pH	Spreadability (cm)	Viscosity (Cps)
F1	Brownish and specific smell	4.95	5.26	15000
F2	Brownish and specific smell	4.63	5.00	30000
F3	Brownish and specific smell	4.67	7.93	82000

Table 2. Quality of human sperm following incubation with gel of durian bark extract

Treatment	Motility (%)	Movement Velocity (µm/s)	Viability (%)	Normal Morphology (%)
Control (Fresh sperm + HBSS)	80.00 ± 8.944a	0.64 ± 0.476^a	79.67 ± 4.179a	84.17 ± 6.242^a
Gel without extract	5.50 ± 3.619b	0.54 ± 0.406^a	73.67 ± 8.756a	89.83 ± 2.787^a
Bark of durian extract at the concentration of 2%	3.00 ± 1.4142b	0.12 ± 1.469^b	4.33 ± 4.033b	23.17 ± 6.274^b
Gel containing bark of durian extract	0.00 ± 0.000c	0.00 ± 0.000^c	0.00 ± 0.000c	20.17 ± 7.413^b

The assessment of spermicidal activity from the gel of bark durian extract can be seen in Table 2. The present study evaluated the spermicidal properties of the gel with the bark of durian extract. It revealed a reduction ($P < 0.05$) in the percentage of motility, viability, normal morphology, and movement velocity of human spermatozoa. The results indicate that formulation of gel with the bark of durian extract decreased the percentage of motility, movement velocity, and viability of human spermatozoa significantly up to 0% and normal morphology of spermatozoa up to 20.17%. The percentage of motility, movement of velocity, viability, and normal morphology of human spermatozoa in gel preparation with the bark of durian extract have significant differences with the bark of durian extract without gel, gel without extract, and with control (Table 2). Gel with the bark of durian extract has better spermicidal activity than the bark of durian extract without gel.

Phytochemistry screening of the extract of durian barks revealed the presence of alkaloids, flavonoids, tannins, steroids, triterpenoids, and saponins. A large number of plants for spermicidal have been screened, and a majority of plant-derived spermicides reported are triterpene saponins of several structural types, flavonoids, and phenol compounds (Farnsworth and Waller 1982). The saponins of several plants have been reported to produce instant immobilization of human spermatozoa within 20 seconds (Primorac et al. 1985). Most plant-derived spermicides, which caused inhibition of the sperm-specific enzymes acrosin and hyaluronidase, were confirmed to contain flavonoids. It is believed that flavonoids and their derivatives, flavanones, and flavonols, contain hyaluronidase inhibitory activity (Farnsworth and Waller 1982). The results indicate that the gel with the bark of durian extract possesses strong spermicidal activity in vitro. However, the mechanism of action from active components of the bark of durian extract as spermicidal agents should be further evaluated.

Based on the spermicidal activity result, the formulation of gel containing a 2% bark of durian extract possesses appreciable spermicidal potential, which may be explored as an effective constituent of male contraceptives.

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Antimicrobial activity, toxicity and phytochemical screening of selected medicinal plants of Losho, Narok County, Kenya

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Abstract. *Chalo DM, Lukhoba C, Fidahussein DS, Nguta JM. 2017. Antimicrobial activity, toxicity and phytochemical screening of selected medicinal plants of Losho, Narok County, Kenya. Biofarmasi J Nat Prod Biochem 15: 29-43.* In Kenya, microbial infections are a major cause of morbidity. The effectiveness of antibiotics is threatened by the increase of resistance of pathogenic microbes against most available drugs because new pathogens continue to emerge. Nowadays, herbal remedies offer hope since they are readily available and cheap. The aim of this research was to investigate the activity of antimicrobial, the lethality of brine shrimp, and the phytochemical composition of crude extracts of four selected plants, namely *Schrebera alata* (Oleaceae), *Ormocarpum kirkii* (Papilionoideae), *Helichrysum forskahlii* (Asteraceae) and *Cussonia holstii* (Araliaceae) that herbalists medicinally use from Losho, Narok County Kenya for treatment of ear, nose and throat infections, gastrointestinal disorders and skin diseases. Using agar, a qualitative antimicrobial susceptibility test against five microorganisms, methicillin-resistant Staphylococcus aureus, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans was investigated diffusion methods to produce inhibition zones, and the data accrued were analyzed using Analysis of variance. Minimum inhibitory concentrations were determined by the broth microdilution method. The toxicity of the extracts was analyzed using a brine shrimp lethality assay. The median fatal concentration of fifty was determined by data analysis using Finney�"s computer program. Phytochemical screening for flavonoids, sterols, alkaloids, tannins, quinones, terpenoids, and saponins was determined using standard procedures. The observation showed that the organic crude extracts of *H. forskahlii* had the highest inhibition zone against methicillin-resistant *S. aureus* of 19.5 and 18.5 mm in agar well and agar disk diffusion, respectively. In addition, organic extracts of *H. forskahlii* showed the highest antifungal inhibition zone of 8.5 mm in agar well diffusion. Minimum values of inhibitory concentrations varied from 15.625 to 250 mg/mL. Organic crude extracts of *H. forskahlii* and *C. holstii* were highly toxic, with a lethal concentration of 0.009 mg/mL. All plant crude extracts contained flavonoids, sterols, alkaloids, tannins, quinones, and terpenoids. Saponins were present in all the plant extracts except in the organic extracts of *H. forskahlii*. This study promoted the first record of antimicrobial activity, toxicity, and phytochemical composition of *S. alata* and *C. holstii*. The study has shown that *H. forskahlii* and *O. kirkii* possess promising antimicrobial activity against microbes of health importance and could isolate new, safe, and efficacious antimicrobial compounds. Further research should be carried out on *O. kirkii* and *S. alata* to isolate and characterize the compounds responsible for the observed activity.

Keywords: Antimicrobial activity, Brine shrimp lethality assay, Kenya, Losho, Medicinal plants, Narok, phytochemical composition

INTRODUCTION

Microbial infections remain a threat to millions of lives globally (Kalita et al., 2012). Nowadays, there is an increasing problem of antibiotic resistance due to microbial persistence (Kitonde et al., 2013). The rapid rise in microbial resistance to synthetic drugs has urged the formulation of new antimicrobial agents and the evaluation of the efficacy of natural plant products as a substitute for chemical antimicrobial agents (Pandian et al. 2006). Traditional medicine is either the mainstay of health care delivery or serves to complement it worldwide (WHO 2008). The World Health Organization in 2008 estimated that up to 80% of the population in some developing countries use traditional medicine. Traditional medicine has been recognized as a part of primary health care programs in many African countries. In Kenya, rich pharmacopeia systems have been documented for communities like Maasai, Gusii, Luo, Abaluhyia, and Kikuyu (Kokwaro 2009).

According to WHO (2002) and Pandey et al. (2011), a medicinal plant contains substances that can be used for therapeutic purposes or precursors of chemo-pharmaceuticals semi-synthetic new drugs. Phytochemicals offer a unique platform for structural diversity and biological functionality, which is indispensable for drug discovery. Plants have an almost limitless ability to synthesize secondary metabolites, which may have a defensive role against herbivores, pathogen attacks, and interplay competition. Moreover, these metabolites act as an attractant for pollinators or symbionts. Many naturally occurring compounds in plants possess antimicrobial

functions and serve as antimicrobial agents (Kalita et al., 2012).

Like in other African countries, in Kenya, traditional medicine is practiced to treat sexually transmitted diseases, eye infections, skin-related problems, wounds, gastrointestinal diseases, measles, and snake bites (Njoroge and Bussmann 2007; Kokwaro 2009; Odhiambo et al. 2010). Microbial infections such as tuberculosis, candidiasis, cryptococcosis, and salmonellosis have increased in the recent past, partially due to HIV/AIDS pandemic (Mwitari et al., 2013). At the same time, antibiotics are becoming less and less efficacious against microbial illnesses due to the emergence of drug-resistant bacteria. Natural products of higher plants may give new sources of antimicrobial agents with possibly novel mechanisms of action (Bhalodia and Shukla 2011). According to Kokwaro (2009), overdosed patients, due to the imprecise diagnosis, are worldwide. Toxic effects have been attributed to certain active principles found in plants (Nguta et al., 2011).

The Maasai of Losho, who are mainly pastoralists, not only depends on plants for food, fuel, and wood but also for medicine in rituals and ceremonies (Karehed and Odhult 1997). However, many of these plants have not been investigated for phytochemical composition, antimicrobial, and toxicity activities. Therefore, this study aimed to evaluate the antimicrobial activity, brine shrimp lethality, and phytochemical composition of crude extracts from *Schrebera alata*, *Ormocarpum kirkii*, *Helichrysum forskahlii,* and the the *Cussonia holstii.* These plants are majorly used as traditional medicine for the currency of skin diseases, gastrointestinal tract diseases, and respiratory problems.

Antibiotic resistance has become a global concern due to an increased incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to the indiscriminate use of commercial antimicrobial drugs commonly employed in treating infectious diseases (Adenisa et al. 2000). Drugs from plant sources are keyway to addressing the problem. Also, synthetic antibiotics are not only expensive; but also have side effects in the treatment of infectious diseases (Kone et al. 2004).

Many communities, especially rural areas, still rely on herbal remedies in Kenya. Nowadays, there is little information regarding the literature on medicinal plants in Kenya (Kigen et al., 2013). Several drugs have been derived directly or indirectly from plants, including digoxin, taxol, vinblastine, nabilone, and artemisinin (Cragg and Newman 2005).

The objectives of this research were (i) To evaluate the antimicrobial potential of the crude plant extracts against *Pseudomonas aeruginosa, Escherichia coli,* methicillin-resistant *Staphylococcus aureus* (MRSA)*, Bacillus cereus,* and *Candida albicans*, (ii) To determine the acute toxicity of the crude plant extracts using brine shrimp (*Artemia salina*) lethality assay, (iii) To investigate the presence of major phytochemical constituents in the crude plant extracts.

MATERIALS AND METHODS

Collection of plant materials

Four plants that were selected based on their ethnomedicinal usage were collected from Losho, Narok County, Kenya, with the help of herbalists (Table 1). The bark stems of *Schrebera alata* and *Cussonia holstii* were peeled; aerial parts of *Ormocarpum kirkii* were cut and chopped into pieces, while the whole plant of *Helichrysum forskahlii* (the herb) was uprooted. Their parts of plants were stuffed in a polythene bag, placed in a cooler box, and transported to Nairobi. They were thoroughly washed with running water and dried at room temperature for six weeks after being ground into a fine powder using an electric mill.

Plant specimens were collected in duplicate; one unit of the specimen was used for preliminary identification in the field as previously described (Agnew and Agnew 1994), while the other was pressed and carried to the University of Nairobi Herbarium for authentication and further compared with the available herbarium collections which were permanently prepared.

Preparation of crude extracts

Preparation of organic extract*s.*

According to standard extraction methods, Dichloromethane/methanol (1: 1) was used to extract 50g of ground material by cold solvent percolation. The plant material powder was mixed thoroughly with the solvent, left to stand for 24 hrs, and decanted (repeated twice). The filtrates were pooled and filtered using a Buchner funnel. After the dissolution of the solvents at 40ºC to obtain crude extracts, dichloromethane and methanol extracts were obtained. These were then stored in airtight containers at 4ºC and prepared for bioassay and phytochemical screening (Odhiambo et al., 2014).

Preparation of aqueous extract*s*

The ground materials were extracted by the cold maceration method. To obtain aqueous extracts, fifty grams of ground plant material were extracted with distilled water (500 mL). The aqueous extracts were filtered, and the filtrate was kept in a deep freezer, then lyophilized (freeze-dried), resulting in a dry powder, and then stored in airtight containers at 4°C being prepared for bioassays and phytochemical screening (Odhiambo et al. 2014).

Media preparation

Mueller Hinton Agar

Fifty-eight grams of MHA medium was suspended in 1000 mL distilled water. Heating to boiling to dissolve the medium completely was done, followed by sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mixing was done well before pouring (Bauer et al. 1966).

Table 1. Voucher specimens and plants parts collected from Losho, Narok County, Kenya

Voucher specimen number	Plant species	Family	Part collected
DMC2014/001	*Schrebera alata* (Hochst.) Welw.	Oleaceae	Bark
DMC2014/002	*Ormocarpum kirkii* (Taub.) Engl.	Fabaceae	Aerial part
DMC2014/003	*Cussonia holstii* Harms ex Engl.	Araliaceae	Bark
DMC2014/004	*Helichrysum forskahlii* (J.F. Gmel.) Hilliard & B.L. Burttv.	Asteraceae	Whole plant

Table 2. List of microbes tested in the study

Name of microbe	Microbe type	Gram strain type	Details of strain used
Bacillus cereus	Bacteria	Gram-positive	ATCC 11778
MRSA	Bacteria	Gram-positive	ATCC 1385
Pseudomonas aeruginosa	Bacteria	Gram-negative	ATCC 27823
Escherichia coli	Bacteria	Gram-negative	ATCC 25922
Candida albicans	Fungus	-	ATCC10231

Sabouraud Dextrose Agar

Sixty-five grams of the medium were suspended in one liter of purified water, heated with frequent agitation, boiled for one minute to dissolve the medium completely, then autoclaved at 121°C for 15 minutes. The prepared media was stored at 8-15°C (Murray et al. 2003).

Source of microorganisms

The micro-organisms methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* were obtained from KEMRI, Centre for Microbiology Research (CMR), while *Bacillus cereus, Escherichia coli, and Candida albicans* were from the Department of Public health, Pharmacology, and Toxicology, University of Nairobi, Kenya (Table 2).

Subculturing of test microorganisms.

Subculturing of the test strains was done in the following procedure. Bacteria were proliferated in Muller-Hinton agar for 18hrs, and fungi were proliferated in Sabouraud Dextrose agar for 48hrs to obtain freshly growing strains. The microbial suspensions were standardized according to the Clinical and Laboratory Standards.

Institute procedures (CLSI 2009 for bacteria) and (CLSI 2008 for fungi) with sterile saline to a turbidity equivalent to 0.5 McFarland (approximately 1.5 $\times 10^8$ CFU/mL for bacteria and1.5×10^6 CFU/mL for *Candida* sp.) and stored at 4 ºC until they were used during the antimicrobial test.

Antimicrobial susceptibility testing

Agar diffusion methods which followed National Committee for Clinical Laboratory Standards (CLSI 2009) procedures, were used to evaluate the antimicrobial activities of the crude extracts. Twenty mL of sterile Muller-Hinton Agar and Sabouraud Dextrose Agar were poured into sterile petri plates and allowed to be set. An inoculum suspension was swabbed uniformly to solidify 20 mL Mueller-Hinton Agar (MHA) for bacteria and Sabouraud Dextrose Agar (SDA) for fungi. The inoculum was allowed to dry for 5 min. Three concentrations (400, 200, and 100 mg/mL) of each test extract (organic extracts and aqueous extracts of *S. alata, C. holstii, O. kirkii, and H. forskahlii*) were prepared for susceptibility testing using 1% of DMSO for organic extracts and distilled water for aqueous extracts.

Agar well diffusion. Holes of 10 mm diameter were made in the seeded agar using a sterile cork borer.100 µl of the test extracts were inserted into the wells using a microtiter-pipette and allowed to stand on the bench for 1h for proper disperse into the agar and, after that, incubated at 24hrs at 37ºC. Microbial growth was determined by measuring the diameter of the zone of inhibition in millimeters (mm). For each microbial strain, controls were maintained where pure solvents were used instead of the crude extracts (Parekh and Chanda 2007). The experiment was undergone in triplicates under sterile conditions, and the mean values were obtained.

Disc diffusion. Commercially prepared sterile discs of 6 mm diameter were infused with 100 µl of each crude extract dried and deposited aseptically onto plates inoculated with a 1mL overnight growth test microorganism. Bacterial and fungal cultures were incubated for 24 hrs at 37 ºC for bacteria and 37ºC for 72 hrs for fungi. Chloramphenicol 30µg/mL (for bacteria) and Amphotericin B 30 µg/mL (for fungi) were used as positive controls, while discs with diluting solvents only were used as negative controls. Each extract was tested in triplicate under sterile conditions. Microbial growth was determined by measuring the diameter zone of inhibition in millimeters (Kitonde *et al*., 2013).

Determination of Minimum Inhibitory Concentration (MIC)

The broth microdilution method was utilized to decide the least inhibitory convergence for the exertive unrefined extracts against the test microorganisms. As the National Committee for Clinical Laboratory Standards suggested, the scheme was now the Clinical Laboratory Standard Institute (CLSI) (Ferraro 2003). 0.5 mL of 24 h culture of the test organism (10^7 CFU/mL) changed in accordance with McFarland turbidity standard 0.5 McFarland (around 1.5 $\times 10^8$ CFU/mL for bacteria and 1.5×10^6 CFU/mL for *Candida sp*. Were incubated in serial dilution 250 mg/mL, 125 mg/mL, 62.5 mg/mL, 31.25 mg/mL, 15.625 mg/mL. Incubation was done for 24hrs at 37 ºC. Minimal centralization of the plant separation did not allow any noticeable development of the vaccinated test microorganism in broth culture. The absence of turbidity was viewed as the visual MIC for each situation (Michael et al., 2003). Tubes that were inoculated with microbes alone and media alone served as control. All the experiments were done in triplicates, and the results were recorded.

Determination of acute toxicity of crude extract*s*

The crucial toxicity assay was performed using brine shrimp *nauplii* based on the Meyer method (Nguta et al., 2013). Artificial seawater was prepared by liquefying 38 grams of sea salt in 1 L of distilled water. A tank with the size of 14 cm by 9 cm by 5 cm having two unequal compartment chambers with several holes on the divider was used for hatching. The chambers were filled with artificial seawater. Brine shrimp eggs were placed in the larger compartment, and yeast was added to act as food for the *nauplii*. The larger compartment was then covered with dark background paper while the smaller compartment was illuminated. The incubation was done at room temperature (23-29 ºC) for 48 hrs to allow the hatching process, and *nauplii* were collected in the illuminated section.

Different focuses of the crude extract in ocean water were utilized, i.e., 10, 100, and 1,000 µg/mL, to test for poisonous quality. A stock solvent of 10,000 µg/mL for every crude extract was made ready. For those aqueous extracts, the stock solvent of 10,000 µg/mL was made ready by dissolving 0. 5g of the crude extract in 10 ml of ocean water, while for organic extracts, 0. 1 g of each sample was first disintegrated on 1% DMSO and then diluted using artificial water to 10 ml to aggravate stock solvent.

Ten brine shrimp larvae were drawn from the hatching tank using Pasteur pipettes and placed in each vial. The volume of artificial seawater in each vial containing 10 Brine shrimp salina was expanded to 5mL for vials of 10 and 100 µg/mL of the plant extracts, while for 1,000 µg/mL, it was topped to 4.5 mL. Using micropipettes, 0.5 mL, 0.05 mL, and 0.005 mL were exchanged from the stock solution to the vials containing 5 mL of artificial seawater to make experimental solutions containing 1,000 µg/mL, 100 µg/mL, and 10 µg/mL, respectively (Table 3). Control experiments were done using artificial seawater and DMSO for organic extract and artificial seawater only in the case of aqueous extract (Wanyoike et al. 2004). Three replicates for the three serial dilutions of distinctive unrefined extracts and the control were performed. Surviving *nauplii* were counted after 24hr using a magnifying glass, and the average mortality at each concentration was determined as it was essential for the estimation of LC_{50}.

Qualitative phytochemical screening of crude extracts

In order to recognize a few classes of the auxiliary metabolites in these plants, subjective chemical tests were conducted on all the unrefined plant extracts. Identification was based on a characteristic color change of precipitate or foam development. Alkaloids, flavonoids, saponins, tannins, glycosides, quinines, and terpenoids were detected using standard methods (Trease and Evans 2002) (Table 4).

Table 3. Brine shrimp bioassay set up for each plant extract

Vials	Volume of artificial seawater (mL)	No of Brine shrimp larvae	Volume of stock solution (mL)	Concentration (µg/mL)	Nature of experiment	Final volume in the vial (mL)
1	4.5	10	0.5	1,000	Trial	5
2	4.5	10	0.5	1,000	Repeat	5
3	4.5	10	0.5	1,000	Repeat	5
4	5	10	0.05	100	Trial	5
5	5	10	0.05	100	Repeat	5
6	5	10	0.05	100	Repeat	5
7	5	10	0.005	10	Trial	5
8	5	10	0.005	10	Repeat	5
9	5	10	0.005	10	Repeat	5
10	5	10	0	0	Control	5
11	5	10	0	0	Control	5
12	5	10	0	0	Control	5

Table 4. Detection for phytochemicals

Phytochemical test	Detection
Test for saponins (Foam test)	1g of each extract was shaken with distilled water in a test tube for 15mins. Bubbles that persist on warming were taken as preliminary evidence for the presence of saponins.
Test for sterols (Salkowaski test)	1g of each extract was dissolved in 2 mL of chloroform, and 2 mL of concentrated sulphuric acid was added from the side of the test tube. The test tube was shaken for a few minutes. The development of red color in the chloroform layer indicated the presence of sterols.
Test for alkaloids (Dragendorffs' test)	1 g of each extract was dissolved in 5 mL of hydrochloric acid (1.5% v/v) and permeated. These filtrates were then used for testing alkaloids. Dragendorffs' reagent was added into 2mL of filtrate. The formation of orange-brown precipitate indicated the presence of an alkaloid.
Test for tannins	1g of each extract was stirred with 10mL of distilled water, filtered, and 1mL of 5% ferric chloride was added to the filtrate. A blue-black, green, or blue-green precipitate was taken as evidence of the presence of tannins.
Test for flavonoids	In a test tube with 1 g of each extract, a few drops of dilute sodium hydroxide (NaOH) were added and shaken. An intense yellow color was produced in the plant extract, which became colorless on adding a few drops of dilute acid, indicating the presence of flavonoids.
Test for quinones	1g of extract was shaken with 1mL of concentrated sulphuric acid(H_2SO_4). The formation of red color shows the presence of quinones.
Test for terpenoids	5g of each extract was mixed with 2 mL of chloroform. 3mL of concentrated sulphuric acid (H_2SO_4) was added to form a layer. A reddish-brown precipitate coloration at the interface formed indicated the presence of terpenoids.

Data analysis

A statistical program for social sciences (statistical analysis software) was used to do a statistical analysis of antimicrobial activity. ANOVA software was used to determine whether there were significant differences in the mean diameter of inhibition zones in various concentrations. Once the means were different, the Dunnett test was then used for some inhibitory comparisons to determine whether inhibition of different treatments differed from the inhibition induced by positive controls. The significance level used in the analysis was 0.05. The lethal concentration (LC_{50}), 95% confidence interval of the selected plants was determined using the Finney (1971) computer program.

RESULTS AND DISCUSSION

Yields of extracts from test plants

The resulting dry powders were weighed and expressed as percentages. Organic extracts yielded a higher percentage than the water extracts, except for *C. holstii* (Table 5).

Antimicrobial activity of the crude extracts against selected microorganisms

Antimicrobial activity of the crude extracts on MRSA

Crude extracts of *O. kirkii* and *H. forskahlii* had inhibition zones of 15 mm at 100 mg/mL in agar well diffusion and disc diffusion (Figures 1 and 5). At 200 mg/mL, only organic extracts of the four plants were active against this microbe in both agar well and disk diffusion (Figures 2 and 7). Effective growth inhibition was recorded at higher concentrations of each extract (Figures 3 and 6). Of all the aqueous extracts tested in both agar well and disk diffusion, only active *H. forskahlii* resisted these tested bacteria at concentrations of 100, 200, and 400 mg/mL (Figure 4). In both agar-well diffusion and disk diffusion, among the tested plant extracts, only organic extracts were active against MRSA at all the concentrations tested (Figures 8 and 9). Inhibition of MRSA growth by various extracts from four selected plants significantly ($P \leq 0.05$) differed from one another in both agar well and disc diffusion methods (Table 6). The levels of comparative significance of everything except *H. forskahlii* organic plant extract at the test were all under 0.05. Subsequently, the concentrates had development inhibitions of MRSA, which were fundamentally not quite the same as that of the positive control at $P \leq 0.05$. In any case, organic extracts of *H. forskahlii* at 400 mg/mL demonstrated no critical distinction between the positive control at $P \geq 0.05$ in both agar well and disk diffusion.

Antimicrobial activity of the crude extracts on P. aeruginosa

H. forskahlii organic extracts had a breadth of 6 mm in agar disk diffusion at 200 mg/mL (Figure 10). Organic extracts of *S. alata* demonstrated antibacterial action at 400 mg/mL in both agar-well and disc dispersion methods (Figures 11 and 13). Among the fluid extracts tested, only *O. kirkii* indicated antibacterial activity against this bacterial strain at 400 mg/ml in the two analyses with an inhibition zone of 10 mm (Figure 12). Of all the tested extracts, *H. forskahlii* organic extracts had the most remarkable inhibition breadth of 10 mm in agar well (Figures 14 and 15). Organic extracts of *C. holstii,* and *O. kirkii* were not exertive against this microorganism. Moreover, *H. forskahlii* organic extracts had an inhibition zone of 5.5 mm at 400 mg/mL in agar well dispersion (Figure 14). Development inhibitions of *P. aeruginosa* by the different extracts of the four chosen plants in the two experiments were found to be fundamentally different from each other at 95% confidence intervals ($P \leq 0.05$) (Table 6). The criticalness levels of all the plants compared with the positive control at 400, 200, and 100 mg/mL were all under 0.05, demonstrating that every one of the extracts had development inhibitions of *P. aeruginosa,* which were significantly different from that of chloramphenicol which was utilized as a positive control.

Antimicrobial activity of the crude extracts on Bacillus cereus

Only organic extract of *H. forskahlii* indicated antibacterial activity against this Gram-positive bacteria with an inhibition zone of 6 and 7 mm in disk diffusion at 200 and 400 mg/mL, respectively (Figures 16 and17). In contrast, in agar-well diffusion at similar concentrations, inhibition zone of 4.5 mm and 5 mm separately appeared by similar extracts (Figures 18 and 19). The positive control had an inhibition breadth of 19 mm against this microorganism. All the tested extracts demonstrated no action at 100 mg/mL in both agar well and disk diffusion methods (Figures 21 and 22). The growth inhibitions of *B. cereus* by the different extracts in the two experiments were fundamentally unique at ($P \leq 0.05$) (Table 6). Their criticalness levels of correlation of all the plants with the positive control at coalescence, 400 and 200ml, were all under 0.05. Consequently, every extract had development inhibitions of *B. cereus,* which were altogether not the same as that of the positive control at $P \leq 0.05$ in both agar well and disk diffusion methods (Figure 20).

Antimicrobial activity of the crude extracts on E. coli

Only natural coalescence of *H. forskahlii* was exertive against this Gram-negative bacterial strain at 200 and 400 mg/mL in the disc diffusion method (Figures 23 and 24). Also, of all the tested extracts, only natural extracts of *H. forskahlii* were exertive at 400 mg/mL (Figure 25). Inhibition zones of 6 mm were noted at 400 mg/mL in both agar well and disc diffusion method (Figures 26 and 27). Growth inhibition of *E. coli* by the different coalescences of the four chosen plants in the two methods was observed to be essentially different from each other at 95% certainty intervals ($P \leq 0.05$) (Table 6). The criticalness levels of all the plants compared with the positive control at 400, 200, and 100 mg/mL were all under 0.05, demonstrating that every extract had growth inhibition of *E. coli* which was significantly different from that of Chloramphenicol which was utilized as a positive control in both agar well and disk diffusion.

Table 5. Percentage yields of extracted crude plant extracts

Plant species	Part used	Solvent	Extraction type	% yield to weight of the dry powered plant
Ormocarpum kirkii	Aerial part	Water	Freeze drying	3.79
		Dichloromethane-methanol (1: 1)	Rotary evaporator	3.94
Schrebera alata	Bark	Water	Freeze drying	9.35
		Dichloromethane-methanol (1: 1)	Rotary evaporator	13.23
Cussonia holstii	Bark	Water	Freeze drying	4.76
		Dichloromethane-Methanol (1: 1)	Rotary evaporator	4.47
Helichrysum forskahlii	Whole plant	Water	Freeze drying	6.32
		Dichloromethane-Methanol (1: 1)	Rotary evaporator	10.74

Note: Percentage yields of crude extract (% yields) = extracted weights/initial weights x 100 (All weights in grams)

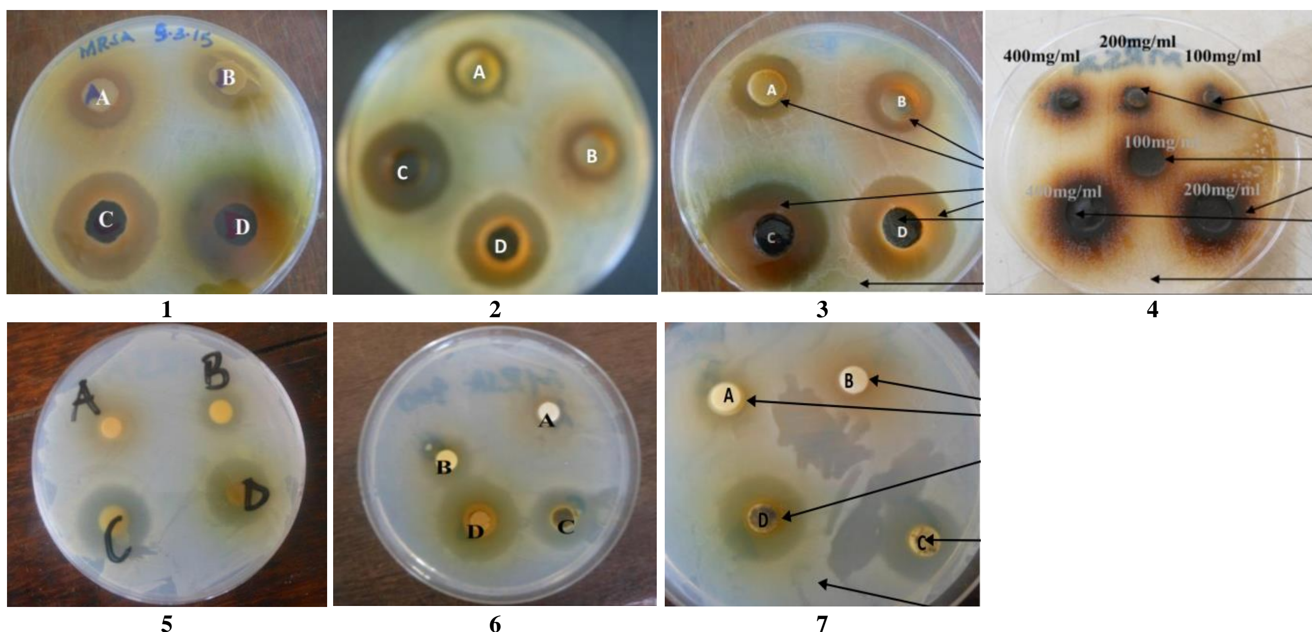
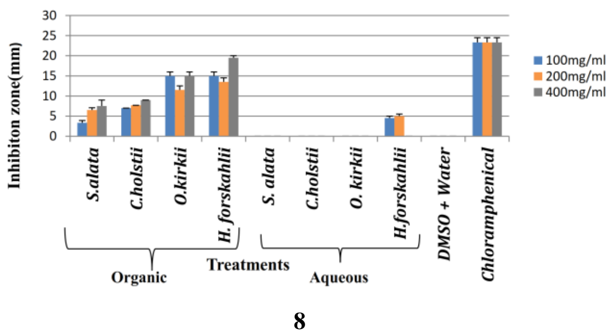


Figure 1. Antibacterial activity of organic extracts 100 mg/mL in a plate of MRSA in agar well diffusion method. Note for all disc-plates: A. Organic *Cussonia holstii,* B. Organic *Schrebera alata,* C. Organic *Ormocarpum kirkii,* D. Organic *Helichrysum forskahlii,* E. Aqueous *Cussonia holstii,* F. Aqueous *Schrebera alata,* G. Aqueous *Pseudomonas aeruginosa,* H. Aqueous *Ormocarpum kirkii.*
Figure 2. Antibacterial activity of organic extracts 200 mg/mL in a plate of MRSA in agar well diffusion method
Figure 3. Antibacterial activity of organic extracts (400 mg/mL) in a plate of MRSA in agar well diffusion method
Figure 4. Antibacterial activity of aqueous extracts of *H. forskahlii* in a plate of MRSA in agar well and disk diffusion method at various concentrations.
Figure 5. Antibacterial activity of organic extracts (100 mg/mL) in a plate of MRSA in agar disc diffusion method
Figure 6. Antibacterial activity of organic extracts (400 mg/mL) in a plate of MRSA in agar disc diffusion method
Figure 7. Antibacterial activity of organic extracts (200 mg/mL) in a plate of MRSA in agar disc diffusion method



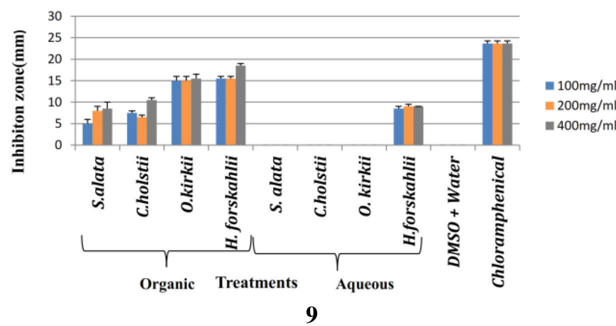


Figure 8. Growth inhibition of the crude extracts on MRSA in agar disc diffusion
Figure 9. Growth inhibition of the crude extracts on MRSA in agar well diffusion

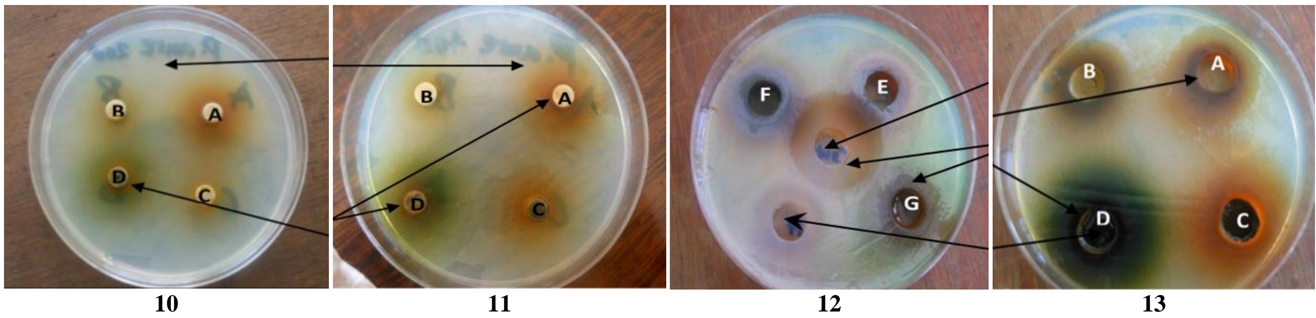
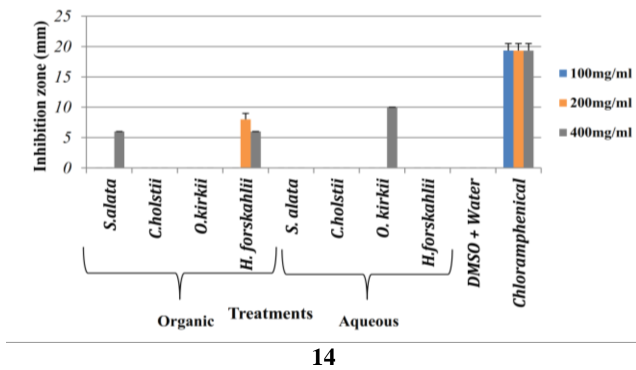


Figure 10. Antibacterial activity of organic extracts (200 mg/mL) in a plate of *P. aeruginosa* in agar disc diffusion method
Figure 11. Antibacterial activity of organic extracts (400 mg/mL) in a plate of *P. aeruginosa* in agar disc diffusion method
Figure 12. Antibacterial activity of aqueous extracts (400 mg/mL) in a plate of *P. aeruginosa* in agar well diffusion method
Figure 13. Antibacterial activity of organic extracts (400 mg/mL) in a plate of *P. aeruginosa* in agar well diffusion method



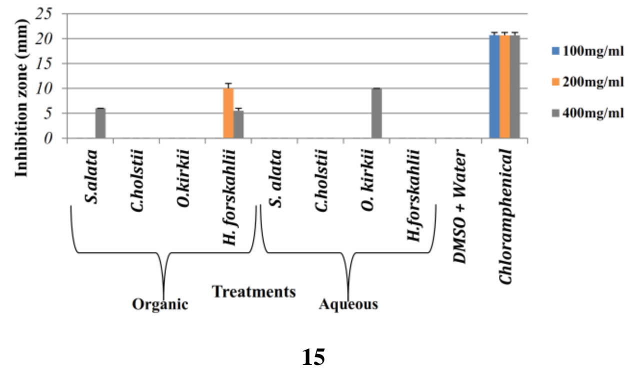


Figure 14. Growth inhibition of the crude extracts on *P. aeruginosa* in agar disc diffusion
Figure 15. Growth inhibition of the crude extracts on *P. aeruginosa* in agar well diffusion

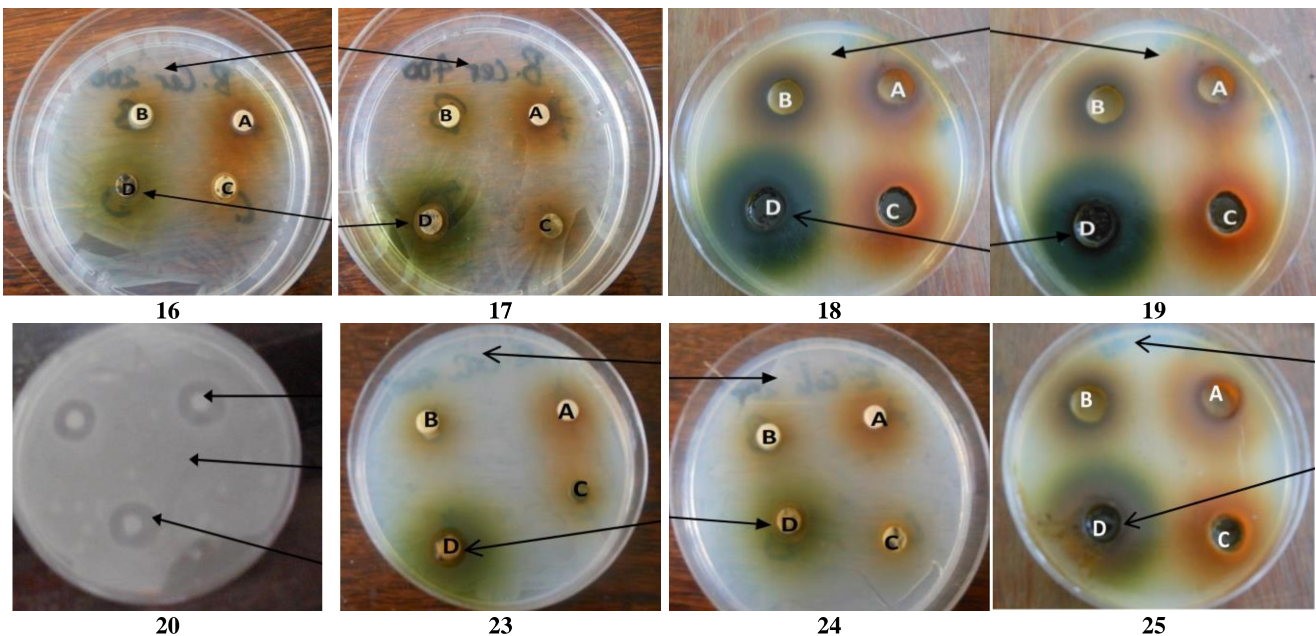
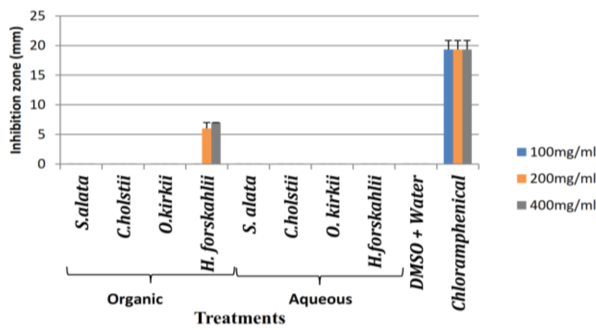


Figure 16. Antibacterial activity of organic extracts D (200 mg/mL) in a plate of *B. cereus* in agar disk diffusion method
Figure 17. Antibacterial activity of organic extracts D (400 mg/mL) in a plate of *B. cereus* in agar disk diffusion method
Figure 18. Antibacterial activity of organic extracts D (200 mg/mL) in a plate of *B. cereus* in agar disk diffusion method
Figure 19. Antibacterial activity of organic extracts D (400 mg/mL) in a plate of *B. cereus* in agar disk diffusion method
Figure 20. Antibacterial activity of chloramphenicol in a plate of *B. cereus* in agar diffusion method
Figure 23: Antibacterial activity of organic extracts D (400 mg/mL) in a plate of *E. coli* in agar disk diffusion method
Figure 24. Antibacterial activity of organic extracts D (200 mg/mL) in a plate of *E. coli* in agar disk diffusion method
Figure 25. Antibacterial activity of organic extracts D (400 mg/mL) in a plate of *E. coli* in agar well diffusion method



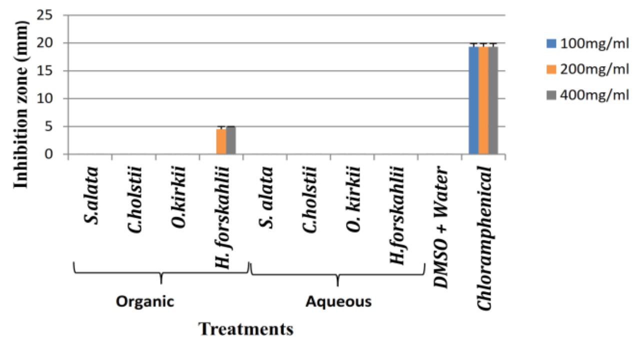


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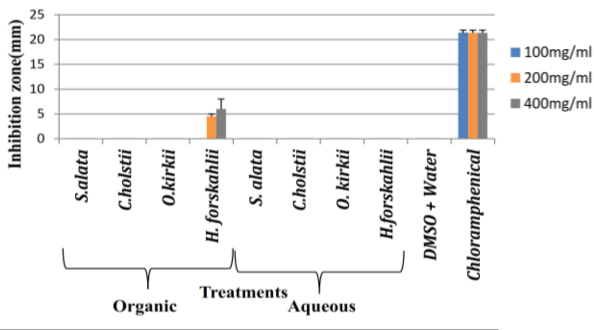
Figure 21. Growth inhibition of the crude extracts on *B. cereus* in disc diffusion
Figure 22. Growth inhibition of the crude extracts on *B. cereus* in agar well diffusion



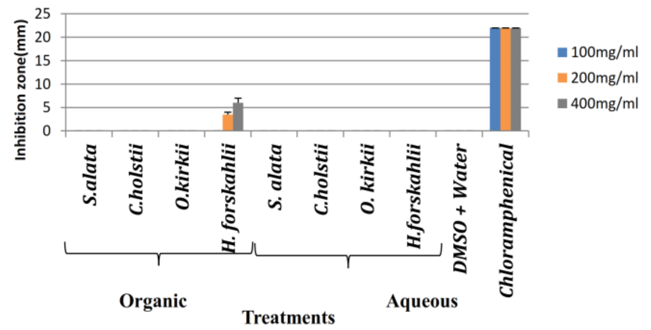


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Figure 26. Growth inhibition of the crude extracts against *E. coli* in agar disc diffusion
Figure 27. Growth inhibition of the crude extracts on *E. coli* in agar well diffusion

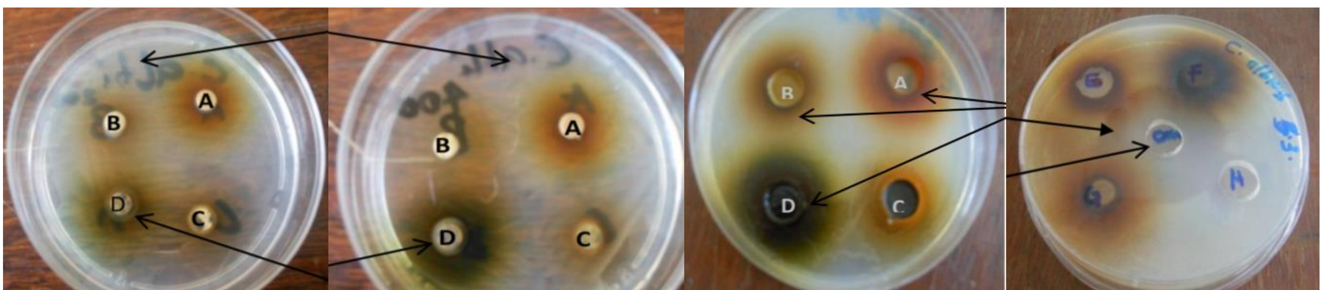

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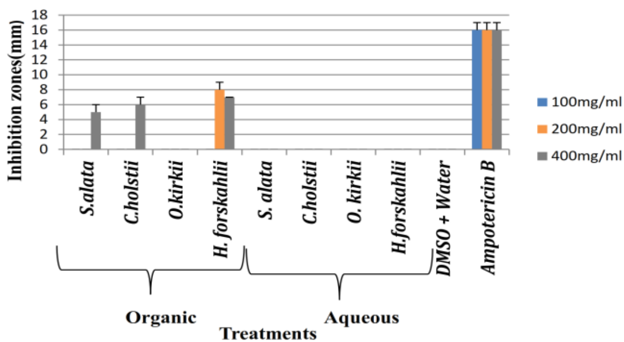
Figure 28. Antibacterial activity of organic extracts (200 mg/mL) in a plate of *C. albicans* in agar disc diffusion method
Figure 29. Antibacterial activity of organic extracts (400 mg/mL) in a plate of *C. albicans* in agar disc diffusion method
Figure 30. Antibacterial activity of organic extracts (400 mg/mL) in a plate of *C. albicans* in agar well diffusion method
Figure 31. Antibacterial activity of organic extracts (400 mg/mL) in a plate of *C. albicans* in agar well diffusion method



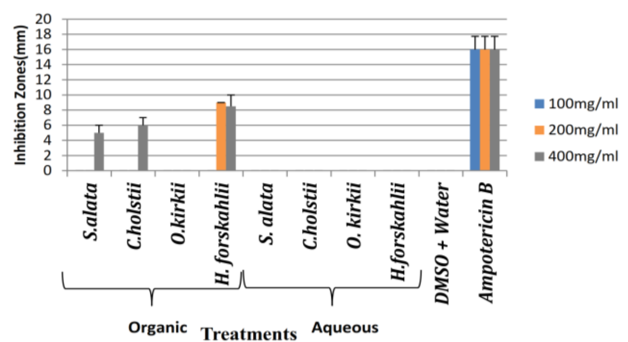


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Figure 32. Growth inhibition of the crude extracts on *C. albicans* in disc diffusion
Figure 33. Growth inhibition of the crude extracts on *C. albicans* in agar well diffusion

Antifungal activity of the crude extracts on Candida albicans

In the agar disc method, *H. forskahlii* had antifungal activity at 200 and 400 mg/mL (Figures 28 and 29). Thus, inhibition zones were noted in organic extracts of *H. forskahlii* at 400 mg/in agar well diffusion (Figure 30). Amphotericin B (positive control) had an inhibition breadth of 16 mm against *C. albicans* (Figure 31). Furthermore, at 400 mg/mL, organic extracts of *S. alata, C. holstii*, were exertive against this fungi strain (Figures 32 and 33). The most elevated antifungal activity with an inhibition zone estimation of 8.5 mm was seen in organic extracts of *H. forskahlii* in agar well diffusion. It was clear that growth inhibition of *C. albicans* by different extracts from the four plants was fundamentally different from each other in both experiments. ($P \leq 0.05$) (Table 6). Comparison of growth inhibition by all plants with the positive control at 400, 200, and 100 mg/mL proved that all the extracts had growth inhibition of *C. albicans* which were fundamentally different from that Amphotericin B utilized as a positive control in both agar well and disc diffusion.

Minimum inhibitory concentration (MIC) of plant extracts against the test microorganism

Results of least inhibitory focus (MIC) of plant extracts against the test microorganisms appeared in Table 7. MIC values differed with plant specimens from 250 to 15.625 mg/mL. The MIC values of the test extract additionally shifted against various test pathogens. The outcomes obtained from these examinations uncovered that MRSA was the most-touchy bacteria at the lower MIC value of 15.625 mg/mL by the biggest number of crude extracts.

Toxicity of the crude plant extracts on brine shrimp larvae

Each plant's mean lethality in the three concentrations (1 mg/mL, 0.1 mg/mL, and 0.01 mg/mL) were fed into the Finney computer program estimate (LC_{50}) of the crude plant extracts. The results are displayed in Tables 8 and 13.

Consideration of toxicity was based on Nguta et al. (2011), where LC_{50} ranging between 0-0.1 mg/mL indicated high toxicity, LC_{50} between 0.1-0.5 mg/mL indicated moderate toxicity, LC_{50} between 0.5-1 mg/mL indicated weakly toxicity and LC_{50} over 1 mg/mL indicated non-toxic nature of the extract. Aqueous crude plant extracts of *O. kirkii* (0.412 mg/mL) and *S. alata* (0.317 mg/mL) were moderately toxic. In contrast, aqueous extract of *C. holstii* (0.544 mg/mL) had weak toxicity, while aqueous extracts of *H. forskahlii* were non-toxic with LC_{50} of 1.206 mg/mL. Organic crude extracts of *H. forskahlii* and *C. holstii* were highly toxic (0.009 mg/mL. In contrast,e organic extracts of *O. kirkii* (0.207 mg/mL) and *S. alata* (0.399 mg/mL) were moderately toxic.

Table 6. MRSA, *P. aeruginosa, B. cereus E. coli,* and *C. albicans* inhibition at different concentrations by various extracts

Method	Concentration in mg/mL		
	100	200	400
MRSA			
Disc diffusion	F=519.27	F=325.75	F=466.22
	P=0.00	P=0.00	P=0.00
Agar well diffusion	F=663.63	F=516.84	F=469.74
	P=0.00	P=0.00	P=0.00
P. aeruginosa			
Disc diffusion	-	F=1.07	F=2.38
	-	P=0.00	P=0.00
Agar well diffusion	-	F=1.08	F=2.38
	-	P=0.00	P=0.00
B. cereus			
Disc diffusion	-	F=345.60	F=504.91
	-	P=0.00	P=0.00
Agar well diffusion	-	F=1.93	F=3.40
	-	P=0.00	P=0.00
E. coli			
Disc diffusion	-	F=2.33	F=320.31
	-	P=0.00	P=0.00
Agar well diffusion	-	F=5.75	F=1.47
	-	P=0.00	P=0.00
C. albicans			
Disc diffusion	-	F=437.33	F=278.22
	-	P=0.00	P=0.00
Agar well diffusion	-	F=305.00	F=121.02
	-	P=0.00	P=0.00

Table 7. Minimum inhibitory concentration (MIC) in mg/mL

Plant	Solvent	*E. coli*	*P. aeruginosa*	MRSA	*B. cereus*	*C. albicans*
O. kirkii	Organic	-	-	31.25	-	-
	Aqueous	-	250	-	-	-
S. alata	Organic	-	15.625	15.625		62.5
	Aqueous	-	-		-	-
C. holstii	Organic	-	-	15.625	-	62.5
	Aqueous	-	-	-	-	-
H. forskahlii	Organic	62.5	31.25	15.625	31.25	31.25
	Aqueous	-	-	15.625	-	-

Table 8. Mortality data of aqueous extracts in mg/mL

Aqueous plants extracts	Concentration (mg/mL)	Mean ± SD	LC_{50} mg/mL
O. kirkii	0.01	1.67±1.53	0.42
	0.1	4.00±2.00	
	1	9.00±1.00	
H. forskahlii	0.01	0	1.21
	0.1	0	
	1	5.67±1.53	
C. holstii	0.01	0	0.54
	0.1	1.00±0.00	
	1	9.00±1.00	
S. alata	0.01	0	0.32
	0.1	4.33±2.08	
	1	10.00±0.00	

Table 9. Mortality data of organic extracts in mg/mL

Organic plants extracts	Concentration (mg/mL)	Mean± SD	LC_{50} mg/mL
O. kirkii	0.01	2.33±1.53	0.21
	0.1	8.33±1.53	
	1	9.67±0.58	
H. forskahlii	0.01	10.00±0.00	0.01
	0.1	10.00±0.00	
	1	10.00±0.00	
C. holstii	0.01	10.00±0.00	0.01
	0.1	10.00±0.00	
	1	10.00±0.00	
S. alata	0.01	3.67±5.51	0.40
	0.1	2.20±2.00	
	1	7.33±4.62	

Phytochemical constituents of the crude plant extracts

The plant extracts showed a positive test for the existence of flavonoids, sterols, alkaloids, tannins, quinones, and terpenoids (Figures 35, 36, 37, and Table 10). Saponins existed in all the extracts screened except organic extracts of *H. forskahlii* (Figure 34).

Discussion

The percentage yield of the plant extracts

The aggregate sum of unrefined extract acquired with the different solvents demonstrates that methanol: DCM (1: 1) was quantitatively better for all plants' extraction than refined water. Organic extracts of *O. kirkii*, *S. alata,* and *H. forskahlii* had higher yields than aqueous extracts. However, aqueous extracts of *C. holstii* had higher yields than organic extracts of a similar plant. The productivity of methanol in the extraction of phytochemicals has been reported in other studies (Ezekiel et al., 2009). These results appeared to be consistent with others confirming methanol as a decent solvent for extraction of bioactive mixtures from plants as it gave the most elevated yield in the three studied plants. Thus, Dichloromethane extracts were less polar mixtures, while methanol extracts were more polar mixtures.

Antimicrobial activity of plant extracts

In the present study, in vitro antimicrobial activities of four chosen therapeutic plants were evaluated against two Gram-positive bacteria (MRSA, *B. cereus*), two Gram-negative bacteria (*E. coli, P. aeruginosa*), and a fungus (*C. albicans*) uncovered that they have potential antimicrobial substances against a majority of the tested microorganisms.

The tested plant extracts were more exertive against Gram-positive bacteria compared to Gram-negative. The most sensitive bacterium was MRSA which was hindered by the unrefined organic extracts of all chosen plants. Generally, Gram-negative bacteria are more resistant than Gram-positive bacteria (Nurul et al., 2010; Darah et al., 2011; Nor et al., 2012). The higher affectability of Gram-positive bacteria (MRSA) could be because of the introduction of the external peptidoglycan layer (Korir et al. 2012), while Gram-negative bacteria bear an additional outer membrane (OM) which incorporates the lopsided circulation of the lipids with phospholipids and lipopolysaccharide (LPS) situated in the inner and outer leaflets, respectively can act as an extra boundary which inhibits the development of foreign substance into the cell (Pages et al. 2008).

These results of both agar diffusion methods were found not to differ significantly from each other (P>0.05). This was in concurrence with Parekh and Chanda (2007). More so, the principle of the agar well diffusion is the same as that of the agar disk diffusion method (Ncube et al. 2008).

Table 10. Relative abundance of detected phytochemical in crude plant extracts

Plant	Crude extracts	Sterols	Alkaloids	Saponins	Flavonoids	Tannins	Quinones	Terpenoids
O. kirkii	Organic	++	+	+	+++	+	+++	+++
	Aqueous	+++	++	++	+++	+	+++	+++
S. alata	Organic	+++	+++	++	+++	+++	+++	+++
	Aqueous	+++	+	++	+++	++	+++	+++
C. holstii	Organic	+++	++	+++	+++	+++	++	+++
	Aqueous	+++	+	+	+++	+	+++	+
H. forskahlii	Organic	+++	++	-	+++	+	++	+++
	Aqueous	+++	++	+++	+++	+++	+++	++

Note: +++: strong presence, ++: moderate presence, +weak presence,-: not detected

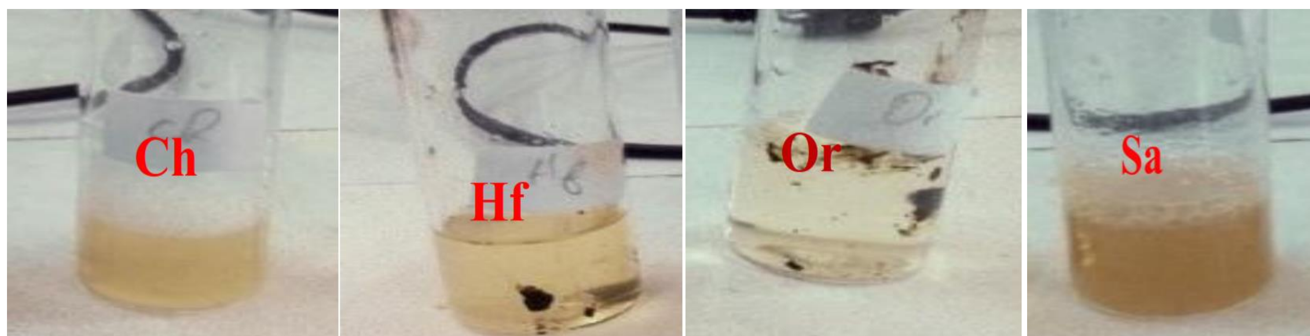

Figure 34. A photograph shows the existence of saponins in organic extracts. Sa indicates the moderate presence of saponins, Ch: organic *Cussonia holstii* Sa: organic, *Schrebera alata* Or: organic *Ormocarpum kirkii* Hf: organic *Helichrysum forskahlii*

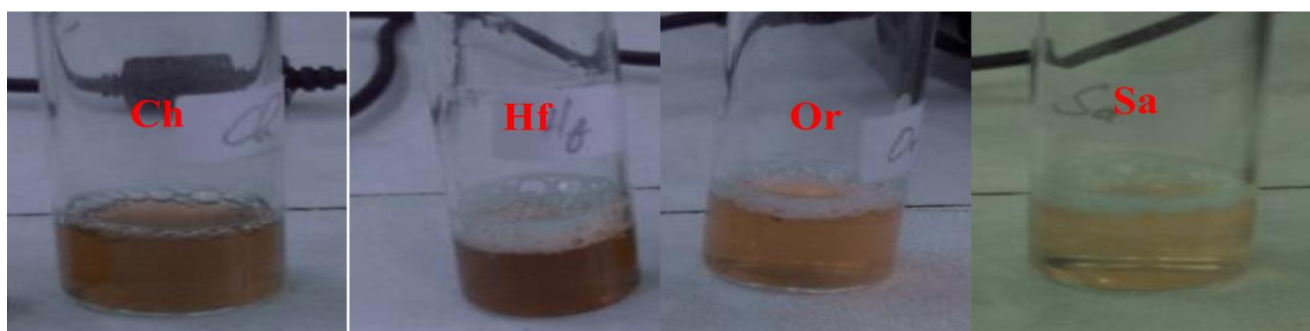

Figure 35. A photograph shows the existence of saponins in aqueous extracts. Ch: aqueous *Cussonia holstii,* Sa: aqueous *Schrebera alata,* Or: aqueous *Ormocarpum kirkii*, Hf: aqueous *Helichrysum forskahlii*, Hf indicates a strong presence of saponins

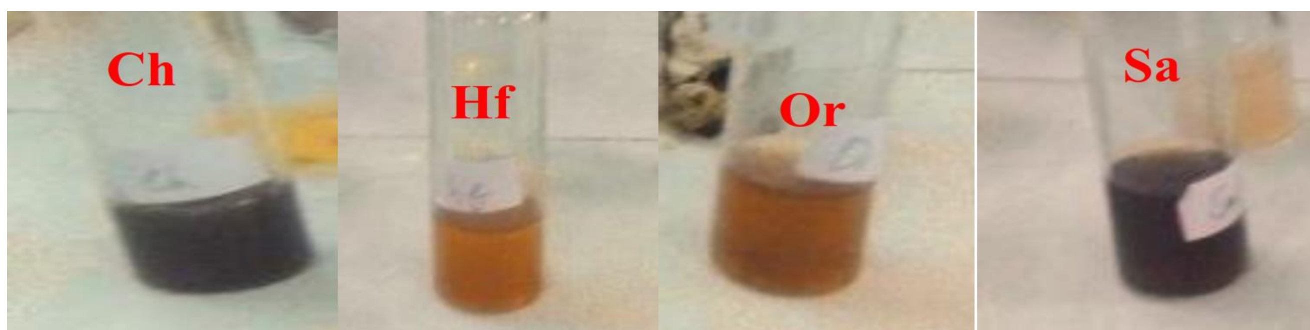

Figure 36. A photograph shows the existence of tannin in the organic extract. Ch: organic *Cussonia holstii,* Sa: organic *Schrebera alata,* Or: organic *Ormocarpum kirkii,* Hf: organic *Helichrysum forskahlii.* The dark green /deep blue in Ch and Sa indicates a strong presence of tannins.

Figure 37. A photograph shows the existence of tannin in aqueous extracts**.** Ch: aqueous *Cussonia holstii,* Sa: aqueous *Schrebera alata,* Or: aqueous *Ormocarpum kirkii*, Hf: aqueous *Helichrysum forskahlii*. The dark green /deep blue in Hf indicates a strong presence of tannins.

Antibacterial activity of organic plant extracts

From this examination, it is clear the dichloromethane: methanol (1: 1) solvent extracts of all tested plants were more potent than their relating aqueous extracts against all the tested microbes. This examination is quite compelling, given that, traditionally, the preparation of herbal remedies is often with water. This might have resulted from the lower solubility of the active constituents in the aqueous solution. Cowan (1999) demonstrated that aqueous extraction could be ineffective because water-soluble compounds might interrupt the antimicrobial effect. In addition, antimicrobial phytochemicals are soluble in moderately polar solvents. Clarkson et al. (2004) clarified that the inactivity of water extracts may have been because they (extracts) were not prepared according to the traditional methods, which in some cases involved boiling for several hours. All tested organic plant extracts showed antibacterial activity against most bacteria employed in this study. Among the tested plant extracts, only organic extract from *H. forskahlii* at 400 mg/mL against MRSA had similar activity as the positive control (P>0.05). Investigation in this study also revealed that the sampled plants differ in their activities against the tested pathogens. The results also clearly showed that antibacterial activity varied with the species of the plant and the solvents used for extraction. In addition, all extracts exhibited concentration-dependent activity at tested concentrations; higher activity was observed at high concentrations (400 mg/mL) in both agar well and disk diffusion method. Organic extracts of *H. forskahlii* showed significant antimicrobial activity against the tested pathogenic organisms. The antibacterial activity of *H. forskahlii* demonstrated a broad spectrum compared to other sampled plants. It justified its use in treating various diseases such as stomach and diarrhea (Kajangwe et al. 2008). The largest inhibition zones of extracts of the tested plant were recorded in agar well (19.5 mm) and disk diffusion (18.5 mm) by organic extract of this plant at 400 mg/mL. More so, only the above diameters had no significant difference in activity as Chloramphenicol (P>0.05). Noteworthy inhibition diameters were shown against *B. cereus, E. coli,* and *P. aeruginosa* at 200 and 400 mg/mL by organic extracts of the same plant. *H. forskahlii* organic extracts showed antibacterial activity against all tested microorganisms. This antibacterial activity of *H. forskahlii* agrees with Kajangwe et al. (2008) and Al-Rehaily et al. (2008). In the present study, the organic crude extracts of *S. alata* showed inhibitory activity against MRSA and *P. aeruginosa.* Against MRSA, *S. alata* recorded an inhibition zone at 100, 200, and 400 mg/mL in both agar well and disk diffusion. Organic extracts of *S. alata* showed antibacterial activity only at 400 mg/mL against *P. aeruginosa.* The sensitivity of *S. alata* to MRSA and *P. aeruginosa* showed activity similar to another species of the same genus *Schrebera swietenioides* powdered leaf extracts, which exhibited potent inhibitory activity against *S. aureus* (Mahida and Mohan 2007; Niranjan et al. 2010). More so, Nanyingi et al. (2008) report the use of this plant in Samburu for candidiasis and toothache. Organic extracts of *C. holstii* were active against MRSA in both agar well and disc diffusion methods. The antibacterial activity of this plant against MRSA was observed at all the tested concentrations. The highest antibacterial inhibition activity (10 mm) was shown in agar well diffusion at 400 mg/mL. This may explain why *Cussonia* species are used in African traditional medicine for several diseases such as pain, inflammation, traditional management of ear, nose, and throat (ENT) diseases, gastrointestinal problems, malaria, and sexually transmitted diseases (Njoroge and Bussmann 2007), De Villiers et al. 2010). However, this study did not agree with other members of the same genus *C. spicata, C. paniculata,* and *C. arborea* methanolic extracts, which show activity against *E. coli* and *P. aeruginosa* (De Villiers et al. 2010). The organic extracts of *O. kirkii* were only active against MRSA. This is in line with another species of the same genus, *Ormocarpum trichocarpum,* which showed antibacterial activity (Chukwujekwu et al., 2013; Pazhanisamy and Ebenezer, 2013).

Antibacterial activity of aqueous plant extracts

Aqueous extracts of *S. alata* and *C. holstii* had no antimicrobial activity against all the tested microbes. In contrast, aqueous extracts of *H. forskahlii* had activity in all concentrations of 100,200,400 mg/mL in both agar well and disc diffusion. This observation agrees with Kajangwe et al. (2008); Al-Rehaily et al. (2008), showing this plant is a potential antimicrobial source. Aqueous extracts of *O. kirkii* were active against only Gram-negative bacteria, *P. aeruginosa.* This was in line with another species of the same genus, *O. trichocarpum,* which showed antibacterial activity (Chukwujekwu et al., 2013, Pazhanisamy et al., 2013).

Antifungal activity of plant extracts

The antifungal activity also varied with the species of the plant and the solvents used for extraction. In addition, all the tested extracts exhibited concentration-dependent activity at tested concentrations; higher activity was observed at high concentrations (400 mg/mL) in both agar well and disk diffusion methods. *C. albicans* was not sensitive to any aqueous extract but showed antifungal activity in organic extracts of *S. alata*, *C. holstii,* and *H. forskahlii*.

The highest antifungal activity with an inhibition zone value of 8.5 mm was observed in organic extracts of *H. forskahlii* in agar well diffusion at 400 mg/mL with a MIC value of 31.25 mg/mL. This study was in agreement with another member of the same species, *H. italicum* essential oil, which shows demelanizing activity against *Aspergillus niger* (Milos et al. 2014). Among the *C. holstii* extracts tested for antifungal activity, only the organic extracts were active against *C. albicans* in both agar well and disc diffusion methods with a MIC of 62.5 mg/mL. This is in agreement with another member of the same genus, *C. bancoensis,* which reports the antifungal activity of methanol, ethyl acetate, and petroleum ether extracts of the stem bark against the same microorganism and minimum inhibitory concentrations ranging from 0.625 mg/mL to 2.5 mg/mL (Mireku et al. 2014). Njoroge and Bussman (2007) argue that *C. holstii* is used for the traditional management

of ear, nose, and throat (ENT) diseases in central Kenya. *O. kirkii* extracts had no antifungal activity, which was against Maregesi et al. (2008), which shows hexane root extracts of *O. kirkii* against *C. albicans* had a MIC of 1 mg/mL. Antifungal activity of *Schrebera alata* was reported for the first time in this study, though, in Samburu, studies show that the root and bark of this plant are pounded or chewed as a treatment for candidiasis and toothache (Nanyingi et al. 2008).

Minimum Inhibitory Concentration

Interestingly, MIC values of less than 100 mg/mL were observed in broth micro dilutions but not an AST, and this could be due to the microdilution method providing a potentially useful technique for determining MICs increased sensitivity for small quantities of extracts which is important if the antimicrobial is scarce as is the case for many natural products (Ncube et al. 2008). More so, dilution testing methods may be quantitative (MIC), in addition to qualitative (susceptible, intermediate, and resistant), whereas disk methods are only qualitative method (Miller 2005)

Organic and water H. forskahlii extracts were more active against MRSA at lower concentrations, as shown by the MIC value of 15.625 mg/mL compared to other test microbes. However, Kajangwe et al. (2008) showed the essential oil from *H. forskahlii* presents activity against *E. coli* (MIC between 0.2 and 0.8 mg/mL), which varied with the study MIC value (62.5 mg/mL) against the same bacterium strain. *S. alata* recorded a MIC value of 15.625 mg/mL against *P. aeruginosa* and MRSA, which was in line with another member of the same genus, *S. swietenioides,* against *S. aureus* (Mahida and Mohan 2007). The activity of *C. holstii* against the MRSA recorded a MIC value of 15.625 was in line with De Villiers et al. (2010) and Mireku et al. (2014) that some other species of the same genus against *E. coli* and *P. aeruginosa*. MIC value (31.25 mg/mL) was revealed by organic extracts of *O. kirkii* against MRSA in line with Maragesi et al. (2008). However, the lack of antibacterial activity showed by the same plant against *B. cereus* from this study did not agree with Maregesi et al. (2008) earlier report on MeOH and n-Hexane root extracts of the same species exhibited antibacterial activity against this microorganism at 0.625 and 0.250 mg/mL.

Brimp shrimp test

Aqueous extracts of *H. forskahlii* were exertive against MRSA and displayed a non-toxic impact on brine shrimps with LC_{50} of 1.207 mg/mL. This helps inherent selectivity of the plant extracts for the currency of bacterial illnesses. Outstandingly, aqueous extracts, in most cases, are the ones utilized by traditional practitioners. Consequently, the coalescence of this plant's crude extract at the employed concentration was sheltered, thus legitimizing the continued utilization in customary pharmaceuticals.

McLaughlin et al. (1998) quoted that a plant with less toxicity and equivalently good coalescence of exertive phytochemicals makes it a good plant for use in customary pharmaceuticals compared to one with high toxicity and less toxicity coalescence of exertive phytochemicals.

The middling toxic impact of *O. kirkii* extracts was observed, namely, on aqueous crude plant extracts, it was 0.411 mg/mL, while organic extracts were 0.207 mg/mL. This study reports for the first time the toxicity of this plant. However, it is not in accordance with another member of the same genus, *Ormocarpum trichocarpum* (0.072 mg/mL), which is poisonous (Moshi et al. 2006). Mbaya (1976) reported that the bark of *S. alata* has some toxicity because of its impact on the liver. Mbaya's results concur with the results of this study which demonstrates the middling toxicity of inorganic extract of *S. alata* (0.399 mg/mL) and aqueous extract of *S. alata* (0.317 mg/mL). *C. holstii* organic extracts uncovered high toxicity (0.009 mg/mL). This was similar to Adepado et al. (2008) on *Cussonia paniculata*, a member of the same genus, which showed that the plant had caused 80% mortality in rats. However, the fluid extracts of this plant showed weak toxicity quality (0.543 mg/mL). Extracts of the plants from organic solvents could have shown higher toxicity because the trace amounts of the solvents are still in them. Practically, it needs thorough "washing" to remove the solvents.

Phytochemical vetting

Phytochemical vetting of bioactive constituents demonstrated that the extracts were rich in auxiliary metabolites. All plant extracts exhibited nearness of flavonoids, sterols, alkaloids, tannins, quinones, and terpenoids. This study's higher number of phytochemicals in organic extracts most likely clarified their comparatively better antimicrobial potential. Phytochemical vetting of extracts of *H. forskahlii* uncovered the existence of flavonoid sterols, alkaloids, tannins, quinones, and terpenoids in both water and aqueous extracts, while saponins were missing in organic and extracts of this plant. Flavonoids and terpenoids phytoconstituents in the stem bark of *H. forskahlii* had been recorded before (Al-Rehaily et al. 2008; Kajangwe et al. 2008).

Even though *O. kirkii* had low antimicrobial activity, it was wealthier in secondary metabolites, such as flavonoids, alkaloids, tannins, quinones, and terpenoids saponins in all the extracts. From *O. kirkii*, other observers have detached a series of known flavonoids and biflavonoids, as well as chamaejasmin, biliquiritigenin, and isovitexin naringenin (Dhoogle et al. 2010; Xu et al. 2011). A phytochemical examination of *C. holstii* uncovered the existence of saponins, flavonoids, alkaloids, tannins, quinones, and terpenoids. Pentacyclic triterpenoid, i.e., hederagenin, has been archived for its antitrichomonas action (He et al. 2003). S. alata extracts displayed flavonoids, alkaloids, tannins, quinones, and terpenoid saponins in all extracts. The phytochemicals of this plant were first recorded in this study. Nevertheless, the existence of antibacterial alkaloids in another member of the same genus, Schrebera swietenioides by Niranjan et al. 2010 concurs with this study. The existence of secondary metabolites in this plant may attribute to the recognized biological activity (Odhiambo et al., 2014). Flavonoids, saponins, tannins, and

terpenoids have been reported to have antimicrobial activity and are currently utilized for cough and diarrhea (Talib and Mahasneh, 2010; Khan et al., 2012). Cytotoxicity, antiviral and antimicrobial activities of alkaloids and flavonoids have also been accounted for (Ozcelik et al. 2011). Antibacterial activity and cytotoxic impacts of steroids have been recorded in their usage as arrow poisons (Doughari 2006). The existence of antibacterial substances in higher plants are well entrenched (Bhalodia and Shukla 2011). Plants have given a source of inspiration for novel medication mixtures as plants derived medicines have made a huge contribution to human health.

In conclusion, the bioactivity of organic and aqueous extracts of *S. alata, O. kirkii*, *C. holstii,* and *H. forskahlii* against bacterial and fungal strains is critical, with only organic extracts of *H. forskahlii* displaying the most elevated antimicrobial activity similar to a positive control (Chloramphenicol) against MRSA. Generally, flavonoids, sterols, alkaloids, tannins, quinones, and terpenoids were available in all four plant species and tested positive for saponins content except for organic extracts *of H. forskahlii,* which is a lack saponin. Organic crude extracts of *H. forskahlii* and *C. holstii* were found to be highly toxic (0.009 mg/mL). The study also shows that only aqueous extracts of H. forskahlii of the evaluated crude extracts were non-toxic to *Artemia salina*, ascertaining value to the selected medicinal plant for continued use in ethnomedicine. Among the studied plant extracts, methanol extracts of *H. forskahlii* had overriding toxicity over other plant extracts. From this study, *H. forskahlii* is a better source of antimicrobial agents. Hence, it can be mainly of interest in developing new chemotherapeutic drugs. For the first time, this study reports an antimicrobial activity and toxicity of *Schrebera alata* and *Cussonia holstii.*

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