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RESEARCH PAPER

The Detection of Phytochemical Properties from Freeze Dried *Ottochloa nodosa* (Kunth) Dandy Extracts and Its Potential as a Bacterial Inhibitor

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Abstract

This study aimed to detect the secondary metabolites and chemical components in the freeze-dried extract of *Ottochloa nodosa* (Kunth) Dandy. In addition, discovering the extract could have potential as a bacterial inhibitor by determination of the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) carried out in extracts. Determination of antimicrobial potential of freeze-dried extracts was conducted through phytochemical screening, gas chromatography-mass spectrometry (GC-MS) test, agar disc diffusion and broth dilution methods. The study shows this extract has bacterial inhibitors' potential since it contained phytochemical compounds such as alkaloid, saponin, flavonoid and tannin, while 9 chemical components have been displayed through GC-MS analysis. The disc diffusion method was done at different concentrations, at 25, 50 and 100 mg/ml. The result also shows that the extracts are able to inhibit the gram-positive and gram-negative bacteria. Freeze-dried extract presented significant differences in 100 mg/ml and 50 mg/ml, p=0.05. The MIC value was found at 2.0 mg/ml in the agar plate containing methicillin-resistant *S. aureus* (MRSA), *Aeromonas hydrophila* and *Staphylococcus aureus*, while *Vibrio parahaemolyticus* was at 5.0 mg/ml. While the MBC value showed 2.0 mg/ml in *MRSA* and *S. aureus* (since the plates containing *A. hydrophila* and *V. parahaemolyticus* had bacterial growths, thus, it demonstrated that the plant extracts were only able to inhibit the bacterial growth but could not kill them). In conclusion, the freeze-dried method can be applied for water removal during plant extraction and possess bacterial inhibition activity. This study implicated that *O. nodosa* extract is a natural source of antibiotic substances for bacterial infection treatment.

Keywords *Ottochloa nodosa*, secondary metabolites, bacterial inhibitor, freeze-dried, zone inhibition

INTRODUCTION

Infectious diseases remain a public health priority, both in Malaysia and globally. They are widely expressed their concern about this abjection matter since it is able to distract the life quality of those got infected, either humans or animals. Disastrously, it can be transmitted from one individual to another as those pathogenic microorganisms, such as bacteria, viruses,

parasites or fungi are easy to grow and find everywhere. They may be in water, air, soil, humans and animal bodies (NIH, 2009). Accordingly, among the biggest challenges of the 21st century is to discover better protection against infectious diseases which have always been a scourge to human beings and their companion animals. The most exciting and challenging time for scientific discovery is to exploit the natural substances in the production process. The objective is to produce a useful product for the benefit of all.

Thus, plants are considered a vital source for natural product development and are explored continuously especially therapeutically for human well-being. They are known in containing a variety of secondary metabolites with antimicrobial properties, such as tannins, terpenoids, alkaloids, and flavonoids (Chakraborty and Brantner, 1999; Cowan, 1999; Bisignano et al., 2000; Setzer et al., 2000; Al-Momami et al., 2007; Bouzada et al., 2009; Sohail et al., 2011). That special features are believed can contribute great significance to the treatment of various microbial infections. Moreover, instead of acting as antimicrobial, they are also contributing to other beneficial medical cases for instance having the ability to act as anti-cancer, antioxidant, antidiarrheal, analgesic and wound healing (Cosa et al., 2006). Shakinaz et al (2020) also proved in their research that natural sources such as plants contribute to various industries for instance food, chemical and pharmaceuticals.

Around 20000 plant species are used in traditional medicines and they are prospective reservoirs for new pharmaceuticals (Amor et al., 2009). Many of them and their extracts which are known as therapeutic phytochemical may lead to the development of novel drugs (Azwanida, 2015). It also may function as a defense mechanism against many microorganisms, insects and herbivores (Tanaka et al., 2000). Various studies have been done in different countries to verify plants have a role as a bacterial inhibitors. The traditional healers have applied the plant-based treatment to prevent or cure the infection (Johnson et al., 2011). Besides, a review related to the potential of secondary metabolites as an antibacterial agent has been studied (Compean and Ynalvez, 2014). People are easily exposed to bacterial infections since microorganisms are available naturally in our surrounding environment. They can easily enter our bodies, animals as well as others around us. For instance, bacteria might be infected the food during food processing (Hatab et al., 2016) such as during harvesting, slaughtering or packaging, then, causing health problems. Therefore, scientists aim to extract biological compounds found in plants since they showed good prospects in medical fields.

Since the phytochemicals components and antimicrobials activities of local nature plants which known have a great significance of medicinal infective diseases with minimum or maybe possibility no side effects compared to the current antimicrobial agents such as antibiotics (Samiha et al., 2020). They updated on the adverse effects of antimicrobial therapies in community practice which found that severe and serious harms have been described for commonly used antibiotics. Besides, the current knowledge of harms from antibiotics that are regularly used in family medicine is also discussed in their study. Moreover, Shekhar and Petersen (2020) mentioned the dark side of antibiotics; adverse effects on the infant's immune defense against infection. Thus, *Ottochloa nodosa* that belongs to the Poaceae family has been targeted in this study. Fundamentally, its common names are slender panic grass, *rumput rawa* and *rumput sarang buaya*. In global distribution, *O. nodosa* occurs in India, Indonesia, Malaysia, Myanmar, Guinea, Philippines, Sri Lanka, Thailand, Vietnam, Africa, Australia, and the Pacific Islands (Bisignano et al., 2000). It spreads naturally, but it can be planted by seeds or rooted culms. Moreover, it contains nitrogen concentrations range typically between 1.1% and 1.3%, with DM (dry matter) digestibilities between 38% and 50% (Manidool, 1989). It becomes problematic for humans and environment since it was regarded as a troublesome weed. The rapid growth rates of this plant are increasing and expanding from time to time making the public find that the grass is affecting the people and ecosystem in the area especially in terms of the cost to remove the grass and the development of aquatic plants and animals.

Hence, this research study is an effort to contribute and assist in solving the conjecture about the above issues which are also related to the development of bacterial inhibitors from natural sources and explore more profiles about this plant species.

MATERIALS AND METHODS

Plant collection and preparation

O. *nodosa* was collected at the surface of Tasik Raban, Lenggong, Perak and brought to the laboratory on the same day. The fresh plant was rinsed thoroughly several times with running tap water and then freeze-dried. Freeze dry is a method that applied the sublimation principle, in which a solid form is directly converted into a gas phase (Liu et al., 2008). Then, it was kept in the container, sealed and labeled and put in the freeze-drying machine for three days (72 hours) before proceeding to the next process. During this process, the samples were frozen at -21°C in a freeze dryer machine and were freeze-dried to a moisture content of 5-6% (w. b.) at an absolute pressure of 85-90 Pa with a chamber temperature of 20°C and a condenser temperature of -48°C.

Nutrient agar preparation

28 grams of NA was mixed with 1liter distilled water and stirred with a magnetic stirrer on an electric hot plate. The mixture was sterilized by autoclaving at 121°C for 15 minutes. After cooling to a temperature around 48°C-45°C, NA medium was dispensed into sterile Petri plates. Then, the Petri plates were sealed, labeled and kept in an inverted position at 4°C.

Bacterial sample

The bacteria in this study were *Escherichia coli, Salmonella typhi, Vibrio parahaemolyticus, Aeromonas hydrophila, Staphylococcus aureus, Streptococcus pyogenes, Bacillus cereus,* and *MRSA*. They were obtained from the laboratory of the Department of Biology, Sultan Idris Education University. The bacteria were subcultured in a laminar flow disinfected using 70% ethanol.

Extraction of crude from plant

About 20 g plant sample was extracted with 200 ml of methanol, n-hexane, and acetone for 72 hours. The crude extracts were filtered by using Whatman filter paper No. 1 and concentrated on a rotary evaporator. The crude was suspended in water-saturated in a separatory funnel. Methanol, n-hexane and acetone portions were separated and collected from the aqueous portion. The extracts of the crude plant were autoclaved, then collected and preserved in a refrigerator (Ndip et al., 2007).

Experimental control

For the agar disc diffusion method, the experiment control was tested by a disc that dissolved in methanol solution. For the broth dilution method, the experimental control contains the mixture of medium nutrient solution without extract of crude extract plants.

Phytochemical screening

Phytochemical analysis was performed to screen the extract of *O. nodosa,* for the presence of tannins, saponins, flavonoids, and alkaloids. The qualitative phytochemical analysis was carried out by using the standard procedure to identify the constituents (Harborne, 1973; Trease and Evans, 1989a; Sofowara, 1993).

(i) Saponin test

About 5.0 ml of the extract of *O. nodosa* was boiled in 20 ml of distilled water in a water bath and filtered. Then, 10 ml of the filtrate was mixed with 5.0 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously. After that, the formation of emulsion, which confirms a positive presence of saponin was observed.

(ii) Tannin test

One (1.0) ml of *O. nodosa* extract was boiled in 20 ml of water in a test tube. Then the sample was filtered. A few drops of 0.1% ferric chloride were added. The changes of color sample for brownish green or a blue-black coloration indicate the presence of tannins.

(iii) Alkaloids test

One (1.0) ml of the extracts was stirred with 5.0 ml of 1% aqueous hydrochloric acid (HCL) on a steam bath and filtered while hot. Distilled water was added to the residue and 1.0 ml of the filtrate was treated with a few drops of Mayer's reagent. The formation of a cream color gives a positive test for alkaloids.

(iv) Flavonoids test

Three (3.0) ml of 1% aluminum chloride solution were added to 5.0 ml of each extract. A yellow coloration was observed indicating the presence of flavonoids. 5.0 ml of dilute ammonia solution were added to the above mixture followed by the addition of concentrated sulfuric acid. A yellow coloration that disappeared on standing indicates a positive test for flavonoids.

Analysis of extract using GC-MS

Methanol, n-hexane, and acetone as solvent extraction had been involved in order to determine the chemical components contained in *O. nodosa*. The extracts were sent to the chemistry lab for analyzing using GC-MS, model Agilent 5975. The extract was analyzed using the following condition: column: *Dimethylpolysiloxane DB-I ciated fused silica capillary column* (30 m x 0.25 mm); carrier gas: He (1 mL/min); injector temperature: 250°C; detector temperature: 200°C; column temperature: 35-180°C at 4°C/min and 180-250°C at 10°C/min; mass spectra: electron 70 Ev. The chromatogram result was then analyzed. Moreover, their molecular structure, molecular mass, and molecular formula were obtained from NIST web (Table 1, 2 and 3).

Paper disc diffusion method

The Kirby-Bauer and Stokes' methods are usually used for antimicrobial susceptibility testing (Lalitha, 2004). Disc made from filter paper (Whatman No 1) which has a diameter 5.5 mm. The disc was soaked in a solution that has a different concentration of crude plant extracts of *O. nodosa* and placed on the agar surface in the Petri dish. The plates were stored inverted and incubated at 37°C for 24 hours. Then, the diameter of the inhibition zone for every disc was observed, measured and recorded. The diameter showed the potential of crude extract of plants to inhibit the bacteria and function as a bacterial inhibitor. Besides, the control plates were also prepared for each test organism without the addition of extracts.

Broth dilution method

(i) Minimum Inhibitory Concentration (MIC)

Serial dilution of the crude plants representing different concentrations was added to a growth medium in separate test tubes. The test bacteria were inoculated in the test tube. The test tube was incubated overnight. The changes in color solution in test tubes were observed and recorded. The analysis was done by observing the changes in broth dilution. It was compared with the control group. The negative inhibitor showed a clear color but for the positive; the

broth dilution becomes cloudy (Rollins et al., 2003). MIC was recorded that the lowest concentration of extract showed no visible growth of the broth.

(ii) Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentration (MBC) was determined by aseptically inoculating aliquots of culture from MIC tubes that show no growth, on sterile nutrient agar plates. The tubes were incubated at 37°`C for 48 hours. MBC was recorded as the lowest concentration of extract showing no bacterial growth. The analysis of MBC was determined by comparing the experimental group and control group.

Statistical analysis

Independent Samples T-Test ($p<0.05$) has been used in this study to determine the significant difference in extract from *O. nodosa* in diameter of inhibition zone in control and experimental group as antibacterial agent.

RESULTS AND DISCUSSION

Bioactive constituents in *O. nodosa* crude extract

The phytochemical screening test shows the secondary metabolites that are presence in *O. nodosa* plant crude extracts are saponin, tannin, flavonoid and alkaloid. The presence of saponin is observed by the formation of a stable froth on the filtrate (Ashour et al., 2019). Saponins are glycoside compounds (Tanaka et al, 2000). The antibacterial activity of saponins is affected by factors such as the aglycone, number, position, and chemical structure of sugar chains (Rakhimov et al., 1996). At the same concentration, saponins able to show more antimicrobial activity against gram-positive and gram-negative bacteria. Saponins act as antibacterial by blocking the membrane ion channel on cell membrane permeability (Santos et al., 2014). Then, it caused the reduction of nutrient intake from the cell, blocks genetic information for reproduction, and attacks the bacteria. It was proven by the inhibition zone on the agar plate that resulted in this present study when the freeze-dried crude extract of *O. nodosa* was tested to against the bacteria (Figure 1 and 2). The result shows that *S. aureus, MRSA, A. hydrophila,* and *V. parahaemolyticus* were inhibited due to their cell walls have been ruptured by the interaction of saponin. This result also is supported by Karlina et al. (2013), saponin is able to suppress the growth of bacteria by reducing the surface tension of the cell walls and causing the cell walls lysis.

While the presence of tannins in the sample is observed by changes in a color sample for brownish green or a blue-black coloration after an addition of a few drops of 0.1% ferric chloride. Tannins are polyphenols that bind and precipitate protein. The mode of action is a possibility due to the ability of these bioactive constituents to inactive microbial adhesions, enzymes and envelope transport protein that was mentioned by Shito (2006). In this study, *MRSA* is found to have been inhibited after being incubated 24 hours by 2 mg/ml *O. nodosa* crude extract (value of MIC), as well as MRSA, also was killed by 2 mg/ml of crude extract (MBC value) (Table 4.3). This finding is supported by Takashi et al (1995) who reported that tannin has been classified as bacteriostatic or bactericidal against bacteria by its activity that able to against *MRSA*. The mechanism of action is by synthesis with the cell wall and membrane disruption.

Moreover, the presence of alkaloids is detected by testing the plant extract with Mayer's reagent. Turbidity of the resulting precipitate indicated the presence of alkaloids (Harborne, 1973; Trease and Evans, 1989a; Sofowara, 1993). Alkaloids are also called heterocyclic nitrogen compounds. Codein and heroin were both derivatives of morphine. Diterpenoid alkaloids were commonly isolated from the plants of the Ranunculaceae or buttercup family, commonly found to have antimicrobial properties. Besides, he also mentioned that a

glycoalkaloid, Solamargine from the berries of *Solanum khasianum* and other alkaloids might be useful against HIV infection as well as intestinal infections associated with AIDS. Besides, the major classes of the antimicrobial compound in alkaloids are berberine, quaternary alkaloids that proved have antibacterial (Dai and Mumper, 2010). The mechanism of action in berberine is intercalated with DNA (Robert and Wink, 1998).

Furthermore, flavonoids are detected by observation of the yellow coloration disappeared on standing after a few seconds. Flavonoids are hydroxylated phenolic substances molecules include occur as an aromatic ring on either side of a 3-carbon ring (Dixon et al, 1983). Multiple combinations of hydroxyl groups, sugars, and oxygen and methyl groups attached to these structures create the various classes of flavonoids. They are known to be synthesized by the plant in response to antibacterial infection. Thus, it is to be an effective antibacterial substance against a bacterial. The mechanism of action can be classified as the inhibition of nucleic acid synthesis, synthesis of cell wall, cytoplasmic membrane function and energy metabolism (Cushine and Lamb, 2005). Flavonoids present in this study are able to support the crude extracts against the bacteria tested. *S. aureus*, *MRSA*, *A. hydrophila*, and *V. parahaemolyticus* since the interaction of bacterial cells with antibacterial compounds caused the cell structure of bacteria damaged and patterns of inactivation of bacteria may affect, thus the function of cellular metabolism was disrupted.

Significantly, the benefits in terms of the medicinal effects of plant materials typically result from the combination of secondary products present in the plants. *O. nodosa* crude extract was proved to have potential as a bacterial inhibitor as it showed many benefits especially against bacterial infections. The bioactive compounds have great potential in pharmaceutical and industrial applications. These compounds promote the health of the cardiovascular system and provide protection against several types of cancer (Mello et al., 2008). Besides, the statement has been supported by Venugopal and Liu (2012), since most of the phytochemicals from plant sources such as phenolic and flavonoids have been confirmed to have a positive impact on health and cancer prevention. Additionally, those secondary metabolites have been reported to confer resistance to plants against bacteria, fungi and pests (Alabi et al., 2012) and therefore explained the demonstration of antibacterial activity by the plant that used in this study.

Chemical components in *O. nodosa* extract using GC-MS

GC-MS analysis for freeze-dried *O. nodosa* crude extracts presented 9 active components has been obtained from this plant. There were 9,12-octadecadienoate acid (*Z, Z*)-, 6-octadecanoic acid, methyl, 9,12-heptadecadienoate, *trans*-13-octadecanoic acid, 9,17-octadecadienal, *cis*-vaccenic acid, butyl 9,12-octadecadienoate, octadecanoic acid, and oleic acid (Table 1, 2 and 3).

Table 1. Chemical components obtained from methanol extract of *O. nodosa*

Components	**Peak area, %**	**Molecular Formula**	**Molecular Mass**
9,12-octadecadienoic acid (Z, Z)-	32.25	$C_{18}H_{32}O_2$	280.45
6-octadecanoic acid	26.84	$C_{18}H_{36}O_2$	284.48
methyl 9,12-heptadecadienoate	12.17	$C_{19}H_{34}O_2$	294.47
trans-13-octadecanoic acid	13.96	$C_{19}H_{36}O_2$	296.49
9,17-octadecadienal	2.35	$C_{18}H_{32}O$	264.45
cis-vaccenic acid	1.59	$C_{18}H_{34}O_2$	282.46
butyl 9,12-octadecadienoate	1.36	$C_{22}H_{40}O_2$	336.55
octadecanoic acid	1.13	$C_{18}H_{36}O_2$	284.48

Table 2. Chemical components obtained from n-hexane extract of *O. nodosa*

Components	Peak area, %	Molecular formula	Molecular mass
9,12-octadecadienoic acid (Z, Z)-	23.71	$C_{18}H_{32}O_2$	280.45

Table 3. Chemical components obtained from acetone extract of *O. nodosa*

Components	Peak area, %	Molecular formula	Molecular mass
cis-vaccenic acid	40.90	$C_{18}H_{34}O_2$	282.46
oleic acid	38.77	$C_{18}H_{34}O_2$	282.47
9,12-octadecadienoic acid (Z, Z)-	17.22	$C_{18}H_{32}O_2$	280.45

Importantly, the data obtained in this present study were supported by the previous research report conducted on the same family (Poaceae). *Urochloa paucispicata* contained fatty acid methyl esters which showed the presence of palmitic, oleic, linoleic and stearic acids that are significant components of the lipid fractions (Morrone et al., 2000). Then, declared that an active component of 9,12-octadecadienoic acid *(Z, Z)*, 49.75% which is present in plant extract has the potential as antimicrobial (Manilal et al., 2009). Moreover, Poaceae plants such as *Desmostachya bipinnata*, *Hordeum vulgare* and *Drepanostachyum falcatum* showed a total of 10, 32, and 28 chemical compounds (Nepal et al., 2018). The major compounds included n-hexadecanoic acid, benzoic acid, and ethyl ester showed antibacterial activity.

Furthermore, Faustino et al. (2019) reported that halophytic grasses (a green plant that grows naturally on the earth's surface and has very thin leaves and grows close together in large numbers). The halophytes, Poaceae (Gramineae) family, possessed antibacterial and antifungal activities because of chemical components such as fatty acids that are presented in their extracts. Additionally, according to Goerch et al (2019), oleic acid in *Eragrostis teff* (an Ethiopian native grass) extract exhibited high nutritional value that strengthened its potential as a food supplement to promote and maintain health. So, that study supported this research finding and consequently was able to contribute to the medical application and accordingly have a potency for commercialization as a new alternative for bacterial inhibitor.

The potential of *O. nodosa* as a bacterial inhibitor

Two (2) types of bacteria that have been tested in this study, gram-positive and gram-negative bacteria are actually to determine the degree of difficulties to kill the bacteria or inhibit their growth. Three (3) different concentrations (25, 50, 100 mg/ml) of plant extracts were against eight bacteria (*MRSA, Salmonella typhi, Escherichia coli, Vibrio parahaemolyticus, Bacillus cereus, Streptococcus pyogenes, Aeromonas hydrophila,* and *Staphylococcus aureus*). Zone of inhibition was observed and recorded after 24 and 48 hours in units of a millimeter (mm) as shown in Figures 1 and 2.

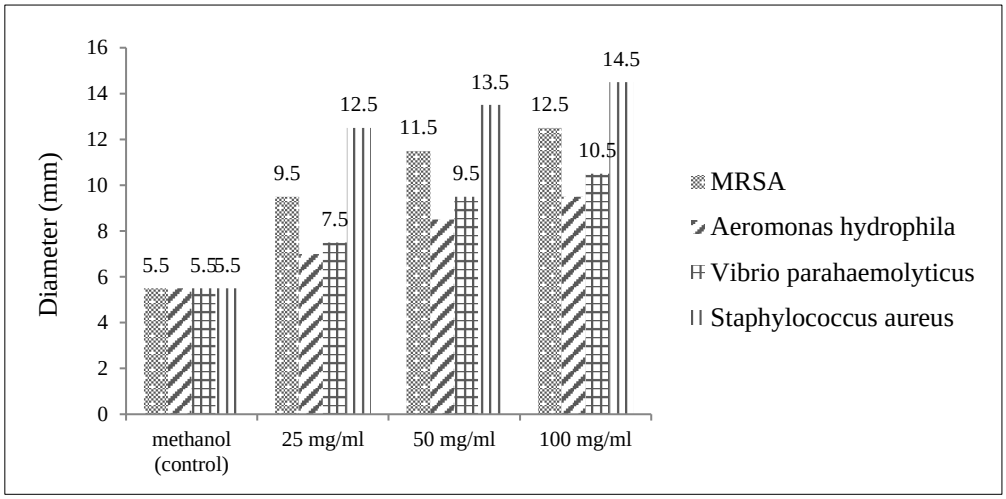

Figure 1. Diameter of inhibition zone (mm) of bacteria on different concentrations of freeze-dried *O. nodosa* plant extracts after 24 hours.

Figure 1 were demonstrated that *S. aureus* showed the higher diameter of zone inhibition (14.5 mm in 100 mg/ml), (13.5 mm in 50 mg/ml) and (12.5 mm in 25 mg/ml), followed by *MRSA* (12.5 mm in 100 mg/ml), (11.5 mm in 50 mg/ml), and (9.5 mm in 25 mg/ml). *V. parahaemolyticus* showed their inhibition zone were 10.5 mm in 100 mg/ml), (9.5 mm in 50 mg/ml), and (7.5 mm in 25 mg/ml). While *A. hydrophila* gave (9.5 mm in 100 mg/ml), (8.5 mm in 50 mg/ml), and (7.0 mm in 25 mg/ml). The other four bacteria tested (*E. coli, S. typhi, S. pyogenes and B. cereus*) gave the negative results in the agar disc diffusion test for those three different concentrations and did not show in this graph. The control group (methanol) showed that the diameter of the inhibition zone remained 5.5 mm after 24 hours.

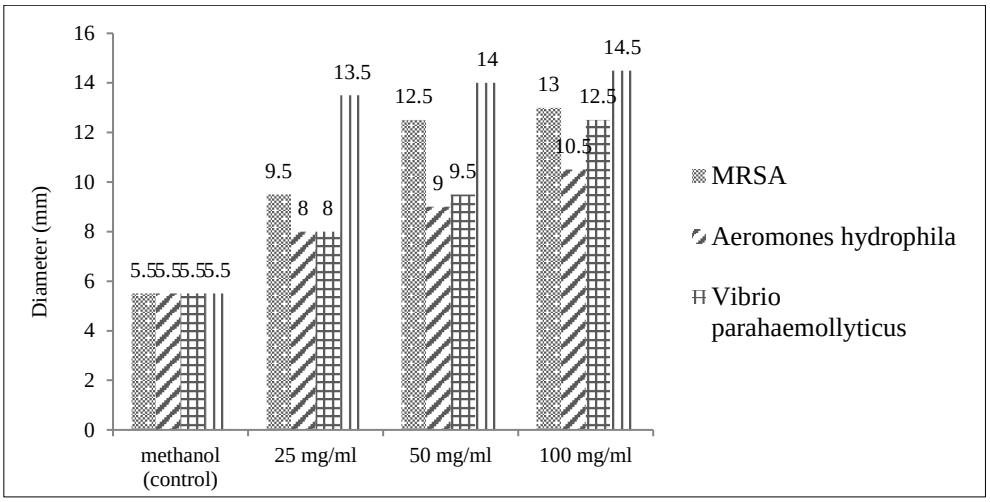

Figure 2. Diameter of inhibition zone of bacteria on different concentration of freeze dried *O. nodosa* extracts after 48 h

Moreover, the observation was proceeding for 48 hours (Figure 2). Two different times were tested to compare the changes in the inhibition zone, either increase, decrease, or remain. It showed that most inhibition zone of each bacteria tested was increased from the 24 hours observation. *S. aureus* (14.5 mm in 100 mg/ml), (14.0 mm in 50 mg/ml), and (13.5 mm in 25 mg/ml). *MRSA* (13.0 mm in 100 mg/ml), (12.5 mm in 50 mg/ml), and (9.5 mm in 25 mg/ml). Then, *V. parahaemolyticus* (12.5 mm in 100 mg/ml), (9.5 mm in 50 mg/ml), and (8.0 mm in 25 mg/ml), while *A. hydrophila* (10.5 mm in 100 mg/ml), (9.0 mm in 50 mg/ml), and (8.0 mm in 25 mg/ml). The other four bacteria tested (*E. coli, S. typhi, S. pyogenes and B. cereus*) gave the negative results in the agar disc diffusion test for those three different concentrations and did not show in this graph. The control group remained unchanged, 5.5 mm. Significantly, all the data that shown approved that *O. nodosa* crude extract through disc diffusion method, have a potential as a bacterial inhibitor since *MRSA, Vibrio parahaemolyticus, Aeromonas hydrophila* and *Staphylococcus aureus* were inhibited after 24 hours of observation. The evidence was also displayed by the formation of the clear zone for the bacteria tested (Figure 3). This finding then revealed that both conditions of *O. nodosa* extract possessed an effective and equipotent bacterial inhibitor against both gram-negative and gram-positive bacteria. Also, it was indicated that this plant could be a source of bioactive substances which could be of a broad spectrum of activity.

It showed that different test bacteria species showed a variation in their resistance and sensitivity to the plant extract. Besides, the observation time also affects the value of the inhibition zone. The possible reason for the different bacteria species tested gave the various activity possibly lies in their morphological differences.

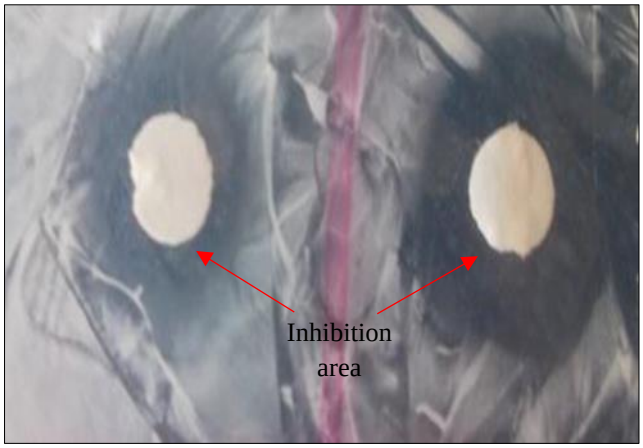

Figure 3. Diameter of inhibition zone of *S. aureus* after 48 h on different concentrations of *O. nodosa* extract

For example, *Aeromonas hydrophila*, gram-negative bacteria have an outer phospholipid membrane making their cell wall impermeable. On the other hand, *S. aureus*, gram-positive bacteria lack this membrane and are thus more permeable. In addition, during 24 and 48 hours (Figure 1 and 2) observation, the diameter of the inhibition zone for control (methanol) remained at 5.5 mm while the extract (50 mg/ml and 100 mg/ml) showed it can inhibit the bacterial growth. The value of significant for 50 mg/ml was 0.044 and 100 mg/ml was 0.048 which p=0.05 (Table 4). Thus, the hypothesis, there was a significant difference between the control group and experimental group in determining the potential of crude extracts from freeze-dried *O. nodosa*.

Table 4. Comparison of diameter zone of inhibition on experimental group and control group in different concentrations of *O. nodosa* extract

	Control group (methanol)	**Experimental group (25mg/ml)**	**Experimental group (50mg/ml)**	**Experimental group (100mg/ml)**
Mean	5.500	6.083	6.750	7.167
Standard deviation		1.429	1.604	2.041
Significant		0.122	*0.044	*0.048

Replicate=3, Df=5, p=0.05; *Significant concentration of *O. nodosa* extract

(ii) The Minimum Inhibitory Concentration (MIC)

The observation of the test tube after 24 hours has been recorded as listed in Table 5. In 0.01 mg/ml, all the bacteria showed the result very cloudy (++) that similar to the control test tube.

Table 5. The observation of MIC after 24 h

Conc. of crude extracts (mg/mL)	**Growth of bacterial**			
	MRSA	***A. hydrophila***	***V. parahaemolyticus***	***S. aureus***
10	-	-	-	-
5	-	-	-	-
3	-	-	+	-
2	-	-	+	-
1	+	+	++	+
0.1	++	++	++	++
0.01	+++	+++	+++	+++
control	+++	+++	+++	+++

Clear= (-), cloudy= (+), very cloudy= (++), most cloudy= (+++)

Test on *MRSA*, *A. hydrophila*, and *S. aureus* indicated that the concentration of crude extract from *O. nodosa* in 2 mg/ml, 3 mg/ml, 5 mg/ml, and 10 mg/ml test tubes are clear. It confirmed that the extraction of those concentrations could inhibit bacterial growth. But, concentrations of 1.0 mg/ml, 0.1 mg/ml, and 0.01 mg/ml showed the presence of bacterial

growth, and the test tubes become cloudy. The result is similar to the control test tube. It proved that the bacteria was growing in the test tube. Fundamentally, minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after incubating overnight. The results presented that the value of MIC from *O. nodosa* plant extract for *Staphylococcus aureus, MRSA* and *Aeromonas hydrophila* was 2.0 mg/ml while the MIC value for *Vibrio parahaemolyticus* was 5.0 mg/ml. The result proved that the low doses of bacterial inhibitor were required to inhibit *Staphylococcus aureus, MRSA,* and *Aeromonas hydrophila,* while the higher dose was needed to inhibit the growth of *Vibrio parahaemolyticus*. Gram-positive bacteria such as *S. aureus* were inhibited more easily than *V. parahamolyticus*, gram-negative bacteria by crude extracts may be due to the bacteria itself characteristics.

The major difference between the two groups is the thickness of the cell wall and the presence of an outer membrane that is gram-negative only (Prochnow et al., 2016). The existence of the murine layer in gram-negative bacteria covered by a lipopolysaccharide layer which makes the bacterial cells more resistant to the plant extracts. Besides, the MIC results from this study showed that by increasing concentration has increased efficiency in inhibiting the organism used. Gram-negative bacteria are much more dangerous and labeled as pathogenic compared to gram-positive bacteria so it needs higher concentration to inhibit their activity. According to Bais et al. (2002) and Hasan et al. (2007), since the mechanism of action of this extract was not understood yet, they proposed that its action against the bacteria and fungi may be due to the inhibition of cell wall formation in the cell resulting in a leakage of cytoplasmic constituents by the bioactive components of the extract. Then, the value of MIC was different among others possibly due to the reason of certain bioactive compounds within the plant that enable it to inhibit the bacteria growth at a low concentration (Abdullah et al., 2012). Furthermore, the determination of MIC value is very important in analytical laboratories to validate the resistance of a microorganism to an antimicrobial agent as well as knowing the MIC value for a certain bacterial inhibitor was beneficial in order to save the cost and amount the extract that need to be used by a scientist in the pharmaceutical industry as well as to prevent any side effects such as allergen problems if it was used in excessive quantities.

(iii) The Minimum Bactericidal Concentration (MBC)

This study was proceeding for the determination of MBC since the ability of bacterial inhibitors is just not to inhibit but their ability to kill the bacteria. It was important to investigate whether the *O. nodosa* crude extract was bactericidal (bacteria-killing) or bacteriostatic (bacteria–inhibiting). The broth dilution method was performed from the MIC test by sub-culturing to agar media without antibiotics and incubated for 24 hours. Table 6 showed that no bacteria grew on an agar plate with *S. aureus* or *MRSA* after being incubated 24 hours. It indicated that the lowest concentration that has the potential to kill the *S. aureus* and *MRSA* was 2 mg/ml. While there were bacterial growths on the agar plate containing *A. hydrophila* and *V. parahaemolyticus* after an incubation period. Thus, it demonstrated that the plant extracts could inhibit bacterial growth only but could not kill them.

Table 6. The observation of bacteria growth of MBC after 24 h from MIC test tube with no bacterial growth

Type of bacteria	**The growth of bacteria in different concentration of crude extract**			
	Concentration (mg/ml)			
	10	**5**	**3**	**2**
MRSA	-	-	-	-
A. hydrophila	+	+	+	+
V. parahaemolyticus	+	+	+	+
S. aureus	-	-	-	-

No bacterial growth= (-), bacterial growth= (+)

The plate that contained *Staphylococcus aureus* and *MRSA* which also showed the value of MBC, proved that *O. nodosa* crude extract is bactericidal (bacteria-killing) on both organisms, while *Aeromonas hydrophila* and *Vibrio parahaemolyticus* were bacteriostatic (bacteria –inhibiting). Bactericidal (bacteria-killing) such as *MRSA*, have a thinner cell wall composed of cellulose-like structure sugar polymer covalently bound to short peptide units in layers. The peptidoglycan structure is made of repeating units of N- acetylglucosamine linked b-1, 4 to N-acetylmuramic acid (NAG-NAM). The peptide begins with L-Ala and ends with D-Ala. Diaminopimelate (DAP) provides a linkage to the D-Ala residue on an adjacent peptide. The bacterial cell wall is completed when a cross-link of two peptide chains attached to polysaccharides backbone is formed. The catalyst in the cross-linking is enzyme transpeptidase. The terminal alanine from each peptide is hydrolyzed and then one of the alanine is joined to lysine through an amide bond. Thus, the values of MIC and MBC, it able to indicate that either the plant extract was low effective on bacteria or the bacteria have the potential for developing antibiotic resistance.

CONCLUSION

The primary phytochemical analysis revealed that the freeze-dried *O. nodosa* crude extracts contained some secondary metabolites such as saponin, tannin, flavonoids, and alkaloids, which could be responsible for the observed antimicrobial property. Those bioactive compounds are known to act by a different mechanism and exert antimicrobial action. Besides, the GC-MS analysis were displayed that this plant extract contained 9,12-octadecadienoic acid (*Z, Z*), 6-octadecanoic acid, *trans*-13-octadecanoic acid, 9,17-octadecadienal, *cis*-vaccenic acid, butyl9,12-octadecadienoate, octadecanoic acid, methyl 9,12-heptadecadienoate and oleic acid. They were also contributing to the role of bacterial inhibitors. This extract showed the potency of antimicrobial activity against both gram-positive and gram-negative bacterial strains tested through inhibition zone's results that obtained from the agar disc diffusion test. Furthermore, the MIC value was found within the range (2.0-5.0 mg/ml) as the minimal concentration required for inhibition, while the value of MBC revealed that *MRSA* and *S. aureus* were bactericidal (bacteria-killing), while *A. hydrophila* and *V. parahaemolyticus* were bacteriostatic (bacteria-inhibiting) in this research. The findings supported the previous indication that this extract contained active constituents responsible for antimicrobial activity.

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REFERENCES

Abdullah, S., Shaari, A.R. & Azimi, A. (2012). Effect of drying methods on metabolites composition of misai kucing *(Orthosiponstamineus*) leaves. *APCBEE Procedia*, *2*, 178-182.

Alabi, O.A, Haruna, M.T., Anokwuru, C.P., Jegede, T., Abia, H., Okegbe, V.U., & Ehsan, B.E. (2012). Comparative studies on antimicrobial properties of extracts of fresh and dried leaves of *Carica papaya* (L) on clinical bacterial and fungal isolates. *Advances in Applied Science Research*, *3*(5), 3107-3114.

Al-Momami, W., Abu-Basha, E., Janakat, S., Nicholas, R.A. & Ayling, R.D. (2007). *In vitro* anti mycoplasmal activity of six Jordanian medicinal plants against three Mycoplasma species. *Tropical Animal Health and Production*, *39*, 515-519.

Amor, I.L.B., Boubaker, J., Sgaier, M.B., Skandrani, I. & Bhouri, W. (2009). Phytochemistry and biological activities of Phlomis species. *Journal of Ethnopharmacology, 125*,183-202.

Ashour, A.S., El Aziz M.M.A, & Melad, A.S.G. (2019). A review on saponins from medicinal plants: chemistry, isolation, and determination. *Journal of Nanomedicine Research*, *8(1),* 282-288.

Azwanida, N.N. (2015). A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Medicinal and Aromatic Plants Research*, *4*, 196.

Bais, H.P., Walker, T.S., Schweizer, H.P. & Vivanco, J.M. (2002). *Plant Physiology and Biochemistry*, *40*, 983-995.

Bisignano, G., Sanogo, R., Marino, A., Aquino, R., D'Angelo, V., Germanò, M.P., De Pasquale, R., & Pizza C. (2000). Antimicrobial activity of *Mitracarpus scaber* extract and isolated constituents. *Letters in Applied Microbiology*, *30*, 105-108.

Bouzada, M.L.M., Fabri, R.L., Nogueira, M, Konno, T.U.P., Duarte, G.G., & Scio, E. (2009). Antibacterial, cytotoxic and phytochemical screening of some traditional medicinal plants in Brazil. *Pharmaceutical Biology*, *47*, 44-52.

Chakraborty, A. & Brantner, A.H. (1999). Antibacterial steroid alkaloids from the stem bark of *Holarrhena pubescens*. *Journal of Ethnopharmacology*, *68*, 339-344.

Compean, K.L. & Ynalvez, R.A. (2014). Antimicrobial activity of plant secondary metabolites: A review. *Research Journal of Medicinal Plants*, *8*(5), 204-213.

Cosa, P., Vlietinck, A.J., Berghe D.V., Maes, L. (2006). Anti-infective potential of natural products. How to develop a stronger in vitro 'proof-of-concept'. *Journal of Ethnopharmacology*, *106*, 290-302.

Cowan, M.M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, *12(4)*, 564-582.

Cushine, T.P.T. & Lamb, A.J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, *26*, 343-356.

Dai, J. & Mumper, R.J. (2010). Plant phenolics: extraction, analysis and their antioxidant and anti-cancer properties. *Molecules*, *15*, 7313-7352.

Dixon, R.A., Dey, P.M. & Lamb, C.J. (1983). Phytoalexins: enzymology and molecular biology. *Advances in Enzymology*, *55*, 1-69.

Faustino, M.A.F., Faustino, M.A.F., & Pinto, C.G.A. (2019). Halophytic grasses, a new source of nutraceutical? A review on their secondary metabolites and biological activities. *International Journal of Molecular Sciences*, *20*, 1-30.

Goerch, M.C.D.S., Schafer, L., Tonial, M., Oliveira, V.R.D., Ferraz, A.B.D.F., Fachini, J., Silva, J.B.D., & Harborne, J. (1973). *Phytochemical methods: a guide to modern techniques of plant analysis*. Chapman & Hall, London.

Hatab, S., Athanasio, R., Holley, R., Rodas-Gonzalez, A., & Narvaez-Bravo, C. (2016). Survival and reduction of shiga toxin-producing *E. coli* in a fresh cold-pressed juice treated with antimicrobial plant extracts. *Journal of Food Science*, *81*, 1987-1995.

Johnson, D.B., Shringi, B.N., Kumar, D., Sai, N. & Chalichem, S. (2011). Screening of antimicrobial activity of alcoholic & aqueous extract of some indigenous plants. *Journal of Pharmaceutical Sciences*, *1(2)*, 186-193.

Karlina, C.Y., Ibrahim, M. & Trimulyono, G. (2013). Aktivitas antibakteri ekstrak herba krokot (*Portulaca oleracea* L.) terhadap *Staphylococcus aureus* dan *Escherichia coli*. *E- Journal Unesa LenteraBio*, *2*, 87-93.

Lalitha, M.K. (2004). *Manual on antimicrobial susceptibility testing*. American Society for Microbiology, USA.

Liu, Y., Zhao, Y., & Feng, X. (2008). Exergy analysis for a freeze-drying process. *Applied Thermal Engineering*, *28*, 675-690.

Manidool, C. (1989). *Natural grassland and native grasses of Thailand.* Technical bulletin no 1301-26-32.

Manilal, A., Sujith, S., Sengah, S., Selvin, S.K., & Shakir, C. (2009). Cytotoxic potentials of Red Alga, *Laurencia brandenii* collected from the Indian Coast. *Global Journal of Pharmacology*, *3*(2), 90-94.

Mello, V.J., Gomes, M.T, Lemos, F.O., Delfino, J.L, Andrade, S.P., Lopes, M.T., & Salas, C.E. (2008). The gastric ulcer protective and healing role of cysteine proteinases from *Carica candamarcensis*. *Phytomedicine*, *15*, 237-244.

Morrone, O., Vega, A.S., & Maier, M. (2000). Elaiosomes in *Urochloa paucispicata* (Poaceae): anatomy and chemical composition. *Flora*, *195*, 303-310.

Ndip, R.N., Malange, A.E., Tarkang, A.E., Mbullah, S.M., Luma, H.N., Malongue, A., Ndip, L.M., Nyongbela, K., Wirmum, C., & Efange, S.M. (2007). *In vitro* anti *Helicobacter pylori* activity of extracts of selected medicinal plants from North West Cameroon. *Journal of Ethnopharmacology*, *4*, 452-457.

Nepal, P., Singh, M., Baniya, A., Singh, S., Sainju, H.K., Shresta, R. (2018). Comparative antioxidant, antimicrobial, and phytochemical assessments of leaves of *Desmostachya bipinnata* L. Stapf, *Hordeum vulgare* L. and *Drepanostachyum falcatum* (Nees) Keng f. *Nepal Journal of Biotechnology*, *6*(1),1-10.

NIH. (2009). *Understanding microbes in sickness and in health.* National Institute of Allergy and Infectious Disease, U.S. department of health and human services. NIH publication no.09-4914.

Prochnow, A.M., Maryse, C., Hong, J. & Murphy, A.B. (2016). Gram positive and gram- negative bacteria differ in their sensitivity to cold plasma. *Scientific Reports*, *6*, 38610.

Rakhimov, R.R., Benetis, N.P., Lund, A., Hwang, J.S., Prokofer, A.I., & Lebeder, Y.S. (1996). Intramolecular and reorientation dynamics of bis(triphenylphosphine)-3,6-di-tert-butyl-4,5-dimethoxy-o-semiquinone complex of copper (I) in viscous media. *Chemical Physics Letters*, *225*, 156-162.

Robert, M.F. & Wink, M. (1998). *Alkaloids: biochemistry, ecology and medicinal applications.* New York, Springer.

Rollins, D.M., Temenak, J.J., Shields, P., & Joseph, S.W. (2003). Microbial pathogenesis laboratory manual, 2nd Edition.

Samiha, M., Dickinson, J.A., & Somayaji, R. (2020). Update on the adverse effects of antimicrobials therapies in community practice. *Mohsen, S., Dickinson, J. A., & Somayaji, R. (2020).* Update on the adverse effects of antimicrobial therapies in community practice. *Canadian Family Physician Medecin de Famille Canadien, 66*(9), 651-659.

Santos, C.C., Guiné, R.P., & Barros, A. (2014). Effect of drying temperature on the phenolic composition and antioxidant activity of pears of *Rocha variety* (*Pyrus Communis* L.). *Journal of Food Measurement and Characterization, 8*(2), 105-112.

Schito, G.C. (2006). The importance of the development of antibiotic resistance in *S. aureus*. *Clinical Microbiology and Infection, 1,* 3-8.

Setzer, W.N., Setzer, M.C., Bates, R.B., & Jakes, B.R. (2000). Biologically active triterpenoid of *Syncarpia glomulifera* bark extract from Paluma, North Queensland, Australia. *Planta Medica, 66*, 176-177.

Desa, S., Khalaf, A.S.A., Baharum, S.N., & Mudhafar, M. (2020). Identification of bioactive compounds from seeds oil of agricultural waste in Malaysia. *International Journal of Pharmaceutical Sciences Review and Research, 13*, 1-8.

Shekhar, S. & Petersen, F. C. (2020). The dark side of antibiotics: adverse effects on the infant immune defense against infection. *Frontiers in Pediatrics*, 8, 54440.

Sofowara, A. (1993). *Medicinal plants and traditional medicine in Africa*. Spectrum Books Ltd, Ibadan, Nigeria.

Sohail, M.N., Karim, A., Sarwar, M., & Alhasin, A.M. (2011). Onion (*Allium cepa* L): An alternate medicine for Pakistani population. *International Journal of Pharmacology, 7,* 736-744.

Takashi, O., Cai, Z., Toda, M., Hara, Y., & Shimamura, T. (1995). Appearance of antibacterial activity of oxacillin against *MRSA* in the presence of catechin. *Kansenshogaku Zasshi, 69,* 1126-b4.

Tanaka, O., Ikeda, T., Ohtani, K., Kasai, R., & Yamasaki, K. (2000). Antiyeast steroidal saponins from *Yucca Schidigeria* (*Mohave Yucca*), a new anti-food-deteriorating agent. *Journal of Natural Products, 63,* 332-338.

Trease, G.E. & Evans, W.C. (1989a). *A textbook of pharmacognosy*. Bailliere Tindall Ltd, London.

Vaghasiya, Y., Dave, R. & Chanda, S. (2011). Phytochemical analysis of some medicinal plants from Western region of India, *Research Journal of Medicinal Plants, 5*, 567-576.

Venugopal, R. & Liu, R.H. (2012). Phytochemicals in diets for breast cancer prevention; the importance of resveratrol and ursolic acid. *Food Science and Human Wellnesslness, 1*, 1-13.