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Antibacterial Property of *Moringa oleifera* Seed Extract: Comparative Study of Crude Extract and Membrane Processed Extract

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In this research, the antibacterial property of both crude and membrane processed seed extracts were investigated against *Escherichia coli, Pseudosomonas aeruginosa Bacillus subtilis* and *Staphylococcus aureus*. The defatted seeds were prepared with water and the water extract obtained was passed through 0.45μm microfiltration membrane system. The antibacterial activity was done using the agar well diffusion. The effect of concentration of the extracts were determined, the Minimum Inhibitory Concentration (MIC) as well as Minimum Bactericidal Concentration (MBC) was carried out. The result showed that the diameter inhibition zone ranges from 13mm to 30mm and increase in the seed extracts showed a corresponding increase in the inhibition of the bacterial strains. Statistical difference was found (P = 0.002) when the mean of antibacterial activity of the membrane processed seed extract against the bacterial strains was compared with the crude seed extract. Statistical results showed that membrane processed extract was more effective than the crude extract. The results of MIC and MBC values of membrane processed extract seed obtained shows that it was bactericidal against *Pseudosomonas aeruginosa, Bacillus subtilis*, and bacteriostatic against *Escherichia coli Staphylococcus aureus* respectively.

Key words: *Moringa oleifera*, microfiltration, antibacterial activity, MIC, MBC.

Water is essential to life and all forms of life depend on it. The availability of good drinking water has been the most critical factor for survival throughout the development of life. Safe drinking water remains inaccessible for about 1.2 billion people in the world, and about 400 deaths of children (below age 5) are recorded on hourly toll from biological contamination of drinking water. Consumption of unsafe, untreated water contaminated with pathogens has led to about 1.5 million deaths each year especially in the developing countries and a number of these problems are detection of coliform bacteria in drinking water[1,2].

Disinfection is an essential part of drinking water treatment, which is usually done at the end of the drinking water treatment train. Commonly used disinfectant such as chlorine produces disinfection by-products, which are carcinogenic and harmful. Trihalomethanes including carcinogen chloroform, halo acetone derivatives that include dichloro and bromo-acetonitrile are among the by-products detected. These compounds are formed when chlorine reacts with organic compounds in water such as amino acids[3]. These chlorinated compounds are very difficult to degrade and can cause the same hazards as chlorine itself. It is becoming increasingly

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difficult to ignore the potentially hazardous by-products that emerge when using conventional disinfectants, thus there is a need to explore organic alternatives for water treatment[4].

*Moringaoleifera*is a well-known plant material locally used for the treatment of drinking water. Its seed kernels contain a significant amount of oil that is commercially known as Ben oil or Behen oil. The oil extracted from the seeds can be used for food preparation, lubrication of machines and cosmetics. *Moringaoleifera* seed's oil content and its properties show a wide variation depending mainly on the species and environmental conditions. The oil contains high level of oleic acid of about 78.59% and about 21.41% of others such as palmitic acid, stearic acid, behenic acid and arachidic acid[5]. The seed extracts contain active agents having excellent coagulation properties[6]and an active antimicrobial agent ascribed to plant synthesized derivatives of benzyl isothiocyanates known as 4 (á-L- rhamnosyloxy) benzyl isothiocyanate was identified and about 8-10% of this compound is present in both defatted (after removing oil) seed and crude seed[5]. This antimicrobial active agent has been reported to exert in vitro bactericidal activity against both gram positive and gram-negative bacteria in raw water[7]. While exhaustive research has been done on the use of the seed extract as a great pharmaceutical substitues for treating infectious ailment, little research as been conducted on its application as a disinfectant in water. Although most resarch has reported the use of the crude seed extract in the coagulation of water by removing suspended particles and reducing harmful bacteria cells[8]. The major setback in using crude *moringaoleifera* seed extract for water treatment is the high organic load released as a result of the oil. The presence of these organic matters leads to problem of colour, taste, odour and even regrowth of microorganisms during storage[6]. Removing these organic loads is facilitated using microfiltration membrane system to enhance isolation and purification of bioactive compounds.

So far, there has been little research on disinfectant ability of the seed extracts after removing the organic matter by microfiltration membrane system. Hence, antibacterial activity of extracts obtained using microfiltration membrane system has never been explored. The objectives of this research are to check for the effect of increase in concentration of crude seed extracts and membrane processed extracts against the selected microbes, compare the antibacterial activity of the crude seed extract and membrane processed seed extracts on two gram-positive bacterial strains namely *Staphylococcus aureus*, *Bacillus subtilis* and two gram-negative bacterial strains *Eschreiashia coli*, *Pseudomonas aeruginosa* by usingpaired *t* test analysis in IBM SPSS software, to determine the minimum inhibitory concentration as well as the minimum bactericidial concentration of the extract that inhibited the microbes most was determined.

MATERIALS AND METHODS

Processing of *Moringaoleifera*seeds powder

Good quality dry seeds of *Moringaoleifera* were selected and the seed coat and wings were removed manually. The kernel was ground to fine powder using the coffee mill attachment of the *Khind*domestic food blender. The grounded seed powder was then sieved through 210µm sieve and appropriate quantity of the powder was weighed.

Crude *Moringaoleifera* seed extract

Moringaoleifera seed powder is weighed with no defatting (removal of oil) and added to 1 liter of distilled water and mixed at high speed of 6000 rpm in a centrifuge for 10 minutes then filtered to remove un-dissolved particles with the filtrate used to make stock solution of 50mg/mL.

Oil extraction from *Moringaoleifera* seed

Oil was removed from the seed by using electro thermal soxhlet extractor (ROSS, UK) and the procedure used is as follows: weighing of 10 gm of *Moringaoleifera*seed powderand setting it in the thimbles of the electro thermal soxhlet extraction chamber; adding 170 ml of normal hexane in the heating chamber; Evaporating of hexane within three cycles each for 30 minutes to ensure the extraction of oil from the; drying of *Moringaoleifera*cake residue from the soxhlet thimbles and weighting the dry sample[9]. The *Moringaoleifera*cake residue stock after oil extraction was used for aqueous extraction.

Microfiltration membrane extraction of defatted *Moringa*seed powder

This was carried out by adding 5gm of defatted *Moringaoleifera* seed powder to 100mL,

mixed at high speed of 6000 rpm in centrifuge for 10 minutes. This was filtered through Whatman filter paper No. 1 to remove un-dissolved particles. The filtrate was passed through 0.45micron microfiltration membrane system (Quix Stand Benchtop System). The permeate from the membrane system was collected to make stock solution of 50mg/mL[6].

Bacterial strains

Bacteria strains (*E.coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*) were locally obtained from stock present in the Microbiology laboratory of Biotechnology Department, International Islamic University Malaysia and maintained on LB (Merck, Germany) slants. The strains were sub-cultured very two weeks and stored at 4°C.

Preparation of inoculum

The microorganisms used were taken from a pure culture of the respective bacteria strains grown on slants and inoculated into 10mL of rich medium Luria-Bertani (LB) broth (Merck, Germany). The broth suspension was incubated at 37°C overnight and the growth obtained was used as inoculums. Their inoculum density was set to 0.1 $OD_{600.}$

Preparation of media

The preparation ofLB broth (Merck, Germany) was carried out according to manufacturer's instruction. 25 grams of LB broth was weighed and dissolved in 1 Litre of distilled water. Then the solution was autoclaved at 121 ^{0}C for 15minutes. After autoclaving, the solution was stored at 4 °C in the chiller. Also, preparation of LB agar was done according to manufacturer's instruction. 37 grams of LB agar was weighed and dissolved in 1 Litre of distilled water. The solution was autoclaved for 15 minutes at 121 ^{0}C. The solution after autoclaving was allowed to cool down then poured into petri dish to solidify under aseptic conditions.

Effects of various concentrations of seed extracts

The effects of seed extracts on the bacterial strains were also studied. In this experiment, different concentrations of the seed extracts (1-5mg/ml) were tested against all the bacterial strains (0.1 OD) using the agar well diffusion test. The diameter zone of inhibition in millimeter (mm) was used to determine the effect of various concentration of the seed extract.

Determination of Antibacterial activity of *Moringaoleifera*seed extracts

The antibacterial activity of *Moringa* seeds extract was done against four selected bacteria strains using the agar well diffusion method. The LB agar plates were seeded with suspension (10^6cfu/mL) of the bacterial strains. Wells of 9mm in size was dug inside the seeded agar plate and 100µL of various concentrations of *Moringa*seeds extracts was pipette inside the wells. Negative control using distilled water was used and chlorine 5% was used as positive control to determine the sensitivity of the bacterial strains. The plates were incubated at 37^{0}C for 24 hours. The antibacterial activity was evaluated by measuring the diameter of inhibition zones in mm. The procedure was repeated thrice for each of the extract solutions produced.

Determination of Minimum Inhibitory Concentration (MIC)

This is expressed as the lowest dilution that inhibited growth judged by lack of turbidity in the tube. This was done using the two-fold broth dilution method[10]. Thirteen screw-capped test tubes (13mm × 100mm) were sterilized and numbered individually. 1 mL of LB broth was introduced into tubes 2 to 11. To tube 12, 2 mL of LB broth was introduced. 1 mL of sample will be introduced to tube 1 and 2 and vortex for 5 seconds. 1 mL of the sample will be withdraw and introduced to tube 3. This process will be continued until the last tube. To tube 13, 1 mL of chlorine was added as positive control. The tubes were incubated for 18hours at 37°C. Initial concentration of 50mg/mL was diluted to 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.09 and 0.048mg/mL in LB broth. All the tubes were inoculated with 0.1mL of 0.1 OD bacterial strains and incubated for 18hrs.

Determination of Minimum Bactericidal Concentration (MBC)

This was determined by collecting loopful of broth form the MIC test determination. 0.01ml of contents of MIC tubes was sub-cultured by streaking on LB nutrient agar plates. Then the plates were incubated at 37 °C for 24 hours. After the incubation, the concentration with no visible growth was noted as the minimum bactericidal concentration. The tube with the lowest concentration of *moringa* extract that gave no visible growth or turbidity was selected.

Statistical analysis

IBM SPSS 20 for windows software was used for the statistical management. The diameter zone of inhibition for the crude extract and membrane processed extract was compared using the paired t-test to determine their statistical significance. The level of significance was set at 0.05 for the statistical test.

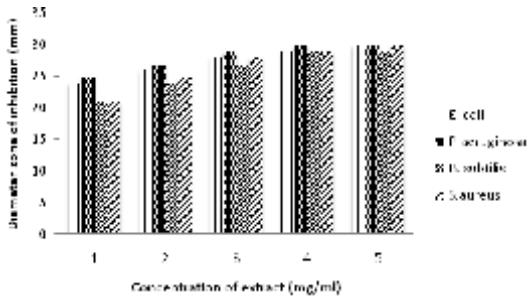

Fig. 1. Effect of membrane processed seed extract concentration on bacterial strains

RESULTS

Effect of seed concentration

The antimicrobial susceptibility testing is important to confirm susceptibility to chosen antimicrobial agents, or to detect resistance in individual bacterial strains[11]. The concentrations of the membrane-processed seed extracts produced

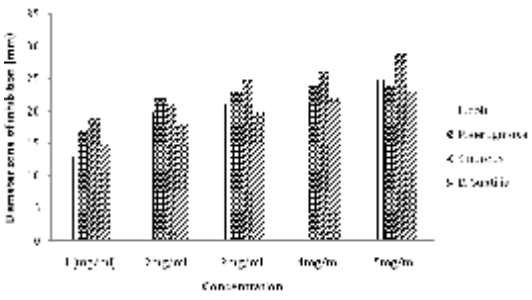

Fig. 2. Effect of crude seed extract concentration on bacterial strains

ranged from 1mg/ml to 5mg/ml were tested on four bacterial strains by using the agar well diffusion method and their diameter zone of inhibition was measured in millimeters (mm). Figure 1 and Figure 2 shows the effect of the seed concentration of the membrane processed extract and crude extract respectively.

Comparison of crude extract and membrane processed seed extract

The comparison between the crude extract and processed membrane extract was evaluated using IBM SPSS software by comparing the mean of diameter zone of inhibition of the all microorganisms. The result shown in table 1 shows the sample statistics as obtained using IBM SPSS.

The paired correlation results obtained from IBM SPSS is shown in table 2

Minimum inhibitory concentration and minimum bactericidal concentration

This is usually referred to as the optimum concentration required for effective inhibition of pathogens. For compounds that require long

Table 1. Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Crude	25.75	4	1.70783	.85391
	Membrane	28.50	4	1.29099	.64550

Table 2. Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Crude - Membrane	-2.75	.50	.25	-3.54561	-1.95439	-11.0	3	.002

contact time for bacterial inactivation, Minimum Inhibitory Concentration (MIC) is commonly used to characterize their effect on controlling microbial growth[11]. The results of the MICs of the membrane processed seed extract as well as the MBCs against the bacterial strains are shown in Table 3 below.

Table 3. MIC and MBC of membrane processed seed extract

Bacterial strains	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
E.coli	0.78	3.125	4
P.aeruginosa	1.56	3.125	2
B.subtilis	3.125	6.25	2
S.aureus	3.125	12.5	4

DISCUSSION

As a general rule, plant seed extracts are considered active and effective against both bacteria and fungi when the zone of inhibition is greater than 6mm[12]. Thus, the diameter zone of inhibition ranges from 13mm to 30mm which indicates the seed extracts are very active and effective against all the bacterial strains as shown in Figure 1 and Figure 2. The seed extracts (both crude and membrane processed) inhibited the bacterial strains to a greater extent regardless of the concentration thus this indicates that the bacterial strains are susceptible to the extracts. This is in close agreement with earlier research conducted by[13,14,15] where similar phenomenon was observed. Research conducted earlier have shown that the seed contains mostly proteins[16,17] hence the results obtained indicates that the extract possesses antibacterial property which might be due to the proteins present which are lipophilic in nature that bind within the cytoplasmic membrane of the microbes which led to their death[18]. Membrane processed extracts inhibited more of the microbial strains than the crude extract as shown in Fig. 1. This could be as a result of more isolation of the bioactive compounds form the seed extract in which the microfiltration process removes impurities from the seed extract. Increasing the concentration of the extracts to 5mg/mL led to a corresponding increase in the diameter zone of inhibition indicating that the bacterial cells are killed. This result corroborate the findings of[19] whose findings also showed that more bacterial strains were killed as the concentration of *Moringaoleifera* seed extract increased. Generally, gram positive bacteria should be more susceptible since they have an outer peptidoclycans layer which is an ineffective barrier[20], however, the results of this study was contradictory. Thus, it is believed that polyssacharide capsular material present in these microorganisms is responsible for their resistance[21].

For the comparison of the mean of diameter zone of inhibition for the crude extract and membrane processed extract for the gram negative bacteria strains (*E.coli* and *P.aeruginosa*) were only considered since they were mostly inhibited, the results are summarized as shown in Table 1 and Table 2 using a correlated group *t* test. The results summarized in Table 3 shows a negative value of mean difference, standard deviation, t-value which has no effect because these parameters are reported in their absolute value. The result for the mean paired group difference is 2.75 with standard deviation of 0.5 and a t-value of 11 as summarized in Table 2. The P value obtained is less than 0.05 (P =0.02) shows that the mean of the extracts are significant hence membrane processed extracts is more effective than the crude extract.

From the results, membrane processed extracts inhibited the microorganisms more as indicated from the diameter zone of inhibition hence its MIC and MBC was determined. The values of MIC obtained were 0.78mg/ml, 1.56mg/ml, 3.125mg/ml and 3.125mg/ml for *E.coli, P.aeruginosa, B. subtilis,* and *S.aureus* respectively as summarized in Table 3. The MBC values obtained were 3.125mg/mL, 3.125mg/mL, 6.25mg/mL, and 12.5mg/mL for *E.coli, P.aeruginosa, B.subtilis,* and*S.aureus* respectively. The evaluation of the bactericidal and bacteriostatic capacity of the seed extract was determined according to the ratio MBC/MIC. If the ratio MBC/MIC is 1 or 2, the effect is considered bactericidal and when the ratio is 4 or 16, it is considered bacteriostatic[21]. As shown in Table 3, the extract was bactericidal against *P.aeruginosa*

and *B.subtilis* while bacteriostatic effect against *E.coli* and *S.aureus.*

CONCLUSIONS

The results showed that the membrane processed extracts of *Moringaoleifera* seed possess antibacterial property and the diameter zone of inhibition ranges from 13mm to 30mm. At high concentration of the seed extract 5mg/ml, the diameter zone of inhibition was about 30mm for all the bacterial strains and the diameter zone of inhibition increased as the concentration of the seed extracts increased. Statistical difference between the crude extract and the membrane processed extract was significant ($P<0.05$). The membrane processed seed extract showed high diameter zone of inhibition as a result of reduced organic matter which enhanced the isolation of more bioactive compound present in the seed hence increased the inhibition against the bacterial strains. The membrane processed extract was bactericidal against, *Pseudosomonas aeruginosa*, *Bacillus subtilis*, and bacteriostatic aganist *Escherichia coli Staphylococcus aureus* respectively.

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