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A Comparative Effect of the Alcoholic and Aqueous Extracts of *Acacia mearnsii* De Wild on Protein Leakage, Lipid Leakage and Ultrastructural Changes in Some Selected Bacterial Strains as Possible Mechanisms of Antibacterial Action

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In this study, the possible mechanisms of action of acetone, methanol and aqueous extracts of *Acacia mearnsii* were investigated. The influences of these extracts on the ultrastructures, protein and lipid leakages of five different bacteria were determined. The results showed that the different extracts had varied effects on the different isolates treated while the untreated isolates remained intact. On the extract-treated isolates, the extracts caused significant ultrastructural changes, protein and lipid leakages. There were disruptions in the outer wall and cytoplasmic membranes, especially, at the polar regions of the cells, whole cell collapse and presence of extruded cellular materials situated close to the collapsed end of the cytoplasm. The morphological changes and the observed leakages showed significant antibacterial effect and membrane disruptions resulting in leakages and efflux of disintegrated cellular materials. The distinct morphological changes such as cell elongation and roughening of the surfaces suggested that the extract may have interfered with bacterial cell wall synthesis leading to cell deformations. While aqueous extract was the most effective in causing protein leakages, methanol extract was the leading cause of lipid leakages. The leakages were time and concentration dependents and, in some instances, significantly different from extract to extract. The possible mechanism of action involved in the lipid and protein leakages in the bacterial cells could be attributed to lipid peroxidation and protein oxidation owing to the antioxidants activities of the extracts being beyond protective levels. The study showed that the different extracts of *A. mearnsii* had bactericidal effects against the test isolates, caused ultrastructural changes and leakages resulting from the disruption of the cytoplasmic membranes of the bacterial populations.

Key words: Scanning electron microscopy, ultrastructure, leakages, Lipid peroxidation, mechanism of action.

Medicinal plants have been the traditional source of raw materials for medicine readily available for use by the local populace. In many developing countries, using medicinal plants as an alternative medicine is well known[1,2]. They have been used to treat infectious diseases in most parts of the world for thousands of years[3] because of their antiseptic and bactericidal properties[4] and their eventual important roles in drug development in pharmaceutical industry[5]. In many parts of the world, the extracts of medicinal plants are used as antibacterial, antifungal and antiviral agents[6]. The successful prediction of their antimicrobial activities has been largely dependent on the type of solvent used in the extraction procedure. While alcohol and aqueous extraction methods are often adopted

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by traditional medical practitioners, the degree of antibacterial activities of medicinal plants have been attributed to the solubility of their phytochemicals in the solvents used in the extraction procedures[7]. Since medicinal plants could be a rich sources of antimicrobial agents[8] and the systematic screening of these plants may result in the discovery of novel effective compounds[9,10], determining their mechanisms of action becomes necessary for the proper elucidation of the degree of their bactericidal activities.

Acacia mearnsii de Wild, Fabaceae, indigenous to South Eastern Australia, was introduced to South Africa over 150 years ago for the tanning industry[11]. Being an invasive plant known as a threat in South Africa, it is considered an economic plant requiring scientific intervention for control[11,12] because it affects hydrological balances in areas where found[13]. Although it is a plant of economic value because of its high tannin content, there is a dearth of information on its pharmacological importance. However, its ethnopharmacological importance as a medicinal plant with astringent property and significant antimicrobial activities had been reported by Olajuyigbe and Afolayan[14,15]. Resulting from the reported antimicrobial activities, we have, in this study, made a comparative analysis of the possible mechanisms of the antibacterial activities of this plant. Hence, this study was designed to investigate the comparative effects of the alcoholic and aqueous extracts of *A. mearnsii* on protein leakage, lipid leakage and ultrastructural changes in some selected bacterial strains. Since knowledge on the mechanisms of action may allow the rational development of antimicrobial agents that target cell functions, visual information may be useful in providing insight on the ultrastructural changes in the cell, assist in characterizing the type and magnitude of changes occurring in cell composition in response to treatments with the extracts and help to understand how and why a treatment is bactericidal or bacteriostatic against a particular organism.

MATERIALS AND METHODS

Bacteria used for the study

The bacteria used in this study included *Escherichia coli* (ATCC 8739), *Shigella flexneri* (KZN), *Proteus vulgaris* (ATCC 6830), *Staphylococcus aureus* (ATCC 6538) and *Bacillus pumilis* (ATCC 14884). These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa.

Collection of plant material

The bark materials of *Acacia mearnsii* De Wild were collected in August, 2010, from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen (OLAJ Med 2010/01) was prepared and deposited in the Griffen Herbarium of the University.

Extract preparation

The bark sample was air-dried at room temperature and pulverized using a milling machine. The extracts of the bark material were prepared according to the description of Basri and Fan[16]. About 100 g of the pulverized sample was extracted with 500 ml of acetone, methanol and water for 48 h with shaking (Stuart Scientific Orbital Shaker, UK). The extracts were filtered through Whatman No. 1 filter paper. While the alcoholic extracts were concentrated under reduced pressure at 40^{o}C using a rotary evaporator (Laborota 4000 – efficient, Heldolph, Germany), the aqueous extract was obtained by freeze drying. The crude alcoholic extracts collected were allowed to dry at room temperature to a constant weight. The extracts were redissolved in their respective solvents before being diluted with sterile distilled water to the required concentrations for bioassay analysis.

The reconstituted extract solution was sterilized by filtering through 0.45 µm membrane filter and tested for sterility after membrane filtration by introducing 2 ml of the extract into 10 ml of sterile nutrient broth before being incubated at 37^{o}C for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period.

Determination of the effect of alcoholic and aqueous extracts on cell morphology

Overnight broth cultures of the different bacteria strains were standardized at 10^6 cfu/ml using McFarland Nephe-lometer standard [17]. One milliliter of the adjusted bacterial culture was added to 9 ml of each of the plant extracts at 4 x MIC and kept at laboratory temperature (28^{o}C) for 2 h.

Untreated controls were prepared in Mueller Hinton broth medium and incubated for the same period of time. The bacterial cells were harvested by centrifuging at 10000 rpm for 10 min and subsequently washed with phosphate buffer (pH 7.2). The bacterial pellets were re-suspended in 2.5% glutaraldehyde and kept for 2 h to fix the bacterial cells. The fixed bacterial cell suspensions were centrifuged at 10000 rpm for 10 min, washed and re-suspended in buffer (pH 7.2) from where the cells were then deposited on a 0.45-µm-pore-size membrane filter (Schleicher & Schuell, Dassel, Germany). The bacterial cells on membrane filters were dehydrated with a graded series of acetone/water washes (20%, 50%, 70%, 90% and 100% acetone) to gradually remove water from the cells without physical damage. The dehydrated bacterial cells were mounted on a stub, allowed to dry before being sputtered with a small amount of gold/palladium using Ion coater: EIKO IB.3 to avoid charging in the microscope. Microscopy was performed with a JEOL JSM-6390LV Scanning Electron Microscope (Japan). Secondary electron images were taken with an accelerating voltage of 15kV.

Determination of Protein leakage from bacteria cells

To determine the leakage of intracellular materials from the cells, the bacteria cells were treated with the 1 x MIC and 2 x MIC of the extract and the samples were incubated at 37°C for 120 min. Immediately the bacteria was introduced into the extract solutions and at an interval of 1 h incubation period, 1 ml of the bacteria-extract mixture was centrifuged at 12000 rpm for 10 min. Ten microlitre (10 µl) of the supernatant was dispensed into 96 - well microtitre plate and 250 µl of Bradford's reagent was added[18]. The mixtures were incubated at room temperature for 15 min with intermittent shaking. The amount of protein released from the cells was determined spectrophotmetrically at 525 nm with Bovine serum albumin (BSA) as a standard.

Determination of Lipid leakage from bacteria cells

Phospho-vanillin was prepared as previously described by Frings and Dunn[19]. 0.6 g of Vanillin (Sigma Chemical Co., St. Louis, Mo. 63118) was dissolved in 10 ml of absolute ethanol before diluting to 100 ml with distilled water. This solution was mixed with 400 ml of concentrated phosphoric acid with constant stirring before being stored at room temperature in a brown bottle. The lipid leakage assay was carried out using the method described by van Handel and Day[20] and Kaufmann and Brown[21]. Briefly, bacterial cells were harvested from an overnight broth culture by centrifuging at 10000 rpm for 10 min and used to prepare 10^8 cfu/ml. The standardized cell suspension was treated with 1 x MIC and 2 x MIC of the extract and the samples were incubated at 37°C for 120 min. After incubating at 37°C for 30 min, each cell suspension was sampled at 30 min interval and centrifuged at 10000 rpm for 10 min. 0.2 ml of sulfuric acid was added to replicated small portions of the supernatant (10 µl) in tubes and heated in water bath for 10 min at 100°C. Vanillin-phosphoric acid reagent was added to the mixture before being removed from water bath, vortexed and allowed to cool while the optical density was measured at 525 nm. The concentration of lipid leakage was estimated from linoleic acid standard curve used as standard.

Statistical analysis

Data were expressed as means ± standard deviations (SD) of three replicate determinations and then analyzed by SPSS V.16 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL). One way analysis of variance (ANOVA) and the Duncan's New Multiple-range test were used to determine the differences among the means. P values < 0.05 were regarded to be significant.

RESULTS

In this study, the possible mechanisms of action of acetone, methanol and aqueous extracts of *Acacia mearnsii* was investigated. The influences of these extracts on the ultrastructures, protein and lipid leakages from five different bacterial isolates including *Escherichia coli* (ATCC 8738), *Shigella flexneri* (KZN), *Proteus vulgaris* (ATCC 6830), *Staphylococcus aureus* (ATCC 6538) and *Bacillus pumilis* (ATCC 14884) were determined. The results showed that the different extracts had varied effects on the different isolates. The effects of the different extracts on the surface morphology of the different bacteria during its logarithmic growth phase were shown in Figures 1 – 5 where the degree of antimicrobial activities on the surface morphology of individual

cells and bacterial populations were indicated. In Figures 1 - 5, the broth-cultured, harvested and untreated bacteria retained their morphological features. *E. coli*, *S. flexneri*, *P. vulgaris* and *B. pumilis* cells were typically rod-shaped and the cells surfaces were relatively smooth while the *S. aureus* was typically spherical in shape. All the *E. coli* cells treated with acetone extract (Fig. 1B) showed significant effect of the extract. Most cells were collapsed with mild depressions on their surfaces. While some cells were wrinkled with rough surfaces, others had pores depicted by scattered dots of extruded intracellular materials on their surfaces while some intact but affected cells bulged with round tapering ends. As a result of the depressions, the extruded cellular materials were either clustered at one of the polar end of the cell or sparsely scattered on the cell surfaces. In *E. coli* treated with methanol extract (Fig. 1C), higher percentages of cells were structurally and badly damaged. Many cells had holes or pores on their surfaces but with a distinct pore at a polar end of the cell or just a little away from the polar end. Some cells were collapsed in the middle without extending to the polar ends. Some cells were elongated with wrinkled surfaces. Many cells had the extruded cellular materials scattered on cell surfaces or cluster to a spot in some cells. Where the extruded materials accumulated on a location, they were rarely found on other parts of the cell

Table 1. Comparative analysis of the protein leakage ability of each extract from each bacterial strain

	Mean ± Standard Deviation of the protein leaked (µg/ml) from each bacterial strain									
	E. coli		*S. aureus*		*B. pumilis*		*P. vulgaris*		*S. flexneri*	
Extract	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC
Methanol	357.08 ± 29.75	443.02 ± 39.74	394.58 ± 15.58	476.83 ± 45.93	319.00 ± 21.62	397.66 ± 32.18	364.50 ± 20.68	410.91 ± 27.93	364.59 ± 15.46	404.75 ± 20.07
Aqueous	349.42 ± 41.03	393.00 ± 37.21	471.17 ± 41.24	577.17 ± 35.23	413.09 ± 13.22	481.00 ± 12.06	419.17 ± 19.18	460.83 ± 31.34	372.16 ± 26.8	408.58 ± 31.21
Acetone	359.08 ± 40.08	453.83 ± 44.72	386.83 ± 14.17	492.75 ± 66.71	385.83 ± 33.78	489.42 ± 55.73	351.34 ± 13.10	426.83 ± 44.83	359.02 ± 24.79	434.00 ± 36.89
	p value = 0.928	p value = 0.133	p value = 0.003	p value = 0.045	p value = 0.001	p value = 0.014	p value = 0.001	p value = 0.182	p value = 0.726	p value = 0.371

Table 2. Comparative analysis of the protein leakage ability of the extracts from each bacterial strain at different time interval

Mean ± Standard Deviation of the protein leaked (µg/ml) from each bacterial strain at different time interval										
	E. coli		*S. aureus*		*B. pumilis*		*P. vulgaris*		*S. flexneri*	
Extract	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC
Time	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC
1 h	308.89 ± 16.01	371.91 ± 29.95	390.33 ± 34.53	459.33 ± 68.44	341.89 ± 55.17	412.44 ± 60.65	355.67 ± 35.51	391.22 ± 24.51	337.67 ± 5.21	381.00 ± 18.50
2 h	344.44 ± 48.81	437.00 ± 26.31	412.67 ± 41.64	500.78 ± 56.50	372.56 ± 40.91	457.11 ± 44.22	375.89 ± 30.72	425.44 ± 27.81	360.89 ± 17.99	414.44 ± 10.56
3 h	376.67 ± 20.83	449.33 ± 35.10	422.67 ± 37.60	525.22 ± 50.85	384.33 ± 45.62	464.33 ± 46.94	385.22 ± 40.80	441.22 ± 32.07	374.48 ± 3.21	418.67 ± 12.34
4 h	371.33 ± 29.08	461.56 ± 40.61	444.44 ± 72.79	577.00 ± 44.70	391.78 ± 55.49	490.22 ± 64.69	396.56 ± 37.52	473.56 ± 24.14	388 ± 9.85	449.00 ± 32.04
	p value = 0.098	p value = 0.044	p value = 0.617	p value = 0.151	p value = 0.641	p value = 0.424	p value = 0.588	p value = 0.035	p value = 0.002	p value = 0.022

surfaces and vice versa. Few cells, also, remained intact with rough surfaces. In *E. coli* treated with aqueous extract (Fig. 1D), the pores were more prominent on cell surfaces with a larger hole at the opposite polar ends. Both scattered and accumulated extruded cellular materials were found on most cells contrary to those of methanol extract-treated *E. coli*. The clustered or accumulated cellular materials were not restricted to the polar end of the cells where found and were more whitish on the cell surfaces than those of acetone extract-treated *E. coli*. The cells of *S. flexneri* treated with acetone extract (Figure 2B) were constricted, shorter and superficially depressed at the centre. Pores and extrusion of cellular materials were not found on the cell surfaces. In Figure 2C, *S. flexneri* treated with methanol extract were mostly shorter, accumulated extruded cellular materials were found mostly at the middle part of the cells or at the polar ends in few cases while cells were collapsed or depressed at the tip or a polar end. In *S. flexneri* treated with aqueous extract (Fig. 2D), there were constrictions only. No collapsed cells or pores indicated by extruded materials were observed. When *Proteus vulgaris* was treated with acetone extract (Figure 3B), the extracellular matrices were

Table 3. Comparative analysis of the bacterial susceptibility to each extract based on quantity of protein leaked

	Mean ± Standard Deviation of the bacterial susceptibility in response to extract treatments					
Organisms	AMM-MIC	AMM-2xMIC	AMW-MIC	AMW-2xMIC	AMA-MIC	AMA-2xMIC
E. coli	357.08 ± 29.75	443.02 ± 39.74	349.42 ± 41.03	393.00 ± 37.21	359.08 ± 40.08	453.83 ± 44.72
S. aureus	394.58 ± 15.58	476.83 ± 45.93	471.17 ± 41.24	577.17 ± 35.23	386.83 ± 14.17	492.75 ± 66.71
B. pumilis	319.00 ± 21.62	397.66 ± 32.18	413.09 ± 13.22	481.00 ± 12.06	385.83 ± 33.78	489.42 ± 55.73
P. vulgaris	364.50 ± 20.68	410.91 ± 27.93	419.17 ± 19.19	460.83 ± 31.34	351.34 ± 13.10	426.83 ± 44.83
S. flexneri	364.59 ±15.46 p value = 0.003	404.75 ± 20.07 p value = 0.028	372.16 ± 26.80 p value = 0.000	408.58 ± 31.21 p value = 0.000	359.02 ± 24.79 p value = 0.256	434.00 ± 36.89 p value = 0.266

Key: AMM = Methanol extract; AMW = Aqueous extract; AMA = Acetone extract

Table 4. Comparative analysis of the lipid leakage ability of each extract from each bacterial Strain

	Mean ± Standard Deviation of the protein leaked (µg/ml) from each bacterial strain									
	E. coli		*S. aureus*		*B. pumilis*		*P. vulgaris*		*S. flexneri*	
Extract	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC
Methanol	169.75 ± 40.68	210.15 ± 50.65	216.13 ± 28.70	252.32 ± 35.56	149.00 ± 28.14	172.75 ± 25.31	224.65 ± 21.35	309.40 ± 40.42	109.06 ± 32.63	140.49 ± 45.22
Aqueous	169.32 ± 64.30	207.78 ± 69.06	159.44 ± 47.42	201.16 ± 39.11	124.40 ± 27.70	194.16 ± 51.75	81.85 ± 40.12	109.63 ± 47.47	107.31 ± 32.50	134.33 ± 34.64
Acetone	152.71 ± 27.82	229.46 ± 31.07	148.10 ± 15.29	208.57 ± 39.66	88.61 ± 48.43	133.71 ± 48.43	90.68 ± 41.45	128.12 ± 52.81	98.00 ± 52.60	148.32 ± 61.19
	p value = 0.844	p value = 0.819	p value = 0.038	p value = 0.178	p value = 0.111	p value = 0.192	p value = 0.000	p value = 0.000	p value = 0.918	p value = 0.920

destroyed. The treated cells were intact, thinner and longer than the broth-cultured, harvested and untreated cells. When treated with methanol extract (Fig. 3C), most cells were badly damaged. The cells that were not totally damaged were shorter and collapsed while others were twisted. There were pores but without attachment of extruded cellular materials to the cell surfaces. Affected but intact cells were swollen at one end and taper towards the other end. Contrary to what were obtained with methanol extract-treated *P. vulgaris*, its treatment with aqueous extract (Fig. 3D) resulted in many cells having significant puffy-like depressions with few cells having a pore at the polar end while many cells were unaffected and intact.

On treating the *B. pumilis* cells with acetone extract (Fig. 4B), a significant elongation of the cells occurred. Each cell collapsed and became flat-shaped dead cells with irregular edges. In methanol treated cells (Fig. 4C), the cell elongation effects were greater than was observed with acetone extract-treated isolates and no pores or aggregated cellular materials were recorded. In

Table 5. Comparative analysis of the lipid leakage ability of the extracts from each bacterial strain at different time interval

Mean ± Standard Deviation of the protein leaked (µg/ml) from each bacterial strain at different time interval										
	E. coli		*S. aureus*		*B. pumilis*		*P. vulgaris*		*S. flexneri*	
Extract	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC	MIC	2 x MIC
30 min	113.03 ± 7.46	154.04 ± 29.62	140.97 ± 33.89	178.30 ± 33.74	79.02 ± 43.66	114.73 ± 35.64	87.71 ± 92.18	123.1 ± 119.7	58.57 ± 14.86	84.44 ± 1.36
60 min	152.77 ± 12.39	203.11 ± 27.72	166.57 ± 35.55	205.80 ± 24.46	109.48 ± 33.22	157.90 ± 34.25	128.71 ± 86.69	172.7 ± 111.1	88.10 ± 17.73	126.04 ± 9.01
90 min	175.67 ± 10.71	235.22 ± 8.27	180.46 ± 46.37	232.08 ± 28.72	134.77 ± 29.92	180.72 ± 29.50	144.35 ± 76.46	200.9 ± 101.1	127.00 ± 2.07	162.71 ± 8.65
120 min	214.22 ± 42.73	270.81 ± 15.31	210.23 ± 44.27	266.58 ± 25.78	159.41 ± 17.37	214.14 ± 33.20	168.81 ± 64.88	232.9 ± 110.0	145.49 ± 9.35	191.00 ± 30.50
	p value = 0.004	p value = 0.001	p value = 0.279	P value = 0.027	p value = 0.075	p value = 0.035	p value = 0.672	p value = 0.673	p value = 0.000	p value = 0.000

Table 6. Comparative analysis of the bacterial susceptibility to each extract based on quantity of lipid leaked

Mean ± Standard Deviation of the bacterial susceptibility in response to extract treatments						
Organisms	AMM-MIC	AMM-2xMIC	AMW-MIC	AMW-2xMIC	AMA-MIC	AMA-2xMIC
E. coli	169.73 ± 40.68	210.15 ± 50.65	169.32 ± 64.30	207.78 ± 69.06	152.71 ± 27.82	229.46 ± 31.07
S. aureus	216.13 ± 28.70	252.32 ± 35.56	159.44 ± 47.42	201.16 ± 39.11	148.10 ± 15.29	208.57 ± 39.66
B. pumilis	149.00 ± 28.14	172.75 ± 25.31	124.40 ± 27.70	194.16 ± 51.75	88.61 ± 48.43	133.71 ± 48.43
P. vulgaris	224.65 ± 21.35	309.40 ± 40.42	81.85 ± 40.12	109.63 ± 47.47	90.68 ± 41.45	128.12 ± 52.81
S. flexneri	109.06 ± 32.63	140.49 ± 45.22	98.00 ± 52.60	148.32 ± 61.19	107.31 ± 32.50	134.33 ± 34.64
	p value = 0.000	p value = 0.000	p value = 0.088	p value = 0.100	p value = 0.047	p value = 0.008

Key: AMM = Methanol extract; AMW = Aqueous extract; AMA = Acetone extract

aqueous extract treated cells (Fig. 4D), cell elongations and death almost equal to that of methanol were observed in the affected cells. The effects of methanol and aqueous extracts on the cells were similar but seemed greater than those observed with acetone treated isolates. In Fig. 5B, C and D, *S. aureus* treated with both aqueous and alcoholic extracts were not lysed or remained intact. They were inflamed and had rough surface.

A comparative analysis of the protein leakage ability of acetone, methanol and aqueous extracts of *A. mearnsii* was considered in this study. Each data in Table 1 represented results of protein leaked from each bacterium treated with each extract

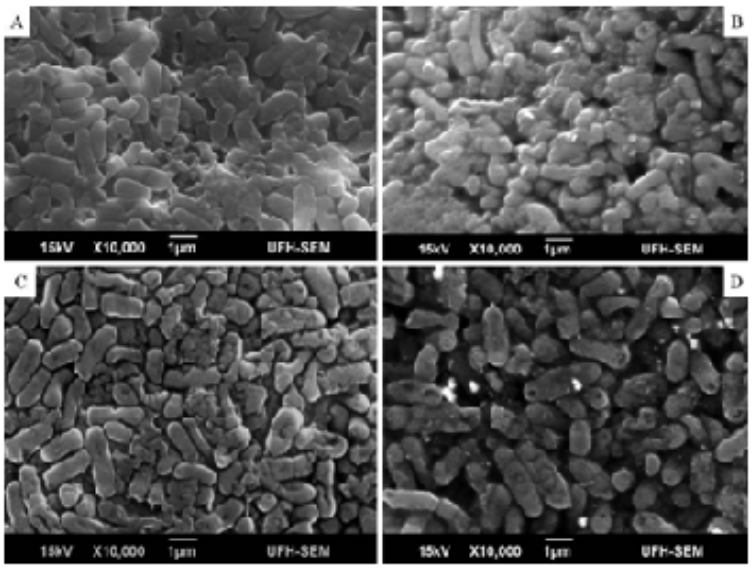

Fig. 1. Influence of alcoholic and aqueous extracts of *A. mearnsii* on *E. coli*. A = Untreated *E. coli*; B = *E. coli* treated with acetone extract; C = *E. coli* treated with methanol extract; D = *E. coli* treated with aqueous extract

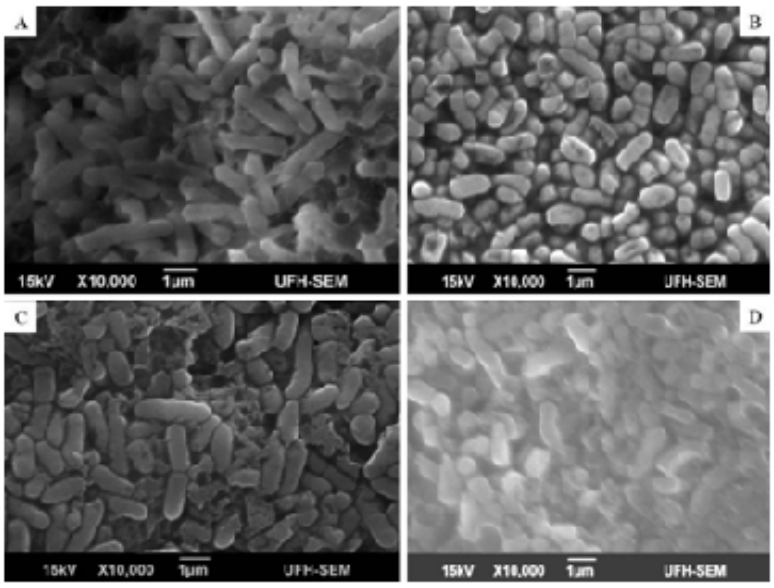

Fig. 2. Influence of alcoholic and aqueous extracts of *A. mearnsii* on *S. flexneri;* A = Untreated *S. flexneri*; B = *S. flexneri* treated with acetone extract; C = *S. flexneri* treated with methanol extract; D = *S. flexneri* treated with aqueous extract

with consideration for the most effective extract. The results showed that the protein leakage ability of the extracts was concentration dependent as the effects of 2 x MICs was significantly higher than those obtained from the MICs of the extracts. On treating the bacterial strains with the MICs and 2 x MICs of each extract, the ability of the extracts to release protein leakages from *S. aureus* (471.17 µg/ml) ($p = 0.003$ @ MICs and $p = 0.045$ @ 2 x MICs), *P. vulgaris* (419.17 µg/ml) ($p = 0.001$ @ MICs) and *B. pumilis* (481.00 µg/ml) ($p = 0.001$ @ MICs and $p = 0.014$ @ 2 x MICs) were significantly different from each other while those obtained from other extract-treated organisms were not

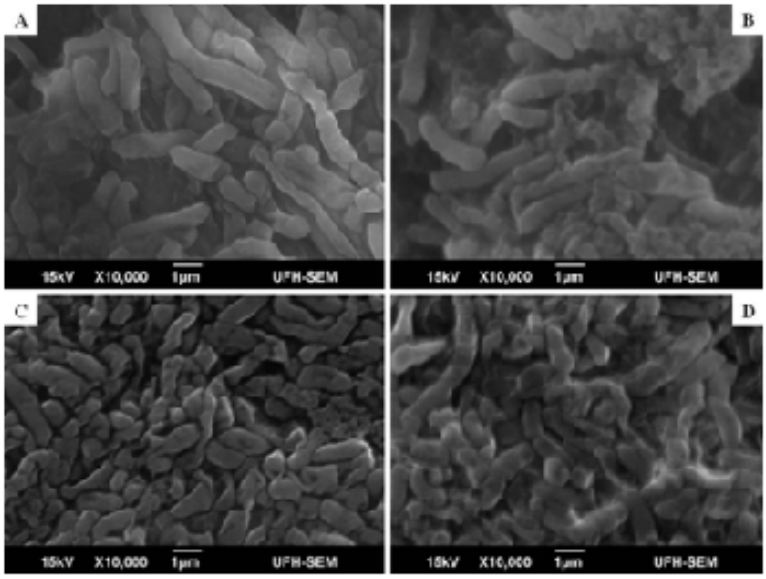

Fig. 3. Influence of alcoholic and aqueous extract of *A. mearnsii* on *P. vulgaris*. A = Untreated *P. vulgaris*; B = *P. vulgaris* treated with acetone extract; C = *S P. vulgaris* treated with methanol extract; D = *P. vulgaris* treated with aqueous extract

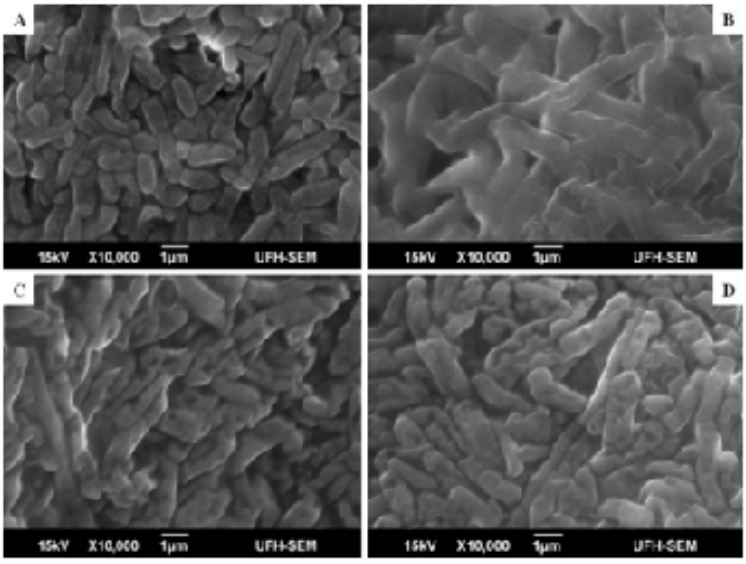

Fig. 4. Influence of alcoholic and aqueous extracts of *A. mearnsii* on *B. pumilis*. (A) = Untreated *B. pumilis*; (B) = *B. pumilis* treated with acetone extract; C = *B. pumilis* treated with methanol extract; D = *B. pumilis* treated with aqueous extract

significantly different from each others. Of the three extracts at 2 x MIC, aqueous extract caused a release of the highest quantity of protein from *S. aureus* (577.17 µg/ml), followed by acetone extract (*S. aureus* – 492.75 µg/ml) and the least protein leakage, at this concentrations, was from the methanol extract (476.83 µg/ml). Of the MIC-treated strains, aqueous extract released more protein from *P. vulgaris* (419.17µg/ml) and *B. pumilis* (481.00 µg/ml) at 2 x MIC in comparison to those of other organisms apart from *S. aureus*. For the methanol extract and apart from the *S. aureus*, highest protein was released from *S. flexneri* (364.59 µg/ml) and *E. coli* (443.02 µg/ml) respectively with the MIC and 2 x MIC treatments in comparison with other isolates. The acetone extract, however, showed that its protein leakage ability, at both MIC and 2 x MIC, was the highest in *S. aureus*, followed by that of *B. pumilis* > *E. coli* > *S. flexneri* > *P. vulgaris* (Table 1).

In Table 2, results of protein leaked from each organism treated with each extract with a consideration for the sampling time interval were indicated. The protein leakage ability of the different extract at different sampling time interval showed that the activities of the extracts to cause protein leakages were time dependent and significantly different on time basis. With the exception of the protein leaked from *S. flexneri*, at both MIC and 2 x MIC, there were no significant differences in the protein leakage ability of the different extracts from the other bacterial strains by considering the different sampling time intervals. Bonferoni's multiple comparison test comparing protein leakages between two paired bacteria over the sampling periods showed that protein leakages from *E. coli* compared with *S. aureus*, *E. coli* and *B. pumilis*, *S. aureus* and *P. vulgaris*, *S. aureus* and *B. pumilis*, *S. aureus* and *S. flexneri* as well as *B. pumilis* and *S. flexneris* were significantly different over the sampling periods. Those of *E. coli* compared with *P. vulgaris, E. coli* and *S. flexneri*, *B. pumilis* and *P. vulgaris* as well as *P. vulgaris* and *S. flexneri* were not significantly different. From Table 3, the protein leakage results indicated that *S. aureus* had the highest quantity of protein leaked when treated with all the extracts. While *B. pumilis* (319.00 µg/ml) had the least quantity of protein leaked by the MIC of methanol extract, other organisms released varied quantity amount of protein leaked by other extracts at different concentrations. The statistical analysis showed that the amount of protein leaked from each bacterial strain by aqueous and methanol extracts was significantly different while the protein leaked from bacteria treated with acetone extract

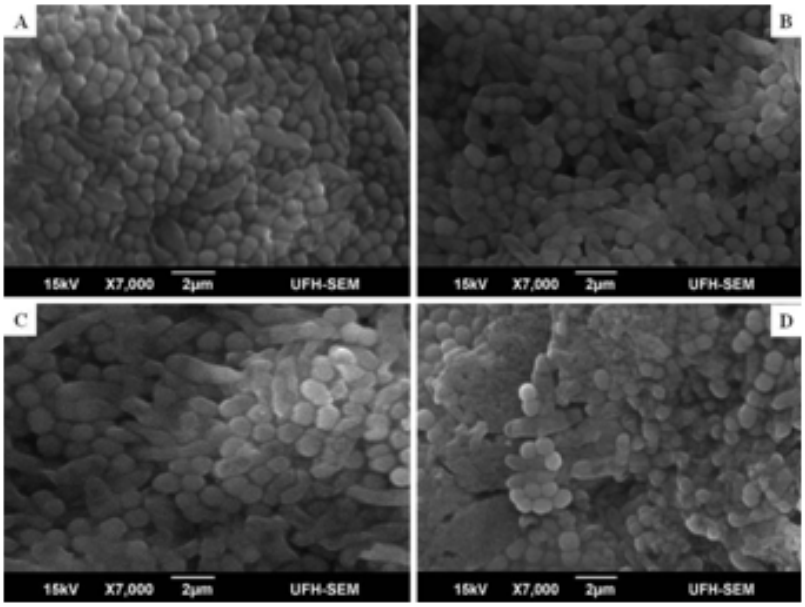

Fig. 5. Influence of alcoholic and aqueous extracts of *A. mearnsii* on *S. aureus*.
A = Untreated *S. aureus*; B = *S. aureus* treated with acetone extract; C = *S. aureus* treated with methanol extract; D = *S. aureus* treated with aqueous extract

was not significantly different from each other. Based on the amount of protein leaked, the bacterial membranes could have been more disrupted by aqueous extract, followed by methanol extract while the acetone extract was the least.

The lipid leakage abilities of the different extracts are as presented in Tables 4 – 6. In Table 4, lipid leakages from each organism treated with each extract were analyzed with consideration for the most effective extract. The results showed that the ability of the extracts to cause lipid leakages was concentration dependent and significantly different for extract treated *S. aureus* ($p = 0.038$ at MICs) and *P. vulgaris* ($p = 0.000$ at MICs and 2 x MICs). The effects of the extracts were not significantly different from each other for the other bacteria treated with the different MICs and 2 x MICs. Generally, the average values of lipid leaked at MICs from the organisms ranged between 81.85 and 224.65 µg/ml. At 2 x MICs, the average values of lipid leaked ranged between 109.63 and 309.40 µg/ml. For methanol, lipid leaked ranged from 109.06 to 224.65 µg/ml at the MIC and 140.49 to 309.40 µg/ml at the 2 x MICs, followed by acetone extract with lipid leaked ranging from 88.61 to 152.71 µg/ml at the MIC and from 128.12 to 229.46 µg/ml at the 2 x MICs and least with aqueous extract with lipid leaked ranging from 81.85 to 169.32 µg/ml at the MIC and from 109.63 to 207.78 µg/ml at the 2 x MICs. With consideration for their lipid leakage ability at the MICs, methanol extract may have indicated higher lipid leakage ability, followed by acetone extract and the least activity from the aqueous extract.

From Table 5, the lipid leakage ability of the extracts was analyzed with consideration for their activity over the sampling periods to determine their effectiveness. Here, the lipid leakage ability of all the extracts was time dependent. The ability of the extracts to cause leakages in the sampling period was significantly different with time in *E. coli* and *S. flexneri* but not significantly different with time in *P. vulgaris* treated at the different concentrations. While the lipid leakage ability of the extracts was not significantly different with time in *B. pumilis* and *S. aureus* treated at MICs, it was significantly different when they were treated with the 2 x MICs of the extracts. Generally, the average values of the lipid leaked ranged between 58.57 and 214.22 µg/ml in the bacteria treated with the MICs while the values ranged between 84.44 and 270.81 µg/ml at 2 x MICs over the sampling period. At 30 min sampling period, the average values of lipid leaked ranged between 58.57 and 178.30 µg/ml while at 120 min, the lipid leaked ranged between 145.49 and 270.81 µg/ml. Based on the lipid leakage profiles from each extract-treated organism in Table 6, the various concentrations of lipid leaked at the different concentrations showed that lipid leakages resulting from the aqueous extract was not significantly different while those of methanol and acetone extracts were significantly different. However, the quantity of lipid leaked from each bacterium was in the following order: *P. vulgaris* > *S. aureus* > *E. coli* > *B. pumilis* > *S. flexneri*. With the different p = values, the different extracts of *A. mearnsii* had bactericidal effects that resulted from the disruption of the cytoplasmic membrane of the bacterial cells while methanolic extract had the highest lipid leakage ability, followed by acetone extract and least activity was recorded with aqueous extract.

DISCUSSION

In this study, the different extracts had different effects on the different extract-treated isolates while varied degrees of morphological changes were observed on the affected cells and bacterial populations. Of notes were the shapes, colours, distribution and locations of the extruded cellular materials on the cell surfaces. The diversity in the features of these extruded cellular materials showed that the extracts contain different phytochemicals producing different effects at specific sites on the bacteria species. The points at which the cellular materials were located on the cell surfaces signify the points at which there are leakages resulting from the pore forming activities of the extracts. Having extruded cellular materials scattered on some cells' surfaces and/or congealed at either or both polar ends of the cells showed that the extracts attacked a particular component of the cells located at the points of extrusions. The differences in the colour and appearances of the extruded cellular materials at different locations on the cell surfaces implied that different components of the extracts attacked different cellular and cytoplasmic materials in a particular bacterial strain

resulting in the colour variations. Comparative analysis of the differently affected cell morphology showed that some cells had extruded cellular materials being attached to cell surfaces, other cells had their cellular materials totally washed into the medium environments. The congealed cellular materials may be attributed to the astringent effect of the tannin contents of the plant. The observed distinct morphological changes such as cell elongation and roughening of the surfaces suggested that the extract interfered with bacterial cell synthesis leading to cell deformations. These findings agreed with a previous report on food-borne pathogens treated with epigallocatechin gallate[22]. These responses in agreement with other researches on the antimicrobial activity of phenolic compounds affecting cellular membranes[23,24] could be attributed to the membrane permeating agents present in the different extracts as observed in spheroplasts of Gram-negative and intact Gram-positive bacteria by Papo *et al.*[25].

Also, determining the protein and lipid leakage abilities of the extracts showed a possible mechanism of action of the different extracts. It showed that the ability of the extracts to cause leakages may or may not be significantly different from each other depending on the types of bacteria being treated. While the leakages were time and concentration dependents, the degree of each leakage from each bacterial strain indicated the rate and extent to which the extracts were able to disrupt the bacterial membranes. Consequently, while aqueous extract was the most active in causing protein leakages, methanol extract was the leading cause of lipid leakages. The ultrastructural changes and the leakages resulting from treating the bacteria strains with the different extracts showed the dynamic natures of the extracts as antibacterial agents.

In eliminating the side effects of antibacterial agents, studying their modes of action becomes essential to foresee the damages done to the microorganisms while the knowledge of the mechanisms of action of these agents could help to define or understand and predict the effects of the antibacterial agents in some metabolic pathways. Consequently, several mechanisms of action including destabilization of cytoplasmic and plasma membranes, inhibition of extracellular microbial enzymes and metabolisms and deprivation of the substrate required for microbial growth[26] have been reported. Although the antimicrobial properties of plants are due to quinine, berberine, tannins, alkaloids, flavanoids, terpenoids, carotenoids, coumarins, curcumines, saponins, etc.[27,28], Vaara[29] and Tsuchiya and Iinuma[30] indicated that these phytochemicals disrupt the plasma membrane by localized hyper-acidification and disruption of membrane transport and/or electron transport. While Sikkema *et al.*,[31] implicated their ability in causing structural and functional damages to plasma membrane, they cause leakages of ions, adenosine triphosphate (ATP), nucleic acids and amino acids[32-35]. Damage to the membrane functions was reported as a mechanism of action for phenols and phenolic compounds[31,36-39] Tannins form irreversible complexes with proline-rich proteins[40], cause polymerization through oxidation reactions[41] and inactivate microbial adhesions, enzymes, cell envelope transport proteins and mineral uptake[42]. While saponins interfere with membrane integrity[43], thymol inhibits ATP-generating pathways and causes membrane perforation as its principal mode of action[44]. In acting on the permeability barriers in cytoplasmic membrane, an increase in cytoplasmic membrane permeability is shown by leakages of the important intracellular materials[45]. Since a mixture of phenolic compounds acts synergistically better than its individual components separately [46], the observed morphological changes and leakages, in agreement with earlier reports, could be attributed to the effect(s) of one or many phytochemicals acting singly or in synergy with others to effect different mechanisms of actions.

Considering the leakages recorded in this study, lipid peroxidation, a continuous process in bacterial cells maintained at low level by antioxidants[47], could be implicated. Although phytochemicals with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems[48] and *Acacia mearnsii* possessed antioxidant properties[49], the antioxidant activities of this plant could have enhanced membrane free radical reactions beyond the protective level to cause lipid peroxidation[50]. Consequentially, from the lipid and protein leakages in the bacterial cells, it may be hypothesized in agreement with Cerutti [50] that

the extracts of *A. mearnsii* attacked the DNA, proteins, lipids, polyunsaturated fatty acids of cells and initiate lipid peroxidation in the cytoplasmic membranes as its possible mechanisms of actions. As a result of this possible lipid peroxidation whose products are toxic to cells[51,52], polysaccharide fatty acids could have been biodegraded to a variety of reactive (C_3 - C_9) aldehyde products including 2-propenal, 4-hydroxynonenal and malondialdehyde which are able to damage proteins[53]. These aldehydes could have attacked the base and sugar moieties of DNA to produce single and double strand breaks in the backbone, allowed joining of base and sugar groups and cross-links to other molecules to obstruct replication[54,55]. The oxidation of protein could have resulted in damages such as oxidation of sulfhydryl groups, reduction of disulphides, oxidative adduction of amino acids residues close to metal-binding sites through metal catalyzed oxidation, reactions with aldehydes, modification of prosthetic groups of metal groups or metal clusters, protein-protein cross-linking and peptide fragmentation. While some amino acids were oxidized to carbonyl derivatives[56], 4-hydroxynonenal produced during the peroxidation of polyunsaturated fatty acids reacted with the sulfhydryl groups of protein to form stable covalent thiolether adducts carrying a carbonyl function [53,57, highly oxidized proteins inhibited preoteases to degrade other oxidized proteins[58,59] while *in vivo* protein carbonylation was generally used as a marker of irreversible and unrepairable oxidative protein damage[60]. As a result of these effects, the lipid peroxidation and protein oxidation are considered as the possible mechanisms of action of these extracts. They could have caused degradation of the cell wall, damage cytoplasmic membrane proteins and lipids as well as leaked cell contents to cause morphological changes observed and deaths of the bacterial cells.

CONCLUSION

This study showed that the different extracts of *A. mearnsii* caused ultrastructural changes and leakages resulting from the disruption of the cytoplasmic membranes of the bacterial cells. While the possibility of using different mechanisms of action, due to the presence of different phytochemicals, may not be underestimated, indicating lipid peroxidation and protein oxidation as possible mechanisms of antibacterial action may not be an overstatement. Resulting from being able to disrupt the outer membranes, the extracts were considered being hydrophobic as they were able to partition the lipids of bacterial cell membranes to effect ultrastructural changes and rendered them more permeable to the influx of the phytochemicals in the extracts. The differences in the ultrastructural damages and the leakages caused by the different extracts could be due to the differences in the type, quantity and degree of bioactivity of each of the phytochemical components extracted by each solvent. Although this study implicated lipid peroxidation and protein oxidation as the possible mechanisms of action resulting from the possibility that the antioxidative properties of this plant could have gone beyond protective levels due to high concentrations, further quantitative and determinative studies on the proteins and lipid components released from cells would be able to elucidate the sources and types of released cellular materials.

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