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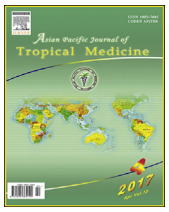

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Time-kill kinetics and biocidal effect of *Euclea crispa* leaf extracts against microbial membrane

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ABSTRACT

Objective: To evaluate antimicrobial potential of the fractions partitioned from *Euclea crispa* leaf extract and determination of their impact on cell membrane disruption.
Methods: Antimicrobial potentials were evaluated via susceptibility test, determination of minimum inhibitory concentrations (MICs) and time-kill kinetics of the potent fractions. Degree of membrane disruption was determined by the amount of proteins and nucleotides released from within the cells and SEM images of the membrane after 120 min of treatment.
Results: The largest inhibition zone (25.5 ± 0.50 mm) was obtained by ethylacetate fraction against *Aeromonas hydrophilla* at 10 mg/mL. The lowest MIC (0.16 mg/mL) was exhibited by n-butanol and ethylacetate fractions against test bacteria while all fractions exhibited MIC values between 0.31 and 1.25 mg/mL against susceptible yeast. n-Butanol fraction achieved absolute mortality against *Bacillus pumulis* (*B. pumulis*) and *Klebsiella pneumoniae* (*K. pneumoniae*) after 90 and 120 min contact time respectively at 1 × MIC. Total mortality also achieved by n-hexane fraction against *B. pumulis* and *K. pneumoniae* after 90 and 120 min respectively at 2 × MIC. Ethylacetate fraction achieved absolute mortality against both bacteria after 120 min at 2 × MIC. n-Hexane fraction achieved total mortality against *Candida albicans* after 120 min at 1 × MIC. Maximum amount of proteins (0.566 μg/mL) was released from *K. pneumoniae* by n-butanol fraction at 2 × MIC after 120 min of treatment while the maximum amount of nucleotides released (4.575 μg) was from *B. pumulis* by n-hexane fraction under similar condition.
Conclusion: This study suggests the leaf of *Euclea crispa* a source of bioactive compound with membrane attack as one of the mechanisms of its biocidal action.

1. Introduction

Several plant extracts have exhibited potential against various infectious agents and thus has found useful as therapeutic agents in folkloric remedies. Circulation of multidrug resistant (MDR) pathogens presents a major pitfall in combating infectious diseases and therefore results in global medical predicament with high rate of morbidity and mortality. It has been reported from different studies that MDR is caused by prolonged abuse of antibiotics both in the clinical practices and in agricultural feeds [1]. In addition to significant increment in the costs and side effects of newer drugs, resistance to antibiotics is a limiting factor in the war against infectious diseases. As resistant strains of bacteria continue to increase there is no significantly different newer drugs to remedy this problem [2].

Plant derived bioactive compounds are widely in use in most pharmaceutical industries due to their therapeutic efficacy and there are several indications from ethno-botanical records pointing to the fact that potent medicinal plants may be a source of affordable drugs that may be readily available cross varying societal classes [3]. *Euclea crispa* (family Ebenaceae) (*E. crispa*) is as one of the most common trees in South Africa. *Euclea* species are extensively in use traditionally against wide range of ailments such as gonorrhoea, leprosy, scabies, diarrhoea and wound infections [4]. Hot water extracts of the root of this

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plant is used as antitussive [5] and the infusion from the roots is used in the treatment of leprosy by the people of Nhema communal area, Zimbabwe [6]. It has previously been established by Pretorious *et al.* [7] that leaf extracts of *E. crispa* possess growth inhibiting potential against both bacteria and fungi.

In this study, we investigated comprehensive antimicrobial potentials of the *E. crispa* leaf extracts and also determine the probable mechanism of its biocidal actions.

2. Materials and methods

2.1. Collection of plant sample

Fresh leaves sample of *E. crispa* (Thunb.) (Ebenaceae) were collected during the month of April 2015 at Puthaditjhaba area, Qwaqwa, Free State, South Africa and identified by Prof. Rodney Moffet. The plant sample was authenticated at University of the Free State herbarium with herbarium collection of Taylor and Van Wyk, 1994 with reference number: 6404000-400. It was then oven-dried (40 °C) until constant weight, ground into fine powder and stored in an air tight container for further use.

2.2. Microorganisms

Microbial isolates used in this study include typed strains as well as locally isolated pathogens (LIPs). The LIPs which are comprise of both clinical and environmental isolates were collected from the culture collection of microbiology division, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State. The isolates were maintained on nutrient agar (bacteria) and yeast malt agar (yeast) medium. This includes Gram positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus pumilis* (ATCC 14884), *Enterococcus faecalis* and *Listeria* sp.; Gram negative bacteria: *Klebsiella pneumoniae* (ATCC 13047) [*K. pneumoniae* (ATCC 13047)], *Escherichia coli* (ATCC839), *Shigella sonnei* (ATCC 29930), *Proteus vulgaris* (ATCC 6830), *Acinetobacter calcoaceuticus anitratus*, *Aeromonas hydrophilla*, *E. faecalis*, *Salmonella typhi*, *Salmonella typhimurium, Shigella flexineri*, *Plesiomonas shigeloides* and *Pseudomonas aeruginosa* as well as yeast: *Candida albicans* (different strains) (*C. albicans*) *Candida rugosa*, *Cryptococcus neoformans*, *Trichophyton mucoides*.

2.3. Extraction of the plant sample

Exactly 600 g of the ground plant sample was extracted in methanol and sterile distilled water (3:2, v/v) for four days with regular agitation at intervals. Supernatant collected was filtered and the filtrate was concentrated *in vacuo* and lyophilized. The yield obtained was 0.2 g/g plant material.

2.4. Solvent partitioning of the extract

Exactly 110 g of the leaf extract was dissolved in 250 mL of sterile distilled water and then partitioned into n-hexane, chloroform, ethylacetate, n-butanol and aqueous fractions in order of the solvents polarity starting with n-hexane (4 × 200 mL). The resulting n-hexane fraction was concentrated *in vacuo* and lyophilized, the residue (8.20 g) was kept in an air-tight container. The resultant aqueous phase was re-concentrated *in vacuo* and further extracted with chloroform (4.25 g), ethylacetate (21.84 g) and n-butanol (27.72 g) using similar procedure. The remaining aqueous fraction was lyophilized to yield 40.32 g powder which was also kept in the freezer for further use.

2.5. Phytochemical screening of the leaf extract

Small portion of the leaf extract was subjected to phytochemical screening using the standard methods in testing for alkaloids, tannins, flavonoids, steroids, saponins, reducing sugars and cardiac glycosides [8–10].

2.6. Susceptibility testing

This was determined via agar-well diffusion method as previously described [11,3]. Exactly 0.1 mL of 24 h old standard inoculums (0.5 McFarland) was inoculated into molten Mueller–Hinton agar (Oxoid, UK) for the bacteria and Potato dextrose agar (Oxoid, UK) (PDA) for the yeast. This was poured into Petri dishes and allowed to set before wells were bored into the agar medium using a sterile cork borer (6 mm). The wells were carefully filled up with prepared solution of the extract at a concentration of 10 mg/mL. The plates were allowed to stand on the laboratory bench for about 2 h before incubated at 37 °C and 25 °C for the bacterial and yeast isolates respectively for 24 h, after which the plates were observed for the zones of inhibition. The susceptibilities of the isolates to the leaf extract were compared with that of ketoconazole, nystatin, streptomycin (1 mg/mL) and tetracycline (0.1 mg/mL) purchased from Sigma Aldrich. Sterile distilled water and 10% methanol were used as control and the experiment was carried out in replicates of three.

2.7. Determination of the minimum inhibitory concentrations (MICs)

The MICs of the potent fractions and that of the standard drugs used were determined using the standard method of European Committee for Antimicrobial Susceptibility testing by agar dilution [12,13]. Two-fold dilution of the extract was prepared in sterile distilled water and 2 mL of different concentrations of the aliquot was added to 18 mL of sterile molten Nutrient Agar (Oxoid, UK) and PDA for the bacteria and yeasts respectively to give final concentrations ranging from 0.08 to 10.0 mg/mL. The mixture was poured into sterile Petri dishes and allowed to set. Surfaces of the media were allowed to dry before streaking with 24 h old standard inoculums and then incubated at 37 °C and 25 °C respectively for 48 h. The plates were subsequently examined for the presence or absence of growth. The MIC was taken as the lowest concentration that inhibits the growth of the isolates. Sterile agar medium plate without the extract served as control. The experiment was carried out in three replicates.

2.8. Determination of killing rate

The killing rate by the potent fractions was determined as described by Odenholt *et al.* [14] and Akinpelu *et al.* [13] with slight modifications. This was carried out against *Bacillus*

pumulis, *K. pneumoniae* and *C. albicans* as representative isolates. Nutrient broth cultures (24 h) of the isolates were standardized and viable counts were determined. Then 5 mL of the known cell density of the bacterial suspension was added to 45 mL of different concentrations of the extracts relative to the MIC. The resulting suspensions were mixed and held at room temperature while the killing rate was determined over 2-h period. A volume of 0.5 mL was taken from each suspension at intervals and transferred into 4.5 mL Nutrient broth recovery medium containing 3% Tween80. This was serially diluted in sterile physiological saline (0.9% NaCl) and 0.1 mL of the final dilution was plated out onto Nutrient agar (bacteria) and PDA (yeast), incubated at 37 °C and 25 °C respectively for 48 h. The control experiment was set up without inclusion of extracts. Viable counts were made in triplicate and a decrease in the number of colony forming units indicates killing by the extracts.

2.9. Analysis of scanning electron microscopy (SEM)

Sample preparation was carried out as described by Kockro *et al.* [15] and Xiao-Nan *et al.* [16] with slight modifications. Standardized nutrient broth cultures (24 h) of the representative isolates were washed (0.9% NaCl) and 1 mL of the washed cell suspension was treated with 9 mL of the extract at a concentration of 1 × MIC over a period of 2 h. The mixture was centrifuged, the cells washed three times with 0.05 M phosphate buffer solution (pH 7.3) and the pellet was fixed in 2.5% (v/v) glutaraldehyde. The fixed cells were dehydrated with different concentration of ethanol. These were critical point dried using Tousimis critical point dryer (Rockville, Maryland, U.S.A.) and then mounted before coated with gold (BIO-RAD, Microscience Division Coating System, London, UK). The samples were then observed under a scanning electron microscope (JSM-7800F Extreme-resolution Analytical Field Emission SEM).

2.10. Determination of protein leakage

Washed cells of the representative isolates were standardized (0.5 McFarland) and exposed to various concentrations of the extracts relative to the MIC at different time intervals over a period of 2 h. Each suspension was then centrifuged at 7000 rpm and the protein concentration in the supernatant was determined [17]. Bradford reagent (0.4 mL) was added to 1.6 mL sample (0.2 mL supernatant + 1.4 mL sterile distilled water) and optical density of the resulting solution was measured at 595 nm within 5 min. Protein quantity of each sample was determined from the equation of the best-fit linear regression obtained from the Bovine Serum Albumin (BSA) standard curve.

2.11. Determination of nucleotide leakage

This was determined using the method described by Joswick *et al.* [18] and Akinpelu *et al.* [13] with slight modifications. Washed suspensions of the representative isolates (0.5 McFarland) were exposed to various concentrations of the fractions at regular time intervals over a period of 2 h. Each suspension was then centrifuged at 10 000 rpm and the absorbance of the supernatant measured at 260 nm using a UV spectrometer. Sterile distilled water inoculated with the same inoculums was used as control.

2.12. Statistical analyses

Data were expressed as means ± SD (standard deviation) of three replicates and were statistically analyzed using one way analysis of variance (ANOVA). Means were separated by the Duncan multiple test using SPSS. Values were considered significant at $P < 0.05$.

3. Results

Phytochemicals screening of the leaf extract of *E. crispa* reveals the presence tannins, flavonoids, steroids, saponins, reducing sugars and cardiac glycosides. No trace of alkaloids was revealed among the phytochemicals tested.

The antimicrobial susceptibility profile indicates clear zones of inhibition of different width by respective fractions at a concentration of 10 mg/mL against all the test bacterial isolates (Table 1). Widest zones of inhibition (≥20 mm) were expressed by the fraction partitioned into ethelyacetate against 55% of the isolates and closely followed by that of n-butanol against 50% of the bacterial isolates. The zones of inhibition expressed by all the fractions at 10 mg/mL against the yeast isolates ranged between (16.3 ± 0.6) mm and (22.0 ± 0.0) mm (Table 2). MICs of the potent fractions are shown on Tables 3 and 4. The MICs exhibited by fractions partitioned into n-butanol and ethylacetate ranged between 0.16 and 1.25 mg/mL as compared with that of streptomycin (0.008 and 0.125 mg/mL) and tetracycline (0.006 and 0.025 mg/mL). Moreover, the fractions partitioned into n-hexane and chloroform showed MICs range of between 0.31 and 2.50 mg/mL while that partitioned into water was between 1.25 and 2.50 mg/mL all against the bacterial isolates. On the other hand, the fractions exhibited lowest MIC values of 0.31 mg/mL and the highest values of 1.25 mg/mL against test yeast isolates as compared with ketoconazole and nystatin (0.25 and 0.13 mg/mL) except for the chloroform fraction that was only active against 10% of the yeast isolates.

The fraction partitioned into n-butanol was able to achieve absolute mortality rate against *B. pumulis* at a concentration of 1 × MIC after 90 min of contact time and after 120 min against *K. pneumoniae* under the same condition. When the concentration was increased to 2 × MIC, total mortality was also achieved by the fraction partitioned into n-hexane after 90 min of contact time against *B. pumulis* and after 120 min against *K. pneumoniae* while the same rate was also achieved by the fraction partitioned into ethylacetate after 120 min of contact time against both representative bacterial isolates. The mortality rate by the fractions partitioned into water and chloroform were 96.1 and 97.5% respectively after 120 min against *B. pumulis* and 95.4 and 94.9% against *K. pneumoniae* (Figures 1 and 2). On the other hand against *C. albicans* (Ho316), the fraction partitioned into n-hexane was able to achieve total mortality after 120 min of contact time at a concentration of 1 × MIC while the mortality rate by those partitioned into n-butanol, ethylacetate and water were 97.2%, 97.9% and 95.7% respectively under similar condition. When the concentration was increased to 2 × MIC all the fractions achieved total mortality rate except the aqueous fraction with 96.5% after 120 min of contact time (Figure 3).

Table 1

The sensitivity patterns of zones of inhibition of fractions from *E. crispa* leaf extract and standard drugs against test bacterial isolates (Zones of inhibition, mm[a]).

Bacterial isolates	BUT (10 mg/mL)	ETH (10 mg/mL)	HEX (10 mg/mL)	AQU (10 mg/mL)	CHL (10 mg/mL)	STP (1 mg/mL)	TET (0.1 mg/mL)	MET (10%)
Aeromonas hydrophilla	16.0 ± 0.0$^{\#}$	25.5 ± 0.5$^{\alpha}$	16.0 ± 1.0$^{\#}$	15.7 ± 1.2$^{\#}$	15.7 ± 0.8$^{\#}$	24.8 ± 0.3$^{\alpha}$	25.0 ± 0.0$^{\alpha}$	0
Acinetobacter calcaoceuticus anitratus	19.7 ± 1.2$^{\#}$	19.7 ± 0.8$^{\#}$	19.7 ± 1.2$^{\#}$	18.0 ± 0.0$^{\#}$	18.3 ± 0.6$^{\#}$	25.0 ± 0.0$^{\alpha}$	26.0 ± 1.0$^{\alpha}$	0
Bacillus pumilis (ATCC 14884)	20.0 ± 1.0$^{\#}$	21.5 ± 0.5$^{\#}$	15.5 ± 0.5$^{\alpha}$	22.3 ± 0.6$^{\#}$	15.7 ± 0.8$^{\alpha}$	24.7 ± 0.8$^{\beta}$	28.0 ± 0.0$^{\beta}$	0
Escherichia coli (ATCC839)	19.8 ± 0.3$^{\#}$	20.0 ± 1.0$^{\#}$	17.7 ± 1.2$^{\#}$	20.0 ± 0.0$^{\#}$	16.3 ± 0.6$^{\alpha}$	27.0 ± 0.0$^{\beta}$	24.0 ± 1.0$^{\beta}$	0
Enterobacter faecalis	18.0 ± 0.0$^{\#}$	19.5 ± 0.5$^{\#}$	16.3 ± 0.6$^{\alpha}$	17.8 ± 0.3$^{\#}$	15.0 ± 0.0$^{\alpha}$	25.3 ± 0.6$^{\beta}$	28.0 ± 0.5$^{\beta}$	0
Enterococcus faecalis	20.3 ± 0.6$^{\#}$	20.0 ± 0.0$^{\#}$	15.7 ± 1.2$^{\alpha}$	18.0 ± 0.0$^{\#}$	18.0 ± 1.0$^{\#}$	25.8 ± 0.3$^{\beta}$	25.8 ± 0.3$^{\beta}$	0
Klebsiella pneumoniae (ATCC 13047)	18.3 ± 0.6$^{\#}$	17.7 ± 1.2$^{\#}$	16.0 ± 0.0$^{\alpha}$	17.7 ± 0.8$^{\#}$	16.0 ± 0.0$^{\alpha}$	26.0 ± 0.0$^{\beta}$	24.0 ± 0.0$^{\beta}$	0
Klebsiella pneumoniae	18.0 ± 0.0$^{\#}$	15.5 ± 0.5$^{\#}$	16.0 ± 0.7$^{\#}$	16.0 ± 1.0$^{\#}$	16.0 ± 1.1$^{\#}$	23.3 ± 0.6$^{\alpha}$	26.7 ± 0.8$^{\alpha}$	0
Listeria sp	21.5 ± 0.5$^{\#}$	21.5 ± 1.2$^{\#}$	17.7 ± 1.2$^{\alpha}$	21.5 ± 0.5$^{\#}$	16.3 ± 0.6$^{\alpha}$	29.0 ± 1.0$^{\beta}$	27.3 ± 0.6$^{\beta}$	0
Shigella sonnei (ATCC 29930)	19.8 ± 0.3$^{\#}$	20.0 ± 1.0$^{\#}$	15.7 ± 0.8$^{\alpha}$	18.3 ± 0.6$^{\#}$	16.3 ± 0.6$^{\alpha}$	27.0 ± 1.0$^{\beta}$	24.8 ± 0.3$^{\beta}$	0
Shigella flexineri	19.5 ± 0.5$^{\#}$	20.0 ± 0.0$^{\#}$	16.3 ± 0.6$^{\alpha}$	20.0 ± 0.0$^{\#}$	15.5 ± 0.5$^{\alpha}$	22.7 ± 1.2$^{\#}$	26.0 ± 0.5$^{\beta}$	0
Salmonella typhimurium	21.3 ± 0.6$^{\#}$	18.0 ± 0.0$^{\alpha}$	17.5 ± 0.5$^{\alpha}$	16.0 ± 0.0$^{\alpha}$	15.7 ± 1.2$^{\alpha}$	25.0 ± 1.0$^{\beta}$	24.8 ± 0.3$^{\beta}$	0
Salmonella typhi	18.0 ± 1.0$^{\#}$	18.0 ± 1.0$^{\#}$	15.7 ± 0.8$^{\#}$	15.8 ± 0.3$^{\#}$	16.0 ± 0.0$^{\#}$	26.0 ± 0.0$^{\alpha}$	26.5 ± 0.5$^{\alpha}$	0
Staphylococcus aureus (ATCC 6538)	17.7 ± 1.2$^{\#}$	20.0 ± 0.0$^{\#}$	17.7 ± 1.2$^{\#}$	17.7 ± 1.2$^{\#}$	16.0 ± 1.0$^{\#}$	26.0 ± 1.0$^{\alpha}$	23.7 ± 1.2$^{\alpha}$	0
S. aureus (OK 2a)	16.0 ± 0.0$^{\#}$	19.5 ± 0.5$^{\alpha}$	18.0 ± 1.0$^{\alpha}$	15.5 ± 0.5$^{\#}$	16.8 ± 0.3$^{\#}$	25.0 ± 0.0$^{\beta}$	25.0 ± 0.0$^{\beta}$	0
S. aureus (OK2b)	19.7 ± 1.2$^{\#}$	18.0 ± 0.0$^{\#}$	18.0 ± 1.0$^{\#}$	15.8 ± 0.3$^{\alpha}$	15.0 ± 0.5$^{\alpha}$	24.0 ± 0.0$^{\beta}$	25.7 ± 1.2$^{\beta}$	0
Plesiomonas shigeloides	15.5 ± 0.5$^{\#}$	15.5 ± 0.5$^{\#}$	18.0 ± 1.0$^{\#}$	16.0 ± 0.0$^{\#}$	16.3 ± 0.6$^{\#}$	24.0 ± 0.0$^{\alpha}$	26.0 ± 0.0$^{\alpha}$	0
Proteus vulgaris (CSIR 0030)	15.5 ± 0.5$^{\#}$	18.3 ± 0.6$^{\#}$	15.8 ± 0.3$^{\#}$	16.0 ± 1.0$^{\#}$	17.5 ± 0.5$^{\#}$	28.7 ± 1.2$^{\alpha}$	26.3 ± 0.6$^{\alpha}$	0
Proteus vulgaris	19.7 ± 1.2$^{\#}$	18.3 ± 0.6$^{\#}$	17.5 ± 0.5$^{\#}$	18.0 ± 0.0$^{\#}$	16.0 ± 0.0$^{\alpha}$	25.7 ± 1.2$^{\beta}$	25.0 ± 0.0$^{\beta}$	0
Pseudomonas aeroginosa	16.0 ± 0.0$^{\#}$	15.8 ± 0.3$^{\#}$	17.0 ± 0.0$^{\#}$	15.7 ± 1.2$^{\#}$	14.7 ± 0.8$^{\#}$	26.0 ± 1.0$^{\alpha}$	25.0 ± 1.0$^{\alpha}$	0

Superscripts across the row are significantly different ($P < 0.05$); ATCC = American type culture collection, CSIR = Council for scientific and industrial research, BUT = n-Butanol fraction, ETH = Ethylacetate fraction, HEX = n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, MET = methanol, 0 = Not sensitive, mm[a] = Mean of three replicates.

Table 2

The sensitivity patterns of zones of inhibition of fractions of *E. crispa* leaf extract and standard drugs against test fungal isolates (Zones of inhibition, mm[a]).

Fungal isolates	BUT (10 mg/mL)	ETH (10 mg/mL)	HEX (10 mg/mL)	AQU (10 mg/mL)	CHL (10 mg/mL)	NYS (1 mg/mL)	KET (1 mg/mL)	MET (10%)
Candida albicans	20.0 ± 1.0$^{\#}$	19.7 ± 1.2$^{\#}$	19.5 ± 0.5$^{\#}$	20.0 ± 1.0$^{\#}$	0	18.0 ± 0.0$^{\#}$	22.0 ± 0.0$^{\alpha}$	0
Candida albicans (CBS8758)	0	0	0	0	0	24.3 ± 0.6$^{\alpha}$	18.0 ± 1.0$^{\#}$	0
Candida albicans (Ho314)	0	19.7 ± 0.8$^{\#}$	0	0	0	22.8 ± 0.3$^{\alpha}$	18.3 ± 0.6$^{\#}$	0
Candida albicans (Ho315)	0	16.3 ± 0.6$^{\#}$	0	0	0	24.0 ± 1.0$^{\alpha}$	23.3 ± 0.6$^{\alpha}$	0
Candida albicans (Ho316)	20.7 ± 1.2$^{\#}$	21.5 ± 0.5$^{\#}$	19.0 ± 1.0$^{\#}$	19.3 ± 0.6$^{\#}$	0	21.7 ± 1.2$^{\#}$	20.7 ± 0.8$^{\#}$	0
Candida albicans (Ho317)	0	0	0	0	0	23.7 ± 0.8$^{\#}$	18.3 ± 0.6$^{\alpha}$	0
Candida albicans (Ho318)	17.7 ± 0.8$^{\#}$	18.0 ± 1.0$^{\#}$	16.3 ± 0.6$^{\#}$	0	0	22.0 ± 1.0$^{\alpha}$	0	0
Candida rugose	22.0 ± 0.0$^{\#}$	22.3 ± 0.6$^{\#}$	19.5 ± 0.5$^{\#}$	19.7 ± 1.2$^{\#}$	18.0 ± 1.0$^{\alpha}$	19.5 ± 0.5$^{\#}$	22.3 ± 0.6$^{\#}$	0
Cryptococcus neoformans	19.5 ± 0.5$^{\#}$	20.3 ± 0.6$^{\#}$	20.0 ± 0.0$^{\#}$	17.7 ± 0.8$^{\#}$	0	22.3 ± 0.6$^{\alpha}$	19.5 ± 0.5$^{\#}$	0
Trichophyton mucoides	18.3 ± 0.6$^{\#}$	20.0 ± 0.0$^{\#}$	17.8 ± 0.3$^{\#}$	18.0 ± 0.0$^{\#}$	0	18.0 ± 0.0$^{\#}$	18.7 ± 0.8$^{\#}$	0

Superscripts across the row are significantly different ($P < 0.05$); CBS = Centraalbureau voor Schimmelcultures, The Netherlands, Ho = Strain numbers for locally isolated strains held in the UNESCO MIRCEN Yeast Culture collection at the University of the Free State, BUT = n-Butanol fraction, ETH = Ethylacetate fraction, HEX = n-Hexane fraction, AQU = Aqueous fraction, CHL = Chloroform fraction, NYS = Nystatin, KET = Ketoconazole, MET = methanol, 0 = Not sensitive, mm** = Mean of three replicates.

The SEM images of the representative isolates are shown in Figures 4–6. Considering the intact anatomical structure of the cell membrane of representative isolates (control), the three most active fractions selected were able to impact an obvious level of cell membrane disruption at various degrees. The degree of membrane disruption is proportional to the quantities of intracellular materials leakages.

The maximum amount of proteins released from *B. pumulis* (0.526 μg/mL) was by the fraction partitioned into water after 120 min of treatment at a concentration of 2 × MIC (Figure 7) while the maximum from the *K. pneumoniae* (0.566 μg/mL) was by the fraction partitioned into n-butanol (Figure 8) and that of the *C. albicans* (0.543 μg/mL) was by the fraction partitioned into n-hexane under similar condition of 2 × MIC and 120 min of treatment (Figure 9). Likewise, maximum nucleotide leakage of 4.575 μg and 4.434 μg were obtained from *B. pumulis* and *C. albicans* (Ho316) by the n-hexane fraction at 2 × MIC, respectively (Figures 10 and 12) while the maximum nucleotide leakage from *K. pneumoniae* was 4.071 μg by the fraction partitioned into n-butanol (Figure 11) at 2 × MIC and after 120 min of treatment.

Table 3

The minimum inhibitory concentrations (MICs) of the fractions of *E. crispa* leaf extract and standard drugs against test bacterial isolates (mg/mL).

Bacterial isolates	BUT	ETH	HEX	AQU	CHL	STREP	TET
Aeromonas hydrophilla	0.63	0.63	0.63	1.25	2.50	0.063	0.013
Acinetobacter calcaoceuticus anitratus	0.63	0.31	0.63	2.50	0.63	0.063	0.025
Bacillus pumilis (ATCC 14884)	0.63	0.16	0.63	1.25	0.31	0.016	0.013
Escherichia coli (ATCC839)	1.25	0.63	2.50	2.50	0.63	0.016	0.013
Enterobacter faecalis	0.63	0.31	0.63	2.50	0.63	0.016	0.013
Enterococcus faecalis	0.63	0.63	0.63	2.50	0.63	0.016	0.013
Klebsiella pneumoniae (ATCC 13047)	0.31	0.31	0.63	1.25	1.25	0.016	0.013
Klebsiella pneumoniae	1.25	0.63	0.63	2.50	1.25	0.063	0.013
Listeria sp.	0.31	0.31	0.63	1.25	0.63	0.008	0.013
Shigella sonnei (ATCC 29930)	0.63	0.31	1.25	1.25	1.25	0.016	0.013
Shigella flexineri	0.63	0.63	0.63	1.25	0.63	0.031	0.025
Salmonella typhimurium	0.63	0.63	0.63	1.25	1.25	0.016	0.025
Salmonella typhi	0.63	0.31	0.63	1.25	1.25	0.016	0.013
Staphylococcus aureus (ATCC 6538)	0.63	0.31	0.63	1.25	0.63	0.016	0.013
S. aureus (OK 2a)	0.63	0.31	1.25	2.50	1.25	0.063	0.006
S. aureus (OK2b)	0.63	0.31	0.63	2.50	2.50	0.016	0.025
Plesiomonas shigeloides	0.63	0.31	0.31	2.50	1.25	0.016	0.025
Proteus vulgaris (CSIR 0030)	0.63	0.31	0.63	1.25	0.63	0.016	0.013
Proteus vulgaris	1.25	0.63	1.25	1.25	1.25	0.125	0.025
Pseudomonas aeroginosa	0.16	0.63	0.63	1.25	0.63	0.016	0.013

ATCC = American type culture collection, CSIR = Council for scientific and industrial research, EC = *E. crispa* extract, BUT = n-Butanol fraction, ETH = Ethylacetate fraction, HEX = n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, STREP = Streptomycin, TET = Tetracycline.

Table 4

The minimum inhibitory concentrations (MICs) of the fractions of *E. crispa* leaf extract and standard drugs against test yeast isolates (mg/mL).

Fungal isolates	BUT	ETH	HEX	AQU	CHL	NYS	KET
Candida albican	0.63	0.63	0.63	1.25	ND	0.25	0.13
Candida albicans (CBS8758)	ND	ND	ND	ND	ND	0.13	0.25
Candida albicans (Ho314)	ND	0.63	ND	ND	ND	0.25	0.25
Candida albicans (Ho315)	ND	1.25	ND	ND	ND	0.13	0.13
Candida albicans (Ho316)	0.63	0.31	0.63	1.25	ND	0.25	0.25
Candida albicans (Ho317)	ND	ND	ND	ND	ND	0.13	0.25
Candida albicans (Ho318)	1.25	0.63	1.25	ND	ND	0.13	ND
Candida rugosa	0.31	0.31	0.63	0.63	1.25	0.13	0.13
Cryptococuss neoformans	0.63	0.63	0.63	1.25	ND	0.13	0.13
Trichophyton mucoides	0.31	0.31	0.63	1.25	ND	0.25	0.25

CBS = Centraalbureau voor Schimmelcultures, The Netherlands, Ho = Strain numbers for locally isolated strains held in the UNESCO MIRCEN Yeast Culture collection at the University of the Free State, BUT = n-butanol fraction, ETH = Ethylacetate fraction, HEX = n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, NYS = Nystatin, KET = Ketoconazole, ND = Not determined.

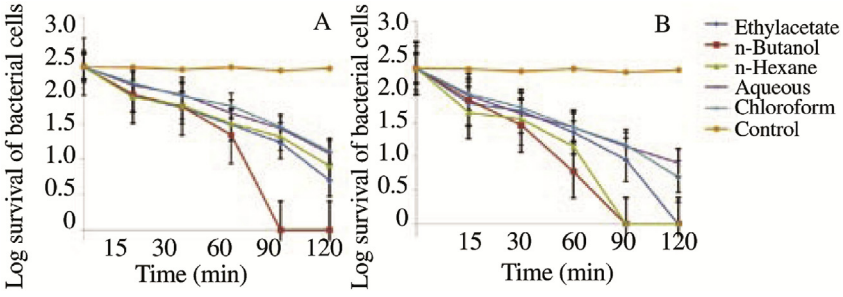

Figure 1. The extent and the rate of killing of *Bacillus pumulis* by ethylacetate, n-butanol, n-hexane, aqueous and chloroform fractions and control at 1 × MIC (A) and 2 × MIC (B).

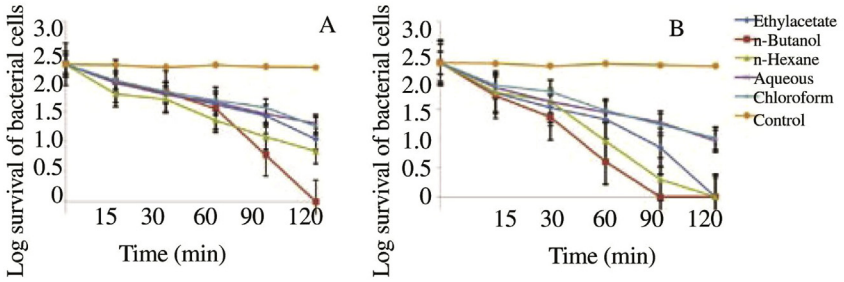

Figure 2. The extent and the rate of killing of *Klebsiella pneumonia* by ethylacetate, n-butanol, n-hexane, aqueous and chloroform fractions and control at 1 × MIC (A) and 2 × MIC (B).

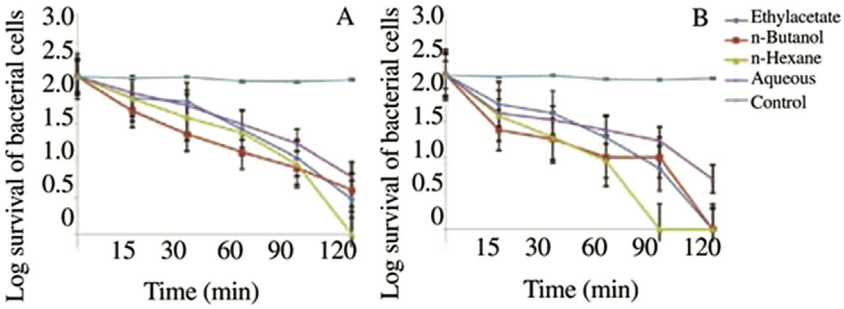

Figure 3. The extent and the rate of killing of *Candida albicans* by ethylacetate, n-butanol, n-hexane and aqueous fractions and control at 1 × MIC (A) and 2 × MIC (B).

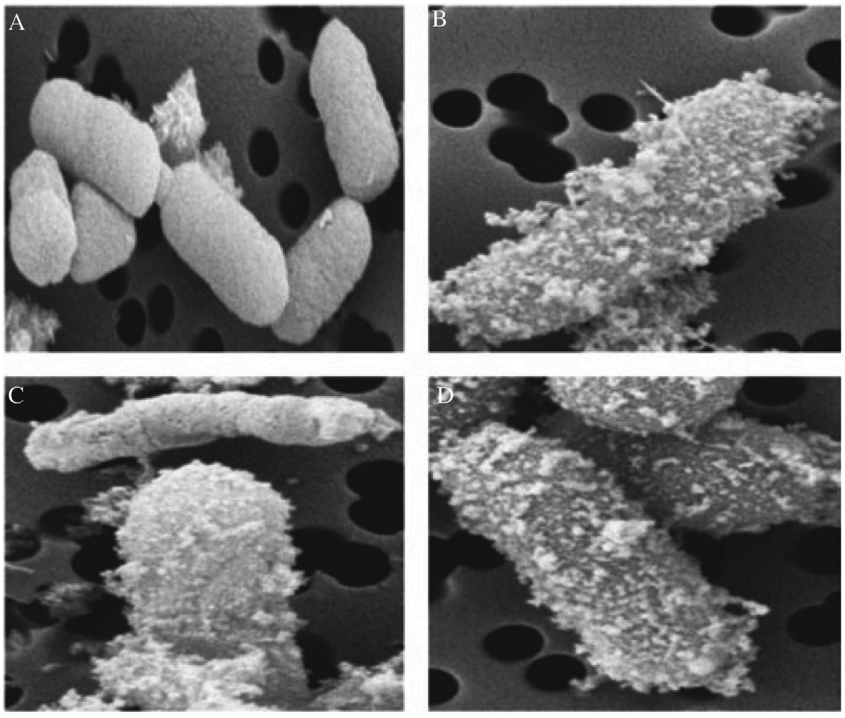

Figure 4. SEM images (1 μm, ×15 000) showing effect of ethylacetate (B), n-butanol (C) and n-hexane fractions (D) and control (A) against *Bacillus pumulis* at 1 × MIC after 120 min of exposure.

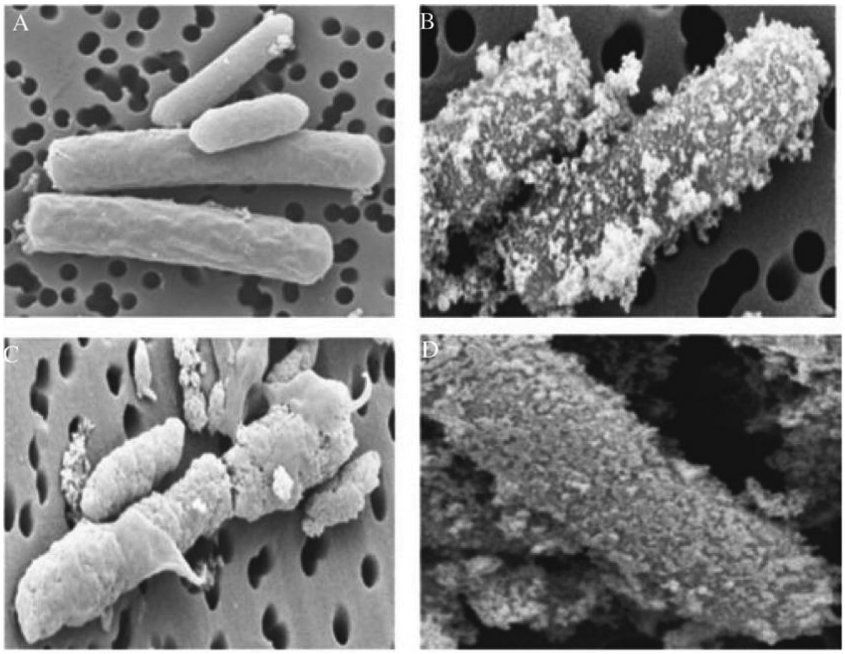

Figure 5. SEM images (1 μm, ×15 000) showing effect of ethylacetate (B), n-butanol (C) and n-hexane fractions (D) and control (A) against *Klebsiella pneumoniae* at 1 × MIC after 120 min of exposure.

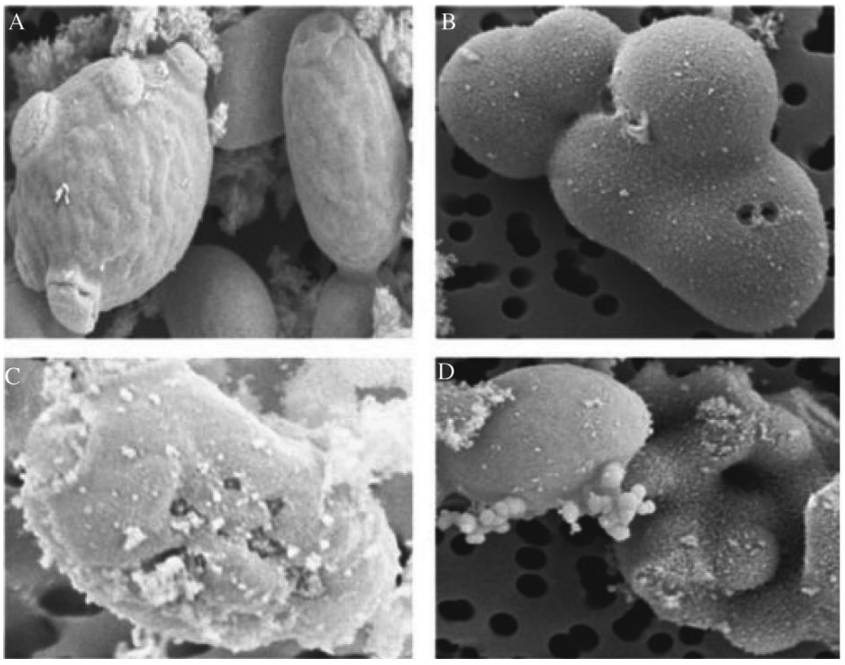

Figure 6. SEM images (1 μm, ×15 000) showing effect of ethylacetate (B), n-butanol (C) and n-hexane fractions (D) and control (A) against *Candida albicans* (Ho316) at 1 × MIC after 120 min of exposure.

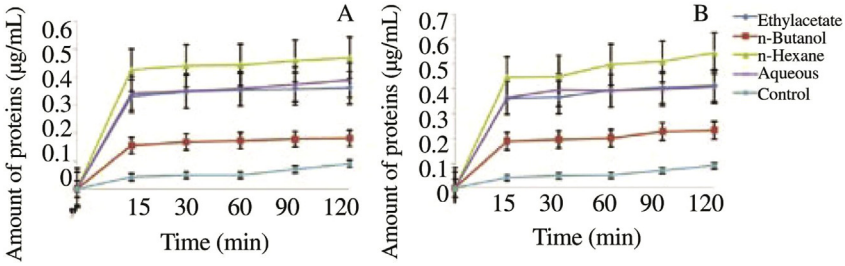

Figure 7. Leakage of proteins from *Bacillus pumulis* by ethylacetate, n-butanol, n-hexane, aqueous and chloroform fractions and control at 1 × MIC (A) and 2 × MIC (B).

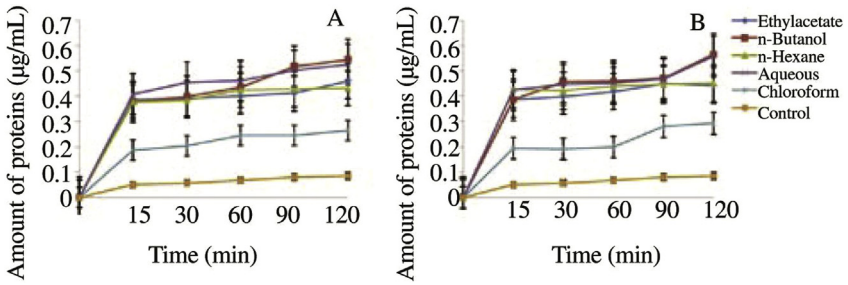

Figure 8. Leakage of proteins from *Klebsiella pneumoniae* by ethylacetate, n-butanol, n-hexane, aqueous and chloroform fractions and control at 1 × MIC (A) and 2 × MIC (B).

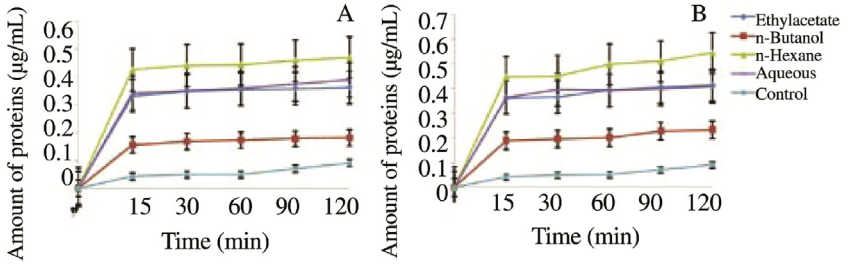

Figure 9. Leakage of proteins from *Candida albicans* by ethylacetate, n-butanol, n-hexane and aqueous fractions and control at 1 × MIC (A) and 2 × MIC (B).

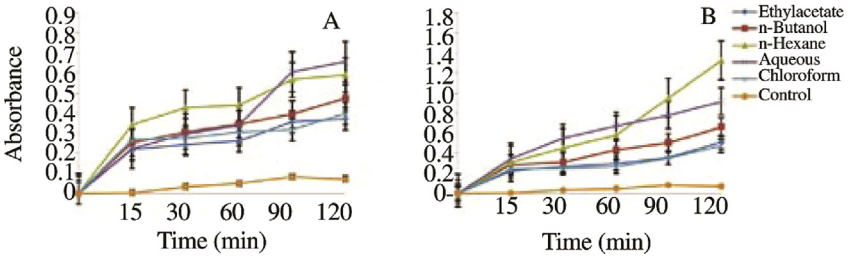

Figure 10. Leakage of nucleotides from *Bacillus pumulis* by ethylacetate, n-butanol, n-hexane, aqueous and chloroform fractions and control at 1 × MIC (A) and 2 × MIC (B).

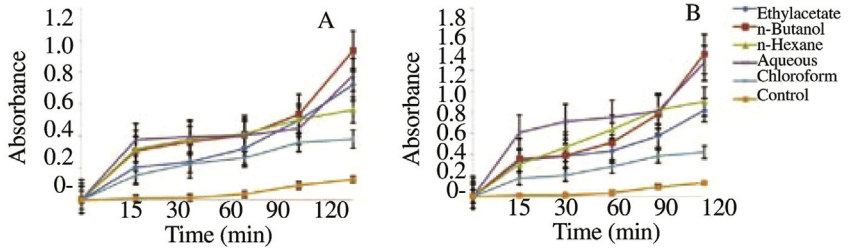

Figure 11. Leakage of nucleotides from *Klebsiella pneumoniae* by ethylacetate, n-butanol, n-hexane, aqueous and chloroform fractions and control at 1 × MIC (A) and 2 × MIC (B).

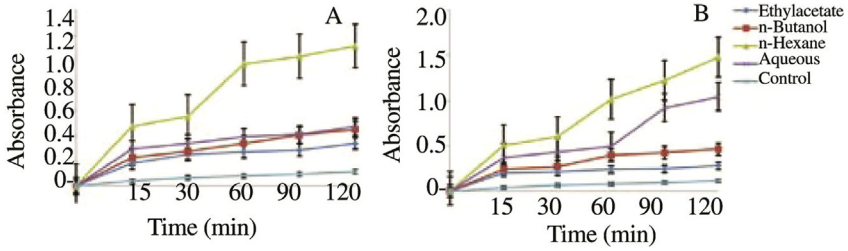

Figure 12. Leakage of nucleotides from *Candida albicans* by ethylacetate, n-butanol, n-hexane and aqueous fractions and control at 1 × MIC (A) and 2 × MIC (B).

4. Discussion

Phytochemical screening of the leaf extract of *E. crispa* reveals the presence tannins, flavonoids, steroids, saponins, reducing sugars and cardiac glycosides all of which have been implicated with antimicrobial properties. Fractions from the leaves extract demonstrated significant antimicrobial potentials with broad spectrum. *S. aureus*, *E. faecalis*, and *Listeria* sp. were among the susceptible pathogens. *S. aureus* has been implicated in bacteremia associated with high morbidity and mortality, often results into infective endocarditis [19]. Pathogenicity of *E. faecalis* ranges from life threatening ailments to less severe disease conditions [20] while *Listeria* sp. is a causative agent of listeriosis with meningoencephalitis and septicaemia as clinical symptoms [21]. The significant activity shown by the extracts against the test pathogens largely premised on the bioactive principles revealed to have been present in the extract. Hence, leaf extracts of *E. crispa* could be a good source of readily available drug against the aforementioned ailments in folklore remedy. The growth of *E. coli* a common causative agent of diarrhoea and urinary tract infections [13] and species of *Salmonella* and *Shigella* were also significantly inhibited by the extracts. This thus validates traditional use of the leaf of *E. crispa* as antidiarrhoeal agent by the people of eastern Free State, South Africa. Moreover, antifungal capability of the plant extract was also investigated. *Cryptococcus neoformans* an emerging yeast pathogen of man has been reportedly responsible for annual deaths of about six hundred thousand immuno-compromised individuals [22]. *C. albicans,* a notorious pathogenic yeast, due to its inherent resistance to antimicrobial therapy accounts for large number of fungal infections in the skin, digestive tract and bloodstream [23]. The susceptibility of these pathogens to the leaf extracts further affirm its efficacy in the management of infectious diseases by the people of Lesotho as earlier reported by Moteetee & Van Wyk [24].

All fractions from the leaf extract exhibited notable antimicrobial activities against some of the test bacterial and fungal isolates with low MIC values which compared favorably with standard antimicrobial drugs used as positive control. The lowest MIC (0.16 mg/mL) was exhibited by the ethylacetate and n-butanol fractions against *B. pumilis* (ATCC 14884) and *Pseudomonas aeroginosa* respectively, thus suggesting both solvents as probable choice of solvent in extraction of most active compounds from this plant. This may be an advantage in the production of antimicrobial compound of natural origin to combat emerging cases of multidrug resistant microbial pathogens. On a general note, ethylacetate fraction showed the best activity with the highest MIC values of $\leq$0.63 mg/mL against all test isolates. This corroborates with the finds of Magama *et al.* [4] where zones of inhibition by different fractions of *E. crispa* were observed against certain human pathogens. Although n-hexane fraction appeared as the most potent in terms of the cell membrane attack considering the amount of intracellular components leakages couple with the SEM imaging which is more prominent against the *C. albicans* tested.

However, we have come to the conclusion that ethylacetate, n-butanol and n-hexane fractions are generally the most active fractions after considering the rate at which each of the potent fractions was able to bring about depression in the number of survival cells after treatment.

The leaf extract of *E. crispa* could be a good source of bioactive agents against wider range of infectious diseases which maybe applicable in the alternative therapy practices as it is readily available and as well in the pharmaceutical industries. Furthermore, membrane disruption has also been confirmed in the course of this study as one of the mechanisms of biocidal action of the leaf extract.

Conflict of interest statement

The authors declare no conflict of interests.

Acknowledgement

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