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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*

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