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Research Article

Antibacterial capacity of *Leucodon immerses*

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ABSTRACT
Antibacterial activity of ethanol extracts of a moss species, *Leucodon immerses,* collected from Gilan province, North of Iran was analyzed. Antimicrobial activity was tested against four microorganisms including one Gram-positive: *Staphylococcus aureus* and three Gram-negatives: *Klebsiella oxytoca*, *Escherichia coli* and *Pseudomonas aeruginosa*. It was shown that the antibacterial effect of ethanol extracts was higher against the Gram negative (*Klebsiella oxytoca*, *Escherichia coli*, and *Pseudomonas aeruginosa*) compared to its effect on Gram positive (*Bacillus subtilis*, *Staphylococcus aureus*) bacteria tested.

Keywords: Mosses; ethanol extracts; *Leucodon immerses*; antibacterial activity; Gram negative.

1. INTRODUCTION

Mosses are non-vascular plants in the land plant division Bryophyta. They are a few centimeters tall herbaceous non-woody plants that absorb water and nutrients mainly through their leaves and harvest sunlight to create food by photosynthesis. They can, some times, become troublesome weed in containerized nursery operations and greenhouses. As vigorous moss growth can inhibit seedling emergence and penetration of water and fertilizer to the plant roots. Their nice appearance and fast growth is amazing when used in garden decorations. Traditionally, mosses have been known for their various medicinal effects. Their earliest medicinal applications go back to World War I, when *Sphagnum* mosses were used as first-aid dressings on soldiers' wounds for treatment of bacterial infections[1]. Some moss species are genetically investigated and their genes identified aiming to understand their potential for use in human health[2]. Some types of mosses are even used as bioreactors to produce biotechnologically engineered pharmaceutical products[3]. The damp nature of deep forests in North of Iran has created a suitable environment for widely growth of various moss species. As *Leucodon immerses* was a common type of mosses in this area and, based on our literature survey, its antibacterial activity was not reported in scientific literature, the aim of this study was investigating the antibacterial activity of this species on a variety of Gram positive and negative bacterial strains. The activity was then compared with a few known potent synthetic antibiotics.

2. MATERIALS AND METHODS

2.1. Collection of moss

Leucodon immerses was collected from deep forests of Gilan province (North of Iran). They were transferred to the research laboratory, separated from wastes, washed, and dried in oven at 37ºC for 48 hours and kept in dry closed container until extraction process.

2.2. Preparation of ethanol extracts from mosses

In this study, the solvent of choice for extraction was 70% ethanol. 10 g of the dried moss was incubated in 200 ml of 70% ethanol at 60° C in flask for 48 hours. The resulting mixture was then filtered through a No. 2 Whatman filter paper. This was followed by addition of 200 ml 70% ethanol to the filtrate and incubation at 60° C for another 48 hours. Both supernatants were added together and the extract obtained in this way was concentrated in a Rotary type apparatus (vacuum distillation) and the almost dry mixture was stored at 4° C[17]. The concentrated extract was incubated at 50° C until dryness. For antibacterial tests, a stock solution of 200 mg/ml was then prepared using dimethyl sulfoxide (DMSO) as solvent[18]. Later, 100, 50, 25, 12.5 and 6.25 mg/ml solutions were prepared from the stock solution using DMSO.

2.3. Preparation of growth medium

Antimicrobial studies were carried out using four microorganisms including one Gram-positive: *Staphylococcus aureus* (PTCC 1133) and three Gram-negatives: *Klebsiella oxytoca* (PTCC 1402), *Escherichia coli* (PTCC 1553), and *Pseudomonas aeruginosa* (PTCC 1558). The bacterial strains were obtained from Persian Type Culture Collection (PTCC) centre in Tehran and stored frozen at -80 °C. The bacterial vials were opened as recommended by PTCC and the strains were grown in nutrient broth followed by incubation at 37 °C for 4 hours. Disk diffusion method was used in order to *in vitro* compare the antibacterial activity of moss extracts with synthetic antibiotics. Practically, broth subcultures were prepared by inoculating, with one single colony from a plate in a test tube containing 5 ml of sterile nutrient broth. The tubes were then incubated at 37 °C for at least 24 hours[4].

The bacterial culture was allowed to reach a concentration of 10^8 CFU/ml (Colny Forming Unit/ml). Each suspension was spread on Muller Hinton Agar medium by sterile swabs. Whatman filter paper disks (diameter 6 mm) dipped in extracts with a range of concentration and two known antibiotics, i.e. gentamicin and ampicillin (Padtan Teb™, Iran) were placed on the agar surface. The diameter of inhibition zone was measured after incubation of all plates at 37°C for 24 hours. The inhibition zones (including disk diameter) less than 10 mm were considered as negative. The resulted inhibition zones were the average of three measurements.

2.4. Measurement of Minimum inhibitory concentration (MIC)

The experiment was performed by micro dilution broth method in 96 wells plat. 50 ml of extract was added to the first well and serial dilutions were prepared down to the last well, 50 ml of microbial suspension diluted to 0.5 Mac Farland tube was then added to each well. The positive control was microbial suspension and Muler hinton broth medium, while the negative control contained the plant extract and Muler hinton broth medium. The optical density (OD) was immediately measured at 630 nm using ELISA reader (Stat Fax 2100). The second OD was measured after 24 hours. MIC was considered as minimum concentration at which reduction of OD was observed[5].

2.5. Measurement of MBC

In order to find the MBC, all clear wells from MIC measurement were further grown on Muler hinton broth medium. The culture medium with no bacterial growth was considered as MBC[5].

2.6. Statistical analysis

The student t-test was used with the GraphPad PRISM® software (GraphPad Software, Inc., San Diego, CA, USA) and $p \leq 0.05$ differences were considered as significant.

3. RESULTS AND DISCUSSIONS

Antimicrobial properties on various dilutions of ethanol extract was evaluated by measuring the diameter of inhibition zone as well as calculating minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The results were then compared to the two synthetic antiobiotics. Table 1 shows the diameter (mm) of inhibition zone and Table 2 represents the values of MIC and MBC of extracts on tested bacteria.

Table 1: Growth inhibition zone (mm) of different bacterial strains in the presence of moss extracts

Effector / Bacteria	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	Gentamicin	Ampicillin
K. oxytoca	14	12	10	10	9	24	12
E. coli	13	--	--	--	--	23	13
P. aeruginosa	15	12	--	--	--	20	7
S. mutance	26	23	22	21	19	31	22

Table 2: MBC values for different bacterial strains in the presence of moss extracts

Bacteria / Factor	*K. oxytoca*	*E. coli*	*P. aeruginosa*	*S. mutance*
MBC (µg/ml)	100	--	--	25
MIC (µg/ml))	50	--	100	12.5

The use of medicinal plants with antibacterial properties is drawing extensive attention during the recent decays[6, 7]. There are a wide variety of medicinal plants grown in Northern part of Iran some of which have been studied previously within our research group. These include peanuts[8], citrus peels[9], *Crocus Sativus* flowers[10], leaves and flowers from *Mentha pulegium*[11] and tea leaves[12]. The purpose of this study was evaluation of antimicrobial effects of ethanol extracts of moss species, *Leucodon immerses*, collected from Gilan province, North of Iran against gram positive and gram negative microorganisms. A selection of bacterial strains was used

for this purpose including *E. coli*, *K. oxytoca* and *P. aeruginosa* and *S. mutance*. The results obtained from inhibition zone diameter, MBC and MIC experiment support each other. The ethanol extracts of this species of moss can be used as a strong, reliable natural antimicrobial agent. As expected, the Gram-positive bacteria were more sensitive than Gram-negative ones due to protection provided by hydrophobic lipopolysaccharide in the outer membrane[13]. Our results showed that ethanolic extracts of *Leucodon immerses* had inhibitory or germicidal effect depending on the type of organisms . A significant antimicrobial activity against Gram-positive bacteria was observed especially on *S. mutance*, the bacteria moslty involved in oral infection and periodontal problems (Table 1). It was observed that a 100mg/ml solution of ethanolic extract could almost exhibit activity slightly less than gentamicin and more than ampicillin (see Table 1). According to this research, the antimicrobial effect of a concentrated solution from *Leucodon immerses* extract (100mg/ml) was as strong as ampicillin to affect the growth of all tested bacteria. The results obtained in our research is supported by observations reported for mint extracts (Cárdenas-Ortega et al., 2005). In Iranian cultural medicine, moss extracts is used against bacterial infectious diseases[14] which could be a practical support for the present study. The increasing antibiotic resistance of pathogens together with undesirable side effects of synthetic antibiotics suggested the use of *Leucodon immerses* and other medicinal plant preparations as natural alternatives. However, further research is required to evaluate the practical values of therapeutic application. In agreement to our design, the antimicrobial activity of ethanolic extracts of 15 Indian mosses has been investigated against five G(+) and six G(−) bacterial strains[15]. They have also reported that the ethanolic extract was mostly effective on G(+) bacterial strains. Using a disk diffusion method, methanol extracts of ten moss species of *Hylocomium splendens* were evaluated[16]. In agreement to our results, most of moss species showed antibacterial activity against Gram (+) bacteria. They have also observed that antibacterial activity against *Staphylococci* was enhanced considerably by UV-A light. It has been found that the phytochemical composition of moss species extracts could vary by changing the solvent used for extraction[17]. They reported that among the five different extracts, ethanol and chloroform extracts show the presence of maximum number (4 each) of compounds. The results on antimicrobial studies show that all tested bacteria, i.e. *S. aureus*, *E. coli* and *C. albicans* were resistant to the ethanol extract, but highly susceptible to petroleum ether extract.

CONCLUSIONS

Based on the results obtained from this study and supported by the traditional use of *Leucodon immerses* from Gilan province, it is concluded that anti bacterial activity is mostly shown on Gram (+) bacteria. We observed that *S. mutance* could by inhibited by ethanolic extracts of this moss species. However, as the extraction solvent plays important part in the phytochemical composition of extract and, therefore, its medicinal properties, more investigations are to be done using other solvents with decreasing polarity. It is also suggested that various mosses in the place be identified and screened for antibacterial as well as antioxidant properties.

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