

Assessment of antimycotic activity of seabuckthorn (*Hippophae rhamnoides*) leaf extracts against common fungi associated with skin dermatitis

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Abstract

Aim: To study the antimycotic activity of seabuckthorn leaf extracts against common fungi associated with skin infection.

Materials and Methods: A total of 31 isolates were collected from the skin cases of animals and the leaves of seabuckthorn collected from the Lahaul and Spiti valley of the Himachal Pradesh. The extracts from the leaves were extracted according to standard procedures. Antimycotic activity was examined by employing agar disc diffusion method on Roswell Park Memorial Institute medium (RPMI). Different concentrations of leaf extracts i.e. 0.50%, 2%, 3%, 4%, 5%, respectively were used.

Results: The study revealed that the inhibitory effect of SBT leaf extract at 5% concentration was observed to be almost 80% in 12 hrs of reading as compared to the standard drugs used as positive control against fungal isolates.

Conclusion: Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antimycotic activity assay. In the present study, a wide range of pathogenic microorganisms were recovered from the clinical samples, including bacteria and fungus. The seabuckthorn methanolic leaf extracts (hot and cold) were studied for their antimycotic activity against common fungal pathogens.

Keywords: antimycotic, disc diffusion, seabuckthorn leaf extract

Introduction

“Antimicrobial resistance is the global problem that needs urgent action” [1]. It is undiluted truth that pain, disease and death are always associated with the human and animal life. The humans of early ages used therapeutic agents available easily to them and thus, plants have been used as remedies since time immemorial. The plants are therefore, man's oldest resources of useful drugs. The history of the medicine and pharmacy begins with Hippocrates, the father of medicine. Medicinal herbs for the treatment of diseases have actively been practiced since the beginning of human civilizations. The skin is supposed as an organ of considerable elasticity and tensile strength that provides a barrier to the external environment. Its prime function is to prevent the invasion of bacteria and viruses and thus to maintain internal homeostasis. One of the major concerns in healthcare today is the ineffectiveness of antibiotics towards new and re-emerging diseases due to the developed resistance in many pathogens. Thus, there is an urgent need to identify novel anti-microbial molecules as leads for effective drug development. Seabuckthorn (*Hippophae species*) is one among the widely known important medicinal plants. The genus name *Hippophae* is classical Latin for "shining horse," a name that was given in ancient times after it was found that feeding the leaves to horses improved their health and made their hair shiny. The plant has got tremendous potential

for therapeutic utilization in human and Veterinary medicine, as it is reported to possess many bioactive substances notably lipids, fatty acids, vitamins (both fat soluble and water soluble), flavonoids, tannins, phenols, steroids, 5-hydroxytryptamine etc. [2]. The presence of these bioactive substances in the plant contributes enormously to its various properties like antibacterial, antifungal, immunostimulant, anti-inflammatory, anti-radiation, antioxidant, hepatoprotective, cytoprotective, anticancerous, anti-atherosclerosis, anti-diabetic, anti-ulcer as well as wound healing actions [3].

In India, seabuckthorn is found in Himalayan states of Himachal Pradesh, Ladakh (J&K), Uttanchal, Sikkim and Arunachal Pradesh. There are about 12,000 ha of seabuckthorn under thick forests, mainly in Ladakh. In Himachal Pradesh, seabuckthorn grows in Lahaul-Spiti, upper Kinnaur and Pangi in Chamba district. Both *H. rhamnoides* and *H. Salicifolia* have been found in Chango area of upper Kinnaur [4]. Seabuckthorn has also been found in high altitude areas of Shimla and Kullu districts. "India has world's second largest resource of seabuckthorn. Natural products will always be considered as one of the major sources of new drugs in the future because it offers structural diversity, and most are small in size. Some experts believe that plants may well hold the secret to combat diabetes, cancer and AIDS etc. The Hiporamin a tannin fraction from SBT possessing a wide spectrum of antiviral activities and mild antimicrobial activities [5].

The present study was carried out to determine the *in vitro* antimycotic activity of methanolic extracts of seabuckthorn leaves against common fungal pathogens associated with skin dermatitis [6].

Materials and Methods

Plant material: The seabuckthorn leaves were collected from the Lahaul and Spiti valley of the Himachal Pradesh. The leaves were than shade dried and then brought to the Department of Veterinary Microbiology, DGCN COVAS, Palampur.

Fungal cultures: The standard strains of *Microsporium gypseum* and *Trichophyton rubrum* were obtained from the Institute of Microbial Technology, Chandigarh (IMTECH). In addition, clinical isolates of *Microsporium gypsum*, *Trichophyton rubrum*, *Aspergillus* spp., *Mucor* spp. *Microsporium canis*, *Candida* spp., *Rhizopus* spp. and *Absidia* spp. were isolated from clinical cases of skin and wound infections from Veterinary Clinic, College of Veterinary and Animal Sciences, Palampur, H.P.

Identification of fungi: Identification of fungi was done on the basis of macroscopic and microscopic morphology/characteristics.

Macroscopic morphology: Textures, topography, growth rate, obverse and reverse side pigmentation of colonies were noted.

Microscopic characteristics: Microscopic characteristic of fungi e.g. budding cells, hyphal structures, macroconidia, microconidia and other mycotic structures were examined.

Drug sensitivity profile of fungal isolates: Drug sensitivity of fungal isolates was observed by disc diffusion method as recommended by the NCCLS (M38-A standard) [7]. The fungal isolates after identification were subjected to *in-vitro* antifungal susceptibility testing by disc diffusion methods. The fungal isolates were grown on PDA slants for one week at 35°C and then flooded with approximately 1ml of sterile NSS. The resultant liquid was allowed to stand in a test tube to allow the heavier particles to settle down. The supernatant was taken out and mixed for 10 min. and the opacity adjusted to 0.4 McFarland barium sulfate opacity standards. A sterile swab was used to streak the liquid on to the RPMI agar plates. Antifungal discs were placed on the agar and plates were incubated at 35°C. Results were interpreted 24 hrs and 48 hrs. Antifungal discs and their concentration have been shown in Table-1.

Protocol for inoculums preparation for fungus: Inoculums consists of non-germinated conidial suspension

Step 1: Growing of fungus on potato dextrose agar slants 7 days at 35°C

Step 2: Flooding of slant with approximately 1 ml of sterile NSS and subsequently withdrawal of suspension

Step 3: Heavy particles allowed settling for 3 to 5 min

Step 4: Upper suspension of non-germinated conidia/sporangiospores/hyphal fragments mixed for 15 seconds

Step 5: Turbidity measured at 530 nm

Step 6: Diluted 1:50 in RPMI 1640 medium

Table-1. Antifungal discs used and their concentration

Antifungal agents	Symbol	Conc./disc
Ketoconazole	Kt	10 µg
Miconazole	Mi	10 µg
Nystatin	Ns	100 units
Amphotericin-B	Ap	100 units
Itraconazole	It	10 µg
Fluconazole	Fu	10 µg
Clotrimazole	Cc	10 µg

Preparation of leaf extract

Grinding of selected plant material: After drying of leaves at 37°C for 3 to 5 days, there were grounded in the grinding machine made for the laboratory. Exposure to sunlight was avoided to prevent the loss of active compounds [8].

Extraction methods: In this study, we used two protocols of extraction: Soxlet extraction and Cold percolation.

Soxlet extraction method: A thimble was prepared by using whatman filter paper. 30 gm of dried powder of seabuckthorn leaves was kept in thimble for extraction. 400 ml of 100 per cent of methanol was used for this process and left for 8 hours in soxlet apparatus at 60°C. Filtered the residue by using whatman filter paper no.1. The extracted liquid was subjected to rota-evaporation or water bath evaporation to remove the excess of methanol. The semi solid extract produced was kept in the deep freezer at -80°C overnight.

Cold extraction method: 100 gm of seabuckthorn leaves powder was mixed with 400 ml of 100 per cent methanol and left for 24 hours in orbital shaker at 25 to 30°C at 120 rpm. Filtered the residue by using triple whatman filter paper no.1. Dried the filtrate by the rotator vacume pressure for recovery of methanol. The semi solid extract produced was kept in the deep freezer at -80°C overnight.

Result and Discussion

Isolation pattern in fungal dermatitis: *Microsporium gypseum* was major fungus isolated (16.13%) followed by *Aspergillus* spp. (16.13%). Dermatophytes accounted for 36.00 per cent of the total fungal isolates. Other molds and yeasts accounted for 64.00 per cent. Isolation pattern has been shown in Table-2. Other dermatophytes isolated were *Microsporium canis* (9.67%), *Trichophyton rubrum* (6.45%), *Epidermatophyton floccosum* (3.23%). Among non-dermatophytes *Aspergillus* species and *Mucor* spp. were the more frequently isolated fungi with prevalence of 16.13% and 12.9%, respectively. Other fungi isolated were *Rhizopus* spp. (9.67%), *Candida* spp. (16.13%), *Absidia* spp. (9.67%), respectively.

Table-2. Isolation pattern in fungal dermatitis

Organism isolated	No. of isolates	Per cent isolates
<i>Aspergillus</i> spp.	5	16.13
<i>Mucor</i> spp.	4	12.9
<i>Microsporium gypseum</i>	5	16.13
<i>Microsporium canis</i>	3	9.67
<i>Trichophyton rubrum</i>	2	6.45
<i>Epidermophyton floccosum</i>	1	3.23
<i>Candida</i> spp.	5	16.13
<i>Rhizopus</i> spp.	3	9.67
<i>Absidia</i> spp.	3	9.67
Total	31	100

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antimycotic activity assay [9]. In the present study, a wide range of fungal isolates were recovered from the clinical samples, including dermatophytes and other zygomycetes. The seabuckthorn methanolic leaf extracts (hot and cold) were studied for their antimycotic activity against common skin dermatophytes. These included 31 fungal isolates. Both SBT leaf extracts (hot and cold) showed significant antimycotic activity against the common fungal isolates. The antifungal activity of SBT leaf extracts (Hot and Cold) against skinborne fungi is presented in Table-3 and 4 as zone of inhibition (in mm). Antimycotic activity of leaf extracts were tested with a comparable concentration of antibiotics ranging 10 to 30 µg/disc. The leaf extract was found significantly effective against *M. gypseum* and *T. rubrum*. The inhibitory effect of SBT leaf extracts at 5 per cent concentration was observed to be almost 50 per cent compared to the standard drugs used as positive control against *M. gypseum* and *T. rubrum*

(Table 3, 4 and Fig. 1, 2). The cold methanolic extract was found to be most significant as compared to the hot methanolic extract. Cold methanolic extract at 5 per cent concentration was giving 10.13 mm zone of inhibition against *M. gypseum* and *T. rubrum* while hot was almost giving 9 mm zone of inhibition. Many reports revealed, that plant metabolites appear to be one of the better alternatives as they are known to have minimal environmental impact [10]. Hence, the plants can be used to discover bioactive natural products that may serve as leads in the development of new pharmaceuticals research activities [11]. In 2010 Sule [12] the *in vitro* investigation of ethanolic activities of extracts of *Senna alata* leaf was carried on Dermatophytes which include the genera *Trichophyton*, *Microsporium* and *Epidermatophyton* and their results showed that the leaf exudates and the ethanolic extract of the leaf of *Senna alata* L. Exhil: had marked antifungal effects on *Microsporium canis*, *Trichophyton mentagrophytes* and *Epidermatophyton* spp. The ethanolic extract showed the highest zone of inhibition on *Trichyophton* spp. and *Epidermatophyton* spp. 20.50 and 20.00 mm zone of inhibition, respectively.

Conclusion

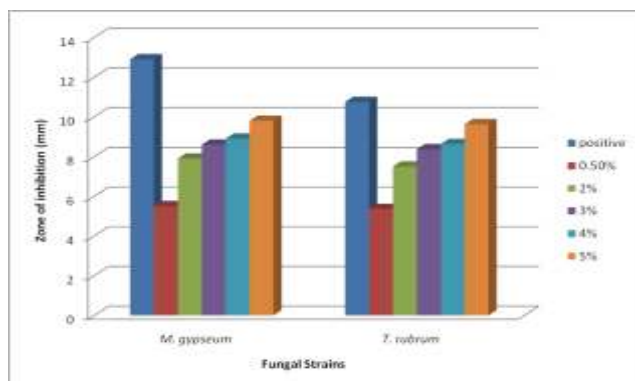
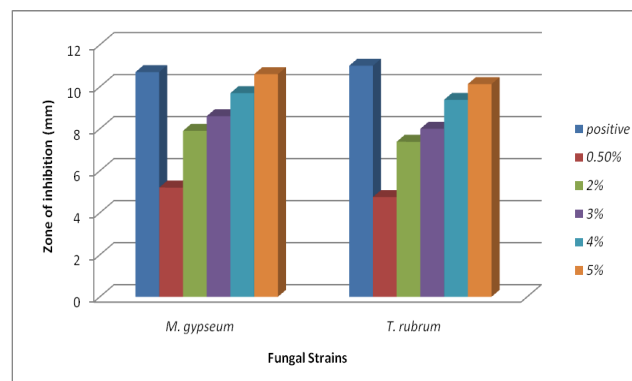
In this study, the extract of seabuckthorn leaves extract having high potential of antimycotic activity. It showed varying degrees of activities against all the tested dermatophytes with better antifungal activity against *M. gypseum* and *T. rubrum*. The phytochemical studied revealed the presence of important secondary metabolites (alkaloids, saponin, tannin and steroids), thus indicating the therapeutic potential of seabuckthorn leaf.

Table-3. *In-vitro* antimycotic activity with hot methanolic extract indicating the zone of inhibition in mm (Mean ± S.E.)

S.No.	Genus spp.	Positive control	Concentration of leaf extract					Negative control
			0.5%	2%	3%	4%	5%	
1.	<i>M. gypseum</i>	12.9±0.4819	5.5±0.4014	7.9±0.2769	8.6±0.1633	8.9±0.2333	9.8±0.2000	Resistant
2.	<i>T. rubrum</i>	10.75±0.3134	5.36±0.3239	7.5±0.2673	8.37±0.1830	8.63±0.1830	9.63±0.1830	Resistant

Table-4. *In-vitro* antimycotic activity with cold methanolic extract indicating the zone of inhibition in mm (Mean ± S.E.)

S.No.	Genus spp.	Positive control	Concentration of leaf extract					Negative control
			0.5%	2%	3%	4%	5%	
1.	<i>M. gypseum</i>	10.7±0.2134	5.5±0.3266	7.9±0.1795	8.6±0.1633	9.7±0.1528	10.6±0.1633	Resistant
2.	<i>T. rubrum</i>	11.0±0.2673	4.75±0.2500	7.38±0.1830	8.0±0.1890	9.38±0.1830	10.13±0.2266	Resistant

Fig. 1. *In-vitro* antimycotic activity with SBT leaves hot methanolic extractFig. 2. *In-vitro* antimycotic activity with SBT leaves cold methanolic extract

It showed the presence of bioactive compounds as well as the antifungal properties of methanolic extracts. However, this finding provides an insight into the usage of this plant in treatment of fungal infections.

Authors' contribution

MS designed the study and all the other authors performed research experiments. MS drafted and revised the manuscript. All the authors read and approved the final manuscript.

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Competing interests

Authors declare that they have no competing interest.

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