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Research Article
Antifungal Activity of Methanolic Extracts of Leaves of *Eucalyptus citriodora* and *Saraca indica* Against Fungal Isolates from Dermatological Disorders in Canines

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Abstract

Background and Objective: *Saraca indica* and *Eucalyptus citriodora* have been used in traditional medicine against variety of health disorders. Present study was undertaken with the objective of evaluating the *in vitro* antifungal activity of methanolic extracts of leaves of *Saraca indica* L. (SI) and *Eucalyptus citriodora* (EC) individually and both in combination (SI+EC; 1:1 w/w) against the fungal isolates obtained from male dogs suffering with general dermatitis and recurrent chronic pyoderma. **Methodology:** Antifungal activity of SI (20 mg mL^{-1}), EC (20 mg mL^{-1}) and SI+EC (10+10 = 20 mg mL^{-1}) extracts was evaluated by disc diffusion method against standard culture of *Microsporum* sp. and clinical isolates of dermatophytes, *Candida* and *Aspergillus sp.* fungus obtained as the mixed culture from clinical cases under study. Fluconazole (25 µg $disc^{-1}$) was used as the positive control while methanol as the negative control. **Results:** All the three extracts (SI, EC, SI+EC) showed promising and prolonged antifungal activity against different fungal micro-organisms. **Conclusion:** This study evidently demonstrated promising antifungal activity of the methanolic extracts of *Saraca indica* and *Eucalyptus citriodora* leaves.

Key words: Anti-fungal, *Eucalyptus*, *Saraca*, ashoka, methanolic extract, disc diffusion method

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Use of medicinal herbs for treatment of skin diseases, including pyoderma, general dermatitis and mycotic infections, is an age-old practice in different parts of the world[1]. Antimicrobial compounds isolated from certain plants have been found to be certain secondary metabolites that serve as the defense agents against invading microorganisms; hence should be investigated for their anti-microbial properties including antibacterial, antifungal and antiviral efficacy[2]. Many plants extracts possess diverse antimicrobial activities which include antibacterial, antiviral, antifungal, anthelminthic, antimalarial and against other protozoa but investigations regarding antimycotic activity are fragmentary and meager[3].

Saraca indica (family Caesalpiniaceae) has been used in traditional medicine against variety of health disorders. Leaves, stem, stem bark, flowers, pods and seeds of this plant contain array of phytochemical compounds which include glycosides, tannins, saponins, flavonoids and sterols. Procyanidin b2, oxyprocyanidin B, leucocyanidin, epicatechin and catech in the bark and oleic, linoleic, palmitic and stearic acids, P- sitosterol, quercetin, kaempferol-3-O-P-D-glucoside, quercetin- 3-o-P-D-3-o-P-D-glucoside, apigenin-7-o-P-D-glucoside, pelargonidin-3,5-diglucoside, cyaniding-3 etc. in flowers have been identified[4]. High Performance Thin Layer Chromatography (HPTLC) studies have shown the presence of catechin and flavonoids in stem bark of *Saraca indica* (*S. asoca*). Oleic, linoleic, palmitic, stearic, catechol, epicatechol and leucocyanidin in seeds and pods while quercetin, quercetin-3-o-alpha-l-rhamnoside, kaempferol 3-o-alpha-L-rhamnoside, cetyl alcohol and beta-sitosterol in leaves and stem. These phytochemical constituents impart beneficial antimicrobial activity, fungitoxic, anthelmintic, larvicidal, CNS depressant, antiulcer, anti-inflammatory, analgesic and antipyretic activity when used properly[5-8]. Some researchers have shown the antifungal activity of methanolic as well as hot aqueous extracts of *S. asoca* leaves, flowers and bark against variety of fungal species such as *Candida* sp., *Alternaria alternata, Colletotrichum gloeosporioides, Drechslera spicifera, Alternaria cajani, Helminthosporium* sp., *Bipolaris* sp., *Curvularia lunata, Aspergillus flavus, A. fumigates* and *Fusarium* sp.[9].

Eucalyptus citriodora (family Myrtaceae) is affluent in biologically active secondary metabolites, namely: polyphenols, flavonoids, gallotannins and ellagitannins, cyclic polyketones, simple acylphloroglucinols, complex acylphloroglucinol derivatives, deroxylonals A and B, grandinol, homograndinol, triterpenes and many terpenes particularly citronellal, g-phellandrene, g-terpineol, piperitone, g-and fleudesmols, aromadendrene, globulol and spathulenol[10]. Eucalyptol is the chief constituent of essential oils obtained from *E. globulus*[11]. The oils, bark and leaves from certain species have shown efficacy in treatment of cold, influenza, toothaches, snake-bites, fevers, mechanical wounds, diarrhoea etc.[12,13]. Crude methanolic extract of *Eucalyptus* containing deroxylonals A and B has been shown to possess anti-viral effect against Epstein-Barr virus and antibacterial effect against *Staphylococcus aureus* and *Bacillus subtilis* but not against Gram-negative bacteria, yeast or fungi in literature[10,14].

In view of the very common occurrence of *Saraca* and *Eucalyptus* plants in almost whole of the country, availability of leaves of both these plants in abundance, very common occurrence of dermal infections in humans and animals, especially canine pyoderma, present study was undertaken to investigate the *in vitro* antifungal activity of methanolic extracts of leaves of *Saraca indica* L. (Ashoka tree) and *Eucalyptus citriodora* L. individually and both in combination.

MATERIALS AND METHODS

Plant material and preparation of the extracts: Leaves of *Saraca indica* and *Eucalyptus citriodora* were collected from DUVASU campus and authenticated by Prof. A. K. Agrawal, Head, Department of Botany, B. S. A. Degree College, Mathura, UP, India based on the taxonomic features of the whole plant material including leaves. Leaves were shade dried after proper cleaning. Methanolic extracts of coarsely ground leaves of both these plants were prepared using soxhlet apparatus by hot percolation method and the extracts obtained were concentrated to dryness using rotatory evaporator under reduced pressure and low temperature (<40°C). Extracts were kept in air-tight containers and stored at 4°C for further studies.

Isolation of the fungus: Fungi were isolated from the clinical cases of canines suffering with chronic dermatophytosis, scrotal dermatitis, alopecia and general dermatitis which were brought to the Teaching Veterinary Clinical Complex of the Institute for treatment. Samples were collected using sterile swab and inoculated into Sabouraud broth and incubated at room temperature. When fungal growth appeared, culture was inoculated over Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) plates for isolation and identification of the fungal isolate using standard methods. Isolated fungal culture was maintained over Sabouraud

Dextrose Agar (SDA) slants at 4°C and sub-cultured intermittently. Before start of the experiment, isolate of *Microsporum* was inoculated over SDA plate and incubated at 25°C for 7-10 days to obtain young and actively growing culture consisting of the mycelia and used as the standard fungal culture.

Evaluation of *in vitro* antifungal activity: Efficacy of the methanolic extracts of leaves of *Saraca indica* (SI), *Eucalyptus citriodora* (EC) and mixture of both these extracts (SI+EC; 1:1; w/w) was evaluated against *Microsporum* fungal culture isolated from the clinical cases and considered as the standard culture. *In vitro* antifungal activity was evaluated against the fungal isolates obtained from four clinical cases of dogs. Anti-fungal efficacy of all the three test extracts was tested initially qualitatively to observe the anti-fungal effect of test extracts by painting the surface of sterile SDA agar with 200 µL of each of these extracts in three different plates. Extracts were allowed to dry at room temperature. After ten minutes, 72 h old fungal cultures (hyphae formation stage) from Sabouraud's broth of standard fungal culture and clinical isolates were inoculated over these plates. Test plates were incubated at room temperature for 7-20 days and results recorded up to 21 days post-inoculation.

For quantitative or dose-dependent assessment of the anti-fungal activity of all the three test extracts, disc diffusion method[15] was employed. From the Sabouraud's broth culture of clinical isolates, 900 µL culture of each was poured over SDA plates and spread uniformly. Fluconazole (FLC = 25 µg disc, HiMedia, India) was used as the positive control while the non-medicated sterile discs (HiMedia, India) impregnated with methanol were used as negative control. Five blank sterile discs (Himedia) were loaded with 20 mg (1000 µL) of the test extracts each, namely-*Saraca indica* (SI), *Eucalyptus citriodora* (EC) and mixture of both these extracts (SI+EC; 10+10 = 20 mg) and allowed to get dry. Each disc was loaded with 4 mg per disc of the test extracts. For each clinical sample, five SDA plates-one each for SI, EC, SI+EC, positive control (FLC) and negative control (methanol) were used. These plates were incubated at room temperature at 22-25°C for 24-72 h up to 42 days and results recorded post-incubation at different time intervals up to 42 days post-inoculation to observe for long-term antifungal effect, if any, of the test plants extracts.

RESULTS

Isolation of fungal species: Laboratory examination of the skin swab samples collected from different dermatological disorders from clinical cases of canines included in the present study revealed involvement of several species of fungi, namely: *Microsporum nanum*, *Microsporum gypseum*, *M. canis*, *Aspergillus niger*, *Rhizopus*, *Alternaria*, *Candida*, *Penicillium* and more than two types of dermatophytes (based on macro-conidial morphology and cultural characteristics).

***In vitro* anti-fungal activity:** All the three test extracts (SI, EC and SI+EC) reduced the fungus growth in plates but to different extents depending on the test extracts as shown in the Fig. 1a, b, 2a and Table 1. Comparison of the efficacy of different extracts against both types of cultures i.e. standard

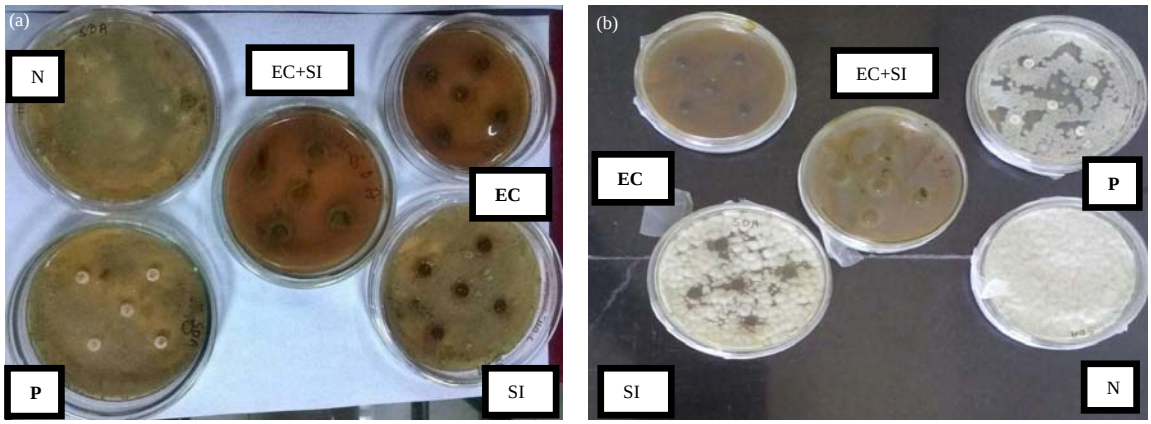

Fig. 1(a-b): Effect of treatment with methanolic extracts of leaves of *Eucalyptus citriodora* (EC), *Saraca indica* (SI) and combination of these both (SI+EC) on growth of mixed fungus culture isolated from clinical cases of canines after (a)7 and (b) 15 days

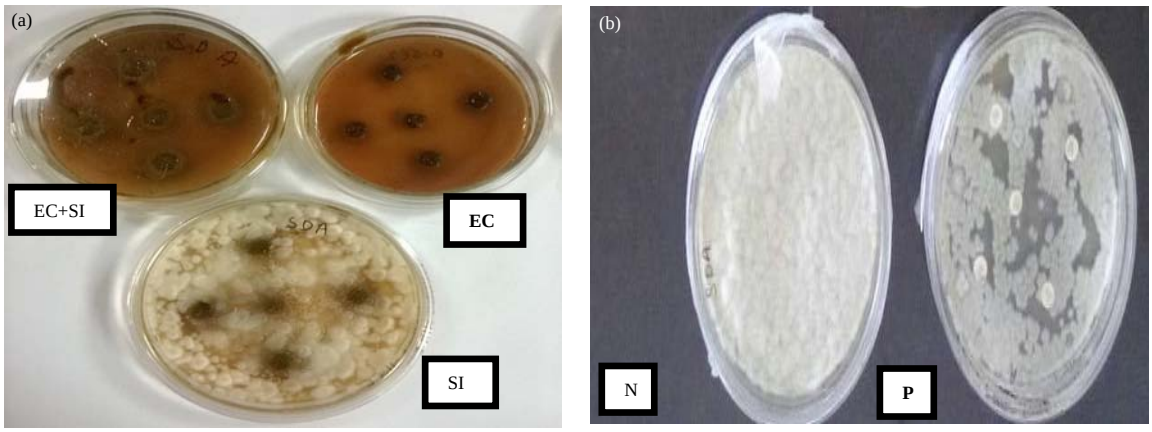

Fig. 2(a-b): Effect of treatment with methanolic extracts of leaves of *Eucalyptus citriodora* (EC), *Saraca indica* (SI) and combination of these both (SI+EC) (a) After day 21 on growth of clinical fungus and (b) Positive and negative controls after 21 days

Table 1: Anti-fungal efficacy of test extracts against standard laboratory fungus culture and clinical fungus isolates from canine's dermatological disorders

	Appearance of fungus growth with the progression of time	
Types of plant extract	Standard laboratory fungus culture	Clinical fungal isolates
Saraca indica	Mild growth appeared after 3 days	Mild growth appeared after 7-8 days
Eucalyptus citriodora	Mild growth appeared after 5 days	Mild growth appeared after 15 days
Saraca indica+ *Eucalyptus citriodora*	Mild growth appeared after 6 days	No growth up to 20 days, mild growth appeared after 20 days but zone of inhibition was clear and intact up to 42 days
Fluconazole	Mild growth appeared after 2 days in the zone of inhibition	Mild growth appeared after 2-3 days and plate surface was completely covered with fungal growth up to 15 days
Methanol	Fungus growth appeared from first day and completely covered the plate	Fungus growth appeared from first day and completely covered the plate gradually

culture and clinical isolates revealed that combination of the two extracts (SI+EC) was most effective followed by EC and SI as it produced the largest zone of inhibition (Fig. 2a) and also the zone of inhibition persisted comparatively for a longer duration compared to the individual test extracts (SI or EC) or the positive control (fluconazole) as fungal colonies started appearing after two-three days in positive control and from first day onward in negative control group as shown in Table 1 and Fig. 2b.

Compared to the efficacy of test extracts against clinical isolates, mild fungal growth appeared against all the three extracts after 3-6 days in standard culture and it was earliest i.e. after three days in SI extract, five days in EC and six days in SI+EC extracts. However, no growth appeared even up to fifteen days in clinical isolates culture treated with SI+EC extracts or EC extract. But fungal growth started appearing after seven days in SI extract-treated culture plates. Further, SI+EC completely inhibited the growth of dermatophytes and other fungal species isolated from all the clinical cases up to twenty one days as no fungal growth appeared over SDA plates. In negative control, positive control and SI extract-treated clinical isolates of fungal culture, SDA plates were completely covered with fungal growth after twenty one days. But in the clinical isolates plates treated with SI+EC, only mild fungal growth appeared after 21 days and the mild zone of inhibition of 10-12 mm persisted around the discs even up to 42 days in case of combination treatment i.e., SI+EC extracts.

Comparison of the antifungal potency of different test extracts against the standard fungus culture and clinical isolates revealed that the test extracts (SI and EC) in combination were most potent and effective followed by EC and SI extracts. Persistence of the zone of inhibition even up to 42 days suggested prolonged anti-fungal effect of all three test extracts.

DISCUSSION

Medicinal and aromatic plants widely constitute major source of natural phytomedicines and are employed in alternative or complementary medicinal therapies. Researchers studied the antifungal activity of leaf extracts

of various species of eucalyptus (*Eucalyptus globulus, E. maculata* and *E. viminalis*) and stated that these extracts significantly inhibited the growth of dermatophytic fungus *Trichophyton mentagrophytes*, causative agent of athlete's foot[14]. Our observations on antifungal activity of Eucalyptus and Saraca leaves methanolic extracts individually and in combination are also in agreement of these findings as our test extracts inhibited the growth of *Microsporum* sp., *Aspergillus* sp. and other dermatophytic fungal agents. Findings of the present study are also in confirmation with the earlier reports describing high antifungal activity of volatile oils and extracts from stems, leaves and flowers of *Eucalyptus sideroxylon* and *Eucalyptus torquata* against *Candida albicans, A. flavus* and *A. niger*[16].

Strong antifungal activity of methanolic extracts of the stem bark of *Saraca indica* against *Candida albicans* and *Cryptococcus albidus* with MIC ranging from 0.5-2% and 1-3%, respectively has been reported[17]. In the current study, we too observed promising anti-fungal activity of methanolic extract of the leave of *Saraca indica* against *Candida* and other fungal species and our observations are in agreement with the previous findings detailing the antifungal activity of methanolic extract of *Saraca asoca* and *Saraca indica* against *Candida* and *Aspergillus* spp.[8]. Results of the current study also confirmed the prior report documenting the antifungal efficacy of methanolic extract of *Saraca asoca* leaves against *Alternaria alternate*, *Aspergillus flavus*, *A. fumigates* and many other fungal species[8]. Similarly our findings are in agreement with the earlier reports describing antifungal activity of leaf extract of *Saraca indica* against *Candida* and *Aspergillus* spp[18].

Therefore, our findings validate the strong and promising antifungal activity of methanolic extracts of leaves of *Eucalyptus citriodora* and *Saraca indica* and these extracts seem to be enriched with anti-fungal phytobiomolecules and these seem to hold promising potential in the treatment of fungi involved in canine dermatological disorders. However, results of *in vitro* studies should be correlated with the results of *in vivo* studies especially from clinical recovery perspective. Further studies on identification of the active phyto-constituents responsible for antifungal activity and possible mechanism of action are warranted to exploit its use in drug development.

CONCLUSION

Study concluded that methanolic extracts of leaves of *Saraca indica* and *Eucalyptus citriodora* seem to be enriched with an array of phyto-constituents having promising antifungal activity and both these plants need to be explored for development of effective natural pharmaceuticals against canine dermatological disorders after unraveling their detailed phytochemistry and mechanism of action.

SIGNIFICANCE STATEMENT

Current *in vitro* study evidently validates the strong and significant antifungal activity of methanolic extracts of leaves of *Eucalyptus citriodora* and *Saraca indica* and these extracts seem to be endowed with potent anti-fungal phytobiomolecules and both these hold promising potential for developing herbal formulations for treatment of dermatological disorders in canine and even human beings.

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