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ANTIBACTERIAL, ANTIOXIDANT AND PHYTOCHEMICAL EVALUATION OF AN ENDANGERED MEDICINAL PLANT *SARACA ASOCA*

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ABSTRACT

S.asoca is an ancient Ayurvedic medicinal tree that is used worldwide to treat various kinds of human disorders. The present research evaluates the anti-microbial activity of leaf (Methanol) endophytic extracts of the *S. asoca* plant by the disc diffusion method against *E. coli, P. syringae P. aeruginosa*, and *B. subtilis, S. aureus, K. sinensis*. Methanolic extract of leaves showed the activity against these bacterial species. The secondary metabolites investigation of leaves of endophytic extract shows the presence of steroids, terpenoids, tannins, Alkaloids, flavonoids, triterpenoids, cardiac glycosides, etc. The anti-oxidant assay of *Rhizopus sps* and *Trichoderma sps* species shows maximum absorbance at 300µg/ml concentration with its IC_{50}.

INTRODUCTION

S. asoca Roxb.Willd medicinal herb belongs to the Caesalpiniaceae family. *S. asoca species* is currently listed as a "globally vulnerable"species by the IUCN 2013. (http://www.iucnredlist.org/apps/redlist/details/34623/0). *S. asoca* is a small evergreen tree 7-10cm high. Leaves are paripinnate whole plant contains glycoside principles, non-phenolic, sapogenetic glycoside, sterols, and aliphatic alcohols.

Fig.1*S.asocatree*

Fig.2.*S.asocaflower*

The two famous formulations of *S. asoca* are ashokarishta and ashokaghirta bark usedand prescribed against several ailments like leucorrhoea, hematuria, menorrhagia, and diseases of the genito-urinary system of females. 'Ashoka' bark has been reported to havea domestic demand greater than 15,000 metric tonnes in India(1).

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STUDY SITE

The study area is in the Shaakarghatta region in Bhadravati taluk, Shivamogga district of Karnataka. Shankarghatta is at 13°44'1" N and 75°37'45"E and hasan elevation range of 680 meters.

MATERIALS AND METHOD

- Isolation of endophytic fungi from medicinal plants:
- Surface Sterilization of Plant Material:

The epiphytic fungi and other contaminations are removed by using surface sterilization of samples. The plant material was washed in running tap water to remove unwanted debris and finally washed with distilled water. Plant material is immersed in 70% ethanol for 1-3min and 4% aqueous solution of mercuric chloride (1g/ml) again and finally rinsed 4-5 times with sterile distilled water. Plant materials were selected by aseptic cutting using scissors and inner tissues were excised. Later the segments were rinsed with sterile distilled water for

5 min and were blotted on blotting paper. Plant materials were cut into small pieces of 1cm long and 3-4mm broad in size. All

the work was performed in the Laminar airflow(2).

- Inoculationof explants on PDA media.

After successive surface sterilization of leaves of *S. asoca*, parts were aseptically cut into small pieces and placed in four pieces on the solidified Potato Dextrose Agar (PDA) media. The inoculated plant implants were incubated until distinguishable fungal endophytes grew.

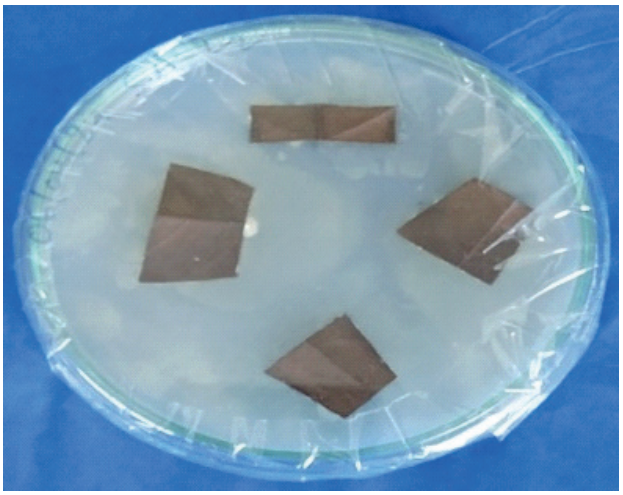


Fig 3. *S. asoca* leaves inoculated on PDA media

- Identification of endophytic fungi:Cultures on PDA media were assessed according to their morphology, colony appearance, mycelium color and structure, the shape of conidiomata, conidia and conidiophore (size, color, ornamentation, etc), and characters of conidiogenus cells were observed for morphological classification of isolated fungi using a compound microscope with 10X and 40X objective lenses for magnification (2).
- Mass production of identified fungi: Identified fungal species were cultured on PDB broth for large-scale cultivation. The inoculated flasks were incubated at room temperature (26+2°C) for 7-21 days and allowed to grow the fungal mats. Mats are used as plant extracts for their antibacterial activity.

ANTIBACTERIAL ACTIVITY

Antibacterial activity was shown by using the culture method in whic hendophytes and test microbes were inoculated on the same plate. Antibacterial activity was tested against bacteria like *E. coli, P. syringae, P. aeruginosa B. subtilis, S. aureus,* and *Knoellia sinensis*.

1.Test bacterial culture

Preserved bacterial cultures were revived in nutrient broth and incubated at 37°C for 24 hours. To avoid contamination, 5mm disks were prepared using Whatman filter paper (40 No) and autoclaved.

2. Disk diffusion assay

In the disk diffusion method the bacterial suspension of 24 hrs old culture of pathogenic bacteria was spread on sterile MHA(Muller Hinton Agar) with a sterile cotton-tipped swab to form an even lawn. Sterile paper disks (6mm in diameter) dipped with extract were placed on the surface of each MHA Media using a sterile pair of forceps. The incubated plates were aerobically at 37°C for 24 hours.The diameter of the inhibition zone was measured after 48 hrsincubation using a ruler.(2)

ANTIOXIDANT ACTIVITY ASSAY OF FUNGAL CRUDE EXTRACTS:The antioxidant activity was determinedusing the radical 2,2- diphenyl-1-Picrylhydrazy(DPPH) radical scavenger assay. The 0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of methanol. The reaction components were incubated in dark conditions for 20 minutes and maintained at room temperature. The absorbance of the mixture was read at 513nm. 3 ml of DPPH was taken as control.

% RSA = (Abs of control)- (Abs of sample)/Abs of control*100

QUALITATIVE PHYTOCHEMICAL ANALYSIS

The leaf extract was tested for biological compounds using the following Standard methods. (3)

1. Test for Steriods: To take 1 ml of fungal crude extract from the endophyte is dissolved using 1 ml of chloroform and 2-3 ml of acetic anhydride, 1-2 drops of conc. Sulphuric acid is added. upper layer turns red and the Conc. sulphuric acid layer shows yellow with green fluorescence indicating the presence of Steriods.
2. Test for Terpenoids: To take 2 ml of extract and 2 ml of chloroform is added, 3 ml of Conc. sulphuric acid. If a reddish-brown colour appears indicates the presence of terpenoids.
3. Test for Tannins: To take 1 ml of extract and 1% Ferric chloride solution to produce a green or brownish-green, blue color.
4. Test for Alkaloids: To take 2ml of extract, a few drops of Wagner's reagent (1.27g of iodine and 2g of Potassium iodide were dissolved in 5 ml of water and made up to 100ml with distilled water) were added by the side of the test tube. A reddish-brown precipitate appears in the presence of alkaloids.
5. Test for Saponins: To take 1 ml of extract and 20 ml of distilled water and keep on stirring for 15 minutes. If foam formsappear the presence of saponins.
6. Test for Flavonoids: To take 2ml of plant extract and 1ml of dilute ammonia and mix well 1 ml of Conc.Sulphuric acid, the formation of a yellow color indicates the presence of flavonoids.
7. Test for Triterpenoids: To take 2ml of plant extract mixed with 5 drops of Conc. sulphuric acid, if a greenish-blue color appears, indicates the presence of triterpenoids.
8. **Test for Carbohydrates**

- Benedict's test: Benedict's reagent is mixed with 2ml of fungal crude extract from the endophytes and kept for boiling in the water bath and observed for reddish brown precipitate which indicates the presence of carbohydrates.

9. Cardiac glycosides: To take 5 ml of extract, it was treated with sodium nitroprusside in pyridine and sodium hydroxide. The presence of cardiac glycosides indicates the formation of pink to red color.
10. Test for Amino acids:

- Ninhydrin test: To 1ml extract, 0.25% ninhydrin reagent was added and boiled for few a minutes formation of a blue color appears in the presence of amino acids.

RESULT AND DISCUSSION

Table 1. Shows different endophytic fungi which are obtained from *S. asoca* plant. All these fungi contain secondary metabolites.

Table 2. Shows the zone of inhibition obtained during the antimicrobial activity of the asoca plant using Gram-positive and Gram-negative bacterial cultures.

Table 3. Shows the anti-oxidant activity of the asoca plant using the DPPH method.

The *S. asoca* leaf extracts show the presence of fungal species such are *Rhizopus sps, A. fumigatus,* and *Trichoderma sps.* These species are used to test the secondary metabolites were the presence of secondary metabolites. The leaf extracts are exposed to the presence of phytochemicals such as alkaloids, flavonoids, triterpenoids, steroids, terpenoids, cardiac glycosides, tannins, amino acids, carbohydrates, and saponins. In *Rhizopus sps* amino acids areabsent were methanol as the solvent. The saponins are absent in *Rhizopus sp, A. fumigatus,* and *Trichoderma sp.*

Isolation and identification of secondary metabolites:

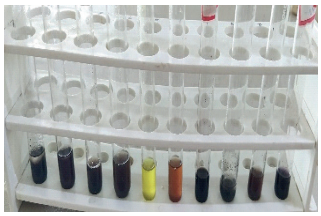


Fig 4. *Rhizopus species*

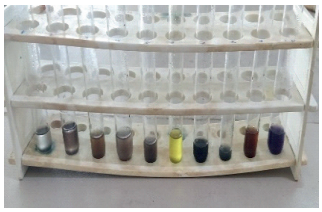


Fig 5. A. *fumigatus*

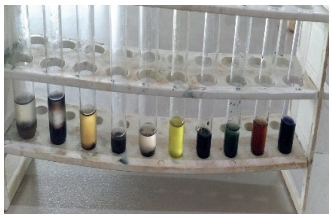


Fig 6.*Trichodermas pecies*

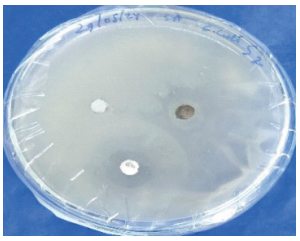
Table 1 Phytochemical analysis of secondary metabolites from endophytic fungal species of leaves

Secondary Metabolites	Endophytic fungal of leaves		
	Rhizopus sps	Aspergillusfu-migatusps	Trichoder-ma sps
Alkaloids	+	+	+
Flavonoids	+	+	+
Triterpenoids	+	+	+
Steroids	+	+	+
Terpenoids	+	+	+
Saponins	-	-	-
Carbohy-drates	+	+	+
Cardiac gly-cosides	+	+	+
Tannins	+	+	+
Amino acids	+	+	+

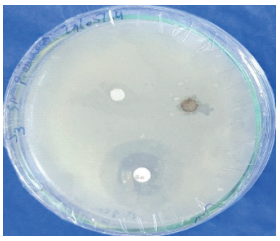
(+ Indicates Present),(- Indicates Absence)

Table 2 Antibacterial activity of S. asoca (fungal extracts)

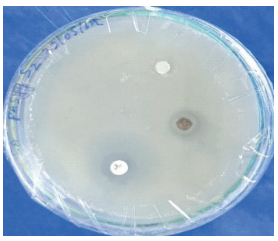
Organism	Fungal extracts in solvents (methanol)		
	Zone of inhibition (mm)		
	Rhizopus sps	*A. fumigatus*	*Trichoderma sps*
S. aureus	13	10	13
B. subtilis	10	10	10
Knoellia sinensis	10	15	11
E. coli	13	10	10
P. syringae	15	10	15
P. aeruginosa	14	10	10



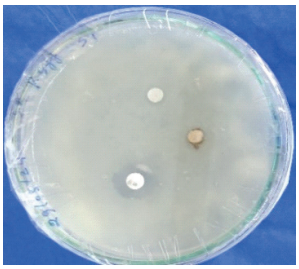
a.*S. aureus*



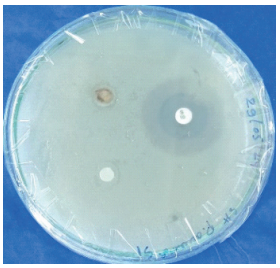
b. B. subtilis



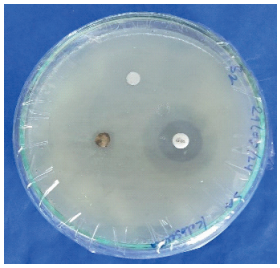
c. Knoellia sinensis



d. *E. colie.*



P. syringaef.



P. aeruginosa

Fig. 4 Illustrate antibacterial activity against pathogenic bacteria

Antibacterial activity

There are six pathogenic bacteria were used in the screening

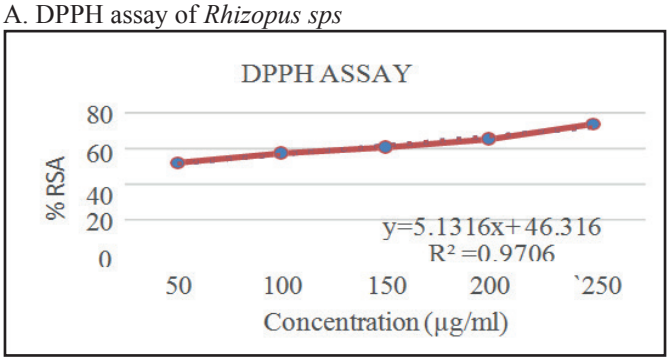
of the antibacterial activity of the methanol extracts of endophytes. The antimicrobial activity of *Rhizopus* extracts of leaves was the highest *P. syringae* with a zone of inhibition of 15mm. while the lowest activity was noticed with *Rhizopus* extract against *S. aureus, B. subtilis, Knoellia sinesis, E. coli,* and *P.aeruginosa.* The Trichoderma sps extracts of the leaf hadthe highest activity against all microorganisms and the highest on *P. syringae* with a zone of inhibition of 15mm. The *A. fumigatus* extract had the least activity against all bacterial species. Antibacterial activity of leaves in Rhizopus sps extract was highest on *B. subtilis* and *K. sinensis* with the zone of inhibition is 15mm and no antimicrobial activity against another microorganism Trichoderma extract showed the highest activity against *B. subtilis, E. coli,* and *P. aeruginosa* the zone of inhibition was 10mm and showed against other microorganisms. The Rhizopus extract has the highest activity against all microorganisms, the highest zone was showed against *P. syringae* with a zone of inhibition of 15mm and the lowest activity showed *B. subtilis and K. sinesis* with a zone of inhibition is 10mm. *A. fumigatus* extract exhibited a zone only against *B.subtilis, S.aureus, E. coli, P. syringae,* and *P. aeruginosa* with a zone of inhibition of 10mm (Table 2).

The antibacterial activity of leaf extracts revealed the presence of alkaloids, flavonoids, steroids, teriterpenoids, terpenoids, carbohydrates, cardiac glycosides, and tannins(4). *S. asoca* has an antibacterial activity against plant pathogens such as *S. aureus, B. subtilis, E. coli, K. sinesis, P. syringa*e, *P. aeruginosa.*

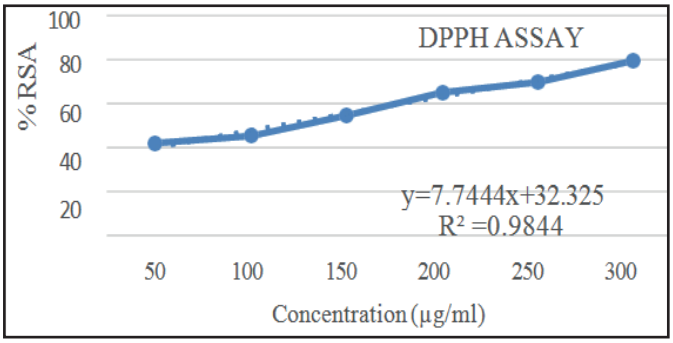
Antioxidant Assay by DPPH Method

The antioxidant assay of Rhizopus sps and Trichoderma sps. Trichoderma sps shows maximum absorbance at 300µg/ml concentration RSA value shows 79.60 with IC_{50} value at 34 the Rhizopus sps shows 300µg/ml concentration RSA value shows 73.68 with IC_{50} value at 49.

A. DPPH assay of *Rhizopus sps*



B. DPPH assay of *Trichoderma sps*



CONCLUSION

S. asoca is a panacea in ayurvedic medicine. *S. asoca* is one of the universal plantsthat contains medicinal activities and is a source of various compounds. The present study gives information on various phytochemical components such as carbohydrates, flavonoids, saponins, tannins, cardiac glycosides steroids, triterpenoids, amino acids, and alkaloids. *S. asoca* fungal extracts have antibacterial activity against various microorganisms. Ashoka is a nontoxic traditional plant it also used for many medicinal purposes. The antibacterial activity of fungal extracts were three fungal extracts used to test antibacterial activity against pathogenic bacteria. *Rhizopus sps, A. fumigatus*, and *Trichoderma sps* these extracts were used. Rhizopus spsand Trichoderma sps show a maximum zone of inhibition(15mm) then, *A. fumigatus* shows the lowest zone of inhibition(10mm). The antioxidant assay of *Rhizopus* sps and *Trichoderma sps. Trichoderma sps* shows maximum absorbance at 300µg/ml concentration RSA value shows 79.60 with IC_{50}value at 34 the rhizopus sps shows 300µg/ml concentration RSA value shows 73.68 with IC_{50}value at 49 and *Aspergillus sps*.

STATEMENTS AND DECLARATION

- Funding -"This research received no external funding"
- Conflicts of Interest - "The authors declare no conflict of interest"
- Acknowledgment - None

Reference

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