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Research Article

Isolation and Characterization of *Saraca asoca,* Secondary Metabolites by *Rhizopus sps* Using Various Analytical Techniques, Including FTIR, NMR, and HPLC

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Abstract

Ashoka (*Saraca asoca*) is a conventionally used medicinal plant in India, known for its curative properties. Secondary metabolites, which are the bioactive compounds produced by living organisms, can unveil symbiotic relationships in nature. The present study elucidated that secondary metabolites show the presence of various bioactive compounds, where the *Saraca asoca* endophyte extract was tested against various bacteria like *E. coli*, *S. aureus*, *B. subtilis*, *K. pneumoniae*, *P. aeruginosa,* and *P. syringae, which* showed the zone of inhibition. The characterization of endophytic extract is used for various analytical techniques, such as FTIR, which is used to detect the presence of functional groups like ketones, Alkenes, Aromatic Compounds, Nitro Compounds, Alkyl and Aryl Halides. HPLC analysis showed the presence of Caffeine, its Ret Time at 3.033, and NMR results showed the Chemical shift at 4.391, an ArOH type of bond present. It was the Phenol, whereas others showed 3.769 and its bond structure is $ArNH_2$, it belongs to Aniline, 2.510 showed the bond structure RC≡C-H, it belongs to Alkynyl. Bioactive compounds are used to synthesize novel drugs. These drugs showed antioxidant and anticancer activity.

Keywords: HPLC; NMR; FTIR; Caffeine; Pharmaceuticals.

Introduction

Saraca asoca is a legendary and sacred tree that is both ornamental and medicinal. Medicinal plants have been a part of our lives and utilized in traditional medicine from time immemorial (Mohan *et. al.,* 2016). *S. asoca* is native to the Indian sub-continent, though also found in the Indo-Malaysian region and Sri Lanka. In India, it is mainly found in the peninsular region extending into the Western Ghats, Eastern Ghats, and the Sub-Himalayan tracks, growing at an elevation of approximately 750 m. A large number of herbal medicines and plant-based drug formulations of immense therapeutic value are described in Indian folk medicine. Even today, plant-based drugs are popular over modern synthetic drugs mainly due to their negligible side

effects, low cost, and easy availability. Ayurveda, a traditional medicinal practice based on human physiology, employs a large number of medicinal plants and herbs, either exclusively or in combination, for overall health (Borokar *et.al.,* 2017). According to World Health Organization , in developing countries like India, almost 70% of the population is dependent on traditional herbal medicines for their primary health care needs (Kumar *et.al.,* 2023).

Materials And Methods

Collection of Plant Material

The collection of plant material is done at the Shanakarghatta region in Bhadravati taluk, Shivamogga district of Karnataka. The plant material was cleaned with distilled water and mercuric chloride after cleaning with running tap water to get rid of any undesired detritus. Aseptic procedures were used to study plant materials, and sterile scissors were used to remove interior tissues.

Identification and Preparation of a Wide Scale of Rhizopus sps by Using PDB Broth

Materials from plants were chopped into 1 cm long and 3–4 mm wide tiny bits. It was done under a laminar airflow. Conidia, conidiophores, mycelium color, colony appearance, and morphology were used to identify cultures on PDA media. Samples were examined for magnification by using the compound microscope's 10x and 40x objective lenses.

The identified fungal species were cultured on a wide scale using PDB broth.To allow the fungal mats to develop, the inoculation flasks were kept at room temperature (26°C) for seven to twenty-one days.

Mats were employed in this research as endophytic extracts. Following storage in an airtight poly-tube, the endophytic extract was sent to additional testing for GC-MS analysis to determine the substances it contained.

Preliminary Phytochemical Tests of Secondary Metabolites

Secondary metabolites found in methanolic extracts of *Rhizopus sps* of *S. asoca* were identified using qualitative testing. Standard procedures were used to document the presence of cardiac glycosides, amino acids, terpenoids, triterpenoids, alkaloids, flavonoids, tannins, carbohydrates, and steroids (Garima Bartariya *et.al*., 2017).

Test for Steroids*:* 1 ml of fungal crude extract from the endophyte was taken and it was dissolved using 1 ml of chloroform and 2-3 ml of acetic anhydride. Thereafter, 1-2 drops of conc. sulphuric acid were added. upper layer turns red, and the Conc. sulphuric acid layer shows yellow with green fluorescence, indicating the presence of Steroids.

Test for Terpenoids*:* 2 ml of extract was taken and 2 ml of chloroform was added, 3 ml of Conc. sulphuric acid. If a reddish-brown colour appears indicates the presence of terpenoids.

Test for Tannins*:* 1 ml of extract was taken and 1% Ferric chloride solution was added to produce a green or brownish-green, blue color.

Test for Alkaloids*:* 2ml of extract was taken, a few drops of Wagner's reagent (1.27g of iodine and 2g of Potassium iodide dissolved in 5 ml of water and made up to 100ml with distilled water) were added to the side of the test tube. A reddish-brown precipitate appears in the presence of alkaloids.

Test for Saponins*:* 1 ml of extract was taken and 20 ml of distilled water was added, then stirred for 15 minutes. If foam appears, the absence of saponins.

Test for Flavonoids*:* 2ml of plant extract was taken, 1ml of dilute ammonia was added and mixed well. Then 1 ml of Conc. sulphuric acid was added, the formation of a yellow color indicates the presence of flavonoids.

Test for Triterpenoids*:* 2ml of plant extract was taken & mixed with 5 drops of Conc. sulphuric acid, if a greenish-blue color appears, it indicates the presence of triterpenoids.

Test for Carbohydrates*:*

Benedict's test: Benedict's reagent is mixed with 2 mL 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.

Cardiac glycosides: 5 ml of extract was taken, it was treated with sodium nitroprusside in pyridine and sodium hydroxide. The presence of cardiac glycosides indicates the formation of a pink to red color.

Test for Amino Acids:

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

Antibacterial Activity

The fungal extract of *Rhizopus sps* was tested against Gram-positive, *Staphylococcus aureus*, *Bacillus subtilis, Knoellia sinensis*, and Gram-negative bacteria- *Pseudomonas syringae*, *Pseudomonas aeruginosa,* and *Escherichia coli* (Dabur *et.al.,* 2007), (Athiralakshmy *et.al.,* 2016) by the disc diffusion method. The sterile discs were dipped in the endophytic extracts. The MHA (Muller Hinton Agar) media, along with forceps and petri plates, were autoclaved at 121˚C for 15 minutes. Thereafter, autoclaved media were kept in the Laminar Air Flow Chamber, then media were Poured to paired Petri plates, and allowed to solidify for 1

hour. Around 10µl of bacterial inoculum was inoculated on the solidified media and spread using cotton swabs. Consequently, the discs immersed in the endophytic extracts of *Rhizopus* sps and placed on MHA media, discs immersed in double-distilled water were used as a negative control, and Streptomycin discs showed antibiotic activity, were used as a positive control. All the plates were incubated for 24 hours at 37°C. The zone of inhibition surrounding the discs was measured to confirm the antibacterial effect.

NMR and FTIR Analysis

FTIR is an analytical technique used to identify organic, polymeric, and, in some cases, inorganic materials. Currently, the functional groups of the endophytic extract of *Rhizopus sps* are being studied using FTIR. But I have used the FTIR model in Agilent Technologies, and also its range is 4,000.00 – 650.00 cm-1. The functional groups present in this extract are C=C (Alkenes), C=C (Ketones), C=C (Aromatic compounds), C-F (Alkyl and Aryl Halides), NO_2 (Nitro Compounds). These functional groups may also be used in the preparation of drugs like antibiotics and antifungal drugs. An analytical method that enables quantitative and non-invasive studies of molecular structure and chemical reactions is nuclear magnetic resonance spectroscopy (NMR). NMR is primarily related to the magnetic properties of certain atomic nuclei; notably the nucleus of the hydrogen atom, the proton, the carbon, and an isotope of carbon. NMR spectroscopy has enabled researchers to study molecules by recording the differences between the various magnetic nuclei (Altemimi *et.al.,* 2017). We have used a NMR BRUKER model. The result showed the presence of functional groups, like at chemical shifts (ppm) 4.391, its bond structure is ArOH, and the compound is Phenol, an 3.769, its bond structure is $ArNH_2$, and the compound is Aniline, an 2.510 its bond structure is RC≡C-H, and the compound is Alkynyl.

HPLC Analysis By Endophytic Extract

To separate, identify, and quantify chemicals in liquid samples, high-performance liquid chromatography (HPLC) is a commonly used analytical method. Chemicals scattered in a liquid sample can be separated using HPLC, enabling both qualitative and quantitative analysis of the sample's constituent parts and their relative concentrations. To identify important peaks in the crude extract, an analytical HPLC Dionex P580 HPLC system connected to a photodiode array detector was used (Pushkar *et. al*., 2015). The HPLC analysis was conducted by the procedures described by (Eze *et al*., 2018). With an injection volume of 20 µL, a mobile phase comprising the sample was used for separation with a 12:85:3 ratio in an isocratic manner. Both the standard and the sample were run for 18 minutes at a flow rate of 0.7 mL/min with a diode array detector (DAD) with a detection wavelength of 280 nm (Sil *et.al*., 2022). The endophytic extract of *Rhizopus sps* extracts tested by using HPLC analysis were the results showed the presence of Caffeine, it's retention time of 3.033.

Results and Discussion

Identification of Endophytic fungi as Rhizopus sps and Mass-culture using PDB broth

By examining its morphological characteristics under a phase contrast and brightfield microscope, the endophytic fungus that was isolated from *S. asoca* was determined to be *Rhizopus sps* (Fig. 1). Additionally, the isolated endophytic fungus was mass-cultured on PDB, and the extracts were used for additional research.

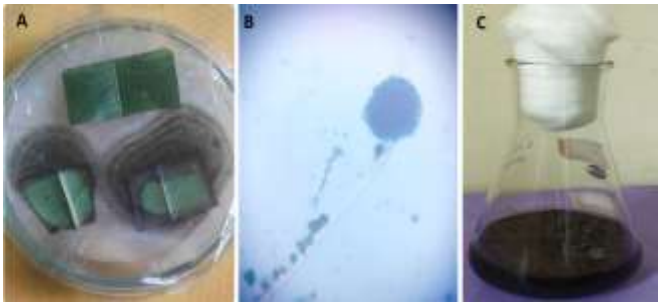

Fig. 1: Fungal endophyte *Rhizopus sps* A).Photographic image of PDA showing the growth. B). Microscopic image at 40x. C) Mass culture on PDB

Preliminary tests of Secondary metabolites:

To verify that secondary metabolites present in *Rhizopus* sps preliminary experiments were conducted using methanolic extracts. Alkaloids, flavonoids, tannins, terpenoids, steroids, carbohydrates, and amino acids were reported in the tests. (Fig. 2 and Table 1). One way to learn more about certain bioactive substances that an organism produces is through qualitative study of secondary metabolites (Smitha & Thondaiman, 2016). By determining the presence of secondary metabolites such as flavonoids, terpenes, tannins, alkaloids, etc., we can comprehend the potential therapeutic benefits they may have in the treatment of different illnesses. It creates a path for more investigation to pinpoint the specific bioactive substances found inside them and their noteworthy biological effects (Sumangala *et.al.,*2017).

Fig. 2: Qualitative analysis of fungal extract to confirm the presence of Secondary metabolites.

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

S.N.	Secondary Metabolites tests Samples	*Rhizopus sps*
01	Alkaloids	+
02	Flavonoids	+
03	Carbohydrates	+
04	Saponins	-
05	Glycosides	+
06	Tannins	+
07	Terpenoids	+
08	Steroids	+
09	Cardiac glycosides	+
10	Aminoacids	+

(+ Indicates Present),(- Indicates Absence)

Antibacterial activity of endophytic extract:

The antibacterial activity of *Rhizopus sps*. methanolic extract was tested against six bacterial species, including Gram-negative bacteria *P. syringae*, *E. coli*, and P. *aeruginosa* [Fig. 3(a) (b) (c)] and Gram-positive bacteria *S. aureus*, *B. subtilis* and *K. sinensis* [3. (d) (e) (f)]. *Rhizopus species* inhibited all of the bacteria, although *P. syringae* with a diameter of 15 mm and *P. aeruginosa* (14 mm) were the most strongly inhibited, followed by *S. aureus* (13 mm), *E. coli* (13 mm), *B. subtilis* (10 mm), and *K. sinensis* (10 mm) was the least inhibited. According to the results, *Rhizopus sps*. Endophytic extracts have the potential to produce antibiotic compounds and may be selected for additional study and the identification of possible bioactive components.

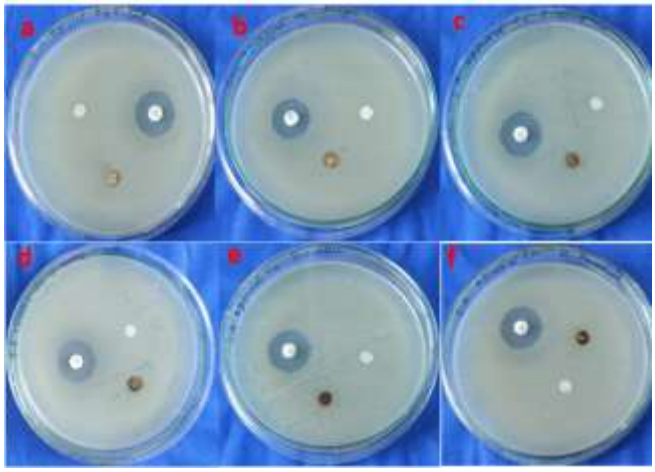

Fig. 3: Antibacterial activity of *Rhizopus sps* against a) *E. coli*, b) *P. syringae*, c) *P. aeruginosa*, d) *S. aureus*, e) *B. subtilis*, f) *K. sinensis*

NMR and FTIR analysis of endophytic extracts:

Currently, the functional groups of the endophytic extract of *Rhizopus sps* was being studied using FTIR. But we have used the FTIR model in Agilent Technologies, and also its ranged 4,000.00 – 650.00 cm-1. The functional groups present in this extract was C=C (Alkenes), C=C (Ketones), C=C (Aromatic compounds), C-F (Alkyl and Aryl Halides), NO_2 (Nitro Compounds). Data shown in Table 2 shows the NMR BRUKER model showing the presence of functional groups, like at chemical shifts (ppm) 4.391, its bond structure was ArOH, and the compound was Phenol, at 3.769, its bond structure was $ArNH_2$, and the compound was Aniline, at 2.510 its bond structure was RC≡C-H, and the compound was Alkynyl (Fig. 4 & 5)

Table 2: NMR analysis by *Rhizopus sps*

Chemical Shift (δ)ppm	Type of Bond	Description
4.391	ArOH, N, O, F, Cl(Heterane)	Phenol
4.378	ArOH	Phenol
3.769	$ArNH_2$	Aniline
3.171	$ArNH_2$	Aniline
2.510	RC≡C-H	Alkynyl

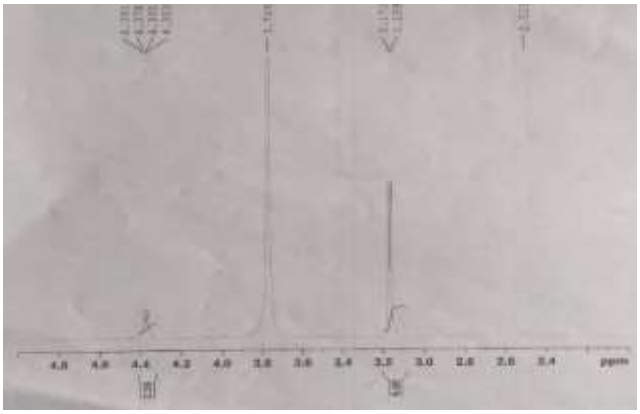

Fig. 4: NMR analysis of endophytic extracts:

HPLC Analysis of endophytic extract:

To separate, identify, and quantify chemicals in liquid samples, high-performance liquid chromatography (HPLC) is a commonly used analytical method. The endophytic extract of *Rhizopus sps* extracts tested by using HPLC analysis were the results showed the presence of Caffeine, it's a retention time of 3.033 (Fig. 6).

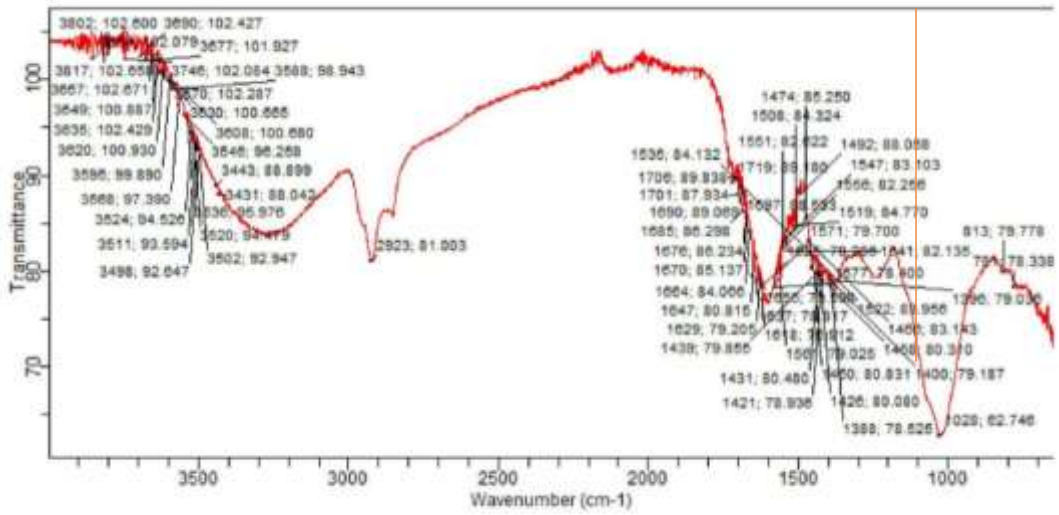

Fig. 5: FTIR analysis of endophytic extracts graph by side view.

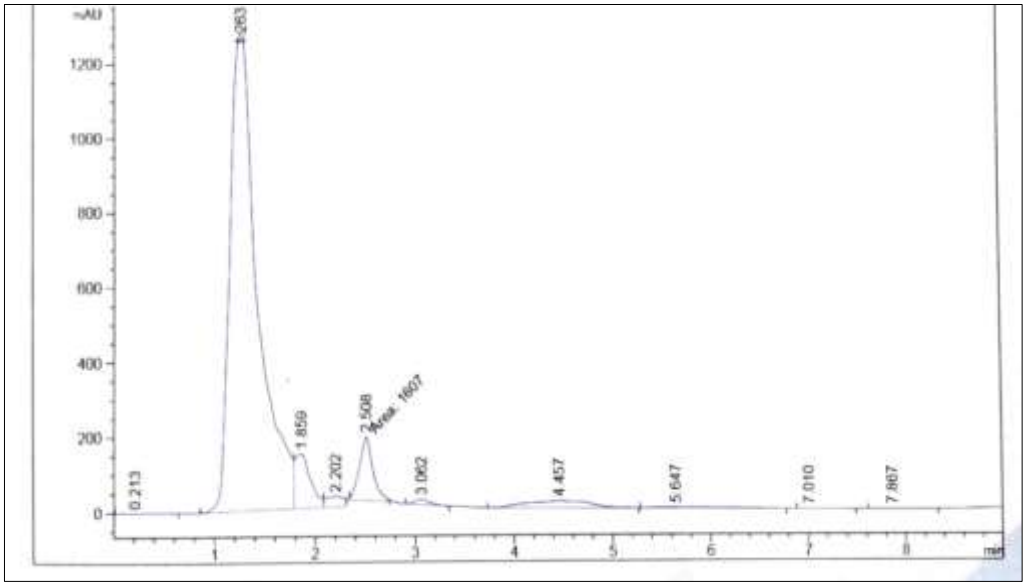

Fig. 6: HPLC analysis of endophytic extracts.

Conclusion

Since the beginning of time, medicinal plants have been a part of our lives and used in traditional medicine. Natural symbiotic interactions can be revealed by secondary metabolites, which display the bioactive substances generated by living things. *E. coli*, *S. aureus*, *B. subtilis*, *K. pneumoniae*, *P. aeruginosa*, and *P. syringae* were among the bacteria tested against the *S. asoca* endophytic extract, which demonstrated the zone of inhibition. This study clarified that secondary metabolites indicate the presence of various bioactive compounds. HPLC analysis results showed the presence of Caffeine, its Ret Time at 3.033. NMR results showed at Chemical shift 4.391, ArOH type of bond present, and it is present in the Phenol, whereas other Chemical shift 3.769 and its bond structure is $ArNH_2$, it belongs to Aniline, 2.510 shows the bond structure RC≡C-H, it belongs to Alkynyl the presence of bioactive. The FTIR functional groups present in this extract are C=C (Alkenes), C=C (Ketones), C=C (Aromatic compounds), C-F (Alkyl and Aryl Halides), NO_2 (Nitro Compounds). The present study showed the synthesis of novel drugs. These drugs showed antioxidant, anticancer activity.

Authors' Contribution

Both authors made an equal contribution to the research, data analysis and manuscript production process. Final version of the manuscript was approved by both authors.

Conflict of Interest

Authors declare there is no conflict of interest with the present publication.

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