

**IN VITRO SCREEENING OF PHYTOCHEMICALS AND
ANTIBACTERIAL ACTIVITY OF SARACA INDICA ROXB. AGAINST
MULTIPLE DRUG RESITANT (MDR) BACTERIA**

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ABSTRACT

The objective of this study is *in vitro* screening of phytochemical and antibacterial activity of *Saraca indica* leaves extract against multiple drug resistant bacteria isolated from clinical samples. Antibacterial activity of five different solvent extracts (Methanol, acetone, ethanol, petroleum ether and n-Hexane) were prepared by using Soxhlet extractor. *In-vitro* antibacterial activity was performed by agar well diffusion method. The phytochemical analysis of both methanolic and ethanolic extract of leaves of *Saraca indica* revealed the presence of Saponins, Glycosides, Polyamides, Alkaloids, Flavonoids, Phenolic substance, Di & Tri-terpenes and Tannins. The methanolic extract

were highly effective against *E. coli* (30 mm) followed by *S. pneumoniae* (28 mm), *S. aureus* (26 mm), *E. cloacae* (26 mm) and *K. pneumoniae* (22 mm). The methanolic extract also showed 21 mm zone of inhibition against *S. typhi*, *A. baumannii* and *E. faecium*. The lowest MIC value was found against *S. pneumoniae* (0.79 mg/ml) and whereas 1.56mg/ml MIC value were observed against *E.coli*, *P. aeruginosa*, *S. typhi* and *A. baumannii*. The highest zone of inhibition of ethanolic extract was found against *E. coli* (22 mm) followed by *S. aureus* (20 mm), *K. pneumoniae* (19 mm) and 18 mm zone of inhibition was observed against *P. aeruginosa*, *P. mirabilis*, *E. faecalis*, *S. pneumoniae* and *E. cloacae*. *S. typhi* and *A. baumannii* showed 16mm zone of inhibition against leaf extract of *Saraca indica*. The lowest MIC value of ethanolic extract of leaves of *Saraca indica* was found against *E. coli* (0.79 mg/ml) while *S. aureus* and *K. pneumoniae* showed 1.56mg/ml. In the present investigation methanol was the best solvent for the extraction of active component of plants. *Saraca indica* can be used as antibacterial agent for the treatment of infectious diseases.

KEYWORDS: Antibacterial activity, MDR, *Saraca indica* Roxb, Soxhlet Apparatus

INTRODUCTION

Saraca indica belongs to the family Caesalpiniaceae is commonly known as Asoka, Sita Asoka and Haempushpam. It is a evergreen tree called in english Asok tree. It is also known as Kankeli (Sanskrit), Ashoka (Assamese), Ashoka (Bengali), Ashoka (Gujrati), Ashoka (Hindi), Ashok (Marathi), Ashoka (Oriya), Ashok (Punjabi), Asogam (Tamil), Ashokapatta (Telugu). It is found in India, China, Ceylon and Malaysia. It occurs almost throughout India up-to an altitude of 750 m in the central and in the eastern Himalayas and Khasi, Garo and Lushai hills, wild in Chittagong, Bihar, Orissa, Konkan, Deccan, and Mysore. It has become quite scarce in several localities and is reported to be threatened in North Eastern Region of India. *Saraca indica* is a small to medium sized tree handsome evergreen tree quite beautiful when it full bloom its height about 7-10 cm. It cultivated the up to the altitude 750 meters. Leaves are paripinnate 15-20cm long and the leaflets 6-12, oblong and rigidly sub-coriaceous. Leaves are narrowly lanceolate, cork like at the base and with a short pestistipules are intra-petiolar and completely united. The bark is dark brown or grey or almost black with warty surface. Stem bark are irregular and rough due to the presence of rounded or projecting lenticels. Flowers are fragrant. Flowers are polygamous apetalous, yellowish orange turning to scarlet, in short laterally placed corymbose, axillary panicles, bract small, deciduous, calyx petaloid. Seed are 4-8, ellipsoid-oblong and compacted. ^[1]

The phytochemistry show in the bark of plant presence of epicatechin, catechin, procyanidin p2, 11 – deoxyprocyanidin B, leucocyanidin etc. the flower part of plant contain oleic, linoleic-palmitic and stearic acids, P- sitosterol, quercetin, kaemferol-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. ^[2] Seeds and pods contain oleic, linoleic, palmitic, stearic, catechol, epicatechol and leucocyanidin. Leaves and stem found to contain quercetin, quercetin – o-alpha-l-rhamnoside, kaempferol 3-o-alpha-L-rhamnoside, ceryl alcohol and beta-sitosterol. ^[4] It is already known that the beneficial effects of medicinal plant materials typically result from the combinations of secondary products present in the plant. The antimicrobial activity is due to presence of alkaloids, steroids, tannins, phenols, flavonoids, steroids, resins, fatty acids and gums. ^[1]

The principal constituents of this tree are a steroid component and a calcium salt. The earliest chronicled mention is in the Ayurvedic treatise, the Charka Samhita (100 A.D.), in which

Asoka is recommended in formulations for the management of pain with relation to uterus (Gynecological) as Anodynes. The Bhavprakash Nighantu, commonly known as the Indian Materia Medica (1500 A.D.), cites the plant as a uterine tonic that is effective in regularizing the menstrual disorders. ^[4] Useful parts of the plant are barks, leaves, flowers and seed. The plant is useful in dyspepsia, fever, burning sensation, colic, ulcer, menorrhagia, leucorrhoea, pimples, etc. the bark used for the pharmaceutical preparations, is bitter, astringent, sweet refrigerant, anthelmintic, styptic, stomachic, constipating, febrifuge and demulcent. Even the juice of leaves, mixed with cumin seeds, is used for the treatment of stomachalgia. Some workers have mentioned that both ethanolic and water extracts of bark of *Saraca indica* are effective in vitro against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Klebsiella pneumoniae*. ^[2] In addition, some other researchers have also indicated that those extracts of the leaves of the plant show antibacterial activity only against *E. coli*. Moreover, both the methanolic and water extracts of its leaves are effective against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*. ^[3]

MATERIALS & METHODS

1) Collection of Plant Materials

The plant materials were collected from different areas of Nagpur city. This plant then authenticated from P.G. Department of Botany, R.T.M. Nagpur University, Nagpur. Leaves were collected washed with sterile distilled water and air dried at room temperature. Dried leaves were coarsely powdered using a mortar and pestle and were further reduced to powder using an electric blender. The powder was transferred into closed containers for further use.

2) Herbal preparations

The dried plant materials (20 gm) were extracted with 200 ml of each solvent separately by using Soxhlet extractor for 2 to 5 h at a temperature not exceeding the boiling point of the Solvent. The solvents used for the study were methanol, ethanol, petroleum ether, acetone and n-hexane. The extracts were filtered and then concentrated to dryness. The extract were transferred to glass vials and kept at 4°C before use. The extracts were dissolved in 20% aqueous dimethyl sulfoxide (DMSO) to produce a stock solution of 100 mg/ml. The stock solutions were stored in a refrigerator until needed.

Phytochemical analysis

The phytochemical screening of all the extracts was carried out to determine the presence of the following compounds; alkaloid, flavonoids, polyuronides, reducing sugars, cyanogenic glycoside, saponins, terpenes, anthracenosides, phytosterols and phenols as described below.
[5]

3.1 : Saponins (The Froth test)

2 ml of the extract was added to distilled water and shaken vigorously. A froth (foam) that persisted for more than 10 minutes indicated the presence of saponins.

3.2: Glycosides

To the solution of extract in glacial acetic acid few drops of ferric chloride and conc. H_2SO_4 are added and observed for reddish brown coloration at the junction of 2 layers and bluish green color in upper layer.

3.3: Polyuronides / Polyamides

Ten milliliters of acetone was added to 2ml of the extract in a test tube. The appearance of a precipitate indicated the presence of polyuronides.

3.4: Reducing sugars

Two milliliters of the extract was diluted in 2ml of distilled water and Fehling's solutions (A+B) added to the mixture. A brick red precipitate after standing in the heat or water bath indicated the presence of reducing sugars.

3.5: Alkaloids

Twenty milliliters of the alcohol extract was evaporated to dryness on a water bath. Five to ten milliliters of 10% hydrochloric acid (HCl) and $CHCl_3$ were added to the extract. Concentrated ammonia was added to the aqueous layer to obtain a pH of between 8 and 9. The solution was then extracted in a separating tube with chloroform or ether. The a polar solvent was evaporated to dryness in an evaporated dish in a water bath and the residue was dissolved with 5ml of HCl (2N) and the solution was divided into three separate test tubes. Two to three drops of Mayer's reagent was added to one and the same amount of Bertrand's reagent to the other, while the third test tube served as a reference. The appearance of an opalescent or yellow-white precipitate with the reagents indicated the presence of alkaloids.

3.6: Anthracenocides

Four milliliters of the extract was concentrated to 2ml with 2ml of 25% of ammonia solution added and shaken. A cherry red colour of the alkaline layer indicated the presence of emodols (aglycones of anthracenosides) in an oxidized form–Borntrager's reaction.

3.7: Flavonoides

Five milliliters of the extract was evaporated to dryness. The residue was dissolved in 2ml of 50% methanol by heating and 4 grams of metal magnesium and 6 drops of concentrated HCl added. A red solution indicated the presence of flavonoids, while an orange solution indicated the presence of flavones.

3.8: Phenolic substances

Two to three drops of 10% Ferric chloride solution was added to 5ml of extract in a test tube and observed. Dark Green color was develops indicated positive results.

3.9: Sterols and Triterpenes

Ten milliliters of the extract was evaporated to dryness. The residue was dissolved in 0.5ml of acetic aldehyde and 0.5ml of CHCl_3 added and transferred into a dry test tube. About two milliliters of concentrated sulphuric acid (H_2SO_4) was added to the bottom of the tube using a pipette. A brownish red or violet ring at the contact zone of the two liquids indicated the presence of sterols and triterpenes. The greenish and brownish red (wine) nature of the supernatant indicated the presence of sterols and triterpenes respectively.

3.10 : Test for Tannins

To 0.5 ml of extract solution 1 ml water and 1-2 drops of ferric chloride solution was added. Blue color was observed for garlic tannins and greenish black for catecholic tannin.

3.11 : Test for amino acids

1 ml of plant extract adds 2 ml of Ninhydrins. For positive results indicates forming purple color.

3.12 : Test for proteins

1 ml of dilute extract add 1 ml of 5% CuSO_4 add 1% of 1ml of NaOH. Deep blue color indicates positive results.

3.13 : Test for Saponin

To 50 mg powder and add 20 ml distilled water shake for 15 minutes. Forming 2 cm foam was produced in measuring cylinder indicated positive results.

4. Bacterial isolates

Multiple drug resistant bacteria were isolated from different clinical specimen such as urine, blood, wound swabs/pus, cerebrospinal fluid and sputum. The MDR strains were identified on the basis of their morphology, cultural, biochemical characteristics as well as antibiotic susceptibility test. These all MDR bacteria were resistant to more than 10 antibiotics. The MDR strains used for the antibacterial activity were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella spp.*, *Enterococcus faecalis*, *Citrobacter freundii*, *Acinetobacter baumannii*, *Streptococcus pneumoniae*, *Enterococcus faecium* and *Enterobacter cloacae*.

5. Determination of the potency of the herbal preparation

The agar diffusion method was used to investigate the antibacterial activity of the crude extracts. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterilized swab was aseptically dipped into the suspension. The dried surface of a Mueller-Hinton agar plates were inoculated by streaking the swab over the entire sterile agar surface with bacteria. A sterilized cork borer of an internal diameter of about 6 mm was used to punch holes in the medium and plant extracts were dispensed into the respective labeled holes. 20 % v/v DMSO was used as negative controls. Triplicates of each plate were made and the procedure was repeated for the other microorganisms. The plates were kept in the refrigerator for about 4 hours for complete diffusion of the extract and incubated at 37°C for 24 hours. After the incubation period, the diameter of each zone of inhibition was measured in millimeters (mm) with zone measuring scale.

6: Determination of minimum inhibitory concentration (MIC) of the crude extracts

MIC for each test organism was determined by following the modified agar well diffusion method. A twofold serial dilution of each extract was prepared by first reconstituting the dried extract (100 mg/ml) in 20% DMSO followed by dilution in sterile distilled water (1:1) to achieve a decreasing concentration range of 50mg/ml to 0.195 mg/ml. A 100 µl volume of each dilution was introduced into wells (in triplicate) in the agar plates already seeded with 100µl of standardized inoculum (10^6 cfu/ml) of the test microbial strain. All test plates were incubated aerobically at 37°C for 24 hrs and observed for the inhibition zones. MIC, taken as the lowest concentration of the test extract that completely inhibited the growth of the

microbe, showed by a clear zone of inhibition (>8mm), was recorded for each test organism. [6,7]

RESULTS & DISCUSSION

Saraca indica Roxb. is used in traditional medicine against different ailments like, gynaecological disorders, uterine fibroids, burns, diarrhoea. So far there are no reports showing the antimicrobial activity of the leaves of this tree. Hence an attempt was made to study the antimicrobial activity of the leaves of this tree. The phytochemical analysis of ethanolic extract of leaves of *Saraca indica* revealed the presence of Saponins, Glycosides, Polyamides, Alkaloids, Flavonoids, Phenolic substance, Di & Tri-terpenes and Tannins whereas methanolic extract also showed the presence of same secondary compounds with the presence of tannins instead of alkaloids which present in ethanolic extract. [Table No. 1] The study conducted by Nayak Sarojini (2011) also showed the similar results for the presence of phytochemicals in both ethanolic and methanolic extract. [8] Many study conducted by different researchers showed that the presence of secondary component in both ethanolic and methanolic extract which shows antibacterial activity. [9,10] In the presence study, antibacterial activity of leaves of *Saraca indica* tested against multiple drug resistant bacteria isolated from clinical specimen. This study showed that the methanolic extract showed highest antibacterial activity against all tested bacterial compared with other solvents. The methanolic extract were highly effective against *E. coli* (30 mm) followed by *S. pneumoniae* (28 mm), *S. aureus* (26 mm), *E. cloacae* (26 mm) and *K. pneumoniae* (22 mm). The methanolic extract also showed 21 mm zone of inhibition against *S. typhi*, *A. baumannii* and *E. faecium*. [Table No. 2] The highest antibacterial activity of all the extracts tested was found against *S. aureus* for methanol extract and it was strongly correlated with the results of Nayak Sarojini et al (2011). [8] This may be due to the presence of flavonoids, glycosides, saponins and steroids. The lowest MIC value was found against *S. pneumoniae* (0.79 mg/ml) and whereas 1.56mg/ml MIC value were observed in *E.coli*, *P. aeruginosa*, *S. typhi* and *A. baumannii*. [Table No. 3] Pal SC et. al. (2007) also showed that the methanolic extract showed their activity against Gram positive bacteria. [11] The ethanolic extract also showed better antibacterial activity against all MDR strains. The highest zone of inhibition found against *E. coli* (22 mm) followed by *S. aureus* (20 mm), *K. pneumoniae* (19 mm) and 18 mm zone of inhibition was observed against *P. aeruginosa*, *P. mirabilis*, *E. faecalis*, *S. pneumoniae* and *E. cloacae*. *S. typhi* and *A. baumannii* showed 16mm zone of inhibition against leaf extract of *Saraca indica* [Table No. 2] The lowest MIC value of ethanolic extract of leaves of *Saraca*

indica L. was found against *E. coli* (0.79 mg/ml) while *S. aureus* and *K. pneumoniae* showed 1.56mg/ml. [Table No. 3] Moreover, the study conducted using ethanolic extract was more effective in cases of *S. aureus* and *E. coli*. It has been reported that different solvents have different extraction capabilities. ^[12,13] Effectiveness of *S. indica* ethanol extract was appeared to be due to the involvement of alkaloids, flavonoids, glycosides and tannins. Seetharam et. al. (2003) found that ethanolic extract of the leaves of plant was active against *E. coli* and *S. aureus*. ^[14]

The other solvent extract also showed good antibacterial activity against tested MDR strains. Petroleum ether showed moderate antibacterial activity against all tested bacteria. This extract was found to be effective against *S. aureus* and *A. baumannii* (18 mm) whereas *E.coli*, *K. pneumoniae*, *S. typhi*, *C. freundii*, *S. pneumoniae* showed 16 mm and *P. aeruginosa*, *P. mirabilis* showed 15mm zone of inhibition. Acetone extract also showed good inhibitory activity against *E.coli*, *K. pneumoniae* (18 mm) followed by *S. aureus* (17 mm), *P. mirabilis* (17 mm), *A. baumannii* (17 mm) and *P. aeruginosa*, *E. faecalis*, *C. freundii*, *S. pneumoniae* showed 16 mm zone of inhibition. [Table No. 2] n-Hexane showed least inhibitory activity against all tested bacteria. [Table No. 2] Moreover, this study found that the methanolic extract was more effective in case of all tested bacteria. This plant extract can be used as an alternative for the treatment of infectious diseases caused by multiple drug resistant bacteria.

Table No. 1: Phytochemical analysis of *Saraca indica* Roxb.

Sr. No.	Phytochemical analysis	<i>Saraca indica</i> L.	
		Solvents	
		Methanol	Ethanol
1.	Saponins	+	+
2.	Glycosides	+	+
3.	Polyamides	+	+
4.	Reducing Sugars	+	-
5.	Alkaloids	-	+
6.	Steroids	+	-
7.	Flavonoids	+	+
8.	Phenolic substance	+	+
9.	Di & Tri-terpenes	-	+
10.	Tannins	-	+
11.	Amino acids	-	+
12.	Proteins	-	-

Table No. 2 : Antibacterial activity of *Saraca indica* Roxb. (Ashoka) against multiple drug resistant (MDR) bacteria

Plant Part used	Solvent	Zone of inhibition in mm											
		<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>S. typhi</i>	<i>E. faecalis</i>	<i>C. freundii</i>	<i>A. baumannii</i>	<i>S. pneumoniae</i>	<i>E. faecium</i>	<i>E. cloacae</i>
Leaves	Ethanol	20	22	18	19	18	16	18	17	16	18	15	18
	Methanol	26	30	23	22	20	21	18	20	21	28	21	26
	Petroleum Ether	18	16	15	16	15	16	14	16	18	16	12	14
	Acetone	17	18	16	18	17	15	16	16	17	16	14	15
	n-Hexane	12	10	12	NZ	NZ	11	12	14	12	11	10	10

NZ – No Zone

Table No. 3 : MIC of *Saraca indica* Roxb. (Ashoka) against Multiple drug resistant (MDR) bacteria

Plant Part used	Solvent	Minimum Inhibitory Concentration in mg/ml											
		<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>S. typhi</i>	<i>E. faecalis</i>	<i>C. freundii</i>	<i>A. baumannii</i>	<i>S. pneumoniae</i>	<i>E. faecium</i>	<i>E. cloacae</i>
Leaves	Methanol	3.125	1.56	1.56	3.125	3.125	1.56	12.5	3.125	1.56	0.79	3.125	6.25
	Ethanol	1.56	0.79	1.56	1.56	6.25	3.125	6.25	3.125	3.125	3.125	6.25	6.25

CONCLUSION

The extract studies have displayed variable antibacterial activities most probably due to the differences in the phytochemical constituents extracted by five different solvents used in this study. Systematic screening of plants may lead to the discovery of novel active compounds.

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