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Comparative study upon the antibacterial activity of flavonoids, alkaloids, steroids, Polar and non-polar solvent extracts of *Saraca asoca* Bark

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Abstract
Exposure of pathogenic microorganisms to the newly invented different kinds of drugs, results in the increased in antibiotic resistant strains of pathogenic organisms. Thus, a variety of new antimicrobial agents are needed to combat the decreasing efficacy of existing antibiotics. In the present work effort in this direction was carried out to assess the antimicrobial activity of *Saraca asoca* bark extracts against some multidrug resistant pathogenic bacteria. Water, methanol, petroleum ether, flavonoids, alkaloids and steroids extracts were examined for antimicrobial activity using 'Disc Diffusion Assay'. Minimum inhibitory concentration, Minimum bactericidal concentration, Total activity and Activity index were taken up and statistically were analyzed. Maximum zone of inhibition observed for bound flavonoids and methanolic extracts. The lowest MIC value of bound flavonoids 0.078 mg/ml was recorded against *Pseudomonas aeruginosa* whereas, lowest MBC observed was 0.156 mg/ml, indicating significant antimicrobial potential of test extracts. The results of the above study revealed that the bound flavonoid and methanolic extracts were exhibited significant antibacterial activity which might be helpful in preventing the progress of various diseases and can be used in an alternative system of medicine.

Keywords: Bark, flavonoids, alkaloids, steroids, minimum inhibitory concentration, minimum bactericidal concentration and activity index

Introduction
Plants synthesize a dazzling array of compounds, called secondary metabolites in addition to primary metabolites. Many secondary metabolites are "antibiotic" in a broad sense, protecting the plants against fungi, bacteria, animals, and even other plants [1]. These secondary metabolites are present in parts of the plant like flower, leaf and bark etc. Since time immemorial bark of the plant has been used as a remedy for various diseases; bark is the outermost layer of stems and roots of woody plants. Bark in plants includes various trees, woody vines, and many shrubs. Bark refers to all the tissues outside the vascular cambium and is a nontechnical term. We use different plant products and the products derive from bark are wall coverings spices, flavorings, tanbark, various hallucinatory chemicals and cork. The barks are the abundant sources of tannins and other compounds. Tannins inhibit the growth of various fungi, yeast, bacteria and virus [2]. Infectious diseases are handled using different kinds of antibiotics available in the market. Although pharmacological industries have been a producer of new antibiotics in the last three decades; resistance to these drugs in microorganisms has increased. In general, microorganisms have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [3]. Besides antibiotics have also been reported to affect our normal metabolism adversely if are used continually. Hence there is an urgent need to develop new herbal drugs to cure diseases.

Saraca asoca is an important indigenous plant with lots of traditional importance belonging to the family fabaceae. It is the wonderful herb that claims to cure several diseases according to ayurvedic medicine. It mainly contains tannin, saponin, glycosides, sterol and flavonoids[4]. It is widely used in Ayurveda to improve the complexion of the body, in blood disease, treat painful conditions, improves digestion and assimilation, to kills all infectious agents, inflammation and also as a CNS depressant. It is commonly called asoka, the Asoka tree is considered sacred throughout India. This tree has many folklorical, religious and literary associations in the religions. Due to its high value and handsome appearance this tree is found close to the temples throughout India [6]. The bark of plant contain (-) epicatechin, procyanidin p2,11'-deoxyprocyanidin B, (+) catechin, (24, £)- 24- methyl-cholesta-5-en-3p-ol(22 E,21 £)-24-ethycholesta-5, 22 dien-33-ol, (24 £)-24 ethylcholesta-5-en-3-p-ol, leucopelargonidin-3-O-p-D glucoside, leucopelargonidin and leucocyanidin [7].

Many studies have been done on medical properties of *Saraca asoca*: Chloroform, methanol, aqueous and ethanolic extracts of the stem bark of *Saraca indica* have been investigated for their antibacterial and antifungal activity [8]. Antioxidant potential of various extracts i.e. ethanolic, hydroalcoholic and acetone prepared by different extraction methods of stem bark of *Saraca asoca* have been studied by using DPPH (1,1, diphenyl-2 picryl hydrazyl) *in-vitro* model [9]. Methanolic extract exhibited potential antimicrobial activity against gram positive, gram negative bacterial and fungal species. So far there are no reports showing the comparative study upon antibacterial activity of flavonoids, alkaloids, steroids, polar and non-polar solvent extracts of *Saraca asoca* bark. Hence in present study an attempt was made to compare the antimicrobial activity of different extracts of the bark of *Saraca asoca.*

Materials and Methods

Collection of Plant Material

Bark of *Saraca asoca,* was collected from the eastern region of Rajasthan (Jaipur). Plant was identified by the senior taxonomist of the Department of Botany, University of Rajasthan and Voucher specimen no: RUBL211459 was submitted to the Herbarium, Botany department, University of Rajasthan.

Selected Test Pathogens

Seven pathogens, in total were screened which include *Escherichia coli* (MTCC 46), *Pseudomonas aeruginosa* (MTCC 1934), *Raoultella planticola* (MTCC 2271), *Enterobacter aerogenes* (MTCC 2822), *Bacillus subtilis* (MTCC 121), *Agrobacterium tumefaciens* (MTCC 431) *Staphylococcus aureus* (MTCC 87). The pathogens were procured from IMTECH (Chandigarh, Punjab, India). Bacterial strains were grown and maintained on Muller-Hinton Agar medium.

Preparation of Extracts

Flavonoid extraction: Bark of *Saraca asoca* was collected; shade dried, finely powdered and extracted using the method of Subramanian & Nagarjan [10]. Hundred grams of finely powdered sample was soxhlet extracted with 80% hot methanol (500ml) for 24 h and filtered using separating funnel. The filtrate obtained was re- extracted successively with petroleum ether. Three fractions formed, first fraction of petroleum ether, second fraction of ethyl and third fraction of ethyl acetate. Fraction of petroleum ether was discarded due to being rich in fatty substances, where as fractions of ethyl ether and ethyl acetate were further analyzed for free and bound flavonoids respectively. Using 7% H_2SO_4, ethyl acetate fraction of the sample was refluxed for the hydrolysis for 2 h (for removal of bounded sugars) and again filtrate was extracted in separating funnel with ethyl acetate. Extract with ethyl acetate thus obtained was washed with distilled water upto neutrality. Ethyl ether contains free flavonoids and ethyl acetate fractions contains bound flavonoids were dried in vacuo and weighed (Table 3). The extracts were stored at 4 °C and were re-suspended in acetone to get 10mg/ ml concentration for antimicrobial assay.

Alkaloids Extraction: Alkaloids were extracted from bark of *Saraca asoca* by well established method [11]. Finely powered sample (100g) of plant was extracted in 20ml methanol after shaking of 15 min. After filtration, filtrates kept for drying then residual mass were addressed with 1% H_2SO_4 (5ml. 2 times). Extraction was then performed in 10ml chloroform ($CHCl_3$) using separating funnel. Organic layer of chloroform was rejected and the aqueous layer was basified with 30% NH_4OH (PH=9- 10). Then again, extraction was done in 10ml chloroform and the organic layer of chloroform which formed as the lower layer was collected in a flask and steps was repeated with fresh chloroform. Extracts thus obtained was then dried in vacuum for further use.

Steroid Extraction: Steroids were extracted from bark of *Saraca asoca* by well established method[12] after preliminary detection of steroids. Finely powdered sample (100g) of plant parts were extracted in petroleum ether for 2-4hr. After filtration, residual mass was treated with 15% ethanolic HCl for 4hr. further extraction was then carried out in ethyl acetate and to neutralize the extract it was washed in distilled water. Neutral extract was then passed over Sodium sulphate to remove moisture contents and was dried in vacuum. Chloroform was used for reconstitution of extract, filtered and dried for further use.

Extraction in different polar and non-polar solvents: Powdered bark of *Saraca asoca* (20 g) was taken in three flasks. Water, methanol and petroleum ether were used as solvent. The powdered material and solvents were taken in 1:10 ratio. These were kept at soxhlet unit for 24 hours. Extracts thus obtained were filtered by using filter paper (Whatman No. 1) and the filtrate was subjected to evaporation to obtain dried extract. The residual extracts were stored in refrigerator at 4 °C in small and sterile glass bottles. The percentage yield of each dried plant extract was calculated.

Antimicrobial susceptibility testing

Disc diffusion assay [13] was carried out for screening. Muller-Hinton agar base plates were seeded with the bacterial inoculums (inoculum's size 1×108 CFU/ml). Sterile filter paper discs of 6mm diameter (Whatmann no.1) were impregnated with 100µl of each of the extract of concentration (10mg/ml) to build up a final concentration of 1 mg/disc. To remove residual solvent, discs were left to dry in vacuum as solvent might interfere with the determination. Dried discs with extract were then placed on the seeded agar plates. To avoid any mistake each extract was verified in triplicate along with streptomycin (1mg/disc) as standard for bacteria. The plates were kept at 4 °C for diffusion of extract. Thereafter were incubated at 37 °C for 24 hours. Activity index for each extracts was calculated (Table 3) by the standard formula viz

$$\text{Activity Index} = \frac{\text{IZ produced by extract}}{\text{IZ produced by Standard}}$$
$$\text{where, IZ} = \text{inhibition zone}$$

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) was determined for each plant extracts showing antimicrobial activity against test pathogens. For the determination of MIC, Broth microdilution method was used [14]. To make the final concentration of 10mg/ml, the plant extracts were re suspended in acetone (as acetone has no activity against test microorganisms). Two fold serially diluted extracts were added to broth media for 96-wells of micotiter plates. Thereafter 100µl inoculum

(1×10^8 CFU/ ml) was added in each well. Bacterial suspensions were served as negative control, while broth containing standard drug was used as positive control. To allow growth of organisms and check by the tested extracts microtiter plates were incubated at 37 °C for 24 hours. Each extract was assayed in duplicate and each time two sets of microplates were prepared. One was kept for incubation while another was kept at 4 °C for comparing the turbidity in the wells of microplate. The MIC values were taken as the lowest concentration of the extracts in the well of the microtiter plate that showed no turbidity after incubation. Using visible growth of microorganisms, the turbidity of the wells in the microtiter plate was interpreted. The minimum bactericidal concentration (MBC) was determined by subculturing 50 µl from each well showing no noticeable growth. Least concentration of extract showing no visible growth on sub culturing was taken as MBC.

Total activity (TA) determination

Total activity is the volume at which test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g [15].

Statistical Analysis

Mean value and standard deviation were calculated for each test bacteria. Data were analyzed by one-way ANOVA and *P* values were considered significant at $P<0.05$.

Results

In vitro. antibacterial activity of flavonoids, alkaloids, steroids, polar and non-polar solvent extracts of *Saraca asoca* bark were assessed, using seven different pathogens by the presence or absence of zone of inhibition (Table 2). Further minimum inhibitory concentration and minimum bactericidal concentration were performed for those extracts which showed zone of inhibition against particular pathogen (Table 2) Quantity of extracts per gram of dried plant material (Table 1), Total activity and Activity index were also calculated and tabulated (Table 3).

Antibacterial Activity

In the present investigation a total seven extracts were tested, among which each of the extracts were active against one of the tested pathogens. Maximum zone of inhibition observed for bound flavonoids against *Pseudomonas aeruginosa* (IZ= 20) (Table 2). Significant activity was recorded in case of bound flavanoids against all tested pathogens except *Agrobacterium tumefaciens* and *Staphylococcus aureus,* whereas average activity was recorded for methanolic extract against all tested pathogen except *Escherichia coli* and *Pseudomonas aeruginosa.* Free flavonoids and steroids were effective against only two tested pathogens and alkaloids has shown zone of inhibition only against one out of seven tested pathogens. In case of water extract best zone of inhibition was observed against *Bacillus subtilis* (IZ=12). Bioactive nature of petroleum ether extract was observed against *Pseudomonas aeruginosa* (IZ=10) and *Staphylococcus aureus* (IZ=10). Among all the seven different extracts, bound flavonoids and methanolic extracts were observed as most bioactive substance, as it has shown activity against five, out of seven pathogens.

MIC and MBC

MIC and MBC were performed (Table 2) for plant extracts which had shown activity, in diffusion assay. The MIC and MBC of free flavonoids were 0.312mg/ml and 0.312mg/ml, respectively for both *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The range of MIC and MBC of bound flavonoids recorded was 0.078mg/ml to 1.25mg/ml and 0.156mg/ml to 1.50mg/ml, respectively. The lowest MIC and MBC value were recorded in case of bound flavonoids i.e 0.078 mg/ml and 0.156mg/ml respectively against *Pseudomonas aeruginosa*, indicating significant antimicrobial potential of test extracts. Out of all tested extracts, alkaloids observed to be very less effective and showed activity against *Bacillus subtilis* with MIC and MBC value 0.625mg/ml and 1.25mg/ml, respectively. *Pseudomonas aeruginosa, Bacillus subtilis, Enterobacter aerogenes, Staphylococcus aureus* and *Raoultella planticola* were observed to be most susceptible organism in the investigation followed by *Agrobacterium tumefaciens* and *Escherichia coli.*

Total activity and Activity index

TA and AI were also calculated and tabulated (Table 3). Maximum values of TA were recorded against *Enterobacter aerogenes, Bacillus subtilis, Raoultella planticola and Agrobacterium tumefaciens* for methanolic extract whereas, significant values of TA were calculated against *Bacillus subtilis* and *Staphylococcus aureus* for water and methanolic extracts respectively. In present work best AI was observed for bound flavonoids extract against *Pseudomonas aeruginosa* (AI=1±0.064).

Table 1: Quantity of extract mg/g dried bark powder.

Extracts	Free flavonoids	Bound flavonoids	Alkaloids	Steroids	Water extract	Methanolic extract	Petroleum ether extract
Quantity (mg/g)	1	2.5	65	4.5	42	126	3

Table 2: IZ, MIC and MBC of all extracts against tested pathogens.

Extracts/ Pathogens/ Parameters		*Enterobacter aerogenes*	*Bacillus subtilis*	*Raoultella planticola*	*Agrobacterium tumefaciens*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
FF	IZ	--	--	--	--	--	14	12
	MIC	--	--	--	--	--	0.312	0.312
	MBC	--	--	--	--	--	0.312	0.312
BF	IZ	10	13	16	--	13	20	--
	MIC	0.625	0.312	0.156	--	0.312	0.078	--
	MBC	1.25	0.625	0.312	--	0.312	0.156	--
A	IZ	--	10	--	--	--	--	--
	MIC	--	0.625	--	--	--	--	--
	MBC	--	1.25	--	--	--	--	--
S	IZ	10		15	--	--	--	--
	MIC	0.625	--	0.312	--	--	--	--
	MBC	0.625	--	0.312	--	--	--	--
W	IZ	--	12	--	--	--	7	--
	MIC	--	0.312	--	--	--	1.25	--
	MBC	--	0.312	--	--	--	1.25	--
M	IZ	12	15	13	13	--	--	7
	MIC	0.312	0.312	0.312	0.312	--	--	1.25
	MBC	0.312	0.312	0.625	0.625	--	--	1.50
PE	IZ	--	--	--	--	--	10	10
	MIC	--	--	--	--	--	0.625	0.625
	MBC	--	--	--	--	--	1.25	1.25

FF- Free Flavonoids, BF- Bound flavonoids, A- Alkaloids, S-Steroids, W-Water, M-Methanol, PE- Petroleum Ether, IZ- Zone of Inhibition, MIC- Minimum Inhibitory Concentration and MBC- Minimum Bactericidal Concentration

Table 3: Total Activity and Activity Index of the bark extracts of *Saraca asoca*

Extracts/ Pathogens/ Parameters			*Enterobacter aerogenes*	*Bacillus subtilis*	*Raoultella planticola*	*Agrobacterium tumefaciens*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Quantity of extract mg/g dried plant part	FF 1	TA	--	--	--	--	--	8.01	8.01
		AI	--	--	--	--	--	0.7± 0.023	0.444± 0.065
	BF 2.5	TA	1.6	3.21	6.41	--	3.21	12.82	--
		AI	0.667± 0.038	0.5± 0.003	0.727± 0.057	--	0.867± 0.059	1± 0.064	--
	A 65	TA	--	104	--	--	--	--	--
		AI	--	0.385± 0.055	--	--	--	--	--
	S 4.5	TA	7.2		14.42	--	--	--	--
		AI	0.667± 0.023	--	0.682± 0.057	--	--	--	--
	W 42	TA	--	134.62	--	--	--	33.6	--
		AI	--	0.462± 0.029	--	--	--	0.35± 0.06	--
	M 126	TA	403.84	403.84	403.84	403.84	--	--	100.8
		AI	0.8± 0.028	0.577± 0.033	0.591± 0.026	0.464± 0.064	--	--	0.259± 0.051
	PE 3	TA	--	--	--	--	--	4.8	4.8
		AI	--	--	--	--	--	0.5± 0.037	0.37± 0.062

FF- Free Flavonoids, BF- Bound flavonoids, A- Alkaloids, S-Steroids, W-Water, M-Methanol, PE- Petroleum Ether, Total activity (TA) = Extract per gram dried plant part/MIC,
Activity Index (AI) = IZ produced by extract/ IZ produced by standard, IZ = inhibition zone.

Discussion

It has been found that in comparison to all tested extracts bound flavanoids extract showed effective bioactivity against most of the tested pathogens followed by methanolic extract. Although most of the tested extract found effective against the tested pathogens but alkaloids remained less effective and showed activity against only one pathogen. The lowest MBC and MIC value of bound flavonoids against *Pseudomonas aeruginosa,* indicates significant antimicrobial potential. Water extracts activity against *Pseudomonas aeruginosa* and *Bacillus subtilis* supported the previous results of the Sainath *et al.* [8] but not against *Staphylococcus aureus* and *Escherichia coli.* Similarly methanolic extracts activity against *Staphylococcus aureus* and *Bacillus subtilis* also supported the results of Sainath *et al.* [8] but not for *Pseudomonas aeruginosa.* In literature, it has been found that petroleum ether extract remained inactive against most of the tested pathogens [16]. But in present work bioactive nature of the petroleum ether extract has been found against *Pseudomonas aeruginosa* and *Staphylococcus aureus.* These minor changes in results from previous studies might be due to change in concentration or methods used for extraction in

different laboratory conditions. In our knowledge upto now there is not any study has been performed related to the antibacterial nature of flavonoids, alkaloids and steroids of *Saraca asoca* bark with MIC and MBC. Therefore, in literature meager data has been found for the comparision of present study. Most of the research has been restricted on determination of IZ of crude extracts without calculating AI, MIC, MBC and TA. Determination of MIC and MBC has now become an inevitable step in antimicrobial studies in order to establish their antimicrobial activity so as to explore them at industrial level for production of drugs, which could replace the existing ones. Hence, most of the studies carried out so far could only reveal their antimicrobial activities, but are not helpful for establishing them as antibiotic. A continuous research for getting new antimicrobial agents is the need of the present scenario, either by designing and synthesizing new agents, chemically or through the search of new natural sources for antimicrobial agents [17]. The present investigation is of great significance as far as the future drugs are concerned and advocates the use of selected plant material for pharmaceutical purpose and to for prepare antimicrobial drugs for resistant pathogens, of course after clinical trials.

Conclusion

Saraca asoca, traditional medicinal plant of India, is the rich source of bioactive compound. As now, little work has been done on the biological activity and hence extensive investigation is needed to exploit the bioactive compounds for medicinal purpose. The results of the above study revealed that the bound flavonoid and methanolic extracts were exhibit significant antibacterial activity which might be helpful in preventing the progress of various diseases and can be used in alternative system of medicine.

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