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In vitro antimicrobial activity and phytochemical analysis of two species of *Saraca asoca*

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Abstract: *Saraca asoca* is used worldwide to treat various kinds of human disorders and regarded as a universal panacea in the ayurvedic medicines. The present research evaluates the anti-microbial activity of bark (aqueous, methanol and acetone) extracts of two plant species *Saraca asoca caesalpiniaceae* and *Saraca asoca leguminosae* by the agar well diffusion assay against *E. coli* (Gram negative bacteria) and *Bacillus subtilis* (Gram positive bacteria). Different fraction of both *S. asoca* species was tested. Out of three fraction viz. aqueous, acetone and methanolic extract of bark had showed the activity against both tested bacterial species. The phytochemical investigation of different fraction of bark extract shows the presence of tannins, phenols, saponin, flavonoids and glycosides. The *Saraca asoca caesalpiniaceae* bark extracts shows significant antibacterial activity as compared to *Saraca asoca leguminosae* which might be due to the presence of higher concentration of phenolic compound which was estimated 1.239µg/ml and 1.129 µg/ml respectively.

Keywords: *Saraca asoca caesalpiniaceae* • *Saraca asoca leguminosae* •antimicrobial action • phytochemical screening •*Bacillus subtilis* • *Escherichia coli*

Introduction

The increasing resistance in pathogenic bacterial species due to the evolution in a microorganism or transfer of resistance gene through vertical transfer or horizontal transfer of gene (Srivastava et al., 2016). The development of resistance in microorganism is not problem of Indian subcontinent alone, but it is a global problem. The researchers are working on reversal of antibiotic resistance, but still there is no method yet developed to prevent or reverse the resistance in microorganism. However, the increase faith shown in Ayurvedic medicine or Herbal drugs in these days by Indian and other countries lead the screening of different plant species to decipher the antibacterial, antioxidant, antiviral properties from plant source.

Saraca asoca is the most ancient tree of India and commonly known as Ashok, it is a Sanskrit word which means no-grief (Panchawat et al., 2010). Asoka is a legendary and a sacred tree of India and considered as a sacred tree by Hindus and

Buddhists possesses various medicinal uses and also known as sita ashok (Mathew et al., 2005). *Saraca asoca* is distributed in evergreen forests of India up to an elevation of about 750 meters. The plant requires slightly acidic to neutral soils for good growth with medium to deep well drained fertile soils. It grows well in tropical to sub-tropical situations under irrigation (Pradhan et al., 2009). *Saraca asoca* is one of the universal plant having medicinal activities. The stem bark of the tree is the principal constituent of several Ayurvedic preparations which are widely prescribed in haematuria, dyspepsia, fever, burning sensation, visceromegaly, colic, ulcers, menorrhagia, metropathy leucorrhoea, pimples and other diseases of the female urogenital system (Suja et al., 2012).

The Saraca asoca bark extracts tested for antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus aureus, Klebsiella aerogenes, Proteus vulgaris* at 4 mg/ml using agar well diffusion method. The ethanol and distilled water extracts showed significant broad spectrum antibacterial activity (Pankaj et al. 2010). *Saraca asoca* ethanol and aqueous extracts of bark was subjected to antibacterial activity on agar plate with different micro-organisms such as *B. subtilis, E. coli, Salmonella* Typhi, *S. aureus* and *Agrobacterium tumefaciens.* Plant extracts show significant zone of inhibition against all the microorganisms except *A. tumefaciens* (Sainath et al., 2009). Both species of *Saraca asoca* leaf extract also possess antibacterial activity against *E. coil* and *B. subtilis* (Patni and Chandra, 2016)

The extracts of *Saraca asoca* bark also reported to have antimicrobial activity against *Propionibacterium acnes* and *S. epidermidis* with different MIC. The ethanolic extract (MIC 100 µg/ml) show better antimicrobial activity as compared to aqueous extract (125 µg/ml). Nayak *et al*. (2011) reported that the zone of inhibition was largest when the ethanolic extract was used against *E. coli,* whereas it was least in case of *S. aureus.* The methanolic extract produced maximum zone of inhibition against *S. aureus* and the minimum zone was found in case of *B. subtilis.* Chloramphenicol (positive control) produced maximum and minimum zones of inhibition against *S. aureus* and *B. subtilis,* respectively. So, methanolic as well as the ethanolic extracts had more potent antimicrobial property than the positive control.

Phytochemicals are the chemical compounds that occur naturally in plants (phyto means "plant") and plant foods that works with nutrients and dietary fibre to protect against various diseases. The plant shows antimicrobial activity due to the presence of the phytochemicals (phenolics, flavonoids, glycosides, tannins, alkaloids and saponins). Phytochemicals reducing the risk of many diseases, including cancer, heart disease, stroke, high blood pressure, cataracts, osteoporosis and urinary infections etc (Ayyappan et al., 2010). They have complementary and overlapping mechanisms of action, including antioxidant affects, modulation of hormone metabolism and antibacterial andante effect. It is also reported that, most of the phytochemicals have antioxidant activity and protect our cells against oxidative damage and reduce the risk of diseases.*Saraca asoca* is reported to contain phenolics, glycoside, alkaloids, flavonoids, tannins steroids and saponins. These phytochemical constituents are responsible for the pharmacological actions like antibacterial, antiulcer, larvicidal chemo protective activities, spasmogenic, oxytocic, uterotonic, anti-implantation, anti-tumour, antiprogestational, antiestrogenic activity against menorrhagia and anti-cancer (Preeti et al., 2012; Saha et al., 2012).

Previous phytochemical screening revealed the presence of tannins, proteins, steroids, glycosides, carbohydrates, saponins, flavonoids in different extracts of the flower of *Saraca asoca.* These results show that flowers of *Saraca asoca* contain a number of chemical ingredients, which may be responsible for the various pharmacological actions (Saha et al., 2011). It has been observed that most active phytochemicals present in the flowers are flavonoids, steroids, tannins and glycosides. These phytoconstituents may be responsible for various pharmacological actions of this plant part, like

antibacterial, antiulcer, anticancer, larvicidal and chemo protective activities (Pal et al., 1985).

The present study was investigated to evaluate the antimicrobial activity of both species of Sarca asoca and also its phytochemical constituent which is responsible for it medicinal plant.

Materials and Methods

Plant Material

The plant varieties i.e. bark of *Saraca asoca caesalpiniaceae* and *Saraca asoca leguminosae* were collected from the herbal garden of Lovely Professional University.

Microorganisms

Bacterial strains (*Bacillus subtilis* (MTCC-121), *Escherichia coli* (MTCC-120) were obtained from MTCC Chandigarh. Both bacterial strain were maintain in the laboratory on the surface of Nutrient Agar Slant and preserved in the refrigerator at 4±1^{o}C and taken out and bring it at room temperature when antibacterial activity was performed.

Extract preparation

Aqueous, methanolic and acetone extracts of the bark of the both plant species was prepared by soxhlet method. The plant bark was sterilized and air dried and grounded with sterile mortar pestle. The powdered plant bark (20gm) was extracted for 8 hours with methanol (200ml) in Soxhlet apparatus. Same procedure was followed for preparation of acetone and aqueous (distilled water) plant extracts of both plant species.

Phytochemical analysis

i. *Qualitative estimation of phytochemical constituents*

Freshly prepared extracts were subjected to standard phytochemical analysis to find the presence of phytochemical constituent's. The method described by Odebiyi and Sofowora (1978) were used to test for the presence of flavonoids, tannins, glycosides, phenolics and saponins.

Tannins: To the 2ml of plant extract, 2ml of 0.1% ferric chloride was added. Formation of black precipitate indicates the presence of tannins.

Phenols: Plant extracts (2ml) were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Flavonoids: 2ml of the plant extracts were treated with 3-4 drops of concentrated HCL and magnesium ribbon was added to it. Formation of red colour indicates the presence of flavonoids.

Glycosides: To the 2ml of the plant extract, glacial acetic acid, ferric chloride ($FeCl_3$) and concentrated HCL was added drop wise. Formation of reddish brown colour indicates the presence of glycosides.

Saponins: 0.5 gm of the plant extract was shaken with 2 ml of water. Formation of foam and it persists for ten minutes it indicates the presence of saponins.

ii. *Quantitative estimation of phytochemical constituents*

Phenolic estimation: Phenolic estimation: Phenols were estimated by the procedure described by (Sadasivam and Manickam, 1997).

1 g leaf tissue was grinded in 5 ml 80% methanol. The extract was agitated at 70ºC for 15 minutes. Now this methanolic extract was used for estimation of total phenols.

To the 1 ml sample 5 ml distilled water was added to make the final volume 6 ml. To this 250 µl Folin's reagent was added and the mixture was incubated for 3 min at room temperature. After incubation, 1 ml 20% sodium carbonate and 1 ml distilled water were added and the solution was incubated for 1 hr at room temperature. Absorbance was recorded at 725 nm. The amount of total phenols was estimated from the standard curve and expressed as µg phenol g^{-1} fresh weight.

Flavonoids estimation: Flavonoids were estimated by the procedure given by (Boham and Kocipaiabyazan, 1974).

1 g leaf tissue was grinded in 5 ml 80% methanol. The extract was agitated at room temperature for 1 hour. Now this methanolic extract was used for estimation of total phenols.

10 gm of the plant sample was extracted repeatedly with 100ml of 80% methanol at room temperature. Then the whole solution was filtered through filter paper. The filtered solution was then transferred into a flask and evaporated into dryness over hot waterbath and weighted to a constant weight. The remaining content after evaporation was flavonoids. And the total amount of flavonoids present in the plant extracts was determined.

Antimicrobial assay

i. Preparation of Inoculum

The ideal inoculum after overnight incubation gives the even semi confluent growth. Too heavy inoculum may reduce the size of inhibition zone by many antimicrobial agents from plant source. Using a straight wire touch 5-10 well isolated colonies of tested microorganism against which antimicrobial activity to be tested was inoculate on the Mueller Hinton Broth Medium. Incubate at 35-37^{o}C for 4 – 6 hour. The density of the inoculums is adjusted to 10^{8} cfu/ml by comparing with that of 0.5 Mc Farland Standard. 0.1 ml of the original cultures (about10^{6}-10^{7} cells) were added into sterile duplicate sets of Petri dishes and 25 ml of the molten (45±1^{o}C) Mueller Hinton Agar (HiMedia, Ltd) were poured into Petri dishes. The methanol extract (0.1ml) were placed in wells (8mm diameter) cut in the agar media and plates were incubated at 37± 1^{o}C. The resulting inhibition zones obtained with bacteria were recorded after 24 hour (Singh et al., 2009).

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent (plant extract) that will inhibit the visible growth of a microorganism after overnight incubation. The MIC of plant extract was determined as per the method described by Chandra et al. (2016).

Results and Discussion

Antimicrobial analysis of *Saraca asoca* bark against two bacterial strains *(Bacillus subtilis and Escherichia coli)* was tested. The zone of inhibition (36.2 ± 0.2mm) was largest when methanolic bark extract of *Saraca asoca caesalpiniaceae* (100mg/ml) was used against *Escherichia coli* and produced least zone of inhibition against *Bacillus subtilis* (26.6±0.4mm) (Table -1). Acetone bark extract of *Saraca asoca caesalpiniaceae* (100mg/ml) produced largest zone of inhibition against *Escherichia coli* (34.2 ± 0.4mm) and produced zone of inhibition against *Bacillus subtilis* (17.0±0.3mm). Aqueous bark extract of *Saraca asoca caesalpiniaceae* (100mg/ml) produced significant zone of inhibition when used against *Bacillus subtilis* (21.2±0.7mm) but produced least zone of inhibition when used against *E.coli* (9.0±0.04 mm). The methanolic bark extract of *Saraca asoca leguminosae* (100mg/ml) shows significant zone of inhibition against *B. subtilis* (15.9±0.6mm) as compare to the *E. coli* (10.9 ± 0.0mm). Similarly but lesser to the methanolic bark extract, acetone bark extract of *Saraca asoca leguminosae* (100µg/ml) shows significant zone of inhibition against *B. subtilis* (11.6±0.0mm) as compare to the *E. coli* (9.5 ± 0.2mm). Aqueous bark extract of *Saraca asoca leguminosae* show maximum zone of inhibition against *E. coli* (12.0 ± 0.7mm) and showed zone of inhibition against *B. subtilis* (10.8±0.7mm). The maximum zone of inhibition of bark extract of plant *Saraca asoca caesalpiniaceae* followed a trend, [Methanol (36.2 ± 0.2mm) > acetone (34.2 ± 0.4mm) > aqueous (9.0 ± 0.04mm)] against *E. coli,* and against *B. subtilis* the bark extract of *Saraca asoca caesalpiniaceae* followed the trend, [methanol (26.6±0.4mm) > aqueous (21.2±0.7mm) > acetone (17.0±0.3mm)]. Similarly the bark extract of plant *Saraca asoca leguminosae* shows the zone of inhibition in methanol (10.9 ± 0.0mm), aqueous (12.0 ± 0.7mm) and acetone (9.5 ± 0.2mm) against *E. coli,* and against *B. subtilis* the bark extract of *Saraca asoca leguminosae* followed the trend, (methanol) (15.9±0.6mm) > acetone (11.6±0.0mm) >aqueous (10.8±0.7mm).

Table 1 Antimicrobial activity of different fraction of *Saraca asoca (S. asoca caesalpiniaceae* and *S. asoca leguminosae)*

Solvent	Positive control (Kanamycin)	*E. coli*	Positive control (Gentamicin)	*B. subtilis*	Negative Control
	Saraca asoca caesalpiniaceae				
	Zone of Inhibition (in mm)				
Aqueous	30.0 ± 0.2	9.0 ± 0.04	20.0 ± 0.4	21.2±0.7	-
Acetone	30.0 ± 0.2	34.2 ± 0.4	20.0 ± 0.4	17.0±0.3	-
Methanol	30.0 ± 0.2	36.2 ± 0.2	20.0 ± 0.4	26.6±0.4	-
	Saraca asoca leguminosae				
Aqueous	30.0 ± 0.2	12.0 ± 0.7	20.0 ± 0.4	10.8±0.7	-
Acetone	30.0 ± 0.2	9.5 ± 0.2	20.0 ± 0.4	11.6±0.0	-
Methanol	30.0 ± 0.2	10.9 ± 0.0	20.0 ± 0.4	15.9±0.6	-

ZOI: Zone of inhibition, Zone of inhibition in mm (millimetre) are mean of inhibition of three replicates and -, = no zone of inhibition

Similar results have been found by Pal *et al.* (1985) in which the methanolic extract was active against *E. coli.* Preeti *et al.* (2012), also showed that the methanolic extract (100µg/ml and 200µg/ml) gave maximum zone of inhibition in opposition to *Staphylococcus aureus* and minimum *in case* of *B. subtilis.* Sainath *et al.* (2009) also concluded that methanolic as well as aqueous showed comparable antimicrobial activity towards both Gram positive and Gram negative organisms and fungi. *Saraca asoca caesalpiniaceae* methanol bark extract shows maximum zone of inhibition with MIC (5 mg/ml) against *E. coli* (15.0mm) followed by acetone extract (15.5mm, 5mg/ml). Methanolic bark extract of *Saraca asoca leguminosae* shows antimicrobial activity with MIC (10mg/ml) against *E. coli* (8.5mm). Acetone bark extract of *Saraca asoca leguminosae* shows zone of inhibition with MIC (30 mg/ml) against *B. subtilis* (8.5mm) and shows zone of inhibition against *E.coli.* (8.5mm, 30mg/ml).The minimum inhibitory concentration of plant species are summarized in Table 2.

Table 2 Minimum inhibitory concentration determination of *Saraca asoca caesalpiniaceae* and *Saraca asoca leguminosae* bark extracts by four fold serial dilution method.

Extracts	Bacteria tested	MIC values are in mg/ml Bark extracts (ZOI in mm)							
		Saraca asoca caesalpiniaceae				***Saraca asoca leguminosae***			
		30mg/ml	**20mg/ml**	**10mg/ml**	**5mg/ml**	**30mg/ml**	**20mg/ml**	**10mg/ml**	**5mg/ml**
Methanol	***E .coli***	29.0	25.0	20.0	15.0	14.5	10.5	8.5	NA
Acetone		24.5	24.0	23.0	15.5	11.6	10.0	9.0	NA
Methanol	***B. subtilis***	30.0	26.0	21.5	9.5	10.0	8.5	NA	NA
Acetone		28.5	23.0	NA	NA	8.5	NA	NA	NA

ZOI = Zone of inhibition, MIC = minimum inhibitory concentration

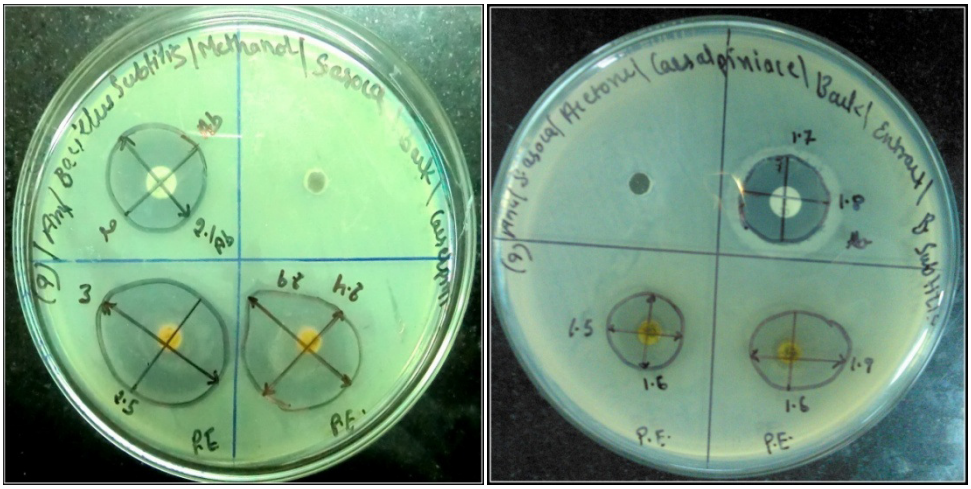

Figure 1 Showing antimicrobial activity of *Saraca asoca caesalpiniaceae* methanolic and acetone bark against *B. subtilis* (MTCC 1220)

Antimicrobial analysis of *Saraca asoca* bark against two bacterial strains *(B. subtilis and E. coli)* was tested. The zone of inhibition (36.2 ± 0.2mm) was largest when methanolic bark extract of *Saraca asoca caesalpiniaceae* (100mg/ml) was used against *E. coli* (Fig 1) and produced zone of inhibition against *B. subtilis* (26.6±0.4) (Fig. 2). Acetone bark extract of *Saraca asoca caesalpiniaceae* (100mg/ml) produced largest zone of inhibition against *E. coli* (34.2 ± 0.4mm) (Fig 1) and produced zone of inhibition against *B.subtilis* (17.0±0.3mm) (Fig 2).

The methanolic bark extract of *Saraca asoca leguminosae* (100µg/ml) shows significant zone of inhibition against *B. subtilis* (15.9±0.6mm) (Fig. 4) as compared to the *E. coli* (10.9 ± 0.0mm). Similarly, but lesser to the methanolic bark extract, acetone bark extract of *Saraca asoca leguminosae* (100mg/ml) shows significant zone of inhibition against *B. subtilis* (11.6±0.0mm) (Fig. 4) as compared to the *E. coli* (9.5 ± 0.2mm).

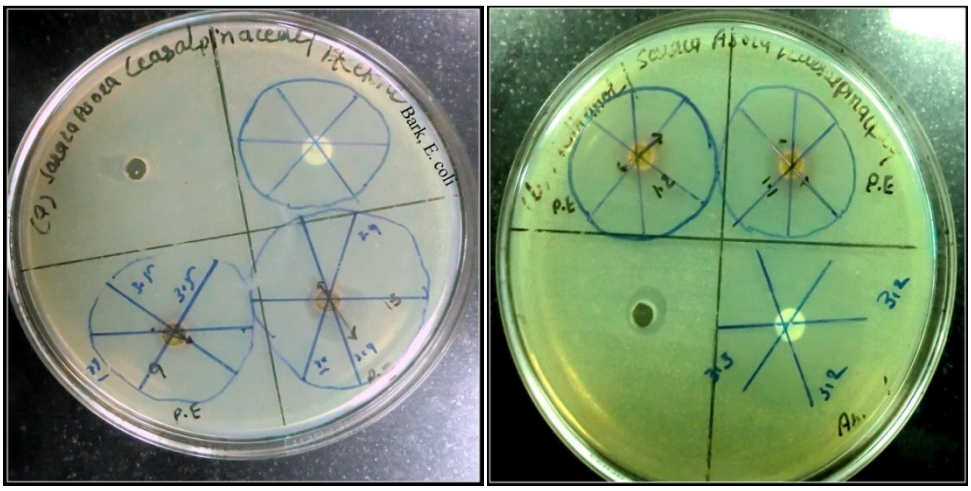

Figure 2 Showing antimicrobial activity of *Saraca asoca caesalpiniaceae* acetone and methanol (bark) extract against *E. coli (*MTCC120)

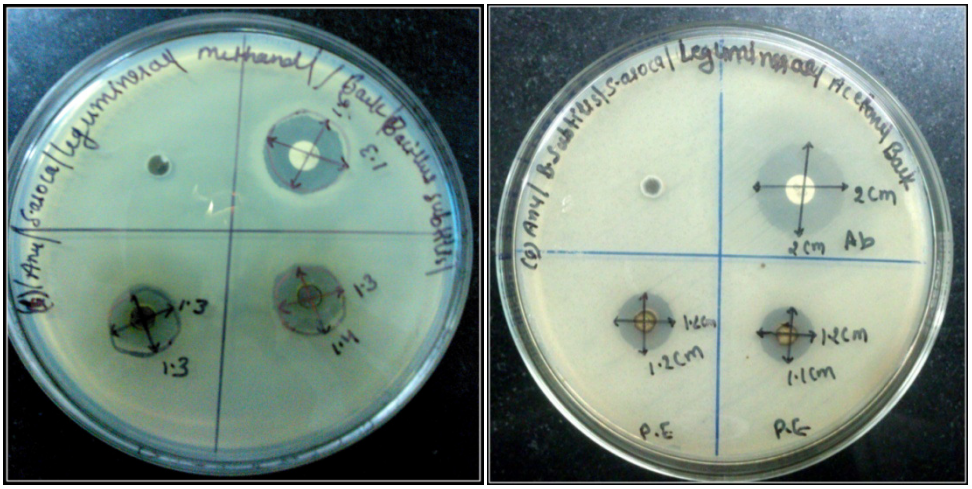

Figure 3 Showing antimicrobial activity of *Saraca asoca leguminosae* acetone and methanol bark extract against *B. subtilis* (MTCC121)

The antimicrobial activity of the plant is due to the presence of several phytochemical constituents like alkaloids, flavonoids, saponins, glycosides, steroids. The phytochemical constituents of the selected plants investigated are summarized in Table 3. Preeti *et al* .(2012 reported that the methanolic extract was relatively more potent against *Staphylococcus aureus* this may be due to the presence of steroids. The methanolic extract was effective against different strains of bacteria which may be due to the presence of flavonoids, glycosides, saponins and steroids. Preliminary phytochemical studies revealed the presence of flavonoids, glycosides, tannins and saponins which were present in the extracts. Preliminary phytochemical screening of the methanol extract showed presence of flavonoids, saponins, tannins, and glycosides. Tannins have been found to form irreversible complexes with proline rich protein resulting in the inhibition of cell protein synthesis. So, the herbs and medicinal plants that have tannins as their main components are astringent in nature and are used for treating intestinal disorders such as diarrhoea and dysentery. (Singh and Thalwal, 2013). By the quantitative analysis it has been concluded that the concentration of flavonoids occurs more in *Saraca asoca caesalpiniaceae* bark extract 438µg/ml as compare to the *Saraca asoca leguminosae* bark extract (182.4µg/ml). The total concentration of phenolic compound present in *Saraca asoca caesalpiniaceae* bark extract is 1.239µg/ml and phenolic compound present in *Saraca asoca leguminosae* bark extract is 1.129µg/ml.

Table 3 Phytochemical screening of aqueous, acetone and methanol extracts of *Saraca asoca caesalpiniaceae* and *Saraca asoca leguminosae* bark extracts.

S. No.	Compound	Bark extracts					
		Saraca asoca caesalpiniaceae			*Saraca asoca leguminosae*		
		Aqueous	Acetone	Methanol	Aqueous	Acetone	Methanol
1.	Tannins	+	+	+	-	+	+
2.	Flavonoids	-	+	+	-	+	+
3.	Phenolics	+	+	+	-	+	+
4.	Glycosides	+	-	+	+	-	_
5.	Saponins (foam test)		+			+	

+, = Positive (presence) -, = Negative (absence)

From the work performed, it is concluded that the bark extracts (methanol, acetone and aqueous) both the plant species i.e. *Saraca asoca caesalpiniaceae and Saraca asoca leguminosae* has potential activity against tested bacterial strains (*Escherichia coli and Bacillus subtilis*). Phytochemical analysis of the plant shows the presence of phytochemical components (glycosides, phenolics, tannins, saponins and flavonoids. Therefore the active phytochemical component of *Saraca asoca* bark could be of interest for further development of medicinal products.. Antimicrobial components present in the *Saraca asoca caesalpiniaceae* and *Saraca asoca leguminosae* may be of great use for the development of pharmaceutical industries as a therapy against various diseases. The results of the antimicrobial study support the development of new antimicrobial drugs from both the plant species *(Saraca asoca caesalpiniaceae* and *Saraca asoca leguminosae).*

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