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RESEARCH ARTICLE

Evaluation of antioxidant activity, total phenolic content, total flavonoids, and LC-MS characterization of *Saraca asoca* (Roxb.) De.Wilde

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Abstract

Antioxidant potential, total phenolic and total flavonoid content of *Saraca asoca* (Roxb.) De.Wilde leaf and bark was determined. Phenolic characterization of the extracts was carried out by Liquid Chromatography-Mass Spectrometry (LC-MS). Bark and leaves were suspended in four different solvent systems viz: methanol, ethanol, distilled water and ethyl acetate. The LC-MS characterization detected the presence of gallic acid, catechin and quercetin in considerable amounts, while other compounds like tannic acid, vanillin, catechol, p-coumaric acid and caffeic acid were present in traces. 2, 2 diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Superoxide radical scavenging activity (SO) and Ferric reducing antioxidant power (FRAP) was used to determine the antioxidant activity. Distilled water leaves and methanol bark showed highest phenolic content, whereas ethanol leaves and methanol bark showed maximum flavonoid content. Methanol leaves and bark showed maximum DPPH and SO radical scavenging activity, ethanol leaves and distilled water bark exhibited highest ABTS activity and ethanol leaves and bark showed maximum FRAP reducing assay activity. The present study is the first comparative analysis between the leaf and bark of the different solvent system in *Saraca asoca*.

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INTRODUCTION

Saraca asoca (Roxb.) De.Wilde belongs to the family Caesalpiniaceae which is an evergreen tree most commonly known as Ashoka and it is a native to Indian subcontinent regions. It is found densely in Central and Eastern Himalayas, Kerala and the Western Ghats of Maharashtra. Saraca grows in rain forest regions up to an elevation of 750m and is distributed in the central areas of the Deccan plateau.

Ayurveda and Unani practitioners regarded it as a miracle tree for women because its medicinal properties solved many gynaecological issues (Panchawat and Sisodia, 2010). Bark of *Saraca asoca* had profound antimicrobial activity against a wide range of bacterial pathogenic organisms (Seetharam et al. 2003). Its flowers gave relief from uterine toxicity, improper digestion, stomach pain, constipation, hemorrhagic dysentery, and diabetes (Pal et al. 2014). Flower and leaves of the plant were used in fever, dipsia, colic, ulcers and pimples (Pal et al. 2014). Ashokarishta is an ayurvedic preparation from *Saraca asoca* to cure various diseases in women. It reduces excessive

bleeding, leucorrhea and headache (Mathew et al. 2005). *Saraca asoca* has being investigated on a large scale by researchers for its anti-inflammatory and analgesic properties (Debnath et al. 2010; Rathee et al. 2010) antipyretic activity (Debnath et al. 2010), chemopreventive activity (Cibin et al. 2010) and molluscicidal activity (Singh et al. 2009). The anthelmintic activities of ethanolic and methanolic extracts of bark were also reported due to the presence of phytochemical constituent such as glycosides, alkaloids, tannin, flavonoids and terpenoids (Nayak et al. 2011). Gallic acid is one of the major compound in antimutagenic and antigenotoxic properties in *Saraca asoca* bark extract (Nag et al. 2013).

Saraca asoca gritha commercial formulation is useful in treating diseases such as leucorrhoea, anorexia, general weakness, less lactation and other gynecological problems (India Abundance, 2014), Femicare tablets are useful for the treatment of amenorrheae, oligomenorrheae, pre-menstrual syndrome, menstrual disorders (eVaidyaji, 2014). *Saraca asoca* was used as a uterine tonic and also in the management of burning sensation, piles and inflammation and thus its anti-inflammatory and analgesic properties were tested and it was observed that the methanolic extract of *Saraca asoca* exhibited significant analgesic and anti-inflammatory action (Debnath et al. 2010; Rathee et al. 2010). Some studies have also reported the evaluation of antipyretic activity of methanolic extracts of Saraca using brewer's yeast induced hyper pyrexia method in Wister albino rats (Sasmal et al. 2012; Varaprasad et al. 2011).

Saraca asoca though it has been extensively used in ayurveda and other medicinal uses, its basic characteristic and antioxidant property of all plant parts are still unreported. Hence in the present study leaf and bark of *Saraca asoca* were subjected to determine the total phenolic content, flavonoids, antioxidant activity and LC-MS in four different solvent systems which consisted of methanol, ethanol, distilled water and ethyl acetate, in order to characterize the underlying biochemical properties that impart such high medicinal values to this plant.

2. Materials and methods

2.1. Plant material

The plant materials such as leaves and bark samples were collected from non-polluted areas of Borivali, Mumbai, Maharashtra (19º 15'N 72º 55'E), India. The harvested materials were air dried completely in a well ventilated and sterilized room.

2.2. Extraction of sample

Extraction of samples was performed in a similar way as described by Chaturvedi et al. 2011. The dried leaf and bark tissues of the plant were crushed into fine powder using liquid nitrogen. Five grams of sample were suspended in 50 ml of four different solvent systems that are distilled water, methanol, ethanol and ethyl acetate respectively followed by overnight extraction. Extracts were filtered using Whatman No.1 paper and the filtrates were concentrated to 10 ml by using rotary evaporator at 40°C. Extracts were resuspended in each extracting solvent to achieve a stock solution of 100 mg/ml.

2.3. Determination of total phenolic content (TPC)

Total phenolic content was determined using Folin-Ciocalteu reagent as described by Ghatak et al. 2014. The reaction mixture consist of solvent extract mixed with FC reagent, after 10 min incubation in dark, sodium carbonate (Na_2CO_3) was added to the mixture which was followed by 90 min incubation at 30°C. Phenolic content of these samples was determined spectrophotometrically using a UV visible spectrophotometer at 760 nm. The standard curve was prepared using gallic acid (0.1mg/mL) as standard.

2.4. Determination of total flavonoid content (TFC)

The total flavonoid content was determined as per Pai et al. 2015. The plant extracts (500µl) was added to 2% aluminium chloride ($AlCl_3$) solution in methanol (500µl) and incubated at 30°C for 10 min. Readings were obtained at 368nm in UV-visible spectrophotometer. The standard curve was prepared considering quercitin as standard compound.

2.5. Determination of DPPH radical scavenging activity

Radical scavenging activity was performed as per Ghatak et al. 2014, where 0.3mM DPPH solution was prepared in methanol, of which 0.5mL of this solution was mixed with 100µL of bark and leaf extracts. The mixture was kept in dark at 37˚C for 30 min. The absorbance was measured spectrophotometrically at 517nm. The ability to scavenge DPPH radical was calculated using following formula:

$$\%\text{Inhibition} = \frac{\text{Absorbance of the control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.6. Superoxide (SO) radical scavenging activity

The superoxide radical scavenging assay was performed as per Mandal et al. 2009, where 1 mL of extract was added to a mix of 0.5mL (0.3mM) Nitro blue tetrazolium (NBT), Nicotinamide adenine dinucleotide (NaDH) (0.936mM), TrisHCl Buffer (16mM) (pH-8.0) and Phenazinemethosulphate (PMS) (0.12 mM). The mixture was left for incubation at 25ºC for 5 min. Followed by absorbance measurement at 560nm spectrophotometrically. Gallic acid was used as a standard. The super-oxide scavenging activity was calculated using the formula:

$$\%\text{Inhibition} = \frac{\text{Absorbance of the control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.7. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out according to the method of Jain et al. 2014. FRAP reagent was prepared using acetate buffer (1.6g sodium acetate and 8 ml acetic acid make up to 100mL) (pH 3.6), 10 mM 2,4,6-Tripyridyl-s-Triazine (TPTZ) solution in 40 mM HCl and 20 mM ferric chloride solution in proportion of 10:1:1 (v/v) respectively. The FRAP reagent was prepared fresh and was warmed to 37ºC in oven prior to use. In total of 100µL extracted samples were added to 3 mL of the FRAP reagent and mixed well. The absorbance was measured at 593 nm using spectrophotometer at 0 min and after 4 min. Standard curve of ascorbic acid was prepared. FRAP reagent was used as a blank for both standard and samples.

FRAP value of sample was obtained using the formula:

$$\%\text{Inhibition} = \frac{\text{Change in absorbance of sample from 0 to 4 mins}}{\text{Change in absorbance of standard from 0 to 4 mins}} * 1000\text{uM}$$

2.8. ABTS radical scavenging assay

ABTS assay was carried out using the method described by Pai et al. 2015 where stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate/ ammonium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12h at 30ºC in the dark. The solution was then diluted by mixing 1 mL ABTS solution with 60 ml methanol to obtain an OD of 0.706±0.001 at 734 nm using the spectrophotometer. Plant extracts (1 mL) were allowed to react with 1 mL of the ABTS solution and the OD was taken at 734 nm after 7 min using the spectrophotometer. All the readings were taken in triplicates. The ABTS scavenging capacity of the extract was calculated as:

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{ABTScontrol - ABTSsample}}{\text{ABTS Control}} \times 100$$

ABTS control is the absorbance of ABTS radical + methanol;

ABTS sample is the absorbance of ABTS radical + sample extract/standard.

2.9. LC-MS characterization

Liquid Chromatography-Mass Spectrometry (LC-MS) for the determination of phenolic compounds in plant extracts were carried out at Reliable Analytical Laboratories, Mumbai, Maharashtra, using Agilent Technologies 6460 triple quadrupole LC/MS. Samples were centrifuged at 12,000 rpm for 10 min before analysis. The HPLC system consisted of two pumps and an automated injector. Separation was achieved on a C-18 column (Agilent Eclipse, 5 µ, 15 cm, 4.6 mm id). Two mobile phases used were: 0.1 % formic acid in water and acetonitrile (Pai et al. 2015).

2.10. Statistical analysis

All the observations were taken in triplicate and the data was presented as ± standard deviation (SD). Analysis of Variance (one way ANOVA) was further performed using SPSS software (version 14.0). Correlation coefficient was calculated using Pearson Product Moment Correlation. A probability of $p < 0.01$ was considered to be statistically significant and correlation coefficient (r) values were determined (Ghatak et al. 2014).

3. Results and Discussion

3.1. Estimation of total phenolic content

Total phenolic content was determined using Folin-Ciocalteau reagent. From the analysis it was observed that distilled water extracts of leaf (175.16±2.3 mg/ml) and methanol extract of bark showed highest phenolic content (119±0.87 mg/ml) (Table 1). The phenolic content decreased in the order of methanol> ethanol> distilled water>

ethyl acetate in the samples of *Saraca asoca*. Hence it was observed that methanol is a good solvent for the extraction of phenols and these results are in agreement with the study performed by (Chaturvedi et al. 2011; Formagio et al. 2014) where methanolic extracts showed highest total phenolic content. Furthermore similar results were also observed in *Mitragyna parvifolia* leaf and bark extracts (Ghatak et al. 2014). The phenolic compounds are very essential secondary metabolites in plant tissues which differ in their concentration during different stages in plant development (Gayathri et al. 2013). Hence phenolic compounds are considered to play very vital roles in plants such as nutrient uptake, photosynthesis, protein synthesis etc (Goleniowski et al. 2013). Usually most of the phenolic compounds are linked to other compounds like esters, cellulose, proteins and lignins (Sadhu et al. 2007). Hence these are very important compounds because their hydroxyl groups confer scavenging ability.

3.2. Estimation of total flavonoid content

Ethanolic extract of leaf (11.33±0.41mg/ml) and methanolic extract of bark (4.62±0.15mg/ml) exhibited highest flavonoid content (Table 2). The results obtained were similar to that in *Mitragyana parvifolia*, wherein the methanolic extracts showed highest flavonoid content (Ghatak et al. 2014). The present investigation results indicate that flavonoid content was mostly abundant in the leaf of *Saraca asoca*, as compared to the bark. Flavonoids occur naturally in plants which not only have positive effect on human health but also possess antibacterial, antiviral and anti-inflammatory, anticancer, and anti-allergic activities (Saeed et al. 2012). Flavonoid also show strong antioxidant activity, hence in our study we observed a strong positive correlation between total flavonoid content and DPPH radical scavenging activity.

3.3. Estimation of DPPH radical scavenging activity

Electron donation capacity of natural products can be easily measured by 2,2-diphenyl-1- picrylhydrazyl radical (DPPH) purple-colour solution (Saeed et al. 2012). The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of colour change can be proportional to the concentration and potency of the antioxidants (Saeed et al. 2012). In the present study DPPH radical scavenging activity was highest in methanolic extract (94.4±1.2%) of the leaf and ethanolic extract of bark (82.06±0.75%) (Figure 1). Distilled water and ethyl acetate extracts showed less antioxidant activity as compared to other solvents. In comparision to *Helicteres isora* (L.) in which maximum DPPH activity was 90% in methanolic extract of leaves (Jain et al. 2014) where as 88% DPPH activity was observed in *Citrullus colocynthis* (Kumar et al. 2008). Higher phenol and flavonoid content in the plant tissue can also lead to increase in antioxidant activity (Ghatak et al. 2014).

3.4. Superoxide anion radical scavenging

Superoxide radical is one of the major biological sources of ROS (Saeed et al. 2012). Although it is a weak oxidant, but it gives raise to powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Saeed et al. 2012). In the present study methanol leaf and bark extract (37.33±1.8%, 30.4±3%) showed maximum anion radical scavenging activity respectively (Figure 2). Superoxide radical scavenging activity decreases in the order of methanol > ethanol > D/W > ethyl acetate. The above results indicate that maximum scavenging activity was observed in the methanol extract of the plant. The obtained results are in complete agreement with the study performed by (Saeed et al. 2012).

3.5. Ferric reducing antioxidant activity

FRAP assay measures the amount of antioxidants based on its ability to reduce Fe^{3+} to Fe^{2+}. Highest FRAP value was estimated in ethanol leaf (1.92±0.4%) and bark extracts (2.83±0.8%) respectively (Figure 3). FRAP values decreased in order of ethanol > methanol > D/W > ethyl acetate (Figure 3). *Saraca indica* bark extracts revealed that ethanol as the best solvent for ferric reducing activity (Gayathri et al. 2013). *Helicteres isora* (L.) exhibited similar results in leaf and fruit extracts (Jain et al. 2014; Polani et al. 2013).

3.6. ABTS scavenging activity

The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, its reduction in the presence of hydrogen-donating antioxidants is measured spectrophotometrically at 734 nm (Saeed et al. 2012). The ABTS scavenging activity is highest in ethanol leaves (89.41±1.5%) and distilled water bark (91.9±1.7%) (Figure 4). The antioxidant activity was observed the lowest in ethyl acetate samples. These results were similar to the study performed by Kumaraswamy et al. 2008, wherein it was reported that aqueous extracts of *Thespesia lampas* gave significant ABTS scavenging activity as in ethanolic extracts.

3.7. LC-MS characterization of polyphenols

Phenolic characterization of the samples was performed. Gallic acid, quercetin, catechin, tannic acid, caffeic acid, catechol, rutin, p-coumaric acid, vanillin have been quantified in *Saraca asoca* by LC-MS analysis. Methanolic extract of leaf contains highest amount of quercetin (520.3±1.9 ppb) and rutin (78.3±0.83 ppb) whereas ethyl acetate extract of leaf showed highest amount of catechol (22.97±1.5 ppb) and p-coumaric acid (180.5±4.3 ppb). Bark methanolic extract exhibited highest gallic acid (685.33±0.88 ppb), tannic acid (72.12±2.4 ppb) and caffeic acid (89.19±1.5 ppb) whereas bark ethanol contains highest catechin (630.4±2.4 ppb). Vanillin was highest in bark distilled water (44.02±3.3 ppb) whereas ethyl acetate extract showed traces of tannic acid, gallic acid, caffeic acid, rutin, and vanillin (Table 3). Similar kind of studies was performed by Pai et al. 2015 on Indian beer samples.

3.8. Correlation analysis between phenolic content, flavonoid content, and antioxidant assays

A correlation analysis was performed using Pearson correlation coefficient to check linear correlation among the assays. It was observed that there is a strong positive correlation between total phenol content and DPPH antioxidant capacity ($r=0.99$, $p<0.01$) as well as with SO radical scavenging activity ($r=0.98$, $p<0.01$). Furthermore strong negative correlation was observed between total phenol content and ABTS scavenging activity ($r= -0.91$). Interestingly we observed negative correlation between total phenol content and total flavonoid content ($r= -0.88$) and there was also negative correlation between total flavonoid and ABTS radical scavenging assay($r= -0.87$, $p<0.001$) (Table 4). Similar correlation was done by Ghatak et al. 2014 in *Mitragyana parvifolia* leaves and bark extracts.

Figure 1. Percent DPPH radical scavenging activity of leaf and bark extracts in *Saraca asoca*

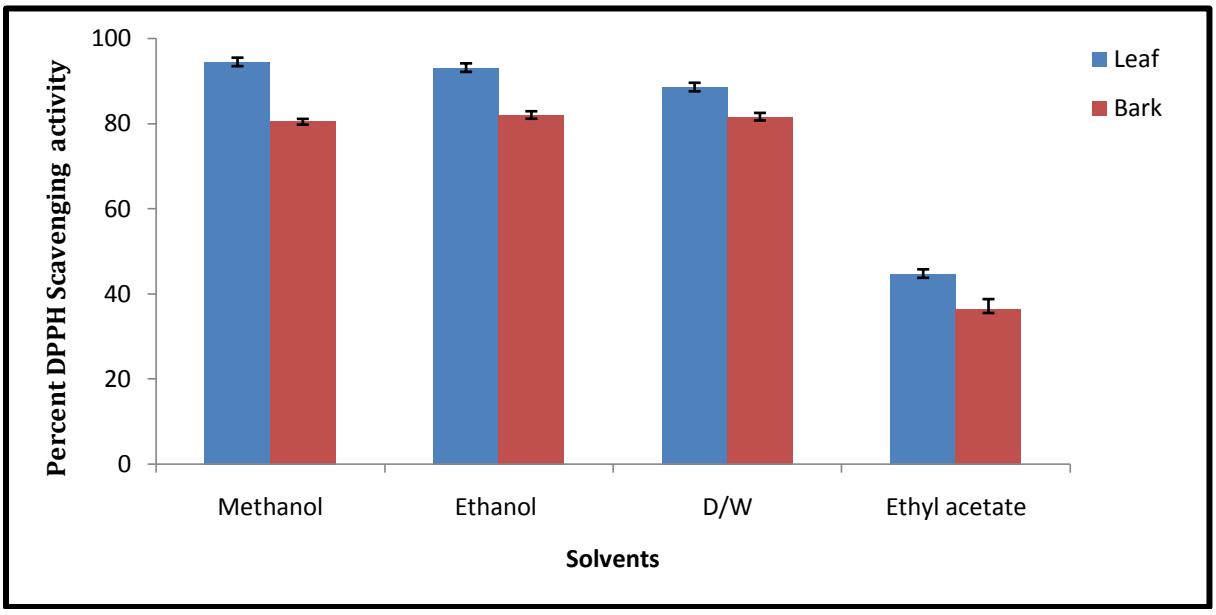

Figure 2. Percent Superoxide (SO) radical scavenging activity of leaf and bark extracts in *Saraca asoca*

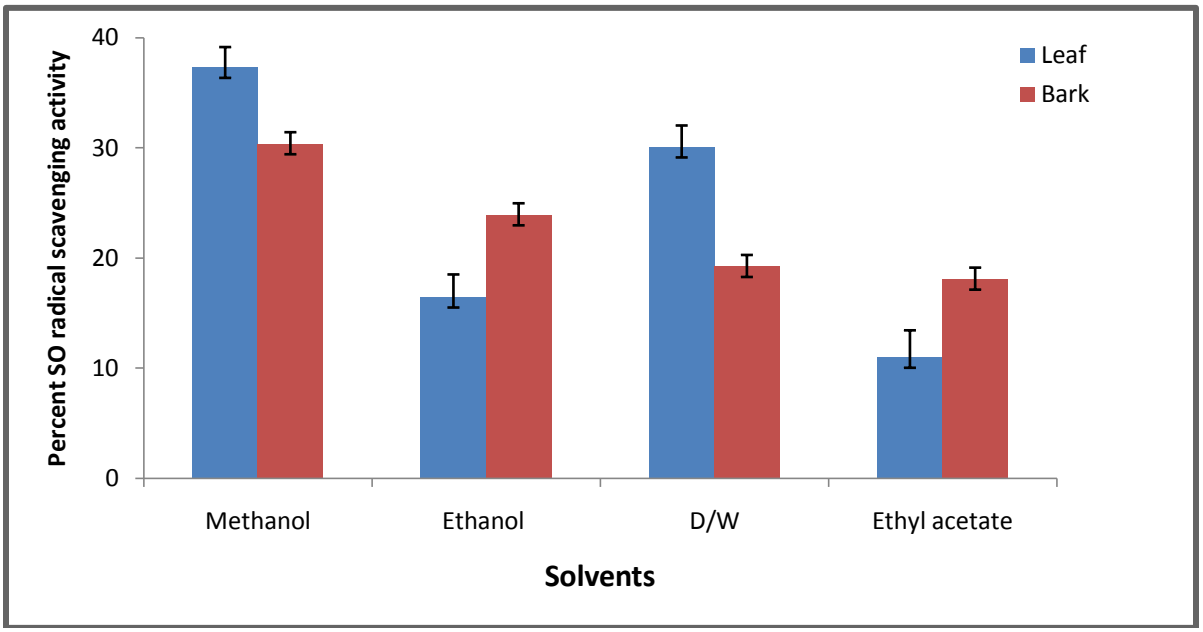

Figure 3. Percent FRAP antioxidant assay of leaf and bark extracts in *Saraca asoca*

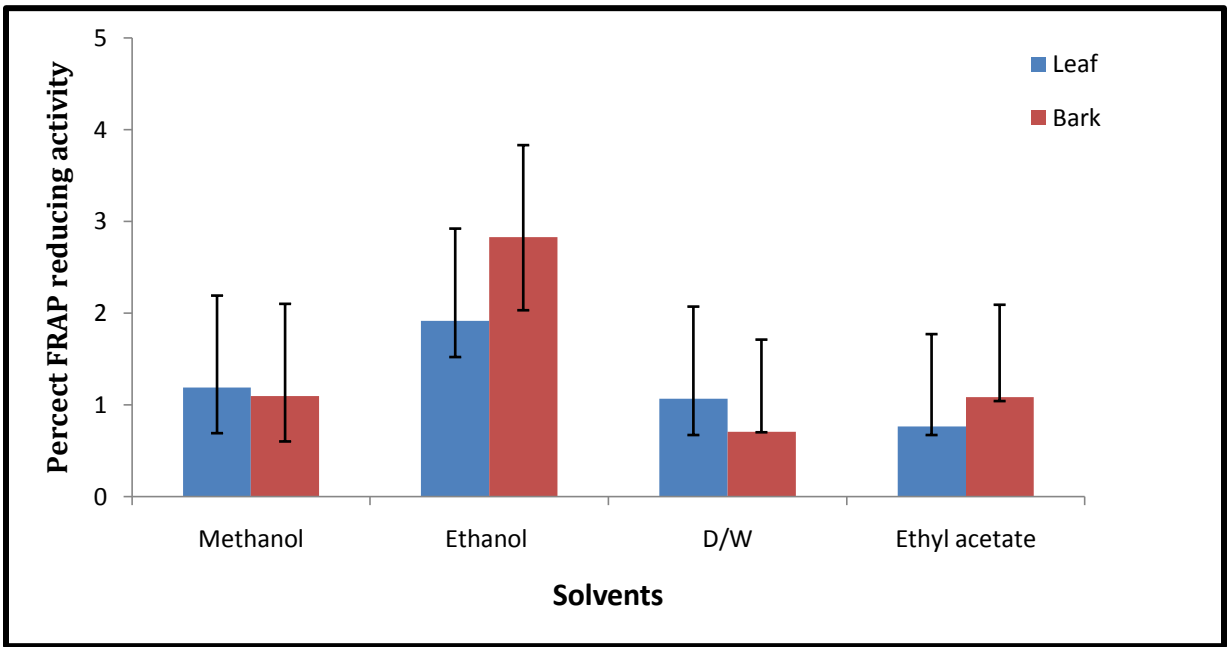

Figure 4. Percent ABTS radical scavenging activity of leaf and bark extracts in *Saraca asoca*

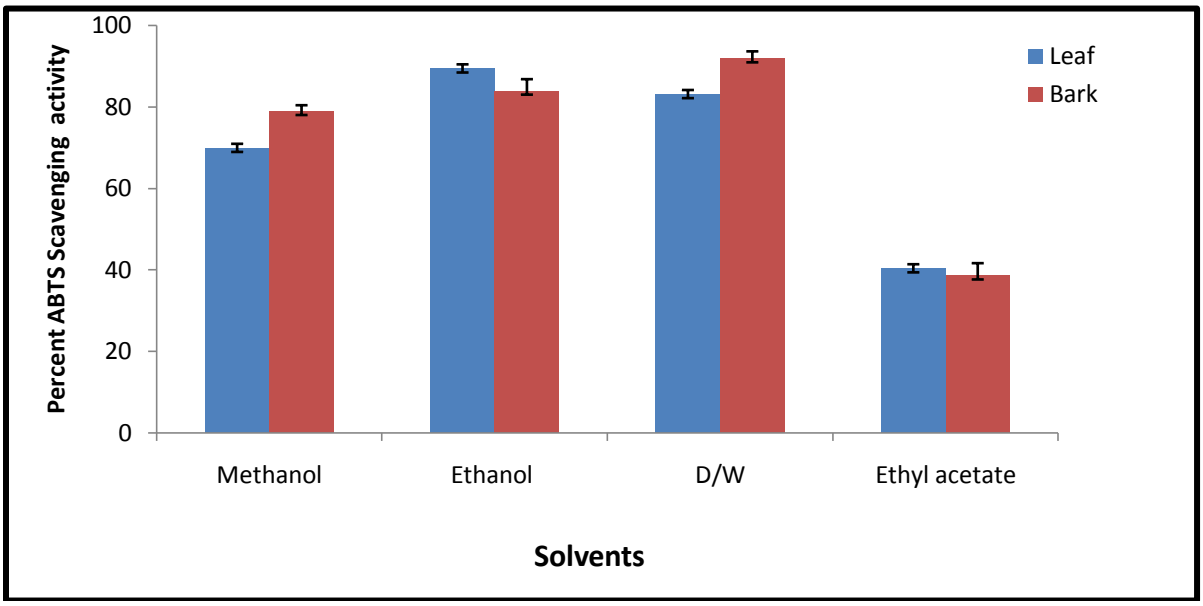

Table1. Total phenolic content (mg/ml) in leaf and bark of *Saraca asoca*

Parts of Plant	Methanol	Ethanol	D/W	Ethyl acetate
Leaf	159.23±0.58	156±1	175.16±2.3	29.33±0.75
Bark	119±0.87	104.93±1.01	69.46±0.61	23.5±0.5

Values represent mean ± standard deviation (SD)

Table 2.Total flavonoid content (mg/ml) in leaf and bark of *Saraca asoca*

Parts of plant	Methanol	Ethanol	D/W	Ethyl acetate
Leaf	5.75±0.46	11.33±0.41	6.05±0.16	2.06±0.25
Bark	4.62±0.15	3.36±0.61	2.32±0.12	1.16±0.15

Values represent mean ± standard deviation (SD)

Table 3.**Phenolic characterization of leaf and bark samples in *Saraca asoca* by LC-MS**

Parts of plant samples	Gallicacid (ppb)	Quercetin (ppb)	Catechin (ppb)	Tannicacid (ppb)	Caffeicacid (ppb)	Catechol (ppb)	Rutin (ppb)	p-coumaric acid (ppb)	Vanillin (ppb)
Leaf(MeOH)	230.2±2.4	520.3±1.9	67.23±0.53	9.33±2.7	18.47±0.11	2.33±0.18	78.3±0.83	2.7±2014	5.94±0.88
Leaf (EtOH)	15.59±0.3	7.4± 2.35	507.48±1.7	12.11±0.3	---	18.57±1.2	10.33±0.2	2.09±0.7	7.30±1.2
Leaf (D/W)	438.84±1.3	28.94±1.5	272.62±3.4	45.25±2.7	0.5±0.12	17.37±3.3	5.83±2.1	0.94±0.8	12.07±0.3
Leaf (EA)	18.37±0.23	---	9.33± 0.4	---	---	22.97±1.5	---	180.5±4.3	0.54±0.1
Bark(MeOH)	685.33±0.88	93.45±0.32	380.23±3.1	72.12±2.4	89.19±1.5	5.33±0.88	18.67±1.5	31.1±1.4	5.33±1.3
Bark (EtOH)	243.53±1.7	14.13±0.88	630.40±2.4	46.53±3.2	---	18.57±0.87	10.33±1.4	2.09±1.6	7.30±0.6
Bark (D/W)	---	20.19±1.5	44.17±1.2	46.53±2.2	---	2.7±0.77	0.82±0.01	0.47±0.03	44.02±3.3
Bark (EA)	11.12±0.27	---	---	45.12±3.8	18.13±2.4	---	34.5±1.8	---	7.8±0.67

Values represent mean ± standard deviation (SD)
ppb - parts per billion
MeOH-Methanol, EtOH-Ethanol, D/W-Distilled water, EA-Ethyl acetate

Table 4.**Correlation between total phenolic content, total flavonoid content, and antioxidant assays**

Comparison n=6	TPC	TFC	DPPH	SO	ABTS
TPC	1	-0.88	0.99*	0.98*	-0.91
TFC	--	1	0.87*	0.86	-0.87*
DPPH	--	--	1	0.91	-0.98*
SO	--	--	--	1	-0.78
ABTS	--	--	--	--	1

*Indicates significance at $p<0.01$, n = number of samples in triplicate (considering extract of bark and leaves). TPC = Total phenol content, TFC = Total flavonoid content, SO = Superoxide, DPPH = Antioxidant activity, ABTS = Antioxidant activity

4. Conclusion

Saraca asoca plant has been extensively used in ayurveda and other medicinal uses. Though its plant parts have been used extensively for the traditional and commercial medicinal formulations but its biochemical properties were very much unknown. In the present study most of its biochemical aspects are evaluated. In summary, the bark of *Saraca asoca* may be considered as a good source of phenols and natural antioxidants as compared to leaves. Further investigation also lead us to conclude that methanol is the best solvent system for the extraction of phenols and flavonoids compare to other solvent systems. The study requires further investigation in phytochemical properties, bioactive compounds, and neutraceutical values present in the plant parts.

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Author Contributions
A.G. and N.D. conceived and designed the experiment; A.G., S.N. and S.K. collected the plant samples; S.N., A.V., S.S. and K.S. performed the experiment; S.K. did the LC-MS analysis of the data; A.G., P.C. and N.D analysed the data. All authors read and approved the final manuscript.

Conflict of Interest
The authors declare that they have no competing interests.

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