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Extraction, quantification and antioxidant activities of flavonoids, polyphenols and pinitol from wild and cultivated *Saraca asoca* bark using RP-HPLC-PDA-RI method

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ABSTRACT

Saraca asoca (Roxb.) De Wilde Syn. *Saraca indica* Linn belonging to the family *Leguminosae*, is one of the ancient medicinal plant of India and its bark is used in Ayurvedic preparations. In the present work, the barks were collected from mature trees of different geographical locations. Besides, one of the best chemotype is cultivated under different fertilizer treatments and the barks from 4 year old plants were taken up for analytical studies. The barks were extracted with methanol as well as aqueous-methanol solvent systems, and chemical compositions were compared using RP-HPLC-PDA-RI analysis. Different classes of compounds viz. pinitol, flavonols and polyphenols were detected through the analytical protocol developed by our laboratory. It was found that the barks collected from Uttar Pradesh (BLA, BLK), Sirsi, Karnataka (BSS) and Empress Garden, Pune (BEG) gave higher yield of the extracts and were more enriched with the above classes of compounds. For getting barks in a short period of time, the BSS plant saplings were experimentally cultivated under different fertilizer treatments. It was found that the plants treated with a combination of fertilizers such as poultry waste + biofertlizer, vermicompost + biofertlizer and farm yard manure + biofertlizer + diammonium phosphate yielded reasonably higher amounts of extracts, which were chemically close with the parent bark extracts. Among these treatments, the vermicompost + biofertlizer gave pinitol (729 mg), flavonoids (2872 mg) and polyphenols (1150 mg) in 100 g bark extracts. The antioxidant activities were evaluated by using 1,1-diphenyl-2-picrylhydrazyl radical-scavenging assay and interestingly it was noticed that the extracts contained improved percentage of flavonoids and pinitol showing better activity.

1. Introduction

Saraca asoca (Roxb.) De Wilde Syn. *Saraca indica* Linn, locally named as Ashoka belongs to the family *Leguminosae* (Anon., 2006; Hattori et al., 1995). It is distributed in India, Bangladesh, China, Pakistan and Malaysia (Sainath et al., 2009; Sadhu et al., 2007). This plant is very well known for its medicinal values in the Indian traditional medicine system and Ayurvedic preparation. In some of the Indian treatise like Charak Samhita and Bhavprakasha Nighantu, this plant has been recommended for various gynecological disorders. It is extensively used in the pharmaceutical preparations like asokarishta and asokagirtha. Most of this plant parts like stem, bark, flowers, leaves and fruits are used in Ayurveda for their medicinal properties. The barks are used for the treatment of menorrhagia, uterine infections, rheumatic arthritis, hemorrhoids, dysmenorrhoea and bacterial infections (Misra, 2013; Pradhan et al., 2009; Mohod et al., 2014). The bark extracts of *S. asoca* have been evaluated for several biological activities like anti-inflammatory, antimicrobial, anti-cancer, anti-tumour, anti-bacterial, anti-progestational, anti-estrogenic locomotor and antioxidant activities (Pradhan et al., 2009; Satyavati et al., 1970; Saravanan et al., 2011; Mukhopadhyay and Nath, 2011; Saha et al., 2013; Yadav et al., 2013). The bark extract is rich in active ingredients (Mukherjee et al., 2012; Sadhu et al., 2007). In our systematic analytical study, it has been found that the bark extract contains

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a potent anti-diabetic and anti-inflammatory cyclitol, generally known as pinitol (Ahmad et al., 2016a). Previously, our group has reported the *n*-hexane, methanol and 60% aqueous-methanol extracts of bark and characterized the compounds using HPLC analysis. In this work, we had reported total 17 compounds belonging to the classes of flavonoids, polyphenols and pinitol (Ahmad et al., 2016a,b).

The bark of this plant is used in the Ayurvedic and modern herbal medicine systems with profound application for curing the complex gynecological diseases. It is found throughout India (Ahmad et al., 2016a), especially in Konkan, Deccan, Karnataka, Maharashtra, central and north India, and eastern Himalayas up to an altitude of 750 m. Remarkably, there are several medicinal formulations using this bark as one of the ingredients and it is available commercially by major ayurvedic/herbal pharmaceutical companies (Singh et al., 2015). The demand of this bark powder by Indian pharmaceutical companies was more than 5000 t in the year 2000 and it was about 11000 t in 2011. It is estimated that *S. asoca* bark demand is increasing with an annual growth rate of 15% (Anon., 2004). In the current herbal marketing there happens to be mixing of adulterants with authentic *S. asoca* bark due to the shortage of this plant material. In the present study, the chemical variations in the barks collected from different geographical locations are analyzed. Due to its slow growing behavior, this plant takes several years for getting the bark material profitably. Therefore, present work is focused on different fertilizer treatment of best plant selected on the basis of higher extracts along with enriched bioactive compounds in the extracts. The barks of these experimental plants were harvested in a span of 4 years to assess the quality.

There are many disorders appearing in humans due to the free radical generation including arthritis, atherosclerosis, ischemia and reperfusion injury, central nervous system injury, cancer, gastritis and AIDS (Pourmorad et al., 2006; Singh et al., 2015). The aqueous extract of *S. asoca* bark inhibited protease activity of Human Immuno Deficiency virus type 1 (HIV-1) (Kusumoto et al., 1995). Further, AIDS patients easily get infected by herpes simplex virus (HSV) and it was validated that the bark extract showed potent inhibitory *in vivo* and *in vitro* activity against HSV-1 at a concentration of 100 μg/ml (Hattori et al., 1995). Due to the suppressed immune system, the consumption of natural antioxidants to control the free radical scavengers might be useful against complex health related issues. Presently, the synthetic antioxidants such as butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), tertiary butylated hydroquinone and gallic acid esters have associated with a number of health related issues (Pourmorad et al., 2006). Therefore, it is recommended for replacing the above synthetic antioxidant with natural products. Flavonoids are anti-inflammatory in nature to inhibit free radical scavenging of hydrolytic and oxidative enzymes, thus act as natural antioxidant. The phenolic compounds in the natural products have potential antioxidant activity (Pourmorad et al., 2006). Generally, antioxidant activity is related to the biological actions of flavonoids and polyphenols (Gryglewski, 1987). Keeping these facts in mind, the antioxidant activities of the experimental bark extracts have now been studied using DPPH free radical scavenger assay.

2. Experimental

2.1. Plant material

Randomly selected bark samples from ten years old (approx) wild growing *S. asoca* trees were collected from different parts of India representing various geographical locations such as south, central western and north regions. The plant materials were deposited in the CSIR-CIMAP herbarium for further use. From Northen India, the locations selected for collection of the barks are Lalitpur (Uttar Pradesh) (24.6°N, 78.4°E) and Lucknow (Uttar Pradesh) (26.8°N, 80.9°E) with voucher specimen number (VSN: S020 and S021), respectively. Similarly, the barks collected from central and western India are Empress Garden, Pune, (Maharashtra) (18.5°N, 73.8°E) (VSN: S015); Dapoli, Kudavare, Ratnagiri district, Pune (Maharashtra) (17.7°N, 73.2°E) (VSN: S017); Phansad, Dharangan, Pune (Maharashtra) (18.4°N, 72.9°E) (VSN: S016); Tillari, Kolhapur, (Maharashtra) (15.7°N, 74.1°E) (VSN: S022) and Anand (Gujarat) (22.6°N, 72.9°E) (VSN: S019). The places from southern India where the barks were collected are Sirsi, (Karnataka) (14.6°N, 74.8°E) (VSN: S014) and Kannur, (Kerala) (11.8°N, 75.3°E) (VSN: S018).

The Sirsi, (Karnataka) plant saplings were cultivated in experimental farm, which had undergone treatment with different fertilizers as presented in Table 1. The barks of these trees were harvested after 4 years, and processed further for preparation of the extracts as well as analysis purpose. The fertilizer treatments were carried out in duplicate on separate saplings and data presented were on an average basis.

2.2. Reagents and solutions

A HPLC grade methanol (*Merck, LiChrosolv*) was used for chromatography. Water (*Merck, LiChrosolv*), CF_3COOH (*Merck, LiChrosolv*) and triethylamine (*Merck, LiChrosolv*) were used throughout the analysis. The amounts of flavonoids were calculated from the calibration curve of standards such as (+)-catechin hydrate, (−)-epigallocatechingallate, (−)-gallocatechingallate, (−)-epicatechin, (−)-gallocatechin, (−)-catechin, (−)-epigallocatechin, (−)-epicatechingallate, (−)-catechingallate, tannic acid, lyoniside, pinitol and gallic acid, earlier isolated from *S. asoca* in our laboratory (Ahmad et al., 2016b), and purchased from Sigma Aldrich, Bengaluru.

2.3. Analysis of organic fertilizers

The pH of these organic fertilizers was determined using pH meter. The nitrogen content of farm yard manure (FYM), vermicompost, poultry and biofertilizers were estimated by Kjeldahl titration process. Whereas ICP-MS (PerkinElmer, Optima 5300 V, USA) was used for the analysis of other elements in these samples as per our previous study (Mohanty et al., 2015). Ash content of all samples were determined in laboratory muffle furnace as per ASTM 3174-04 (2004) method. The minerals were extracted from the ash by adding 20 ml of 2.5% HCl and reduced the volume to ~7 ml. For quantification, it was transferred to a 25 ml volumetric flask and make-up the mark with de-ionized water. ICP analysis of elements present in the ash was determined by comparing with standard sample viz. Mg, Ca, P, Mn, Zn, Cd, Co, Cr, K, Na, Ni and Pb using calibration curve with multiple standards. A full scan m/z 10–250 was carried out for quantification study.

2.4. Sample extract preparation

Approximately 400 g of air dried *S. asoca* bark was grinded and kept in air tight poly bag for further use. The grinded bark (40 g) was extracted with methanol and water methanol (3:2) three times each for 24 h at room temperature as per the procedure reported earlier (Ahmad et al., 2016a). The solution was filtered and solvent was evaporated under reduced pressure to give crude extracts of methanol and aqueous-methanol extracts. For tannin removal, methanol and aqueous-methanol bark extracts were followed by the column chromatography packed with Polyamide 6 using methanol as mobile phase. The fractions were collected and solvent was distilled under reduced pressure to obtain tannin free

Table 1
Chemical compositions of *S. asoka* bark collected from different geographical locations and fertilizer treatment of BSS saplings.

Samples code	Treatments	Yield of extracts (g/100 g)		Pinitol (mg)		Flavonoids (mg)		Benzenoids			
								Tannic acid (mg)		Lyoniside (mg)	
		MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME
T1	Control	2.50	1.20	175.0	66.4	251.1	96.6	148.5	26.3	20.2	7.2
T2	Urea (2 g/plant)	3.17	1.36	254.4	88.1	499.0	180.9	116.9	16.2	3.5	0.5
T3	DAP (2 g/plant)	3.78	1.54	375.0	104.4	828.8	381.0	131.5	23.2	8.3	1.5
T4	Sampurna (2 g/plant)	4.71	1.69	476.8	148.9	827.6	351.0	170.1	40.0	16.9	5.7
T5	FYM (20 g/plant)	4.21	1.70	471.1	150.6	1008.7	426.0	160.8	38.4	16.4	5.5
T6	Vermicompost(20 g/plant)	4.27	1.80	526.8	173.5	1826.2	670.3	175.1	44.4	10.2	2.9
T7	Poultry (20 g/plant)	4.28	1.81	544.7	178.4	1572.6	550.4	206.8	59.5	18.2	6.8
T8	Biofertilizer(20 g/plant)	4.22	1.78	492.5	161.2	2176.1	798.3	118.6	29.0	5.9	1.2
T9	FYM + Biofertilizer (20 g/plant)	4.30	1.83	490.5	160.2	2263.8	799.7	116.3	28.4	7.8	3.3
T10	Poultry + Biofertilizer (20 g/plant)	4.72	1.97	555.2	184.9	2602.1	1006.7	150.1	47.9	16.1	6.2
T11	Vermicompost + Biofertilizer(20 g/plant)	4.76	2.02	729.1	205.7	2872.1	1150.8	159.6	50.1	14.2	4.8
T12	FYM + Biofertilizer(19.5 g/plant) + DAP (0.5 g/plant)	4.71	1.99	560.8	188.4	2820.3	1095.5	110.9	28.5	2.6	1.2
BSS	–	6.38	2.82	819.2	210.6	3414.8	1446.3	150.0	31.1	27.1	8.8
BLA	–	6.47	2.96	236.3	73.1	2257.3	903.7	149.0	31.5	27.4	9.1
BLK	–	5.21	2.28	416.3	94.7	3562.9	1462.9	72.0	20.8	32.8	11.1
BEG	–	5.98	2.47	358.1	93.1	3122.7	1156.8	41.8	16.8	13.5	3.7
BKR	–	3.70	1.52	172.3	58.9	2897.5	1121.5	19.9	5.6	6.8	2.3
BPD	–	3.89	1.86	263.5	80.4	2823.7	1140.4	14.5	4.3	26.6	7.7
BTK	–	5.32	2.28	200.4	60.4	2331.4	853.3	8.6	1.2	14.3	6.4
BAN	–	2.35	1.18	84.4	53.6	531.4	215.8	6.1	3.7	6.8	2.4
BKA	–	2.04	1.02	75.2	48.8	348.5	134.3	8.9	3.8	3.9	1.1

Biofertilizer: Azatobacter + Azospirilium + Pseudomonas; DAP: diammonium phosphate, Saplings of Sirsi (BSS), Karnataka is treated with different fertilizer treatment, FYM: Farmyard manure,MEE: Methanol extract; AME: Aqueous methanol extract, T2–T12 are Sirsi collection as treated with different fertilizer treatment and T1 is the control; BLA: Lalitpur; BLK: Lucknow; BSS: Sirsi; BEG: Empress Garden, Pune; BKR: Dapoli, Kudavare, Ratnagiri district, Pune; BPD: Phansad, Dharangan, Pune; BTK: Tillari, Kolhapur; BAN: Anand, Gujurat; BKA: Kannur, Kerala.

extracts of methanol and aqueous methanol. Equal amount (5 mg) of each extract was dissolved in 1 ml of methanol (100%), shaken periodically and filtered using sterile 0.22 μm filters. An aliquot of 20 μl of each sample was injected into the HPLC system for analysis. All the bark extractions are carried out at least three times and average data are presented.

2.5. RP-HPLC-PDA-RI analysis

The RP-HPLC-PDA system was used for the separation and characterization of flavonoids and polyphenols in the investigated bark samples of *S. asoca*. Although, the flavonoids fall under polyphenols, but in order to avoid confusion, we separately named the non-flavonoid compounds as polyphenols (containing polyphenol units). Waters (Milford MA, USA) HPLC system equipped with binary pump, manual injector, photodiode array detector (PDA, model 996), Empower Pro software (Waters, USA) were used for the analysis. RP Column of Sunfire C18 (4.6 mm × 250 mm, 5 μm coating; Waters, USA) make was used for the separation and quantification of compounds.

The stock solution of each flavonoids and polyphenols such as (+)-catechin hydrate, (−)-epigallocatechingallate, (−)-gallocatechingallate, (−)-epicatechin, (−)-gallocatechin, (−)-catechin, (−)-epigallocatechin, (−)-epicatechingallate, (−)-catechingallate, gallic acid, tannic acid and lyoniside were dissolved in methanol, (v/v) at a concentration of 1 mg/ml. Sample analysis was carried out at 30 °C using gradient solvent system. The mobile phases for gradient elution comprised of solvent A (water with TFA) and B (methanol, 100%), mixed according to the following profile: 25% B–35% B (0–25 min), then 35% B–50% B (25–50 min), then 50%–80% B (50–60 min) and 80%–100% B (60–70 min). The injection volume of standards and extracts were 20 μl with 0.8 ml/min flow rate. Signal detection at 276.8 nm was used for the quantification of flavonoids and polyphenols.

For the analysis of pinitol, the above instrument specification equipped with photodiode array (PDA) as well as Refractive Index (RI) detector were used for the analysis. HPLC XBridge Amide column (250 mm × 4.6 mm, 3.5 μm; *Waters*, USA) was used for the analysis of pinitol. The stock solutions of standard pinitol diluted separately in acetonitrile and H_2O 7:3, (v/v) at a concentration of 2 mg/ml. Sample analysis was carried out at 30 °C using isocratic solvent system. The mobile phase for isocratic elution were of A (80:20 acetonitrile and H_2O with 0.2% triethylamine) and B (30:70 acetonitrile and H_2O with 0.2% triethylamine). The mobile phase comprised of 90:10 (solvent A:solvent B) and flow rate was 1 ml/min with 20 min analysis time. The injection volume (20 μl) of standards and plant samples was taken for the analysis. The analysis of pinitol was successfully accomplished under such conditions using RI detector. During analysis, UV/vis spectra were also collected in the range of 210–400 nm for monitoring the pinitol. Thus, UV/vis spectra were assured the presence of pinitol in case of co-elution of any contaminant present in the extracts through RI analysis.

2.6. Determination of antioxidant activity

Antioxidant activity of all *S. asoca* bark extracts was determined by using DPPH radical scavenging activity assay. It was performed according to methodology reported by Brand-Williams et al. (1995). This activity analysis was carried out using 0.5 mM DPPH methanol solution. The samples were reacted with the stable DPPH radical in methanolic solution. The control solution (blank) was prepared by mixing methanol (3.5 ml) and DPPH radical solution (0.3 ml). Different concentrations of extracts and ascorbic acid as 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml and 80 μg/ml were taken and each concentration was treated with 500 μl of DPPH solution for measuring the absorbance after 30 min of reaction. It noticed that the color was changed from deep violet to light yellow at 517 nm (Gupta et al., 2016). The data presented in Table 3 are based

on the average of triplicate readings. The effects of antioxidants on DPPH radical scavenging were thought to be due to their hydrogen-donating ability. The different concentrations of extracts of all bark samples were able to reduce the stable free radical DPPH to the yellow colored 1,1-diphenyl-2-picrylhydrazyl. The radical scavenging activity was then calculated from the equation.

$$\%\text{ of radical scavenging activity} = \text{Abs (control)} - \text{Abs(sample)}/\text{Abs (control)} \times 100$$

Where Abs (control) is the absorbance of the control reaction (containing all the reagents except the extracts), and Abs (sample) is the absorbance of the tested extracts. The IC_{50} value was calculated by using Graph Pad Prism 5 software.

2.7. Statistical analysis

To evaluate the best variety of *S. asoca* among the studied barks, the contents of different secondary metabolites such as pinitol, flavonols and polyphenols in methanol extracts were compared for all the varieties through Principal Component Analysis (PCA) by using the PAST 2.17 software. In another study, the saplings of BSS (bark of Sirsi) were treated with different fertilizer for the period of 4 years to evaluate the quality of barks. This PCA analysis was clarified the role of fertilizer to change the secondary metabolite profiling of treated barks.

3. Result and discussion

The average yield of the wild collected samples viz. BSS (2.55, 1.13 g), BLA (2.59, 1.18 g), BLK (2.08, 0.91 g), BEG (2.39, 0.99 g), BKR (1.48, 0.61 g), BPD (1.56, 0.74 g), BTK (2.13, 0.91 g), BAN (0.94, 0.47 g) and BKA (0.82, 0.41 g) in methanol and aqueous-methanol extracts, respectively. Similarly, the average yield of the cultivated samples viz. T1 (1.0, 0.48 g), T2 (1.27, 0.54 g), T3 (1.51, 0.62 g), T4 (1.88, 0.68 g), T5 (1.68, 0.68 g), T6 (1.71, 0.72 g), T7 (1.71, 0.72 g), T8 (1.69, 0.71 g), T9 (1.72, 0.73), T10 (1.89, 0.79), T11 (1.9, 0.81) and T12 (1.88, 0.8 g) in methanol and aqueous-methanol extracts, respectively. Several experiments were performed to monitor the quality of the bark extracts, one related to the optimization of HPLC parameters and the other concerning the analysis of pinitol, flavonoids and polyphenols in the extracts of *S. asoca*.

3.1. Optimization of HPLC conditions for analysis of flavonoids and polyphenols

Optimization of mobile phase being extremely important step in the method development, the separation of flavonoids and polyphenols were carried out using gradient elution based on water with 0.1% trifluoroacetic acid (TFA) as solvent A and methanol as solvent B. The results showed that best resolution and sharp peaks were achieved with volume ratios of acidified water to methanol from 75:25–65:35 (0–25 min), then from 65:35–50:50 (25–50 min), then from 50:50–20:80 (50–60 min) and then from 20:80–0:100 (60–70 min). The RP C18 column has shown better resolution of peaks in the above analytical conditions. Other chromatographic parameters were optimized as flow rate (0.8–1.0 ml/min) and column temperature (25 °C, 30 °C, 32 °C and 35 °C). The best separation was achieved at flow rate of 0.8 ml/min and at column temperature 30 °C. The wavelength of maximum absorbance of the standard solutions for flavonoids and polyphenols were analyzed by spectral scan in between 200 and 400 nm to obtain optimal detection wavelength at 276.8 nm for final spectra recording. The chromatogram to represent the resolution of flavonoids and polyphenols in the above conditions has been published in our earlier communication (Ahmad et al., 2016b).

For pinitol analysis, the separation was tried through isocratic elution using water and acetonitrile. The results showed that the best resolution and sharp peaks were achieved in water: acetonitrile (80:20) along with 0.2% triethylamine as solvent A and water: acetonitrile (30:70) with 0.2% triethylamine as solvent B at a volume ratio of 90:10 for 20 min analysis time. Two different carbohydrate analysis columns such as XBridge Amide (250 mm × 4.6 mm, 3.5 μm) and Rezex ROA-Organic Acid H+ (300 mm × 7.80 mm, 8%) were tested. Among these two columns, the better separation of peaks was accomplished in XBridge amide column (Tewari et al., 2016). Other chromatographic parameters considered for optimization study were flow rate (0.6–1.2 ml/min) and column temperature (25 °C, 30 °C, 32 °C and 35 °C). The optimum separation was achieved at the flow rate of 1 ml/min and with column temperature of 30 °C.

3.2. Selection of best collected variety

The barks collected from different parts of India have shown significant variation in the yield of the extracts as presented in Table 1. It was observed that, the methanol extracts were (2.04–6.47%) more than twice in compared to the aqueous-methanol extracts (1.02–2.97%). It justified that the highly polar low molecular weight alcohol selectively extracted the oxygenated organic molecules in comparison to the aqueous-methanol solvent system. From Table 1, it clear that the barks collected from Lalitpur, Utter Pradesh (BLA) and Sirsi, Karnataka (BSS) are giving highest extract yield. The yield of methanol and aqueous-methanol extracts were 6.47, 6.38% and 2.96, 2.82% in BLA and BBS samples, respectively. But, BLA and BBS samples contained pinitol (236.3, 819.2 mg), flavonoids (2257.3, 3414.8 mg) and polyphenols (176.4, 177.1 mg) in their methanol extracts, respectively. Overall, the BSS sample contained higher yield of the extracts along with the improved percentage of bioactive components. On the other hand, the BAN (2.35, 1.18%) and BKA (2.04, 1.02%) were resulted poor yield of methanol and aqueous-methanol extracts, respectively. Besides, the BSS extracts contained reasonable amounts of all thirteen detected flavonoids such as (−)-gallocatechin (530.3, 275.8 mg), catechin +(−)-epigallocatechin (331.5, 150.8 mg), (−)-epigallocatechingallate (370.0, 172.2 mg), (+)-afzelechin-3-*O*-L-rhamnopyranoside (780.5, 258.0 mg), (+)-epiafzelechin-3-*O*-β-D-glucopyranoside (260.7, 110.6 mg), (−)-epicatechin (275.4, 121.9 mg), (−)-gallocatechingallate (171.8, 60.5 mg), (−)-catechingallate (140.4, 61.1 mg), epicatechingallate (194.4, 92.2 mg), 5,3′-dimethoxy-(−)-epicatechin (143.7, 67.2 mg), leucocyanidin (169.8, 58.2 mg) and leucopelargonidin (46.3, 17.8 mg) in methanol and aqueous-methanol extracts, respectively (Table 2). Similarly, it contained higher amounts of polyphenols (177.1 mg, 39.9) and pinitol (819.2 mg, 210.6) in methanol and aqueous-methanol extracts, respectively.

3.3. Cultivation of BSS saplings with different fertilizer treatment

It is known that *S. asoca* is a very slow growing plant and it takes several years (nearly 10 years) for getting the profitable amounts of barks. Due to the superior quality of BSS samples, these plant saplings were cultivated in experimental field with the aim to harvest in a shorter span of time with maximum amount of bark. These plants are cultivated in experimental field with different fertilizer treatment as presented in Table 1. The pH of these organic fertilizers was varied in between 6.2–6.7. NPK ratio of FYM (2.3:1.8:1.5), vermicompost (2.9:2.2:1.6), poultry (2.5:1.4:1.5) and biofertilizers (2.7:2.5:1.8). The ratio of P and K were calculated on the basis of PO_4 and K_2O. In general, the chemical fertilizer was applied in the average of 2 g/plant, whereas the organic fertilizer was applied in the approximate dose of 20 g/plant. From the chemical analysis of organic fertilizer point of view, it found that NPK

Table 2
Flavonoids contents in different methanol (MEE) and aqueous methanol (AME) extracts of *S. asoca*.

Sample	(−)-Gallo catechin		Catechin + (−)-Epigallo catechin		(−)-Epigallocatechingallate		(+)-Afzele chin-3-*O*-L-rhamno pyranoside		(−)-Epiafzele chin-3-*O*-β-D-gluco pyranoside		(−)-Epi catechin		(−)-Gallocate chin gallate		(−)-Catechin-gallate		(−)-Epicatechin-gallate		5,3′-di methoxy-(−)-epicatechin		Leuco cyanidin		Leucopelargonidin	
Extract	MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME
T1	25.7	11.8	7.2	0.8	27.7	12.5	16.8	9.3	10.2	5.2	76.7	30.5	8.3	5.7	2.5	0.8	34.5	9.4	29.2	7.4	3.2	1.6	9.1	1.6
T2	88.4	23.2	38.4	10.5	78.6	39.9	41.7	23.7	32.9	9.9	100.5	30.6	16.8	5.1	16.8	5.1	25.0	11.1	45.3	16.5	8.9	4.5	5.7	0.8
T3	119.2	57.2	54.1	19.2	145.0	75.9	163.8	80.2	42.0	11.3	126.6	59.1	33.3	17.2	35.5	14.2	15.7	7.4	49.9	23.1	11.3	6.5	32.4	9.7
T4	123.4	54.0	40.1	14.7	134.1	63.7	187.5	76.6	40.3	17.6	138.5	60.3	9.7	3.5	25.6	10.3	33.7	15.6	36.5	15.1	32.1	11.1	26.1	8.5
T5	190.3	72.0	65.7	20.1	148.2	69.1	190.2	80.3	65.9	25.7	123.8	54.0	14.7	7.5	46.3	21.7	39.6	17.8	45.6	25.1	51.1	23.1	27.3	9.6
T6	373.2	97.1	131.5	32.5	291.6	128.9	415.9	133.0	106.5	33.9	185.9	77.5	57.2	26.0	95.9	54.2	27.3	14.4	53.1	29.8	74.7	37.4	13.4	5.6
T7	277.5	80.8	63.7	20.9	246.2	96.7	327.7	121.6	99.9	30.9	185.9	71.3	58.3	26.9	76.4	37.8	68.3	10.8	61.3	21.2	71.8	21.4	35.6	10.1
T8	352.3	127.3	276.8	124.7	179.4	76.5	487.4	144.1	253.6	121.2	188.3	60.9	59.5	24.5	70.9	30.9	96.2	11.5	78.9	26.9	88.4	36.9	44.4	12.9
T9	342.3	120.6	239.8	115.7	399.4	136.1	527.9	151.6	116.0	31.8	150.5	40.5	65.3	32.9	84.5	39.7	126.3	40.5	111.7	55.9	72.9	25.4	27.2	8.0
T10	485.3	193.1	315.3	136.3	371.6	128.2	489.6	148.5	189.3	83.5	162.3	74.4	85.1	35.4	87.3	38.5	133.7	56.8	117.8	43.9	113.1	49.8	51.7	18.3
T11	513.9	217.3	308.0	131.6	475.2	193.5	528.3	160.7	193.7	85.2	196.1	80.6	114.8	45.9	119.9	50.6	145.9	67.4	125.4	54.4	102.1	45.8	48.8	17.8
T12	345.8	130.2	368.2	141.1	430.4	181.1	615.8	173.1	216.3	112.3	196.8	78.2	92.2	40.5	119.1	51.9	140.5	65.7	120.8	53.0	130.3	53.0	44.1	15.4
BSS	530.3	275.8	331.5	150.8	370.0	172.2	780.5	258.0	260.7	110.6	275.4	121.9	171.8	60.5	140.4	61.1	194.4	92.2	143.7	67.2	169.8	58.2	46.3	17.8
BLA	649.3	285.1	143.2	42.7	133.8	37.9	378.5	139.4	15.9	7.3	292.6	142.1	64.7	24.9	28.3	14.4	177.8	78.4	217.6	80.3	145.5	48.9	10.1	2.3
BLK	688.2	298.1	462.7	178.5	487.1	225.8	752.6	246.8	280.0	125.2	163.1	83.9	188.1	68.3	131.3	59.6	186.1	85.5	205.9	84.3	ND	ND	17.8	6.9
BEG	392.8	143.2	353.9	139.7	392.6	141.8	773.3	192.2	259.7	127.1	284.2	131.2	182.6	70.9	147.6	63.2	186.5	78.5	149.5	69.0	ND	ND	ND	ND
BKR	204.4	88.5	123.5	65.1	523.5	234.8	777.7	257.4	94.3	29.9	268.9	96.7	329.2	145.9	76.6	35.1	137.8	65.9	182.4	44.2	35.1	13.8	144.1	44.2
BPD	200.7	83.1	92.2	34.1	615.3	337.0	798.4	261.3	117.8	46.46	64.67	21.1	398.7	155.4	85.9	54.5	133.1	59.5	178.5	38.2	35.9	13.2	102.5	36.5
BTK	450.8	222.4	238.1	101.3	465.3	173.8	621.9	150.3	56.3	17.1	4.5	1.5	149.5	51.2	122.3	44.3	62.8	23.3	36.9	12.5	107.4	30.5	15.6	7.1
BAN	16.5	8.01	50.1	22.7	187.5	71.1	5.5	0.9	14.9	3.7	59.2	29.4	18.9	6.5	22.8	8.3	35.7	14.9	19.2	6.9	14.6	6.9	86.5	36.5
BKA	13.4	4.44	52.1	22.4	122.4	51.9	ND	ND	0.9	ND	33.5	10.4	14.2	5.7	22.1	8.1	32.4	14.0	0.9	ND	37.3	12.3	19.3	5.1

MEE: methanol extract; AME: Aqueous methanol extract.

ratio was nearly tenfold less in comparison to the chemical fertilizer. Therefore, it was decided to apply organic fertilizer (20 g) ten times more in comparison to the chemical fertilizer (2 g). It is clear that all the fertilizer treated plant gave improved extract yields with proportionally to increase of bioactive components. The yield of the extracts as well as quantitative amounts of pinitol, flavonoids and polyphenols of all the extracts are presented in Table 1. It was observed that the plants treated with mixed fertilizers gave higher amounts of extract along with the enriched bioactive compounds. Especially the plants treated with poultry waste + biofertilizer, vermicompost + biofertilizer and farm yard manure + biofertilizer + DAP yielded higher extracts with enhanced bioactive compounds. In particular, the plants treated with vermicompost + biofertilizer gave improved yield of the extracts (MEE: 4.75%, AME: 2.02%). The MEE and AME contained pinitol (729.1, 205.7 mg), flavonoids (2872.1, 1150.8 mg) and polyphenols (173.8, 54.9 mg), respectively. The amounts of all the identified flavonoids in the extracts are presented in Table 2 for comparison purposes.

It was noticed that the organic fertilizer along with sampurna gave reasonable yield of the extracts; therefore the all three major nutrients (NPK) played the vital role for healthy plant growth. From the ICP analysis, it was observed that the toxic elements such as Cr, Cd and Pb were absent in the organic fertilizers. On the other hand, the important micronutrients such as Ni (25.0, 29.0, 46.2, 39.5 mg/Kg), Mn (75, 80, 102, 111 mg/Kg) and Zn (90, 102, 157, 137 mg/Kg) were present in significant amounts in FYM, poultry, vermicompost and biofertilizer, respectively. As discussed above, the mixed organic fertilizer gave improved yield of the bioactive compounds which might have happened due to the proper nourishment of the plants with micronutrients helping in the sustainable plant growth. Besides, the organic fertilizers contain 25–30% of carbon source as determined by elemental composition analysis. The organic fertilizers especially the biofertilizer enriched with microorganisms viz. *Azotobacter, Azospirilium, Pseudomonas* helped to uptake the nutrients through their interaction in the rhizosphere. *Azotobacter* and *Azospirilium* are well known for their capability of fixing nitrogen and *Pseudomonas* acts as biocontrol to inhibit the growth of plant pathogens. Biofertilizers add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus and stimulating plant growth through the synthesis of growth-promoting substances (enzymes). Such complex mechanism might have helped in holistic plant growth. It is, thus, summarized that the micronutrients, microbial interaction and suitable enzymes have possibly played the synergistic role in enhancing plant growth under mixed organic fertilizer treatments.

3.4. Antioxidant activities

The antioxidant studies revealed that the extracts have potent activities as presented in Table 3. Methanol extracts showed better antioxidant activity as compared to their corresponding aqueous-methanol extracts (Table 3). From the collected barks viz. BLA, BLK, BSS, BEG, BKR and BPD have shown enhanced free radical inhibiting activities (IC_{50}: 3.3–5.8). The methanol extracts of all the samples showed enhanced activities (IC_{50}: 3.2–9.5) as compared to the standard ascorbic acid (IC_{50}: 23.3). Similarly, the bark methanol extracts of the saplings cultivated after the application of mixed fertilizers also have shown equally or better antioxidant activities (IC_{50}: 3.2–6.4). It has been inferred that the antioxidant activity of these extracts might be due to the presence phenolic compounds (Pourmorad et al., 2006). In the present analysis, it can be generalized that the extracts containing higher amount of flavonoids and polyphenols have shown enhanced antioxidant activity. Interestingly, it was observed that the extracts containing higher amount of pinitol with reasonable amount of flavonoids and polyphenols have shown enhanced antioxidant activity. Pini-

Table 3
Antioxidant activity of methanol (MEE) and aqueous methanol (AME) extract of *S. asoca* bark.

Sample	5 μg/ml		10 μg/ml		20 μg/ml		40 μg/ml		80 μg/ml		IC 50	
Extract	MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME	MEE	AME
T1	9.3	1.2	26.3	7.7	54.5	28.9	60.4	50.1	74.7	65.9	8.0	32.1
T2	10	1.7	28.6	19.1	50.8	32.1	61.6	52.7	75.2	68.8	9.6	24.0
T3	10.2	2.0	30.1	6.2	52.1	30.0	62.8	50.6	75.8	68.8	8.4	36.4
T4	10.8	2.2	31.5	6.6	54.2	30.3	63.5	50.8	76.3	70.0	7.4	38.0
T5	11.3	2.8	32.5	7.2	55.5	31.6	64.0	52.2	77.2	70.6	6.8	35.5
T6	11.1	2.6	31.9	6.8	54.5	30.8	63.6	51.8	76.8	70.2	7.3	36.7
T7	12.5	3.0	32.8	7.4	55.8	32.2	64.2	55.4	77.4	70.9	7.2	35.0
T8	13.8	3.5	35.1	8.5	57.8	35.2	68.4	54.1	79.2	72.2	6.6	33.6
T9	14.0	3.9	35.6	9.2	58.3	35.7	68.9	54.7	79.5	72.5	6.4	30.4
T10	13.8	3.8	38.8	10.4	64.9	42.3	76.5	60.2	86.1	74.5	5.2	20.0
T11	25.3	7.2	48.1	15.3	78.2	48.6	85.2	64.6	90.1	78.5	3.2	17.0
T12	18.5	5.3	44.3	11.1	64.3	41.8	75.9	60.1	85.2	73.8	4.5	20.9
BSS	25.2	7.1	47.6	15.1	78.1	48.4	85.1	64.0	90.0	78.1	3.3	17.0
BLA	15.2	4.2	37.2	10.2	62.1	38.1	72.9	56.4	82.2	74.8	5.8	27.9
BLK	19.9	6.1	41.5	13.7	75.2	45.8	84.7	62.8	89.5	74.9	4.4	16.9
BEG	16.5	5.1	40.2	10.7	63.9	40.2	74.5	59.4	84.0	72.3	5.1	21.5
BKR	14.2	4.1	36.2	9.6	60.8	36.1	70.5	55.8	80.6	73.2	5.6	29.3
BPD	15.6	4.6	38.5	10.4	62.6	40.2	73.2	58.5	83.1	75.5	5.6	24.8
BTK	13.6	3.2	34.3	7.9	56.9	33.6	66.5	53.4	78.1	72.2	6.8	35.0
BAN	9.4	1.5	28.4	18.6	50.5	30.8	60.8	50.6	75.1	67.5	9.5	26.1
BKA	9.2	1.0	26.1	6.8	54.2	25.0	60.2	50.0	74.5	63.7	8.1	35.6

Standard (Ascorbic acid): 5 mg/ml (3.3), 10 mg/ml (29.5), 20 mg/ml (78.2), 40 mg/ml (85.0), 80 mg/ml (90.9) IC50 (3.7).

tol, which is known as an anti-diabetic compound along with the flavonoids and polyphenols might have played the synergistic role for enhancing antioxidant activities to reduce the DPPH. On the other hand, aqueous-methanol extracts have shown inferior antioxidant activities due to the poor percentage of the discussed bioactive compounds.

3.5. Statistical analysis

The yields of the methanol extracts were more than twice as compared to the aqueous-methanol extracts. The methanol extracts were also contained higher percentage of pinitol, flavonols and polyphenols. Therefore, the methanol extracts were considered for the statistical analysis. PCA is studied with consideration of all major aspects such as yield of the extracts, antioxidant activities, pinitol, flavonoids and polyphenols contents as presented in Fig. 1a. The PCA result clearly showed that T11 plant, treated with bio-fertilizer and vermicompost produced bioactive compounds enriched extracts in comparatively higher amounts. Furthermore, PCA evaluation of individual flavonoid, the most favorable situation for T11 and closely followed by T12 and T10 treatments (Fig. 1b). Based on the PCA results and scores, we could be able to discriminate different varieties. Sample BLK was rich in flavonoids and pinitol, BLA, BTK, BSS and BEG were found yielded maximum extracts and rich in polyphenols, T11 sample was found best in chemical profiling of extract since, it was rich in all pinitol, flavonoids and benzenoids (Fig. 1c). Among the different collections, BSS has shown superior quality in terms of extracts yield as well as enhanced bioactive compounds. Therefore BSS saplings were considered for fertilizer treatment studies. Yield of extract was identified as PC1 and flavonoid content as PC2, and a variance-covariance ratio of 88.37% and 10.65% was obtained. The experimental plants grown under mixed fertilizers treatment have also shown equivalent activities as compared to its mother plant. Though, the extract yields of the mixed fertilizer treated plants afforded nearly 20% of reduction due to their early harvesting in comparison to its mother plant. But, the accumulations of the discussed bioactive compounds are slightly better as compared to the mother plant. Among all the treatments, T11 plants leading to higher amounts of extracts, which was enriched with bioactive compounds. Similarly, T12 and T10 closely followed the T11 and all three plants have shown best anti-oxidant activities.

4. Conclusions

S. asoca is a commercially important plant in modern Ayurvedic and year old traditional medicine. The present work has addressed the quality control of the extracts by analyzing the marker compounds (flavonoids and polyphenols). Similarly, our group has reported the anti-diabetic pinitol in *S. asoca* bark and method of its extraction as well as analysis protocol has been discussed in this paper. Due to slow growing nature of this plant and high industrial demand of its bark in Ayurvedic preparations, our work now addressed the production of its bark in shorter period without compromising the quality of the extracts. Through the present work, we have been able to select the best one for multiplication among the wild collection of number of barks from different geographical locations of India. Among these collections, the Sirsi, Karnatka (BSS) species gave the reasonably higher amount of methanol extracts (6.38%) followed by pinitol (819.2 mg), flavonoids (3414.8 mg) and polyphenols (177.1 mg). In experimental conditions, the plant saplings were cultivated under chemical/organic/mixture of both fertilizers treatments. It was found that the plants grown under vermicompost and biofertilizer treatment in the average of 20 g/plant gave the improved yield of the extract (4.75%). The above methanol extract contained pinitol (729.1 mg), flavonoids (2872.1 mg) and polyphenols (173.8 mg) as major class of compounds. The antioxidant activity of this extract has shown slightly higher scavenging of DPPH probably due to its improved accumulation of bioactive compounds. The higher pinitol content along with improved flavonoids and polyphenols have shown enhanced antioxidant activity. It was concluded that the *S. asoca* bark methanol extract contained higher amounts of the bioactive compounds than aqueous-methanol extracts having enhanced medicinal properties might be helpful for curing the complex nature of diseases. This is first report on agro-practice of the medicinally and commercially important crop under organic fertilizer treatment to enhance the accumulation of bioactive compounds in shorter duration.

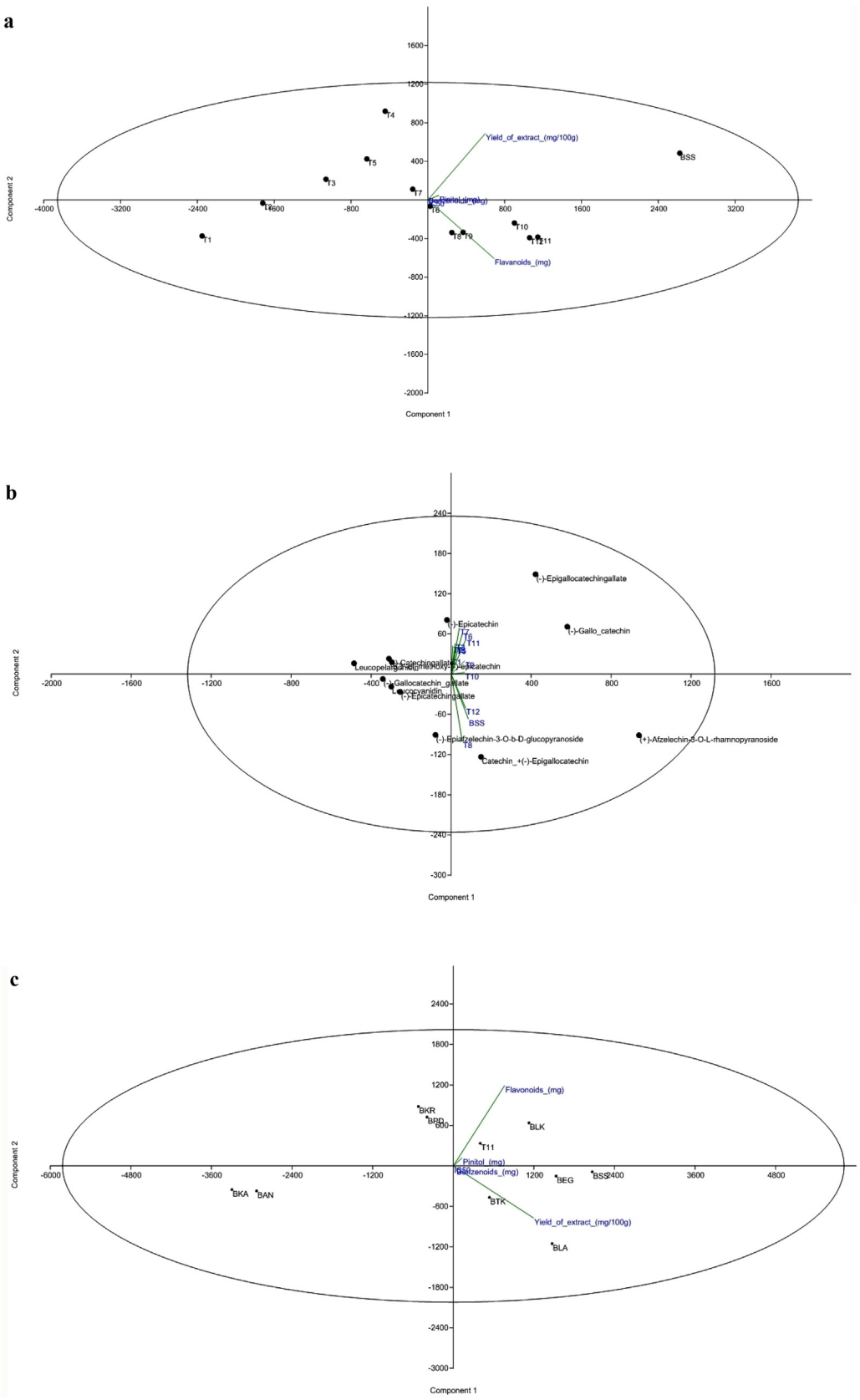

Fig. 1. (a) Principal component analysis for yields of extracts, pinitol, flavonoids and polyphenols in cultivated barks. (b) Principal component analysis of flavonoids in methanol extracts of cultivated barks. (c) Principal component analysis for yields of methanol extracts, pinitol, flavonoids and polyphenols in wild collected barks.

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