Monitoring seasonal variation of epicatechin and gallic acid in the bark of *Saraca asoca* using reverse phase high performance liquid chromatography (RP-HPLC) method

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ABSTRACT

Background: *Saraca asoca* (Roxb.) Wilde (Fabaceae) is a high valued but vulnerable medicinal plant of Western Ghats region. This plant is mainly known for its use in various gynecological disorders. **Objective:** The objective of the present study was to investigate seasonal variation of the polyphenolic compounds *viz*., epicatechin and gallic acid in the bark of *S. asoca* by using Reverse Phase High Performance Liquid Chromatography-Diode Array Detector (RP-HPLC-DAD) method. **Materials and Methods:** The bark was collected in six different *Ritu* (season) *viz*. *Varsha* (monsoon), *Sharad* (autumn), *hemant* (early winter), *Shishir* (winter), *Vasanta* (spring), and *Grishma* (summer) mentioned in Ayurveda. **Results**: The RP-HPLC-DAD analysis indicated that levels of epicatechin and gallic acid in the bark of *S. asoca* vary seasonally. The highest concentration of epicatechin was observed in *Shishir Ritu* (3315.19 ± 165.76 mg/100g) and gallic acid during *Hemant Ritu* (211.90 ± 10.60 mg/100 g). **Conclusions:** In present study, the ability to synthesize and accumulate both the compounds in bark of *S. asoca* varied greatly throughout the seasons. It was also observed that the compound epicatechin was present abundantly as compared to gallic acid throughout the seasons.

Key words: Ayurveda, epicatechin, gallic acid, *Saraca asoca* (Roxb.) Wilde, seasonal variation

INTRODUCTION

Saraca asoca (Roxb.) Wilde (Fabaceae) commonly known as 'Ashoka' is a highly valued medicinal plant categorized 'vulnerable' by International Union for Conservation of Nature [Figure 1]. This plant is mainly known in Ayurveda for its use in gynecological disorders.[1,2] Recent studies revealed that aqueous and alcoholic extracts of the stem bark of *S. asoca* have been reported phenolic glycoside P_2 from phenolic glycoside fraction and also non-phenolic glycosides which have stimulant action on isolated human uterus.[3,4] Moreover, the phenolic glycoside P_2 was inactive on central nervous system (CNS), cardiovascular system, and smooth muscles other than uterus.[3] Furthermore, diverse pharmacological activities *viz*., antibacterial,[5] anticancer,[6] antimutagenic, genoprotective effect,[7] antihyperglycemic,[8] antioxidant,[8,9] molluscicidal,[10] anxiolytic,[11] CNS depressant,[12] anti-pyretic,[13] and analgesic[14] have been reported from different parts of *S. asoca*. This plant is used to treat skin infections, CNS function, genitor-urinary functions, uterus pain during periods, clots, and ammenorhea.[15] Polyphenolic compounds catechin, epicatechin, leucocyanidin,[16] leucopelargonidin,[17] procyanidin B-2, 11'-deoxyprocyanidin B,[18] β-sitosterol,[19] gallic acid, quercetin, (-) 3-O-*p*-D-glucoside,[20] catechol, (-) epicatechol[16-19] apigenin-7-O-β-D-glucoside, cyanidin-3, 5-diglucoside, kaempferol 3-O-β-D-glucoside, pelargonidin-3,5-diglucoside, quercetin and its 3-O-β-D-glucoside, *n*-octacosanol, (−)-procyanidin

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derivatives, methyl- and ethylcholesterol derivatives, kaempferol-3-O-α-L-rhamnoside, amyrin, and ceryl alcohol[19,21] have been identified from the plant of *S. asoca*. Out of the identified compounds, epicatechin and gallic acid are the major polyphenolic bioactive molecules in the bark of *S. asoca*, which have various pharmacological effects. Gallic acid shows evidence of suppression of a high-fat diet-induced dyslipidemia, hepatosteatosis, oxidative stress in rats,[22] and also possess many potential therapeutic properties including anti-cancer, antimicrobial properties,[23] and neuroprotective action.[24] Epicatechin protects endothelial cells against oxidized low-density lipoprotein (LDL) by scavenging free radicals, maintaining nitric oxide synthase,[25] and possess insulin-like activity.[26] Gallic acid and epicatechin have cholesterol-lowering activity by inhibiting pancreatic cholesterol esterase, binding of bile acids, and reducing solubility of cholesterol in micelles.[27]

Ayurveda is the ancient science of medicine. It is suggested that part specific and *Ritu* (season) specific collection of plant capitulate gives maximum efficacy and potency (*Veeryavan*).[28] Ayurveda mentions six *Ritus* (seasons) *viz. Shishira* (winter), *Vasanta* (spring), *Grishma* (summer), *Varsha* (monsoon), *Sharada* (autumn), and *Hemanta* (early winter).[29] The officinal part, stem bark (*Twak*) of *S. asoca* is used for therapeutic purpose.[30] In *Charaka samhita* and *Sushruta samhita*, it has been suggested that the stem bark of medicinal plants should be collected in *Sharada Ritu* (*Ashwin-Kartik*).[31,32] The aim of the present study was to analyse the seasonal variations of the epicatechin and gallic acid in the bark of *S. asoca*.

MATERIALS AND METHODS

Chemicals

Epicatechin [(−)-*cis*-3, 3',4',5,7-Pentahydroxyflavane] [Figure 2a] and gallic acid [3,4,5-trihydroxybenzoic acid] [Figure 2b] were purchased from Natural Remedies, Bangalore, India. The high performance liquid chromatography (HPLC) grade acetonitrile, glacial acetic acid and water were purchased from Fisher Scientific (Thermo electron LLS India Pvt. Ltd.)

Figure 1: Inflorescence of *Saraca asoca*

Plant collection and processing

The plant of *S. asoca* was identified and authenticated at Regional Medical Research Centre (RMRC), Belgaum (Voucher No. RMRC 980). The bark was collected from a single habitat from Belgaum region (N 15.862 E 074.510; elevation: 799M above MSL) during specified *Ritu* (season) as given in Table 1[33,34] from the same trees (n = 3). Flowering trees over 3-meters height were considered as mature plants for collection of bark samples (each tree one bark sample). The collected bark (from 3 trees) was shade dried, powdered, sieved, and stored in cool and dry place until further use.

Extraction of plant material

Extraction was achieved using cold maceration technique for all samples. Two-gram bark powder was accurately weighed and soaked in 20 mL methanol overnight. The mixture was sonicated (Sonicater bath, Bandelin-Sonorex, Germany, 35 KHz) for 30 minutes and filtered using Whatman No. 1 filter paper. The extraction procedure was repeated three times and pooled. The solvent was distilled off using Rotaevaporator at 40ºC. The extracts were stored in sealed vial at 4ºC until analysis. The concentration of 0.5 mg/mL was prepared in methanol and was used for RP-HPLC analysis after filtering through 0.2 µ Nylon filter paper.

Table 1: Seasons as per Indian and English calendars

Year of collection	Indian calendar		English calendar	
	Month	*Ritu*	Month	Season
2012	Shraavana (Sawan) and Bhadrapada (Bhado)	*Varsha*	July to September	Monsoon
2012	Ashwin (Kwar) and Kartika	*Sharad*	September to November	Autumn
2012-13	Margashirsha (Agrahayana, Agahan) and Pausha (Poos)	*Hemant*	November to January	Early winter
2013	Magh and Phalguna (Phagun)	*Shishir*	January to March	Winter
2013	Chaitra and Baisakh	*Vasanta*	March to May	Spring
2013	Jyeshta and Aashaadha	*Grishma*	May to July	Summer

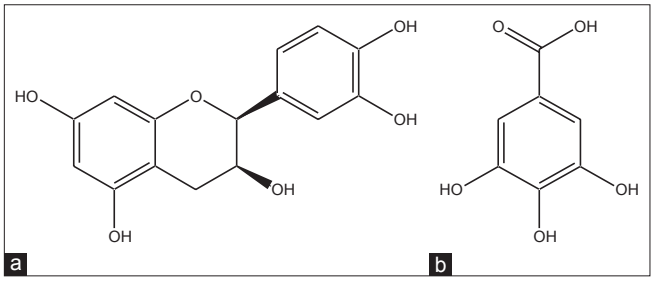

Figure 2: Chemical structure of (a) Epicatechin; (b) Gallic acid

Reverse phase high performance liquid chromatography-diode array detector (RP-HPLC-DAD) analysis

The RP-HPLC analysis was performed on Shimadzu chromatographic system (Model no. LC-20AD) consisting of a quaternary pump, manual injector, degasser (DGU-20A5), and dual λ ultraviolet (UV) absorbance diode array detector (Model No. SPD-M20A). The built in LC (Liquid Chromatography) solution software system was used for data processing. Chromatographic separation was achieved on a Qualisil BDS (Base Deactivated Silica) 250-4.6 mm (5 μm) C18 column. A mobile phase consisting of "A" (acetonitrile), "B" (water), and "C" (glacial acetic acid) were used for separation with 12:85:3 in an isocratic mode with injection volume of 20 μL. The flow rate was 0.7 mL/min and the detection wavelength of diode array detector (DAD) was set 280 nm with 18-minutes run time for both standard and sample.

Calibration and linearity

Calibration and linearity was achieved by accurately weighing epicatechin and gallic acid dissolved it in methanol to obtain mg/mL standard stock solution. The stocks were serially diluted to prepare working solutions (epicatechin: 0.5 to 10 μg/mL; gallic acid: 0.1 to 10 μg/mL) for calibration curves at five concentration levels separately. All solutions were stored at 4ºC temperature. The calibration curve for the standards with above analytical column was established by peak areas and concentrations of working solutions.

System suitability

The system suitability test was assessed by three replicate injections of the standard solutions at a certain concentration. The peak areas of epicatechin and gallic acid were used to evaluate repeatability of the method and their peaks were analyzed for resolution and tailing factors. The limits of detection (LOD) and quantification (LOQ) were determined with the signal/noise method. Signal/noise ratios of 3.3 and 10 were applied for estimating the LOD and LOQ, respectively.

RESULTS

The yields of extracts at different seasons were 34.88 ± 1.74% (*Shishira*); 20.32 ± 1.02% (*Vasanta*); 9.33 ± 0.47 (*Grishma*); 22.34 ± 1.12% (*Varsha*); 19.86 ± 0.99% (*Sharada*); 33.00 ± 1.65% (*Hemanta*). The identification of the two polyphenols *viz*., epicatechin and gallic acid in the extract of *S. asoca*, was done by comparing retention times and UV spectral data with those of authentic standards. The profiles of both samples and standards are presented in Figure 3 (a-e). Profiles with retention time of 16.056 ± 0.158 minutes for epicatechin and 6.208 ± 0.040 minutes for gallic acid was recorded [Figure 3 a and b]. A 5-point standard computer generated linear calibration curve of standard epicatechin and gallic acid within the concentration ranges of 0.5-10 μg/mL and 0.1-10 μg/mL, respectively was obtained with coefficient of determination (R^2) not more than 0.982 [Figure 3 c-d]. The regression equations showed significant relationship between peak area and concentration and this equation was used to estimate contents from samples. LOD of epicatechin and gallic acid were 0.192, 0.490 μg/mL, and LOQ were 0.582, 1.485 μg/mL, respectively. The relative standard deviation (RSD) values were less than 2% indicating method to be precise and reproducible. Validation of method was done by spiking 50 μL (5μg/mL) of standards to equal volume of extracts to obtain recovery within the range of 95-100%.

The profile of epicatechin and gallic acid using RP-HPLC-DAD analysis of the extracts of bark of *S. asoca* was obtained during six different seasons, achieved as the final output of this study. The results of RP-HPLC quantitative analysis of the content yields of epicatechin and gallic acid are presented in Figure 4. The analysis revealed that sample collected during *Shishira Ritu* contained higher level of epicatechin (3315.19 ± 165.76 mg/100g), whereas gallic acid was found in high amount during *Hemanta Ritu* (211.90 ± 10.60 mg/100g). On the other hand, lowest content was recorded during *Grishma Ritu* (409.40 ± 20.47 mg/100g) for epicatechin and *Vasanta Ritu* (30.96 ± 1.55 mg/100g) for gallic acid. More than 80% difference between highest and lowest content in epicatechin and gallic acid were observed [Figure 4].

DISCUSSION

As per Ayurveda, potency of the bark drug corresponding to the action should be at its peak during *Sharada Ritu*. Thus it is expected that the contents responsible for this action must be abundant in the respective season. But in the present investigation *Shishira* and *Hemanta Ritu* were responsible for yielding higher content of epicatechin and gallic acid, respectively. The quantitative and qualitative divergence may be due to the climatic conditions, which in turn may affect the composition and other secondary metabolites of the plants.[35,36] Phenolic compounds are said to be maximum during the summer season which is also evident from the present study.[37] The compounds may accumulate at a particular period in the plants with response to environmental changes.[37,38] Biological activity which is dependent on the chemical composition, is similarly subject to variation depending on the said factors.[39] Ayurveda emphasizes on standardization of the crude drug as well as the end product. The time of collection, place

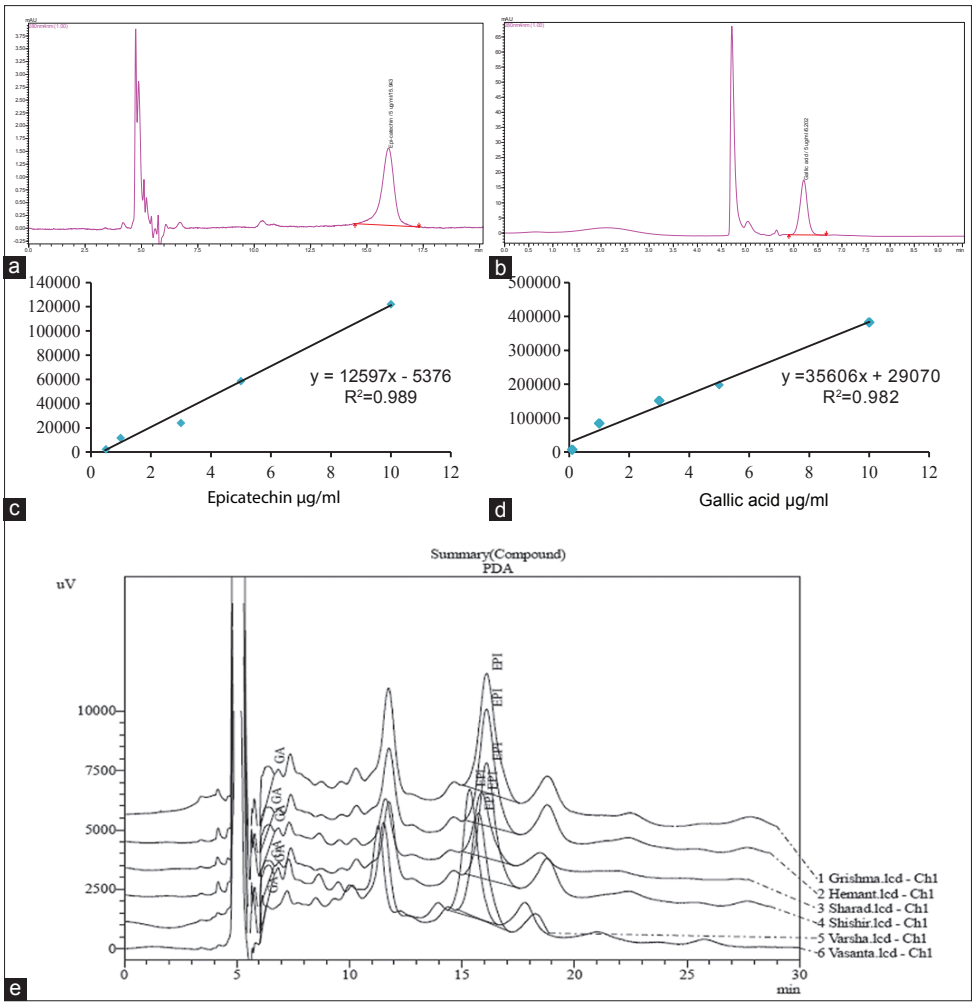

Figure 3: HPLC chromatograms of (a) Standard epicatechin 5 µg/mL; (b) gallic acid 5 µg/mL; (c-d) Five-point calibration curves of the standard epicatechin and gallic acid; (e) HPLC profile of samples collected during different *Ritu* or season. *HPLC:* High performance liquid chromatography

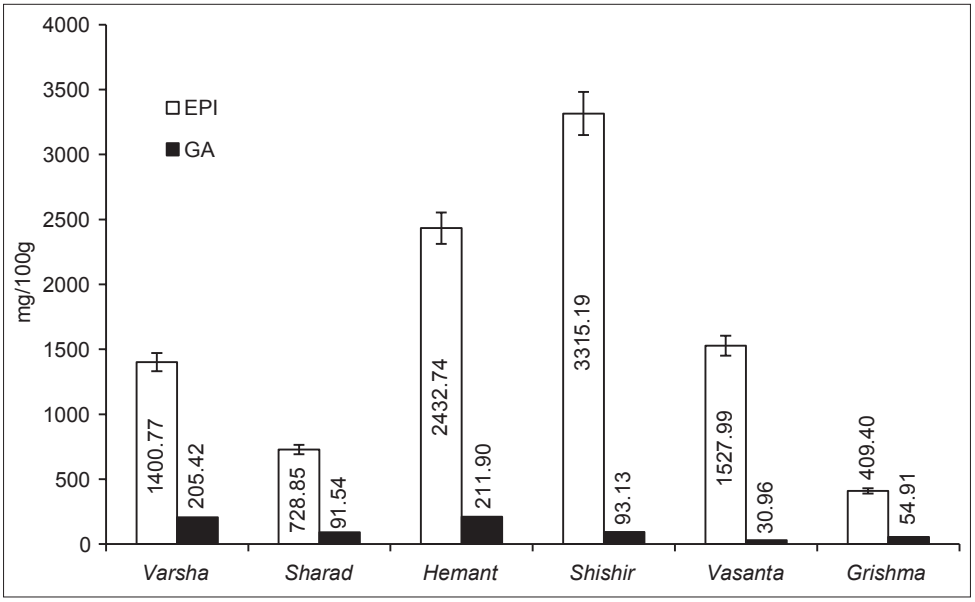

Figure 4: Comparative histogram of epicatechin and gallic acid in the bark of *S. asoca. EPI:* Eicatechin, *GA:* Gallic acid

of collection, methodology is documented in the ancient texts. All these factors are basically being told for utilizing the maximum potency of the medicine.[40] Apart from the phytochemical group of substances typical for a taxon, the chemical outfit depends on the specific genotype, the stage of plant development, influence of environmental factors and the part of the plant.[41] The variation in the secondary metabolites among plants chemotype may occur within different sites. This may also be due to genetic responses to climatic and edaphic factors in formation of secondary metabolites.[42,43]

CONCLUSION

It was observed that *Ritu* or season influences secondary metabolite concentration in the plants or its parts. In present study, the ability to synthesize and accumulate both the compounds in bark of *S. asoca* varied greatly throughout the seasons. Apart from this, it was also observed that the compound epicatechin was present abundantly as compared to gallic acid throughout the seasons. Consequently, to obtain optimum utility of the plant as a source of medicine, the screening for presence of the secondary metabolites during different season is useful. Further studies are required to determine and correlate the biological activities with respect to the concentration of compounds present in the plant/plant parts.

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