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ORIGINAL ARTICLE

Determination of flavonoids, phenolic acid and polyalcohol in *Butea monosperma* and *Hedychium coronarium*by semi-preparative HPLC Photo Diode Array (PDA) Detector

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Abstract The semi preparative HPLC method with PDA Detector was proposed for the determination of one phenolic acid, three flavonoids and one polyalcohol from *Butea monosperma* and *Hedychium coronarium* in gradient elution system. The influence of composition of the mobile phase concentration of the mix modifier and temperature on the separation of gallic acid, quercetin, isobutrin, butrin and eugenol for 90 min was studied. Two different gradient programmes were used to separate these components. The lower limit of quantification of phenolic acid, flavonoids and eugenol is 0.050–0.150 μg/mL and was determined by the least square method and a good correlation was obtained for all separated components.

1. Introduction

The importance of medicinal plants to the health of individuals and communities is known since antiquity. The medicinal value of these plants lies in some chemical substances that produce a definite physiological effect on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, phenolic compounds and flavonoids (Hill, 1952). Among these, flavonoids are the most ubiquitous group of plant secondary metabolites demonstrating a wide range of biochemical and pharmacological effects, including anti-inflammatory (Owoyele et al., 2008), antibacterial (Hernández et al., 2000), antifungal (Li et al., 2005), antioxidant (Bernardi et al., 2007) and anticarcinogenic (Seelinger et al., 2008).

The methanol extract of *Butea monosperma* seeds was tested *in vitro*, showed a significant anthelmintic activity (Prashanth et al., 2001), anticonvulsive (Kasture et al., 2002), hepatoprotective (Wagner et al., 1986), antiestrogenic potential (Johri

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et al., 1991), antifertility activity (Johri et al., 1991), anti-diarrhoeal activity (Gunakkunru et al., 2005) and antifungal activity (Bandara et al., 1989). Isolation and *in vitro* antimicrobial efficiency of *B. monosperma* seed oil were also observed (Redo et al., 1989). "*Pippali rasayana*" the mixture of "Palash" (*B. monosperma*) and "Pippali" (*Piper longum*) is effective against Giardiasis. *B. monosperma* has been also evaluated for antistress activity (Bhatwadekar et al., 1999), antifertility activity (Garg et al., 1969). The effect of oral administration of the aqueous and alcoholic extracts of the leaves was assessed on stress, cognitive function, and anxiety in albino rats (Soman et al., 2004). Ethyl acetate, butanol and aqueous fractions derived from total methanol extract of *B. monosperma* flowers were evaluated for free radical scavenging activities using different *in vitro* models (Lavhale and Mishra, 2007).

Alcoholic bark extract of *B. monosperma* is effective in healing the cutaneous wound in rats (Manikandan et al., 2005). A potential antiviral flavones glycoside from the seeds of *B. monosperma* is also identified and isolated. Three glucosides have been identified as coreopsin, isocoreopsin and sulphurein from the seeds of *B. monosperma*, which were useful in the treatment of various diseases (Yadava and Tiwari, 2005).

The seed of *Hedychium coronarium* is aromatic, carminative and stomachic in nature. The root of *H. coronarium* is effective as anti-rheumatic, excitant and tonic. The ground rhizome is used as a febrifuge. An essential oil from the roots of *H. coronarium* is carminative and has anti-thematic indications. The plant has been used as a remedy for fetid nostril (Matsuda et al., 2002). High performance liquid chromatography is established as the most convenient method, which enables separation and identification of flavonoids using various detection systems (Janeska et al., 2007; Plazonić et al., 2009; Tüzen and Ozdemir, 2003) and developed for qualitative and quantitative analyses of flavonoids in various plant materials (Bobzin et al., 2000; Dubber and Kanfer, 2004). Recently, spectrophotometric determination of secondary metabolites was reported after solid phase extraction methods (Mustafa et al., 2011a,b).

The present study describes a simple, precise, rapid and reproducible method to identify three flavonoids one phenolic acid and one polyalcohol in the methanolic extract of aerial parts of *B. monosperma* and *H. coronarium*. The methanolic extracts were partitioned further to determine the solvent most suitable for the extraction of these flavonoids and phenolic acid.

2. Experimental

2.1. Chemicals and reagents

Three flavonoids were purchased from M/s Sigma–Aldrich Chemical (India) Limited, which are 99% pure. One phenolic acid was purchased from M/s Himedia Chemicals (India), which is 98% pure and one phenolic acid was purchased from M/s Spectrochem (India) Pvt. Ltd., which is 99% pure. These standards were used without further purification. All other chemicals and reagents used were of analytical grade purchased from SD Fine Chemicals India, E-Merck (India) Limited. Gradient grade solvents were also procured from E-Merck (India) Limited and M/s Spectrochem (India) Pvt. Ltd. as and when required.

2.2. Plant material

Roots and flower of *B. monosperma*, were collected from the campus of the Junagadh Agricultural University, India. *B. monosperma* is a species of *Butea* native to tropical southern Asia especially from India, Pakistan, Bangladesh, Nepal, Sri Lanka, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, and western Indonesia. It may be found throughout in India up to a height of 1250 m, except in the arid zones. *H. coronarium* was collected from its native place Amrkantank (Chhattisgarh, India). Both plants were collected under the supervision of botanical experts and kept in passive conditions.

2.3. Extraction procedure

The sample was extracted in 34 mL Accelerated Solvent Extraction (ASE) cell with sample size of 4 g. Plant material, can contain a degree of moisture and it requires pre-treatment to yield a fine and dry sample. The plant material was loaded into an ASE cell. Moisture from samples was removed using a dispersing agent (Ottawa Sand). This dispersing agent prevents the sample from compaction in the extraction cell and increased the surface area, which improved solvent penetration into the sample matrix. Therefore, extraction efficiency and precision were increased in comparison to the conventional method. The ASE method for this extraction was employed at a temperature between 60 and 90 °C. The time and temperature based changes were made for the optimum conditions to yield more active ingredients from the plant material and data were summarized in Table 1.

2.4. HPLC analysis

2.4.1. Preparation of standard solution

Standard stock solutions of the reference compounds were prepared in HPLC grade methanol at a concentration of 1 mg/mL and stored in a refrigerator at −20 °C until use. All standard solutions were filtered through 0.45 μm filters and diluted as necessary with methanol.

2.4.2. Sample preparation

A weighed amount of each extract was dissolved in HPLC grade methanol to give a concentration of 1 mg/mL for HPLC analysis. All samples were stored at 4 °C and were filtered through a 0.45 μm filter before undertaking HPLC analysis.

Table 1 Percentage yield obtained for crude extract with respect to different solvents by using the ASE system.

Plant name	Part used	Total weight (g)	95% Ethanol		Water		Methanol	
			% Weight	g	% Weight	g	% Weight	g
Butea monosperma	Flowers	4.0	12.50	6.25	7.68	3.78	18.80	9.40
	Roots	4.0	5.84	2.92	4.10	2.05	8.88	4.44
Hedychium coronarium	Flowers	4.0	10.76	5.38	3.92	1.96	5.40	2.70

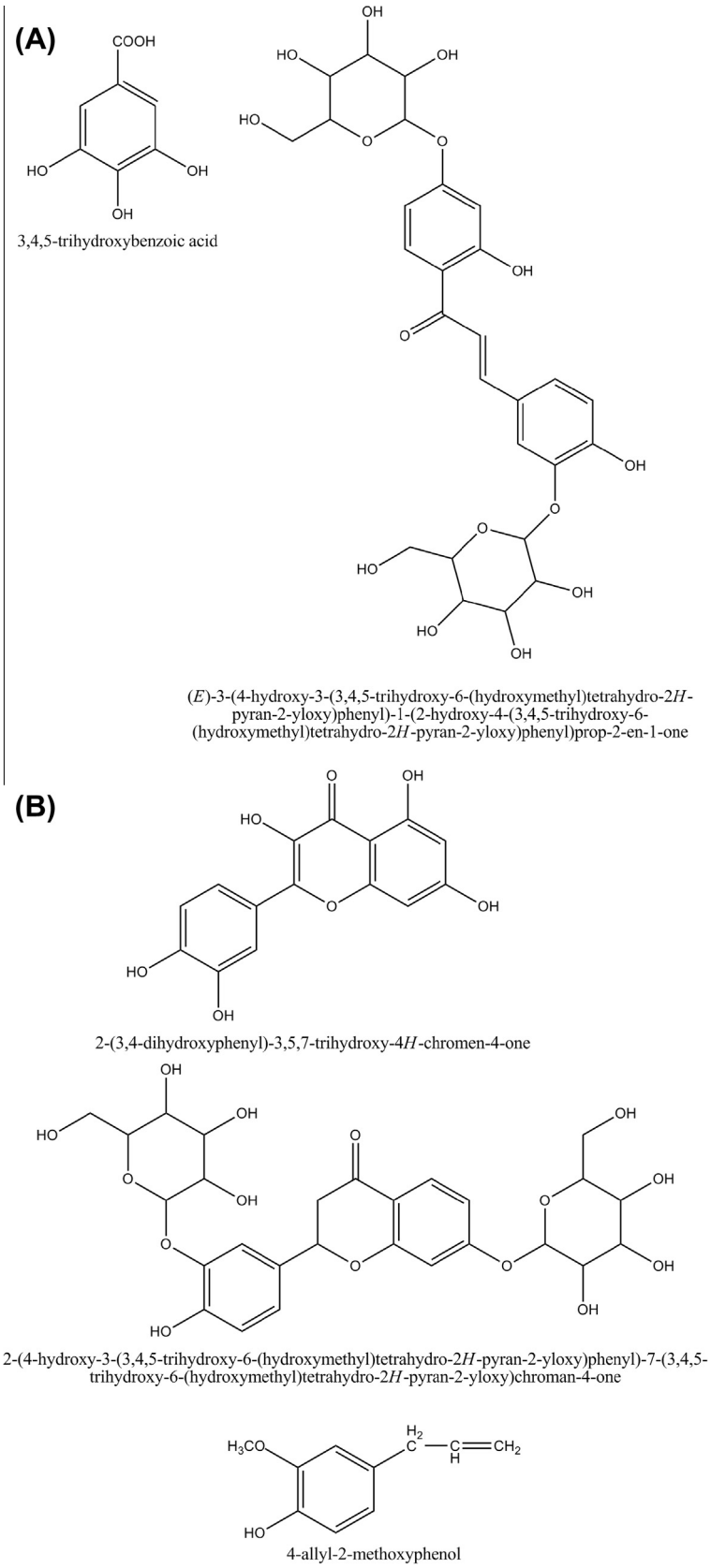

Figure 1 (A and B) Possible structures of all five components separated from extracts of *B. monosperma* and *H. coronarium*.

2.4.3. Apparatus and conditions

Separation of phenolics and flavonoid in the crude extract of *B. monosperma* and *H. coronarium* was achieved on quaternary gradient semi-prep HPLC system with a PDA Detector and 600e Multi Solvent Delivery System from Waters (USA) and a Xterra MSC-18 column (7.8 × 100 mm, 5 μm) with octadecylsilane as a solid support was used to separate the components. The data were analysed and processed using the installed Empower 2 software. The column was maintained at room temperature and the flow rate of the mobile phase was kept at 1.0 mL/min, which consists of methanol + acetonitrile: phosphate buffer. Sample volume of 5000-μL was kept with the help of a Rheodyne 77251 injector and compounds were detected between 190 and 400 nm with 2996 Photo Diode Array Detector. The separated flavonoids, phenolic acid and polyalcohol were initially identified by a direct comparison of their retention times with those of standards. The standard addition spike method was employed to identify each fractions of the crude extract with the aid of increase in the respective peak areas. This procedure was performed separately for each standard. The flavonoids, phenolic acid and polyalcohol contents were calculated from the peak areas of HPLC chromatograms from the five replicate samples.

3. Results and discussion

The effect of solvent on the extraction of flavonoids and phenolic acid, the methanolic extracts of both species was used to identify three flavonoids (quercetin, iso-butrin and butrin) and one phenolic acid (gallic acid) and one polyalcohol (eugenol). Flavonoids, phenolic acid and one polyalcohol occur naturally in plants as glycosides. The extracts were hydrolysed prior to investigation for bracking glycoside linkage, which yields the maximum recovery of active components from the extracts.

The method developed for HPLC fingerprinting provided a quick analysis of the crude extracts of the two species. The compounds were identified a by comparison with the chromatogram of the five reference standards obtained under the specified experimental conditions. The present method was a quick and accurate method for the separation of three flavonoids one phenolic acid and one polyalcohol with a run time of 90 min.

3.1. The selection of the conditions of chromatographic separation

Semi-preparative version of HPLC was used with the column having chemically attached octadecylsilane groups and eluents of different compositions were usually employed in analytical practice for the separation of flavonoids, phenolic and

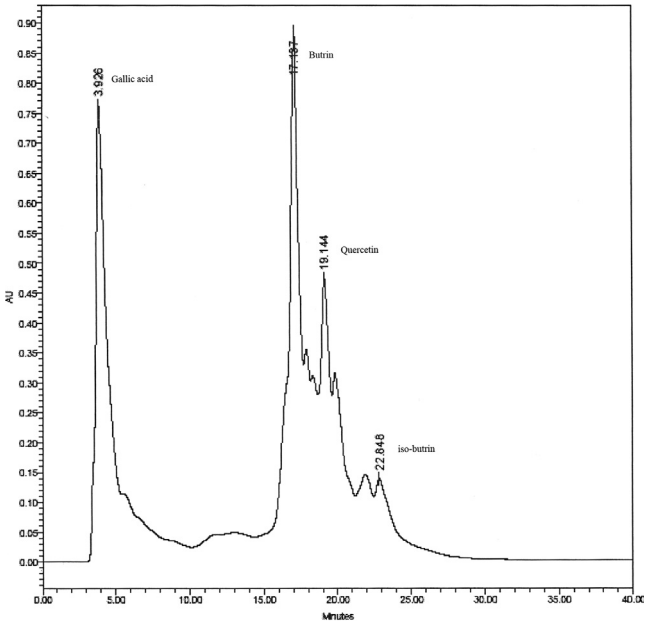

Figure 2 HPLC chromatogram of the extract of *B. monosperma* root with 1 mg/mL concentration in the gradient elution system of eluent A (methanol–acetonitrile) and eluent B (10 mM ammonium acetate with glacial acetic acid to maintain pH 4.14) with a flow rate 1 mL/min and column thermosetting at 30 °C.

Table 2 HPLC chromatographic data for different fraction from *B. monosperma* and *H. coronarium* with respective percentage area of active components in each fraction of flower and roots.

Species	Part used	Sample no.[a]	Gallic acid (t_R = 3.724 min) % Area	Butrin (t_R = 17.365 min) % Area	Quercetin (t_R = 20.288 min) % Area	Iso-butrin (t_R = 28.511 min) % Area	Eugenol (t_R = 3.652 min) % Area
Butea monosperma	Flower	1	ND	67.20	ND	ND	ND
		2	ND	ND	ND	ND	ND
		3	ND	ND	51.67	ND	ND
		4	ND	51.45	ND	1.80	ND
		5	ND	ND	ND	0.51	ND
	Root	1	33.12	ND	ND	ND	ND
		2	45.87	ND	39.43	ND	ND
		3	ND	ND	ND	ND	ND
Hedychium coronarium	Flower	1	ND	ND	ND	ND	56.31
		2	ND	ND	ND	ND	34.28
		3	ND	ND	ND	ND	70.58

ND = not detected.

[a] Five fractions of *B. monosperma* flower; three fractions of *B. monosperma* root and three factions of *H. coronarium* flower were used as different samples.

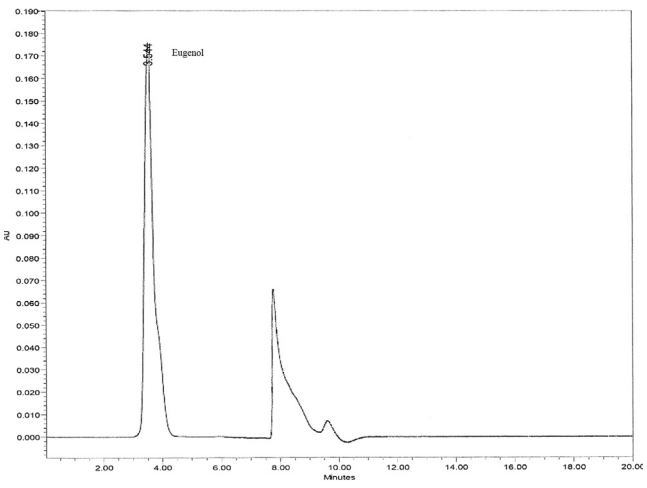

Figure 3 HPLC chromatogram of *H. coronarium* flower eluent A 10% vol. (methanol–acetonitrile) and eluent B 90% vol. (10 mM ammonium acetate with glacial acetic acid to maintain pH 4.14) with flow rate 1 mL/min and column thermosetting at 30 °C.

polyalcohol from *B. monosperma* and *H. coronarium*. The main component of the eluent was usually isopropanol, methanol and acetonitrile. Isopropanol or methanol is normally used to achieve separation of phenolic components but introduction of a small amount of acetonitrile into the mobile phase leads to batter separation of chromatographic peaks.

Peak broadening was one of the possibilities due to dissociation of compounds and hence, pH 4.14 was maintained throughout the experiment by adding glacial acetic acid in eluent B (10 mM ammonium acetate). The gradient programme was employed to elute active ingredients from crude extract of *B. monosperma* and *H. coronarium*.

The gradient programme was initiated with 5% vol. of eluent A and 95% vol. of eluent B (gradient 1) and it was allowed to travel up to 30 min. The 50% vol.:50% vol. of eluent A and eluent B (gradient 5) was achieved with a linear ramp in both solvents and flow rate within 15 min. The gradient was reverted to original conditions within next 5 min. The baseline was allowed to stabilize for 25 min before injecting the actual samples. Separation of active components was achieved successfully within a total run time of 90 min. The result of chromatogram was suggestive that three flavonoids and one phenolic acid were separated with the mobile phase of gradient-1 to gradient-4 in isocratic manner from *B. monosperma*. The possible structures of reported components were depicted in Fig. 1 and corresponding chromatographic data are summarized in Table 2.

Optimization of experimental condition in liquid chromatography is a major task for an analyst. pH of gradient eluent, organic subtract, nature of mobile phase and temperature must be taken into account and therefore, the composition of eluent A was increased from 5% vol. to 40% vol. stated as gradient-4. This was done to improve the resolution of chromatographic peaks. The chromatogram of eluted components was depicted in Fig. 2. The figure suggests that gallic acid was poorly retained in the stationary phase, which indicates lower composition of methanol.

The selectivity and resolution of the tested substances were increased in methanol and a phosphate buffer solution. The temperature of the chromatographic process was varied from 25 to 60 °C at 5 °C/min ramp to separate each elute with batter resolution. Looking to the resolution of each peak of phenolic acid and flavonoids the optimum temperature was found at 30 °C. The chromatogram of actual sample of *H. coronarium* flower was represented in Fig. 3. The separation parameters along with wavelength maximum were summarized in Table 3, which suggest that all five components exhibit UV absorption band via $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions.

4. Conclusion

Finally, optimum conditions for separation of five components; three flavonoids, one phenolic acid and one polyalcohol were achieved successfully. Gradient 3 that is eluent A methanol–acetonitrile and eluent B (10 mM ammonium acetate with glacial acetic acid to maintain pH 4.14) with flow rate 1 mL/min and column thermosetting at 30 °C was used to elute eugenol. The retention time was found to be varied with the presence of the hydroxyl and methoxy group in the structure of phenolic acid. It was observed that retention time of elute increased with less number of the hydroxyl group and increased with addition of the methoxy group. The retention time of phenolcarbonic acids showed the following pattern for separation under the specified experimental conditions. The proposed method of semi-prep HPLC was successfully used for the separation of three flavonoids, one phenolic acid and one polyalcohol with the lower limit of quantification of phenolic acid, flavonoids and eugenol are 0.050–0.150 μg/mL was determined by the least square method. A good correlation was obtained for all separated components. The recovery data of each component with the help of the standard spike addition method were summarized in Table 3.

Table 3 Quantity of components recovered from different extracts of *B. monosperma* and *H. coronarium* after partitioning.

Compounds	*B. monosperma*			*H. coronaium*			UV absorption band (nm)
	Methanol (mg)	95% Ethanol (mg)	Water (mg)	Methanol (mg)	95% Ethanol (mg)	Water (mg)	
Gallic acid	10.55	12.65	16.01	ND	ND	ND	267.5
Butrin	6.75	9.60	12.22	ND	ND	ND	272 and 315
Quercetine	6.82	10.68	14.10	ND	ND	ND	255.7 and 368.5
Iso-butrin	7.62	8.89	6.24	ND	ND	ND	239 and 373
Eugenol	ND	ND	ND	2.22	2.64	4.31	236.8 and 281.7

ND = not detected.
Standard deviation = 0.079 and % RSD = 1.820 for all calculations.

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