ORIGINAL ARTICLE

Antioxidant markers based TLC-DPPH differentiation on four commercialized botanical sources of *Shankhpushpi* (A Medhya Rasayana): A preliminary assessment

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ABSTRACT

Shankhpushpi is a cognition boosting traditional ayurvedic brain supplement. *Convolvulus pluricaulis* (Convolvulaceae), *Evolvulus alsinoides* (Convolvulaceae), *Clitoria ternatea* (Papilionaceae), and *Canscora decussata* (Gentianaceae) are botanical claimants of *Shankhpushpi*. This investigation is to focus the identification of the compound based on biological marker differentiation of four botanical claimants of *Shankhpushpi* for their antioxidant evaluation on thin layer chromatography (TLC) by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. A rapid TLC-DPPH method was developed to identify and differentiate four botanical claimants of *Shankhpushpi* in terms of presence of β-carotene, rutin, scopoletin, chlorogenic acid, and mangiferin. *C. pluricaulis* shows presence of scopoletin; *E. alsinoides* shows presence of β-carotene, scopoletin, and chlorogenic acid; *C. ternatea* shows presence of β-carotene, scopoletin, and rutin; and *C. decussata* shows presence of β-carotene, scopoletin, and mangiferin. The order, they followed, based on their antioxidant potential is β-carotene < mangiferin < rutin < scopoletin < chlorogenic acid. Antioxidants are attributed for their beneficial role in age-related cognition decline. The proposed method provides an edge in terms of identification and quantification of antioxidant constituents in a multi-component system. This method may also provide application for identification of correct plant sources used in the name of *Shankhpushpi* in marketed ayurvedic formulation, food supplement, and extracts.

Key words: Antioxidant, cognition, controversial, separation, *Shankhpushpi*

INTRODUCTION

Recent clinical and animal studies have identified nutritional intervention as a viable method to curtail the cognitive aging process. Further studies investigating nutritional modulation of age-related cognitive decline have focused on foods rich in antioxidants and essential fatty acids.[1] The restorative effects of polyphenols and flavonoid-rich foods on age-related cognitive and motor dysfunctions have been repeatedly proved.[2,3] Clinical studies have shown that antioxidants improve cognition decline particularly in older people.[4,5]

The colorimetric estimation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a simple method for identifying antioxidants but it is not applicable to colored extract/fraction due to interference by pigments, and it measures the total DPPH radical-scavenging activity of the extract/fraction but it lacks the ability to identify the activity of only one or many constituents of the extract/fraction.[6] Thin layer chromatography (TLC) combined with DPPH radical detection of antioxidants *in situ* has been reported and similar methods have been used for the screening of antioxidants

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produced by marine bacteria and antioxidants present in plant extracts.[7] Quantitative free radical-scavenging activity of individual compounds by high-performance thin layer chromatography (HPTLC) with diode array detector has been reported in plant extracts.[8] Abourashed[9] has reported a TLC-DPPH method to screen the free radical-scavenging capacity without any chromatographic development.

In ayurvedic literatures many a times, two or more entirely different plant species are recognized by one common vernacular name, thus raising controversy to the correct identity of the source.[10,11] One such controversial name is *Shankhpushpi*, a "Medhya Rasayana drug" meaning drug which nourishes the brain. The sources comprise of entire herbs with following botanicals viz., *Convolvulus pluricaulis* Choisy. (Convolvulaceae), *Evolvulus alsinoides* Linn. (Convolvulaceae), *Clitoria ternatea* Linn. (Papilionaceae), and *Canscora decussata* Schult (CD) (Gentianaceae).[12-14] *Shankhpushpi* is a cognition boosting ayurvedic medicine mentioned for its therapeutic effect on CNS disorders such as insanity, epilepsy, nervous debility, and memory enhancement.[15-20] Many formulations containing *Shankhpushpi* as a single drug or in combination with other drugs are available in Indian market and *Shankhpushpi* is vigorously advertised for memory enhancement in print and electronic media in India.

As antioxidants are attributed for their beneficial role in age-related cognition decline, identification of free radical-scavenging markers may serve both the purpose: To solve the existing controversy among four botanical claimants of *Shankhpushpi* and determination of antioxidant potency simultaneously. This study was planned to develop a rapid TLC-DPPH method that can be used to identify and differentiate the botanical claimants of *Shankhpushpi* in terms of antioxidant compounds.

MATERIALS AND METHODS

Reagents and Chemicals

DPPH, β-carotene, and rutin were obtained from Himedia Laboratories (Mumbai, India). Mangiferin from Sigma Aldrich (Mumbai, India), chlorogenic acid from Sisco Research Laboratories (Mumbai, India), and scopoletin were obtained as gift sample from Laila Impex Laboratory (Vijayawada, India), other solvents and chemicals were of analytical grade. Pre-coated silica gel $60F_{254}$ TLC plates were purchased from Merck (Darmstadt, Germany).

Plant Material

C. decussata was collected from the Ninai ghat (Gujarat, India) in the month of October and identified by Dr. S.C. Agrawal (Department of Botany, Central Drug Research Institute, Lucknow). Whereas *C. pluricaulis*, *E. alsinoides*, and *C. ternatea were collected from locality of Vadodara (Gujarat, India)* and identified in the Department of Botany, The M S University of Baroda, Vadodara (Gujarat, India). Voucher specimens of all four plants (No. Pharmacy/EA/09-10/10/NS, Pharmacy/CP/09-10/11/NS, Pharmacy/CT/09-10/12/NS, and Pharmacy/CD/09-10/13/NS) have been deposited in Herbal Drug Technology Department, The M. S. University of Baroda, Vadodara (Gujarat, India).

Extraction

All herbs were shade dried at room temperature and coarsely powdered. Accurately weighed 5 g of dried coarse powder of CP, EA, CT, and CD (whole herb) were extracted separately with methanol (3 × 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated on a rotary evaporator, and transferred to a 50-ml volumetric flask and the volume was made up with methanol.[21]

High-performance thin layer Chromatography Equipment

A Camag TLC system equipped with Camag Linomat V (CAMAG, Switzerland) with an automatic TLC sample spotter, Camag glass twin trough chamber (20 × 10 cm), Camag scanner 3, and integrated winCATS 4 software (Synectica Limited, London, UK) were used for the analysis. TLC was performed on 20 × 10 cm pre-coated plate. Samples and standards were applied on the plate as 8 mm wide bands with an automatic TLC sampler (Linomat V) under a flow of nitrogen gas, 10 mm from the bottom and 10 mm from the side and the space between two spots were 15 mm of the plate. The linear ascending development was carried out in a Camag twin trough chamber (20 × 10 cm) which was pre-saturated with 20 ml mobile phase for 20 min at room temperature (25 ± 2°C and 40% relative humidity). The length of the chromatogram run was 8 cm. Subsequent to chromatographic development, TLC plates were dried in current air with the help of an air dryer.[22]

Determination of Radical-scavenging activity by TLC-DPPH Method

The post-chromatographic derivatization was carried out with DPPH (0.2% in methanol) for 3 s in immersion chamber (Camag). The plates were scanned before DPPH and 30 min after DPPH derivatization in absorption–reflection mode using a slit width of 6 × 0.45 mm and data resolution 100 μm/step and scanning speed 20 mm/s at optimized wavelengths. Regression analysis statistical data were generated by GraphPad® 3.0 (San Diego, California, USA) for Windows.

Optimization with Reference Standards

β-carotene, rutin, scopoletin, chlorogenic acid, and mangiferin [Figure 1] were used as reference standards. Stock solutions (12 μg/ml) of all the reference standards were prepared in methanol and different concentrations (300, 600, 900, and 1200 ng/spot) of each reference standards were applied in triplicate on a TLC plate and developed in

respective mobile phase. The separated bands were scanned before and 30 min after DPPH derivatization and a four-level concentration–percentage area reduction curve was constructed. The concentration at 50% reduction in area was calculated for each reference standard from the calibration curve.

$$\text{Percentage area reduction} = \frac{(PA_b - PA_a)}{PA_b} \times 100$$

where PA_b is the peak area of the spot before DPPH derivatization and PA_a is the peak area of the spot after DPPH derivatization.

Identification of Differentiable Antioxidant Markers

All the four botanical claimants were simultaneously developed in various mobile phases and derivatized with DPPH to find the antioxidant markers which would differentiate the claimants. These antioxidant markers found in extracts were identified by co-TLC with standard compounds and further confirmed by R_f comparison, multi-wavelength scanning, and spectral overlay. β-Carotene, rutin, scopoletin, chlorogenic acid, and mangiferin were identified as the differentiable antioxidant markers.

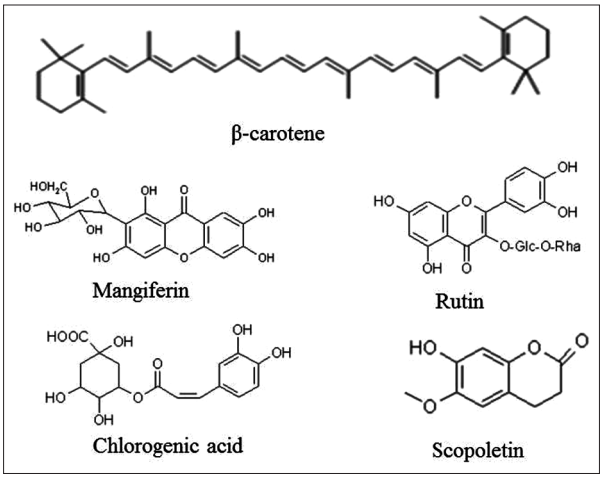

Figure 1: Chemical structures of studied antioxidant compound

TLC-DPPH for *shankhpushpi* claimants

Ten microliters of the extracts and the marketed formulation (Brain Tab, Baidyanath, India) in triplicate were applied on a TLC plate and developed in respective mobile phases along with β-carotene, rutin, scopoletin, chlorogenic acid, and mangiferin (300, 600, 900, and 1200 ng/spot). The identified antioxidant markers in extracts were quantified from the calibration curve of peak area versus concentration of standard compounds. After 30 min, the plates were derivatized with DPPH and the identified antioxidant markers in extracts were again quantified from the calibration curve of percentage area reduction versus concentration of standard compounds.

RESULTS

Densitogram of all reference antioxidants (β-carotene, rutin, scopoletin, chlorogenic acid, and mangiferin) after DPPH derivatization exhibited concentration-dependent reduction in peak area [Figure 2]. Polynomial second-degree calibration equation calculated for the reference standards was found to give satisfactory correlation between concentration and percentage area reduction, with regression coefficients of $r^2 = 0.9987$, 0.9961, 0.9959, 0.9941, and 0.9996 for β-carotene, rutin, scopoletin, chlorogenic acid, and mangiferin, respectively [Table 1, Figure 3]. The antioxidant potency of reference antioxidants was analyzed by calculating the concentration at which there was 50% reduction in area, they followed the order β-carotene < mangiferin < rutin < scopoletin < chlorogenic acid [Table 1]. The mobile phase used, R_f, scanned wavelength, calibration equation, regression coefficients, and the concentration at which 50% reduction in area was obtained are shown in Table 1. β-Carotene was detected in EA, CT, and CD; rutin was detected in CT; mangiferin was detected in CD; chlorogenic acid was detected in EA; and scopoletin was detected in CP, EA, CT, and CD [Table 2]. The marketed formulation (Brain Tab) showed the presence of scopoletin and Mangiferin, which concludes to contain CD as a source of *Shankpushpi*.

The amount of identified antioxidants quantified before derivatization and after DPPH derivatization is shown

Table 1: Chromatographic conditions, linearity parameters, and concentration of 50% reduction in peak area of antioxidants

Antioxidants	Mobile phase	R_f	λ_{max} (nm)	Calibration equation	r^2	Concentration at 50% reduction in peak area (ng)*
β-Carotene	*n*-Hexane/C_6H_6 (9:1)	0.55	425	$Y=-0.18X^2-10.284X+83.735$	0.9987	922.67±1.153
Rutin	EtOAc/HCO_2H/HOAc/H_2O (100:11:11:26)	0.35	380	$Y=4.845X^2-35.585X+91.66$	0.9961	462.33±4.485
Scopoletin	$CHCl_3$/MeOH/toluene (8:1:1)	0.81	350	$Y=1.6425X^2-12.842X+42.533$	0.9959	312.10±1.12
Chlorogenic acid	EtOAc/HCO_2H/HOAc/H_2O (100:11:11:26)	0.45	350	$Y=0.665X^2-13.721X+95.05$	0.994	304.14±0.84
Mangiferin	EtOAc/HCO_2H/HOAc/H_2O (100:11:11:26)	0.50	366	$Y=5.925X^2-47.583X+114.52$	0.9996	521.66±10.41

*Values are expressed as mean±SEM ($n=3$), C_6H_6: Benzene, EtOAc: Ethyl acetate, HCO_2H: Formic acid, HOAc: Acetic acid, H_2O: Water, $CHCl_3$: Chloroform, MeOH: Methanol

in Table 3. The four claimants for *Shankpushpi* were also analyzed similarly.

DISCUSSION

DPPH radical-scavenging compounds appeared as yellow spots against a purple background. When reverse phase TLC plates were used with DPPH as detecting agent, the developing color proved to be very unstable, but in normal TLC plates the coloration produced after spraying with DPPH has been proved to be relatively stable, enabling the identification of radical-scavenging activity after a period of 30 min.[23,24] Densitogram of all reference standards (β-carotene, rutin, scopoletin, chlorogenic acid, and mangiferin) after DPPH derivatization exhibited concentration-dependent reduction in peak area. Polynomial second-degree calibration equation calculated for the reference standards was found to give satisfactory correlation between concentration and percentage area reduction. Concentration-dependent reduction in peak area of all reference standards (β-carotene, rutin, scopoletin, chlorogenic acid, and mangiferin) after DPPH derivatization proved that concentration at 50% reduction in peak area can be used to assess the antioxidant potency of compound. Chlorogenic acid was found to be the most active DPPH radical scavenger and β-carotene exhibited the least activity in this method.

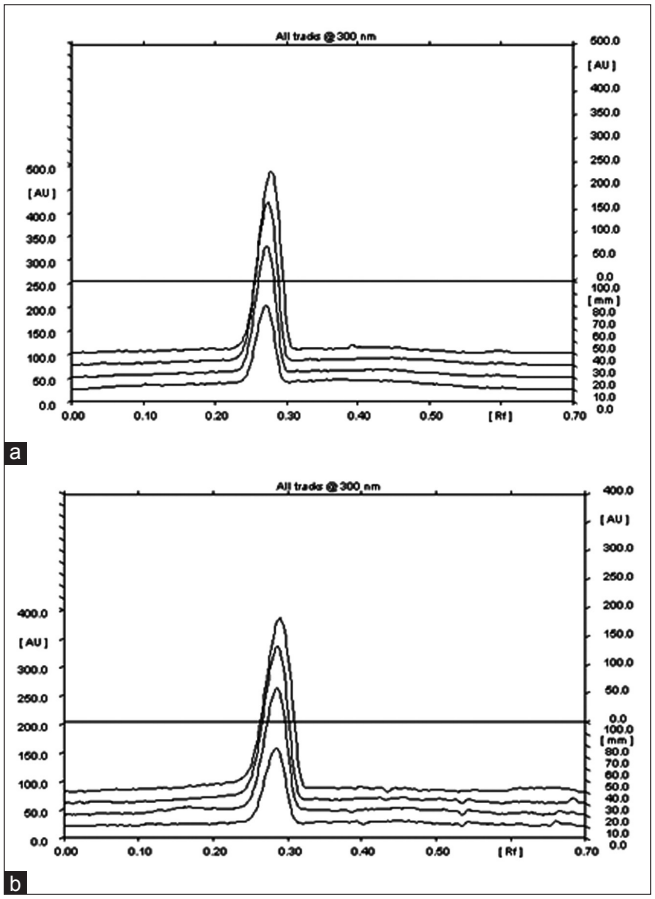

Figure 2: Densitogram showing peak area of β-carotene. (a) Peak area of β-carotene before 2,2-diphenyl-1-picrylhydrazyl (DPPH) derivatization, (b) peak area of β-carotene after DPPH derivatization

The presence and absence of antioxidants, β-carotene, rutin, scopoletin, chlorogenic acid, and mangiferin, can be used to differentiate the four botanical claimants in polyherbal formulation containing *Shankhpushpi* as ingredient. Scopoletin in all four claimants and mangiferin in CD have been reported.[25] β-Carotene in EA, CT, CD; rutin in CT; and chlorogenic acid in EA were identified for the first time by co-TLC. Presence of scopoletin in all four claimants makes it unqualified for differentiation but presence of scopoletin along with presence or absence of rutin, mangiferin, β-carotene, and chlorogenic acid could provide valuable inference. The differentiable antioxidant markers were quantified before and after DPPH derivatization to find whether

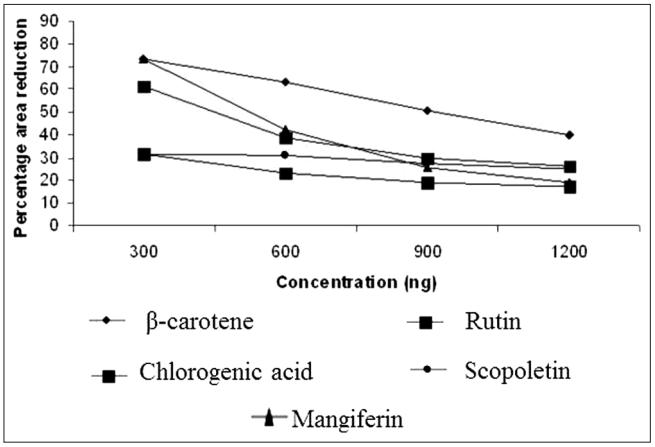

Figure 3: Concentration–percentage area reduction calibration curves

Table 2: Antioxidants for differentiating the four claimants of *Shankhpushpi*

Botanical claimants of *Shankhpushpi* and its marketed formulation	β-Carotene	Rutin	Mangiferin	Chlorogenic acid	Scopoletin
Convolvulus pluricaulis	−	−	−	−	+
Evolvulus alsinoides	+	−	−	+	+
Clitoria ternatea	+	+	−	−	+
Canscora decussate	+	−	+	−	+
Brain Tab	−	−	+	−	+

(+): Present, (−): Absent

Table 3: Identified antioxidant markers to differentiate between the claimants for *Shankhpushpi* and their quantification before and after 2,2-diphenyl-1-picrylhydrazyl (DPPH) derivatization

Claimants of	β-Carotene (ng)		Rutin (ng)		Mangiferin (ng)		Chlorogenic acid (ng)		Scopoletin (ng)	
*Shankhpushpi**	Aqb	Aqa	Aqb	Aqa	Aqb	Aqa	Aqb	Aqa	Aqb	Aqa
CP	–	–	–	–	–	–	–	–	604.41±0.75	590±17.32
EA	564.93±1.39	523.33±27.28	–	–	–	–	985.56±0.72	973.33±70.23	797.23±1.27	816.66±28.86
CT	459.40±3.01	483.33±20.27	766.88±1.60	790±52.91	–	–	–	–	<300	<300
CD	336.79±1.85	356.67±27.28	–	–	994.30±0.46	985.56±0.72	–	–	<300	<300

*Amount quantified is for 10 µl of all samples, Values expressed as mean±SEM (n=3), (–): Absent, Aqb: Amount quantified before DPPH derivatization, Aqa: Amount quantified after DPPH derivatization

identification and quantification can be estimated simultaneously, but the quantification after DPPH derivatization showed a lot of variation. However, quantification after DPPH can be used to assess the relative antioxidant potency within the individual constituents of extracts/fractions. The marketed formulation (Brain Tab) showed only the presence of scopoletin and mangiferin providing the evidence for the presence of *C. decussata*. The analyses of marketed formulation (Brain Tab) proved again the utility of this method in identifying the claimant used as *Shankhpushpi*.

Antioxidants impart direct effects on signaling to enhance neuronal communication, can buffer against excess calcium, enhance neuroprotective adaptations, reduce stress signals, regulates extracellular signal for kinase activation, increases insulin-like growth factor I, and regulates mitogen-activated protein kinase and other signaling pathways at the level of transcription.[26,27] These findings suggest that the putative signal-modifying properties of antioxidants may significantly contribute to the cognitive and behavioral improvement. The identified antioxidant markers, β-carotene, rutin, mangiferin, scopoletin, and chlorogenic acid, have been reported to improve age-related cognition decline.[28-31] Differentiating the four botanical claimants for *Shankhpushpi* in terms of biological markers served both purpose of identification and determination of antioxidant potency.

CONCLUSION

In this study, significant differences in content of β-carotene, rutin, scopoletin, chlorogenic acid, and mangiferin in different varieties of S*hankhpushpi* were found. The concentration–reduction in peak area curves of the samples could be compared to the concentration–reduction in peak area curves of standard antioxidant compound in terms of relative radical-scavenging activities. This proposed method can also be utilized for the bioassay-guided isolation of unidentified natural antioxidants and can be used for selection of potential antioxidants from a group of structurally diverse compounds. The current application also demonstrates the versatility and adaptability of a standard HPTLC system to serve an additional purpose in the drug discovery arena. Although DPPH spectrophotometric methods are ubiquitously available, the proposed method provides an edge in terms of identification and quantification of antioxidant constituent/s in a multi-component system, a simple and cost-effective alternative to the established methods.

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