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Research Article

Evaluation of Antioxidant Properties of Flower Heads of *Sphaeranthus indicus* Linn

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ABSTRACT

Methanol extract (ME) of flowerheads *Sphaeranthus indicus* Linn. and its chromatographic methanol fraction (CMF) were screened for *in-vitro* antioxidant activity subjecting to DPPH free radical scavenging assay, superoxide scavenging assay, *in-vitro* lipid peroxidation and determination of reducing power. Both ME and CMF showed potent *in-vitro* antioxidant activity, whereas, CMF was more effective as free radical scavenger than ME. HPTLC fingerprint were undertaken for identification of active components. Flower-heads of *Sphaeranthus indicus* demonstrated *in-vitro* antioxidant activity in present study.

INTRODUCTION

Experimental evidence suggests that free radicals (FR) and reactive oxygen species (ROS) can be involved in high number of diseases [1]. As plant produce lot of chemical moieties as antioxidants to control oxidative stress caused by sunbeams and oxygen, these can serve as source of new antioxidant compounds [2]. Ayurveda, an ancient Indian system of medicine, follow in prevention and treatment of various diseases a group of large number of medicinal plants which are considered active due to chemical constituents and are designated as '*Rasayana*' as one of the clinical specialties. Rasayana is not only a drug therapy but is a specialized procedure practiced in the form of rejuvenating recipes, dietary regimen promoting good habits. The purpose of rasayana is two-fold: prevention of disease and counteracting aging process, which result from optimization of homeostasis [3]. Sharma et al. reported the strong antioxidant activity shown by rasayana drugs [4]. Around 34 plants are identified as Rasayanas in the Ayurvedic system of medicine [5].

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These plants are described to possess various pharmacological properties such as immunostimulant, tonic, neurostimulant, anti-ageing, anti-bacterial, anti-viral, anti-rheumatic, anti-cancer, adaptogenic, anti-stress etc. Among these plants enlisted, some have been specifically investigated for their well demonstrated antioxidant activity [6].

Sphaeranthus indicus Linn. (Family-*Compositae*) is an herb found mostly in southern India. It is a bitter stomachic, stimulant, alterative, pectoral and demulcent, and externally emollient. Distilled water prepared like rose water from the herb is recommended by Hakims (Physicians of Unani System of Medicine) for bilious affections and for dispersions of various kinds of tumors. Flowers (flower heads) are highly esteemed as alternatives, depuratives, refrigerants and tonics, useful as blood purifiers in skin diseases. The drug is also useful in urethral discharges and in jaundice [7]. It is one of the Rasayana drugs of Ayurveda and the activity is attributed to flower heads of the plant. The objective of the present investigation was therefore set to study the antioxidant activity of flower heads of *S.indicus* using *in-vitro* methods.

METHODS

Plant material

Fresh flower heads of *S.indicus* were collected from the outfield of Baroda city, India and

authenticated in Botany Department of M.S.University, Baroda, India.

Extract preparation and fractionation of extracts

Separate maceration of air-dried, powdered flower heads of *S.indicus* afforded methanol extract (ME) in 6.65% (w/w) yield. M E (40 g) was fractionated by loading on silica gel (100-200 mesh) and eluted with petroleum ether (5.23 g), benzene (5.76 g), chloroform (1.30 g) and methanol (CMF) (24.4 g). ME gave positive tests for alkaloids, phenolic compounds, phytosterols and terpenoids on phytochemical screening, whereas CMF retained mainly phenolic compounds and alkaloids.

TLC finger print profile

HPTLC finger print profile was established for both ME and CMF. A stock solution (1mg/ml) was prepared in methanol. Suitably diluted stock solution was spotted on pre-coated Silica gel G60 F254 TLC plates using CAMAG Linomat V Automatic Sample Spotter and the plates were developed in solvent systems of different polarities to resolve polar and non-polar components of the bioactive fraction. The plates were scanned using TLC Scanner 3 (CAMAG) at 254 nm (absorbance/reflectance mode) and 366 nm (fluorescence/reflectance mode) and Rf values, spectra, λ_{max} and peak areas of resolved bands were recorded. Relative percentage area of each of bands was calculated from peak areas. Developed chromatograms in different solvent systems were then sprayed with phosphomolybdic acid regent and NP-PEG reagent to detect phenolic compounds and flavonoids type of compounds respectively. Figure 1, shows characteristic chromatograms.

***In-vitro* antioxidant activity**

Assay for antiradical activity with DPPH

Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanolic solution of colored 1, 1, diphenyl picryl hydrazine brought about by sample [8]. A stock solution of DPPH was prepared by dissolving 4.4 mg in 3.3 ml methanol. Test medium includes 150µl of DPPH solution along with different concentration of samples in 3 ml methanol. Blank was performed in the same way with no sample added. Decrease in absorbance, in presence of sample was noted after 15 minutes. EC_{50} was calculated as 50% reduction in absorbance brought about by sample compared with blank.

Assay for superoxide radical scavenging activity

The assay was based on capacity of the sample to inhibit blue formazon formation by scavenging the superoxide radicals generated in riboflavin-light-nitro blue tetrazolium (NBT) system [9]. The reaction medium contains phosphate buffer (pH 7.6) 2.5 ml, 100µl riboflavin (20µg), 200µl EDTA (12mM), 100µl NBT (0.1 mg) and different concentration of sample contained in 100µl of methanol. The reaction was started by illuminating the reaction mixture for 5 minutes. The absorbance was measured at 590 nm. Blank was performed in the same way with 100µl of methanol instead of test substance. EC_{50} was calculated as 50% reduction in absorbance brought about by sample compared with blank.

Determination of reducing power

The reducing power of ME and CMF was determined according to the method of Oyaizu [10]. Samples were mixed with 5 ml phosphate buffer (2M, pH 6.6) and 5 ml potassium ferricyanide (1%), the mixture was then incubated at 50^0 C for 20 minutes, 5 ml trichloroacetic acid (10%) was added an the mixture was centrifuged at 4000 rev./ min. The upper 5 ml solution was then mixed with 5 ml distilled water and 1 ml ferric chloride (0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid (0.3 mg) was used as standard.

Measurement of effect on lipid peroxidation on rat liver homogenate

Rat liver homogenate was prepared by homogenizing the tissue in chilled Tris buffer (10mM, pH 7.4) at a concentration of 10% w/v; peroxidation was induced in liver tissue by Iron-ADP complex in the presence of ascorbic acid. The incubation medium constituted 0.5 ml of the liver homogenate (10% w/v), 100 µM $FeCl_3$, 1.7 µM ADP, 500 µM of ascorbate and different concentrations of samples in 2 ml of total incubation medium. The medium was incubated for 20 min. at 37^0C. Extent of lipid peroxidation was measured by estimation of malondialdehyde (MDA) content [11]. Results were expressed in terms of decrease in MDA formation by the sample extract. Ascorbic acid was used as positive control.

RESULTS AND DISCUSSION

The participation of reactive oxygen species in etiology and physiopathology of human disease, such as neurodegenerative disorders, inflammation, viral infection, autoimmune pathologies and digestive system disorders such as gastrointestinal inflammation and gastric ulcers is already evident. The understand role of these reactive oxygen species in several disorders and potential antioxidant protective effect of natural compounds on affected tissues are topics of high current interest. Initially it is necessary to investigate in-vitro antioxidant properties of any natural product or drug to consider it as an antioxidant substance, followed by evaluation of its antioxidant function in biological systems [12].

In the present attempt therefore antioxidant activity of the ME and CMF of flower-heads of *S.indicus* was first evaluated *in-vitro* and the chemical composition of CMF being more active, was undertaken by establishing its TLC fingerprint profile.

In-vitro antioxidant activity

DPPH is a stable free radical in aqueous or ethanol solution and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [13]. In order to evaluate antioxidant potency through free radical scavenging with the test samples, the change in the optical density of DPPH radicals is monitored. Hence, DPPH· is usually used as a substrate to evaluate antioxidative activity of antioxidants [14]. ME and CMF showed a concentration dependent antiradical activity by inhibiting DPPH radical with an EC_{50} value of 806.26 µg/ml and 258.43 µg/ml respectively (Table 1). CMF showed almost three times more inhibitory activity on DPPH stable radical than ME. Standard curcumin showed EC_{50} at 52.71 µg/ml. CMF was found less potent than standard curcumin.

Superoxide radical is known to be very harmful to cellular components as precursor of more reactive oxygen species [15]. ME and CMF were found as scavenger of superoxide radical generated in riboflavin-NBT-light system *in-vitro.* EC_{50} values of ME and CMF were found 687.56 and 107.92 µg/ml respectively. CMF was six times more potent than ME but was lesser than ascorbic acid. EC_{50} of ascorbic acid was 23.52 µg/ml (Table 2).

Table 1: Antiradical activity of ME and CMF of *S.indicus* flowerheads observed with DPPH

Samples	Concentration (µg/ml)	% inhibition	EC 50 (µg/ml)
ME	250	17.81± 1.36	
	500	27.68 ± 1.82	806.26
	750	34.07 ± 0.81	
	1000	71.70 ± 1.69	
CMF	100	25.67 ± 0.20	258.43
	200	42.18 ± 1.43	
	300	54.21 ± 1.49	
	400	72.77 ± 1.40	
Curcumin			52.71

Values are mean ± S.E.M. of three replicate analyses.

Table 2: Superoxide anion scavenging activity of ME and CMF of *S.indicus* flowerheads observed with a riboflavin-light-NBT system

Samples	Concentration (µg/ml)	% inhibition	EC 50 (µg/ml)
ME	250	27.76 ± 1.48	687.56
	500	39.45 ± 2.09	
	750	49.96 ± 1.64	
	1000	69.30 ± 3.76	
CMF	50	30.14 ± 3.16	107.92
	100	51.20 ± 1.84	
	150	62.73 ± 1.70	
	200	76.51 ± 2.22	
Ascorbic acid			23.52

Values are mean ± S.E.M. of three replicate analyses

Table 4: Inhibition of lipid peroxidation induced by iron/ADP/ascorbate system in rat liver homogenate by ME and CMF of *S.indicus* flowerheads

Samples	Concentration (µg/ml)	% inhibition	EC 50 (µg/ml)
ME	200	18.65 ± 0.98	528.03
	400	39.25 ± 1.26	
	600	65.00 ± 2.22	
	800	75.58 ± 0.99	
	1000	80.25 ± 2.56	
CMF	100	23.74 ± 0.87	290.62
	200	40.37 ± 0.91	
	300	52.15 ± 2.43	
	400	66.23 ± 3.05	
	500	73.35 ± 1.29	
Ascorbic acid			30.05

Values are mean ± S.E.M. of three replicate analyses.

Table 3: Reducing power determination of different concentrations of ME and CMF of *S.indicus*

Sample	Reducing powers of different concentrations (mg/ml)			
	0.0	5.0	10.0	15.0
ME	0.028 ± 0.01	0.100 ± 0.07	0.220 ± 0.12	0.313 ± 0.10
CMF	0.028 ± 0.01	0.120 ± 0.09	0.250 ± 0.16	0.353 ± 0.20

Values are mean ± S.E.M. of three replicate analyses.
Ascorbic acid (0.3 mg) was used as standard, giving a reading of 0.430 at 700 nm.

Table5: TLC finger printing profile of bioactive fraction of *S.indicus*

Scanned at	Solvent system 1			Solvent system 2			Solvent system 3		
	Rf	λ_{max}	Relative %	Rf	λ_{max}	Relative %	Rf	λ_{max}	Relative %
254 nm	0.10	319	3.68	0.32	330	64.30	0.23	200	0.98
	0.19	348	1.71	0.45	203	5.31	0.31	323	2.60
	0.24	312	5.90	0.75	200	30.39	0.44	318	8.13
	0.35	607	3.19				0.49	324	1.24
	0.69	580	0.74				0.60	328	12.31
	0.81	233	9.68				0.81	228	47.09
	0.90	332	75.10				0.91	327	27.63
366 nm	0.07	323	8.54	0.09	328	4.87	0.11	324	3.60
	0.11	313	5.96	0.36	329	92.34	0.14	323	1.90
	0.15	335	7.45	0.42	203	3.79	0.35	324	6.90
	0.23	309	23.36				0.38	317	1.21
	0.38	580	30.32				0.49	327	2.17
	0.49	613	24.37				0.56	327	11.11
	0.56	223	7.41				0.72	329	54.14
	0.90	332	20.15				0.78	200	4.35
							0.89	296	13.59
							0.95	228	1.03

Solvent system 1. Toluene/Ethyl acetate (7:3 v/v); Solvent system 2. Ethyl acetate/Methanol/Water (10:1.35:1.00 v/v); Solvent system 3. n-butanol/glacial acetic acid/water (6:2:2 v/v).

The measurement of reductive ability was done by Fe3+-Fe2+ transformation in the presence of samples ME, CMF and standard antioxidant, ascorbic acid [10]. The reducing power is associated with antioxidant activity [14] . As shown in Table 3, ME and CMF had similar reductive capabilities as these showed reducing power comparable with standards at higher concentration i.e. at 15 mg/ml.

Lipid peroxidation is initiated by radicals attacking unsaturated fatty acids, and propagated by a chain reaction cycle [16]. Since unsaturated fatty acids are most important components of biological membranes and impart desirable properties upon the fluidity of cellular membrane structure, the peroxidation of unsaturated fatty acids in biological membranes leads to disruption of membrane structure and function [17]. In particular O^{-}_{2} and ·OH induce various injuries to the surrounding organs and play a vital role in some clinical disorders. Therefore removal of O^{-}_{2} and ·OH is the most effective defense of the living body against disease [18]. Any compound – natural or synthetic – with antioxidant properties might totally or partially alleviate this damage. In the present study ME and CMF showed potent inhibition of lipid peroxidation induced by Iron/ADP/Ascorbate complex in rat liver homogenate. EC_{50} values for ME and CMF were 528.03 and 290.62 µg/ml respectively. Both test samples showed dose dependent inhibition of lipid peroxidation. Standard ascorbic acid showed EC_{50} value 30.05 µg/ml (Table 4).

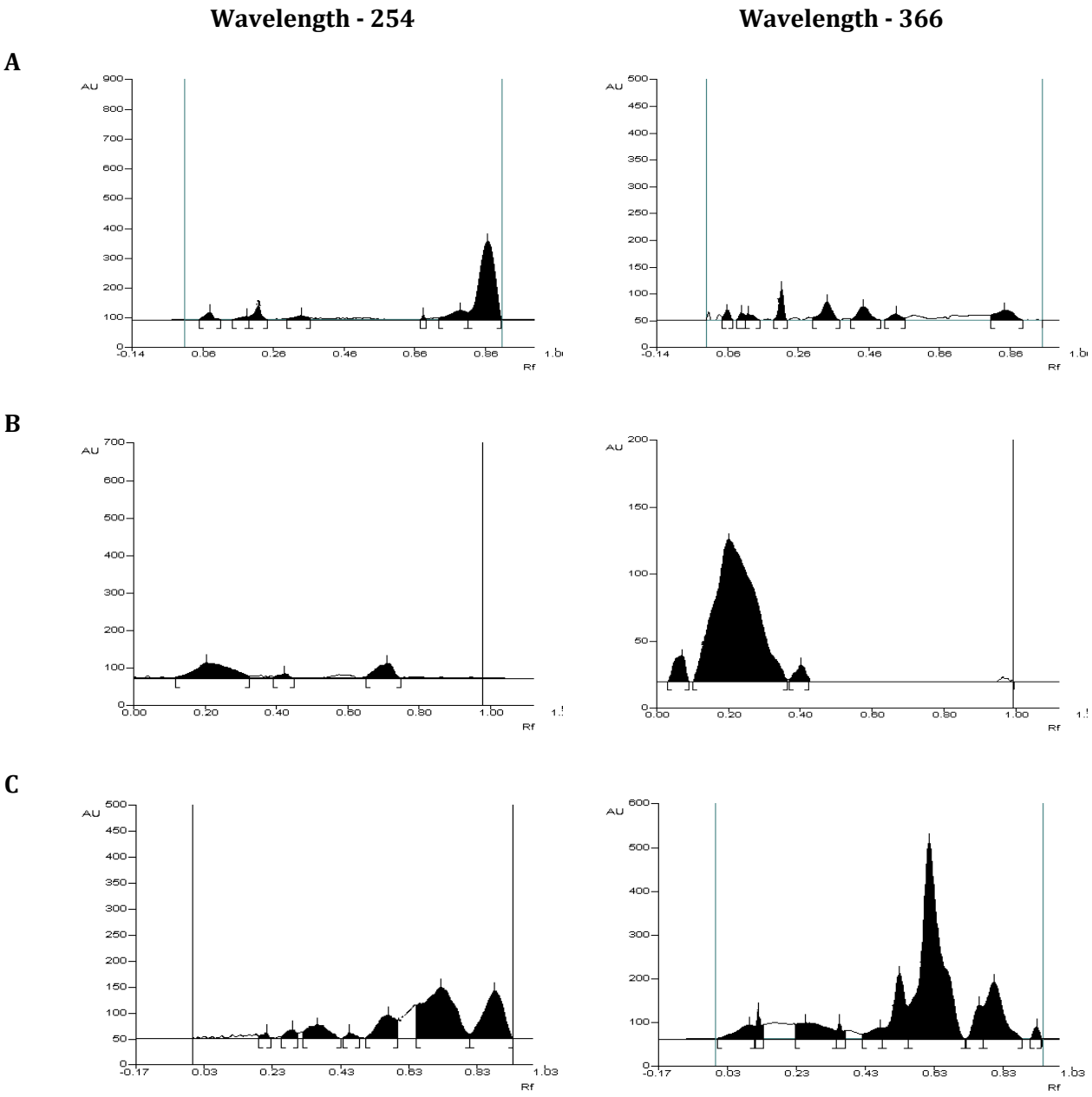

Figure 1: TLC chromatogram of bioactive fraction of *S.indicus* run in three different solvent system and scanned at 254 and 366 nm (solvent systems as in Table 3) **A-** Solvent system 1**,** B- Solvent system 2, **C-** Solvent system 3**.**

Recent studies showed that a number of plants products include polyphenolic substances such as flavonoids and tannins. Those natural antioxidative substances usually have a phenolic moiety in their molecular structure. They have been found among flavonoids, tocopherols and catechines. Organic acids, carotenoids, protein hydrolysates and tannins can act as antioxidants or have synergistic effects when used together with phenolic antioxidants. Phenolic antioxidants are potent free radical terminators [19]. Phenolic compounds, biologically active components, are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups [20]. In present study both the samples revealed the presence of phenolic compounds and flavonoids and one can therefore substantiate their activity pertaining to the chemical composition. Table 5 explains the chromatographic details of CMF which can be utilized further to assess the quality of it.

It was observed during present evaluation of antioxidant activity that CMF had 5-6 times more scavenging activity than ME. Results of preliminary phytochemical work indicated that CMF constituted 61% of ME, rest were non polar components extracted with petroleum ether, benzene and chloroform. All these fractions of ME were ineffective in scavenging free radicals (results not included). Therefore, it can be considered that free radical scavenging activity of ME was due to CMF only. Phytochemical reports on *S.indicus* flowerheads revealed presence of alkaloids [21], eudesmanolides and sesquiterpenoids [22, 23]. The exact mechanism of action, however, could only be unfolded after detailed characterization of active moieties from different fractions. Studies on these lines have already been taken up and shall follow in our future communications.

CONCLUSIONS

The results of present study indicate that flowerheads of *S.indicus* carry certain levels of antioxidant activities. ME and CMF both were effective in scavenging stable free radical DPPH, superoxide ion and possess good reducing power with inhibitory action on lipid peroxidation. The activity was however of moderate nature when compared to different standard antioxidants. Further in-vivo studies on evaluation of drug are warranted and already taken up .It may be concluded that the free radical scavenging activity of flower-heads of *S.indicus* may follow one of the above mechanisms in exhibiting its effectiveness in traditional medicine.

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