ESTIMATION OF TOTAL PHENOLS WITH SPECIAL EMPHASIS TO ANTIOXIDANT POTENTIALS OF FEW HYPERICUM SPECIES

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Summary

In present investigation, the phenolic content and antioxidant activity of leaf extracts (methanolic) of *Hypericum mysorense, H. perforatum, H. japonicum* and *H. patulum* were studied. Various in vitro antioxidant models (ABTS, DPPH, H_2O_2, Nitric oxide, LPO, Alkaline DMSO, pNDA) were carried out for all methanolic extracts of hypericum species. The total phenolic and flavanol content were performed in order to correlate whether the antioxidant activity of extracts is influenced by the concentration of flavonols and/or phenols present. The higher percentage of total phenolic and flavonol content was found to be 24.72 and 101.7 mg/g in *H. mysorense* and *H. japonicum* extract, respectively. The *H. mysorense* exhibited stronger free radical scavenging activity as evidenced by the low IC_{50} values in DPPH (3.35 µg/ml), ABTS (29.5 µg/ml), H_2O_2 (58 µg/ml), and Lipid peroxidation (13 µg/ml) methods, and *H. perforatum* exhibited its potent activity against ABTS (28.5 µg/ml), DPPH (7.5 µg/ml), and *H. japonicum* exhibited its H_2O_2 scavenging activity at 49 µg/ml. The significance of the obtained values are distinguished when compared with the standards (Rutin) used. The results strongly suggest the exploration of effective lead from hypericum species will be alternative choice for the treatment of pathologies caused in the consequences of excess free radical production.

Key words: *Hypericum mysorense, H. perforatum, H. japonicum, H. patulum, antioxidant*

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Introduction

In cellular defense system, scavenging of free radicals is an important issue affiliated by utilization of both exogenous and endogenous antioxidants because the increase in production of free radicals has been reported to cause damage to cell membranes, enzymes, DNA, lipids, and proteins, impairing their function [1]. Although the body possesses defense mechanisms as enzymes and antioxidant nutrients [2], continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them and cause irreversible oxidative damage [3]. The use of spices and herbs as antioxidants in processed foods is a promising alternative to the use of synthetic antioxidants [4] like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), commonly used in processed foods. It has been suggested that there is an inverse relationship between dietary intake of antioxidant rich foods and the incidence of human disease [5].

The genus *Hypericum* contains about 400 species, widely used in the folk medicine [6] and it has attracted much attention in investigation of metabolites, many of which are biologically active compounds with phloroglucinol moiety [7]. It has been reported it contained some antiviral prenylated pholoroglucinol derivatives [8], and also contains variety of compounds such as flavonoids [9], xanthones [10], chromenyl ketones [11], hyperforins derivatives [12], *n*-alkanes [13], napthodianthrones [14] and essential oil [15]. In an earlier study carried out in our laboratory, the methanol extracts of the aerial parts, leaves, and stem of *H. hookerianum* exhibited *in vitro* cytotoxicity against various cancerous cell lines [16]. Hence in the present study the exploration of antioxidative activities of the leaf extract from different Hypericum species such as, *H. mysorense, H. perforatum, H. japonicum* and *H. patulum* were investigated.

Materials and methods

Plant material

The fresh plant material (leaves) was collected from the Government Horticultural Garden, Ootacamund, Tamil Nadu, India, in June 2005. The plant was authenticated by Dr. S. Rajan, Botanical Survey of India, Medicinal Plants Survey and Collection Unit, Ootacamund, Tamil Nadu, India and a voucher specimen was deposited in herbarium of this institute for future reference.

Preparation of plant extracts

Leaves of different species of Hypericum were separated and shade dried. The dried parts were chopped, coarsely powdered, and extracted separately using methanol by soxhlet extraction. The extracts were then concentrated to dryness under reduced pressure and controlled temperature to yield a deep drown–dark brown semisolids. The yields were 24.76%, 11.9%, 9.02% and 8.25% w/w with respect to dried powered material of leaves of *H. mysorense, H. perforatum, H. japonicum* and *H. patulum.*

Preparation of Test and Standard solutions

The extracts and the standard antioxidants (ascorbic acid, rutin, Butylated hydroxyl anisole and α-tocopherol) were dissolved in distilled dimethyl sulphoxide (DMSO) separately and used for except the hydrogen peroxide method where methanol was used to dissolve the extracts in order to avoid the interference noted with DMSO. The stock solutions were serially diluted with the respective solvents to obtain the lower dilutions.

Chemicals

1, 1-diphenyl-2-picryl hydrazyl (DPPH) and 2, 2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma Aldrich Co., St. Louis, USA. Rutin and p-nitroso dimethyl aniline (*p*NDA) were obtained from Acros Organics, New Jersey, USA. Naphthyl ethylene diamine dihydrochloride (NEDD) was obtained from Roch-Light Ltd., Suffolk, UK. Ascorbic acid and Nitro blue tetrazolium (NBT) were obtained from SD Fine Chemicals Ltd., Mumbai, India. Sodium nitroprusside was obtained from Ranbaxy Laboratories Ltd., Mohali, India. Sulphanilic acid was obtained from E-Merck (India) Ltd., Mumbai, India. All chemicals used were of analytical grade.

In vitro antioxidant assays

In all methods, a particular concentration of the extract or standard solution was used which gave a final concentration of 1000 µg/ml to 1.95 µg/ml after all the reagents were added. Absorbance was measured against blank solutions that contain extract or standard, but without the reagents. A control was performed without adding extracts or standards. Percentage scavenging and IC_{50} values ± S.D were calculated.

DPPH method

The hydrogen atoms or electrons donating ability of the corresponding extracts and some pure compounds were measured from the bleaching of purple coloured methanol solution of DPPH. A 10 µl aliquot of the extract (from 21 mg/ml to 21 µg/ml) was added to 200 µl of DPPH in methanol solution (100 µm) in a 96-well microtitre plate (Tarson Products (P) Ltd., Kolkata, India). After incubation at 37°C for 20 min, the absorbance of each solution determined at 490 nm using ELISA reader (Bio-Rad Laboratories Inc, California, USA, Model 550) [17].

Nitric oxide radical inhibition assay

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess Illosvoy reaction [18]. In this investigation, Griess Illosvoy reagent was modified by using NEDD (0.1%, w/v) instead of 1-naphthylamine (5%). The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (1 ml) and extract or standard solutions (1 ml) were incubated at 25ºC for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite was added and mixed with 1ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1ml of NEDD was added, mixed and allowed to stand for 30 min at 25°C. A pink colored chromophore is formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions using spectrophotometer.

Scavenging of Hydrogen peroxide

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of the extract or standard in methanol (1 ml) were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min the absorbance was measured at 230 nm [19].

ABTS radical scavenging activity

ABTS radical cation ($ABTS^{\cdot +}$) was produced by reacting ABTS stock solution (7 mM concentration) with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use [20]. To 0.2 ml of various concentrations of the extract or standards added, 1 ml of distilled DMSO and 0.16 ml of ABTS solution to make a final volume of 1.36 ml. After 20 min of the incubation in dark, the absorbance was measured at 734 nm.

pNDA method

To a reaction mixture containing ferric chloride (0.1 mM, 0.5 ml), EDTA (0.1 mM, 0.5 ml), ascorbic acid (0.1 mM, 0.5 ml), hydrogen peroxide (2 mM, 0.5 ml) and p-NDA (0.01 mM, 0.5 ml) in phosphate buffer (pH 7.4, 20 mM), were added with various concentrations of extract or standard in distilled DMSO (0.5 ml), to produce a final volume of 3 ml. Absorbance was measured at 440 nm [21].

Scavenging of Superoxide radical by alkaline DMSO method

Superoxide is generated according to the alkaline DMSO method. The reduction of Nitro blue tetrazolium (NBT) by superoxide was determined in the presence and absence of the extracts [22]. To the reaction mixture containing 1 ml of alkaline DMSO, 0.3 ml of the extracts in DMSO at various concentrations added 0.1 ml of NBT (0.1 mg) to give a final volume of 1.4 ml. The absorbance was measured at 560 nm.

Lipid peroxidation (LPO) assay

The test samples (100 µl) of different concentrations were added to 1 ml of liposome mixture, control was maintained without test sample. Lipid peroxidation was induced by adding 10 µl $FeCl_3$ (400 mM) and 10 µl L-ascorbic acid (200 mM). After incubation for 1 hour at 37°C, the reaction was stopped by adding 2 ml of 0.25 N HCl containing 15% trichloro acetic acid and 0.375% thio barbituric acid and the reaction mixture was boiled for 15 min then cooled, centrifuged and absorbance of the supernatant was measured at 532 nm [23].

Total Phenol estimation

In a series of test tubes, 0.4 ml of the appropriate dilution of the filtered extract in methanol was taken, oxidized with 2 ml of FC reagent and neutralized with 1.6 ml of standard sodium carbonate. After shaking, it was kept for 2 h and the absorbance was measured at 765 nm using a Shimadzu-UV-160 spectrophotometer (Shimadzu Corporation, Japan). The total phenolic content was expressed as % w/w of the extracts [24].

Total flavonol estimation

The flavonol content was measured using a colorimetric assay [25]. 0.5 ml of the extracts was mixed with 1.5 ml methanol then 0.1 ml of 1 M potassium acetate, after 5 min 0.1 ml of 10% aluminium chloride was added and finally the mixture is diluted with 2.8 ml of distilled water. After 30 min incubation at room temperature, the absorbance of the reaction mixture was measured at 415 nm. The total flavonol content was expressed as mg/g or % w/w of the extracts.

Results

The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability [26]. A higher antioxidant activity in scavenging of stable free radical (DPPH) was found in the leaf extract of *H. mysorense* (IC_{50} = 3.35 µg/ml), which is almost equal or more than that of the standards (Rutin, 3.91µg/ml; Ascorbic acid, 2.69 µg/ml) used. All the extracts failed to scavenge the nitric oxide radicals even at higher concentrations; it reveals that the extracts are inactive against nitrate radicals. All the extracts, even the standards used, found to be inactive against the superoxide radicals produced. Among the studied Hypericum species, *H. japonicum* had the highest hydrogen peroxide radical scavenging activity (49 µg/ml) followed by *H. mysorense* (58 µg/ml), *H. perforatum* (67 µg/ml), and *H .patulum* showed moderate activity at 92 µg/ml.

An ABTS radical scavenging method is the most reliable method involves the determination of the disappearance of free radicals [20]. Among all the extracts tested, *H. Perforatum* and *H. mysorense* extracts showed potent activity, i.e, IC_{50} at 28.5 and 29.5 µg/ml respectively. In pNDA method, the extract of *H. japonicum* showed moderate activity at 790 µg/ml, whereas other extracts failed to inhibit the hydroxyl radicals.

The *H. mysorense* extract significantly inhibited the formation of lipid peroxides in Fe^{2+}-ascorbate induced lipid peroxidation. The IC_{50} value (13 µg/ml) of the extract proved to be potent against lipid peroxides and obtained value reveals that the extract is 7 times more potent than that of standard (α-tocopherol) used, the *H. japonicum* and *H. perforatum* extracts also exhibited better activity at 140 and 235 µg/ml, respectively. The total flavonol profile of the hypericum shows that *H. japonicum* is enriched with higher percentage of flavonols among others tested i.e. 101.7 mg/g of extract, followed by *H .mysorense* shows 88.21 mg/g of extract. The total phenol content of *H. mysorense* and *H. perforatum* shows 24.72 and 16.28 mg/g of extract, respectively.

Discussion and Conclusion

Phenolic compounds are considered as the most important antioxidative components of herbs and other plant materials, and good correlation between the concentration of plant phenolics and the total antioxidant capacity has been reported [27]. The polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants [28] and their epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent [29]. The qualitative phytochemical tests revealed the presence of phenolics and flavonoids. The total phenolic and flavonol contents were determined mainly to correlate with the antioxidant potential of the extracts. The percentage of total phenols in *H. mysorense* is 24.72 mg/g of extract which is greater among all the extracts tested. The antioxidant activity of plant extracts vary with assay methods [30] hence we cross checked antioxidant activities of hypericum with seven methods based on different mechanisms. *H. mysorense* has potent scavenging activity against DPPH, ABTS and lipid radicals. Secondly, *H. perforatum* exhibits its potent scavenging activity against ABTS and moderate activity against DPPH and H_2O_2 radicals. In conclusion, the numerous antioxidant methods firmly support the antioxidative potentials of hypericum extracts, (mainly *H. mysorense and H. perforatum*) observed in the present study is due to the presence of polyphenolic compounds. The plant merits further investigation to determine the exact active constituents responsible for antioxidant activity.

Table 1. *In vitro* antioxidant activity of methanol extracts of leaves of Hypericum species

Sl No.	EXTRACTS / STANDARDS	IC_{50} values ± SE (µg/ml)* by methods							Total flavanol content mg/g*	Total phenol content mg/g*
	EXTRACTS	ABTS	DPPH	Hydrogen peroxide	Nitric oxide	Lipid peroxidation	Alkaline DMSO	p-NDA		
1	*H .mysorense*	29.5±0.9	3.35±0.06	58±1.8	>1000	13±0.42	>1000	>1000	88.21±2.43	24.72
2	*H .perforatum*	28.5±0.8	7.5±0.08	67±2.2	>1000	235±4.6	>1000	970±17.2	76.98±2.1	16.28
3	*H .japonicum*	225±4.2	21.5±0.68	49±1.2	>1000	140±2.93	>1000	790±10.4	101.7±1.41	7.52
4	*H .patulum*	148±2.8	7.73±0.07	92±2.6	>1000	162±2.46	> 1000	> 1000	28.76±0.87	7.94
	STANDARDS									
1	Ascorbic acid	11.25±0.49	2.69±0.05	187.33±3.93	-	-	>1000	-	-	-
2	Rutin	0.51±0.01	3.91±0.1	36.66±0.22	65.44±2.56	-	>1000	>1000	-	-
3	BHA	-	-	24.88±0.16	-	112.66±1.32	>1000	>1000	-	-
4	α-tocopherol	-	-	-	-	91.66±4.92	-	-	-	-

*Average of three determinations, values are mean ± S.E.M., (-) means – Not done

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