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SCREENING FOR ANTIOXIDANT ACTIVITY IN TRADITIONALLY USED MEDICINAL HERBS: COMPARISON OF TOTAL PHENOLS, RADICAL SCAVENGING AND DNA DAMAGE PROTECTING ACTIVITY

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ABSTRACT

Herbal infusions of nine traditionally used medicinal plants were screened for total phenolic content (TPC), radical scavenging activity (RSA) and oxidative DNA damage protecting capacity by chemical assays. The TPC was determined in order to know its correlation with RSA. The extracts of *T. cordifolia* and *C. spectabilis* presented highest TPC values (34.21 ± 0.93 and 28.36 ± 1.05 mg GAE/g) and showed strong RSA (96.29 ± 0.91 and 84.68 ± 0.90 mg TE/g). A significant relationship (R^2 = 0.915, p< 0.01) was found between TPC and RSA indicating that phenolics are contributing to the antioxidant properties of these plants. The qualitative analysis of DNA damage protecting activity revealed the potential of *T. cordifolia*, *V. negundo*, *O. sanctum* and *C. phlomides* extracts as potent DNA damage protecting agent. Present study validates the uses of these herbs in various herbal ailments to treat many illnesses associated with oxidative stress.

KEYWORDS:Antioxidant activity,Total phenolic content, Radical scavenging activity,Oxidative DNA damage, Correlation analysis.

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INTRODUCTION

During normal physiological processes, reactive oxygen species (ROS) are continuously produced and removed by various antioxidant defense mechanisms but the overproduction of ROS induces DNA damage, protein carbonylation and lipid peroxidation which results into many health related problems such as aging, cancer, Parkinson's disease and Alzheimer's disease[1-4]. Many studies in past decades have proven that intake of antioxidants as a dietary supplement is effective in preventing many diseases as they are capable of neutralizing oxygen free radicals and inhibit LDL oxidation and protect against coronary heart disease, cancer and neurodegenerative diseases[5-7]**.** Antioxidant compounds occurring in many plants have been identified as free radical scavengers. These antioxidants are gaining more attention by researchers as a replacement of many synthetic antioxidants such as butylatedhydroxy toluene (BHT) and butylatedhydroxy anisole (BHA) as these are associated with many side effects[8]**.** Among plant derived antioxidants, polyphenols are of great interest to the food industry as they have the capacity to retard oxidative degradation of lipids and scavenging free radicals and hence improving the quality and nutritional value of food[9, 10]. Keeping these points of view some plants have been selected to evaluate total phenolic content and antioxidant activity to find out new sources of natural antioxidants and the scientific validation of their uses in oxidative stress related diseases such as inflammation, neural disorders, diabetes, stomachache and rheumatism. Present study evaluates and compares the total phenolic content (TPC) and radical scavenging activity (RSA) of various herbal infusions which are being used by traditional healers all over the world and also reports the qualitative measurement of oxidative DNA damage protecting capacity which is, to the best of our knowledge, first of its kind as many plants under study have been checked for this activity for the first time.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade purity. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetra methyl chromane-2-carboxylic acid (97%) i.e. TROLOX, 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) were acquired from Sigma-Aldrich chemical Co., MO, USA. Gallic acid & Folin&Ciocalteu's phenol reagent (FCR) were purchased from S.D. Fine Chemicals, Mumbai, India. pBR322 DNA was purchased from Bangalore Genei, Bangalore, India.

Plant Material

Nine medicinal plant species which are used by traditional healers all over the world were selected to test their antioxidant activity and total phenolic content. Their botanical name and traditional uses are given in table 1. These plants were collected locally and taxonomically identified by Dr. M.C. Rajanna, Professor (Curator), Botanical Garden, University of Agricultural Sciences, Gandhi Krishi Vigyan Kendra (GKVK), Bangalore, Karnataka, India. Plant material was washed, dried under shade, powdered and stored in the dark until use.

Table 1
List of herbs used in the present study and their traditional uses. Plant leaves were used to study antioxidant activity.

Plant species	Family	Medicinal uses	Ref.
Andrographis paniculata	Acanthaceae	Hepatoprotective, cholinergic, antispasmodic, stomachic, anthelmintic, alterative, blood purifier, used in jaundice, flatulence, cold and upper respiratory tract infections	11
Annona squamosa	Annonaceae	Insecticide, abortifacient, purgative, antibilious, antiemetic, expectorant, antiulcerogenic, astringent, used for diarrhea and dysentery	11, 12
Cassia spectabilis	Fabaceae	Laxative, purgative, antimicrobial, antipyretic, anti-inflammatory, antiviral, in treatment of cold & flu	13,14, 15,16
Clerodendrum phlomides	Verbenaceae	Used in dyspepsia, stomachache, colic, cholera, dysentery, postnatal fever, during convalescence from measles, in nervous disorders	11
Costus speciosus	Zingiberaceae	Astringent, purgative, depurative, anti-inflammatory (used in gout, rheumatism, bronchitis, asthma, catarrhal fevers, dysuria), anthelmintic, antivermin, maggoticide, antifungal	11
Ficus religiosa	Moraceae	Astringent, antiseptic, alterative, laxative, hemostatic, vaginal disinfectant, used in diabetes, diarrhea, leucorrhoea, menorrhagia, nervous disorders, skin diseases, also applied on ulcers and wounds, laxative	11, 17
Ocimum sanctum	Labiatae	Carminative, stomachic, antispasmodic, antiasthmatic, antirheumatic, expectorant, stimulant, hepatoprotective, antiperiodic, antipyretic, diaphoretic, antimalarial, antistress, adaptogenic, antibacterial, antifungal	11, 17
Tinospora cordifolia	Menispermaceae	Antipyretic, antiperiodic, anti-inflammatory, antirheumatic, spasmolytic, hypoglycemic, hepatoprotective, antacid, antidiarrheal, antidysentric	11
Vitex negundo	Verbenaceae	Used in spermatorrhoea, as a rejuvenating tonic, anti-inflammatory, analgesic, astringent, febrifuge, antidiarrheal	11

Preparation of extracts

Plant infusions were prepared as tea is prepared for human consumption. Briefly, 10 g of dried leaves were placed in a glass flask and steeped in 100 ml. double-distilled water at 95^0 C for 10 min[18]. These infusions were lyophilized and weighed to know the extract yield. Lyophilized extract were stored at 4 ^{0}C until use.

Determination of Total Phenol Content (TPC)

The total phenolic content (TPC) was determined by the Folin-Ciocalteu method using Gallic acid as standard[10]. To the extract, Folin-Ciocalteu reagent and Na_2CO_3 was added and after incubation at room temperature, the absorbance was measured at 760 nm. Results were expressed as mg of gallic acid equivalent/g dry weight (mg GAE/g) using linear equation based on a calibration curve of gallic acid. Gallic acid calibration curve was done between 0.1 to 0.5 mg/ml. Samples were analyzed three times.

Determination of DPPH radical scavenging activity

The spectrophotometric DPPH radical scavenging activity assay was adapted from a standard protocol [19]. To ethanolic solution of DPPH (1.0 X 10^{-4} M), extracts at a fixed concentration (1 mg) were added. After shaking, reaction mixtures were kept for 30 min. the absorbance of reaction mixture was recorded at 517 nm. Decrease absorbance indicates increase of DPPH radical scavenging activity. Results were expressed in mg of trolox equivalent/ g of dry weight (mg TE/g) using a trolox calibration curve from 0.1 to 1 mg/ml. Samples were analyzed three times.

Evaluation of DNA damage protecting activity of various extracts

Oxidative DNA damage protective activity of various extracts was checked by inhibition of the conversion of supercoiled pBR322 plasmid DNA to open circular and further linear forms induced by AAPH. The experimental procedure was conducted according to a standard protocol[20]**.** Briefly, 100 ng of pBR322 DNA was incubated with various extracts and standard antioxidant TROLOX and 10 mM AAPH in phosphate-buffered saline (PBS) (consisting of 137 mMNaCl, 2.7 mMKCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4) for 1 h at 37^0C at a final volume of 25 µl (i.e. 5µl DNA, 10µl AAPH, 10µl

extracts/antioxidants/PBS). After incubation samples were electrophoresed in a horizontal slab gel apparatus in TAE buffer for 1 h. after staining with ethidium bromide the agarose gel was photographed under UV light.

Statistical analysis

All analyses were performed in triplicates. The data were recorded as mean±S.D. and analyzed by SPSS (version 11.5 for windows, SPSS, Inc.). One-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference test were used to compare means among groups. A pearson correlation test was used to study the relationship between TPC and RSA of various extracts. *P* values< 0.05 were regarded as significant and *p* values<0.01 very significant.

RESULTS AND DISCUSSION

1. Total Phenolic content

Plant tissues encounters with different forms of environmental stresses and in response; many compounds such as free phenolic acids or derivatives are synthesized. Different parts of herbal plants possess distinct quality of compounds which are used for cuisine, food preservation and herbal medicine[21].Plant phenolics are important constituents of the human diet and potent antioxidants that can scavenge harmful active oxygen species including $O_2^{\cdot-}$, H_2O_2, $^{\cdot}OH$ and $^1O_2$[22].Therefore, in this study, we calculated the total phenolic content in units of mg Gallic acid equivalent (GAE) of phenolic constituents per gram of the sample (Standard curve equation: y = 0.0023x, r^2 = 0.9928). Table 2 summarizes the total phenolic content in tested herbal infusions. The amount of total phenolics varied markedly in different plants and ranged from 11.59 to 34.21 mg GAE/g of dry material. *Tinospora cordifolia* exhibited the highest amount of total phenolic content (34.21 ± 0.93 mg GAE/g) followed by *Cassia spectabilis* (28.36 ± 1.05 mg GAE/g) (*p* < 0.05) and the lowest in *Clerodendrum phlomides* (11.59 ± 0.85mg GAE/g). It is well documented in several articles that consumption of phytochemicals particularly plant phenolics, has been linked to reduced risk of many diseases such as atherosclerosis, cancer, diabetes, cardiovascular diseases and neurodegenerative disorders[23, 24]. So the high TPC value contributes to the many medicinal properties exhibited by these herbs.

Table 2
Total phenolic content and DPPH radical scavenging activity of herbal infusions.

Plant Species	TPC (mg GAE/g dry weight)*	DPPH (mg TE/g dry weight)*
Andrographis paniculata	22.96 ± 0.94^{c}	51.64 ± 0.47^{e}
Annona squamosa	18.12 ± 0.86^{e}	46.27 ± 1.10^{f}
Cassia spectabilis	28.36 ± 1.05^{b}	84.68 ± 0.90^{b}
Clerodendrum phlomides	11.59 ± 0.85^{g}	38.24 ± 0.81^{g}
Costus speciosus	15.45 ± 0.33^{f}	47.57 ± 0.56^{f}
Ficus religiosa	13.50 ± 1.02^{fg}	39.86 ± 0.49^{g}
Ocimum sanctum	18.85 ± 0.57^{de}	54.35 ± 0.82^{d}
Tinospora cordifolia	34.21 ± 0.93^{a}	96.29 ± 0.91^{a}
Vitex negundo	21.13 ± 0.99^{cd}	63.75 ± 1.45^{c}

**** The values are expressed as means ± SD (n = 3). Values with different lower case letters (^{a,b,c,d,e,f,g}) were significantly different (p< 0.05) analyzed by ANOVA and Tukey's Honestly Significant Difference test.***

2. DPPH radical scavenging activity of various extracts

The main antioxidant action of various antioxidants is radical scavenging activity therefore many methods have been developed to evaluate the antioxidant action of antioxidants by scavenging of free radicals such as, superoxide radical, ABTS (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radicals[25]. DPPH is a stable free radical and

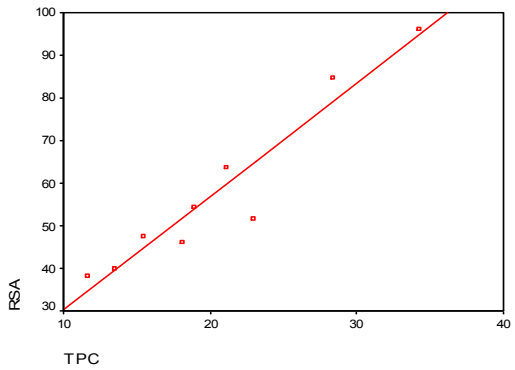
accepts an electron or hydrogen radical to become a stable diamagnetic molecule[26]. A freshly prepared DPPH solution is of deep purple colour with absorption maximum at 517 nm and in the presence of antioxidant this colour disappears due to quenching of DPPH free radicals and convert them into a colourless product i.e. 2,2-diphenyl-1-hydrazine (antioxidant mechanism performed by providing hydrogen atoms or electron)[27]. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. Hence, DPPH is often used as a substrate to evaluate the antioxidant activity of compounds[28]. In the present study, the DPPH method was selected to evaluate the antioxidant activity of various herbal extracts because it is one of the most effective and rapid method to evaluate the antioxidant potential of a given sample[29]. Table 2 summarizes the DPPH Radical scavenging activity exhibited by various herbal extracts. As can be seen from the table, DPPH scavenging effect increases with more total phenolic content hence *T. cordifolia* showed highest activity followed by *C. spectabilis*. However, it should be noted that the *V. negundo* has more scavenging effect than *A. paniculata* although the TPC is more in the later one. Moreover,*A. squamosa* and *O. sanctum* have almost same TPC value but their DPPH radical scavenging effect differs significantly ($p < 0.01$). These results were consistent with previous findings where radical scavenging effect was not increased with high value of TPC[30]. One reason may be due to the synergistic action exhibited by many compounds present in crude extracts which is responsible for the observed activity.

3. Correlation analysis

To correlate the results obtained for TPC and RSA, a regression analysis was performed. A significant correlation was found between TPC and RSA ($R^2 = 0.915$, $p< 0.01$, Figure 1).

Figure 1

Linear correlation between TPC (mg GAE/g) and RSA (mg TE/g). Correlation coefficient R = 0.957. The two tailed p value is < 0.01.



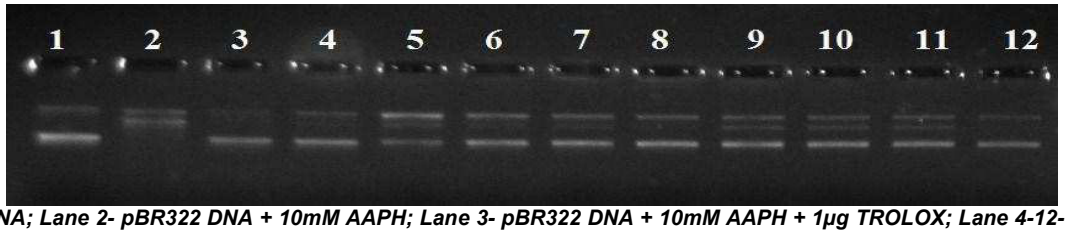
These results suggest the relationship between phenolic compounds and their free radical scavenging capacities and indicate that phenolic compounds are major contributors to the antioxidant potential of these herbs. This result is in agreement with previous studies where a similar correlation (R = 0.939) was found between DPPH radical scavenging activity and total phenolic content[31].

4. Evaluation of DNA damage protecting activity of various extracts

The plasmid or bacteriophage DNA remains in supercoiled form generally. Single-strand breaks cause the formation of circular form and double-strand breaks cause the formation of linear form of DNA[32]. It has been demonstrated that AAPH is able to cause strand breaks in pBR322 DNA and thermal decomposition of AAPH generates alkyl radicals which convert into alkyl peroxyl

radicals (ROO·) after reacting with oxygen. These alkyl peroxyl radicals attack on plasmid DNA which led to oxidative DNA damage[20]. The protective effect of various extracts against oxidative DNA damage is shown in Figure 2.

Figure 2
Electrophoretic pattern of pBR322 DNA (100 ng) after treatment with AAPH and in the presence and absence of TROLOX and various herbal extracts.



Lane 1- pBR322 DNA; Lane 2- pBR322 DNA + 10mM AAPH; Lane 3- pBR322 DNA + 10mM AAPH + 1µg TROLOX; Lane 4-12- pBR322 DNA + 10 mM AAPH + 5 µg of herbal extract (Lane 4- T. cordifolia; Lane 5- C. spectabilis; Lane 6- V. negundo; Lane 7- A. paniculata; Lane 8- O. sanctum; Lane 9- A. squamosa; Lane 10- C. speciosus; Lane 11- F. religiosa; Lane 12- C. phlomides).

Plasmid DNA is mainly of supercoiled form (bottom band in Figure 2.) and open circular (top band) form in the absence of AAPH and in the presence of AAPH the supercoiled form starts converting into the linear form i.e., middle band which is found between supercoiled and open circular (Lane 1 and 2)[1]. As can be seen from the figure that each herbal extract was able to protect DNA from oxidative damage but the effect varies from species to species. The electrophoretic pattern suggests that *T. cordifolia*, *V. negundo, O. sanctum* and *C. phlomides* (Figure 2, Lane 4, 6, 8 and 12 respectively) are more protective than other species (only two forms are seen i.e., supercoiled and open circular). However, it should be noted that *C. phlomides* had lowest TPC value (11.59 ± 0.85 mg GAE/g) but able to protect oxidative DNA damage at the concentration tested (Figure 2, Lane 12) and *C. spectabilis* had good TPC value (28.36 ± 1.05 mg GAE/g) but was found less protective to oxidative DNA damage (Figure 2, Lane 5). This suggests that antioxidant capacity depends upon nature of phytochemicals present in herbal extracts and it may vary in different assays so we need to analyze the overall antioxidant potential of a plant under study by using a variety of assays.

CONCLUSION

The antioxidant capacity of herbal extracts should be analyzed by using a variety of assays in order to establish their potency as antioxidant agents. The present study not only evaluates the antioxidant potential of many traditionally used herbs, but also establishes the relationship between total phenolic content and radical scavenging activity of these herbs. The investigation further supports the view that some plants are promising sources of natural antioxidants. Among these plants *T. cordifolia* and *C. spectabilis* exhibited strong radical scavenging activity and high total phenolic content. The strong correlation between total phenolic content and radical scavenging activity indicates that phenolic compounds are major contributors to the antioxidant potential of these herbs. The oxidative DNA damage protecting activity of many herbs is encouraging which offers their uses in many nutraceutical products. The study also validates their traditional uses as an anti-inflammatory agent as the antioxidant action could be involved in anti-inflammatory mechanism[33]

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