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Investigations on Antioxidant Potential of Phenolic Acids and Flavonoids: The Common Phytochemical Ingredients in Plants

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Abstract

Antioxidant property of the phenolic acids and flavonoids was assessed in multiple antioxidant assays such as 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity and deoxyribose protection assay. In all the assays the antioxidant property was found to be concentration dependent. Among phenolic acids, gentisic acid was reported to be the most effective compound with the IC_{50} values of 3.56, 6.68 and 3.53 µg/ml for DPPH, ABTS and deoxyribose protection activity, respectively followed by the gallic and caffeic acids. These compounds were found to be rather more effective than the standard compounds butylated hydroxytoluene (BHT) and α-tocoferol. Among the flavonoids, catechin hydrate was reported to be the strongest antioxidant compound (IC_{50} 8.34, 4.93 and 5.96 µg/ml) in comparison to BHT (IC_{50} 17.41, 17.12 and 6.1 µg/ml) and α-tocoferol (IC_{50} 10.97, 32.41 and 8.37 µg/ml) for DPPH, ABTS and deoxyribose protection activity, respectively. The total antioxidant activity of these phenolic acids and flavonoids was ascertained to reflect antioxidative comparative capacity of these compounds which have been largely extracted, identified and characterized from natural sources.

Keywords: Phenolic acids; Flavonoids; DPPH; ABTS; Deoxyribose protection assay; Antioxidants

Abbreviations:

DPPH: 1,1-Diphenyl-2-picrylhydrazyl; ABTS: 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); BHT Butylated HydroxyToluene; SD: Standard Deviation; ROS: Reactive Oxygen Species; ABTS•+: ABTS cation radical.

Introduction

Phenolics are structurally diverse, broadly distributed and the most abundantly found secondary metabolites in the plant and microbial species. Phenolic compounds and flavonoids constitute major bioactive components in the medicinal and food plants and thus, comprises essential part of the human diet [1]. They are the secondary metabolic derivatives of pentose phosphate, shikimate and phenylpropanoid metabolic pathway with aromatic rings bearing one or more hydroxyl groups [2]. The structural diversity within different phenolic compounds due to the hydroxyl group substitutions in the aromatic ring makes these compounds biologically more effective and potential [3]. They serve as antioxidants and free radical scavengers and helps in coping with oxidative stress and their harmful effects, leading to the avoidance and control of many frightful diseases and untimely ageing [4]. Plant and microbial phenolics majorly include phenolic acids, flavonoids, tannins, stilbenes and lignins [5]. Most of these compounds potentially possess anticancer, anti-inflammatory, antibiotic, anti-septic, cosmetic properties [6-10] and help in epidemiological disease risks [11]. Phenolic compounds are reported to participate in the broad spectrum biological properties such as morphological development, physiological processes, reproduction and biotic and abiotic stress management in the organisms including humans [12,13].

Phenolic compounds are biologically active antioxidants [14]. Many of these compounds found in vegetables, fruits, cereals, spices and herbs make these crops functional foods for high value nutraceuticals [4,15-17]. Organic extracts of many food crops have shown different levels of antioxidant properties and further have led to the isolation, identification and characterization of phenolic compounds [18]. Chemically, variation in the oxidative substitution and various degree of changes due to hydroxylation, methoxylation, prenylation or glycosylation in the central ring alters the basic molecule of phenolics [19]. Since phenolics have hydroxyl groups which are better hydrogen donors, the chemical species with such structural moieties can act as hydrogen-donor antioxidants by reacting with reactive oxygen or nitrogen species [20]. Phenolics also forms relatively long-lived free radicals due to interaction of hydroxyl groups with the benzene ring pi-electrons. This facilitate radical-mediated oxidation processes that lead to pronounced antioxidant activity [21]. Further, the antioxidant capacity in phenolics is also generated from their ability to chelate with the metal ions that form free radicals [22]. Phenolics also possess the ability to interfere with several enzymes like cytochrome P450 isoforms, lipoxygenases etc. that catalyze radical generation directly or indirectly [23]. The isolation, identification and characterization of biologically active phenolics and flavonoids and the antioxidant activities of the plant extracts or individual compounds based on a few parameters is very well reported from numerous plant sources. In this study, we report antioxidant properties of different phenolic acids and flavonoids in terms of DPPH and ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) as free radical scavenging and deoxyribose activity in order to generate a comparative view on the antioxidant property of these chemical species.

General information

Chemicals: Chemicals like 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), deoxyribose, reference compounds butylated hydroxytoluene (BTH) and α-tocoferol, phenolic acids gallic, caffeic, syringic, t-chlorogenic, ferulic, gentisic (sodium salt), cholic, shikimic, cinnamic, vanillic, p-coumaric and quinic acids and flavonoids naringenin, 3-hydroxyflavone, 7- hydroxyflavone, t-chalcone, hesperetin, flavanone, quercetin, quercetin hydrate, rutin, catechin hydrate, kaempferol were purchased from Sigma Aldrich (USA).

Preparation of stock solution

A stock solution of each phenolics was prepared in absolute methanol by dissolving one mg of the compounds in one ml of the solvent in order to get an stock solution of 1000 ppm. From this stock solution, different concentrations of working solution (e.g., 5, 10, 15, 20, 25, 100, 200, 300, 400 and 500 µg/ml or more) was prepared for experimentation.

Determination of antioxidant property

DPPH scavenging activity: From the stock solutions of the compounds, 5, 10, 15, 20 and 25 µg/ml of final concentrations of pure chemicals was prepared in methanol. One ml from each concentration was added to 3 ml of freshly prepared methanolic solution of DPPH (0.1 mM). The mixture was shaken well on vortex and then kept in the dark at room temp for 30 min., after which the absorbance was measured at 517 nm against the blank containing all the samples except the test solution as described [24]. BHT and beta-carotene was used as positive control for comparison of results. The percent inhibition of DPPH (I%) was calculated as under

$$I\%\ (\text{DPPH radical scavenging activity}) = 1-(A_{\text{sample (t 30 min)}}/A_{\text{control(t0 min)}}) \times 100$$

where $A_{\text{sample (t30 min)}}$=absorbance of sample at 30 minute, $A_{\text{control (t0 min)}}$=absorbance of control at 0 minute.

The IC_{50}, the concentration reflecting 50% inhibition of DPPH, was extrapolated from a graph presenting (I%) i.e., percent inhibition versus concentration of the compounds.

ABTS+ scavenging activity determination

An improved method for ABTS {azinobis (ethylbenzothiazoline-6-sulphonic acid)} scavenging activity was performed to measure total antioxidant property of the phenolic and flavonoid compounds [25]. Stock solution of the compounds was diluted to 5, 10, 15, 20 and 25 µl/ml in methanol. In brief, 7.0 mM ABTS solution and 140mM potassium persulphate solution were mixed and kept in the dark at room temp for 16h so that the absorbance become stable to generate ABTS+ free radical. The ABTS+ radical solution was then diluted with ethanol for an absorbance of 0.70 ± 0.05 at 734 nm. Then, the reaction mixture containing 0.9 ml of ABTS.+ working solution with 0.1 ml of compounds of each concentration was prepared and mixed for 45 seconds and kept at ambient temp. in the dark for 30 minutes. The absorbance was then recorded at 734 nm using spectrophotometer taking methanol as blank. BHT and α-tocoferol again served as positive control. The ABTS+ scavenging activity was calculated as under-

$$\%\text{ABTS radical scavenging activity} = 1-(A_{\text{sample (t30 min)}}/A_{\text{control (t0 min)}}) \times 100$$

where, $A_{\text{sample (t30 min)}}$=absorbance of sample at 30 minute, $A_{\text{control (t0 min)}}$=absorbance of control at 0 minute.

Deoxyribose protection assay: The activity of phenolic acids and flavonoids for deoxyribose protection assay was estimated according to the method [26]. In brief, the reaction mixture containing 450 µl of 0.2 M sodium phosphate buffer (pH=7.0), 150 µl of 10 mM deoxyribose, 150 µl of 10 mM $FeSO_4$, 150 µl of 10 mM EDTA, 150 µl of 10 mM H_2O_2, 500 µl of distilled water and 100 µl of compound solution of different concentrations (10, 20, 30 and 40 µg/ml) was prepared at room temp. The reaction mixture was then incubated at 37°C for 4 h, after which 750 µl of 2.8% TCA and 750 µl of 1% TBA in 50 mM NaOH was further added. The whole mixture was boiled for 10 min and then cooled in water. Absorbance was taken at 532 nm with absolute methanol as blank solution. For control solution, reaction mixture contains absolute methanol in place of any compound. Deoxyribose protection assay (%)=1-($A_{\text{sample (t30 min)}}/A_{\text{control (t0 min)}}$) × 100

where $A_{\text{sample (t30 min)}}$=absorbance of sample at 30 minute, $A_{\text{control (t0 min)}}$=absorbance of control at 0 minute.

Statistical analysis

The experiments were carried out in triplicate. The data is represented as means ± standard deviation (SD). All the statistical analyses were carried out using SPSS 16.0 (SPSS, USA). Pearson's correlation analysis was done to determine statistical significance of variations among the values.

Results and Discussion

Phenolic acids and flavonoids with multiple biological functions are among the most important natural secondary metabolites of the plants. There have been continued surge of interest in the isolation, identification and functional characterization of these bioactive metabolites due to their role in the plant defense, either as preformed chemicals acting as barriers against infections or as signal molecules that induce local or systemic defense mechanisms [27]. In humans, these chemicals exhibit broad range of biological protective activities including those against reactive oxygen species (ROS), ageing and cancer [4]. Since various extracts having different phenolic constituents showing specific antioxidant properties have continuously been isolated and characterized from crops and herbs, it was pertinent to assess multiple antioxidant properties of the most frequently identified phenolic and flavonoid compounds. The study enables a comparative profiling of the antioxidant activity of the phenolics and can be used as a reference for future analysis on these compounds.

Out of 12 phenolic acids tested, gallic and gentisic acids were equally active (IC_{50} 3.53 and 3.56 respectively) in terms of DPPH scavenging. Their DPPH activity was significantly high as compared to that of the established reference antioxidant compounds BHT and α-tocoferol (IC_{50} 17.41 and 10.97 respectively) (Table 1). The DPPH activity of other phenolic acids such as syringic (IC_{50} 5.44), caffeic (IC_{50} 6.34) and t-chlorogenic acid (IC_{50} 6.41) was also significantly higher than the reference compounds. Among the 12 phenolic acids, cholic, shikimic acid, cinnamic and quinic acids did not show any DPPH activity (Table 1). Likewise, flavonoids also showed different levels of DPPH activity. Quercetin hydrate (IC_{50} 4.71) showed maximum activity followed by quercetin (IC_{50} 6.55), catechin hydrate ($IC5_0$ 8.34) and rutin (IC_{50} 9.44). The compounds like naringenin, 7-hydroxyflavone and t-chalcone did not show any activity. Comparative

antioxidant evaluation at different concentrations (5, 10. 15. 20 and 25 µg/ml) as shown in Figure 1 reflected that gallic, caffeic, syringic, t-chlorogenic, ferulic and gentisic acids showed concentration dependent DPPH activity. Similarly, at different concentrations, quercetin, quercetin hydrate, rutin, catechin hydrate and kaempferol showed concentration-dependent antioxidant activity.

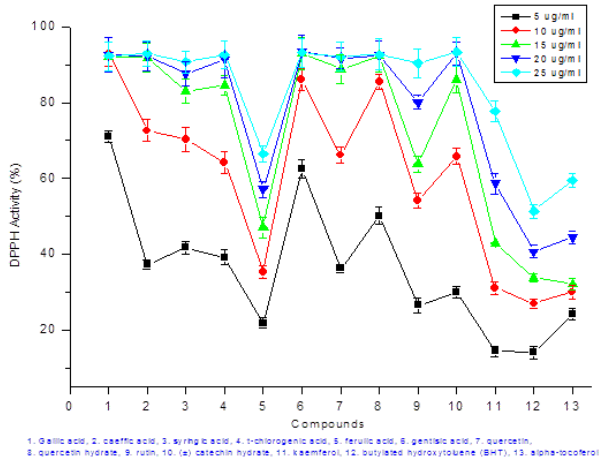

Figure 1: Concentration dependent DPPH activity of phenolic acids and flavonoids.

The ABTS cation radical (ABTS•+) has been used to determine the antioxidant property of fruits, vegetables, beverages, tea, coffee and other foods [28]. Again, since these products are known to possess high content of polyphenolics and flavonoids, these constituents positively add to the antioxidant value to these foods based on the ABTS radical scavenging activity. The results suggested fairly high ABTS radical scavenging activity in some of the phenolic acids and flavonoids we tested. Out of the 12 phenolic acids tested, maximum ABTS activity was reported in gentisic acid (IC_{50} 6.68 µg/ml) followed by p-coumaric (IC_{50} 8.21 µg/ml), gallic acid (IC_{50} 8.85 µg/ml) and ferulic acid (IC_{50} 9.47 µg/ml) (Table 1). Quantitatively, these values reflected significantly higher antioxidant activity in terms of ABTS scavenging activity in the above phenolic acids than the standard compounds BHT and α-tocoferol. Among the flavonoids, maximum activity was observed in catechin hydrate (IC_{50} 4.93 µg/ml). Naringenin, hesperetin and kaempferol showed almost similar antioxidant activity (IC_{50} 8.29, 8.37 and 8.54 µg/ml). Again these compounds showed fairly high ABTS radical scavenging activity than the tested standard compounds. However, most of the tested flavonoid compounds were found to be better ABTS free radical scavengers than the α-tocoferol. Analysis of the antioxidant activity of the highly efficient phenolic acids and flavonoids resulted in the comparative ABTS radical scavenging profile (Figure 2).

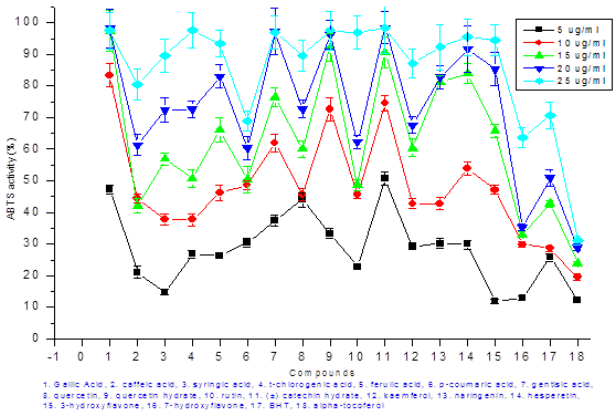

Figure 2: Concentration dependent ABTS activity of phenolic acids and flavonoids.

Concentration dependent activity was observed at various concentrations (5, 10, 15, 20, 25 µg/ml) and the inhibitory impact of the radical's competence was correlated to be concentration dependent.

Besides the DPPH and ABTS free radical scavenging activities which determine antioxidant properties, the protection of the DNA from degradation effects of hydroxyl radical species need to be investigated in order to assess the impact of the free radicals on the DNA protection activity from damages due to oxidation. The antioxidative degradation impact of phenolic acids and flavonoids was therefore, assessed on the simple molecules of deoxyribose sugar (the backbone of the DNA) to ascertain oxidative DNA protection ability of these molecules against damage.

For all the compounds, the effect on deoxyribose protection was observed as concentration dependent percentage of inhibition of the hydroxyl radical in the presence of deoxyribose (Figure 3).

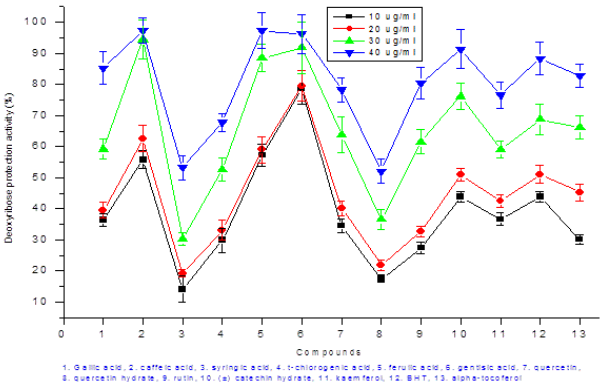

Figure 3: Concentration dependent deoxyribose protection activity of phenolic acids and flavonoids.

Among the tested phenolic acids and flavonoids, gentisic acid and catechin hydrate showed maximum protection of deoxyribose (IC_{50} 3.53 and 5.96 respectively). Other phenolic acids that showed significantly high deoxyribose protection (% inhibition of OH

+radicals) were caffeic (IC_{50} 4.76), ferulic (IC_{50} 4.51), gallic (IC_{50} 7.84) and t-chlorogenic acid (IC_{50} 8.53) (Table 1). The activity of these phenolic acids was found to be almost equivalent to the standard compounds although caffeic and ferulic acids were better antioxidants that standards in terms of deoxyribose protection. Likewise, all the tested flavonoids except catechin hydrate possess lesser antioxidant value in terms of deoxyribose protection assay in comparison to the BHT (Table 1).

Compounds	IC_{50} value		
	DPPH activity	ABTS activity	Deoxyribose protection activity
Gallic acid	3.53 ± 0.24	8.85 ± 0.74	7.84 ± 0.72
Caffeic acid	6.34 ± 0.37	18.04 ± 0.68	4.76 ± 0.37
Syringic acid	5.44 ± 0.53	13.97 ± 0.59	20.70.87
t-Chlorogenic acid	6.41 ± 0.61	13.15 ± 0.71	8.53 ± 0.62
Ferulic acid	11.75 ± 0.45	9.47 ± 0.69	4.51 ± 0.37
Gentisic acid (Sodium salt)	3.56 ± 0.72	6.68 ± 0.39	3.53 ± 0.4
Cholic acid	ND	342.46 ± 6.2	82.9 ± 2.5
Schikimic acid	ND	235.5 ± 4.2	199.3 ± 6.2
Cinnamic acid	ND	342.46 ± 7.3	90.56 ± 2.8
Vanillic acid	416.7 ± 6.2	132.1 ± 3.4	91.1 ± 5.2
p-Coumaric acid	1282.3 ± 12.7	8.21 ± 0.54	194.5 ± 4.8
Quinic acid	ND	663.1 ± 7.2	258.9 ± 6.3
(±) Naringenin	ND	8.29 ± 0.43	80.5 ± 3.9
3-Hydroxyflavone	503.7 ± 8.4	20.83 ± 0.97	216.5 4 ± 4.7
7- Hydroxyflavone	ND	19.35 ± 1.3	96.4 ± 4.8
t-Chalcone	ND	225.7 ± 3.3	542.8 ± 6.1
Hesperetin	363.7 ± 7.2	8.37 ± 0.53	97.1 ± 5.3
Flavanone	2707.8 ± 16.3	525.8 ± 8.4	99.68 ± 3.2
Quercetin	6.55 ± 0.54	13.78 ± 0.93	7.58 ± 0.66
Quercetin Hydrate	4.71 ± 0.49	10.6 3 ± 0.83	20.1 ± 0.84
Rutin	9.44 ± 0.39	14.6 3 ± 1.2	9.87 ± 0.62
(±) Catechin Hydrate	8.34 ± 0.45	4.93 ± 0.31	5.96 ± 0.71
Kaempferol	17.30 ± 1.3	8.54 ± 0.6	7.17 ± 0.46
BHT	17.41 ± 1.6	17.12 ± 1.3	6.1 ± 0.45
α-tocoferol	10.97 ± 1.2	32.41 ± 1.7	8.37 ± 0.72

Table 1: Antioxidant activity of phenolic acids and flavonoids (IC_{50}) in terms of DPPH, ABTS and deoxyribose protection assays.

DPPH free radical method has broadly been employed for the investigation of total antioxidant properties of metabolites or extracts and evaluation of the free radical scavenging activity of the natural antioxidants [29]. DPPH analysis is considered as one of the most suitable and simple colorimetric techniques for free radical scavenging effects of pure compounds or extracts from different biological sources [30]. DPPH free radical reacts with the hydrogen donor to generate reduced state of the DPPH accompanied by the violet color disappearance [31]. From the results, it is clear that the phenolic acids and flavonoids showed strong radical scavenging activity in comparison to the standard compounds like BHT and α-tocoferol.

Antioxidant property of the food ingredients is a basic parameter to assess the oxidative damage and deleterious alterations especially during preservation and storage conditions and loss of nutritional and commercial value of the foods [32]. Flavonoids, anthocyanins,

phenolic acids, vitamins and carotene are considered as some of the most important exogenous compounds that are found in the food plants and herbs as their essential ingredients and therefore add functional value to the products of human intake. There exist several reports on the antioxidant activity of various crops, plants, herbs, spice extracts, fruit juices, beverages and drinks [13,33]. From many of these sources, phenolic acids and flavonoids have been isolated and identified. Therefore, establishing antioxidant potential of phenolic acids and flavonoids from multiple antioxidant tests holds promise and directly correlate with the properties of the food products that possess them in fairly high quantity.

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