JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Properties of Phenolic Compounds from *Pelargonium reniforme*

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Flavonoids and hydrolyzable tannins isolated from *Pelargonium reniforme* were evaluated for their antioxidant ability using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical generating system and a luminol-dependent chemiluminescence assay. In both assays, the polyphenols tested showed higher radical scavenging activities than the reference antioxidant, ascorbic acid (IC_{50} 2.6−32.9 μM vs 40.9 μM in the DPPH test, and 2−25 times stronger effects in the chemiluminescence assay). A comparison of the flavonoids and the tannins showed that the latter have more potential than the former. Structural requirements for marked antioxidant activities of hydrolyzable tannins were the presence of galloyl and hexahydroxydiphenoyl groups, and apparently carbonyl (ester) functionalities in oxidatively modified dehydrohexa-hydroxydiphenoyl moieties. For flavonoids, it appeared that a catechol (3′,4′-dihydroxy) element in the B-ring were important determinants and that *O*-glycosides were more effective than flavone-based C-glucosyls. Conspicuously, introduction of a galloyl group significantly enhanced their potentials. The demonstrated marked antioxidant effects of the polyphenols provide a clue for beneficial effects of *P. reniforme* in the treatment of liver disorders among several ethnic groups in areas of southern Africa.

KEYWORDS: ***Pelargonium reniforme*; Geraniaceae; hydrolyzable tannins; flavonoids; 1,1-diphenyl-2-picrylhydrazyl; chemiluminescence; free radicals; antioxidant**

INTRODUCTION

Numerous lines of evidence have indicated that free radicals play a critical role in a variety of pathological conditions including the processes of aging, cancer, multiple sclerosis, inflammation, coronary heart and cardiovascular diseases, senile dementia, arthritis and atherosclerosis (*1*−*3*). Following the growing realization that a wide range of herbal medicines and foodstuffs may be credited for preventive effects on chronic diseases due to their radical scavenging or antioxidant properties, although their overall function in vivo has yet to be clarified, increasing attention has thus been directed to the development of safe and effective functional foods and the extraction of novel potential antioxidants from medicinal plants (*4*, *5*). With reference to the health beneficial principle of herbal products, polyphenols have been shown to represent effective antioxidant constituents (*6*−*10*).

Pelargonium species (Geraniaceae) are widely used by traditional healers in areas of southern Africa for the treatment of diarrhea, dysentery, fever, respiratory tract infections, liver complaints, and wounds (*11*). Infusions and decoctions of the fleshy tubers are commonly taken, while a traditional method of using Pelargonium roots is to boil the tuber in milk. Furthermore, the roots may also be directly chewed or powdered and mixed with food. Among those species used in the medical systems of several ethnic groups is *Pelargonium reniforme* CURT., a shrublet of up to 1-m height with kidney-shaped ("reniforme") leaves and pink flowers (*12*). This species, indigenous to the eastern parts of the Cape Province, occurs mainly in coastal regions (*13*). Previous studies on the chemistry of the title plant have revealed the presence of coumarins (*14*), tannins (*15*) and flavonoids (*16*). To provide a chemical rationale for the reported health benefits in diseased liver conditions, characteristic phenolic constituents isolated from *P. reniforme* were evaluated for their antioxidant properties using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-generating system and luminol-dependent chemiluminescence.

MATERIALS AND METHODS

Compounds. All compounds tested were isolated from *P. reniforme* CURT. ssp. *reniforme*. Their identification and purity were determined on the basis of NMR data and HPLC analyses (*15*, *16*). The plant material was kindly provided by the pharmaceutical company Dr. Willmar Schwabe, Karlsruhe, Germany.

Chemicals. Ascorbic acid was purchased from Roth (Karlsruhe, Germany) and DPPH (1,1-diphenyl-2-picrylhydrazyl) from Sigma (St. Louis, MO). A commercial kit (photosensitizer luminol (PS-1), PL-1 buffer and PCL-aqua) for assaying luminol-dependent chemiluminescence was obtained from F. A. T. (Berlin, Germany).

Determination of the DPPH Radical. A methanolic solution of a phenolic sample (tannin, flavonoid) at various concentrations (0.2, 2, 5, 10, 15 and 20 μg/mL) was added to a solution of DPPH (9.6×10^{-4}

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10.1021/jf0495688 CCC: $27.50
Published on Web 06/25/2004

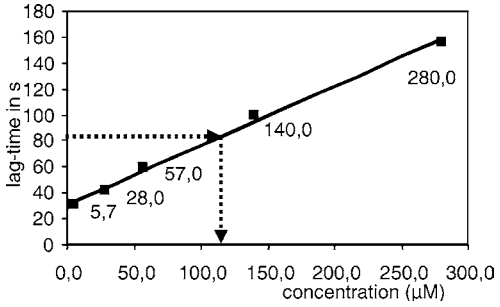

Figure 1. Calibration curve of ascorbic acid for the calculation of ascorbic acid equivs. For example, the concentration of gallic acid exhibiting a lag-time of 84.2 s showed the same radical scavenging activity as 117.0 μM of ascorbic acid.

M) in MeOH (0.5 mL) according to (*17*). The reaction mixture (total volume 2.5 mL) was shaken vigorously and allowed to react at room temperature. After 30 min, remaining DPPH was determined colorimetrically at 515 nm (Shimadzu, Model UV 160-A) by blanking against an appropriate control (2 mL MeOH + 0.5 mL of the DPPH solution). The radical scavenging activities were expressed as IC_{50} values, indicating the concentration of a compound required for 50% reduction of DPPH, calculated from the log-dose inhibition curve. Ascorbic acid was used as a positive control. Due to insufficient sample quantities, the mean values were obtained from duplicate experiments.

Luminol-Dependent Chemiluminescence. Measurements of chemiluminescence were carried out according to the protocol described previously (*18*) using a Photochem instrument (F. A. T.). The assay was based on an antioxidant-sensitive inhibition of a photoinduced autoxidation of luminol, a process associated with chemiluminescence. In the presence of antioxidants, the chemiluminescence was delayed, and the resulting lag-time as a parameter of the radical scavenging activity of a test compound was calculated using Poplab 1.74 software (F. A. T.). For determining lag-times, reaction mixtures contained 10 μL of sample solution (aqueous) at the concentration of 5 μg/mL in a final volume of 2.5 mL of carbonate buffer with luminol (commercial F. A. T. kit). The control sample was prepared without any phenolic sample. The antioxidant capacity of each sample was expressed by the ratio of the concentration of ascorbic acid, which showed the same antioxidant properties, calculated from a calibration curve (5.7, 28.0, 57,0, 140.0, and 280 μM with lag-times of 30.3, 42.2, 59.6, 98.9, and 156.0 s, respectively) (**Figure 1**), relative to that of the test compound. The experiments were performed in duplicate due to insufficient sample quantities.

RESULTS AND DISCUSSION

A series of hydrolyzable tannins and flavonoids (for structures see **Figure 2**) isolated from *Pelargonium reniforme* CURT. ssp. *reniforme* were evaluated for antioxidant activity using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-generating system (*17*) and a luminol-dependent chemiluminescence assay (*18*). As parameters for radical scavenging activity, the IC_{50} value (i.e., the sample concentration causing 50% inhibition of DPPH radical formation) and the ascorbic acid equivalent antioxidant capacity (AEAC) (chemiluminescence assay) were used. As shown in **Table 1**, all phenolic compounds tested in this study exhibited considerable inhibitory activity against the DPPH radical, with IC_{50} values ranging from 2.6 to 32.9 μM, when compared with the IC_{50} 40.9 μM of the reference, ascorbic acid. Regarding AEAC, the phenolic samples showed 2−25 times stronger scavenging effects on the superoxide radical anion than ascorbic acid in the chemiluminescence assay.

Radical Scavenging Effect on DPPH Radical. DPPH is a stable free radical which has commonly been used in antioxidant activity analysis (*8*, *17*, *19*, *20*). This test system can be used for the primary characterization of the scavenging potential of

Table 1. In-Vitro Antioxidant Activities of Tannins and Flavonoids as Determined in the DPPH Radical-Scavenging System and the Luminol-Dependent Chemiluminescence Assay[a]

	DPPH	luminol-dependent chemiluminescence			
compound	IC_{50} (μM)	sample (μM)[b]	lag-time (s)	ascorbic acid equivs (μM)[c]	AEAC[d]
		tannins			
gallic acid	32.9	29.0	84.2	117.0	4.0
methyl gallate	6.9	27.0	118.5	190.3	7.0
glucogallin	9.9	15.0	104.8	157.9	10.5
corilagin	2.7	7.5	85.2	120.5	16.0
phyllanthusiin C	5.8	5.0	63.1	70.5	14.0
brevifolincarboxylic acid	4.6	17.0	61.8	68.2	4.0
		flavonoids			
vitexin	13.1	11.5	43.3	22.7	2.0
isovitexin	16.6	11.5	45.0	28.4	2.5
orientin	19.1	11.0	62.5	68.2	6.3
isoorientin	23.2	11.0	65.9	76.1	7.0
vitexin 2″-gallate	6.8	8.5	85.9	122.7	14.4
isovitexin 2″-gallate	14.7	8.5	61.9	68.2	8.0
orientin 2″-gallate	2.6	8.0	108.9	176.1	22.0
isoorientin 2″-gallate	4.6	8.0	120.2	202.3	25.0
quercetin	5.9	16.5	60.5	62.5	3.8
isoquercitrin	14.6	10.5	70.8	87.5	8.3
rutin	9.1	n.d.	n.d.	n.d.	n.d.
		reference			
ascorbic acid	40.9	28.0	42.2	100.0	1.0

[a] Results presented are the average of two independent experiments; n.d., not determined [b] Corresponding to 5 μg/mL [c] As calculated from the standard curve [d] Ascorbic acid equivalent antioxidant capacity (AEAC) is defined as the ratio of the concentration of equivalent ascorbic acid (μM) relative to that of the test compound (μM)

compounds. In the series of hydrolyzable tannins, all polyphenols tested, except for gallic acid (IC_{50} 32.9 μM), showed pronounced inhibitory activity against the DPPH radical (IC_{50} 2.7−9.9 μM versus 40.9 μM for the reference antioxidant, ascorbic acid) (**Table 1**). The most potent candidate was the ellagitannin corilagin, with an IC_{50} of 2.7 μM.

The significance of ester functionalities for the scavenging of DPPH radicals was evident from the moderate activity of gallic acid (IC_{50} 32.9 μM) and the enhancement in potency upon esterification, as reflected by the efficiency of methyl gallate (IC_{50} 6.9), glucogallin (IC_{50} 9.9), and brevifolincarboxylic acid (IC_{50} 4.6).

Regarding structure−activity relationships for the tested hydrolyzable tannins, some interesting structural features emerged from this study, indicating conspicuous dependency of DPPH radical scavenging capabilities of polyphenols not only on the nature of their galloyl-derived substituents but also possibly on the accessibility of the radical center of DPPH to each polyphenol. For example, comparison of the radical scavenging activity of corilagin with that of phyllanthusiin C indicated that substitution at the 2,4-positions resulted in less activity. Since the number of galloyl groups, hexahydroxydiphenoyl (HHDP) moieties, and ortho-arrangements of free phenolic hydroxyl groups represent important structural features for the scavenging of radicals (*8*, *17*, *19*), perusal of data reported for a series of corilagin derivatives (*8*) provided a possible structural clue. Whereas analogues (geraniin, chebulinic acid, and elaeocarpusin) with a 2,4-bridging DHHP, chebuloyl, or elaeocarpusyl entity showed similar scavenging effects (IC_{50} 2.1−3.1 μM), putranjivain A, a structural variant of elaeocarpusin, was found less potent (IC_{50} 11.2 μM). A closer definition of the 2,4-substituent of the more active candidates revealed the presence of at least one carbonyl/lactone group in each case.

It was notable that the introduction of a 2,4-bridging moiety either maintained or decreased activity of the corilagin derivatives due to the nature of the substituent. Significantly, adding additional phenolic groups to this site of the molecule apparently does not enhance radical scavenging activity.

This finding thus extends the range of known parameters including the number of galloyl, HHDP, and DHHDP moieties, as well as the presence of ortho-dihydroxyl arrangements for appreciable scavengening activity of tannins (*8*, *17*, *19*). It also appeared that an increase in molecular weights did not necessarily enhance radical scavenging activity, at least at this level.

For flavonoids, structure−activity relationships have recently been reviewed (*9*, *10*). Concerning the inhibitory activity of the *Pelargonium* flavonoids against the DPPH radical, all compounds showed stronger scavenging capabilities (IC_{50} 2.6 to 23.2 μM) than ascorbic acid (IC_{50} 40.9 μM). Their antioxidant potentials increased in the order of C-glucosyls (vitexin, isovitexin, orientin, isoorientin) < *O*-glycosides (rutin, isoquercetin) < aglycones (quercetin) < galloylated C-glucosyls (except for isovitexin 2″-gallate) (**Table 1**). Although the presence of an ortho-hydroxy group is generally considered as one of the most important parameters for antioxidant activity, the C-glucosyls orientin and isoorientin having this structural feature were found to be only moderately active. This phenomenon cannot satisfactorily be explained at the present. Conspicuously, the introduction of a 2″-*O*-galloyl entity in members of the *C*-glucosyls significantly increased the activity, indicating again the marked antioxidant potential of pyrogallol elements in molecules (*21*, *22*). An additional interesting structure−activity relationship could also be defined for the C-glucosyls in that C-8 derivatives demonstrated higher scavenging activity than their C-6 counterparts, due possibly to steric differences associated with their accessibility of the radical.

Photochemiluminescent Detection of Antiradical Activity. The method used in this study was based on a temporary inhibition of the photoinduced, oxygen-mediated chemiluminescence emanating from oxidation of luminol (*18*). Luminol is a photosensitizer, generating superoxide radicals, and also a chemiluminogenic probe for free radicals. In the presence of an antioxidant agent, chemiluminescence was delayed and the resulting lag-time was indicative of the radical scavenging potential of this compound. The quantification and comparison of the antioxidant properties of the compounds tested were made using a standard curve for ascorbic acid (**Figure 1**). Here, the antioxidant capacity of a compound represented the amount of ascorbic acid that showed the same antioxidant properties under these experimental conditions. The AEAC was then defined as ratio of the concentration of calculated ascorbic acid equivs relative to that of the test compound.

Starting with the hydrolyzable tannins, these candidates were found to be 4−16 times more active than ascorbic acid. Consistent with the DPPH model, corilagin showed the strongest effect, followed by phyllanthusiin C (**Table 1**), confirming the above structural features for the radical scavenger potential of the ellagitannins. However, gallotannin analogues showed different trends in scavenging activity in either test system. Whereas gallic acid and brevifolincarboxylic acid, each possessing three hydroxyl groups, exhibited marked variation in radical scavenging ability when tested against the DPPH radical, tentatively explained by the presence of carbonyl groups (vide supra), no divergence was observed in the photochemiluminescence measurements. The reason for this peculiar behavior cannot be satisfactorily explained on structural grounds. Differences in solubility (water vs methanol) and stability of the resulting aryloxy radicals in the test systems may rationalize this phenomenon.

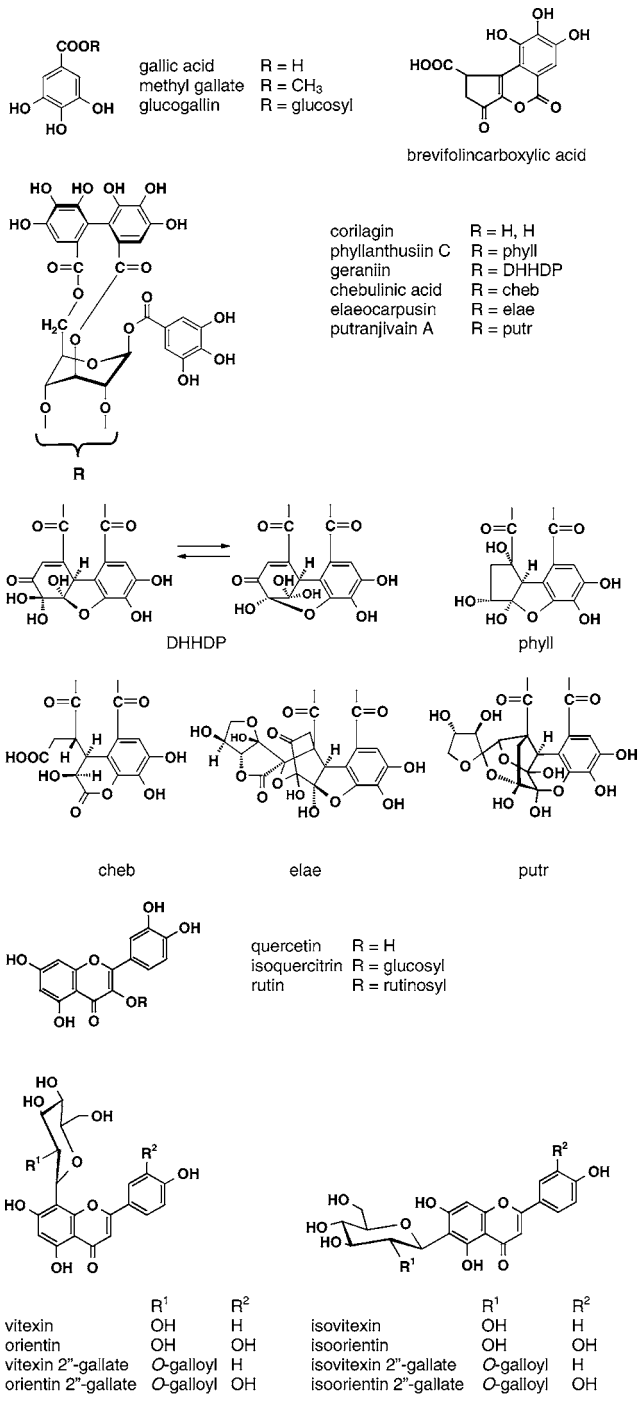

Figure 2. Structures of hydrolyzable tannins and flavonoids.

A more complex picture emerged from examining the antioxidant capacities of the flavonoids. The experimental data showed that all compounds efficiently scavenged radicals and that the galloyl derivatives of the C-glucosyls were the most powerful compounds, consistent with results obtained from the DPPH model system (**Figure 2**). However, the antioxidant capacities of the O-glycoside, C-glucosyls and the aglycone appeared not very distinctive. Within the group of C-glucosyls, members possessing 3′,4′-dihydroxyls (orientin, isoorientin, and their galloyl analogues) consistently exhibited stronger effects than their 4′-monooxygenated analogues. That this hydroxylation pattern represented an important structural feature was also

evident from the activity of isoquercitrin. In contrast, quercetin showed less potential in this model system than in the DPPH assay, which may be explained on the basis of the different experimental conditions used in these in vitro assays.

From this finding, it was evident that antioxidant capacities depend not only on the chemical structure but also strongly on the model system. Accordingly, in vivo settings are essential to prove the therapeutic benefits of antioxidants in free radical diseases, though in vitro studies facilitate the primary evaluation.

***P. reniforme* and Hepatic Disorders.** Several studies on medicinally used plants with marked antioxidant activity demonstrated hepatoprotective properties. Tannins and flavonoids *(23−28)* were frequently identified and assumed to represent the biologically active principle due to their antioxidant potentials. Therefore, the prominent antioxidant capacities of characteristic constituents demonstrated in this study provide a basis for the traditional use of *P. reniforme* in the treatment of liver disorders. It should also be noted that these metabolites occurred in relatively high quantities of ca. 0.03−0.1% related to the dry weight in this species, thus giving credence to their role as potentially antioxidant components. Clearly, further studies using cellular systems are needed to support the medical indication on a rational basis.

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Received for review March 16, 2004. Revised manuscript received May 18, 2004. Accepted May 22, 2004.

JF0495688