



Anti-oxidant activity of polyphenols from *Phyllanthus debilis* Klein ex Willd

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Abstract

Phyllanthus debilis Klein ex Willd. (Euphorbiaceae) usually used as substitute/adulterant to *Phyllanthus amarus*, a hepatoprotective drug. However, the phytochemical investigation of *P. debilis* has not been performed much. In the present study, five compounds were isolated from the ethyl acetate extract of aerial parts of *P. debilis* and they were identified as Gallic acid, Rutin, Corilagin, Furosin and Geraniin. All the constituents have been reported for the first time from this plant. Their structures were elucidated by spectral techniques. Furthermore, the phenolic compounds were tested for antioxidant activity against DPPH (1,1-diphenyl picrylhydrazyl) and by the phosphomolybdenum method. Ascorbic acid is used as the reference standard. From the isolated compounds, the Geraniin had showed highest activity in DPPH assay and Gallic acid in phosphomolybdenum method.

Keywords: *Phyllanthus debilis*; antioxidant activity; polyphenols. DPPH; Phosphomolybdenum complex

1. Introduction

Phyllanthus debilis Klein ex Willd belongs to the genus *Phyllanthus*, which contains of approximately 550 to 750 species and are widely distributed throughout most tropical and subtropical countries. The genus *Phyllanthus* have long been used in folk medicine in India and most other countries for thousands of years for the treatment of a broad spectrum of diseases, such as disturbances of the kidney and urinary bladder, intestinal infections, diabetes, and the hepatitis B virus [1, 2]. Almost all species of *Phyllanthus* found in India are used medicinally, especially in the treatment of

jaundice. The *P. debilis* belonging to the Euphorbiaceae usually used as substitute/adulterant to *P. amarus*, which has been widely used as traditional medicine for various ailments. *P. debilis* has been found to be a better hepatoprotective agent than *P. amarus* [3].

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases as well as in the normal process of aging. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging crucial biomolecules. If they are not effectively scavenged

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by cellular constituents, they lead to disease conditions [4]. There are a number of literature reports on phytochemical analyses of this genus *Phyllanthus* [5-7]. However, the phytochemical investigation of *P.debilis* has not been performed much.

TLC comparisons of *Phyllanthus* species to investigate the antihepatotoxic agents were described [8]. Isolation of lignans from the *P. debilis* was reported in the literature [9]. In this investigation we report the phenolic constituents and their antioxidant activity of *P.debilis* growing in South India.

2. Materials and methods

Plant material: Aerial parts of *P.debilis* were collected in and around Chennai during the rainy season (November 2003). Botanical identification was performed by Dr. D. Narashiman, Department of Botany, Madras Christian College where the voucher specimen has been deposited.

Extraction and isolation: Dried aerial parts of *P.debilis* were ground and extracted at room temperature with methanol. After filtering, the methanol was evaporated, and the extract was redissolved in water, kept at 4°C for 12 h, and filtered again, thus obtaining the crude extract. This crude extract was then partitioned with hexane (200 ml fractions repeatedly until decoloration of the organic solvent), thus obtaining both the hexane fraction and the “clean” or “defatted” crude extract.

The defatted crude extract was then successively partitioned with dichloromethane and ethyl acetate (as for the hexane partition), thus obtaining the dichloromethane, ethyl acetate, and aqueous fractions. The ethyl acetate extract (3.5 g) was subjected to Column Chromatography on Sephadex LH-20 and eluted with 50% methanol in water and increasing amounts of methanol to 100%. Fifteen fractions

of 250 ml each were collected. The fractions having similar TLC pattern were combined, yielded six fractions. These fractions were subjected to rechromatography over Sephadex LH20 using methanol as solvent to afford five compounds: gallic acid (22.5 mg), rutin (16 mg), corilagin (18 mg), furosin (12 mg) and geraniin (14 mg), characterized by spectral methods and reference to literature.

Spectroscopic data

Gallic acid (1) White amorphous powder, ¹H NMR (DMSO-d₆): δ 6.92 (ArH). ¹³C NMR (DMSO-d₆): δ 109.1(2) (ArC), 120.8 (the acid link carbon on the aromatic ring), 138.4, 145.8(2) (hydroxylated carbon atoms) and 167.9 (-COOH).

Rutin (2) Yellow powder, UV (λ_{max}, nm) MeOH=255, 265sh, 353. ¹H NMR (CD₃COCD₃): δ 1.12 (d, J=6.26Hz, H-6’’), 3.55 (dd, J=3.55, 10.45Hz, H_b-6’’), 3.64 (dd, J=1.7, 3.4Hz, H-2’’), 3.80 (dd, J=2, 10.9Hz, H_a-6’’), 4.52 (d, J=1.68Hz, H-1’’), 5.11 (d, J=2.14Hz, H-1’’), 6.20 (d, J=2.1Hz, H-6), 6.41 (d, J=1.98, H-8), 6.87 (d, J=8.4Hz, H-5’), 7.63 (dd, J=2.14, 8.41Hz, H-6’), 7.65 (d, J=2.14Hz, H-2’).

Corilagin (3) White amorphous powder, ¹H NMR (CD₃COCD₃): δ 4.14 (br s, glucose (Glc) H-2), 4.40 (br s, Glc H-6), 4.50 (m, Glc H-5), 4.85 (br s, Glc H-3), 5.1 (t, J=10.9 Hz, Glc H-6’), 6.42 (d, J=1.78 Hz, Glc H-1), 6.70 (s), 6.85 (s) (hexahydroxydiphenoyl (HHDP) protons) and 7.10 (s, galloyl-H). ¹³C NMR (CD₃COCD₃): δ 62.0 (C-4), 64.2 (C-6), 69.0 (C-2), 70.5 (C-3), 75.6 (C-5), 94.0 (C-1) (Glc-C), 108.0, 109.9 (HHDP-C), 111.0 (galloyl-C), 115.9, 116.9 (C atoms linking the diphenyl rings of HHDP), 120.8, 125.5, 125.7 (the ester link carbons on the aromatic rings), 134.6, 135.1, 137.2, 142.6(2), 142.8, 143.2(2), 143.7 (hydroxylated carbon atoms), 163.1, 165.1 and 166.5 (ester CO).

Furosin (4) Yellow crystalline powder, ^1H NMR (CD_3COCD_3): δ 4.05 (dd, $J = 5.5, 10.6$ Hz,), 4.20 (m), 4.45 (brs)-Glc H-3, H-5 and H-6, 5.15 (d, $J = 3.3$ Hz, Glc H-4), 5.34 (d, $J = 3.05$, Glc H-2 and methine), 6.42 (d, $J = 8.4$ Hz, Glc H-1), 6.54 (s, Vinyl), 7.22 (s, galloyl-H) and 7.25 (m, ArH). ^{13}C NMR (CD_3COCD_3): δ 45.9, 51.9 (didehydrohexahydroxydiphenoyl (DHHDP) C-1), 62.8, 63.0, 63.4, 71.2, 72.2, 72.5, 73.6, 77.8, 78.5, 91.8, 92.4 (Glc-C), 92.5, 96.1 (DHHDP C-5), 92.7, 109.0 (DHHDP C-6), 110.41, 110.46 (galloyl C), 113.4, 113.9 (DHHDP C-11), 116.1, 117.8 (DHHDP C-7), 119.3, 120.0 (DHHDP C-12), 120.6, 120.8 (the ester link carbons on the galloyl rings), 125.6, 128.6 (DHHDP C-3), 137.2, 139.0 (DHHDP C-9), 139.5, 145.9, (hydroxylated carbon atoms galloyl rings), 143.0, 146.9 (DHHDP C-8), 145.5, 147.4 (DHHDP C-10), 149.1, 154.6 (DHHDP C-2), 165.5, 165.9, 166.1, 166.2, 166.5 (ester CO), 192.5 and 195.4 (DHHDP CO).

Geraniin (5) Greenish yellow crystalline powder, ^1H NMR (CD_3COCD_3): δ 4.34 (m), 4.45 (m, Glc H-6a), 4.82 (m, Glc H-5), 4.98 (m, Glc H-6b), 5.21 (s, DHHDP H-1), 5.45 (brs), 5.52 (s, Glc H-4), 5.55 (s, Glc H-3), 5.59 (s, Glc H-2), 6.29 (d), 6.58 (s) (DHHDP H-3), 6.59 (brs, Glc H-1), 6.69 (s), 6.71 (s), 7.11 (s), 7.17 (s), 7.22 (s), 7.23 (s), 7.24 (s) and 7.29 (s) (Ar-H from the galloyl, HHDP and DHHDP). ^{13}C NMR (CD_3COCD_3): δ 46.1 (didehydrohexahydroxydiphenoyl (DHHDP) C-1), 63.3, 63.7, 65.9, 69.8, 72.6, 90.7 (Glc-C), 92.4, 96.1 (DHHDP C-5, C-6), 108.0, 110.6 (HHDP-C), 110.9 (galloyl-C), 115.7, 117.1 (C atoms linking the diphenyl rings of HHDP), 113.6 (DHHDP C-11), 115.2 (DHHDP C-7), 119.4 (DHHDP C-12), 120.2, 124.7, 125.7 (the ester link carbons on the HHDP and galloyl rings), 128.7 (DHHDP C-3), 136.5, 137.8, 139.7, 144.8, 145.0, 145.4, 145.7, 145.8(2),

(hydroxylated carbon atoms on HHDP and galloyl rings) 138.8 (DHHDP C-9), 143.3 (DHHDP C-8), 144.5 (DHHDP C-10), 154.5 (DHHDP C-2), 164.8, 165.4, 165.6, 166.2, 168.4 (ester CO) and 191.7 (DHHDP CO).

Assay for DPPH free radical scavenging effect. The method used was adapted from the literature [10]. The phenolic compounds were evaluated in terms of their hydrogen donating or radical scavenging ability using the stable DPPH radical. Ascorbic acid was used as reference material. The samples dissolved in methanol at various concentrations (2-50 μM /mL) were introduced into 10 mL screw-capped tubes. Three milliliters of the freshly prepared solution of DPPH (0.004% in methanol) was added to the sample tube and mixed vigorously for 15 s. The sample tube was then kept at room temperature for 30 min. The absorbance of the sample was measured at 517 nm by spectrophotometer. All tests were performed three times. The DPPH radical scavenging effect was calculated as “inhibition percentage” according to the eq.1.

$$\text{Inhibition percentage (\%)} = \frac{[AC(0) - AA(t)]/AC(0)}{[AC(0) - AA(t)]/AC(0)} \times 100 \quad (1)$$

where AC(0) is an absorbance of control DPPH solution at 0 min and AA(t) is an absorbance of test samples at 30 min.

Evaluation of total antioxidant capacity by phosphomolybdenum method. The total antioxidant capacity of phenolic compounds was evaluated by the literature method [11]. An aliquot of 0.1 ml of sample solution of various concentrations was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). For the blank, 0.1 ml of methanol was used in place of sample. The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled

to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm by spectrophotometer against a blank. Antioxidant capacity was expressed as equivalents of ascorbic acid.

3. Results and discussion

The ethyl acetate fraction of the aerial parts of *P.debilis* yielded five compounds, one phenolic acid, one flavonol-glycoside and three ellagitannins, namely, gallic acid, rutin, corilagin, furosin and geraniin (Fig. 1). The identity of the isolated compounds was confirmed through interpretation of their spectral characters and comparison with the reported data [12-15]. The results for the free radical scavenging activity and total antioxidant activity are shown in Table 1 and Table 2 respectively.

Table 1.
DPPH scavenging activity of the tested compounds

Tested materials	IC50 (μ M)
Gallic acid	8.31
Rutin	9.42
Corilagin	3.05
Furosin	3.75
Geraniin	2.65
Ascorbic acid	8.41

Table 2.
Total antioxidant activity of the tested compounds

Tested materials	Antioxidant activity ^a
Gallic acid	1.10
Rutin	0.27
Corilagin	1.00
Furosin	0.70
Geraniin	1.09

^acompared with ascorbic acid = 1.00.

In DPPH assay, the geraniin had showed highest activity and the scavenging effect in the order: Geraniin > Corilagin > Furosin > Gallic acid > Ascorbic acid > Rutin. DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants [16]. The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction. The compounds were able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine.

It appears that the isolated constituents possess hydrogen donating capabilities to act as antioxidant. Scavenging activity of Ascorbic acid, a known antioxidant used as positive control was lesser than the isolated compounds except Rutin.

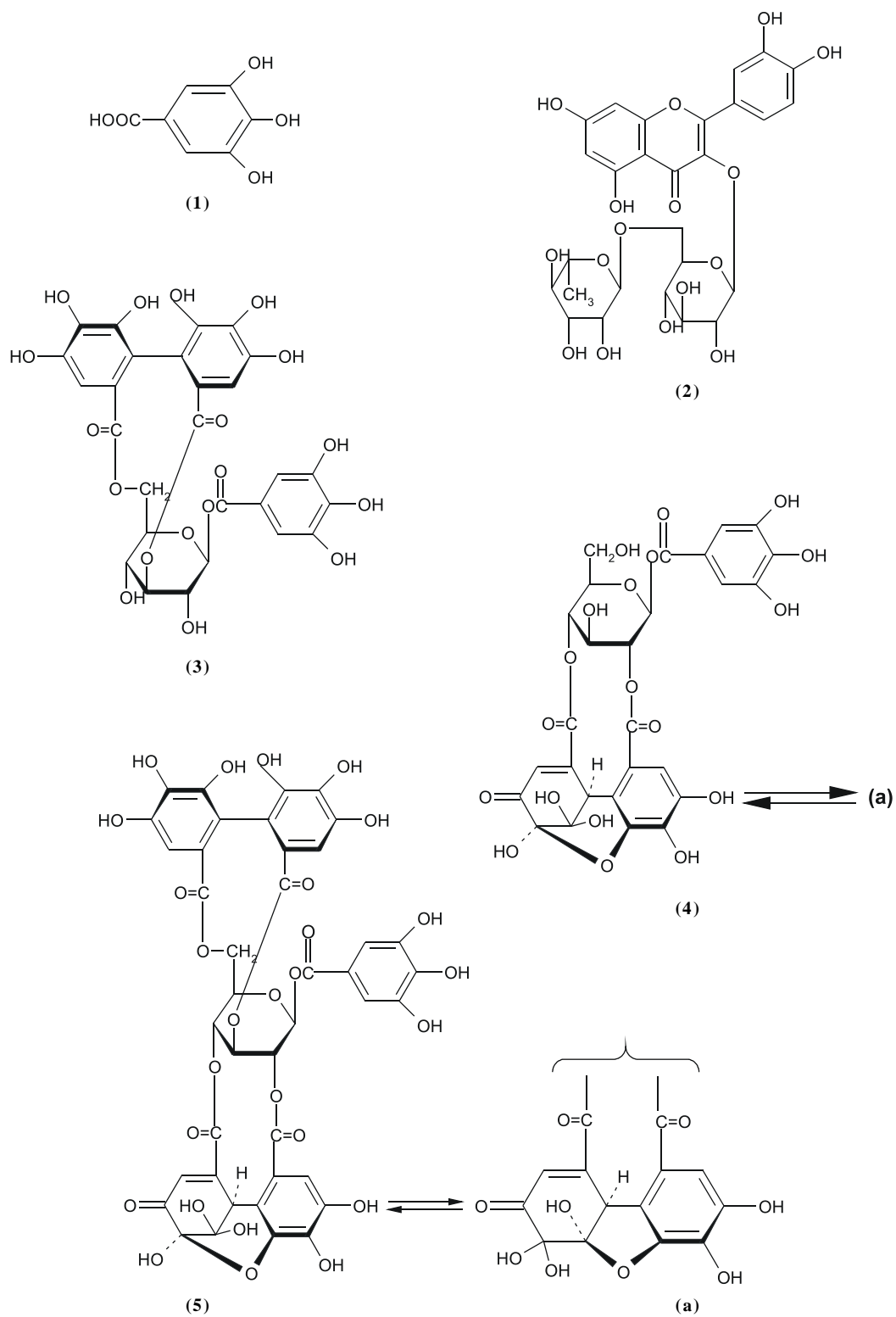
In phosphomolybdenum method, Gallic acid had showed highest activity and the potency was in the following order: Gallic acid > Geraniin > Corilagin > Furosin > Rutin. The assay is based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid.

The assay was successfully used to quantify vitamin E in seeds [11] and, being simple and independent of other antioxidant measurements commonly employed. The result indicates that the compounds possess significant antioxidant capacity when compared with ascorbic acid except Rutin.

4. Conclusion

The antioxidant activity assessments of polyphenols, by measuring their capacity to scavenge the DPPH and to reduce

Structure of the isolated compounds: (Fig.1)



Mo(VI) to Mo(V), showed that the isolated compounds were potent antioxidants. The potency of these compounds could provide a chemical basis to *P.debilis* which can be used for various disorders where free radicals are thought to be involved. This report suggests that *P.debilis* extracts may provide a new source of natural food antioxidants. However, the *in vivo* safety of the extracts as well as

polyphenols needs to be thoroughly investigated in experimental rodent models prior to its possible application as an antioxidant ingredient, either in animal feeds or in human health foods.

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REFERENCES

1. Unander DW, Webster GL, Blumberg BS. (1995) *J. Ethnopharmacol.* 45: 1-18.
2. Calixto JB, Santos AR, Filho VC, Yunes RA. (1998) *Med. Res. Rev.* 18: 225-258.
3. Sane RT, Kuber VV, Chalissery, MS, Menon, S. (1995) *Curr. Sci.* 68: 1243-1246.
4. Halliwell B, Gutteridge JMC, Cross CE. (1992) *J. Lab. Clin. Med.* 119: 598-620.
5. Anjaneyulu ASR, Rao KJ, Row LR, Subrahmanyam C. (1973) *Tetrahedron* 29: 1291-1298.
6. Syamasundar KV, Singh B, Thakur RS, Husain A, Kiso Y, Hikino H. (1985) *J. Ethnopharmacol.* 14: 41-44
7. Huang YL, Chen CC, Hsu FL, Chen CF. (1998) *J. Nat. Prod.* 61: 1194-1197
8. Kuber VV, Chawla JL, Sane RT. (1997) *Indian Drugs* 34: 36-42.
9. Chandrashekar KS, Satyanarayana D, Joshi AB, Subrahmanyam EVS. (2004) *Nat. Prod. Sci.* 10: 101-103.
10. Germano MP, Pasquale RD, D'Angelo V, Catania S, Silvari V, Costa C. (2002) *J. Agric. Food Chem.* 50: 1168-1171.
11. Prieto P, Pineda M, Aguilar M. (1999) *Anal. Biochem.* 269: 337-341.
12. Mabry TJ, Markhm KR, Thomas MB. (1970) *The systematic identification of flavonoids.* Springer-Verlag: Berlin
13. Yoshida T, Okuda T. (1980) *Hetrocycles* 14: 1743-1749.
14. Lim YA, Mei MC, Kusumoto IT, Miyashiro H, Hattori M, Gupta MP, Correa M. (1997) *Phytother. Res.* 11: 22-27.
15. Adesina SK, Idowu O, Ogundaini AO, Oladimeji H, Olugbade TA, Onawunmi GO, Pais M. (2000) *Phytother. Res.* 14: 371-374.
16. Oyaizu, M. (1986) *Jpn. J. Nutrition* 44: 307-315.