

Research Article

An *in-vitro* investigation of the antioxidant activity of *Phyllanthus amarus*

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Abstract

The aim of this work was to investigate the total phenolic content and antioxidant potential of aqueous extract of *Phyllanthus amarus*. The aqueous extract of *Phyllanthus amarus* whole plant was prepared then systematically investigated by various antioxidant assays, including: DPPH- (1, 1-diphenyl-2-picrylhydrazyl radical), Nitric oxide radical inhibition activity, and β -carotene-linoleate method, compared with ascorbic acid and rutin. Its total phenolic content was also measured by Folin-Ciocalteu colorimetric method. *P. amarus* aqueous extract exhibited effective antioxidant activity in dose-dependent manners and its IC₅₀ values were calculated as 4.21 \pm 0.379, 426 \pm 0.512, 126 \pm 0.348 for DPPH, Nitric oxide radical inhibition activity, and β -carotene-linoleate assay respectively. Its total phenolic content was 52.71 \pm 0.214 mg GAE /g. *P. amarus* aqueous extract has effective *in vitro* antioxidant activity which may attribute to its total phenolics, among which phyllanthin and hypophyllanthin can be considered as the active components. The pharmacological effects or healthcare functions of *P. amarus* may result from the synergistic effects caused by the combination of its components and its antioxidant effect plays an important role in the synergistic effects.

Keywords: *Phyllanthus amarus*, total phenolic content, antioxidant, 1, 1-diphenyl-2-picrylhydrazyl radical, β -carotene-linoleate method

1. Introduction

Reactive oxygen species (ROS) such as superoxide radical anion, hydroperoxyl radical are generated in cells from byproducts of metabolism and responsible for the development of a wide number of degenerative diseases such as cardiovascular disease (CVD), diabetic, cirrhosis and several cancers¹. All organisms have their own antioxidant defense system to protect them against free radical damage by enzymes, such as superoxide dismutase and catalase or compounds such as ascorbic acid, tocopherol and glutathione². However, our body system sometimes cannot work efficiently that lead to unavoidable damage caused by oxidation. As a result, consumption of additional antioxidants through herbs, foods or supplements (phytochemicals) are beneficial to defense against the harmful ROS³. A recent investigation on the beneficial effects of fruits, vegetables and spices containing high phytochemical antioxidants were carried out by groups of researchers⁴⁻⁵ concluded that the crude extracts with antioxidant properties are bioprotective in nature and are therefore valuable in the food industry for longer shelf life. Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and Tert-butylated hydroxyquinone (TBHQ) are the common synthetic antioxidants to prevent oxidative process⁶ that have been used in food industry since 1940 but the trend currently is to move towards natural antioxidants as an alternative source⁷⁻⁸ revealed that BHA and BHT were found to promote tumor formation, therefore the use of a natural antioxidant source become crucial.

Phytochemicals are produced by the plant kingdom for defense, protection, cell to cell signaling and as attractants for pollinators⁷ are bioactive non-nutrient plant compounds that have been linked to the reduction of risk related to chronic diseases. Their commercial potential can be utilized in food industry as additives, as antibiotics in pharmaceutical and as essence extract in herbal and cosmetic products. Phytochemicals are structurally diverse, based on their biosynthetic origins they can be classified into basically four classes whose members may exert positive effects on human health; the terpenoids, phenolics and polyphenolics and nitrogen-containing alkaloids and sulphur-containing compounds⁹. Therefore, any research elucidating antioxidant properties of plant extract could prove to be a promising alternative to those antibiotics, which are becoming ineffectual against infections¹⁰.

India is a land of biodiversity; this biodiversity supports numerous species of medicinal plants. One of such genus that have been used for in folk medicine for decades and known to possess great diversity of secondary metabolites is the genus *Phyllanthus* (Euphorbiaceae). It is a very large genus consisting of approximately 550 to 750 species and is subdivided into 10 or 11 subgenera: *Botryanthus*, *Cicca*, *Conani*, *Emblica*, *Ericocus*, *Gomphidium*, *Isocladus*, *Kirganelia*, *Phyllanthodendron*, *Phyllanthus*, and *Xylophylla*.

Phyllanthus amarus Schum. & Thonn. (Euphorbiaceae) also locally known as “Bhumyamalaki”. *P. amarus* is reported to contain variety of phytoconstituents like lignans namely phyllanthin, hypophyllanthin, nirphyllin and phyllnirurin; flavanone glycosides like niranthin, nirtetralin, phylltetralin and lintetralin; a steroidal hormone estradiol; flavanoids like quercetin, quercitrin, and astragalin; triterpenes like phyllanthanol, phyllanthone and phyllanthol are some of the important constituents¹¹. It has been traditionally used for treating kidney and gallbladder stones, liver related diseases and viral infection. Research on *Phyllanthus* sp. has been widely conducted in India¹² and reported that antioxidant activity and hepatoprotective potential found in *P. niruri*. Meanwhile, *P. amarus* was reported to have anti-diabetic, anti-cancer and anti-inflammation properties and was shown to have anti-mutagenic and anti-carcinogenic effects in India¹³. In addition, antioxidant activities from methanol extract of five *Phyllanthus* species in India have been reported¹⁴. Although there has been some reports on the health benefits of *Phyllanthus* sp elsewhere but information regarding the antioxidant activities of this species is very limited. This study was therefore undertaken to determine the total phenol content and antioxidant potential of aqueous extract of *P. amarus*.

2. Materials and Methods

2.1 Plant material

The material was purchased as aerial tender branches of *Phyllanthus amarus* from the local market of Nagpur, and authenticated by Dr. A.A. Fulzele, Dept. of Botany, S. M. Mohota College of Science, Nagpur. A sample specimen No-1102 is stored for future reference in Dept. of Botany, S. M. Mohota College of Science, Nagpur. The aerial tender branches of *Phyllanthus amarus* were cleaned by removing foreign matter, dried and powdered coarsely.

2.2 Chemicals and reagents

DPPH-(1,1-Diphenyl-2-picrylhydrazyl radical), dimethyl sulfoxide, Folin–Ciocalteu reagent, sodium nitro prusside, naphthyl ethylene diamine dihydrochloride, sulphanilic acid reagent, linoleic acid, Tween 40 emulsifier were purchased from Sigma Co. All other chemicals and solvents were of analytical grade.

2.3 Extraction

About 1000 gm of coarsely powdered *Phyllanthus amarus* whole plant was taken in a 2 liter round bottom flask separately and extracted continuously with water by soxhlet extraction. The aqueous extracts were then separated and filtered. The filtrate was then concentrated under reduced pressure and dried in a dessicator.

2.4 Preparation of plant extracts for total polyphenol content

Dry plant extract (1 g) was weighed into a test tube. A total of 10 ml of 80% aqueous methanol was added, and the suspension was stirred slightly. Tubes were sonicated twice for 15 min and one left at room temperature (20 °C) for 24 h. The extract was centrifuged for 10 min and supernatants were collected at 4 °C prior to use within 24 h.

2.5 Estimation of total polyphenol content

Total polyphenol content was measured using Folin–Ciocalteu colorimetric method described previously¹⁵. Plant extracts (100 µl) were mixed with 0.2 ml of Folin–Ciocalteu reagent and 2 ml of H₂O, and incubated at room temperature for 3 min. Following the addition of 1 ml of 20% sodium carbonate to the mixture, total polyphenols were determined after

1 h of incubation at room temperature. The absorbance of the resulting blue colour was measured at 765 nm with a Shimadzu UV-VIS spectrophotometer. Quantification was done with respect to the standard curve of gallic acid. The results were expressed as gallic acid equivalents (GAE), milligram's per 100 g of dry weight (dw). All determinations were performed in triplicate (n= 3).

2.6 Free radical-scavenging ability by the use of a stable DPPH radical

2.6.1 Preparation of test solutions

21 mg of the plant extract was weighed and dissolved in distilled dimethyl sulfoxide (DMSO) separately to obtain a solution of 21mg/ml concentration. This solution was serially diluted separately to obtain lower concentration ranging from 1,000 mg/ml to 0.9765 mg/ml.

2.6.2 Preparation of standard solution

10 mg of each of ascorbic acid and rutin were weighed separately and dissolved in 0.95 ml of DMSO to get 10.5 mg/ml concentrations. This solution was serially diluted with dimethyl sulfoxide to get lower concentrations.

2.6.3 Method

The DPPH radical-scavenging activity was determined using the proposed method¹⁶. The assay was carried out in a 96 well microtitre plate. To 200 µl of DPPH solution, 10 µl of each of the test sample or the standard solution was added separately in wells of the microtitre plate. The final concentration of the test and standard solutions used were 1000 to 1.95 mg/ml. The plates were incubated at 37 °C for 20 minutes and the absorbance of each solution was measured at 490 nm, using ELISA reader against the corresponding test and standard blanks and the remaining DPPH was calculated. IC₅₀ (Inhibitory Concentration) is the concentration of the sample required to scavenge 50 % of DPPH free radicals. The results were corrected for dilution and expressed in µM trolox per 100 g dry weight (dw). All determinations were performed in triplicate.

$$\text{Formula for IC}_{50} \text{ value} = \{ \text{Control} - \text{Sample} / \text{Control} \} \times 100$$

2.7 Nitric oxide radical inhibition activity

2.7.1 Method

The reaction mixture (6 ml) containing sodium nitro prusside (10 mm, 4 ml), phosphate buffer saline (PBS, 1 ml) and 1 ml of extract in DMSO were incubated at 25 °C for 150 minutes. After incubation, 0.5 ml of the reaction mixture containing nitrate was removed and 1 ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 minutes for completion of diazotization, then 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 minutes in diffused light at room temperature. The absorbance of these solutions was measured at 540 nm using ELISA reader against corresponding blank solution. IC₅₀ value obtained is the concentration of the sample required to inhibit 50 % nitric oxide radical. The results were corrected for dilution and expressed in µM trolox per 100 g dry weight (dw). All determinations were performed in triplicate.

2.8 β-carotene-linoleate method

2.8.1 Preparation of Test and Standard solution

Different concentrations 10-200 µg/ml of extracts and ascorbic acid (Standard) prepared in methanol.

2.8.2 Method

The antioxidant activity of extract was evaluated by the beta-carotene-linoleate model system¹⁷. A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in the 10 ml of chloroform. This solution (2 ml) was pipetted into a 100 ml round bottom flask. After chloroform was removed under vacuum, 40 mg of purified linoleic acid, 400 mg of Tween 40 emulsifier, and 100 ml of aerated distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into test tubes containing different concentrations of the extracts (0.2 ml). As soon as the emulsion was added to each tube, the zero time absorbance was measured at the 470 nm using a spectrophotometer. The tubes were placed at 50 °C in a water bath, and measurement of absorbance was recorded after 2 h; a blank, devoid of beta-carotene, was prepared for background subtraction. The same procedure was repeated with the ascorbic acid, as a positive control. The results were corrected for dilution and expressed in µM trolox per 100 g dry weight (dw). All determinations were performed in triplicate. Antioxidant activity was calculated using the following equation:

Antioxidant activity = (β -carotene content after 2h of assay/ Initial beta-carotene content) X 100

3. Result and Discussion

3.1 Total phenolic content (TPC) determination and total antioxidant activities analysis

The TPC assay is a common assay widely used to estimate relative amounts of phenolic compounds present in an extract. The TPC results were expressed as mg gallic acid equivalent as this compound represents the most simple form of a phenolic compound. Phenolic compounds present in the extract undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the TPC reagent¹⁸. Depending on the number of phenolic groups present, different response can be observed in terms of the color change due to oxidation of the TPC reagent. This color change is detected by a spectrophotometer and quantified in term of mg gallic acid equivalent per dry weight plant. The results for the TPC analysis indicate that aqueous extract of *Phyllanthus amarus* contain significant amounts of phenolic compounds 52.71 ± 0.214 (Table 1) as revealed by the concentrations when compared to the positive control.

The three antioxidant assays that have been used in the study were DPPH scavenging assay, Nitric oxide radical inhibition activity, and β -carotene-linoleate method. 1,1-Diphenyl 1-2-picryl hydrazyl is the main chemical used in the DPPH assay and it is used widely for the determination of free radical scavenging activity of antioxidant compounds⁴. It is also one of the few known stable and commercially available organic nitrogen free radical¹⁹. When this compound is added to the plant extracts containing antioxidant compounds, diphenyl picryl hydrazyl is reduced to diphenyl picryl hydrazine and a color change is observed in the process where the color fades from purple to yellow. This then can be measured using UV-VIS spectrophotometer at absorption 515 nm. The antioxidant activity can be expressed in various ways and one of the most common ways is to express by referring it to a common reference standard. One common reference standard used for this purpose is (S)-(-)-6 hydroxy-2,5,7,8-tetra methyl chroman-2-carboxylic acid, also known as Trolox. Therefore, all the antioxidant activities for DPPH assay was expressed as mg Trolox equivalent per g dry weight of plant.

Sodium nitro prusside (SNP) in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of Griess Illosvoy reaction. In the present investigation, Griess Illosvoy reagent is modified by using naphthyl ethylene diamine dihydrochloride (NEDD) (0.1 % W/V) instead of 1-naphthylamine (5 %). Scavengers of Nitric oxide compete with oxygen leading to reduced production of Nitric oxide.

β -carotene-linoleate model measure the prevention of bleaching of β -carotene by an antioxidant. β -carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of beta-carotene and linoleic acid, which generates free radicals. This linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated beta-carotene molecules. As a result, beta-carotene will be oxidized and broken down in part; subsequently, the system loses its chromophores and characteristic orange color, which can be monitored spectrophotometrically at 470 nm. The presence of different antioxidants can hinder the extent of beta-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system.

P. amarus aqueous extract exhibited effective antioxidant activity in dose-dependent manners and its IC₅₀ values were calculated as 4.21 ± 0.379 , 426 ± 0.512 , 126 ± 0.348 for DPPH, Nitric oxide radical inhibition activity, and β -carotene-linoleate assay respectively (Table 1). These result suggested that antioxidant capacity in *Phyllanthus amarus* was due to the contribution of phenolic compounds.

Table 1. Determination of total phenolic content and antioxidant activity analysis

Plant material	Total phenolic content (TPC) ^a	DPPH Scavenging assay (DPPH) ^b	Nitric oxide scavenging assay (NOS) ^c	β -carotene linoleate assay(BCL) ^d
<i>P. amarus</i> Aq. extract	52.71 ± 0.214	4.21 ± 0.379	426 ± 0.512	126 ± 0.348
Positive control	58.05 ± 0.261	2.75 ± 0.228	68.5 ± 0.426	91.65 ± 0.219

All values are the means of three measurements.

a express as mg GAE/g dry weight, 100 μ g/mL of 3-hydroxyphenylacetic acid as positive control

b, d express as mg TE/g dry weight, 10 μ g/mL of ascorbic acid as positive control

c express as mg TE/g dry weight, 10 μ g/mL of rutin as positive control

4. Conclusion

The aqueous extract of *Phyllanthus amarus* whole plant possesses an effective *in vitro* antioxidant activity which can contribute to its medicinal or health-care effects. Its antioxidant activity may result from the radical-scavenging & reducing power, and can attribute to the total phenolics, among which phyllanthin and hypophyllanthin is regarded as important bioactive compounds.

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