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Phytochemical and Antioxidant Studies on Fruits of *Phyllanthus* Species

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ABSTRACT

The genus *Phyllanthus* (Euphorbiaceae) is widely used in the Indian system of traditional medicine and is reported to have various biological activities. In this study, the polyphenol and flavonoid contents of *Phyllanthus acidus*, *Phyllanthus emblica* and *Phyllanthus fraternus* were quantitated. The fruits of *Phyllanthus acidus*, *Phyllanthus emblica* and *Phyllanthus fraternus* were collected and shade dried. The shade dried plant material were extracted with 95% methanol under sonication and the antioxidant activities were investigated using *in vitro* assays along with the determination of phytochemical constituents (total polyphenol and total flavonoid). Their ability to salvage were extensively investigated with in vitro DPPH$^{\bullet}$ scavenging assays. The extract exhibited significant antioxidant properties and the observed biological activities provide scientific validation of ethno medicinal use of this plant.

Keywords: *Phyllanthus, antioxidant, ultrasonication, DPPH, polyphenolics.*

Introduction

Oxidative stress is a state of disequilibrium between oxidants and antioxidants in favour of the oxidants, potentially leading to damage [1]. The human body possesses numerous antioxidant defenses and repair mechanisms against oxidative stress. Numerous experimental and epidemiological studies have shown that a wide variety of phytochemicals such as phenolics, flavonoids, isoflavones, flavones, anthocyanins, catechins, isocatechins and carotenoids are able to prevent or slow down oxidative stress-induced circumstances. High consumption of fruits and vegetables is associated with low risk for the diseases such as coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer, which is ascribed to the antioxidant vitamins and other phytochemicals [2-4]. There is a great deal of interest in edible plants that contain antioxidants and health – promoting phytochemicals, in view of their health implications.

The plants of the genus *Phyllanthus* (Euphorbiaceae) are widely distributed in most tropical and subtropical countries, and have long been extensively used in folk medicine in India and most other countries for thousands of years in 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 [5, 6]. Several reasons contribute to this, such as (a) their greater distribution in many tropical and subtropical countries, (b) the great number of species in this genus, (c) their broad therapeutic use in folk medicine (Triphala), and (d) the large variety of secondary metabolites present in such plants [7].

The three species of *Phyllanthus*, *Phyllanthus emblica* L. (*P. emblica*), *Phyllanthus acidus* (L.) Skeels (*P. acidus*) and *Phyllanthus fraternus* G.L.Webster (*P. fraternus*) are locally available and used extensively, hence were chosen for the present study. The aim of the study was to investigate the *in vitro* antioxidant capacities of the methanol extracts of three *Phyllanthus* species. The total phenolic and flavonoid content were quantified; their attributions to salvage the reactive oxygen species (ROS) were estimated by DPPH scavenging potential and antioxidant capacity by phosphomolybdenum and ferric reducing assays.

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Materials and Methods

Drugs and chemicals

DPPH, quercetin, and gallic acid were purchased from Sigma

Aldrich, USA. Organic solvents and HCl, sodium nitrite, aluminum chloride, NaOH, Na_2CO_3 and Folin-Ciocalteu reagent were purchased from Merck, India.

Plant material

The fruits of *P. emblica*, *P. acidus* and *P. fraternus* were collected in and around the University of Hyderabad, Hyderabad, authenticated and has been preserved in our laboratory for future reference with voucher specimen no. UoH-MDP-00010, UoH-MDP-00011, UoH-MDP-00012.

Preparation of the extracts and phytochemical screening

The fruits of *P.emblica* and *P.acidus* and *P.fraternus* were collected, chopped to pieces and taken in liquid nitrogen and finally blended to coarse powder and preserved at -20 °C. The powdered material was then extracted with 95% methanol under sonication for 30 min. The organic solvent was removed under reduced pressure (40 ^{o}C) using a rotary vacuum evaporator (Buchii, USA), and further to remove minor quantities of polar solvent the extract was lyophilized. Hence, brown semi solid extract of *P. emblica* (PefM), *P. acidus* (PafM) and greenish gummy of *P. fraternus* (PffM) were obtained and preserved at −20 °C for further analysis. Phytochemical screening of secondary metabolites in all three extracts was carried out as described by Harbone [8].

Determination of total phenolic contents

The amount of total soluble phenolic content in all three extracts (PefM, PafM, PffM) was estimated using Folin-Ciocalteu reagent [9] with minor modifications. Three different concentrations of plant extract (100, 200 and 300 µg) were taken for this assay and the volume was made upto 100 µl by adding double distilled water. To these plant extracts 100 µl of Folin-Ciocalteu reagent was added, vortexed and incubated at room temperature for 10 mins. To this mixture, 300 µl of 20% Na_2CO_3 was added, thoroughly vortexed and the volume of the reaction mixture was adjusted to 1 ml with double distilled water. This reaction mixture was then incubated for 2 hours in dark and then the absorbance was read against blank at 765 nm using UV-Vis spectrophotometer. Gallic acid was used to plot a standard curve and the total phenolic content was expressed as mg of gallic acid equivalents (GAE)/g dry weight (DW).

Determination of total flavonoids

Total flavonoid content in each extracts was estimated by following the method of Veronica *et al.* [10] with minor modifications. Three different concentrations of plant extract (100, 200 and 300 µg) were taken and the volume was made upto 100 µl by adding double distilled water. To these extracts, 30 µl of 5% sodium nitrite ($NaNO_2$) added and incubated for ten mins at room temperature. This was followed by addition of 60 µl of 10% $AlCl_3$ and ten mins incubation. Subsequently, 350 µl of 1M NaOH added and the volume was made up to 1ml using double distilled water. These samples were left as such at room temperature for half an hour and the absorbance was read against blank at 510 nm. Quercetin was used to plot a standard curve and the total flavonoid content was expressed as quercetin equivalents (mg QR/ gram of dry weight).

Phosphomolybdenum assay

The total antioxidant activities of the test extracts were assessed by green phosphomolybdenum complex according to the method of Prieto *et al.* [11]. Three different concentrations of extracts (100, 200 and 300 µg) were taken from the stock solution (20 mg/ml) and a standard (ascorbic acid) was also run along with these extracts and made up to 100 µl with double distilled water. 1ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added to the samples followed by incubation of 90 min at 90 °C. After cooling to room temperature, the absorbance was measured against blank at 695 nm. Ascorbic acid (AA) was used to plot a standard curve and the reducing capacities of the analyzed extracts were expressed as mg of ascorbic acid equivalents (mg AAE)/ g of dw.

Ferric-reducing/antioxidant power (FRAP) assay

The Fe^{3+} reducing power of the extracts were determined by the method of Oyaizu *et al.* [12] with slight modifications. Three different concentrations of plant extracts (100, 200 and 300 µg) and standard (ascorbic acid) were mixed with 250 µL of 0.2 M phosphate buffer (pH 6.6) and 250 µl of potassium ferricyanide (1 %), and then was incubated at 50 °C for 30 min. Later, 250 µl of trichloroacetic acid (10%) was added to the mixture and then immediately centrifuged at 5000 rpm for 10 min. 500 µl of the upper layer solution was taken into fresh tubes in which 400 µl of 0.2 M phosphate buffer already present and followed by the addition of 100 µl of $FeCl_3$ (0.1 %). The absorbance was read against blank at 700 nm and the reducing power of the extracts was expressed as mg AAE/g of dw.

DPPH• radical scavenging activity

The free radical scavenging activity of the extracts was measured in term of hydrogen donating or radical scavenging ability using the stable radical DPPH described by Cuendet *et al.* [13]. 0.004% w/v of DPPH radical solution in methanol was used for conducting this assay. 900 µL of this solution was added to 100 µL of extract solution containing 10–200 µg/mL of dried extract. The absorbance change of extracts to that of blank was read at 517 nm after 30 min of incubation. Methanol (95 %), DPPH solution and ascorbic acid were used as blank, control and reference respectively.

Calculations and statistical analysis

The percentage inhibitions of radicals and of the extracts were calculated using the formula:

$$\text{Percentage inhibition} = (A_{control} - A_{sample}) / A_{control} \times 100$$

All results are expressed as mean ± standard deviation (SD) values average from 3 to 4 independent experiments performed in duplicate. IC_{50} value (the concentration of the extracts required to scavenge 50 % of radicals) was calculated for PafM,

PefM, and PffM. Statistical tests as well as mean and SD calculations and graphical representation of the results were performed using GraphPad Prism v5 and Sigmaplot v11.0 software's.

Results and Discussion

Metabolites from medicinal plants have been a source of a variety of compounds for therapeutic use. Genus *Phyllanthus* (*Euphorbiaceae*) which is widely distributed has long been and is still being used in folk medicine to treat diseases and also as important ingredient in food in many tropical and subtropical countries. Substantial progress on their chemical and pharmacological properties, as well as a few clinical studies of some *Phyllanthus* species has been made [7].

A comparative study of antioxidant activity of fruits of three species of *Phyllanthus*, *P. emblica* and *P. acidus* and *P. fraternus* was carried out in present study. Study was initiated by rapid freezing followed by lyophilization of fruits collected to avoid the rapid changes of secondary metabolites and retain them substantially as vital by freeze drying using liquid nitrogen. By this the secondary metabolites can be preserved and stabilized and to retain their antioxidant properties for accurate analysis and study. Ultra sonication method is very simple, easy to operate, short time, better extraction for both thermo labile and stable phytoconstituents. Hence, from the freeze dried fruits of three species of *Phyllanthus* the secondary metabolites were extracted in 95% methanol under ultrasonication in good yields. In the present study, the extraction was carried out under ultrasonication using 95% methanol, removed the solvents, preserved in -20 ºC and the extracts of *P. emblica*, *P. acidus* and *P. fraternus* fruits were designated as PefM, PafM, PffM. The extracts were further analyzed for determination of phytochemicals such as phenolic and flavonoid content, their antioxidant capacity and free radical scavenging potential. These three different extracts were subjected to phytochemical screening to check the presence of different phytoconstituents and found the presence of phenolics, flavonoids, glycosides, tannins, terpenoids, steroids (minor concentration) and test against alkaloids and saponins found to be negative. These extracts were diluted in 20% DMSO prior to carrying out the *in vitro* assays.

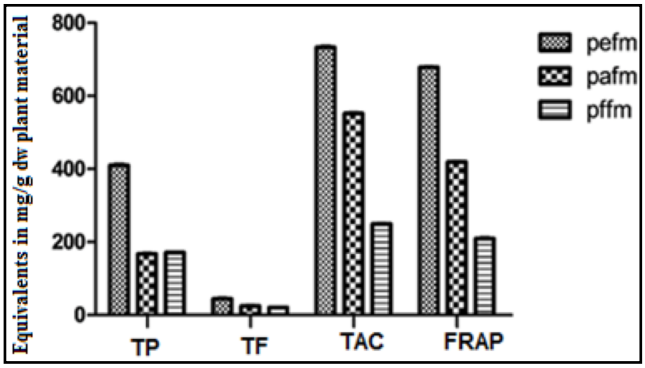

Figure 1: Graphical represenetation of Phytochemical content and antioxidant activities of three different species of fruits *Phyllanthus*. *TP: Total phenolic content; TF: Total flavonoids content; TAC: Total Antioxidant Capacity; FRAP: Ferric reducing antioxidant power.*

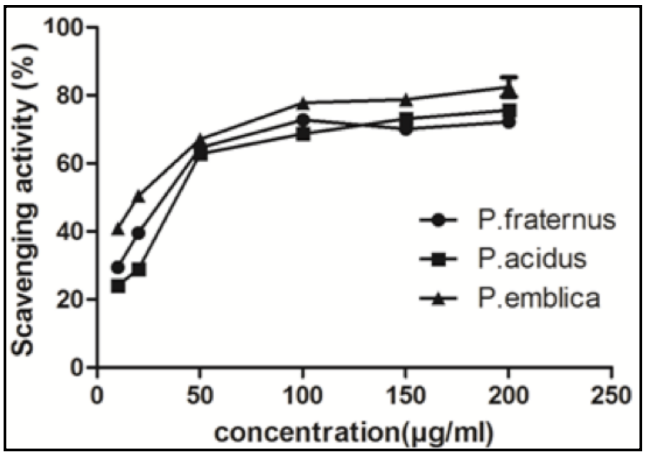

Figure 2: DPPH radical-scavenging activities of *P emblica, P acidus* **and** *P fraternus* **fruits methanolic extracts at different concentrations**

The amounts of total phenolics and flavonoid content in different extracts (PefM, PafM and PffM) were measured and represented in Table 1 and Figure 1. The phenolic content

Table 1: Quantitative estimation of phytochemicals content, antioxidant potential and free radical scavenging activities of *P emblica, P acidus* **and** *P fraternus* **fruits methanolic extracts.**

Type of Extract	Yield (%)	Total Phenols[a]	Total Flavonoid[b]	Total antioxidant capacity[c]	Ferric reducing antioxidant power[c]	DPPH % inhibition 200 µg/ml	DPPH IC_{50} µg/ml
PefM	10.8	409.9 ± 1.9	44.58±0.46	733.02± 2.1	678.13±1.09	87.89 ± 1.45	17.52
PafM	19.77	167.1 ±0.4	24.18±0.49	551.97±1.3	418.83±0.67	75.59 ± 2.98	41.05
PffM	14.09	171.7±0.1	20.33±0.65	249.81±0.63	209.65±0.26	72.25 ± 4.63	32.66
Quercetin	-	-	-	-	-	-	14.34
Ascorbic Acid	-	-	-	-	-	-	6.86

a: Gallic acid; b: Quercetin; c: Ascrobic acid equivalents mg/g dw plant material respectively. Each value is expressed as a mean ± standard deviation (n = 3). PefM : P emblica fruits methanol extract; PafM : P acidus fruits methanol extract; PffM : P fraternus fruits methanol extract.

of the extracts PefM, PafM, and PfwM was found to be in the order of 409.9 ± 1.9, 167.1 ±0.44 and 171.7±0.11 mg GAE/g dw, in terms of gallic acid equivalents respectively. The flavonoid content was found to be 44.58±0.46, 24.18±0.49 and 20.33±0.65 mg/g dw in terms of quercetin equivalents. The results indicate that methanolic extract of *P.emblica* possess higher amount of phenolics and flavonids compared to *P. acidus* and *P. fraternus* methanolic extracts.

In phosphomolybdenum assay, all the three extracts exhibited antioxidant activity. The reducing power of a compound is associated with electron donating capacity and serves as an indicator of antioxidant activity [14, 15]. Results indicated that all three extracts were potent antioxidants with ample antioxidant capacity shown in table 1. PefM found to be higher antioxidant capacity followed by PafM and PfwM with 733.02± 2.1, 551.97±1.3 and 249.81±0.63 mg ascorbic acid equivalent/g dw respectively.

In ferric reducing power assay, the reduction of Fe^{3+} to Fe^{2+} would only happen in the presence of reductants (antioxidants) in samples which thus serve as a significant reflection of antioxidant activity [16]. This can be quantitatively estimated by the formation of Perl's Prussian blue at 700 nm. Higher the absorbance change in the reaction mixture against blank indicates higher reducing ability [17]. Earlier literature studies reported that the presence of phenols and flavonoids exhibited ferric reducing ability [18]. All three extracts showed significant Ferric reducing ability with PefM having higher activity with 678.13±1.09 AAE mg/g dw followed by PafM and PffM with 418.83±0.67 and 209.65±0.26 AAE mg/g dw respectively represented in Table 1. The reducing properties of plant extracts are generally associated with the presence of reductones [18] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [19]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation.

DPPH assay provides basic information on antiradical activity of extracts and its results can indicate the presence of phenolic and flavonoid compounds in plant extracts [20]. Very significant antioxidant activities were found in all the three extracts and positive control, which increased with increasing concentration. DPPH activity values for the extracts are represented in table 1 and figure 2. The PafM, PefM and PffM were able to inhibit the formation of DPPH• radicals with a percentage inhibition of 75.59 ± 2.98, 87.89 ± 1.45 and 72.25 ± 4.63% respectively at the highest concentration of 200 µg/ml with the IC_{50} values of 41.05, 17.52 and 32.66 µg/mL respectively.

Conclusion

The results of experiments in present study reveal that the three selected species of *Phyllanthus* have significant antioxidant activity. The extracts are found to have different levels of antioxidant activity in all the systems tested. The antioxidant activity of the three extracts of *Phyllanthus* are in the order *P. emblica* >*P. acidus* > *P. fraternus*. Among the three species of *Phyllanthus* under study, *P emblica* found to be best with highest content of phenolic and flavonoid content, its antioxidant and free scavenging property. The antioxidative effect is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes [21]. Phenolic compounds have been proved to be responsible for the antioxidant activity of *P. emblica* fruit [22, 23]. Further studies are warranted for the isolation and identification of individual phenolic compounds and also *in vivo* studies are needed for understanding their mechanism of action as an antioxidant better.

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