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Research Article

BIOACTIVE POTENTIAL OF HERBACEOUS *PHYLLANTHUS* SPECIES

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ABSTRACT

Phyllanthus species are the most valued medicinal plants, extensively used in liver disorders. Ethanolic fractions of seven herbaceous species of *Phyllanthus* namely, *P. amarus*, *P. debilis*, *P. maderaspatensis*, *P. virgatus*, *P. urinaria*, *P. scabrifolius* and *P. tenellus* were tested for their *in vitro* antioxidant and DNA protection properties. Antioxidant properties were assessed by 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azino-bis 3-thylbenzthiazoline-6-sulphonic acid (ABTS) and superoxide anion scavenging assay. The total phenolic content in the ethanolic fractions were measured by Folin-Ciocalteu assay. Ethanolic fractions of seven *Phyllanthus* species were tested for pTZ57R/T plasmid DNA protection against hydroxyl radicals. In addition, we have also carried out preliminary *in vivo* longevity studies on *Drosophila melanogaster* supplemented with ethanolic crude extract of *P. amarus* and *P. debilis*. *In* vitro antioxidant assay results for ethanolic fractions of *Phyllanthus* species clearly showed concentration-dependent increase in activity in relation to phenolic compounds. These fractions also protect the pTZ57R/T plasmid DNA damage induced by hydroxyl radicals. Results from preliminary longevity studies in *D. melanogaster* indicates nearly 50% increase in life span in *Phyllanthus* extract treated groups compared to control. In conclusion, our study clearly demonstrated that herbaceous *Phyllanthus* species have good antioxidant, DNA protection and life span extension properties.

Keywords: *Phyllanthus*, Ethanolic fractions, Antioxidant, DNA protection, Longevity.

INTRODUCTION

Antioxidants are micronutrients that have gained importance in recent years due to their ability to neutralize free radicals or their actions. Free radicals have been implicated in the etiology of several major human ailments, including cancer, cardiovascular disease, neural disorders, diabetes and arthritis[1]. Many antioxidant compounds, naturally occurring from plant sources, have been identified as free radical or active oxygen scavengers[2]. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects. In addition, natural antioxidants have the capacity to improve food quality and stability and can also act as nutraceuticals to terminate free radical chain reactions in biological systems and thus may provide additional health benefits to consumers.

It has been reported that antioxidant activity of plants might be due to their phenolic compounds[3]. Phenolics and flavonoids commonly found in both edible and non edible plants. Among the various natural antioxidants, phenolic compounds are reported to have the character of quenching oxygen derived free radicals by donating a hydrogen atom or an electron to the free radical[4]. Also, phenolic compounds of plant materials have been shown to neutralize free radicals in various model systems[5]. One such plant group having high phenolic content is genus *Phyllanthus* L.[6]

Phyllanthus species has a long history in herbal medicine systems in every tropical country where it grows. It is used against biliary and urinary conditions including kidney and gall bladder stones; hepatitis, cold, flu, tuberculosis, and other viral infections; liver diseases and disorders including anemia, jaundice and liver cancer; and bacterial infections such as cystitis, prostatitis, venereal diseases and urinary tract infections. It is also widely employed for diabetes and hypertension as well as for its diuretic, analgesic, stomachic, antispasmodic, febrifugal, and cell protective properties in many other conditions[7].

Aging is an important event in the life of an organism. It is the progressive loss of physiological functions, which eventually may lead to death of the organisms. Even a small change in physiological/aging pathways can lead to extension or reduction of life span. Hence, the process of aging is gaining more attention today. Aging is a whole-organism phenomenon and is, therefore impossible to study *in vitro*. Modeling the progression of human aging is difficult because of the extreme complexity of the process. Thus, the comparative study of aging in model organisms holds the potential to reveal information about the aging process in humans. Hence, many principal animal model systems viz., nematode *C. elegans*, the fruit fly *Drosophila* and the mouse or rat are being used to investigate the molecular-genetic basis of aging. Due to their limitations, *Drosophila* has proved to be the best model system for aging studies. It has been shown that the research finding on flies can tell the probably happening events in the human aging process[8]. Aging of an organism can be detected by longevity. Longevity is the total duration of life span of an individual. It is a good indicator of viability of organisms[9].

Phyllanthus L. is a complex genus and consists of approximately 833 species[10], chiefly distributed in moist humid tropics. Chaudhary and Rao[11] have listed twelve herbaceous *Phyllanthus* species in India. While working on the diversity of herbaceous species of *Phyllanthus* we have collected seven *Phyllanthus* species from different parts of Karnataka (India) and tested for different bioactive potential.

MATERIALS AND METHODS

Plant materials and preparation of extracts

Seven herbaceous *Phyllanthus* species namely, *P. amarus* Schum., *P. debilis* Klein ex Willd., *P. maderaspatensis* L., *P. virgatus* Forst. f., *P. urinaria* L., *P. scabrifolius* Hook.f. and *P. tenellus* Roxb. were collected from different parts of Karnataka. Botanical authentication was done by the first author and herbarium specimens are maintained in the laboratory. Initially we prepared crude extracts from dried aerial parts of all *Phyllanthus* species using non-polar, partially polar and polar solvents separately and tested for their antioxidant potential. Ethanolic crude extracts showed good antioxidant activity. Therefore, we have selected the ethanolic crude extracts of *Phyllanthus* species and further fractionated by using hexane, chloroform and ethanol. These three fractions of seven *Phyllanthus* species were again tested for their antioxidant potential and only ethanolic fractions showed good activity. Therefore, we have selected ethanolic fractions of seven *Phyllanthus* species and tested for different bioactive potential.

Chemicals

1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), nicotinamide adenine dinucleotide reduced (NADH), phenazinemethosulphate (PMS), nitro blue tetrazolium (NBT), ascorbic acid and gallic acid were purchased from Sigma Aldrich. All other chemicals and reagents used were of analytical grade.

Determination of total phenolics

The total phenolic content was estimated using Folin-Ciocalteu calorimetric method of Velioglu *et al*[12]. Test sample (100 µl) was reacted with 0.75 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22º C for 5 minutes. The reaction was neutralized with saturated sodium carbonate (60 g/l) and allowed to stand for 1.5 hours in the dark at 22ºC. The absorbance of the resulting blue colour was measured at 725 nm (Hitachi U-3900 UV/visible spectrophotometer). Total phenolics were quantified by calibration curve obtained from measuring the absorbance of known concentrations of gallic acid standard (25 to 250 µg/ml). The total phenolic contents were expressed as gallic acid equivalence (GAE) in µg.

DPPH radical scavenging assay

The free radical scavenging property of the ethanolic fractions of seven herbaceous *Phyllanthus* species was determined by DPPH method[13]. The $DPPH^{\cdot}$ radical solution was prepared in methanol. The reaction mixture contained 5 µl of test samples and 95 µl of DPPH (300 µM) in methanol. Different concentrations of test samples were prepared and used for $DPPH^{\cdot}$ radical scavenging activity. The reaction for scavenging $DPPH^{\cdot}$ radical was carried out at 37ºC in dark for 30 min and the absorbance was recorded at 517 nm (Spectra max 340, Molecular devises). Percent radical scavenging activity was determined by comparing with a solvent treated control. IC_{50} values were determined, which denote the concentration of extracts required to scavenge 50% DPPH free radicals. Ascorbic acid was used as positive control. Percent scavenging effect was determined by the following equation.

% inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance of control] x100

ABTS radical scavenging assay

ABTS antioxidant activity was measured using Hitachi U-3900 UV/visible spectrophotometer according to the method described by Roberta *et al*[14] with slight modifications. ABTS radical cation ($ABTS^{\cdot+}$) solution was prepared by reacting 7 mM ABTS and 2.45 mM potassium persulfate on incubating the mixture at room temperature in dark for 12 hours The resulting $ABTS^{\cdot+}$ solution was then diluted with methanol to get an absorbance of 0.700 ± 0.005 at 734 nm. Different concentrations of test samples (50 µl) were added to 2.95 ml of $ABTS^{\cdot+}$ working solution to give a final volume of 3 ml. The absorbance was recorded after incubation at room temperature for 30 min at 734 nm. Gallic acid was used as reference standard. The percent inhibition was calculated from the following equation:

% inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance of control] x100

Superoxide radical scavenging assay

Superoxide anion scavenging activity was performed according to the method of Liu *et al*[15] with some modifications. Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). The superoxide radicals were generated in 3 ml of Phosphate buffer (100 mM, pH 8.0) containing 1 ml of NBT (150 µM) solution, 1 ml NADH (468 µM) solution and test sample solution (100 µl). The reaction was started by adding 100 µl of phenazine methosulphate (PMS) solution (60 µM) to the mixture and incubated at 25º C for 5 minutes. The absorbance was read at 560 nm (Hitachi U-3900 UV/visible spectrophotometer) against negative control and ascorbic acid was used as positive control. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula.

% inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance of control] x100

Reducing Power estimation

This estimation of reducing power carried out as described previously by Nagulendran *et al*[16] with slight modifications. About 0.75 ml of test sample solution (1 mg/ml) was mixed with equal volume of 0.2 M phosphate buffer (pH 6.6) and 0.75 ml of 1% potassium ferricyanide, followed by incubation at 50° C for 20 minutes. Trichloroacetic acid (10 %, 0.75 ml) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. Finally, 1.5 ml of the supernatant solution were mixed with equal volume of distilled water. Absorbance was measured at 700 nm (Hitachi U-3900 UV/visible spectrophotometer) after the addition of 0.5 ml of 0.1% $FeCl_3$. Ascorbic acid was used as standard and phosphate buffer was used as blank solution. Increased absorbance of the reaction mixture indicates stronger reducing power.

DNA nicking assay

Extent of protection against pTZ57R/T plasmid DNA damage by ethanolic fractions of seven herbaceous *Phyllanthus* species was tested as described by Jeong *et al*[17] with some modifications. Mixture of 5 µl of test sample (1mg/ml) and 2 µl of plasmid was added to 5 µl of Fenton's reagent (30 mM H_2O_2, 50 µM ascorbic acid and 80 µM $FeCl_3$). The final volume was made up to 15 µl with sterile water followed by incubating for 30 minutes at 37°C. The DNA was analyzed on 1% agarose gel using ethidium bromide staining.

In vivo* longevity studies in *D. melanogaster

Newly emerged male flies were segregated and isolated flies were released into 8 × 2.5 cm individual culture vials containing equal quantities of wheat cream agar medium and food supplement. Flies in each culture vial transferred to fresh culture vials once in every 3 days without being etherized. The experimental cultures were divided into 2 groups.

1. Group 1 culture vials supplemented with yeast solution (control).
2. Group 2 culture vials supplemented with ethanolic crude extract of *P. amarus* and *P. debilis*.

Seventy-five such replicates were maintained in each group until they reach mortality and longevity was recorded in days. Longevity in *Phyllanthus* extracts supplemented group was compared to control group and all the experiments were carried out in two different concentrations (1mg and 5mg/ml).

Statistical analysis

All determinations of antioxidant property of DPPH, ABTS and superoxide assays and measurement of total phenolic contents were done in triplicates. The reported value for each sample was calculated as the mean of three measurements. The correlation coefficients (R), coefficient of determination (R^2) and $p \leq 0.05$ values were calculated using Microsoft Excel 2007.

RESULTS

Determination of total phenolic content

The content of phenolic compounds of ethanolic fractions of seven herbaceous *Phyllanthus* species are shown in Table 1. It is evident that the phenolic content in different species varied considerably. Total phenolic content is expressed as gallic acid equivalence (GAE) in µg. *P. amarus*, *P. virgatus* and *P. tenellus* have 195, 233 and 183 µg in GAE respectively. *P. maderaspatensis* and *P. scabrifolius* have 150 and 140 GAE (in µg), respectively whereas *P. debilis* and *P. urinaria* have lowest phenolic content.

DPPH and ABTS radical scavenging assay

The percentage of DPPH decolorization is attributed to hydrogen donating ability of test compounds. Variable DPPH activity was recorded for the seven *Phyllanthus* species. The ethanolic fractions of *P. amarus*, *P. virgatus* and *P. tenellus* showed highest activity, whereas *P. debilis*, *P. maderaspatensis*, *P. urinaria* and *P. scabrifolius* have shown moderate activity. *P. virgatus* exhibited higher antioxidant activity (IC_{50} = 85 µg/ml) when compared to other species in DPPH model. Reference standard ascorbic acid showed 50% inhibition at 70 µg/ml. The concentration of ethanolic fractions of each *Phyllanthus* species taken to inhibit 50% of DPPH and ABTS radicals are indicated in Table 1. *P. virgatus* exhibited highest ABTS radical scavenging activity (IC_{50}=

80 µg/ml). *P. amarus* and *P. tenellus* also showed good activity. Whereas P. debilis, P. maderaspatensis, P. urinaria and P. scabrifoliusposses moderate activity, Reference standard gallic acid showed 50% inhibition at 30 µg/ml in ABTS model.

Table 1: Antioxidant activity and total phenolic content of ethanolic fractions of herbaceous *Phyllanthus* species

Plants	DPPH assay (IC_{50} value in µg/ml)	ABTS assay (IC_{50} value in µg/ml)	Total Phenolic content (GAE in µg) (1 mg/ml)
P. virgatus	85	80	233
P. amarus	90	90	195
P. tenellus	100	90	183
P. maderaspatensis	125	130	150
P. scabrifolius	150	140	140
P. debilis	200	175	120
P. urinaria	200	180	115

Values represent the mean (n=3)

Relationship among the estimates of total phenolic content with antioxidant DPPH and ABTS assays

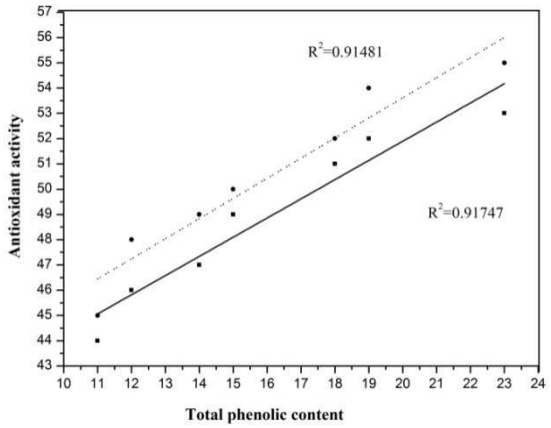

Fig. 1: Correlation between total phenolic content and antioxidant DPPH and ABTS radical scavenging potential of ethanolic fractions of herbaceous *Phyllanthus* species

Linear correlation between the amounts of total phenols and antioxidant capacity (DPPH and ABTS) is found in all seven *Phyllanthus* species (Fig.1). The coefficient of determination (R^2) was 0.91747 and 0.91481 between total phenolic content and antioxidant DPPH and ABTS methods respectively. This positive correlation suggests that the antioxidant capacity of the ethanolic fractions is due to a great extent of phenolic content.

Superoxide radical scavenging assay

The superoxide anion radical scavenging activities of seven *Phyllanthus* ethanolic fractions were assayed by PMS-NADH system are shown in Fig. 2. The activity increased markedly with the increase in concentrations from 100 to 500 µg/ml *P. amarus*, *P. maderaspatensis, P. virgatus* and *P. tenellus* showed the high radical scavenging activity of more than 50% at 180 µg/ml. Whereas *P. debilis*, *P. urinaria* and *P. scabrifolius* showed the IC_{50} value at 250 µg/ml. A dose-dependent inhibition of superoxide anion radical scavenging was observed in all plant ethanolic extracts. Ascorbic acid was taken as a standard and ascorbic acid equivalent (in µg) is plotted.

Reducing power estimation

The reducing capacity of the seven *Phyllanthus* ethanolic fractions were compared to BHT (Fig. 3). An increase in absorbance at 700 nm indicates the reducing power of the ethanolic fractions of *Phyllanthus*. The reducing capacity increased with increasing concentration of test samples. The extracts *P. amarus*, *P. maderaspatensis, P. virgatus* and *P. tenellus* showed good reducing power, whereas *P. debilis*, *P. urinaria* and *P. scabrifolius* showed moderate reducing power. Reducing power showed significant correlation ($p<0.05$) with phenolic content for all ethanolic fractions of *Phyllanthus*.

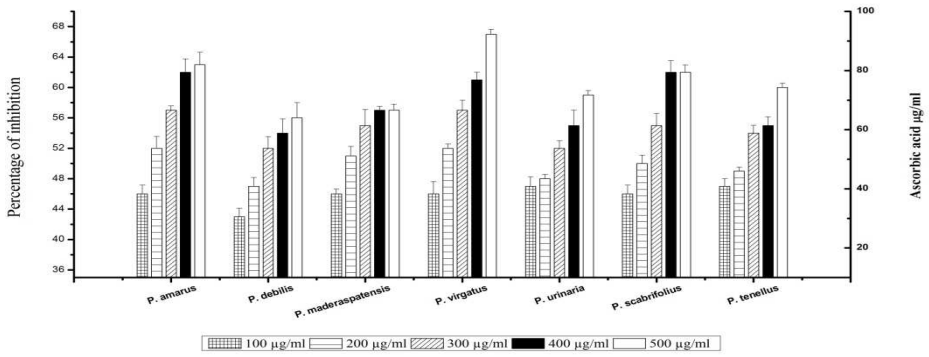

Fig. 2: Ethanolic fractions of herbaceous *Phyllanthus* species showing % inhibition of superoxide anion scavenging assay at different concentrations with ascorbic acid equivalent

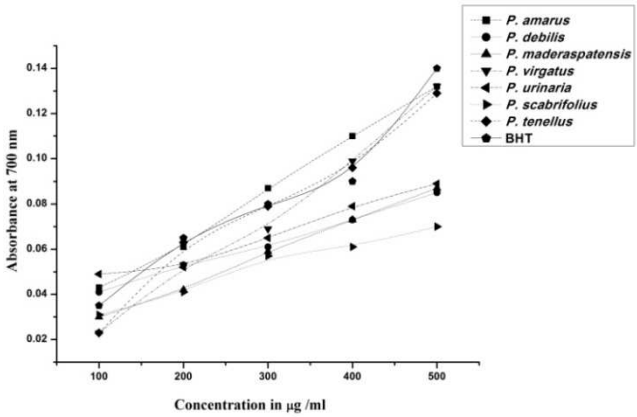

Fig. 3: Reducing power of ethanolic fractions of herbaceous *Phyllanthus* species at different concentrations

DNA nicking assay

Normal pTZ57R/T plasmid (lane 1) showed two bands on agarose gel electrophoresis and plasmid DNA when fragmented using Fenton's reagent, showed two thin bands with smear as shown in lane 2 of Fig. 4. The two bands appearing in lane 3 to 9 are visualized as the normal pTZ57R/T plasmid DNA (lane 1). The role of ethanolic fractions of herbaceous *Phyllanthus* species in preventing DNA damage was assessed. All seven *Phyllanthus* ethanolic fractions showed stronger protective effect against hydroxyl radical released by Fenton's reaction.

In vivo* longevity studies in *D. melanogaster

Longevity can be defined as total lifespan of an organism. It is a good indicator of viability of organisms. Preliminary longevity studies in *D. melanogaster* (Table 2) indicate nearly 50% increase in life span of *Phyllanthus* extracts treated groups compared to control. *P. amarus* and *P. debilis* extract supplemented groups showed nearly similar life span in both the concentrations (1mg and 5mg/ml). The strain with the same letter in the parenthesis are not significantly different at 5% level according to DMRT.

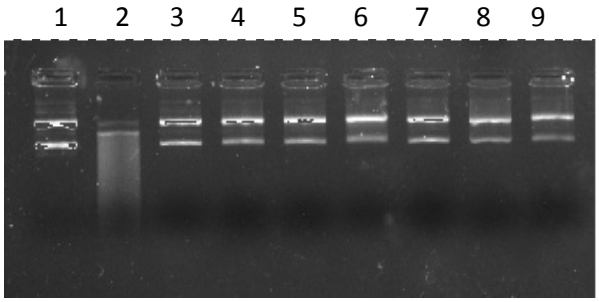

Fig. 4: Effect of ethanolic fractions of herbaceous *Phyllanthus* species against hydroxyl radical-mediated fragmentation. Lane 1: untreated DNA (control), lane 2: Fenton's reagent + DNA, lane 3 to 9: Fenton's reagent + DNA + ethanolic fractions of seven *Phyllanthus* species sequentially represent *P. amarus*, *P. debilis*, *P. maderaspatensis*, *P. virgatus*, *P. urinaria*, *P. scabrifolius* and *P. tenellus*

Table 2: Longevity of *Drosophila melanogaster* in *Phyllanthus* extracts supplemented groups with the control group

Group	Minimum life span (days)	Maximum life span (days)	Mean ± S.E	F value
Control	44	75	58.41 ± 0.92 (c)	
P. amarus (1mg/ml)	61	118	96.48 ± 1.55 (a)	
P. amarus (5mg/ml)	68	116	97.16 ± 1.41 (a)	140.12
P. debilis (1mg/ml)	65	111	89.70 ± 1.26 (b)	
P. debilis (5mg/ml)	65	110	88.52 ± 1.48 (b)	

df = (4, 374)

DISCUSSION

Medicinal plants are considered to be the best source for antioxidant compounds[18]. As plants produce significant amount of antioxidants to prevent the oxidative stress caused by photons and oxygen, they are considered as potential source of antioxidant compounds[19]. Our study clearly demonstrated that the ethanolic fractions of seven herbaceous *Phyllanthus* species have significant antioxidant property when assessed by DPPH, ABTS, Superoxide radical scavenging and reducing power assays. Antioxidant activity of *Phyllanthus* species are in the order *P. virgatus* > *P. amarus* > *P. tenellus* > *P. maderaspatensis* > *P. scabrifolius* > *P. debilis* > *P. urinaria*. A high linear correlation was observed between DPPH and ABTS radical scavenging activity with total phenolic content of *Phyllanthus* species. This positive correlation suggests that the antioxidant capacity of the ethanolic fractions of herbaceous *Phyllanthus* species is due to their phenolic content. This data is in accordance with earlier reports, which claimed that high total phenol content increases the antioxidant activity[20]. Therefore, our results support the role of phenolics as a source of natural antioxidants.

Antioxidants have a major role in hepatitis, liver protection and aging[21]. They are very good scavengers for the reactive oxygen species that prevents the damage in many cellular components such as DNA, proteins, and lipids[22]. Plasmid DNA damage by hydroxyl radicals generated by Fenton's reagent caused DNA fragmentation.

However, ethanolic fractions of herbaceous *Phyllanthus* species prevented such damage as evident in the agarose gel. This property also could be attributed to antioxidant activity of *Phyllanthus* species. Finally, longevity studies in *D. melanogaster* confirms the life span extension properties of *P. amarus* and *P. debilis.* Therefore, herbaceous *Phyllanthus* species could be targeted as a source of potential anti-aging agent. Similarity in life span of *D. melanogaster* treated with *P. amarus* and *P. debilis* extract validates the substitution of *P. debilis* as a source of *Bhumyamalaki* (*P. amarus*) in South Indian traditional medicine.

In conclusion, our study clearly demonstrated that herbaceous *Phyllanthus* species have good antioxidant, DNA protection and life span extension properties. Further studies are warranted for the isolation and identification of bioactive compounds.

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