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Original Article

ASSESSMENT OF *IN VITRO* ANTIOXIDANT POTENTIAL AND QUANTIFICATION OF TOTAL PHENOLS AND FLAVONOIDS OF AQUEOUS EXTRACT OF *PHYLLANTHUS AMARUS*

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ABSTRACT

Objective: The modern research is directed towards finding naturally occurring antioxidants of plant origin. To search for new sources of safe and inexpensive antioxidants, present study was undertaken to quantify total phenols and flavonoids and, evaluation and correlation of antioxidant potential of aqueous extract of *Phyllanthus amarus* Schum & Thonn, (PAAEt) belongs to Euphorbiace family under *in vitro* condition.

Methods: Determination of total phenolic and flavonoid contents was performed by UV-visible spectrophotometer. The antioxidant potential of *P. amarus* was tested with different antioxidant test systems; inhibition of lipid peroxidation (LPO), scavenging of 1,1-Diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide and nitric oxide radicals with reducing power.

Results: The plant extract has appreciable amounts of total phenolic compounds (120±2.0 mg GA eq. g^{-1}) and flavonoids (3.02±0.13 mg Q eq. g^{-1}) of the extract. PAAEt exhibited effective and strong antioxidant activity in dose-dependent manners in inhibiting LPO (IC_{50} 623.4 µg/ml) and scavenging of free radicals such as DPPH (IC_{50} 200.2 µg/ml), hydroxyl (IC_{50} 280.6 µg/ml), superoxide (IC_{50} 201.5 µg/ml), nitric oxide (IC_{50} 408.5 µg/ml) with reducing power (IC_{50} 500.0 µg/ml). PAAEt showed effective and strong positive correlation with reducing power (r^2= 0.972) and LPO inhibition (r^2= 0.964) and free-radicals ($P<0.05$).

Conclusion: Presence of high amount of phenolic and flavonoid compounds and appreciable amounts of IC_{50} for various free radicals and inhibition of LPO and reducing power with significant strong positive correlation for scavenging of free radicals, LPO inhibition and reducing power indicated that PAAEt has strong antioxidant potential.

Keywords: Antioxidant activity, Flavonoids, Free radicals, *Phyllanthus amarus*, Total phenols.

INTRODUCTION

Oxidative stress is an imbalance between the formation and neutralization of prooxidants such as hydroxyl, superoxide, and nitric oxide radicals, etc., which are generated as byproducts of metabolism in the cells. The prooxidants being unstable seeks stability through electron pairing with lipids, proteins and DNA biomolecules in healthy human cells resulting in damage of these biomolecules. The healthy human being has an antioxidant defense mechanism against free radicals, which protect from damage of biomolecules. Sometimes these defense mechanisms which are interrupted by various pathological conditions resulted involvement in the pathogenesis of a wide number of degenerative diseases and syndromes such as diabetes mellitus, cardiovascular disease, hypertension, cirrhosis and cancers [1].

Additional intake of antioxidant supplements is essential to battle oxidative damage. The use of available synthetic antioxidants showed side effects like carcinogenicity [2]. Recently interest has increased towards finding natural antioxidants for use in foods or medicinal materials to substitute synthetic antioxidants. Many natural antioxidant compounds from plant sources have been identified as free radicals or active oxygen scavengers [3]. Numerous studies have confirmed the bioprotective nature of crude extracts of natural foods like fruits, vegetables and spices containing high phytochemical antioxidants [4, 5].

Phyllanthus amarus Schum & Thonn, (Euphorbiaceae) is an annual, profusely branched herb, growing to a height of 10 to 70 cm. *P. amarus* is commonly known as a stone breaker in English, Bhuamla in Hindi, and Nelavusiri in Telugu. It is widespread throughout the tropical and subtropical countries of the world including India, Nigeria, China, Philippine and Cuba etc. It is commonly used to treat problems of liver, stomach, kidney, genitourinary system and spleen in an Indian Ayurvedic system. In Northeastern part of South America *P. amarus* is sold as fresh and dry material in herb market. Decoctions of this herb are used in herbal baths after labor and to treat various diseases and syndromes such as asthma, cramps, stomach ache and uterus complaints [6]. Also, decoction of leaves of *P. amarus* was considered to be a diuretic and was used in the treatment of diabetes, hepatitis, dysentery, menstrual disorders and skin disorders [6]. Most of the medicinal properties of plant origin are due to the synergistic effects of antioxidant phytochemicals. In a previous study from our laboratory the antioxidant potential of aqueous extract of *P. amarus* under *in vivo* condition was evaluated in STZ induced insulin deficient [7] and high fructose diet induced insulin resistant [8] rat models. Numerous studies support that plant extracts exhibit antioxidant property of polyphenols and flavonoids [9, 10]. Now we quantified total phenol and flavonoid contents and, also evaluated and correlated the antioxidant potential of aqueous extract of *Phyllanthus amarus* (PAAEt) by different antioxidant test systems; hydroxyl (OH), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO), superoxide ($^{\cdot}O_2$) radicals scavenging with reducing power and inhibition of lipid peroxidation (LPO) generation under *in vitro* condition.

MATERIALS AND METHODS

Plant extract

An aqueous extract of *Phyllanthus amarus* (Lot No. L5111031) was procured from Chemiloids, manufacturers and exporters of herbal extracts, Ms. Plantex Pvt. Ltd., Vijayawada, Andhra Pradesh (INDIA). The plant was authenticated by Dr. Narasimha Reddy, Taxonomist, Laila Implex R&D Centre, Vijayawada prior to extract preparation. The extract was stored in an airtight container to free from moisture and humidity and then dissolved in water just before use.

Qualitative screening for phytochemicals

The PAAEt was qualitatively screened for the presence of various phytochemical constituents using standard procedures [11-13]. The phytochemical tests were carried out in this work for phenols, alkaloids, flavonoids, saponins, carboxylic acids, tannins, glycosides, steroids, triterpenoids and resins.

Quantitative estimation of phytochemicals

Total phenol content assay

The total phenolic compound of PAAEt was assessed by using Folin-Ciocalteau phenol reagent method [14]. Briefly, 1.0 ml of the extract (1.25 mg) was mixed with 2.5 ml of 10% Folin-Ciocalteau reagent and 2.5 ml of 7.5% sodium carbonate. The contents were thoroughly mixed and allowed to stand for 30 min. The absorbance was read at 750 nm in a spectrophotometer and all estimations were done in triplicates. The total phenol content was expressed as gallic acid equivalents in milligram per gram of the extract using a standard curve generated with gallic acid (range of 50 to 500 µg).

Total flavonoid content assay

The flavonoids content of PAAEt was determined using aluminum chloride colorimetric method [15]. Briefly, 0.5 ml of PAAEt (2.5 mg) was mixed with 3 ml of 95% methanol, 0.1 ml of 10% (w/v) aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The reaction mixture was allowed to stand at room temperature for 30 min and absorbance was measured at 415 nm against blank sample. Calibration curve was prepared using quercetin in methanol (range of 10 to 100 µg). The flavonoid content was expressed as quercetin equivalents in milligram per gram of the extract. All estimations were repeated in triplicates.

Assay of antioxidant potential

Lipid peroxidation inhibition efficacy

Lipid peroxidation was induced by Fe_{3+}ADP-ascorbate system in rat liver homogenate by the method of Sugioka *et al.* [16], and the extent of LPO was estimated by the method of Utley *et al.* [17]. The reaction mixture, containing 1.0 ml of 10% normal rat liver homogenate prepared in 0.15 M KCl, 0.3 ml of 1 mM ferric chloride, 0.3 ml of 17 mM ADP-Na_2, 0.4 m of 3.75 mM ascorbic acid, 0.2 ml of PAAEt at different concentrations (150 to 3000 µg) and 0.8 ml of 0.15 M KCl, was incubated at 37 °C for 20 min. A system devoid of extract served as the control. One ml from each of the test, and control was treated with 4.0 ml of 0.67 % TBA, 2.0 ml of 10% TCA and heated in a boiling water bath for 30 min. After cooling the absorbance of supernatant was read at 535 nm. The ability of PAAEt to inhibit LPO was compared with curcumin as a standard and all the determinations were performed in triplicates.

DPPH radical scavenging activity

The free radical scavenging activity of PAAEt was determined using assay adopted by Okada & Okada [18]. Different concentrations of the extract (50 to 600 µg) and standard curcumin in 0.3 ml of distilled water were separately mixed with 2.7 ml of 1.0 mM DPPH (prepared in 99% ethanol) solution. The mixtures were then mixed vigorously and allowed to stand at room temperature for 30 min. Systems devoid of the extract were considered as control. The absorbance was measured spectrophotometrically at 517 nm. The free radical scavenging capacity was determined by comparing the absorbance of treatment with that of the control. All estimations were performed in triplicates.

The per cent inhibition of DPPH free radical was calculated using the following formula:

$$\text{Per cent inhibition} = (A_{Control} - A_{Sample})/A_{Control} X100$$

Where, $A_{Control}$ is the absorbance of the control reaction

A_{Sample} is the absorbance of the extract

Hydroxyl radical scavenging activity

Hydroxyl radicals are generated in Fenton reaction mixture, which attack the deoxyribose and finally leads to formation of MDA, which can be measured as a pink MDA-TBA chromogen at 535 nm [19]. To 0.5 ml of 40 mM Potassium phosphate buffer (pH 7.4), 0.3 ml of freshly prepared Fenton reaction mixture (16.2 mg of $FeCl_3$, 38.7 mg of EDTA, 17.2 mg of ascorbic acid dissolved in 99.96 ml of distilled water and 0.34 ml of H_2O_2), 0.1 ml of 28 mM 2-Deoxy-D-ribose and 0.1 ml of extract with different concentrations (200 to 1000 µg) were added and incubated at 37 °C for 1 h. Control was maintained by substituting PAAEt with distilled water. One ml from test and control were separately treated with 1.0 ml of 0.67% TBA and heated in a boiling water bath for 30 min followed by cooling and the absorbance was read at 535 nm. All estimations were performed in triplicates. The hydroxyl radical scavenging activity of PAAEt was compared with curcumin as a standard and per cent inhibition was evaluated by comparing the test with control solutions.

Superoxide radical scavenging activity

Superoxide anion scavenging activity of PAAEt was estimated according to the method described by Oktay *et al.* [20]. Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of NBT. The reaction mixture contained 1.5 ml of 32 mM Tris-HCl buffer (pH 8.0), 0.5 ml of 0.3 mM NBT, 0.5 ml of 0.468 mM NADH and 0.5 ml of extract at different concentrations (50-500 µg). The reaction was initiated by the addition of 1.0 ml of 30 µM PMS followed by incubation at 25 °C for 5 min. The absorbance was measured at 560 nm against buffer blank. System devoid of the extract served as control. Decrease in absorbance of the reaction mixture indicates the presence of superoxide anion scavenging activity. Superoxide radical scavenging activity of PAAEt was compared with curcumin as the standard. All estimations were performed in triplicates.

Nitric oxide radical scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution was measured by using Griess reagent [21]. The reaction mixture containing 1.0 ml of 30 mM sodium nitroprusside, 1.5 ml of 10 mM Tris-HCl buffer (pH 7.4) and 0.5 ml of plant extract with different concentration (10 to 1000 µg) was incubated at 25 °C for 150 min. After incubation 1.0 ml of the reaction mixture was treated with 1.0 ml of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diamine dihydrochloride, 2% orthophosphoric acid) and the absorbance of the chromophore was measured at 546 nm against the buffer blank. The system devoid of the extract was served as control. All estimations were carried out in triplicates. The nitric oxide radical scavenging activity of PAAEt was compared with curcumin as the standard.

Reducing power

The reducing power of the extract was determined according to the method described by Oyaizu [22]. Potassium ferricyanide complex gets reduced to reddish brown colored ferrous form with absorbance maxima at 700 nm. The different concentrations of the PAAEt (50 to 500 µg) and standard curcumin in 1.0 ml of distilled water were separately mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), 2.5 ml of 1.0% potassium ferricyanide and incubated at 50 °C for 20 min. 2.5 ml of 10% TCA was added and centrifuged at 1000 x g for 10 min. To 2.5 ml of the upper layer, 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ were added and finally the absorbance was measured at 700 nm against a blank. System devoid of extract was used as a control. The reducing power was determined by comparing the absorbance of treatment with that of the control. All estimations were performed in triplicates.

Statistical analysis

All estimations were carried out in triplicates. Data were presented as mean±SD Statistical analyses were performed by Duncan Multiple Range test (DMRT). A difference with $P<0.05$ was considered statistically significant. The 50% inhibitory concentration (IC_{50}) was calculated from the dose response curve (Origin 91SR Version) obtained by plotting percentage inhibition verses concentrations.

RESULTS

Qualitative screening for phytochemicals

The phytochemical screening of PAAEt revealed the presence of various pharmacological important phytochemical constituents such as phenols, flavonoids, saponins, alkaloids, anthocyanins, anthocyanidins, anthracene glycosides, carboxylic acids, coumarins, triterpenoids, gallic tannins, resins and steroids (table 1).

Quantitative estimation of phytochemicals

Quantitative estimation of phytochemicals in PAAEt revealed the presence of an appreciable amount of polyphenolic compounds 120±2.0 (mg GA eq. g^{-1}) and flavonoids 3.02±0.13 (mg Q eq. g^{-1}) of the extract (table 2).

Antioxidant activity

The plant extract inhibited lipid peroxides generated by the induction of Fe_3+ADP-ascorbate in rat liver homogenate from 3.3 to 62.8% at a concentration of 50 to 1000 µg/ml with IC_{50} 623.4 µg/ml. This effect of PAAEt was compared with standard curcumin, which showed IC_{50} at 128.2 µg/ml concentration (fig. 1a). Thus, PAAEt showed protection against LPO induced by the Fe^{+3}+ADP-ascorbate system in rat liver homogenate.

Decrease in absorbance of the reaction mixture indicates the presence of free radical scavenging activity. Fig. 1b represents DPPH radical scavenging activities of PAAEt and standard antioxidant curcumin. The free radical scavenging activity gradually increased (10.5 to 74.9%) with an increase in concentration of PAAEt (25 to 1000 µg/ml) and showed IC_{50} at 200.2 µg/ml whereas, the standard antioxidant curcumin showed IC_{50} at 48.4.0 µg/ml.

PAAEt at a concentration of 25 to 1000 µg/ml showed 5.2 to 72.5% inhibition of hydroxyl radical formation with IC_{50} of 280.6 µg/ml (fig. 1d) whereas, curcumin at different concentrations ranging from 5 to 150 µg/ml showed 4.7 to 85.1% inhibition of hydroxyl radical formation with IC_{50} of 56.9 µg/ml.

As shown in fig 1c, inhibition of superoxide radical formation was observed from 4.7 to 69.3% at 25 to 1000 µg/ml of PAAEt whereas, curcumin showed 4.5 to 64.9% inhibition at concentrations of 2.5 to 150 µg/ml. The IC_{50} values of PAAEt and curcumin for inhibition of $^{\cdot}O_2$ are 201.5 and 59.3 µg/ml respectively.

Nitric oxide radical was generated at physiological pH from sodium nitroprusside. The nitric oxide radical scavenging activity of PAAEt increased gradually from 8.3 to 67.06% at a concentration of 25 to 1000 µg/ml with an IC_{50} of 408.5 µg/ml (fig. 1e). The standard antioxidant curcumin at different concentrations ranging from 5 to150 µg/ml showed 10.3 to 97.3% inhibition of $NO^{\cdot}$ generation with an IC_{50} of 37.9 µg/ml.

Reducing power of PAAEt increased from 14.2 to 78.5% with an increase in concentration of extract from 50 to 1000 µg/ml and showed 50% of reducing power at 500.0 µg/ml (fig. 1f) whereas, curcumin showed 50% of reducing power at 15.0 µg ml.

Table 3 represents the correlation studies of PAAEt illustrated significant and strong positive correlations ($P<0.05$) with free-radical $DPPH^{\cdot}$ ($r^2=0.735$), $OH^{\cdot}$ ($r^2=0.802$), $NO^{\cdot}$ ($r^2=0.845$), $^{\cdot}O_2$ ($r^2=0.666$) scavenging efficiency, reducing power ($r^2=0.972$) and potent of LPO inhibition ($r^2=0.964$). Reducing power and LPO inhibition showed highest correlation followed by $NO^{\cdot}$, $OH^{\cdot}$, $DPPH^{\cdot}$ and $^{\cdot}O_2$ radicals.

DISCUSSION

Nature has been a source of medicinal treatments from ancient times. Plant based systems continue to play an important role in the primary health care of 80% of the world's underdeveloped and developing countries [23]. Several modern drugs and processed scientific medicines are of plant origin [24]. Many diseases are associated with changes in the oxidative status [25, 1]. Inadequate endogenous antioxidant defense mechanism and carcinogenic effect of available synthetic antioxidants led to much attention being paid on dietary natural antioxidants and numerous natural antioxidants isolated from different varieties of plant materials which are safe and possess various therapeutic properties such as anti-diabetic, anti-obesity, hepatoprotective, anticancer, antiviral, anti-inflammatory, anti-tumor and anti-mutagenic properties [26, 6].

Table 1 represents the presence of various phytochemical constituents in *P. amarus.* Patel *et al.* [6] reported that polyphenols, flavonoids, lignans, alkaloids, hydrolysable tannins (ellagitannins), sterols and triterpenes are the key phytochemical compounds in *P. amarus* which are in accordance with our findings.

Table 1: Qualitative phytochemical profile of aqueous extract of *P. amarus*

Phytochemicals	Presence	Phytochemicals	Presence
Phenols	+	Carboxylic acids	+
Flavonoids	+	Resins	+
Saponins	+	Coumarins	+
Alkaloids	+	Gallictannins	+
Anthocyanins	+	Triterpinoids	+
Anthocyanidins	+	Aucubins & iridoids	+
Anthracene glycosides	+	Steroids	+

+-denotes presence of a compound.

The extract has contained an appreciable amount of polyphenolic compounds 12% (gallic acid equivalents) and flavonoids 0.302% (quercetin equivalents) (table 2) [27]. Reported that aqueous extract of *P. amarus* has considerably high constitutions of phenolic compounds which is in consistence with findings of the present study. Presence of high quantity of phenolic compounds is due to its huge series of molecules containing at least one aromatic ring with hydroxyl group and other constituents along with their functional derivatives.

Table 2: Quantification of total phenols and flavonoids in *P. amarus*

Name of the phytochemical compounds	Quantity
Total phenolic compounds (mg GA eq. g^{-1})	120±2.0
Flavanoids (mg Q eq. g^{-1})	3.02±0.13

Data expressed as mean±SD (n = 3)

Many epidemiological studies suggest that herbs/diets rich in phytoconstituents and antioxidants execute a protective role in health and diseases [28, 29]. Earlier reports indicated that, total phenols and flavonoids (one of the main groups of phenolic compounds) have exhibited antioxidant potential (27, 30). It may be due to redox properties which take part in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen and decomposing peroxides. The extract has shown marked dose dependent increase in antioxidant potential as monitored from scavenging activity of hydroxyl, DPPH, nitric oxide, superoxide radicals with reducing power and inhibition of lipid peroxide generation. The antioxidant activity of PAAEt was compared with

standard antioxidant curcumin. The curcumin showed significantly lower inhibitory concentration (50%) than PAAEt. The different radicals scavenging activities of PAAEt may be referred to the different antioxidant mechanisms for each specific radical.

Lipid peroxidation takes part in a series of free radical mediated chain reaction processes which mainly affect biological membranes of all organs of the body. Lipid peroxides are involved in many pathological events, including metabolic disorders, inflammation and cellular aging [22]. Therefore lipid peroxidation inhibition is of great importance in the induction/onset of diseases involving free radicals. The PAAEt and standard curcumin exhibited gradual inhibition of lipid peroxide generation as their concentrations increased in a dose dependent manner (fig. 1a).

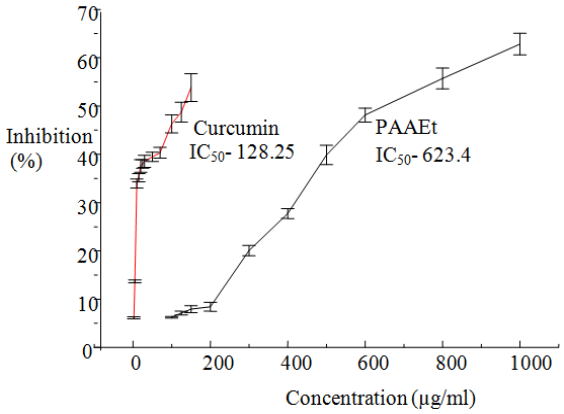

Fig. 1a: Lipid peroxidation inhibition activity of the PAAEt extract and the reference compound curcumin

The results are mean±SD (n=3)

The IC_{50} values of PAAEt and curcumin for inhibition of LPO are 623.4 and 128.25 µg/ml respectively. The aqueous extract of the whole plant of *P. amarus* showed significant lipid peroxidation inhibition against chromium-induced oxidative cellular damage in MDA-MB-435S cells [27] and tannins of ethanolic extract of *P. amarus* against $FeCl_3$ induced lipid peroxidation in mice liver [31], which are in accordance with our findings. Aqueous extract of *P. amarus* exhibited LPO inhibition activity against fructose induced renal damage, which was communicated in our earlier report [8]. This protection could be due to several mechanisms, such as (a) chelation of iron (b) reducing the formation of free radicals (c) scavenging the $^{\cdot}OH$ and $^{\cdot}O_2$ radicals and other reactive oxygen molecules, (d) supplying a competitive substrate for unsaturated lipids in the membrane which are responsible for LPO. Furthermore, the deteriorating effects of hydroxyl, superoxide, nitric oxide and DPPH radicals contribute to LPO. Hence, PAAEt effects on above said free radicals were analyzed.

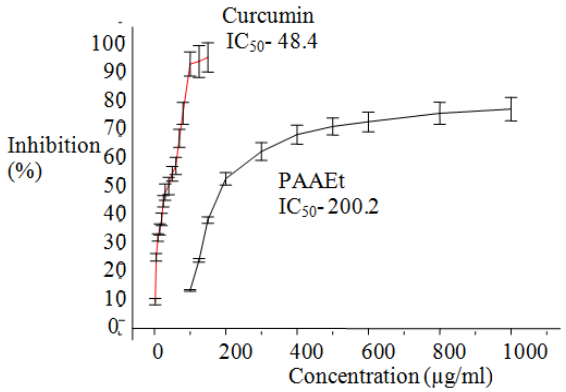

Fig. 1b: DPPH radical scavenging activity of the PAAEt extract and the reference compound curcumin

The results are mean±SD (n=3)

1,1-Diphenyl-2-picrylhydrazyl, a stable free radical, accepts hydrogen or an electron to become a stable diamagnetic molecule [32]. It is widely used to test the free radical scavenging ability of various samples. The free radical scavenging activity gradually increased with increase in concentration of PAAEt and curcumin with IC_{50} at 200.2 and IC_{50} at 48.4.0 µg/ml respectively (fig. 1b). This is in congruent with previous *in vitro* studies [27, 31]. Also, some of its active principle constituents such as geraniin, phyllanthusiin D, repandusinic acid and rutin were tested for DPPH radical scavenging activity as a part of antioxidant potential screening and found remarkable antioxidant activity [33]. Results from the present study suggested that PAAEt has good DPPH radical scavenging activity.

Superoxide radical has been of intense interest due to its production increased under *in vivo* condition in different disease conditions [34]. Flavonoids are effective antioxidants mainly they scavenge superoxide anions [9].

Super oxide radicals are generated in PMS-NADH systems by NADH oxidation and were assayed by the reduction of Nitro blue tetrazolium. The $^{\cdot}O_2$ radical scavenging activities of the both PAAEt and curcumin increased markedly with their increasing concentrations (fig. 1c).

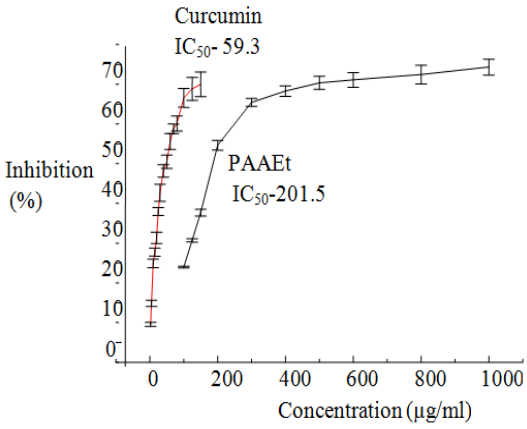

Fig. 1c: Superoxide radical scavenging activity of the PAAEt extract and the reference compound curcumin

The results are mean±SD (n=3)

The superoxide radical is self toxic and generates more toxic species like ˙OH which promote LPO. These hydroxyl radicals are the major active oxygen species causing lipid peroxidation and huge biological damage by abstracting hydrogen atoms from membranes of cells. Hydroxyl radicals are also generated by incubating ferric-EDTA with ascorbate and H_2O_2 at pH 7.4 [19]. Both extract and standard curcumin gradually increased per cent inhibition of hydroxyl radical formation with IC_{50} 280.6 µg/ml and 56.9 µg/ml respectively (fig. 1d).

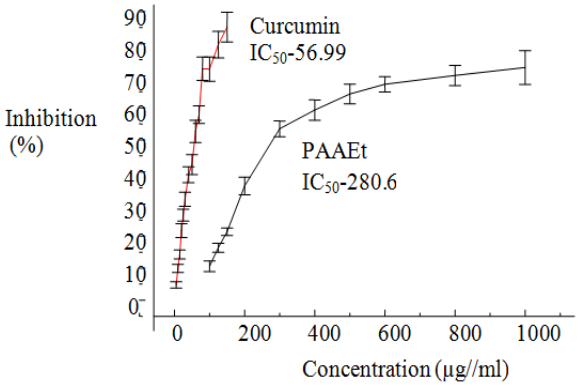

Fig. 1d: Hydroxyl radical scavenging activity of the PAAEt extract and the reference compound Curcumin

The results are mean±SD (n=3)

Ujwala *et al.* [31] reported that an alcoholic tannin extract of *P. amarus* showed superoxide and hydroxide radical scavenging activity, which are in congruent with the present findings. Methanolic extracts of leaves and stem of *P. amarus* were found to have potential superoxide and hydroxide radical scavenging activity [35]. The results of the present findings indicate that PAAEt has remarkable scavenging capacity for superoxide and hydroxide radicals.

Nitric oxide radical plays multiple roles in a variety of biological processes viz. as an effector molecule, vasodilator, neuronal messenger and antimicrobial agent etc, [36]. Nitric oxide reacts with $^{\bullet}O_2$ radical to form peroxynitrite radicals (ONOO-) that cause toxicity to proteins, lipids and nucleic acid molecules [37]. Among the reactive nitrogen species, NO is the most important mediator of oxygen radical injury due to the presence of an unpaired electron which can readily combine with many free radicals. Many tissues are normally exposed to higher concentrations of $NO^{\bullet}$ than oxygen radicals [38]. The plant extract and curcumin showed gradual increase in inhibition of $NO^{\bullet}$ formation as their concentration increased with IC_{50} values of 408.5 and 37.87 µg/ml respectively (fig. 1e).

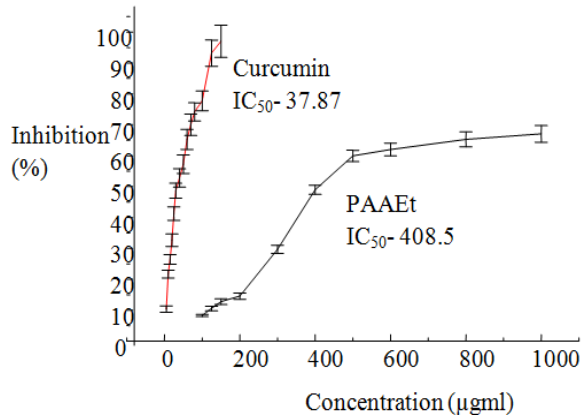

Fig. 1e: Nitric oxide radical scavenging activity of the PAAEt extract and the reference compound curcumin

The results are mean±SD (n=3)

Alcoholic extract of *P. amarus* showed good nitric oxide scavenging activity [31], which is congruent with our present findings. In an earlier study, we reported that PAAEt has DNA protective effect against nitric oxide induced lymphocyte DNA damage by single cell gel electrophoresis method [39] which revealed that PAAEt has higher nitric oxide radical scavenging activity.

Reducing capacity of a compound is one of the best indicators of its potent antioxidant activity. The presence of antioxidants in the sample reduced the Fe^{+3}/ferricyanide complex to ferrous form during the assay. Reducing power of PAAEt gradually increased as the concentration of extract increased. The 50% reducing power of the extract and curcumin are 500.0 µg/ml and 15.0 µg/ml respectively (fig. 1f). Thounaojam *et al.* [40] demonstrated that the methanolic extract of *Sida rhomboidea* has higher reducing potential, is in consistence with the findings of the present study. The results of the present study showed that PAAEt have good reducing potential. Overall, the results from this study revealed that PAAEt has good scavenging activity against above said free radicals with efficient reducing capacity.

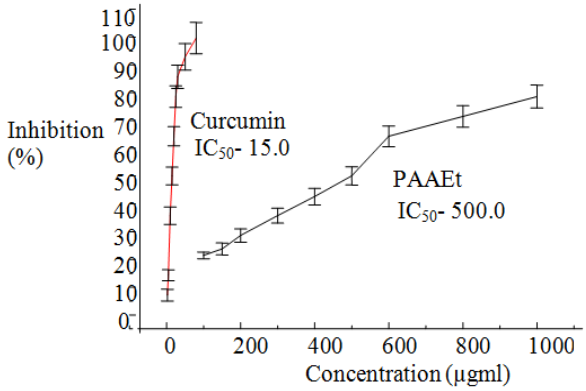

Fig. 1f: Reducing power activity of the PAAEt extract and the reference compound curcumin

The results are mean±SD (n=3)

The PAAEt showed significant and strong positive correlations for scavenging of free-radicals ($OH^{\cdot}$, $DPPH^{\cdot}$, $NO^{\cdot}$ and $^{\cdot}O_2$), reducing power and potent of LPO inhibition (table 3) which indicated that it has strong antioxidant potential.

Table 3: Correlation studies of PAAEt with LPO and various free radicals scavenging

Parameter	Line of regression of Y	Coefficient of determination (r)
% Inhibition of LPO formation	Y = 0.111+0.068x	r^2= 0.964
% Inhibition of DPPH radical formation	Y = 29.05+0.059x	r^2= 0.735
% Inhibition of hydroxyl radical formation	Y = 18.18+0.069x	r^2= 0.802
% Inhibition of superoxide radical formation	Y = 23.73+0.061x	r^2= 0.666
% Inhibition of nitric oxide radical formation	Y = 9.962+0.070x	r^2= 0.845
% Inhibition reducing power	Y = 14.63+0.069x	r^2= 0.972

The PAAEt showed high positive correlation with reducing power (r^2= 0.972) and LPO inhibition (r^2= 0.964). Similarly, PAA*Et* also showed good free radical scavenging activity as monitored from

positive correlation studies for scavenging of free radicals such as $OH^{\cdot}$, $DPPH^{\cdot}$, $NO^{\cdot}$ and $^{\cdot}O_2$. The findings of the present study are in agreement [27], who reported that the total phenol content of *P. amarus* have strong and significant positive correlations to free-radical scavenging potential, lipid peroxidation inhibition capacity against chromium induced oxidative cellular damage in MDA-MB-435S cells [41] also reported a high positive correlation between antioxidant activities and reducing power of certain plant extracts.

Differential scavenging activities of PAAEt against $OH^{\cdot}$, $DPPH^{\cdot}$, $NO^{\cdot}$ and $^{\cdot}O_2$ radicals may be referred to the different radical-antioxidant reaction mechanisms in these assays. Many researchers have reported that total phenols and flavonoids execute strong antioxidant property [42, 27, 43]. Islam *et al.* [43] reported that *in vivo* antioxidant property of lignans (phyllanthin and hyypophyllanthin), gallotanoids (geraniin) and flavonoids (rutin) combinations isolated from *P. amarus* against CCl_4 induced liver damage in a rat model. Some of the active constituents, namely amariin, phyllanthin, repandusinic acid and phyllanthusiin D compouds isolated from *P. amarus* showed remarkable high antioxidant activity [27]. Tannins are the group of polyphenols isolated from *P. amarus* which exhibited strong *in vitro* antioxidant potential [31]. Hence, the effective antioxidant potential of PAAEt might be due to the presence of polyphenols and flavonoids, which occupied the major portion in this extract. The high antioxidant potential of PAAEt is in consistence with reports of Lim & Murtijaya [26], who demonstrated that the aqueous extract of *P. amarus* exhibited strong antioxidant potential than methanolic extract due to greater solubility of compounds, breakdown of cellular constituents as well as hydrolysis of tannins.

CONCLUSION

Total phenolic and flavonoid compounds are the major contributors in the antioxidant capacity of all herbal extracts. Total phenols and flavonoids compounds occupied the major portion in this extract. PAAEt has high antioxidant activity as evident from appreciable IC_{50} for various free radicals with significant and strong positive correlation for scavenging of free radicals, strong reducing power and inhibition of LPO. PAAEt may be suggested as a source of beneficial compounds for human health either in the food industry, as functional food ingredient or in preparation of allopathic medicine to alleviate oxidative stress.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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