Comparisonof *invitro* antioxidant potentials of *Phyllanthusacidus* and *Phyllanthus niruri* leaf extracts

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Abstract

In humans, many ailments are related to the accumulation of oxidative free radicals in the cells.. However, the antioxidants in the biological system scavenge the free radicals and reduce their harmful effects. Therefore, the search for naturally derived antioxidant compounds from plants or herbal sources is imperative. Here, we aimed to investigate the preliminary phytochemicals and antioxidant activity of selected ethanolic leaf extracts of *Phyllanthus acidus (PA)* and *Phyllanthus niruri (PN)*.Standard spectroscopic methods were used to estimate the phytochemicals and antioxidants. We used Folin-Ciocalteu reagent to estimate total phenolics and aluminium-chloride-colorimetric assays to estimate flavonoids.Ascorbic acid was used as standard for estimating free-radical scavenging activities by estimating DPPH, OH*, superoxide, and reducing activity methods.Both the selected plant leaf ethanolic extracts showed significant anti-oxidant activity. However, PN showed a significantly superior effect compared to the extract of PA.In conclusion, the selected plant extracts confirmed the presence of supportive phyto-chemicals such as polyphenols, flavonoids, saponins, alkaloids, glycosides, steroids, and terpenoids. PN showed a superior effect. The high flavonoid and phenolic content may be the cause of this better action.

Keywords: Flavonoids, Phenolics, in vitro antioxidant; *Phyllanthus acidus; Phyllanthus niruri*

Introduction:

There is fascinating evidence linking the build-up of free radicals to several hazardous pathophysiological processes, including diabetes, cancer, cardiovascular, and neuro-degenerative diseases[1-3]. A free radical is quite an unstable molecule having propensity to bind with biological macromolecules like proteins, lipids and DNA causing damage to the cell[1].

Radical induced damage may be aggravated when there is a lesser or lack of antioxidant defence in the cell. Antioxidants taken through diet contribute to defence against cellular oxidations resulted from free radicals. Diets like fruits are rich source of antioxidants and can contribute to cellular antioxidant potentials[4]. Antioxidants reacts with free radicals and negate their detrimental effects on the biological system. These health advantages are mostly attributable to phytochemicals such as polyphenols, flavonoids, carotenoids, and vitamins such as E and C [5].Although phenolic compounds are frequently present in both edible and inedible herbs, grains, fruits, spices, vegetablesand other plant components. Because endemic plants are only found in a few places and are only known to the local community, there is a lack of scientific data on their antioxidant capacities. To uncover prospective sources of natural antioxidants for functional foods and/or nutraceuticals, it is still intriguing and important to examine their qualities[6, 7].

*Phyllanthus acidus*is the largest genus of phylanthaceae which consist of *ca*. species of 1270 that can be sub-divided into 11 sub-genres including Kieganelia, Cicca, Isoclocus, and Emblica located in subtropical regions of the world greater than 10 species. The local name for this plant is PokokCermai, In India, it is named chalameri, haroharoi, and bamboo found in the Caribbean region, south and central America, and Asia. *P. acidus* is an ornamental shrub or tree that can grow upto 10 meters tall, with branches. The bark is rough, the leaf is pinnate 20-10 cm long and the lanceolate, ovate leaflets with short petioles upto 7.5 cm. Leaves are green with smooth upper surface, blue-green with bloom on its outer surface. The tree bears fruit twice a year in the southern part of India[8].

*Phyllanthus niruri*belongs to the Euphorbiaceae family found with more than 600 species it is a tiny erect annual herb native to the Amazon rainforest and other tropical regions such as Southern India and south east asia including China, growing up to 30-40 cm in height. Leaves are sessile, oblong 7–12 cm long. It has tiny, auxiliary, solitary, apetalous, monoecious, greenish blooms. In terms of appearance, phytochemical makeup, and history, *P. amarus* and *P. sellowianus* are closely related to *P. niruri*, but they can be found in drier parts of Brazil and India, even in Florida and Texas. The traditional medicine of South and Southeast Asia has employed the perennial tropical shrub and one of the most significant groups of plants in the genus Phyllanthus (L.). The herb's local name in South India is Bhumyamalaki[9].

Materials and Methods

Plant materials authentication

Dr. Madhava Chetty, Botanist, SVU Tirupathi, AP, Indiaauthenticated the plant materials bearing the specimen numbers 0956 (PA) and 0922 (PN).

Preparation and preliminary phytochemical evaluation of the extracts:

The ethanolic extraction was done as per the standard method [10]thedry leaf materials were sliced and crushed until a coarse powder and about 100g of each plant powder were placed in filter paper and inserted into the Soxhlet apparatus separately and the solvent ethanol(500 mL) was used for extraction using a temperature at 70^0C under atmospheric conditions.The extracted product was dried and purified under a hot-air oven at 40^0C and the percentage yield was calculated. Then the extract was examined for preliminary constituentsby using standard methods[11]. Further the flavonoid and total phenoliccontent was determined by spectrophotometer[12, 13].

Estimation of Total Phenolic Content:

We used Folin-Ciocalteu method to estimate total phenolic portion in the extracts[13].The standard solution of extracts (1:10 v/v) was made with water and an aliquot of extracts (PA and PN) was mixed with the reagent of Folin-Ciocalteu (2mL) and 2 mL of sodium carbonate. Then the tubes were vortexed for fifteen minutes and allowed for 20 min at 25^0C for color formation. Using UV-Spectrophotometer (Shimadzu, USA) the absorbance was measured at a wavelength of 760 nm. The extracted samples were evaluated at a final conc. of 0.1 mg/mL and 0.15 mg/mL. Finally, the total phenolic content was expressed in terms of gallic acid (GA) equivalent, the standard curve: $y = 0.013x + 0.1275$; $R^2 = 0.9978$ µg of GA/g of dry extract. For each concentration, the experiment was repeated thrice.

Estimation of Total Flavonoid Content:

The total flavonoid content was estimated by the aluminium chloride method[13].150 µL of 5% sodium nitrate and 2.5 mL distilled water were added to 0.5 mL of the test sample (extracts, PA and PN). 5 min later, 10% aluminium chloride was added and after 6 min, 1 mLof 0.001 M NaOH and distilled water (0.55 mL) were added to the whole mixture and hold it room temperature for 15 minutes. Then the absorbance of the mixture was measured at a final conc. of 0.1 and 0.15 µg/mL. The total flavonoid portion was expressed in terms of quercetin (QE). The standard curve was obtained as $y = 0.0031x + 0.0653$; $R^2 = 0.9967$ µg of GA/g of dry extract. The experiment was repeated three times at each concentration.

***In-vitro* antioxidant activity:**

DPPH assay: Various concentrations of extracts of PA and PN was added to the test tubes with 1 mL of water. After 30 minutes, 3 mL of DPPH stock solution (0.004%) was added and mixed well. Then the mixture was incubated for 10 min. in a dark place. A control was prepared using methanol (Blank) and DPPH solution. The absorbance was determined at 517nm using a spectrophotometer[14]. The percentage inhibition was calculated by:

(%) Inhibition = (A0 – A1 / A0) × 100

Where; A0 is the absorbance of the control and A1 is the absorbance of the test.

Scavenging of Nitric Oxide Radical: The OH* radical scavenging activity was carried out as per Atere TG et al. [15]. Briefly, the reaction mixture contained 1.0 ml of reagent (3.0 mM deoxyribose, 0.1 mM EDTA,2 mM H2O2, 0.1 mM L-ascorbic acid, 0.1 mM FeCl3.6H2O in 10 mM phosphate buffer, pH 7.4) and various concentrations of the extract (50–350 µg/ml). The reaction mixtures were incubated at 37^0C for1h, followed by the addition of 1.0 ml of 1% (w/v) TBA (in0.25 M HCl) and 1.0 ml 10% (w/v) trichloroacetic acid (TCA). The reaction mixture was heated in a boiling water bath at 100^0C for20 min and the pink chromogen (malondialdehyde-(TBA) adduct) was extracted into 1.0 ml of butan-1-ol and the absorbance (Abs)was read at 532 nm against reagent blank. BHT (butylated hydroxytoluene) served as the positive control.

(%) Inhibition = (A0 – A1 / A0) × 100

Where; A0 is the absorbance of the control and A1 is the absorbance of the test.

Superoxide Radical by Alkaline DMSO Method: The superoxide anion scavenging activity was determined as described elsewhere[16]. In aphenazine methosulfate-nicotinamide adenine dinucleotide(PMS-NADH) system, superoxide radicals were produced by NADH oxidation and quantified by NBT reduction. In this method, radicals were generated in 3 mL sodium phosphate buffer (pH 7.4, 10 mM) comprising of NBT (1mL, 150µM) solution, and various concentrations (25-250µg/mL) in water. Then the reaction was initiated by the addition of PMS solution (1mL, 60µg) to this mixture. This reaction mixture was incubated for 5 minutes at 25^0C. Finally, the absorbance was determined against blank. Ascorbic acid was used as a positive control. The amount of NBT reduction is determined by measuring the absorbance of the reaction mixture and is correlated with the extract's capacity to scavenge superoxideradicals. Using the following formula, the percentage of superoxide radical scavenging was determined:(%) Scavenging = (A0 – A1 / A0) × 100

Reducing Power Assay: The Oyaizu methodwas used to determine the reducing power of ferric ions in the extract[16]. About 0.75 mL of extracts was added to 1%, w/v of potassium hexacyanoferrate

(0.75 mL) and phosphate buffer (0.75 mL, 0.2 M, pH 6.6) at various concentrations before being incubated at 5^0C in a waterbath for 20 minutes. Then the reaction was stopped by the addition of 10 % of TCA (trichloroacetic acid, 0.75mL) solution and then centrifuged for 10 minutes at 3000rpm. The collected supernatant (1.5mL) was mixed with distilled water (1.5mL) and 0.1mL of $FeCl_3$ solution (0.1% w/v) for 10minutes. Finally, the absorbance was measured at 700nm as the reducing power.

Total anti-oxidant Capacity of the Extracts: The total antioxidant capacity was determined by methods discussed by Prieto et al. [15].Briefly, 1 ml of the reagent solution, which was composed of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate, was added to 0.1 ml (0.25 mg/ml) of the extract or standard solutions of ascorbic acid (20-100 g/ml). The reaction mixture was contained in tubes that were incubated in a water bath at 95°C for 90 minutes. Once the combination had cooled to room temperature, it was tested for absorbance at 695 nm in comparison to a blank that contained the reactive mixture with distilled water in place of the extract. In terms of ascorbic acid equivalents, the antioxidant activity of the extracts was expressed.

Statistical analysis:

The findings were presented as mean ± SD. Analyzed by GraphPad Prism 8.01 using nonlinear regression.

Results and discussion:

Preliminary phyto-chemical screening: The extracts were evaluated for qualitative evaluation for the presence of several phyto-chemicals such as phenols, flavonoids, tannins, saponins, alkaloids, glycosides, and triterpenoids. These findings revealed the presence of all the supportive chemicals in both plant extracts shown in **Table 1**. However, the alkaloids were found absent in the extract of *Phyllanthus acidus*(PA). The phenol and flavonoid content were presented in **Table 2 and Fig. 2.**The standard curves for the reference samples were presented in **Fig. 1.**

Radical scavenging activity: The prepared ethanolic extracts of *Phyllanthus acidus*(PA)and *Phyllanthus niruri* (PN)leaf extracts were shown free-radical scavenging activity evaluated by DPPH, Hydroxyl, Super-oxide assays. This antioxidant (free-radical scavenging) activity wasfound to be concentration dependent presented in **Fig.3a-e.** The IC_{50} Values for both extracts were presented in **Table 3**.

In the free radical scavenging activity ofDPPH (2,2-diphenyl-1-picrylhydrazyl) assay, OH* radical assay, and Superoxide assays, both the extracts *Phyllanthus acidus* (PA) and *Phyllanthus niruri* (PN) were shown the concentration-dependent activity. However, the extract of *Phyllanthus niruri*showed a significantly superior effect compared to the extract of *Phyllanthus acidus*(**Table 3 and Fig. 3a-c**).

Reducing power activity: The reducing power activity of extracts and standard is shown in **Fig. 3d.**Both the extracts PA and PN showed concentration-dependent activity. However, the extract of PN showed a significantly superior effect compared to the extract of PA.

Discussion: In the current pharmaceutical research, the pursuit of anti-oxidant phyto-derived chemicals has been greatly increased due to their potent therapeutic benefit in various chronic infectious and metabolic diseases. In the hunt for new therapeutic Phyto-constituents, in this research, we have screened for ethanolic leaf extracts of *Phyllanthus acidus* and *Phyllanthus niruri* belonging to Phyllanthaceae and Euphorbiaceae family respectively.

The highly unstable freeradicals (associated with one or more unpaired electrons) become stable by stealing electrons from other molecules. In the biological system, unstable chemical species produced reacts with by free radicals because of high level of reactivity by the later. Humans were shown to have an increased risk of several chronic conditions when their free-radical levels were elevated due to a malfunctioning endogenous antioxidant defense system, exposure to oxidants in the environment, or damage to cell structures[17]. As a result, we can prevent the advancement of chronic diseases and the dangers that go along with them by giving patients additional research-proven antioxidants or by boosting their antioxidant defense. The effect of therapeutic herbs in reducing tissue damage brought on by free radicals exposes their antioxidant potential[18]. Hence, the current study investigated the anti-oxidant activity of leaf ethanolic extracts of *Phyllanthus acidus* and *Phyllanthus niruri*. The extracts were tested against a variety of free radicals get an insight into variety of mechanismsthat includes free radical scavenging, reducing activity, potential complexing of pro-oxidant metals, and quenching of singlet oxygen[19, 20].

The free-radical (anti-oxidant) activity of the selected plant extracts was assessed by preliminary phytochemical evaluation. These extracts showed concentration-dependent and significant anti-oxidant activity in DDPH, OH*, and Superoxide radical assays. In the DPPH assay, the *Phyllanthus niruri*(IC_{50} = 526.8µg/mL) was shown superior activity compared to *Phyllanthus acidus*(IC_{50} = 910.3µg/mL).The results were presented in **Table 3** and **Fig 3a**.

The hydroxy-radical formation in the biological system of iron-rich tissues via Fenton-reaction contributes to lipidperoxidation and cell damage by DNA destruction, Inflammation[21, 22]and also leads to the development of various cancers [23]. Therefore, hydroxyl-radical (OH) scavenging activity can be significantly considered for the evaluation of various new chemicals as anti-oxidants. In this study, extracts showed the concentration-dependent and significant anti-oxidant activity. However, the extract of *Phyllanthus niruri*(IC_{50} = 518.3 µg/mL) was shown superior activity

compared to *Phyllanthus acidus*(IC_{50} =438.1 µg/mL). The results were presented in **Table 3** and **Fig 3b**.

Being a precursor to more reactive species, superoxide radical is extremely damaging to cellular components and can produce in vivo H_2O_2 through a dismutation reaction. The produced H_2O_2, which is not particularly reactive by itself, further generates OH* radicals in the cells, ultimately leading to cell damage. Hence, for the antioxidant defense mechanism to function, H_2O_2 must be removed from the cell system[24]. In this study, extracts showed the concentration-dependent and significant anti-oxidant activity. However, the extract of *Phyllanthus niruri* (IC_{50} = 9944 µg/mL) was shown superior activity compared to *Phyllanthus acidus* (IC_{50} = >1000 µg/mL). The results were presented in **Table 3** and **Fig 3c**.

The extracts have shown the presence of Phenols, tannins, alkaloids, glycosides, flavonoids, saponins, and terpenoids. Many studies have demonstrated the anti-oxidant capabilities of the majority of these substances throughpolyphenols and flavonoids[25]. Also, the polyphenols like quercetin, caffeic acid, and gallic acid) inhibits the production of H_2O_2 that is brought about by the harm to mammalian cells [26, 27]. The highlevels of flavonoids and phenols in the plants were shown to have better free-radical scavenging capabilities in the current study. Moreover, theplant extractspossess the ability to supply hydrogen or electron atoms necessary for antiradical activity. The results of the current study show that extracts can stabilize free radicals by providing them with an electron or hydrogen.

The various plant extracts contain several scavenging chemicals that may work in concert to increase antiradical activity in a variety of oxidative stress conditions and conditions like diabetes[27]. The varied levels of many bioactive components such as phenols, tannins, and flavonoids) that can donate hydrogen atoms to stabilize the free radicals may account for the differences in antiradical activity across the extracts in the current investigation[26, 27].

Compounds' capacity to mitigate other substances would be a good measure of how effective an antioxidant they are. Hence, in the current study, we evaluated the reducing power of the extract by determining their ability to decrease Fe^{3+} to Fe^{2+} by donating electrons[28]. All extracts demonstrated the maximum optical density (reduction capacity) when compared to ascorbic acid at a test concentration of 1000 g/mL.The presence of reducing agents, such as polyphenols, and the quantity and/or location of hydroxyl groups on polyphenols may be responsible for the extracts' ability to reduce Fe^{3+}[28].In the current study, the total antioxidant activity of the extracts is evaluated and contrasted with that of the ascorbic acid standard. The collected data c showed that the ethanolic extract of *Phyllanthus niruri* had more anti-oxidant activity than *Phyllanthus acidus*.

Several studies have revealed a significant correlation between the amount of total phenol and the anti-oxidant activity of various plant species[29, 30].These outcomes confirm the possible antioxidant

properties of polyphenols. Because polyphenols serve as reduction agents, hydrogen donors, free radical scavengers, singlet oxygen quenchers, and metal chelators, the present study's increased antioxidant activity of the extracts could be explained by their abundance[29]. However, further evaluation with other screening methods is required to confirm the anti-oxidant in the biological system.

Conclusion

The preliminary phyto-chemical investigation, theselected plant's leaf ethanolic extracts contain the supporting phyto-chemicals such as polyphenols, flavonoids, saponins, alkaloids, glycosides, steroids, and terpenoids. The study's findings demonstrated that both plants' leaf ethanolic extracts were shown significantly in vitro anti-oxidant activity. However, *Phyllanthus niruri* showed a significantly superior effect compared to the extract of *Phyllanthus acidus*. It's high phenolic and flavonoid content may be the cause of superior action. However, more research is required to identify other phyto-chemical constituent(s) in these plants with underlying mechanism of action.

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List of Tables and Figures

Tables

Table 1: Preliminary phytochemical findings

Extracts	P	F	T	S	A	G	T
PN	+	+	+	+	+	+	+
PA	+	+	+	+	+	+	+

*PA: Phyllanthus acidus;*PN:*Phyllanthus niruri***(+)** Presence; P=Phenols; F=Flavonoids; T=Tannins; S=Saponins; A=Alkaloids; G=Glycosides; T=Triterpenoids

Table 2. The phenol and flavonoid content

Extracts	TPC (mg/gm)	TFC (mg/gm)
PN	126.947±0.009	27.974±0.004
PA	97.199±0.002	18.23±0.005

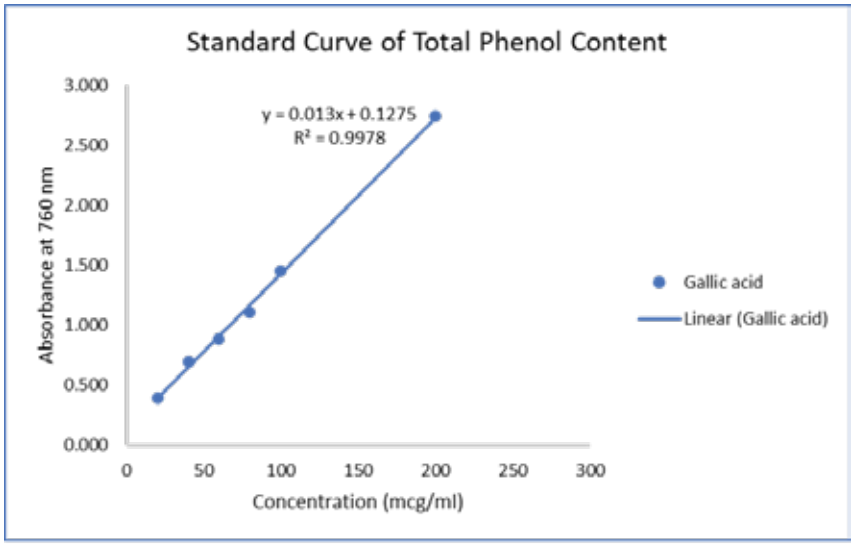
*TPC: Total phenolic content; TFC: Total flavonoid content; PA: Phyllanthus acidus;*PN:*Phyllanthus niruri*

Table 3.IC_{50}Free-radical scavenging activity of extracts

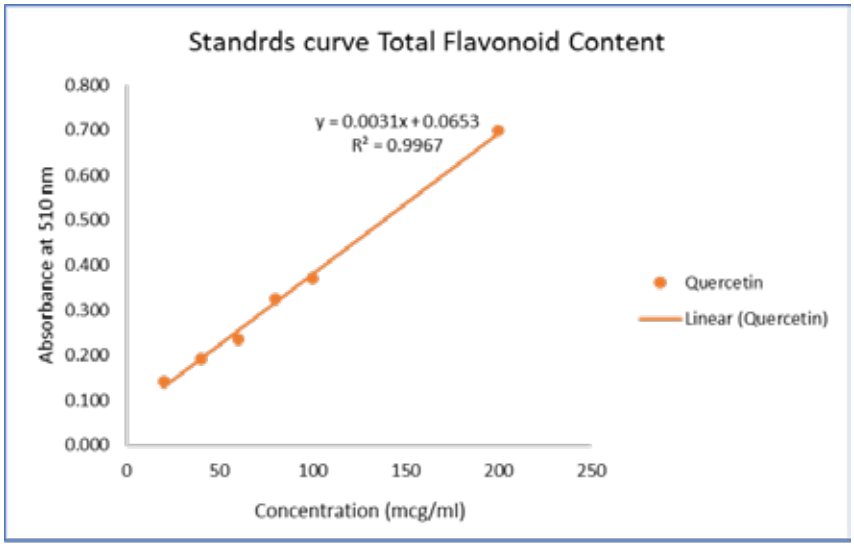
Extracts	IC_{50} (µg/mL)		
	Free-radical scavenging activity		
	DPPH	OH*	Superoxide
PN	526.8	518.3	9944
PA	910.3	438.1	>1000

Values expressed as mean ± SD, (n = 3). *PA: Phyllanthus acidus;*PN:*Phyllanthus niruri*

Figures



(A)



(B)

Fig. 1 Standard curve of (A): Gallic acid and (B): Quercetin

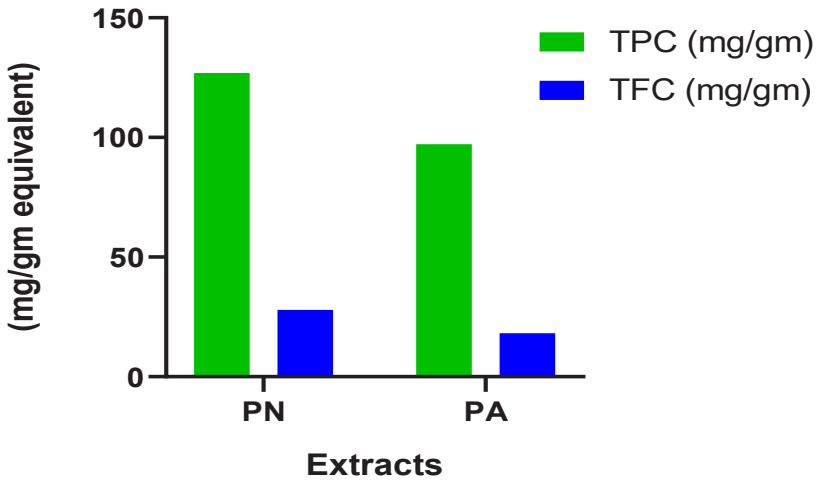


Fig. 2 Total phenol and Flavonoids content: PA: *Phyllanthus acidus;* PN: *Phyllanthus niruri*

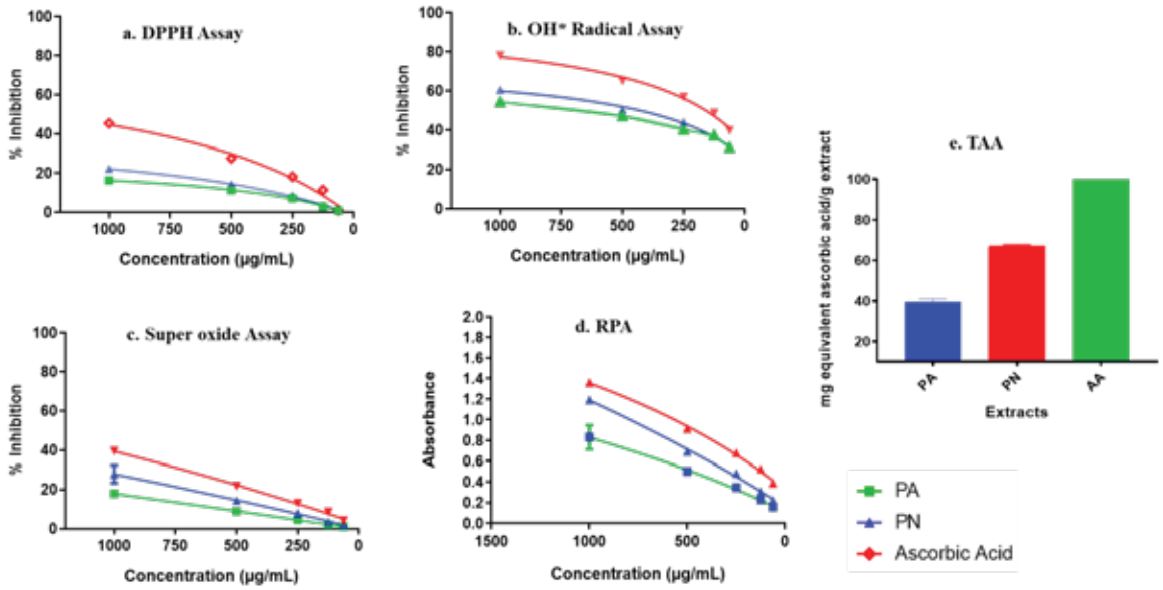


Fig. 3 Free-radical activity of prepared extracts: PA: *Phyllanthus acidus;* PN: *Phyllanthus niruri*