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Research Article

THE STUDY OF *IN VITRO* AND *IN VIVO ANTIOXIDANT* ACTIVITY AND TOTAL PHENOLIC CONTENT OF *PHYLLANTHUS AMARUS* SCHUM. & THONN.: A MEDICINALLY IMPORTANT PLANT

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ABSTRACT

The presence of natural antioxidant activity in plants has been well acknowledged world wide. There is an increasing demand for natural antioxidants to replace synthetic additives in the food and Pharmacological industries. *Phyllanthus amarus* Schum. & Thonn., commonly known as buhi amla, is also one of the traditionally used as medicinal plant. The main goal of this study is to determine the free radical scavenging properties screened for different *in vivo* and *in vitro* plant extraction of *Phyllanthus amarus* Schum. & Thonn. Free radical scavenging activity was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. *In vitro* callus was induced using internodal explant on MS medium fortified with 2,4-D (0.6 mg/l). The analysis was carried out for *in vivo* plant and *in vitro* callus to determine the quantitative phenolic content along with their antioxidant activity of different plant extracts. The result of the present study showed that the methanol extract of *Phyllanthus amarus* Schum. & Thonn., contains highest amount of phenolic compounds and exhibits the greatest anti-oxidant activity in comparison to other extracts. It has been observed that, the *in vitro* plant extract shows more Phenolic contents and revealed better antioxidant activity as compared to *in vivo* plant extraction.

Keywords: *Phyllanthus amarus* Schum. & Thonn., Free radical scavenging activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Phenolic compounds

INTRODUCTION

Approximately 80% of the world total population depends exclusively on plants for their health and healing. Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing such free radical-induced tissue injury[1]. In response to this, increased popularity and greater demand for medicinal plants, a number of conservation groups are recommending that wild medicinal plants be brought into cultivation. All plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. A rich heritage of knowledge to preventive and curative medicines was available in ancient scholastic works included in the Atharva veda, Charaka, Sushruta etc[2].Over 50% of all modern clinical drugs are of natural product origin[3] and natural products play an important role in drug development programs in the pharmaceutical industry[4]. Herbal drugs have gained importance in recent years because of their efficacy and cost effectiveness.

Thus, there is an increasing interest in the measurement and use of plant antioxidant for scientific research as well as industrial (dietary, pharmaceutical and cosmetics) purposes. The antioxidants reactions involve multiple steps including the initiation, propagation, branching and termination of free radicals. Free radicals are created when cells use oxygen to generate energy. These by-products are generally reactive oxygen species (ROS) such as super oxide anion, hydroxyl radical and hydrogen peroxide that result from the cellular redox process. At low or moderate concentrations, ROS exert beneficial effects on cellular responses and immune function but at high levels, free radicals and oxidants generate oxidative stress, a deleterious process that can damage cell structures, including lipids, proteins, and DNA[5]. Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular and neurodegenerative diseases[6]. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced *in situ*, or externally supplied through foods. These antioxidants act as free radical scavengers by preventing and repairing damages caused by ROS, and therefore can enhance the immune defense and lower the risk of cancer and other degenerative diseases[5]. In the continuation of this strategy of new drug discovery, we have studied the plant *Phyllanthus amarus* Schum. & Thonn. for their antioxidant activity and phenol content.

Phyllanthus amarus (Schum & Thonn) is an herb belonging to the family Euphorbiaceae and is commonly used in India and are found in other countries including China, Philippines, Cuba, Nigeria and Guam[7]. It is traditionally used in the treatment of malaria-related symptoms, jaundice, constipation, diabetes, flu, kidney ailments, and chronic dysentery, frequent menstruation, ringworm, ulcers, genito-urinary tract infections, haemorrhoids, and gonorrhoea, hepatic and urolitic diseases. It is reported to possess antimalarial[8], antiviral[l9], antiplasmodial[10], antibacterial[11], anti-inflammatory[12] and antiamnesic activity[13]. It has been employed for treatment of nervous debility, epilepsy and dropsy[14, 15].

An easy, rapid and sensitive method for the antioxidant screening of plant extracts is free radical scavenging assay using 1,1-dipheny l-2-picryl hydrazyl (DPPH) stable radical spectrophotometrically[16]. In the presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases[17]. In particular, despite widespread use of this plant as medicines all over the globe, the literature contains few reports of antioxidant activity[15]. In present study, we carried out a systematic record of the relative free radical scavenging activity of both *in vivo* and *in vitro* models of *Phyllanthus amarus* Schum. & Thonn., using different plant extractions. We have also found the relationship of phenol contents with antioxidant activity.

MATERIAL AND METHODS

Chemical used

All the chemicals and growth regulators used are of high analytical grade. 2, 2-Diphenyle-2-picryl hydrazyl (DPPH) from Sigma Aldrich Co., St. Louis, USA. All solutions were prepared in freshly prepared doubled distilled water. Stock solutions of the test extracts were prepared in methanol, ethanol and Petroleum ether. Appropriate blanks were used for individual assays.

Plant Material

The plant samples i.e. *Phyllanthus amarus* Schum. and Thonn. were collected from the Botanical Garden, University of Rajasthan, Jaipur. Fresh plant was rinsed severally with clean tap water to make it dust and debris free. Then they were spread evenly and dried in the shady condition for 5 to 6 days, until they become crispy while still retaining the green coloration. Dried plants were ground in electric blender to get fine powder for further use.

Callus induction

The young internodes were picked up and kept under running water for about 15-20 minutes to remove soil particles and then rinsed with liquid detergent (Teepol 1%) (v/v)) for 5-10 minutes. They were then rinsed with sterile double distilled water at least thrice to

get rid of teepol. Prior to inoculation, these explants were subsequently surface sterilized in the laminar air flow chamber with 0.1% mercuric chloride ($HgCl_2$ w/v) for 2 minutes, followed by repeated rinsing with sterile double distilled water. The surfaced sterilized explants were finally aseptically inoculated on sterile MS medium[18] comprising 3% sucrose as carbon source and 0.8% agar as solidifying agent. The medium was also supplemented with various growth regulators, which include auxinsLike, 2,4-D, NAA, IAA, IBA in different concentrations(0.2-4.0mg/l). The pH of the media was adjusted to 5.8 before autoclaving. All media were autoclaved at 1.06 kg cm^{-2} and 121ºC for 15 min. The cultures were incubated in growth room at temperature of 25 ± 2 °C, relative humidity 55 ± 5%, and 16-h photoperiod. 20 replicate cultures were established and each experiment was repeated twice and the cultures were observed at regular intervals.

Plant extraction Preparation

The dried and powdered plant samples of *Phyllanthus amarus* Schum. & Thonn (*in vivo*) and callus (*in vitro*) (each 50g) were extracted successively with ethanol, methanol and Petroleum ether (each 400ml.) for 25-26 hrs.,using a Soxhlet apparatus. Then collected solutions were filtered through Whatman No-1 filer paper. The extracts were evaporated to dryness under reduced pressure at 90˚C by Rotary vacuum evaporator to obtain the respective extracts and stored in a freeze condition at −18˚C until used for further analysis.

Phenolic Estimation

To estimate the total phenolic content of plant extracts the protocol of Bray[19] was followed, wherein the standard curve of different concentrations of Catechol was prepared.

500mg of test sample was taken and ground in 10- times volume of 80% ethanol. The homogenate Centrifuged at 10,000 rpm for 20 min., supernatant was saved. Re- extracted the residue with 50-times the volume of 80% ethanol, centrifuged and supernatant was pooled. The supernatant was then evaporated to dryness. After that the residue was dissolved in a known volume of distilled water (5 ml). Pipetted out aliquots (0.2ml) into the test tubes. Made up the volume in each tube to 3ml with water. 0.5 ml of Folin- Ciocalteu reagent was added. After 3 min, 2 ml of 20% Na_2CO_3 solution was also added to each tube. The tube was placed in boiling water for exactly one minute, cooled and measured the absorbance at 650nm against a reagent blank. A standard curve was prepared using different concentration of Catechol.

Antioxidant activity (DPPH free radical scavenging activity) of Different extracts

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples[20]. DPPH is a stable nitrogen centered free radical, the color of which changes from violet to yellow upon reduction either by the process of hydrogen- or electron-donation[21]. Specific compounds or extracts are allowed to react with the stable radical, DPPH•, in methanol solution. In the presence of hydrogen donors, DPPH• is reduced and a stable free radical is formed from the scavenger. The reaction of DPPH• is monitored by the decrease of the absorbance of its radical at 517 nm, but upon reduction by an antioxidant, the absorption disappears[22].

DPPH• + antioxidant ® DPPH-H + antioxidant•

Purple color Yellow color

The antioxidant activity of the plant extracts and the standard was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity by modified method[23].The diluted working solutions of the test extracts were prepared in methanol. Ascorbic acid was used as standard in 1-1000 µg/ml solution and the same concentrations were prepared of the test solutions. 0.002% of DPPH was prepared in methanol and 1 ml of this solution was mixed with 1 ml of sample solution and standard solution separately. Experiment was done in triplicate. These solution mixtures were kept in dark for 30 minutes and optical density was measured at 517 nm using Cecil-Elect Spectrophotometer. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. Methanol (1 ml) with DPPH solution (0.002%, 1 ml) was used as blank. The optical density was recorded and the inhibitory effect of DPPH was calculated according to the following formula[24].

Inhibition (%) = [(Absorbance control –Absorbance sample)/ Absorbance control] × 100

IC_{50} represents the level where 50% of the radicals were scavenged by test samples.

RESULT AND DISCUSSIONS

Callus induction

MS medium supplemented with different concentrations of auxins like 2, 4- D, NAA, IAA, IBA was used for callus induction. The internodal explants showed maximum callus formation on 2, 4- D at 0.6mg/l (Table 1, Fig. 1,2). This gave rise to Green nodulated, fast growing compact callus. Callus obtained from 2, 4- D (0.6mg/l) was further evaluated for its antioxidant activity. Similarly, only auxin 2, 4-D was also used for the induction of normal callus in *Rauvofia serpentina Benth. ex Kurz* as reported by Pant and Joshi[25]. Whereas, in contrast to this, Philomania [26], reported that, for callus induction the combination of two auxins (2,4-D and Kn) was used in plant *Sapindus mukorossi* Gaertn.

Crude extraction content

The significant variation in the yields of *Phyllanthus amarus* Schum. and Thonn. extracts was shown using various fraction of solvents. The yield of extracts of plant using methanol, ethanol, and petroleum ether for *in vivo* samples was 6.96, 5.35, 4.63 respectively. Whereas, for *in vitro* samples it was 6.42, 5.13, 4.25 respectively (Table 2). The variation in yield may be due to the polarity of the solvents used in the extraction process.

Phenol Content

The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity[27,28]. They exhibited antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals[29,30]. The total phenol content in the tissues varied from 195-215 (mg/100gm) respectively**.** The screening of all plant samples revealed that the amount of phenols was higher in the methanol sample as compared to all other plant extraction samples (Table2). The maximum amount of phenols was observed in *in vitro* samples while; the lower amount was observed in *in vivo* samples.

DPPH Assay

DPPH radical is commonly used as a Substrate to evaluate antioxidant activity; it is a stable free radicals that could accept an electron or hydrogen radical to become a stable molecule[31]. The reduction of DPPH radical was determined by the decrease in its absorbance induced by antioxidant at 517nm. Concentration of sample at which the inhibition percentage reaches 50% is its IC_{50} value. IC_{50} value is negatively related to the antioxidant activity, as it expresses the amount of antioxidant needed to decrease its radical concentration by 50%. The lower the IC_{50} value, the higher is the antioxidant activity of the tested sample. In the present study, different extracts of *Phyllanthus amarus* Schum.and Thonn showed potential free-radical scavenging activity. The antioxidant activities of the individual compound, present in the extracts may depend on structural factors, such as the number of phenolic, hydroxyl or methoxyl groups, flavone hydroxyl, keto groups, free carboxylic groups and other structural features[32,33]. It has been shown that the scavenging effect on the DPPH radical increases sharply with the increasing concentration of the sample and standard to a certain extent[34].and hence are strongly dependent on the extract concentration[35]. During the present study, result showed that methanolic extract of *in vitro* grown *P.amarus* sample exhibited significant activity with lowest concentration in IC_{50} (Fig. 3- 8). The IC_{50} value ranged from 55-200 (µg/mg).Ascorbic acid was used as standard with IC_{50} value of 57 µg/mg.

Similarly, the use of ascorbic acid as a standard to determine the IC_{50} value of the extract was found to be use in many plant species like *Psilanthus travancorensis* (Wt. & Arn.) Leroy [36], *Ocimum canum, Ocimum adscendens, Thymus vulgaris and Leucas linifolia* [37] respectively.

There is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidants. The different antioxidant activities of phenolic extracts rich in phenolic compounds can be attributed to different extracting solvent as the antioxidant activity depends on the type and polarity of the extracting solvent, the isolation procedures, the purity of active compounds, as well as the test system[38,39]. In the present experiment, the order of total phenol content was recorded in the order of *In vitro* methanol> *in vivo* methanol> *In vitro* ethanol > *in vivo* ethanol> *In vitro* Petroleum ether >*in vivo* Petroleum ether and for the significant antioxidant activity, the same order was observed followed i.e., *In vitro* methanol> *in vivo* methanol> *In vitro* ethanol > *in vivo* ethanol> *In vitro* Petroleum ether >*in vivo* Petroleum ether (Fig. 9).

In the present study it is observed that the *in vitro* plant extracts exhibit the higher amount of phenol content in comparison to their respective *in vivo* extracts. The total phenol content in the *in vitro* methanol extract showed highest amount of phenol content *i.e.* 212.67 mg/100gm followed by *in vivo* sample of the same extract *i.e. 2*10.45 mg/100gm. Much higher antioxidant activity of the alcoholic preparation have given evident assumption, is more useful than the aqueous one in medical approach[40]. High percent of yield and high value of phenol content in methanolic extracts show that phenolic constituents must be responsible for such properties[41]. The finding is in-agreement with the data of[42,43]. A positive correlation between total phenolic content and antioxidant activity may be present in experiment. The correlation between total phenolic content and antioxidant capacity in the plant samples is possible owing to the presence of following factors, that is may be due to the presence of phenolic compounds or polyphenols or flavonoids or tannins[44,45].

Table 1: Percentage of the callus induction from *Phyllanthus amarus* Schum. & Thonn. under different levels of auxins after 15 days of culture.

Auxin concentration (mg/l)	Response	Remarks
2,4-D		
0.2	C^{+}	Green nodulated, Fast growing compact callus.
0.4	C^{+}	
0.6	C^{+++}	
0.8	C^{++}	
1.0	C^{++}	
2.0	C^{+}	
4.0	C^{+}	
NAA		
0.2	C^{+}	
0.4	C^{+}	Very light green compact callus with moderate growth.
0.6	C^{++}	
0.8	C^{++}	
1.0	C^{++}	
2.0	C^{+}	
4.0	C^{-}	
IAA		
0.2	C^{++}	
0.4	C^{+}	
0.6	C^{+}	Light green friable callus which later turned compact and dark on further subculture.
0.8	C^{+}	
1.0-4.0	C^{-}	
IBA		
0.2-4.0	C^{+}	Pale, creamish yellow callus with very slight growth.

C: Callusing response; C^{-}: No callusing response; C^{+}: Slighting callusing; C^{++}: Moderate callusing; C^{+++}: Maximum callusing

Table 2: Crude extract, Phenol content and IC_{50}% of different solvent of *Phyllanthus amarus* Schum. & Thonn.

Solvent used		Crude extract (gm)	Phenol content (mg/100gm)	IC $_{50}$ %
Methanol	*In vivo*	6.96	210.45	47.88
	In vitro	6.42	212.67	43.39
Ethanol	*In vivo*	5.35	205.32	83.46
	In vitro	5.13	208.09	52.38
Pet Ether	*In vivo*	4.63	199.98	132.46
	In vitro	4.25	203.54	116.82
Standard (ascorbic acid)				57

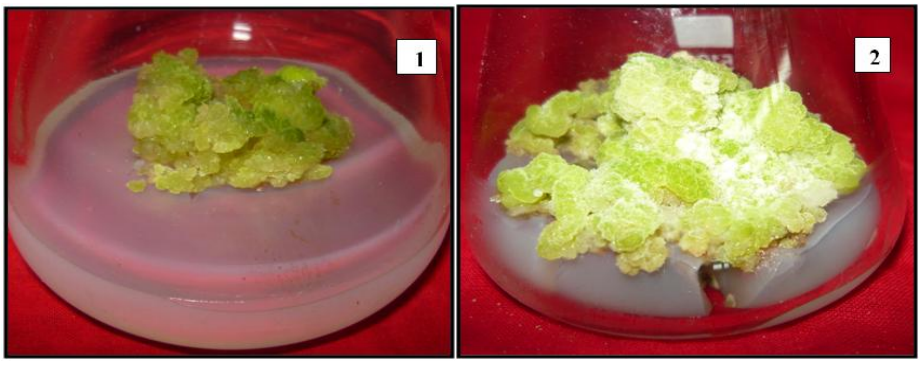

Fig. 1,2 : Callus formation from internodal explant on MS+ 2,4-D (0.6 mg/l) after 1 and 4 weeks, respectively.

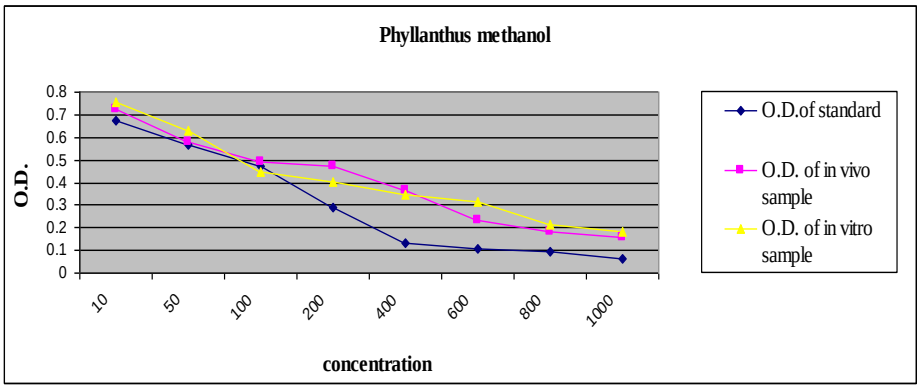

Fig. 3: DPPH scavenging Assay of the Methanol extract of *Phyllanthus amarus* Schum. and Thonn. compared with standard ascorbic acid.

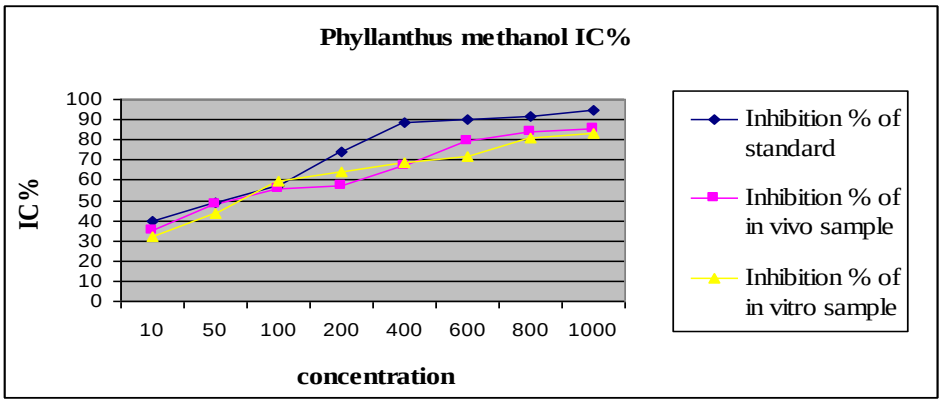

Fig. 4: Evaluation of IC $_{50}$ of the Methanol of extract of *Phyllanthus amarus* Schum. and Thonn. compared with standard ascorbic acid.

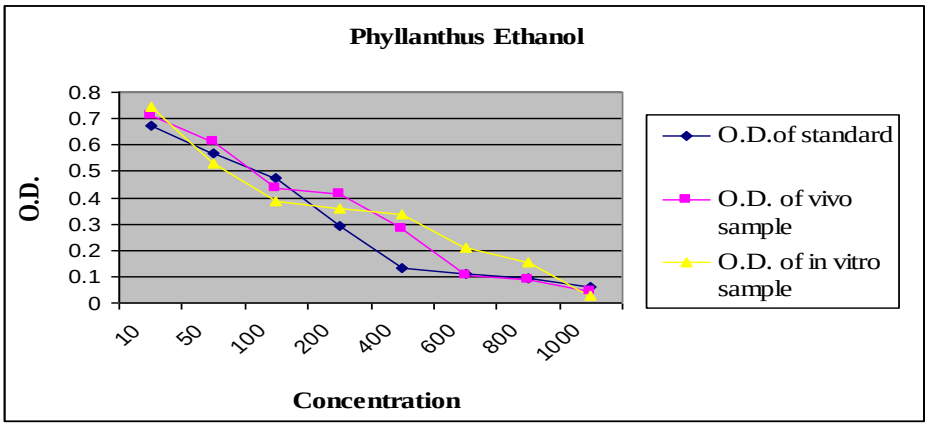

Fig. 5: DPPH scavenging Assay of the Ethanol extract of *Phyllanthus amarus* Schum. and Thonn. compared with standard ascorbic acid.

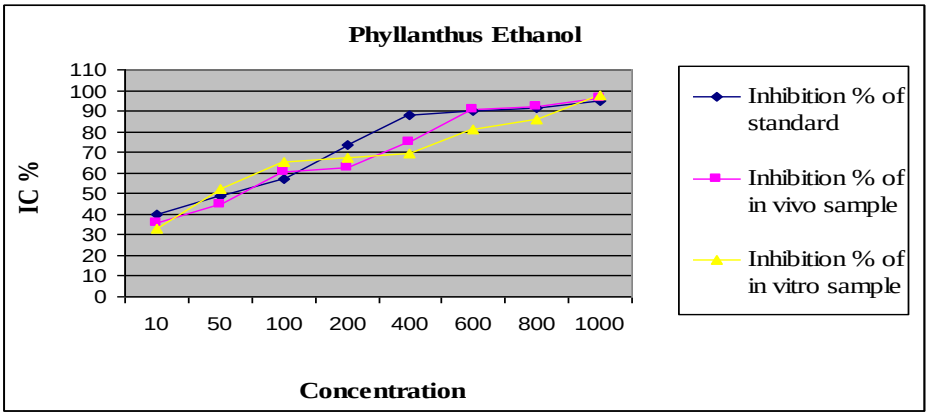

Fig. 6: Evaluation of IC $_{50}$ of the Ethanol extract of *Phyllanthus amarus* Schum. and Thonn. compared with standard ascorbic acid.

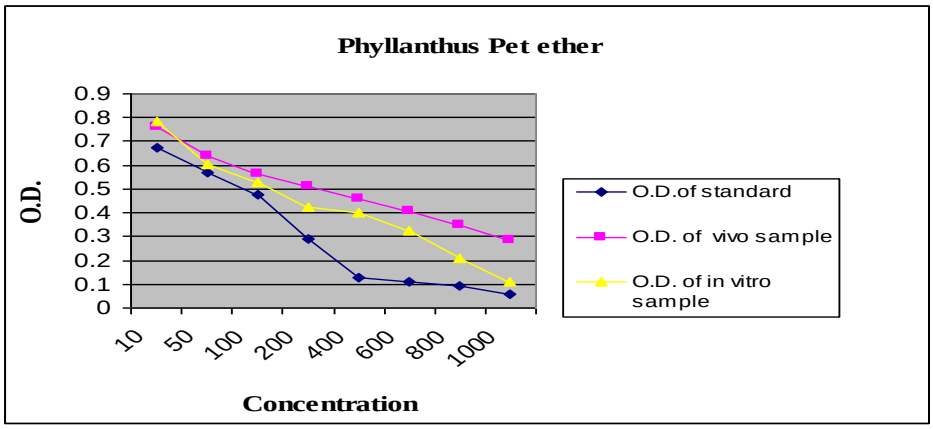

Fig. 7: DPPH scavenging Assay of the Petroleum Ether extract of *Phyllanthus amarus* Schum. and Thonn. compared with standard ascorbic acid.

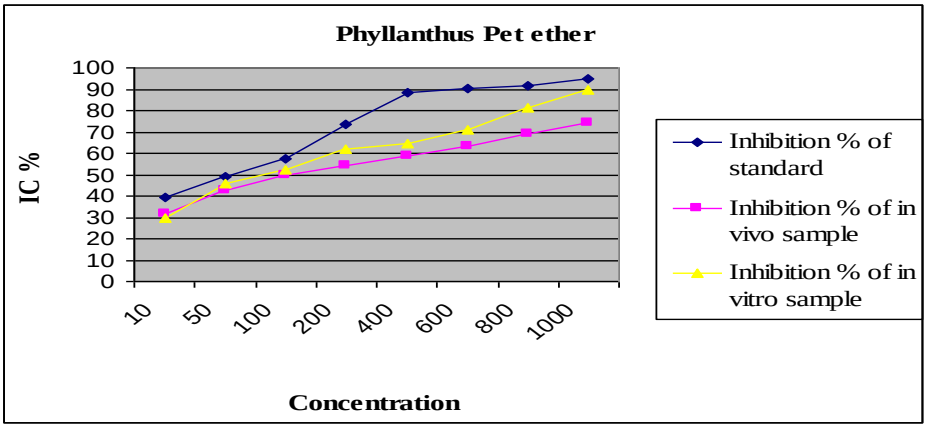

Fig. 8: Evaluation of IC $_{50}$ of Petroleum ether extract of *Phyllanthus amarus* Schum. and Thonn. compared with standard ascorbic acid.

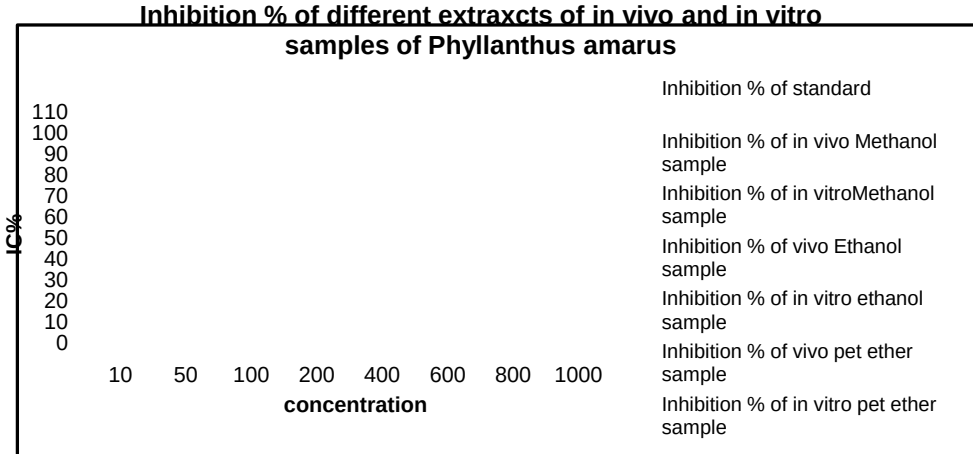

Fig. 9: Combined evaluation of *in vitro* and *in vivo* samples of antioxidant activity of *Phyllanthus amarus* Schum. and Thonn. with different plant extractions using ascorbic acid as standard.

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