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Evaluation of Anti-oxidant potential of root, stem and leaf extracts of *Phyllanthus amarus.*

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ABSTRACT

Present study was aimed for evaluation of free radical scavenging activity of root, stem and leaf extracts of *Phyllanthus amarus* by DPPH and Nitric oxide assays, where ascorbic acid is used as standard. Various polar and non-polar solvents were used for extraction and for determination of anti-oxidant potential of root, stem and leaves of *Phyllanthus amarus.* In both DPPH and NOS assays, dimethyl formamide which is a polar solvent exhibited greater anti-oxidant activity compared to other solvents. The anti-oxidant activity for inhibiting free radicals is expressed in terms of $IC_{50,}$ which was calculated using the concentration *vs* activity curve .The IC_{50} values of various parts of *Phyllanthus amarus* using different solvents ranged from 25.2-123.7 µg/ml in DPPH radical scavenging activity and from 79.74-458.5 µg/ml in Nitric oxide scavenging activity. IC_{50}value of leaf extracts are lower than stem and root extracts which indicates the high potentiality of leaf compared to root and stem in its anti-oxidant properties which may be due to the presence of phenols and flavonoids. Based on IC_{50}values, DPPH assay was found to have better results compared to NOS assay. At 10 µg/ml concentration, dimethyl formamide leaf extracts exhibited inhibitory percentage of 50.75% in DPPH assay and 33.24% in NOS assay.

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Background:

Herbal medicine is used for primary health care by about 80% of people belonging to Asia and Africa is the estimation given by World Health Organization (WHO). Phyto therapy is considered as alternative medicine to Western medicine. Herbal usage is universal for disease treatment mostly in non-industrialized societies [17, 3, 7] as they are affordable in contrast to modern pharmaceuticals which are expensive. Medicinal plants gained recognition in research of present times as they protect human body from damage of free radicals. Medicinal plants contain blend of chemical compounds which may operate singly or in combination to heal diseases and for improving health as they are a good source of anti-oxidants [1]. A wide variety of anti-oxidants naturally occur in nature which differs in composition and properties such as enzymes, vitamins, minerals etc. [13]. Antioxidant activity is not restricted to specific plant part or specific families.

Antioxidants remove free radical intermediates and terminate chain reactions in cell thereby inhibiting other oxidation reactions preventing cell damage. Oxidative stress is caused by low levels of antioxidants or antioxidant enzymes which damages cell structure and function thereby causing many human diseases, including cancers. Natural antioxidants such as fruits, vegetables and plants decrease the effects of aging and promote health. Plants and animals contain large amounts of antioxidants counteracting cell damage and repairing damaged cells. [6].Medicinal plants contain compounds like phenols, flavonoids, and tannins which

act as natural anti-oxidants and help in disease protection. As there is a thirst for novel natural anti-oxidants of plant origin, presence of high level of anti-oxidants in medicinal plants provide enormous scope for therapeutic approach of pharmaceuticals products .Hence present study is aimed to assess anti-oxidant potential of an important medicinal plant *Phyllanthus amarus* using various solvents in root, stem and leaf extracts.

Materials and Methods:

Plant collection: The plants of *Phyllanthus amarus* were collected from fields of Andhra Pradesh, India.

Extract preparation:

Fresh plant material was collected, washed under running tap water and stem, root and leaf parts were separated. They were shade dried to eliminate moisture and powdered. They were stored in air tight bottles for further use in solvent extraction. The solvents used for the present study are Ethylacetate, Di-methyl Formamide, Di-Chloromethane, Chloroform and n-Hexane .For sample preparation, 1gm powder of all plant parts (root, stem, leaves) were extracted in different solvents and were placed on a rotary shaker for 24 h at 190-220 rpm. The filtrates were concentrated using a rota vapour (IKA,Germany) under reduced pressure .

Estimation of anti oxidants in the given plant sample by using ascorbic acid:

Preparation of standard solution:

Asorbic acid was used as standard for estimating the anti-oxidant content. 100 µg of ascorbic acid was dissolved in 1ml of methanol and was taken as standard to get 0.1mg/ml solution. Required quantity of Ascorbic acid was dissolved in methanol to give the concentration of 5, 10, 20, 30, 40 and 50 µg/ml.

DPPH assay:

DPPH method was used for determination of free radical scavenging activity in plant extracts by the procedure of Brand-William and Mensor [14, 4].

Preparation of DPPH solution:

Stock solution was made by dissolving 4.3mg of DPPH (2, 2'diphenyl-1-picrylhydrazyl) in 3.3 ml methanol. It was protected from light by covering with aluminum foil and stored at 20^0C until required.

Estimation of free radical scavenging activity by calibration curve:

Different aliquots of ascorbic acid were taken from the above stock solution in a series of test tubes as 10 µl, 20 µl, 30 µl, 40 µl and 50 µl. The volume was made up to 3 ml with methanol and mixed well. 150 µl DPPH solution was added to each test tube. Blank was prepared by taking DPPH solution and methanol without ascorbic acid. Absorbance was measured after 15 min at 516 nm using UV/VIS spectrophotometer (Shimadzu, UV-1700, Japan). A graph was plotted by taking the optical density values on Y-axis and concentration of Ascorbic acid on X-axis.

Preparation of test sample solution:

10mg of the dried plant extract was dissolved in10 ml of methanol to give concentration of 1mg/ml. Different volumes of test sample were taken in series of test tubes as 10 µl, 50 µl, 100 µl, 200 µl, 300 µl and 500 µl and each tube was made up to 3.0 ml with methanol, followed by addition of 150 µl of DPPH and incubated for 15 min. By comparing the results of measured absorbance at 516nm with standard curve of ascorbic acid, percentage of radical scavenging activity was calculated in root, stem and leaf extracts of *Phyllanthus amarus*.

Statistical analysis:

The percentage of inhibition was calculated by formula and a graph was plotted in comparison to standard. IC_{50} values are defined as the concentration of sample required to inhibit 50 % of the DPPH radical. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of percentage inhibition against sample concentration.

The free radical scavenging activity (FRSA) (% antiradical activity) was calculated using the following equation:

$$\% \text{ antiradical activity} = \frac{\text{Control absorbance-Sample absorbance}}{\text{Control absorbance}} \times 100$$

Calculation:

Test Concentration=O.D of Test/Dilution Factor

Dilution Factor= O.D of Standard / Concentration of Standard.

Nitric Oxide scavenging activity:

NO^+ is generated in biological samples by specific nitric oxide synthases, which metabolizes arginine to citrulline with the formation of NO^+ ***via*** a five electron oxidative reaction [11, 8, 2, 16, 21]. The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO^+. Under aerobic conditions, NO^+ reacts with oxygen to produce stable products (nitrate and nitrite), the quantities of which can be determined using Sulfanilic acid reagent [15].

Reagents:

1. Sodium nitroprusside (100mM)
2. Phosphate buffered saline (pH 7.4)
3. Sulfanilic acid reagent (33 gr of chemical in 20% glacial acetic acid)
4. Naphthylethylene diamine dihydrochloride (0.1% w/v).

Procedure:

0.5 ml of phosphate buffer saline (pH 7.4) was mixed with plant extracts at various concentrations (root, leaf & stem) and the final volume was made upto 2 ml with 10 mM sodium nitroprusside and the mixture was incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added with 1.0 ml sulfanilic acid reagent and incubated at room temperature for 5 min. Finally, 1.0 ml naphthylethylene diamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 546 nm against the reagent blank, in a spectrophotometer. The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylene diamine dihydrochloride was measured at 546 nm. The nitrite generated in the presence or absence of the plant extract was estimated using the same procedure was done with ascorbic acid as standard in comparison to extracts.

Statistical analysis:

The percentage of inhibition was calculated by formula and a graph was plotted in comparison to standard. IC_{50} values are defined as the concentration of sample required to inhibit 50 % of the nitric oxide radical. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of percentage inhibition against sample concentration.

The free radical scavenging activity (FRSA) (% antiradical activity) was calculated using the following equation:

$$\% \text{ antiradical activity} = \frac{\text{Control absorbance-Sample absorbance}}{\text{Control absorbance}} \times 100$$

Calculation:

Test Concentration = O.D of Test/Dilution Factor

Dilution Factor = O.D of Standard / Concentration of Standard.

Results and Discussion:

In both DPPH and NOS assays, radical scavenging activity was found to be highest for leaf extracts compared to root and stem extracts. Among leaf extracts, dimethyl formamide, which is a polar solvent, was found to have excellent radical scavenging activity compared to non-polar solvents that are used. Comparative graphs of DPPH free radical scavenging activity of root, stem and leaf extracts of *Phyllanthus amarus* using different solvents with respect to ascorbic acid were shown in figures 1, 2, 3 and they were shown in figures 4,5,6 respectively for NOS activity.

There was a report on antioxidant activity of leaf extracts of *Pimpinella tirupatiensis* which were evaluated by DPPH (1, 1-diphenyl-2-picryl-hydrazyl), FRAP (Ferric Reducing Ability of Plasma), Nitric oxide scavenging, and Reducing power assays where there was a correlation of phenol and flavonoid content with antioxidant activity [10] .Similar type of studies have been reported in *Torilis leptophilla*, where cytoprotective activity has been studied *in vitro* and *in vivo* to find novel antioxidants for development of food and pharmaceutical industries [18] .*Salacia oblonga* was studied for its phytochemical and free radical scavenging activity, where the phytochemicals of this plant were found to be responsible for antiproliferative activity against breast cancer cell lines (MDA-MB-231) [12]. Prakash Veeru *et al*., scanned various medicinal plants for their antioxidant activity using ascorbic acid as standard for knowing their beneficial effects in drug formulation. [9].Our reports are in corroboration with the reports of s. s .sravanthi pammi *et al.,*where *Phyllanthus amarus* leaf extracts exhibited better antimicrobial activity in dimethyl formamide solvent[19] and this may be due to the detection of maximum percentage of phenols and flavonoids in dimethylformamide solvent in comparison to other solvents[20].

Table: 1. DPPH free radical Scavenging activity of root extracts of *Phyllanthus amarus*

Conc (µg/ml)	EtAc % inhibition	DMF % inhibition	Chloroform % inhibition	Dichloromethane % inhibition	n-hexane % inhibition	Ascorbic acid
10	30.99	42.25	38.03	36.62	33.80	60.91
50	46.48	47.89	43.66	43.66	40.85	70.91
100	53.52	57.75	53.52	49.30	46.48	75.45
200	63.38	61.97	60.56	57.75	56.34	82.73
300	69.01	67.61	70.42	63.38	60.56	90.91
500	73.24	73.24	74.65	69.01	71.83	92.73
IC_{50} (µg/ml)	**87.44**	**77.42**	**88.35**	**110.9**	**123.7**	**8.42**

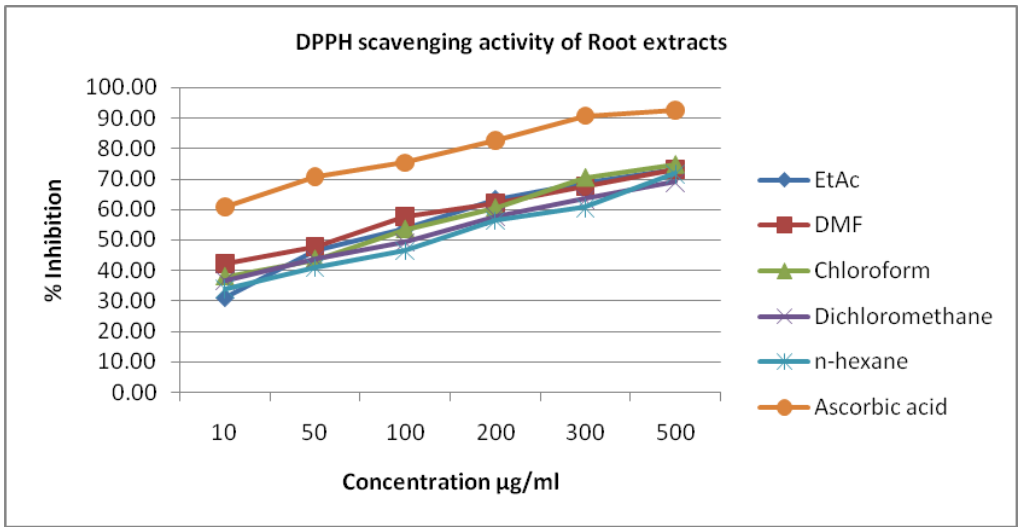

Fig. 1: Comparative graph of DPPH free radical scavenging activity of root extracts of *Phyllanthus amarus* using different solvents

Table: 2. DPPH free radical Scavenging activity of stem extracts of *Phyllanthus amarus*

Conc (µg/ml)	EtAc % inhibition	DMF % inhibition	Chloroform % inhibition	Dichloromethane % inhibition	n-hexane % inhibition	Ascorbic acid
10	44.93	49.28	40.58	47.83	36.23	60.91
50	56.52	63.77	49.28	59.42	42.03	70.91
100	69.57	72.46	57.97	69.57	53.62	75.45
200	75.36	75.36	73.91	73.91	60.87	82.73
300	78.26	79.71	76.81	76.81	69.57	90.91
500	84.06	82.61	78.26	79.71	73.91	92.73
IC_{50} (µg/ml)	**36.22**	**26.3**	**57.53**	**31.26**	**92.02**	**8.42**

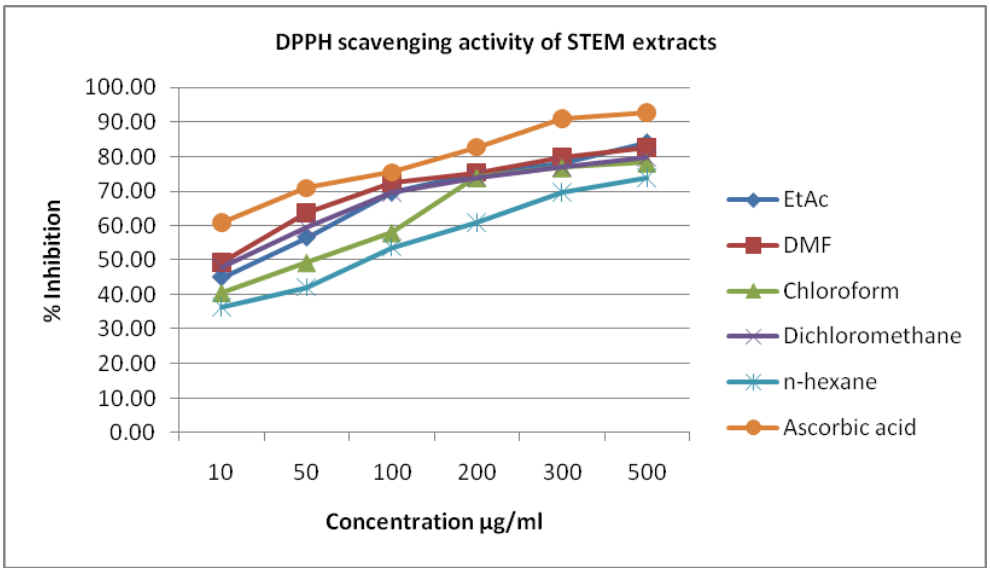

Fig. 2: Comparative graph of DPPH free radical scavenging activity of stem extracts of *Phyllanthus amarus* using different solvents

Table: 3. DPPH free radical Scavenging activity of leaf extracts of *Phyllanthus amarus*

Conc (µg/ml)	EtAc % inhibition	DMF % inhibition	Chloroform % inhibition	Dichloromethane % inhibition	n-hexane % inhibition	Ascorbic acid
10	34.33	50.75	43.28	49.25	40.30	60.91
50	52.24	64.18	61.19	53.73	52.24	70.91
100	61.19	70.15	68.66	62.69	65.67	75.45
200	64.18	73.13	76.12	70.15	73.13	82.73
300	70.15	85.07	82.09	77.61	85.07	90.91
500	76.12	86.57	88.06	85.07	89.55	92.73
IC_{50} (µg/ml)	**66.78**	**25.2**	**31.92**	**43.95**	**42.27**	**8.42**

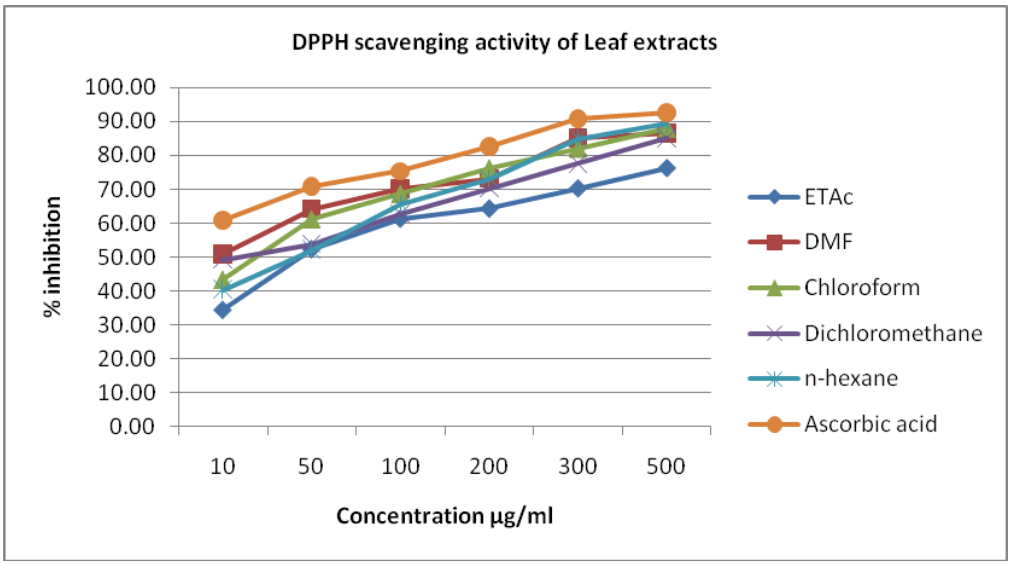

Figure 3: Comparative graph of DPPH free radical scavenging activity of leaf extracts of *Phyllanthus amarus* using different solvents.

Table: 4. Nitric oxide free radical scavenging activity of root extracts of *Phyllanthus amarus*

Conc (µg/ml)	EtAc % inhibition	DMF % inhibition	Chloroform % inhibition	Dichloromethane % inhibition	n-hexane % inhibition	Ascorbic acid
10	6.86	8.09	8.78	13.58	19.07	33.78
50	13.85	20.44	10.84	27.30	30.04	40.51
100	17.70	42.39	18.38	42.39	36.90	48.37
200	23.87	54.73	35.94	47.87	43.76	56.22
300	38.27	61.59	47.60	58.85	50.62	66.32
500	57.20	68.45	58.71	69.82	58.85	68.57
IC_{50} (µg/ml)	**458.50**	**174**	**357.3**	**178.90**	**235.20**	**116.90**

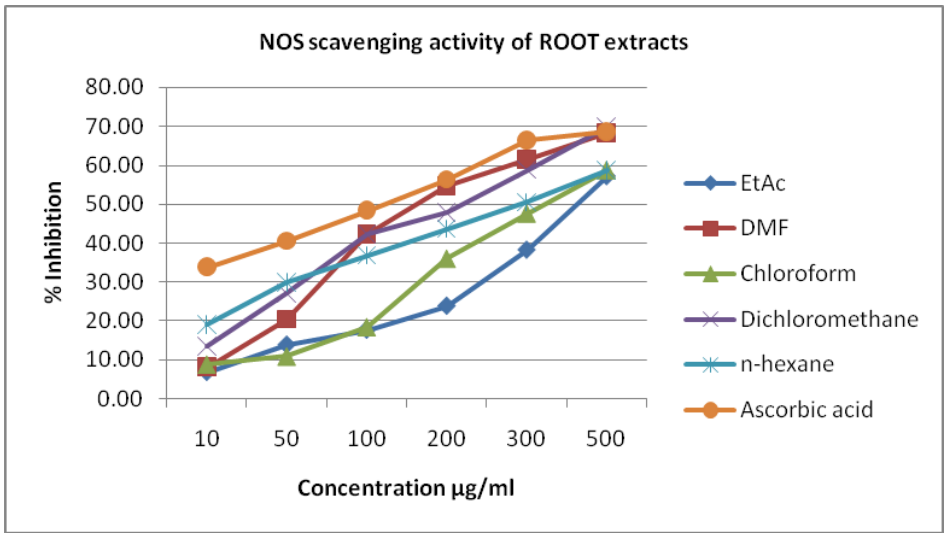

Fig. 4: Comparative graph of Nitric oxide free radical scavenging activity of root extracts of *Phyllanthus amarus* using different solvents.

Table: 5. Nitric oxide free radical scavenging activity of stem extracts of *Phyllanthus amarus*

Conc (µg/ml)	EtAc % inhibition	DMF % inhibition	Chloroform % inhibition	Dichloromethane % inhibition	n-hexane % inhibition	Ascorbic acid
10	22.46	22.46	19.79	18.45	23.40	33.78
50	37.17	43.85	27.81	31.82	35.83	40.51
100	49.20	55.88	42.51	42.51	47.86	48.37
200	58.56	63.90	54.55	54.55	59.89	56.22
300	63.90	67.91	71.93	69.25	61.23	66.32
500	73.26	71.93	75.94	73.26	70.59	68.57
IC_{50} (µg/ml)	**120.20**	**92.74**	**135.60**	**137.60**	**127.7**	**116.90**

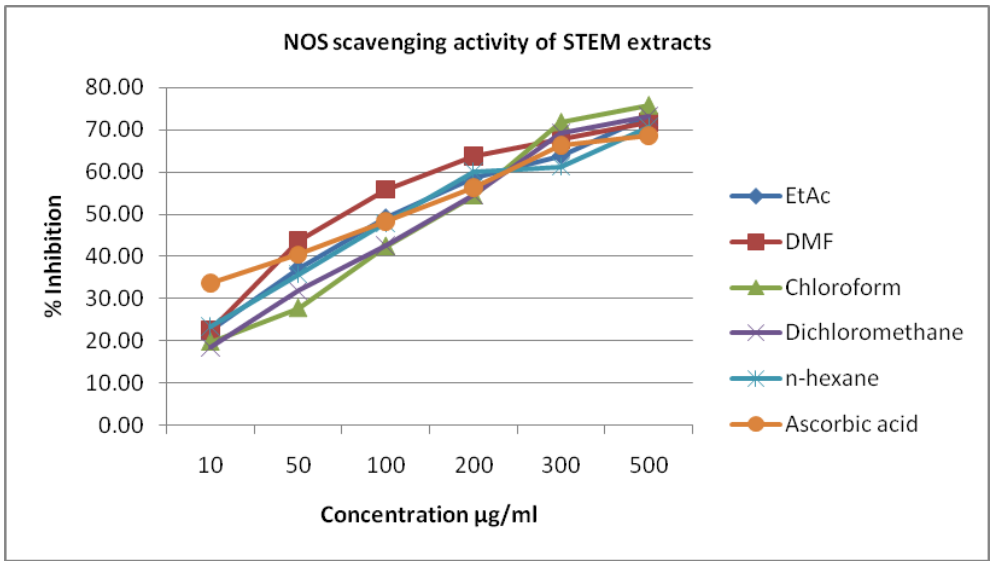

Fig. 5: Comparative graph of Nitric oxide free radical scavenging activity of stem extracts of *Phyllanthus amarus* using different solvents.

Table: 6. Nitric oxide free radical scavenging activity of leaf extracts of *Phyllanthus amarus*

Conc (µg/ml)	EtAc % inhibition	DMF % inhibition	Chloroform % inhibition	Dichloromethane % inhibition	n-hexane % inhibition	Ascorbic acid
10	40.05	33.24	44.14	38.69	35.97	33.78
50	45.50	48.23	49.59	45.50	42.78	40.51
100	55.04	55.04	59.13	50.95	48.23	48.37
200	61.85	64.58	63.22	59.13	57.77	56.22
300	71.39	70.03	68.66	64.58	61.85	66.32
500	75.48	74.11	74.11	70.03	72.75	68.57
IC_{50} (µg/ml)	**80.44**	**79.74**	**81.97**	**100.50**	**112.40**	**116.90**

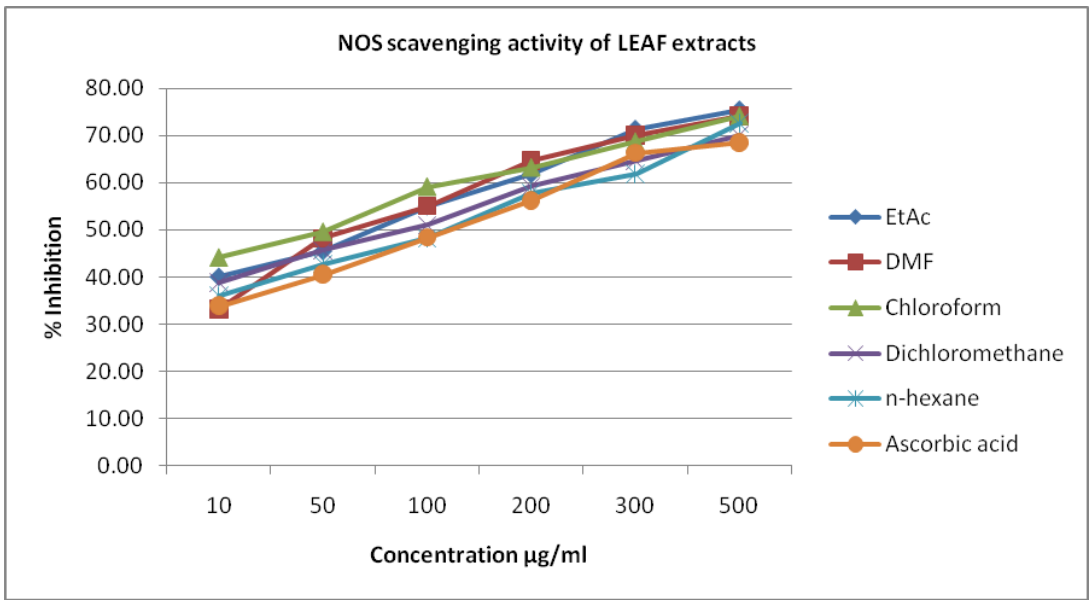

Fig. 6: Comparative graph of Nitric oxide free radical scavenging activity of leaf extracts of *Phyllanthus amarus* using different solvents.

The percentages of inhibition in all the extracts for both the assays were also shown with respect to ascorbic acid and found to be increased gradually with the increase of concentration. The standard IC_{50} Value for ascorbic acid in DPPH and NOS assays were found to be 8.42 µg/ml and 116.90 µg/ml respectively.

Among leaf extracts, in DPPH assay, dimethyl formamide solvent exhibited highest radical scavenging activity by inhibiting DPPH radical significantly with lowest IC_{50} Value of 25.2 µg/ml and ethyl acetate solvent exhibited lowest radical scavenging activity with highest IC_{50} Value of 66.78 µg/ml (Table 3). In NOS assay, dimethyl formamide leaf extract exhibited highest radical scavenging activity by inhibiting NOS radical significantly with lowest IC_{50} Value of 79.74 µg/ml and n-hexane leaf extract exhibited lowest radical scavenging activity with highest IC_{50} Value of 112.40 µg/ml (Table 6).Comparative study of percentage of radical scavenging activity of leaf extracts of *Phyllanthus amarus* in DPPH and NOS assays at various concentrations were shown in figure 7.This indicates that DPPH is a better assay than NOS assay in testing the antioxidant potential of *Phyllanthus amarus*.

Among stem extracts, in DPPH assay, dimethyl formamide solvent exhibited highest radical scavenging activity by inhibiting DPPH radical significantly with lowest IC_{50} Value of 26.3 µg/ml and n-hexane solvent exhibited lowest radical scavenging activity with highest IC_{50} Value of 92.02 µg/ml (Table 2). In NOS assay, dimethyl formamide solvent exhibited highest radical scavenging activity by inhibiting NOS radical significantly with lowest IC_{50} Value of 92.74 µg/ml and dichloromethane solvent exhibited lowest radical scavenging activity with highest IC_{50} Value of 137.6 µg/ml (Table 5).

Among root extracts, in DPPH assay, dimethyl formamide solvent exhibited highest radical scavenging activity by inhibiting DPPH radical significantly with

lowest IC $_{50}$ Value of 77.42 µg/ml and n-hexane solvent exhibited lowest radical scavenging activity with highest IC $_{50}$ Value of 123.7 µg/ml (Table 1). In NOS assay, dimethyl formamide solvent exhibited highest radical scavenging activity by inhibiting NOS radical significantly with lowest IC $_{50}$ Value of 174 µg/ml and ethyl acetate solvent exhibited lowest radical scavenging activity with highest IC $_{50}$ Value of 458.5 µg/ml (Table 4).These results proved the radical scavenging activity of *leaf >stem>leaf.* The solvent used and plant part that is taken influences the extent of inhibition. The occurrence of phenols and flavonoids may be the main reason for excellent anti-oxidant activity in this plant .The amount of phenols and flavonoids in various plant parts (root, stem and leaf) may be the reason for differential radical scavenging activity among plant parts of *Phyllanthus amarus* .Sensitivity of DPPH to light and spectrophotometric sensitivity are the main factors which influence accuracy of present method [5].

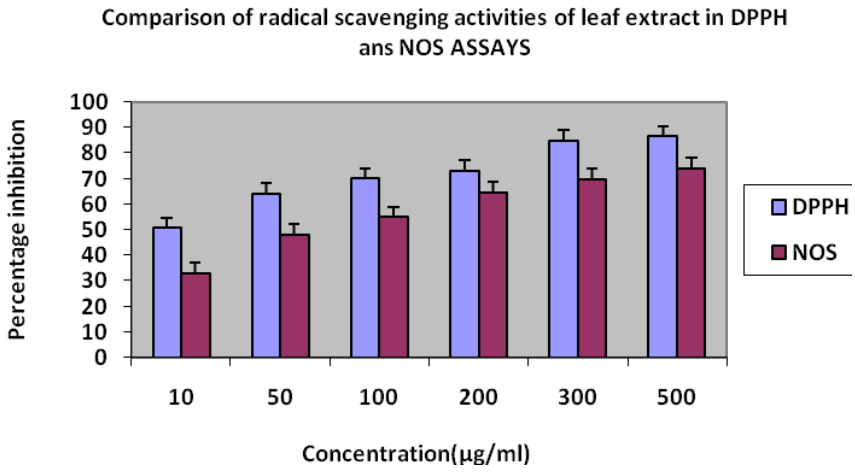

Fig. 7: Comparative study of percentage of radical scavenging activity of leaf extracts of *Phyllanthus amarus* in dimethyl formamide solvent through DPPH and NOS assays at various concentrations.

Conclusion:

This study suggests that many bioactive compounds showing good antioxidant activity were found in plant extracts of *Phyllanthus amarus*. Many of these extracts exhibited considerable antioxidant activity which is comparable to standard ascorbic acid. A range of oxidative stress induced degenerative diseases may be prevented using these plant extracts for the advancement of innovative and more effective natural antioxidants which provides a platform for pharmaceutical industries and researchers for development of valuable medicines.

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