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PROXIMATE, PHYTOCHEMICAL AND *INVITRO* ANTIOXIDANTS ANALYSIS OF *PHYLLANTHUS AMARUS*

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ABSTRACT

ROS are chemically reactive molecules containing oxygen have important roles in cell signalling and homeostasis. It also causes damage of DNA, lipid peroxidation, oxidation of amino acids in proteins, oxidatively inactivate specific enzymes by oxidation of co-factors. Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system which has the ability to readily detoxify the reactive intermediates or to repair the resulting damage. An antioxidant is a molecule that inhibits the oxidation of other molecule. Substances that can reduce the damage by free radicals are known as antioxidants. Types of antioxidants include enzymatic antioxidants and non-enzymatic antioxidants. Many of these phytochemicals have beneficial effects on long-term health when consumed by humans, and can be used effectively to treat diseases. Phyllanthus amarus is one such medicinal plant enriched with antioxidants and macronutrients like iron, calcium, phosphorus, magnesium and copper. Primary metabolites like total carbohydrates, proteins, amino acids, and cellulose and secondary metabolites like phenol were present. Enzymatic antioxidants, like SOD, CAT, NO and non enzymatic like Ascorbic acid, carotenoids were found. Being enriched with macronutrients and primary metabolites along with antioxidants this plant could be used for herbal nutraceuticals.

KEY WORDS: Antioxidants, Macronutrients, micronutrients, ROS.

ABBREVIATIONS: SOD: Super oxide dismutase, CAT: Catalase, NO : nitricoxide, DPPH: 1, 1-diphenyl- 2-picrylhydrazyl, BHA: butylated hydroxyl anisole, BHT: Butylated hydroxyl toluene. DNA: Deoxy ribonucleic acid, H_2O_2: Hydrogen peroxide, SO: Super Oxide.

INTRODUCTION

Antioxidants are the substances that can reduce the damage by free radicals. It includes enzymes and other nutrients such as vitamin C, vitamin E, and beta carotene etc. They are capable of counteracting the damaging effects of oxidation. Antioxidants are also commonly added to food products such as vegetable oils and packed foods to prevent or delay their deterioration from the action of air. Antioxidants may possibly reduce the risks of cancer. Antioxidants clearly slow the progression of age related (macular degeneration) disorders.

Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend themselves against attack from predators such as insects, fungi and herbivorous mammals. Many of these phytochemicals have beneficial effects on long-term health when consumed by humans, and can be used to effectively treat diseases. At least 12,000 such compounds have been isolated. A number estimated to be less than 10% of the total. Chemical compounds in plants mediate their effects on the human body through process identical to those already well understood for the chemical compounds in conventional drugs, thus herbal medicines do not differ greatly from conventional drugs. This enables herbal medicines to be as effective as conventional medicines[1].

Phyllanthus amarus: This medicinal plant is used to treat many diseases. The leaves are used to treat liver disorders particularly Hepatitis B, Jaundice, Intestinal infection and diabetes etc. The major compounds isolated from leaves are hypophyllanthin and phyllanthin. In India it is used to cure appetizer, Asthma, Bronchial infections, diuretics, dyspepsia, fever, jaundice, liver diseases, skin ulcers, sores and swellings.

MATERIALS AND METHODS

For the present study *Phyllanthus amarus* was selected and procured from Gandhi market, Trichy. Stem and leaves of *Phyllanthus amarus* was air dried, powdered coarsely and soaked in water for 24 hours. This aqueous extract was used for the following analysis. Analysis of macronutrients, *invitro* antioxidant assay and phytochemicals screening was carried out.

Determination of Foreign matter, Total ash, Water Soluble Ash, Acid-Insoluble Ash and Moisture Content by the method suggested by Brain[2]. Preliminary Phytochemical screening of the plant extract was carried out as per the methods and tests given by Dey and Raman[3]. Total Protein was estimated by the method adapted by Lowry[4]. Total carbohydrate was estimated by the anthrone method of Hedge[5]. Total Phenols were estimated by the method of Malick and Singh[6]. The free amino acid present in the plant materials was estimated by the method of Moore[7]. Cellulose was estimated by the method adapted by Updegraff[8]. Carotenoids were estimated by the method of Zakaria[9].

The ascorbic acid present in the plant materials was estimated as described by Sathasivam and Manickam[10]. Iron was estimated by the method adapted by Jaffery[11]. Phosphorous was estimated by Fiske and Subba Rao method[12]. Calcium was estimated by the method described by Summerson[13]. The estimation of Magnesium was done by the method of E.E Ludwig, C.R.Johnson[14]. Copper was estimated by method of C.A.Noll, L.D. Betz[15]. Chlorophylls were estimated by the method of Arnon[16]. DPPH radical scavenging activity was assayed by the method of Gyamfi[17]. Reducing power assayed by the method of Yildrim[18]. H_2O_2 scavenging activity was measured by the method of Ruch[19]. Superoxide radical scavenging activity was determined by the method of Halliwell[20]. Nitric oxide scavenging activity was measured by Sreejayan and Rao[21].

RESULTS

In the present study the physiochemical properties of *Phyllanthus amarus* was determined. The foreign matter present was 2.3%, the moisture content was 8.14 %, the total ash was 6.81%, water soluble ash was 4.76%, and acid insoluble ash was 2.27%.

TABLE 1 TEST FOR IDENTITY, PURITY AND STRENGTH

S.No	Parameters	Value(%)W/W
1.	Foreign matter	2.3
2.	Moisture content	8.14
3.	Total ash	6.81
4.	Water soluble ash	4.76
5.	Acid insoluble ash	2.27

Table 2 Preliminary Phytochemical Analysis of *Phyllanthus Amarus*

S.NO	Description	Observation
1.	Saponin	+ve
2.	Protein	+ve
3.	Tannin	+ve
4.	Sugar	Trace
5.	Quinone	+ve
6.	Coumarins	Trace
7.	Flavonoid	+ve
8.	Sterols	+ve
9.	Terpenes	Trace
10.	Lignin	+ve
11.	Alkaloid	+ve

The preliminary phytochemical analysis of *Phyllanthus amarus* revealed the presence of saponin, protein, tannin, quinone, flavonoid, sterols, lignin and alkaloids whereas coumarin, sugar and terpenes were present in trace amount.

TABLE 3 QUANTITATIVE ANALYSIS OF PRIMARY METABOLITES IN *PHYLLANTHUS AMARUS*

S.NO	Parameters	Amount (mg/100g)
1.	Proteins	14
2.	Carbohydrates	22
3.	Phenols	500
4.	Total free amino acids	60
5.	Cellulose	1920
6.	Vitamin-A	16.4
7.	Vitamin-C	72

The quantitative analysis of organic compounds present in *Phyllanthus amarus* was tabulated in Table 3. The amount of protein present in *Phyllanthus amarus* was found to be 14 mg/100g, carbohydrate was 22mg/100g, phenol was 500 mg/100g, free amino acid was 60 mg/100g, cellulose was 1920 mg/100g. Vitamin-A was 16.4 mg/100g, and Vitamin-C was 72 mg/100g.

TABLE 4 LEVELS OF MINERAL CONSTITUENTS IN *PHYLLANTHUS AMARUS*

S. NO	Minerals	Value (mg/100g)
1.	Iron	12.5
2.	Phosphorous	266.6
3.	Calcium	122.2
4.	Magnesium	86
5.	Copper	5.0

Table 4 shows the mineral content of *Phyllanthus amarus*. The iron content was found to be 12.5mg/100g, phosphorous content was 266.6 mg/100g, calcium content of *Phyllanthus amarus* was 122.2mg/100g, Magnesium was 86mg/100g and copper was found only to be 5mg/100g .

TABLE 5 LEVELS OF PIGMENTS IN *PHYLLANTHUS AMARUS* EXTRACT (MG%)

Description	Chlorophyl a	Chlorophyl b	Carotenoid
Phyllanthus amarus	0.0102	0.0619	16.4

Table 5 shows the levels of chlorophylls and carotenoids. The chlorophyll a in *Phyllanthus amarus* found to be 0.0102 mg %, Chlorophyll b was 0.0619 mg% and the carotenoids was 16.4 mg%.

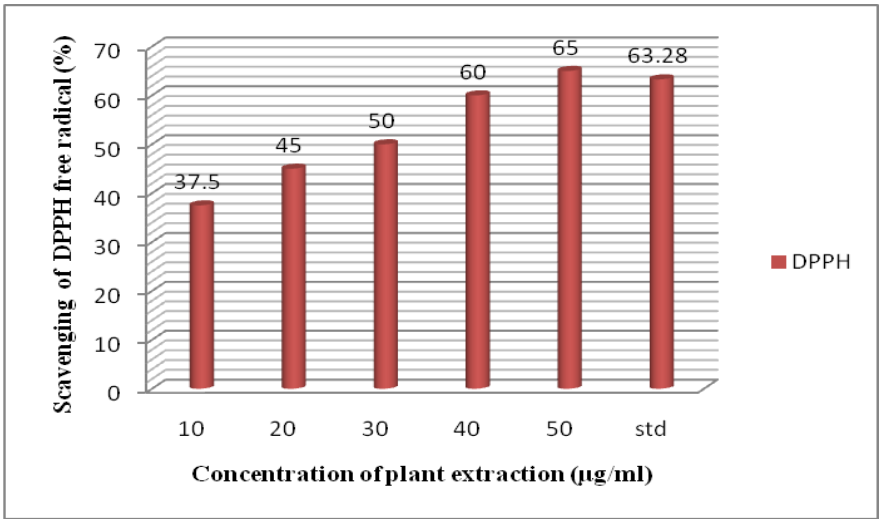

Figure 1 - DPPH Assay Of Aqueous Extract Of *Phyllanthus Amarus*

The DPPH free radical scavenging activity of aqueous extract of *Phyllanthus amarus* was shown in the figure 1. Low concentration (10µg/ml) showed 37.5% of activity whereas at high concentration (50µg/ml) showed 65% of activity and the IC_{50} value was found to be 30µg/ml. The results were comparable with standard ascorbic acid (63.28µg/ml).

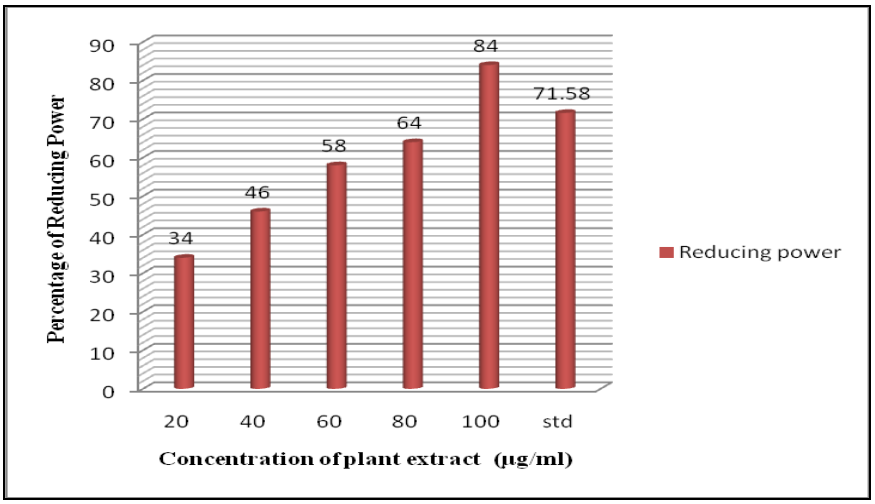

Figure 2- Reducing Power Assay Of Aqueous Extract Of *Phyllanthus Amarus*

The reducing power assay of aqueous extract of *Phyllanthus amarus* was depicted in figure 2. Low concentration (20µg/ml) showed 34% of reducing power while high concentration (100µg/ml) showed 84% of reducing power. IC50 value was found to be 45µg/ml.

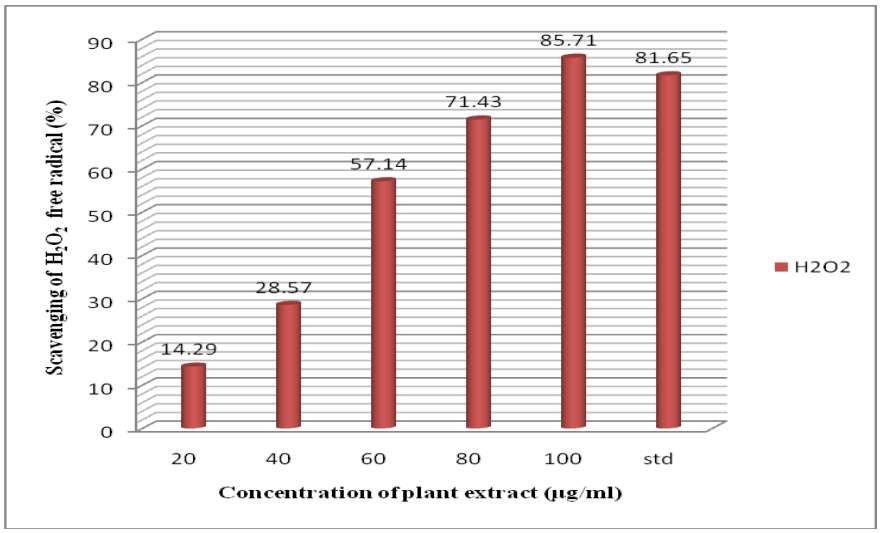

Figure 3 - Hydrogen Peroxide Assay Of Aqueous Extract Of *Phyllanthus Amarus*

The H_2O_2 scavenging potential of aqueous extract of *Phyllanthus amarus* was shown in figure 3. Low concentration of (20µg/ml) extract showed moderate activity whereas at high

concentration the extract showed maximum activity (100µg/ml). The IC_{50} value was found to be 55µg/ml.

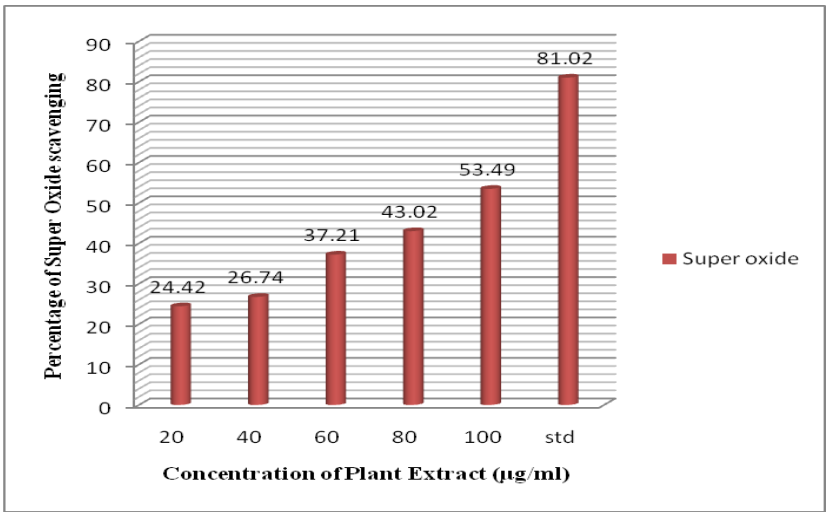

Figure 4 - Superoxide Radical Scavenging Potential Of Aqueous Extract Of *Phyllanthus Amarus*

From the figure 4, it was noticed that the aqueous extract of *Phyllanthus amarus* was capable of inhibiting the superoxide radicals. The high concentration (100µg/ml) showed 53.49% of activity which was comparable to that of standard. The IC_{50} value was found to be 97µg/ml.

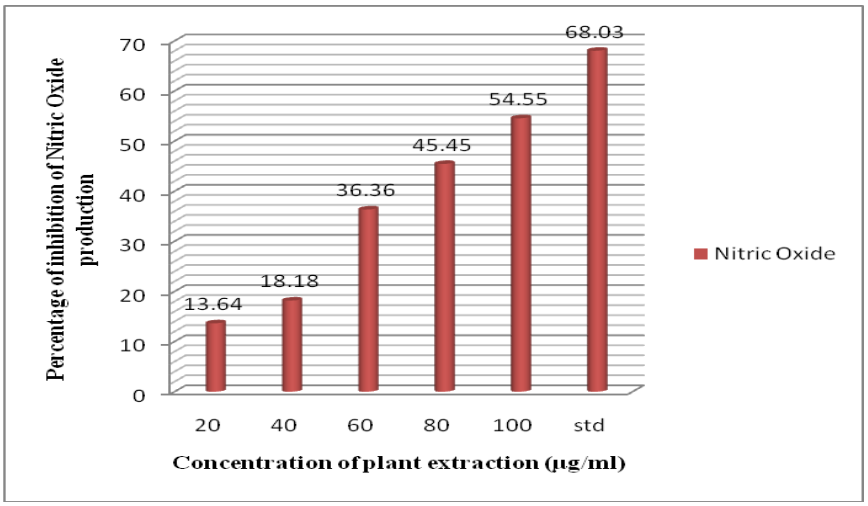

Figure 5 - Nitric Oxide Inhibition Potential Of Aqueous Extract Of *Phyllanthus Amarus*

The NO inhibition potentials of aqueous extract of *Phyllanthus amarus* was depicted in figure 5. The low concentration (20µg/ml) showed 13.64% of activity and high concentration

(100µg/ml) showed 54.55% of NO inhibition potential. The IC_{50} value was found to be 90µg/ml.

DISCUSSION

The use of medicinal plants in the management of various illness is due to their phytochemical constituents and dates back to antiquity[22]. During the last decade, an increase in the use of medicinal plants has been observed in metropolitan areas of developed countries[23].

Dhar[24] reported that many of the active constituents to which the biological activity of *Phyllanthus niruri* has been attributed includes lignans, tannins, coumarins, terpenes, flavonoids, alkaloids, saponins and phenyl propanoids, which have been found in the leaves, stem and roots of this plant. The present study on *Phyllanthus amarus* also revealed the presence of phytoconstituents like saponin, protein, tannin, quinone, flavonoid, sterols, lignin and alkaloids, which were in accordance with their report.

Although, folk history does not have a record of *Phyllanthus amarus* being used as a vegetable, the extract contains a high percentage composition of carbohydrates and crude fibre. This may be the reason why the plant's extract is allegedly used as tonic[25], because of its high and readily available carbohydrate content. The rich amount of carbohydrate, protein, and amino acid present in the plant drug, may be attributed to its nutritional enrichment. The rich cellulose content may be attributed to the pharmacological activity of *Phyllanthus amarus*.

Present data showed that *Phyllanthus amarus* extract possessed high phenolic content and exhibited strong free radical scavenging activity and ferric reducing property. Large quantity of phenolic compounds in *Phyllanthus amarus* extract makes it a strong free radical scavenger, which indicates that *Phyllanthus amarus* extract has good potential as a source for natural antioxidants to prevent free radical mediated oxidative damage.

Presence of minerals such as calcium in the plant can ensure adequate removal of the anti-nutritional factors by the formation of complexes. Complex formation between calcium and oxalate makes more calcium minerals unavailable; however it also ensures excretion of oxalates. Furthermore, steaming or boiling reduces oxalate content of plant extracts to very minimal concentrations[26]. These minerals function, among other areas, in the maintenance of

osmotic pressure and water distribution in the various body fluid compartments. This explains the traditional use of the plant extract in the treatment of oedema, kidney problems and oliguria[27]. Present data showed the presence of macronutrients which are essential for normal body functions for the maintenance of health.

Chlorophyll has been suggested as an effective antioxidant since it scavenges free radicals such as 1, 1-diphenyl- 2-picrylhydrazyl[28]. Carotenes have the ability to detoxify various forms of activated oxygen and triplet chlorophyll that are produced as a result of excitation of the photosynthetic complexes by light. In terms of its antioxidant properties, carotenoids can protect the photo systems in one of four ways – by reacting with lipid peroxidation products to terminate chain reactions or by scavenging singlet oxygen and dissipating the energy as heat or by reacting with triplet or excited chlorophyll molecules to prevent formation of singlet oxygen or by the dissipation of excess excitation energy through the xanthophyll cycle[29]. The free radicals scavenging activity of *Phyllanthus amarus* was due to the presence of pigments like chlorophyll and carotenoids.

The crude extracts of *Datura metel* contain flavonoid, saponins, tannins, phenolics and aromatic compounds. All these bioactive compounds were able to discolour DPPH solution by their hydrogen donating ability[30,31,32,33,34]. From the results it appears that leaves of *Datura* possess hydrogen donating capabilities and it will act as an antioxidant. The antioxidant capacity of *Phyllanthus amarus* could also be due to the presence of secondary metabolites like flavonoid, saponins, tannins and phenolics.

Shankar D. Katekhaye[35] reported that free radical scavenging activity of the methanol and acetone extract of bark and leaves of *Pithecellobium dulce* showed that there was decrease in absorbance of the DPPH radical, which was due to the scavenging of the radical by hydrogen donation. A lower value of IC_{50} indicates a higher antioxidant activity. DPPH radical scavenging activity of each extracts is directly proportional to the concentration of total phenolics including tannins of respective extracts. In the present study the DPPH radical scavenging activity was dose dependent. It was visually noticeable as the color changed from purple to yellow which was due to hydrogen donation. This radical scavenging activity of extracts could be related to the presence of phenolic in the plant extract. The results were in accordance with the above findings.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging. The reducing power assay of aqueous extract of *Phyllanthus amarus* was dose dependent.

Hydrogen peroxide has strong oxidizing properties. It can be formed *in vivo* by many oxidizing enzymes such as superoxide dismutase. It can cross membranes and may slowly oxidize a number of compounds. These results showed that extract had an effective hydrogen peroxide scavenging activity. Hydrogen peroxide itself is not very reactive; however it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells.

Hydrogen peroxide though a weak oxidizing agent is important because of its ability to penetrate biological membranes, once inside the cell it can probably react with Fe^{2+} and Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. Like superoxide anion, all extract showed excellent H_2O_2 scavenging activity. Leaves and stems contain β-sitsterol, stigmasterol, taraxeryl acetate and three cyclopropane compounds and their derivatives responsible for antioxidant activity. The hydrogen peroxide scavenging activity of methanolic extract of stem and leaves of *Hibiscus rosasinensis* was 32.4 ± 0.3, 23.0 ± 0.46 while aqueous extract was 32.9 ± 0.17 and 32.7 ± 0.3 respectively[36]. The H_2O_2 scavenging activity of aqueous extract of *Phyllanthus amarus* was dose dependent and 100µg/ml showed maximum activity which was comparable with standard. The IC_{50} value was found to be 55µg/ml which could be due to bioactive compound present in the plant.
It is well known that nitric oxide has an important role in various inflammatory processes. Sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis. The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO-). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite.

The extract inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. The crude aqueous extract of *Hibiscus rosasinensis* showed a remarkable nitric oxide radical scavenging activity. It is well documented that NO plays a crucial role in the

pathogenesis of inflammation where it is secreted as a mediator, this may explain the use of *Hibiscus rosasinensis* extract for the treatment of inflammatory disease[37,38]. The present study showed that the extract had comparable nitric oxide scavenging activity with the standard ascorbic acid. Nitric oxide inhibition potential of aqueous extract of *Phyllanthus amarus* was dose dependent.

The biological toxicity of superoxide is due to its capacity to inactivate iron–sulfur cluster containing enzymes, which are critical in a wide variety of metabolic pathways, thereby liberating free iron in the cell, which can undergo Fenton chemistry and generate the highly reactive hydroxyl radical. It can also reduce certain iron complex such as cytochrome C. Superoxide anions are a precursor to active free radicals that have potential of reacting with biological macromolecules and thereby inducing tissue damage. It has been implicated in several pathophysiological processes due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation. Superoxide anion plays an important role in the formation of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA.

Rajesh Mandade[38] reported that the crude extract of *Carthamus tinctorius* had inhibited superoxide radical generation by 20 µg/ mL concentration was found as 74.2 ± 3.7 %. On the other hand, at the same concentration, BHA, BHT and α-tocopherol exhibited 76.4 ± 5.3, 72.2 ± 6.4 and 24.1 ± 3.2% superoxide anion radical scavenging activity, respectively. According to their results, *Carthamus tinctorius* had similar superoxide anion radical scavenging activity to BHA and BHT; however, it had higher superoxide anion radical scavenging activity than α-tocopherol. The results of the present study suggested that *Phyllanthus amarus* are good scavenger of superoxide radical. The IC_{50} value was found to be 97µg/ml the antioxidant properties which might be due to flavonoids which are effective mainly via scavenging of superoxide anion radical.

CONCLUSION

The present study was concluded that *Phyllanthus amarus* showed rich potent antioxidant effect in a dose dependent manner with high content of primary metabolites like cellulose, phenolics and appreciable amount of minerals such as iron, phosphorus, calcium and magnesium were observed in the plant which could be the cause of its interesting therapeutic functions. Further studies are essential to treat the herbal drug with preclinical and clinical

trials which would result in cost effective nutraceutical with possessing natural antioxidants. This could help to ameliorate deleterious life threatening disorders.

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