Research Article

IN-VITRO ANTIOXIDANT ACTIVITY OF ISOLATED TANNINS OF ALCOHOLIC AND AQUEOUS EXTRACTS OF DRIED LEAVES OF *PHYLLANTHUS AMARUSSCHONN AND THONN*

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ABSTRACT

Tannins are the most abundant antioxidants in the human diet and they exhibit many biologically important functions which include protection against oxidative stress and degenerative diseases. The stems, infusion of leaves and roots of *Phyllanthus* species are used in folk medicine for treating intestinal infections, diabetes, the hepatitis B virus and disturbances of the kidney and urinary bladder and it has antioxidants activity. The purpose of the present study was to isolate the tannins from alcoholic and aqueous extract of *Phyllanthus amarus* and to evaluate the *in–vitro* antioxidant activity of isolated tannins. Tannins were isolated from both the extracts of *Phyllanthus amarus* leaves by lead acetate method. Both the extracts were used to evaluate the presence /absence of various phytoconstituents and to find out its *in-vitro* antioxidant activity by various models. Comparative antioxidant potential of tannins isolated from both the extracts of *Phyllanthus amarus* leaves were evaluated for the various *in-vitro* antioxidant activity models like *in-vitro* anti-lipid peroxidation activity, nitric oxide, superoxide scavenging, DPPH (1,1-diphenyl-2-picryl-hydrazyl) scavenging activity were evaluated against standard antioxidants such as Curcumin and ascorbic acid were used. Isolated tannins from both the extracts of *Phyllanthus amarus* showed significant *in-vitro* antioxidant activity in all the test systems. Isolated tannins from the alcoholic extract of *Phyllanthus amarus* showed significant *in-vitro* antioxidant activity in all the test systems. The tannins isolated from alcoholic extract of *Phyllanthus amarus* showed good *in-vitro* antioxidant activity for all the antioxidant assays against the tannins isolated from aqueous extract of *Phyllanthus amarus*. Tannins isolated from alcoholic and aqueous extracts of *Phyllanthus aramus* showed significant antioxidant activity in all the test systems. Thus the results indicate that the tannins isolated from alcoholic and aqueous extracts of *Phyllanthus amarus* are a potential source of natural antioxidants.

Keywords: Anti-lipid peroxidation, anti-oxidant activity, DPPH, Superoxide radical, *Phyllanthus amarus.*

INTRODUCTION

Tannins are naturally occurring, high molecular weight polyphenols which can be divided into hydrolysable tannins and condensed tannins. Tannins are the most abundant antioxidants in the human diet and they exhibit many biologically important functions which include protection against oxidative stress and degenerative diseases. The tannin compounds are widely distributed in many species of plants, where they play a role in protection from predation, and perhaps also as pesticides, and in plant growth regulation.

Phyllanthus has been used in Ayurvedic medicine for over 2000 years and has a wide number of traditional uses. The World Health Organization has compiled more than 20 000 medicinal plants used in different parts of the world[1]. Among the medicinal plants more than one hundred botanicals have larger potential for commercial exploitation and could be marketed in the world drug markets[2]. *Phyllanthusamarus*, commonly known as Keelanelli (Tamil), Bhuiaonla (Hindi) belonging to the family Euphorbiaceae occupies a prime position among the commercially cultivated medicinal plants. It has a long history of folk use in drug industry for the treatment of dropsy, urogenital problem, dysentery, diabetes, skin ulcer, dyspepsia, fever, asthma, bronchial infections, tumours and Hepatitis B virus. *Phyllanthus amarus* is believed to possess anti-diabetic, anti-nociceptive, antioxidant, antiseptic, antiviral, contraceptive, diuretic, hypotensive and stomachic properties. It is used according in many countries. Herbal medicines are widely perceived by the public as being natural, healthful and free from side effects[3]. *Phyllanthus amarus* Schum & Thonn is an important medicinal plant species due to its antiviral properties, and thus useful against hepatitis infection. The plant is also highly valuable in the Ayurvedic system of medicine[4]. *Phyllanthus amarus* is a rich source of phytochemicals such as alkaloids, astragalin, brevifolin, carboxylic acids, corilagin, cymene, ellagic acid, ellagitannins, gallocatechins, geraniin, hypophyllanthin, phyllanthin, lignans, lintetralins, lupeols, methyl salicylate, phyllanthine, phyllanthenol, phyllochrysine, phyltetralin, repandusinic acids, quercetin, quercetol, quercitrin, rutin, saponins, triacontanal and tricontanol[5]. The tannin compounds are widely distributed in many species of plants, where they play a role in protection from predation, and perhaps also as pesticides, and in plant growth regulation. All of these chemical components have their useful as well as toxic effects. Hence safety of herbal medicines is still an issue worldwide. Based on growing attention in free radical biology and the lack of efficient therapies for most chronic diseases, the utility of antioxidants in fortification against neurodegenerative diseases is warranted. Oxidation is essential to many living organisms for production of energy to fuel biologics. Oxidative stress

occurs in a cellular system when the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the system. Oxidative stress plays an important contributory role in the process of aging and pathogenesis of numerous diseases like diabetes, cancer, neurodegenerative diseases, and respiratory tract disorders. Improved antioxidant status helps to minimize the oxidative damage, and thus can delay or decrease the risk for developing many chronic age related, free radical induced diseases[6]. Almost all organisms are well protected against free radical damage by antioxidant enzymes such as superoxide radical dismutase (SOD), catalase or chemical compounds such as ascorbic acid polyphenols and glutathione but uncontrolled production of reactive oxygen species (ROS) is responsible for several pathophysiological processes[7]. Tannins were found to be the major phytoconstituents in individual alcoholic extract of *Phyllanthus amarus.* Hence isolation of tannins from this extract was undertaken in order to check whether they possess any *in-vitro* antioxidant activity or not. Majority of disease condition like atherosclerosis, hypertension, ischemic disease, Alzheimer disease, parkinsonism, cancer diabetes mellitus, and inflammatory conditions are being considered to be preliminary due to imbalance between prooxidant and antioxidant homeostasis[8]. Stress, smoking, drugs and diet generates excessive free radicals in the human body. However there are defence mechanisms, which scavenge the free radicals and protect the body from attack of free radicals. These free radicals are important factors for several pathological conditions such as cardiovascular diseases, inflammation, atherosclerosis and degenerative diseases[9]. Antioxidant acts as a major defence against radical – mediated toxicity by protecting the damages caused by free radicals[10]. In the present study, antioxidant properties of *Phyllanthus amarus* including its reducing power. The antioxidant may reconcile its effect by unswervingly reacting with ROS, quenching them or chelating the catalytic metal ions. Certain plants show antioxidant activity because of their phenolic constituents [(11)]. Flavonoids are a broad class of secondary metabolites widely distributed in plants. Flavonoids basically have the antioxidant and the chelating abilities. Numerous synthetic antioxidants e.g. BHA and BHT are commercially available but are pretty perilous and noxious, restricting its use in foods. Therefore the importance of searching natural antioxidants has significantly increased in the current years. The present study was undertaken to investigate the *in-vitro* antioxidant activity of isolated tannins of leaves of *Phyllanthus amarus.*

MATERIALS AND METHODS

Standards and reagents

Ascorbic acid, Potassium ferricyanide, ferrous chloride, ferric chloride, NBT, DPPH, PMS, napthyl ethylene diamine dihydrochloride, TCA, NADH, TBA, were obtained from sigma. All other chemicals and solvents are of analytical grade.

Sample collection and extraction

All the leaves of *Phyllanthus amarus* Schonn and Thonn were procured from the local market of Mumbai and authenticated at Nicholus Piramal Pvt.Ltd. India. The leaves were shade-dried, coarsely powdered using a cutter mill and stored in an air-tight, light resistant container for further use. Air–dried leaves of *Phyllanthus amarus* were defatted with Pet.ether (60-80°C) by using Soxhlet extraction apparatus for 48 hours. All the solvents were removed from the Pet.Ether extracts under reduced pressure. All the defatted leaves were air dried and then used individually for alcoholic and aqueous extraction. Individual alcoholic extraction was carried out by using Soxhlet extraction apparatus for 48 hours. Individual aqueous extraction was carried out by using reflux extraction apparatus for 8 hours. All the extracts were removed by using reduced pressure. All the extracts were freeze dried and stored in air tight container. Tannins were found to be the major phytoconstituents in individual alcoholic and aqueous extracts of *Phyllanthus amarus*. Hence isolation of tannins from this extracts were undertaken in order to check whether they possess any *in - vitro* antioxidant activity or not.

Isolation of Tannins

1g of individual alcoholic and aqueous extract of *Phyllanthus amarus* were shaken with (100ml) of ethanol on mechanical shaker for 30 min. The solution of extract was treated with lead acetate solution (10%) to precipitate tannins as a lead tannate. The suspension was centrifuged for 10 minute to allow the solvent to separate out. The supernatant was decanted off and the precipitate was washed with distilled water. The water was removed by centrifugation followed by decantation. The precipitate was suspended in ethanol and hydrogen sulphide gas was passed through it to remove excess of lead. The lead sulphide precipitate formed was filtered off and the resultant light yellowish coloured filtrate was evaporated to dryness on the water bath to constant weight. The total tannin content extract were determined.

Preliminary phytochemical evaluation

All the extracts means aqueous and alcoholic extracts of *Phyllanthus amarus* were evaluated qualitatively for the presence or absence of various phytoconstituents[12].

Assay of *in-vitro* antioxidant activity

In-vitro antioxidant activity by DPPH method

To determine the *In-vitro* antioxidant activity of isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus*, a method based on reduction of methanolic solution of colored free radical DPPH was used. Hydrogen atom or electron donating abilities of corresponding extracts like alcoholic and aqueous extracts of *Phyllanthus amarus* and pure compound (synthetic compound like ascorbic acid) were measured from the bleaching of purple colored methanol solution

of DPPH. This spectroscopic assay uses the stable radical DPPH as a reagent. The decrease in absorbance of DPPH is proportional to concentration of free radical scavenger added to DPPH reagent solution. Absorbance was taken at 517nm and ascorbic acid was used as a standard solution.[13] The percent inhibition activity was calculated as $(A_0 - A_1)/A0*100$, where A0 is the absorbance without sample. And A_1 was the absorbance with sample.

$$\text{DPPH Scavenged (\%)} = \frac{(A_O - A_1)}{(A_O)} \times 100$$

Where, A_O = Absorbance of control reaction

A_1 = Absorbance of test reaction

Assay of Nitric Oxide Scavenging Activity

Griess reagent containing 1% sulphanilamide, 2% Phosphoric acid and 0.1% Napthyl-ethylene diaminedihydrochloride. Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction. Sodium nitroprusside in aqueous solution, at physiological pH spontaneously generate nitric oxide, which interact with oxygen to produce nitrite ions that can be estimated by use of the Griess reagent. Scavengers of nitric oxide (plant extracts of tannins) compete with oxygen leading to reduced production of nitric oxide. The absorbance of chromophore created during diazotization of nitrite with sulphanilamide following coupling with naphthyl ethylene diaminedihydrochloride was read at 540 nm and compared to the absorbance of standard. The decrease in absorbance of Griess reagent at its absorption maximum of 540nm is proportional to the concentration of free radical scavenger added to the Griess reagent solution[14]. Ascorbic acid was used as a standard solution. (NO radical scavenging activity was calculated by using following equation:

$$\text{NO Scavenged (\%)} = \frac{(A_{cont.} - A_{test})}{(A_{cont.})} \times 100$$

Where, $A_{cont.}$ = Absorbance of control reaction

A_{test} = Absorbance of test reaction

Lipid peroxidation method

Mice liver homogenate was used to check the inhibition of lipid Peroxidation. During the aerobic incubation of tissue homogenate with *in-vitro* free radicals generated by ferrous ascorbate system, malondialdehyde was formed which on reaction with thiobarbituric acid produces a pink color. Formation of this thiobarbituric acid reacting substance (TBARS) was monitored at 532 nm. Curcumin was used as a reference standard. The method was based on the formation of malondialdehyde during lipid peroxidation. The content of malondialdehydes was estimated as thiobarbituric acid reacting substances (TBARs) by monitoring their colour intensity at 532 nm.

Materials: (AR Grade) 0.15 M Potassium chloride Tries buffer Ferrous sulphate (10 µM) Ascorbic acid (100 µM) Thiobarbituric acid reagent (TBA).

Method: The liver was quickly removed and chilled in ice-cold saline. After washing with 0.9 % ice-cold saline, the liver was homogenised in 0.15 M Potassium chloride to get 10% (w/v) liver homogenate. Fresh liver homogenate (0.2ml) was mixed with 0.15 mM Potassium chloride (0.1ml) and Tries buffer (0.4 ml). The test extracts were then added in various concentrations. *In-vitro* lipid peroxidation was initiated by addition ferrous sulphate (10 µM) and ascorbic acid (100 µM), 0.1 ml each. After incubation for 150 min at 37°C reaction was terminated by addition 2ml of ice cold 0.25N, HCL containing 15% TCA, 0.38% TBA and 0.2 ml of 0.05% (BHT) butylated hydroxyl toluene. These reaction mixtures were heated for 60 min at 80°C. They were also cooled and centrifuged at 5000 rpm for 15mins. The absorbance of the supernatant was measured at 532nm in all reagent except liver homogenate and drug. Identical experiments were performed to determine the normal (without drug and ferric chloride) and induced (without drug) lipid peroxidation. Curcumin was used as a reference standard[15].

$$\text{\% ALP} = \frac{\text{Ferric chloride O.D. – Sample O.Dample O.D}}{\text{Ferric Chloride O.D. – Normal O.D}}$$

Assay of Superoxide Radical Scavenging Activity

The effect of superoxide radical production was evaluated using nitroblue tetrazolium reduction method (Nishikimi *et al.*, 1972). The reaction mixture consisted of 1ml NBT solution (156 µM), 1ml NADH (468µM) and 1ml of sample extracts. The reaction was started by adding 100 µl. Phenazinemethosulphate (60 µM PMS) in phosphate buffer pH 7.4) to the reaction mixture. The reaction was incubated at 25°C for 5 min. and the absorbance was measured at 590 nm was measured against blank. Decreased absorbance of reaction mixture indicates increased superoxide anion scavenging activity. Mixture without sample was used as a control and mixture without PMS was used as a blank. The scavenging activity was calculated as follows:[16]

$$\text{Scavenging activity (\%)} = \frac{\text{1- Abs of sample –Abs of Blank}}{\text{Abs of Control}}$$

Hydroxyl radical scavenging activity

Hydroxyl radical (OH^-) are generally form Fe^{2+} -ascorbate, EDTA – H_2O_2 system (Fenton's reaction) which attack the deoxyribose and set off a series of reaction that eventually results in the formation of malondialdehyde (MDA), measured as a pink MDA-TBA chromogen at 535nm. Reaction mixture (1ml) contained deoxyribose (2.8 mM), KH_2PO_4-KOH (20mM) pH 7.4, $FeCl_3$ (100mM), EDTA (104 µM), H_2O_2 (1mM) and ascorbate (100µM) reaction mixture was incubated at 37°C for 1 hour and colored developed as degradation by the isolated tannins

from alcoholic and aqueous extracts of *Phyllanthus amarus* over the control was measured[16]. Curcumin was used as a positive control[17]. The % inhibition was measured by the formula. The OH^- radical scavenging activity of extract is reported as % inhibition of deoxyribose degradation and is calculated as:

$$OH^- \text{ Scavenged (\%)} = \frac{(A_{cont.} - A_{test})}{(A_{cont.})} \times 100$$

Where, $A_{cont.}$ = Absorbance of control reaction

A_{test} = Absorbance of test reaction

Determination of H_2O_2 radical scavenging activity

A solution of H_2O_2 was prepared in PBS (pH 7.4). H_2O_2 concentration was determined spectrophotometrically, by measuring absorption with extinction coefficient for H_2O_2 of 81 $m^{-1}cm^{-1}$. Extracts (100-500 µg/ml) in distilled water were added to H_2O_2 solution (0.6 ml, 40 mM). Absorbance of H_2O_2 at 230 nm was determined 10 min later against a blank solution containing PBS without H_2O_2[18]. The percentage of H_2O_2 scavenging of both the extracts and standard compounds were calculated as follows:

$$H_2O_2 \text{ Scavenged (\%)} = \frac{(A_{cont.} - A_{test})}{(A_{cont.})} \times 100$$

Where, $A_{cont.}$ = Absorbance of control reaction

A_{test} = Absorbance of test reaction

Statistical analysis

The results are presented as Mean ± SEM one –way analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons, was used for Statistical evaluation. The p-value less than were considered as significant.

RESULTS

From the preliminary phytochemical evaluation (table 1) it was proved that isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus* leaves has Alkaloids as confirmed by using the Mayer's reagent and Dragendorff's reagent. Glycoside was found in both the extracts of tannins of *Phyllanthus amarus* by Fehlings and Legal test. Flavonoids (in alcoholic and aqueous extract). Fixed oils were also presents (in alcoholic and aqueous extracts). Carbohydrates were found in aqueous and alcoholic extracts. Amino-acids were found in alcoholic and aqueous extracts. Lignan like Phyllanthin and Hypophyllanthin were found in alcoholic and aqueous extracts of *Phyllanthus amarus.* Steroids and phenols were found in alcohol and aqueous extracts of *Phyllanthus amarus* by performing various tests.

DPPH radical scavenging activity

DPPH radical scavenging activities of isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus* were presented in Fig 1. Results showed that the radical scavenging activity of isolated tannins of alcoholic and aqueous extract of *Phyllanthus amarus* increased with increasing concentration and were high for aqueous extracts. Aqueous extract of *Phyllanthus amarus* is comparatively less potent than alcoholic extract. Isolated tannins of aqueous extracts of *Phyllanthus amarus* shows IC_{50} 26.34 at a concentration level of 1mg/ml and R^2 was found to be 0.9961. Alcoholic extract of *Phyllanthus amarus* showed IC_{50} 14.35 at a concentration of 0.5mg/ml respectively and R^2 was found to be 0.9946. However Ascorbic acid is used as a standard and its radical scavenging activity was found to be more potent. The IC_{50} Values of standard ascorbic acid was found to be 2.49 at the concentration of 100 µg/ml. Alcoholic extract of *Phyllanthus amarus* was found to be more potent and it has good *in-vitro* antioxidant activity.

Table 1: Evaluation of alcoholic and aqueous extracts of dried leaves of *Phyllanthus amarus*

Phytoconstituents	Alcoholic extract of *Phyllanthus amarus*	Aqueous extract of *Phyllanthus amarus*
Alkaloids	+	+
Carbohydrate	+	+
Gums	+	+
Flavonoids	+	+
Proteins/Amino acids	+	+
Phytosterol	+	+
Saponins	+	+
Phenolic compounds tannins	+	+
Glycosides	+	+
Lignan (Phyllanthin and Hypophyllanthin)	++	++

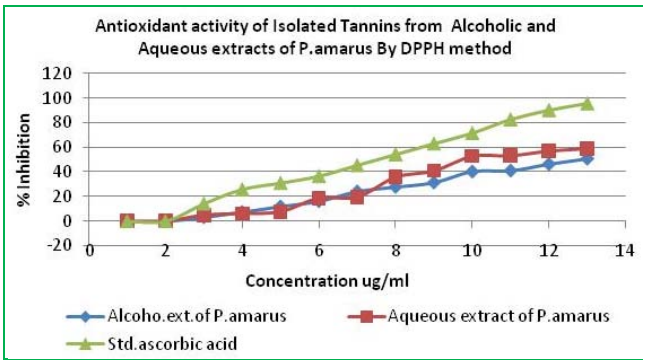

Figure 1: Antioxidant activity of alcoholic and aqueous extract of *Phyllanthus amarus* along with Standard by DPPH method [Comparative graph of isolated tannins with Standard (Ascorbic acid) by DPPH Method.]

Nitric oxide scavenging activity

In the present study, the nitrate produced by the incubation of solution of sodium nitroprusside in standard phosphate buffer at 25°C, was reduced by the alcoholic and aqueous extract of *Phyllanthus amarus* and all exhibited a NO – Scavenging dose response curve. Absorbance of the chromophore formed by the diazotization of nitrite with sulphanilamide with subsequent coupling with napthyl ethylene diaminedihydrochloride was read at 546 nm. Nitric oxide determination activities of isolated tannins from alcoholic

and aqueous extracts of *Phyllanthus amarus* were presented in Fig 2. Percentage inhibition was calculated by comparing the results of the test with those of the control that i.e. ascorbic acid. The corresponding IC_{50} value for NO-Scavenging abilities was calculated from the linear range to concisely compare the relative NO-Scavenging potencies of various extracts of isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus.* IC_{50} values for the isolated tannins of alcoholic extract of *Phyllanthus amarus* was found to be 17.31 at a concentration range of 0.5mg/ml and R^2 was found to be 0.9989 and IC_{50} value of isolated tannins of aqueous extract of *Phyllanthus amarus* was found to be 13.76 and R^2 was found to be 0.9982 at a concentration of 1mg/ml. Ascorbic acid was used as a standard and its IC_{50} value was found to be 10.28 at a concentration of 100 µg/ml. Alcoholic extract of *Phyllanthus amarus* showed good nitric oxide scavenging activity against tannins isolated from aqueous extract of *Phyllanthus amarus.*

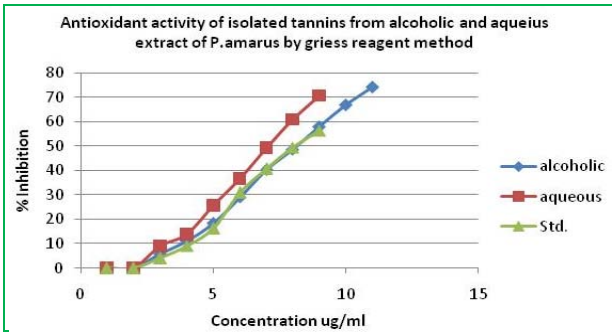

Figure 2: Antioxidant activity of alcoholic and aqueous extract of *Phyllanthus amarus* along with Standard by Griess reagent method [Comparative graph of isolated tannins with Standard (Ascorbic acid) by Griess reagent Method.]

Lipid peroxidation methods

Decomposition of lipid membrane in the body leads to the formation of malondialdehyde (MDA) along with other aldehydes and enals as the end product. These react with thiobarbituric acid to form coloured complexes. Hence, these are called as the Thiobarbituric Acid Reactive Substances (TBARS). The complex of TBA-MDA is selectively detected at 532 nm using UV spectrophotometer. Lipid peroxidation involves series of free radical mediated chain reaction processes, which also associated with several biological damages. Lipid peroxidation mainly affects biological membranes of especially liver, brain, spinal cord, containing highly oxidizable PUFA (polyunsaturated fatty acids). Malondialdehyde (MDA) is volatile β-scission product formed as major product of lipid peroxidation along with other aldehydes and ketones. These substances are mainly measured by (TBA) thiobarbituric acid, so called as thiobarbituric acid reactive substances (TBARS). Measurements of MDA levels in biological fluids have been extensively used as measure of lipid peroxidation. Inhibitory effects of ascorbic acid, *Phyllanthus amarus* extracts on TBARS formed in mice liver induced by $FeCl_3$ *in - vitro* were studied.

Mice liver homogenate was used to check the inhibition of lipid peroxidation. During the aerobic incubation of tissue homogenate with *in-vitro* free radicals generated by ferrous ascorbate system, malondialdehyde was formed which on reaction with thiobarbituric acid produces a pink colour. Formation of this thiobarbituric acid reacting substance (TBARS) was monitored at 532 nm. Lipid Peroxidation method of isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus* was presented in Fig 3. Curcumin was used as a reference standard. The IC_{50} value of Curcumin was found to be 2.40 at a concentration of 100µg/ml. IC_{50} value of tannins isolated from aqueous extract of *Phyllanthus amarus* was found to be 45.38 at a concentration range of 1mg/ml and R2 was found to be 0.9927. IC_{50} value of tannins isolated from alcoholic extract of *Phyllanthus amarus* was found to be 30.09 at a concentration range of 0.5 mg/ml and R2 was found to be 0.9934. Hence, Alcoholic extract of *Phyllanthus amarus* shows good lipid peroxidation activity against aqueous extract of *Phyllanthus amarus*, it has good potency. Curcumin was used as a standard and it has good potency and good lipid peroxidation activity against the two extracts of *Phyllanthus amarus*. The results showed inhibition of TBARS formation in mice liver homogenate increased by increasing concentrations.

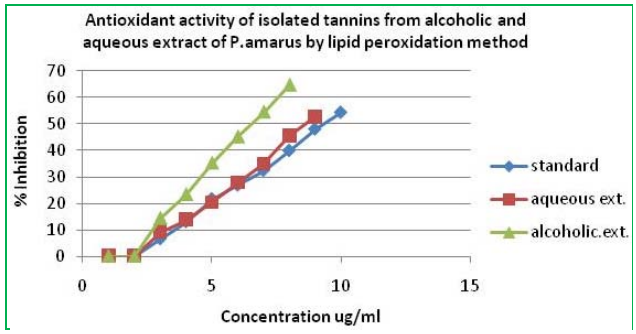

Figure 3: Antioxidant activity of alcoholic and aqueous extract of *Phyllanthus amarus* along with Standard by Lipid Peroxidation method [Comparative graph of isolated tannins with Standard (Curcumin) by Lipid Peroxidation method.]

Assay of Superoxide Radical Scavenging Activity

In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen, by PMS-NADH coupling reaction, reduces NBT (yellow dye) to blue-colored product called formazon. Drugs possessing superoxide scavenging activity decreases the reduction of NBT, which is a measure of superoxide anion scavenging activity that is indicated by reduction in absorbance at 560 nm. Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive species. One risk of the superoxide generation is related to its interaction with nitric oxide to form peroxinitrite which is a potent antioxidant that causes nitrosative stress in the organ systems. Superoxide radical scavenging activities of isolated tannins from aqueous extract of *Phyllanthus*

amarus and tannin isolated from alcoholic extract of *Phyllanthus amarus* were expressed as IC_{50} value. SOD radical scavenging activities of isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus* were presented in Fig 4. IC_{50} value of isolated tannins from alcoholic extract of *Phyllanthus amarus* was found to be 84.36 at a concentration range of 0.5mg/ml and R2 was found to be 0.9926. IC_{50} value of isolated tannins from aqueous extract of *Phyllanthus amarus* was found to be 79.47 at a concentration range of 0.5mg/ml and R^2 was found to be 0.9949. The extracts show dose dependent free radical scavenging activity. The scavenging activity of *Phyllanthus amarus* alcoholic and aqueous extracts and standard ascorbic acid (IC_{50} values 49.32, 52.55, 67.16 respectively) at a concentration range of 0.5 mg/ml, 1mg/ml and 100µg/ml respectively. Alcoholic extract of *Phyllanthus amarus* showed good superoxide radical scavenging activity against the aqueous extract of *Phyllanthus amarus*. Ascorbic acid used as a standard and it is more potent for the extracts.

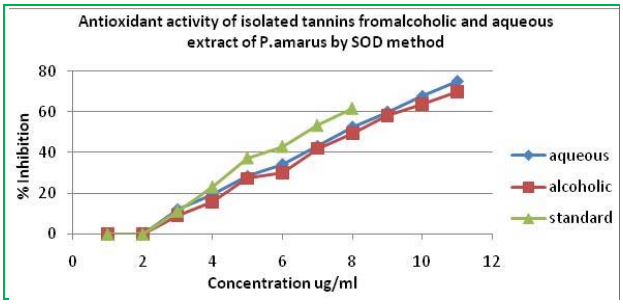

Figure 4: Antioxidant activity of alcoholic and aqueous extract of *Phyllanthus amarus* along with Standard by SOD method [Comparative graph of isolated tannins with Standard (ascorbic acid) by SOD method.]

Hydroxyl radical scavenging activity

All the extracts like isolated tannins of alcoholic and aqueous extracts of *Phyllanthus amarus* showed potent hydroxyl radical scavenging activity. The scavenging ability of hydroxyl radicals, various extracts were effective in order of their IC_{50} values. Hydroxyl radical scavenging activities of isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus* were presented in Fig 5. IC_{50} value of standard vitamin C was found to be 49.63 at a concentration range of 100µg/ml. IC_{50} value of isolated tannins from aqueous extract of *Phyllanthus amarus* was found to be 22.23 at a concentration range of 1mg/ml and IC_{50} Value of isolated tannins from alcoholic extract of *Phyllanthus amarus* was found to be 25.56 at a concentration range of 0.5mg/ml. It shows moderate hydroxyl radical scavenging activity of both the extracts.

Hydrogen peroxide radical scavenging activity

The results of the hydrogen peroxide scavenging activity of aqueous, alcoholic extracts and Vitamin E showed good potency respectively. Both exacts are good H_2O_2 scavenging activity. Hydrogen peroxide radical scavenging activities of isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus* were presented in Fig 6. IC_{50} value of vitamin E used as a standard was found to be 473.48 at a concentration of 100µg/ml. The activity was found to be very less concentration so that the standard Vitamin E was found to be more potent as compared to both the extracts of isolated tannins from alcoholic and aqueous extract of *Phyllanthus amarus.* IC_{50} values of isolated tannins from alcoholic extract of *Phyllanthus amarus* was found to be 5.92 at a concentration of 0.5mg/ml. IC_{50} values of isolated tannins from aqueous extract of *Phyllanthus amarus* was found to be 6.49 at a concentration of 0.5mg/ml. Among alcoholic and aqueous extracts, again alcoholic extract was found to be more potent because it gave results at 0.5mg/ml.

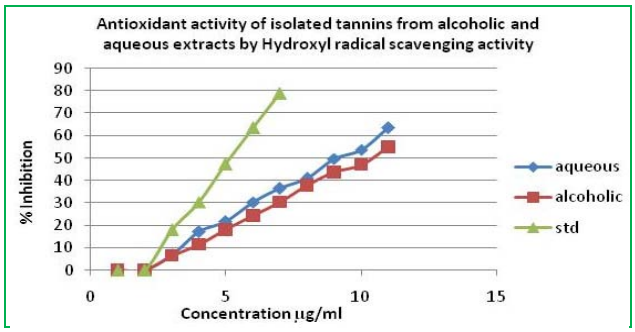

Figure 5: Antioxidant activity of alcoholic and aqueous extract of *Phyllanthus amarus* along with Standard by Hydroxyl Radical Scavenging activity [Comparative graph of isolated tannins with Standard (ascorbic acid) by Hydroxyl Radical Scavenging method.]

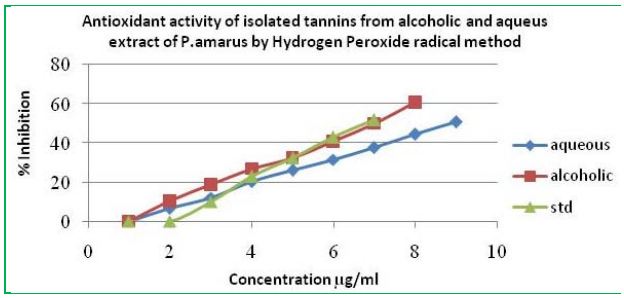

Figure 6: Antioxidant activity of alcoholic and aqueous extract of *Phyllanthus amarus* along with Standard by Hydroxyl Radical Scavenging activity [Comparative graph of isolated tannins with Standard (ascorbic acid) by Hydrogen Peroxide Radical Method.]

DISCUSSION

The present study indicates the *in-vitro* antioxidant activity *of isolated tannins from alcoholic and aqueous tannins of Phyllanthus amarus* is one of the widely used drugs in various Ayurvedic and herbal formulations. Traditionally this plant has been recommended mainly for gastric liver damage. Phytochemical analysis were confirmed the presence of phenolics like flavonol glycosides and the glycosides, lignan. The results from the current study clearly justified the use of *Phyllanthus amarus* leaves extracts in various pathologic conditions. It gives protection against various free radicals by inhibiting the DPPH radical, superoxide anion, nitric oxide, decreasing the lipid peroxidation level. Different models used to evaluate the antioxidant activity suggest that *Phyllanthus amarus alcoholic tannins and isolated tannins from aqueous extract showed* good source of natural

antioxidants as compared to the isolated tannins from aqueous extract of *Phyllanthus amarus*. Hence, on the basis of the present study it may be concluded that there is corroboration of traditional claims of *Phyllanthus amarus.*

DPPH radical scavenging activities of different extracts were presented. Results showed that the radical scavenging activity of isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus* increased with increasing concentration and were high for alcoholic extracts. Griess reagent method, all the extracts of *Phyllanthus amarus* exhibited a NO – Scavenging dose response curve. Absorbance of the chromophore formed by the diazotization of nitrite with sulphanilamide with subsequent coupling with napthyl ethylene diaminedihydrochloride was read at 546 nm. Percentage inhibition was calculated by comparing the results of the test with those of the control that i.e. ascorbic acid. The corresponding IC_{50} value for NO-Scavenging abilities was calculated from the linear range to concisely compare the relative NO-Scavenging potencies of various extracts of *Phyllanthus amarus*. Ascorbic acid used as a standard and it showed good potency. Alcoholic extract of *Phyllanthus amarus* showed good nitric oxide scavenging activity against aqueous extract of *Phyllanthus amarus.* Mice liver homogenate was used to check the inhibition of lipid peroxidation. During the aerobic incubation of tissue homogenate with *in-vitro* free radicals generated by ferrous ascorbate system, malondialdehyde was formed which on reaction with thiobarbituric acid produces a pink colour. Formation of this thiobarbituric acid reacting substance (TBARS) was monitored at 532 nm. Curcumin was used as a reference standard. Hence, Alcoholic extract of *Phyllanthus amarus* shows good lipid peroxidation activity against aqueous extract of *Phyllanthus amarus*, it has good potency. Curcumin was used as a standard and it has good potency and good lipid peroxidation activity against the two extracts of *Phyllanthus amarus.* The results showed inhibition of TBARS formation in mice liver homogenate increased by increasing concentrations. The highly reactive OH^- radical can cause oxidative damage to DNA, lipid and proteins. The effect of extract on the inhibition of free radical mediated deoxyribose damage was assessed by means of iron (II) dependent DNA damage assay. The Fenton reaction generate OH^- radical, which degrade deoxyribose sugar of DNA using Fe^{2+} salt as an important catalytic component. Ferric – EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radical was formed in free solution and was detected by their ability to degrade 2-decoy-2-ribose into fragments that formed a pink chromogen upon heating with TBA at low pH. All the extracts like alcoholic and aqueous extracts of *Phyllanthus amarus* showed potent hydroxyl radical scavenging activity. The scavenging ability of hydroxyl radicals, various extracts were effective in order of their IC_{50} values. IC_{50} value of standard vitamin C was found to be 49.63 at a concentration range of 100µg/ml. IC_{50} value of aqueous extract of *Phyllanthus amarus* was found to be 20.88 at a concentration range of 1mg/ml and IC_{50} Value of alcoholic extract of *Phyllanthus amarus* was found to be 27.42 at a concentration range of 0.5mg/ml. It shows moderate hydroxyl radical scavenging activity of both the extracts. In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen, by PMS-NADH coupling reaction, reduces NBT (yellow dye) to blue-colored product called formazon. Drugs possessing superoxide scavenging activity decreases the reduction of NBT, which is a measure of superoxide anion scavenging activity that is indicated by reduction in absorbance at 560 nm. One risk of the superoxide generation is related to its interaction with nitric oxide to form peroxi nitrite which is a potent antioxidant that causes nitrosative stress in the organ systems. Superoxide radical scavenging activities of aqueous and alcoholic extract of *Phyllanthus amarus* were expressed as IC_{50} value. The extracts showed dose dependent free radical scavenging activity. Alcoholic extract of *Phyllanthus amarus* showed good superoxide radical scavenging activity against the aqueous extract of *Phyllanthus amarus* Ascorbic acid used as a standard and it is more potent for the extracts. H_2O_2 radical it not very reactive but it can sometimes be toxic to cell because it generates OH^- radical in the cell. The results of the hydrogen peroxide scavenging activity of aqueous, alcoholic extract and Vitamin E showed good potency respectively. Both exacts are good H_2O_2 scavenging activity. The activity was found to be very less concentration so that the standard Vitamin E was found to be more potent as compared to both the extracts of *Phyllanthus amarus.* The results from the current study clearly justified the use of *Phyllanthus amarus* leaves extracts in various pathologic conditions. It gives protection against various free radicals by inhibiting the DPPH radical, griess reagent, lipid peroxidation method, hydroxyl radical, superoxide anion, hydrogen peroxide method, Different models used to evaluate the antioxidant activity suggest that *Phyllanthus amarus alcoholic extracts showed* good source of natural antioxidants as compared to the aqueous extract of *Phyllanthus amarus*. Hence, on the basis of the present study it may be concluded that there is corroboration of traditional claims of *Phyllanthus amarus.* These results suggest that the higher levels of antioxidant activity were due to presence of phenolic components. These findings are in accordance with the earlier report on total phenolics and antioxidant activity in isolated tannins from alcoholic and aqueous extract of *Phyllanthus amarus*. Tannins are very important plant constituents because of their scavenging ability due to their hydroxyl groups. The phenolics compounds may contribute directly to antioxidative actions. It is known that polyphenolic compounds have inhibitor effects on mutagenesis and carcinogenesis in humans. Phenolics compounds from plants are known to be good natural antioxidant. However, the activity of synthetic antioxidants was often observed to be higher than that of natural antioxidant.

CONCLUSION

The present investigation on the *in-vitro* antioxidant activities of isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus* leaves were found to be more potent antioxidants. The investigation indicates that the *Phyllanthus amarus isolated tannins from* aqueous extract of *Phyllanthus amarus* was found to be less potent comparatively to be isolated tannins from alcoholic extract of *Phyllanthus amarus*, The DPPH free radical assay, nitric oxide, *in-vitro* lipid peroxidation method, superoxide radical scavenging method, hydroxyl radical, hydrogen peroxide methods were found to be good antioxidant activity for both the extracts. Different models used to evaluate the antioxidant activity suggest that isolated tannins of *alcoholic extract of Phyllanthus amarus showed* good source of natural antioxidants as compared to the isolated tannins from aqueous extract of *Phyllanthus amarus.* Hence, on the basis of the present study it may be concluded that there is corroboration of traditional claims of *isolated tannins of Phyllanthus amarus.*

In the present study, there exist a positive correlation between total phenolic content and the antioxidant activity which is in accordance where the earlier findings. We found higher *in-vitro* antioxidant activity with higher polyphenolics compounds. The higher radical scavenging efficacy of isolated tannins from alcoholic and aqueous extract of *Phyllanthus amarus* may be due to their retention of antioxidant phytochemicals in this extracts. Strong evidence supports these findings that the isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus* enhance the antioxidants effects *in – vitro* models. These results suggest that isolated tannins from alcoholic and aqueous extracts of *Phyllanthus amarus* may offer effective protection from free radicals and support that is a promising source of natural antioxidants.

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