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Original Article

ASSESSING THE ANTIOXIDANT POTENTIAL OF *PHYLLANTHUS ACIDUS* BARK EXTRACTS

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ABSTRACT

Objective: The study was performed with a view to evaluate the traditional use of *P. acidus* bark as an antioxidant agent.

Methods: The antioxidant activity of *P. acidus* bark extracts was evaluated by *in vitro*, *in vivo* and electrochemical methods. The extracts were checked for scavenging and nullifying radicals. The behaviour to protect erythrocytes when exposed to oxidative stress was observed. The redox potential of extracts were analysed using a cyclic voltammeter. Qualitative analysis of the extracts was done to support their antioxidant property. The marker enzymes in the liver were profiled to see the action of ethanol extract against CCl_4 induced stress.

Results: The ethanol extract (PAE) and aqueous extract (PAA) of *P. acidus* exhibited significant antioxidant property, to support this, phenolic content of the extracts were high [152.12± 0.079 µg mg^{-1} (PAE) and 219.81± 0.19µg mg^{-1} (PAA) gallic acid equivalent]. The extracts scavenged >90% of ABTS radicals at low concentrations (50µg of PAE showed 97% of inhibition and 20µg of PAA could scavenge 91% of radicals). The ethanol extract showed better effect than the aqueous extract in protecting the erythrocytes under oxidative stress on exposure to H_2O_2. The metal chelating activity of PAE was thrice better than PAA. The animals pre-treated with PAE showed the markers restoring to normal levels. The extracts showed potential electro catalytic effect during the electro chemical oxidation of dopamine. HPLC analysis of the extracts indicated the presence of known antioxidant molecules like gallic acid, ellagic acid, coumaric acid, hydroxyl benzoic acid, rutin, quercitin, myrcetin and lutolin.

Conclusion: The study indicates the richness in phenolic and flavonoid compounds in the extracts. It provides a scientific support for the traditional use of *P. acidus* as a natural antioxidant.

Keywords: *Phyllanthus acidus*, Oxidative stress, Free radicals, cyclic voltammeter, Erythrocyte protection, SOD, Lipid peroxidation.

INTRODUCTION

Reactive oxygen species (ROS) are generated as by-products of biological processes/reactions or from exogenous factors [1]. ROS cover a wide range of chemical components including superoxide anion, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite [2]. Their overload causes oxidative stress, which is implicated in the etiology of many human pathogenic conditions and degenerative diseases including cancer, cardiovascular disease, neural disorders, diabetes and arthritis [3, 4, 5].

Aerobic organisms have developed their own efficient enzymatic and nonenzymatic self-defensive network during the course of evolution against oxidative stress to maintain cellular homeostasis [6]. Cells are equipped with several defence systems against free radical damage, including oxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT), or antioxidands such as α-tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione [7,8].

Antioxidants are micronutrients that have gained importance in recent years due to their ability to neutralize free radicals or their actions. Among the various natural antioxidants, phenolic compounds are reported to have the power of donating a hydrogen atom or an electron to oxygen derived free radicals and quench them [9, 10]. Amongst the most important exogenous antioxidants, vitamin E, vitamin C, β-carotene, vitamin E, flavonoids, vitamin D and vitamin K are well known [11]. The total antioxidant potential of nutritional supplements depends on the synergic and redox interaction among the different molecules present in food [12, 13].

Phyllanthus acidus is a member of the complex genus phyllanthaceae. Plants of genus *Phyllanthaceae* have high phenolic content [14]. Commonly known as star gooseberries, it is used in Indian colloquial practices to treat a number of illnesses. The fruits are used as a blood purifier and appetite stimulant. The plant parts are used to treat bronchitis, biliousness and treat digestive disorders. Leaves are used to treat sciatica, lumbago and rheumatism, while the seeds are used as a cathartic and the root as a purgative [15, 16]. *P. acidus* contains 4-hydroxybenzoic acid, caffeic acid [17], adenosine, kaempferol and hypogallic acid [18]. The plant extracts and the isolated compounds show numerous activities. It has been assessed for antinematodal activity [19], dye Brill Red 5B was isolated from leaf aqueous solution [20].

Leaf extracts from *P. acidus* showed strong antibacterial activity [21, 22] and hepatoprotective effect against acute liver damage induced by carbon tetrachloride [23]. Extract and compounds of the plant induce airway chloride secretion, considered a potent treatment for cystic fibrosis [18]. The triterpenoids Phyllanthusols A and B isolated from the plant are proposed as possible antitumor agent [24]. The aim of the study was to evaluate the *in vitro* and *in vivo* antioxidant property and protective effects of *P. acidus* bark extracts against induced oxidative damage and to study the electro chemical property of the extracts.

MATERIALS AND METHODS

Chemicals

2,2-Diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide phosphate reduced (NADH), phenazine methosulphate (PMS), (α-napthyl)-ethylenediamine, thiobarbituric acid (TBA) were obtained from Sigma Chemical Co. (St. Louis,MO,USA). Butylated hydroxyl anisole (BHA), sulfanilamide, sodium nitroprusside (SNP), trichloroacetic acid (TCA), Folin–Ciocalteu reagent were purchased from Merck (Germany). Ferrozine, gallic acid, ascorbic acid, ferrous chloride were procured from Himedia Pvt. Labs. (Mumbai, India).

All other chemicals and solvents used were of analytical grade. Water was purified by a Mili-Q water purification system (Millipore, Bedford, MA, USA).

Plant material and preparation of extracts:

Stem bark of *P. acidus* was collected from Balehonnur forest area of Western Ghats, Chikkamagalur (Dist.), Karnataka. Stem bark was cleansed thoroughly, shade dried, pulverized mechanically and sieved (sieve no. 10/44). The extract was defatted using petroleum ether in Soxhlet apparatus. Further, hot extraction was carried out with defatted material (600 g) successively with chloroform (1.5 L, 45°C, ≈ 15 cycles) and ethanol (2 L, 50°C, ≈15 –17 cycles) to get ethanol extract. The plant material (100g) in water (200ml) was heated to 65°C for 15 mins, sieved to get the aqueous extract. The extracts were dried in vacuum. Ethanolic extract (PAE) and aqueous extract (PAA) were stored in desiccators to avoid oxidation until further studies.

Qualitative and Quantitative analysis of phenolics, flavonoid

Qualitative analysis of the extracts

The extracts were qualitatively examined for various secondary metabolites using standard tests as described by Khanna and Kannabiran (2006) [25].

Assessment of total phenolic and flavonoid content in the extracts

The total phenolic content was determined by Folin–Ciocalteu method [26]. PAA and PAE were dissolved in methanol (1mg/ml) and then the extract solution (0.2ml) was mixed with 2ml of the Folin–Ciocalteau reagent (1:10 dilution) and 2ml of sodium carbonate (7.5%) (added 2 min after the Folin–Ciocalteau reagent). After initial mixing the reaction mixture was incubated at room temperature for 90 mins. The optical density samples were measured at 765nm and total phenolic contents were expressed as mg gallic acid equivalent.

Flavonoid content was measured using a modified colorimetric method [27]. Solutions of PAA and PAE (0.2ml, 1 mg/ml) were added to test tubes and the volume was made up to 5ml. Sodium nitrite solution (5%, 0.3 ml) was added followed by Aluminium chloride (0.3ml of 10%). After 6 min, 0.5ml of 1M sodium hydroxide was added. The mixture was diluted with 2.4ml of distilled water. The absorbance of the mixture was measured at 510 nm. The data was compared to a standard curve prepared using quercetin. The flavonoid contents were expressed as mg quercetin equivalent.

HPLC–UV analysis

Phenolic acids and flavonoids in PAA and PAE were analyzed by HPLC (Model LC-10ATVP, Shimadzu Corp, Kyoto, Japan) on a reversed phase Shimpak C18 column (250 mm ×4.6 mm). Phenolic content in both the extracts were detected using octadecylsilyl silica gel as stationary phase. 125 mg of the samples in 25 ml of methanol were injected to the column. Solvent system consisting of [A] phosphoric acid:water (0.5:99.5, v/v), [B] cetonitrile was used as mobile phase at a flow rate of 1 ml min^{-1}.

Phenolic acid standards such as gallic acid, coumaric acid, ellagic acid, ferulic acid, mandelic acid and vanillic acids were employed for identification of phenolic acids present in PAE and PAA by comparing the retention time under similar experimental conditions. The detector used for analysis was UV detector at 220 nm.

Flavonoid content in both the extracts was detected using octadecylsilyl silica gel as stationary phase. Solvent system consisting of methanol, water and phosphoric acid (100:100:1, v/v) was used as mobile phase at a flow rate of 0.5 ml min^{-1}. Rutin, quercetin, myricetin, kaempferol, luteolin were used as reference standard to identify the flavonoids in PAE and PAA. The detector used for analysis was UV detector at 350 nm.

***In vitro* antioxidant assays**

Total antioxidant capacity

Phosphomolybdenum method was used to determine the total antioxidant capacity of PAE and PAA [28]. 300µl of PAE and PAA at different concentrations (50, 100, 200 µg) were combined with 3 ml of reagent mixture (4 mM ammonium molybdate, 0.6 M sulfuric acid and 28 mM of sodium phosphate). Test tubes were kept for incubation at 95°C for 90 min and allowed to cool. Absorbance of the content was measured at 695 nm against blank. Antioxidant capacity of each extract was expressed as equivalents of ascorbic acid.

Total reductive capability

Total reductive capacity of PAE and PAA was determined using the method of Oyaizu (1986) [29]. 1 ml of PAE and PAA at different concentrations (50, 100, 200 µg) were mixed with phosphate buffer (2.5 ml, 0.2 M pH 6.5) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. At the end of the incubation period, trichloroacetic acid (2.5 ml, 10%) was added and centrifuged at 3000 rpm for 10 min. To 2.5 ml of supernatant, 2.5 ml of water and ferric chloride (0.5 ml, 0.1%) were added. The absorbance of the reaction mixture was measured at 700 nm against blank. Increased absorbance of the reaction mixture indicated increased reducing power. Total reducing capacity of each extract was expressed as equivalents of quercetin.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

PAE and PAA were screened for free radical scavenging activity by DPPH method [30]. Free radical scavenging activity of the extracts was carried out on the basis of ability to scavenge stable DPPH radical. Each extract at different concentrations(100, 200, 300, 400, 500 µg) were added to 3 ml of 0.004% DPPH in methanol and the mixtures were incubated at room temperature in dark condition for 30 min. The scavenging activity of extracts against DPPH radical was determined by measuring the absorbance at 517 nm. DPPH radical scavenging activity of BHT was assayed for comparison. Radical scavenging perecentage and IC_{50} were calculated.

Superoxide anion radical scavenging assay

Superoxide anion radical scavenging activity of PAE and PAA was measured using a slightly modified method of Nishimiki *et al.* (1972) [31]. All the reagents were prepared in phosphate buffer (pH 7.4). 1 ml of NBT (156 µM), 1 ml of NADH (468 µM) and 2 ml of extracts at different concentrations were added to each test tube. The reaction was initiated by adding 100 µl of PMS (60 µM) and incubated at 25°C for 5 min followed by the measurement of absorbance at 560 nm against blank. Decreased absorbance of the reaction mixture indicated increased super oxide anion radical scavenging activity. Gallic acid was taken as reference standard. The percentage inhibition and IC_{50} were calculated.

Lipid peroxidation inhibition assay

TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which was detected using the method of Halliwell and Guttridge (1989) [32]. 10% of chicken liver homogenate in 0.15 M potassium chloride was prepared. 0.5 ml of liver homogenate and 1 ml of PAA and PAE at different concentrations were taken in test tubes. Lipid peroxidation was induced by adding ferrous sulphate (50 µl, 0.07 M) and incubated at room temperature for 30 min. The reaction was stopped by adding chilled acetic acid (1.5 ml, 20%, pH 3.5) containing 20% TCA followed by the addition of TBA (50 µl, 0.8% TBA in 1.1% SDS). The content was incubated in boiling water bath for 60 min. After cooling, 5 ml of butanol was added and centrifuged at 3000 rpm for 10 min. Absorbance of the organic supernatant was measured at 532 nm. IC_{50} and percentage of inhibition of lipid peroxidation were calculated.

ABTS radical scavenging activity

ABTS radical scavenging activity for PAE and PAA were screened according to modified method of Roberta *et al*., 1999 [33]. The activity of the extracts was compared to BHA standard. The ABTS radical was generated by mixing equal volume (v/v) of 7 mM ABTS and 2.6 mM potassium persulfate and incubating in dark, overnight at room temperature. Prior to assay, ABTS was mixed with methanol (1:60, v/v) to get working reaction mixture of ABTS. 150 µl of each extract (PAA and PAE) at different concentrations were added to test tubes and volume in each test tube was made up to 3ml by adding reaction mixture of ABTS and the mixtures were incubated at room temperature in dark for 2 h. The scavenging activity of extracts

against ABTS radical was determined by measuring the absorbance at 734 nm. Radical scavenging activity and its IC_{50} were calculated.

Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by Fenton reaction system and the scavenging capacity towards the hydroxyl radicals was measured by using a deoxyribose method [34]. The reaction mixture contained 0.8 ml of phosphate buffer solution (50 mM, pH 7.4), 0.2 ml of samples at different concentrations. Sequentially, 0.2 ml of EDTA (1.04mM), 0.2 ml of FeCl3 (1mM), and 0.2 ml of 2-deoxyribose (60mM) were added. The reaction was started by adding 0.2 ml of ascorbic acid (2mM) and 0.2 ml of H_2O_2 (10 mM). Incubation was for an hour at 37°C. 2ml of cold thiobarbituric acid (10 g L^{-1}) was added to the reaction mixture followed by 2 ml of HCl (25%). The mixture was heated at 100°C for 15 min and then cooled. The absorbance of solution was measured at 532 nm. The scavenging percentage and its IC_{50} were calculated according to the formulae.

Metal chelating activity

Metal chelating activity of the extracts was measured according to the method of Dinis *et al*., (1994) [35]. About 3 ml of extracts and EDTA at different concentrations were taken in different test tubes followed by the addition of ferrous chloride (50 µl, 2 mM) and ferrozine (20 µl, 5 mM). Tubes were allowed to stand for 10 min at room temperature. Absorbance of reaction mixture was measured at 562 nm against blank. EDTA was used as standard for comparison. Percentage of metal chelation and IC_{50} were calculated.

***In vitro* inhibition of erythrocyte haemolysis assays**

Preparation of erythrocytes

2 ml of blood from healthy volunteer was collected in tubes containing sodium heparin. Erythrocytes were isolated and stored according to the method described by Yang *et al*. (2006) [36]. Briefly, blood samples collected were centrifuged (1500 xg, 10 min) at 4°C, erythrocytes were separated from the plasma and buffy coat. The sample was washed thrice by centrifugation (1500xg, 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes were stored at 4°C and used within 6 h for further studies.

Inhibition of erythrocyte haemolysis

The inhibition of induced erythrocyte haemolysis was evaluated according to the method described by Tedesco *et al*., (2000) [37] with slight modifications. 50 µl of PAA and PAE at different concentration were added to 100 µl of 5% (v/v) suspension of erythrocytes in PBS. To each tube 100 µl of 100 µM H_2O_2 (in 0.1 M PBS pH 7.4) was added. A negative control was maintained by adding erythrocytes suspension and 100 µl of l00 µM H_2O_2. The reaction mixture was incubated at 37°C for 3 h and diluted with 8 ml of PBS and centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 540 nm to determine the percentage of haemolysis. The inhibitory effect of the extracts was compared with standard antioxidant BHA. To assess the possible haemolysis, percentage of haemolysis and IC_{50} was calculated by taking haemolysis caused by 100 µM H_2O_2 as 100%.

Protective effect on erythrocytes structural morphology

To evaluate the protective effect of PAA and PAE, extracts (50 µl, 1mg ml^{-1}) were added to test tubes containing erythrocyte suspension (50 µl) in PBS followed by the addition of and 100 µl of 100 µM H_2O_2 in PBS (pH 7.4). Simultaneously, negative control (erythrocytes+H_2O_2) and positive control (erythrocyte suspension alone) were maintained for comparative evaluation. The mixture was incubated for 60 min. at 37°C. After incubation, the incubate was centrifuged at 3500 rpm for 10 min. and the cell pellets were inspected for morphological changes using a Nikon Eclipse E600 microscope (at 100x magnification) connected with Nikon Coolpix digital camera.

Calculations

1. Percentage of inhibition was calculated according to the formula:

$$\% \text{ inhibition} = \left[\frac{\text{Acontrol} - \text{Atest}}{\text{Acontrol}}\right] \times 100$$

Where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance of the extract reaction.

2. IC_{50} value was calculated using the formula:

$$IC50 = \left[\left(\frac{\sum C}{\sum I}\right) \times 50\right]$$

Where $\sum C$ is the sum of extracts concentrations used to test and $\sum I$ is the sum of percentage of inhibition at different concentrations.

Cyclic voltametric studies

Apparatus and preparation of bare carbon electrode

Cyclic voltammetry study was performed with Model CHI-660c electrochemical work station, equipped with a personal computer. A conventional three electrode cell was employed throughout the experiments. The electrochemical cell contained carbon paste electrode (CPE) as a working electrode, platinum counter electrode (PCE) and saturated calomel electrode (SCE) as reference. The state of dopamine in a potential difference is as below.

The bare carbon electrode was prepared by mixing of graphite powder and silicon oil at a ratio of 70:30 (w/w) in an agate mortar until a homogenous paste was obtained. The paste was packed into the homemade cavity (3 mm in diameter) and then smoothened on a butter paper. The electrical contact was provided by a copper wire connected to the paste at the end of the tube and cyclic voltamogram was produced using 10 mm Dopamine.

The experiment was repeated with a modified carbon paste electrode, in which the carbon paste was blended with extracts and then used in filling the electrode. The electrochemical response of Dopamine (0.1mM in phosphate buffer solution, pH 7.2) at modified CPE was observed at a scan rate 100 mVs^{-1}.

***In vivo* antioxidant property**

Experimental animals and treatment protocol

Wister strain albino rats of 180-220 g body weight were used in the present study. All animals were kept in polyacrylic cages and maintained under standard housing conditions. The animals were allowed to get acclimatized to the laboratory conditions for 7 days before the commencement of the experiment. The animals were divided into six groups of six animals in each. Group I served as control, Group II was administered with CCl_4 (negative control), Group III was administered with silymarin (25 mg/kg body weight) and the Groups IV, V and VI were administered with PAE. The ethanol extract was suspended in 2% DMSO and was administered orally to Groups IV, V and VI at dose of 50, 100 and 200 mg/kg body weight for 7 days. Stress was induced in animals of groups II, III, IV, V and VI by intra peritoneal administration of CCl_4, dose of 1.25 ml/kg (1:1in olive oil) on the seventh day, past an interval of 6 h after the administration of last dose of extract/saline on the 7th day . Animals were sacrificed after 24 h of CCl_4 administration and liver samples were collected for further studies. (The study received clearance from the Institutional Ethical Committee (Registration Number 144/1999/CPCSEA/SMG) under the certification Ref No. NCP/IAEC/CL/203/01/2013-14).

Assays of hepatic antioxidant enzymes

Catalase (CAT)

Catalase activity was estimated according to Aebi, 1984 [38]. Briefly, 10 µl of liver homogenate was added to reaction mixture consisting of 490 µl of 50 mM phosphate buffer (pH- 7.0) and 500 µl of 30 mM H_2O_2. The decrease in the absorbance at 240 nm was recorded for 3 min. and CAT activity was measured by using molar extinction co-efficient 43.6 M^{-1} cm^{-1}.

Superoxide dismutase (SOD)

To determine the SOD activity, the riboflavin -NBT assay was adapted [39] with slight modification. The homogenate (0.1ml) was

mixed with 0.1ml of 67 mM phosphate buffer (pH 7.8) containing 0.01M EDTA and 0.1 mL of 1.5 mM NBT. 3ml of 1.2 mM riboflavin was added followed by incubation at 37°C for 5 min. The reaction mixture was illuminated with a 25 W light tube for 15 min. The inhibition of NBT reduction was determined at measuring the absorbance at 560 nm. A control (water instead of NBT), test, standard (without homogenate) and blank (water instead of NBT and homogenate) were maintained simultaneously. Unit of SOD activity was expressed as the amount of enzyme required to inhibit the reduction of NBT using the formula:

$$\text{SOD activity} = \text{Decrease in OD} \times \frac{2}{(S - B)}$$

Where Decrease in OD= (S-B)-(T-C). Where, S - Absorbance of standard, B - Absorbance of blank, T - Absorbance of test and C - Absorbance of control.

Glutathione S-transferase (GST)

Glutathione S-transferase activity was estimated according to Warholm *et al*., (1985) [40] by measuring the CDNB–GSH conjugate formed using 1-chloro-2, 4-dinitrobenzene as substrate. Liver homogenate (10 µl) was mixed with reaction mixture consisting of 890 µl of 0.1 M sodium phosphate buffer (pH 6.5), 50 µl of 20 mM GSH, 50 µl of 20 mM CDNB. The change in the absorbance at 340 nm was recorded for 3 min. and the GST activity was measured using molar extinction co-efficient of 0.0096 $\mu M^{-1}cm^{-1}$.

Measurement of Lipid peroxidation

Level of lipid peroxidation in liver homogenate was measured as TBARS, Berton *et al*., (1998) [41]. Briefly, 0.5 ml of liver homogenate was mixed with ferric chloride (100 µl, 0.2mM) and incubated at 37 °C for 30 min. Followed by the addition of 2 ml of TCA-TBA-HCl reagent (15% TCA, 0.30% TBA in 0.25 N HCl) containing 0.05% BHT and heated for 60 min. in boiling water bath. Reaction mixture was cooled to room temperature, centrifuged and absorbance of the supernatant was measured at 532 nm. The TBARS was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Estimation of nitric oxide (NO)

Nitrite determination Griess assay was employed to determine the concentration of nitrite (NO^{2-}), the more stable metabolite of NO, according to Lee *et al*. (2006) [23]. An equal volume of Griess reagent was mixed with the liver homogenate. The developed colour was measured at 550nm.The amount of nitrite in the homogenate was calculated based on the standard curve of sodium nitrite. Percentage of the NO inhibition was calculated by using the formula: NO inhibitory %= (NO^{2-} control) – (NO^{2-} sample)/ (NO^{2-} control) X 100. Control was CCl_4 treated animals.

Total peroxidase

The peroxidase assay was carried out according to the method of Nicholos (1962) [42]. To 0.5ml of liver homogenate, 1ml each of KI (10mM) and sodium acetate (40mM) solutions were added and the absorbance was read at 353 nm. 50µl of H_2O_2 (15mM) was added and the change in the absorbance in 3min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change the optical density by 1 unit/min. The specific activity was expressed in terms of units per milligram of protein.

Statistical analysis

All data are expressed as mean ± SEM. Values of $p< 0.05$, $p< 0.01$ and $p< 0.001$ were considered as significant, very significant and highly significant respectively. Statistical analysis was performed using one way ANOVA followed by Dunnet's multiple comparison tests. Data was computed for statistical analysis using GraphPad Prism 5.

RESULTS AND DISCUSSION

As plants produce significant amount of antioxidants to prevent the oxidative stress caused by photons and oxygen, they are considered as potential source of antioxidant compounds [43]. The antioxidative system protects the organisms from ROS induced oxidative damage. They are very good scavengers for the reactive oxygen species that prevents the damage in many cellular components such as; DNA, proteins, and lipids [44].The use of synthetic antioxidants has limitations and hence natural antioxidants have gained importance [45].

Qualitative and quantitative analyses

The results of the tests support the presence of a number of significant secondary metabolites. The ethanol extract includes terpenoids, tannins, saponins, glycosides, alkaloids, flavonoids, organic acids, quinones and coumarins. The aqueous extract lacked tannins, alkaloids and organic acids. Total phenolic content in extracts was expressed as equivalent to gallic acid (EGA). The total phenolic content in PAE and PAA was found to be 152.12± 0.079 and 219.81± 0.19 µg mg^{-1} of dry extract, respectively. Analysis of flavonoid (expressed as equivalent to quercetin) has revealed 65.82± 0.166 µg mg^{-1} in PAE and in PAA, 341.75±0.208 µg mg^{-1} of dry extract.

HPLC UV analysis

The analysis of phenolics and flavonoids has been considered important for the medicinal plants to evaluate their antioxidant property [46]. HPLC was employed to characterize the important antioxidant molecules present in PAE and PAA in comparison with various standard phenolic and flavonoid molecules. UV spectra of these peaks together with the analysis of retention time of standard molecules under identical experimental conditions indicate the presence of important antioxidant phenolic acids; gallic acid, coumaric acid respectively (Table 1).

Gallic acid, ellagic acid, hydroxy benzoic acid and three unknown peaks were detected in PAE at 220 nm (Fig. 1B) with retention time of 1.96, 5.68, 8.27 min, respectively. Among them, gallic acid (120.49 mg g^{-1}) was the most abundant phenolic constituent present in PAE. PAA showed peaks at 220 nm with retention time of 2.95, 4.20 min (Fig. 1D) and three unknown peaks.

UV spectral peaks of PAE and PAA at 350 nm showed the presence of rutin, quercetin, myrcetin and lutolin in PAE and PAA showed the presence of rutin, quercetin (Fig. 1A and 1C, Table 1). To summarise, the chromatogram analysis of the spiked extracts compared to standard phenolic and flavanoid compounds revealed the possible presence of gallic acid, ellagic acid, hydroxyl benzoic acid, coumaric acid, rutin, quercetin, myrcetin and lutolin (Table 1). The presence of various significant antioxidant molecules could be responsible for the antioxidant and protective activity showed by the extracts of *P. acidus*.

Table 1: It shows quantitative HPLC analysis of PAE and PAA

S. No.	Phenolic compounds			Flavonoids		
	Standard	PAE (mg g^{-1})	PAA (mg g^{-1})	Standard	PAE (mg g^{-1})	PAA (mg g^{-1})
1	Gallic acid	120.4	11.79	Rutin	51.44	283.93
2	Coumaric acid	-	13.62	Quercetin	26.02	89.71
3	Ellagic acid	15.3	-	Myrcetin	8.0	-
4	Hydroxyl benzoic acid	53.2	-	Kaempferol	-	-
5	Vannilic acid	-	-	Luteolin	9.6	-

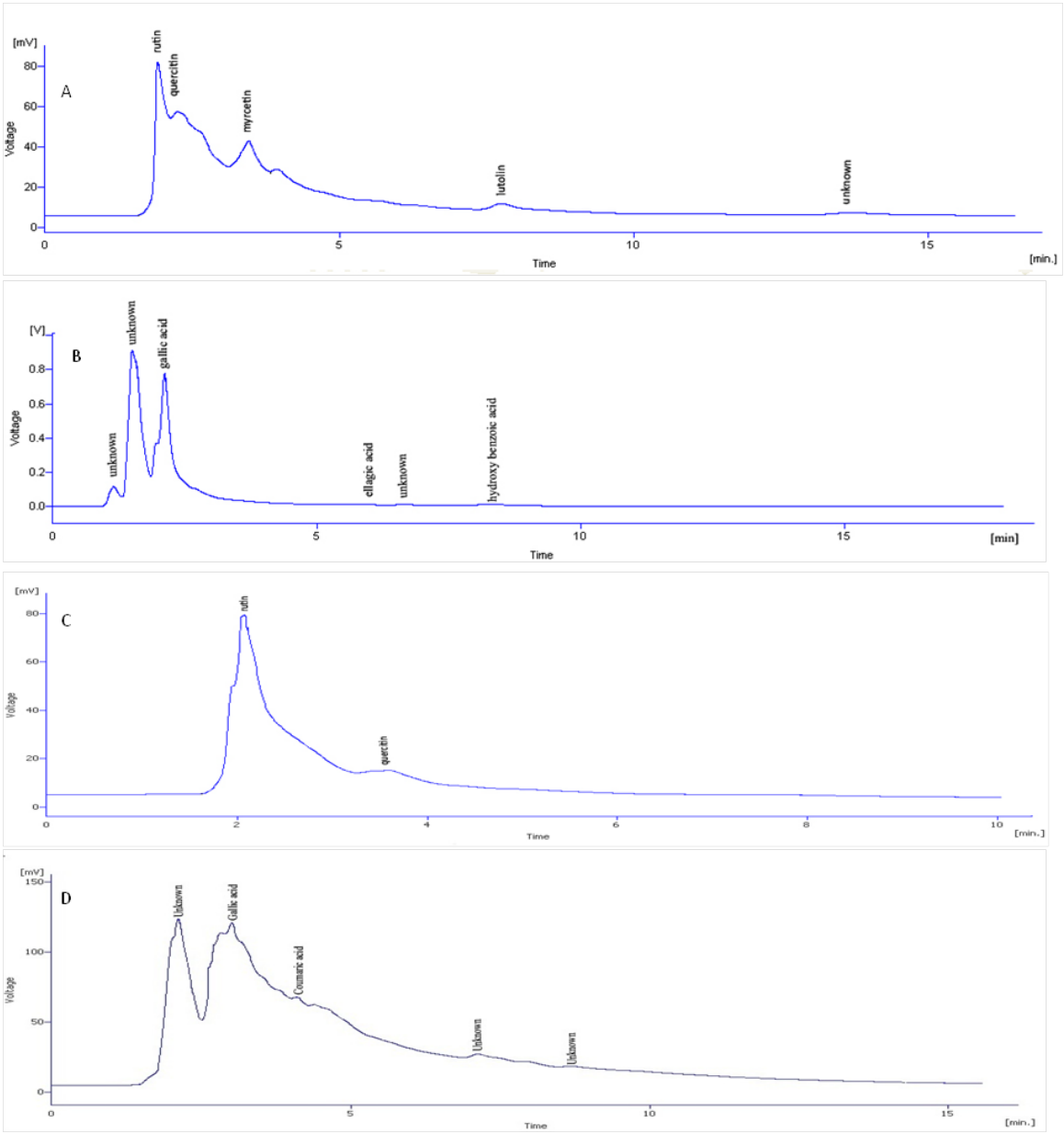
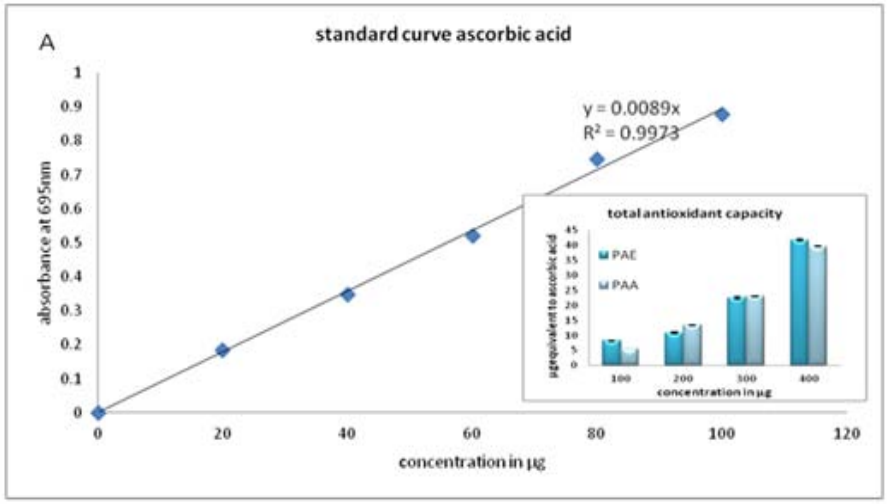


Fig. 1: It shows RP-HPLC Chromatograms of Phenolic and Flavonoid contents of PAE (A, B) and PAA (C, D)



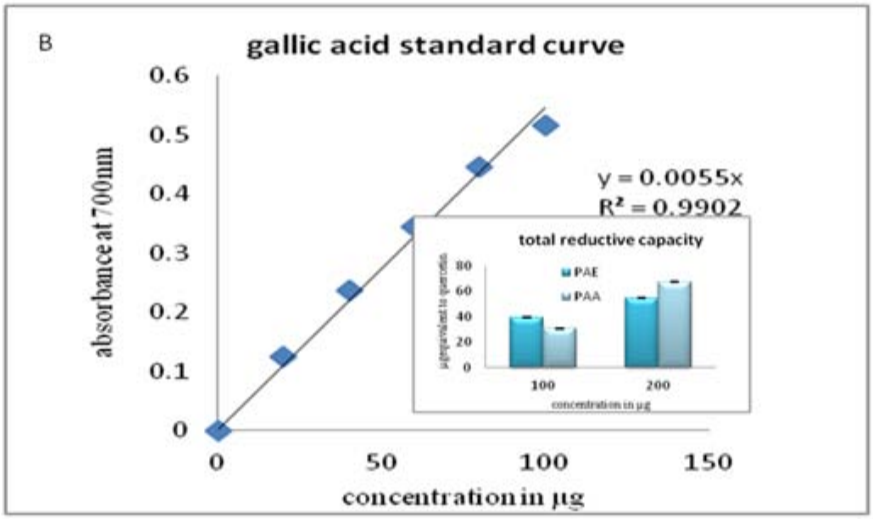


Fig. 2: The figure shows plots of total antioxidant capacities (A) and total reductive capacities (B) of ethanol and aqueous extracts of *Phyllanthus acidus*.

Table 2: It shows the *in vitro* antioxidant activity of ethanol and aqueous extracts of *P. acidus.*

Activity	Extract concentrations in µg		% inhibition		IC_{50} in µg	
	PAE	PAA	PAE	PAA	PAE	PAA
DPPH radical scavenging assay	100	100	37.95± 0.725	45.23±0.105		
	200	200	67.39± 0.695	69.45± 0.33	169.8±1.151	195.33±1.010
	300	300	84.8± 0.01	85.21± 0.055		
	400	400	95.31± 0.085	91.73± 0.365		
		500		92.21± 0.155		
ABTS radical scavenging assay	10	4	25.75± 0.05	29± 0.1	19.452±4.35	9.39±2.25
	20	8	50.65± 0.65	52.2± 0.6		
	30	12	69.95± 1.95	67± 0.4		
	40	16	90± 1.0	80.6± 0.7		
	50	20	97.8± 0.7	90.65± 0.45		
Superoxide radical scavenging assay	100	100	20.1±2.49	84.1±1.6	229±13.04	136±2.805
	200	200	52.3±6.4	91.64±0.43		
	300	300	67.15±2.55	94.32±0.72		
	400	400	78.64±1.6	97.955±0.055		
Hydroxyl radical scavenging	50	50	27.58±0.375	34.85±0.635	115±2.425	106±1.885
	100	100	42±1	45.75±0.49		
	150	150	65.25±0.45	67.44±0.44		
	200	200	81.8±0.6	88.44±0.32		
Lipid peroxidation inhibition assay	100	100	45.99±0.09	15.3±0.2	476±1.04	686±1.02
	500	500	57.9±0.45	41.2±0.3		
	1000	1000	64.2±0.2	60.16±0.33		
	1500	1500	32.1±0.3	35.21±0.19		
Metal chelating activity	200	1000	8.215±0.215	20.24±0.24	755±1.445	4136±2.425
	400	2000	24.05±0.045	22.2±0.7		
	600	3000	47.15±0.45	25.5±0.4		
	800	4000	56.51±0.11	45.2±0.6		
	1000	5000	64.18±0.28	68.19±0.485		
	1500		97.85±0.345			
Inhibition of erythrocyte heamolysis	200	200	18.22±0.315	14.69±0.3445	919±3.005	1330±2.147
	400	400	20.09±0.885	20.06±0.52		
	600	600	24.47±0.35	21.95±0.0515		
	800	800 1000	28.86±0.86	24.91±0.6845		
	1000		71.5±0.595	31.12±0.5465		

The values reported are average values of three independent experiments, values are mean ± SEM.

In vitro antioxidant studies

Total antioxidant and reductive capacities of both PAA and PAE were performed. The antioxidant capacity was expressed as equivalent to ascorbic acid and the reductive capacity was expressed in equivalents to quercetin. Both the extract showed significant total antioxidant and reductive activity (Figure 2).

DPPH is a stable free radical. The delocalisation of the spare electron imparts purple colour. The percentage of DPPH decolourization is attributed to the hydrogen donating ability of the test compounds. In the present study, PAA and PAE showed significant radical scavenging (IC50 169.8±1.151, 195.33±1.010 µg) (Table 2). Similarly the reduction of blue coloured ABTS radical to colourless by PAA and PAE was notable (IC50 19.452±4.35, 9.39±2.25 µg) (Table 2).

The scavenging activity of the extracts can be credited due to the presence of phenolic compounds which are known as potent antioxidants.

Both PAE and PAA of *P. acidus* bark were capable to scavenge the superoxide radical and hence inhibit the formation of blue NBT. The IC_{50} values of quenching the radicals were found to be 229±13.04 µg and 136±2.805 µg (Table 2) for ethanol and aqueous extracts respectively. Similarly, hydroxyl radical scavenging was found to be concentration dependant on the extracts. Their IC_{50} values were 115±2.425µg and 106±1.885µg (Table 2) for PAE and PAA.

Like all other assays, in the present study, PAE and PAA showed dose dependent lipid peroxidation inhibition. PAA and PAE showed the same path in lipid peroxidation inhibition assay. The trend line increased until it showed an approximate 60% inhibition but, it dropped down as the concentration increased further. The metal chelating activity of the extracts was very apart. The PAE showed an IC_{50} of 755±1.445 µg whereas the same was 4136±2.425 µg for aqueous extract. The metal chelating power of PAE was 5 times stronger than the PAE. The action of PAE over PAA in inhibition of erythrocyte haemolysis was conspicuous. The image shows that the ethanol extract has considerable affect in protecting morphology of erythrocytes over aqueous extract.

The antioxidant activity of antioxidants has been attributed by various mechanisms, among which a few of them are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [47]. The antioxidant activity of PAE and PAA increased with increasing amount of sample. The extracts showed notable radical scavenging activity. The extracts have protective effect against erythrocytes exposed to H_2O_2 action. It inhibited the haemolysis and as well as maintained the morphology of the erythrocytes. Metal chelating capacity of the extracts was significant demonstrating an effective capacity of iron binding. The study suggests that its action as antioxidant may be related to its iron-binding ability. H_2O_2 is not a very strong oxidizing agent, and has not been shown to react directly with polyunsaturated fatty acids in the absence of metals [48]. However it is seen that in RBC's, haemoglobin could facilitate hydrogen peroxide–induced lipid peroxidation [49]. It can be concluded that, due to the action of the extracts on metal ons there is a check on lipid peroxidation. Also, the extract directly inhibits peroxidation of lipid molecules. These reasons can cumulatively account for the protection of erythrocytes when exposed to H_2O_2. It is also evident in the study that the aqueous extract has lesser chelating activity and as well as lesser ability to protect erythrocytes from being damaged (Figure 3).

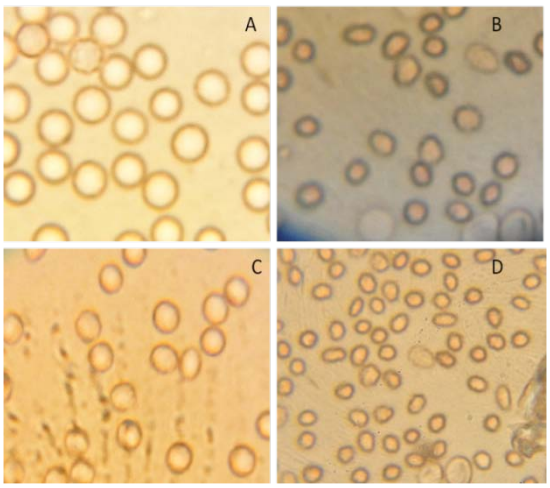


Fig. 3: It shows the protective effect of extracts against H_2O_2 induced oxidative stress on human erythrocytes; (A) Normal Erythrocytes, (B) Erythrocytes + H_2O_2, (C) Erythrocytes + Ethanol Extract + H_2O_2, (D) Erythrocytes + Aqueous Extract + H_2O_2

C V studies

Cyclic voltammetry is a type of potentiodynamic electrochemical measurement. In cyclic voltammetry, the potential of a working electrode is linearly scanned from an initial value to a final value and back, while recording the respective current intensity. The current at the working electrode is plotted versus the applied voltage to give typical cyclic voltammogram. The antioxidant property of PAE and PAA was evaluated by an electrochemical approach. To substantiate the beneficial activity of extracts, a new advanced electrochemical method cyclic voltammetry was employed. Figure 4 illustrates the electro catalytic oxidation of dopamine on PAE and PAA modified CPE in PBS in pH-7.0. Dopamine is electro active on bare CPE. It is oxidized and reduced quasi-reversibly showing the oxidation reduction peak heights of the same concentration dopamine (dotted line). The modified carbon paste shows much higher peaks when compared to the bare CPE (continuous line). Dopamine oxidation peak became much sharper and the current response was enhanced. The cyclic voltammograms obtained at a scan rate of 0.1 V s^{-1} showed cathodic peak potential shifting towards anodic direction with broad peak potential as the oxidized antioxidant molecules characteristically exhibited decreased cathodic current with reciprocal increased anodic current.

Generally, electrolysis occurs at the surface of electrode in response to a change in potential in order to maintain the surface concentration of redox species at the values required by the Nernst equation. Therefore, the rate of change of potential is directly proportional to the rate of electrolysis and the current. Antioxidants are oxidized at an electrode and enhance anodic potential [50]. Interestingly, in the present study broad peak was observed towards anodic potential, which represents the antioxidant property of PAE and PAA and the electro chemical behavior is attributed to the presence of antioxidant molecules, flavonoids and polyphenols. The extract modified carbon paste electrode shows sensor for the estimation of dopamine, it also portrays the biological oxidation potential which relate to the nature of specific molecules.

***In vivo* antioxidant studies**

Liver is the central organ to metabolize all foreign compounds. As most of the orally ingested chemicals and drugs are metabolised into toxic intermediates, liver is the target organ for toxicity caused by xenobiotics and drugs. These toxicants damage liver by producing ROS. To encounter the oxidative stress, antioxidant defence mechanism operates in our body to detoxify or scavenge ROS [51]. CCl_4 is one of the most studied hepatotoxicant. The hepatotoxicity of CCl_4 results from its metabolic conversion to free radical product $CCl^{\bullet}{}_3$ by Cyt P-450 to a highly reactive trichloro methyl free radical. This radical reacts with oxygen to form a more reactive trichloromethylperoxy radical which leads to elicit lipid peroxidation, elevation of hepatic enzymes and reduction in protein synthesis, finally resulting cell death.

The SOD, CAT, GST are major markers and molecules that defence against oxidative stress. The SOD in rat liver homogenate under stressed condition decreased pointing the impaired functioning towards scavenging superoxide radicals. The rats pre-treated with extract were found to restore the values closer to control values. Similarly, the estimations of CAT and NO showed lower values or inflamed condition when compared to the treated rats where the values shifted towards normal (Table 3). The lipid peroxidation, estimated as MDA in homogenates showed significantly increased level of MDA in CCl_4 treated rats. The groups pre-treated with the extract showed lower values of MDA close to the normal values SOD eliminates radicals derived from peroxidative processes converting it to H_2O_2. This is rapidly converted to water and oxygen by CAT. NO or reactive nitrogen species are formed during their reaction with oxygen or with superoxides. These compounds are responsible for altering the structural and functional behaviour of many cellular components. NO is also implicated for inflammation, cancer and other pathological conditions [52]. In the graph (Figure 5) SOD versus lipid peroxidation there is a perfect

negative correlation, with R^2 value 0.9274 showing that the correlation is highly significant. As the SOD increases the lipid peroxidation decreases, this states that the oxidative stress is diminished and the cell is restored to safe levels. Strong scavenging of superoxide, hydroxyl and nitric oxide radical will be helpful in protecting free radical induced diseases.

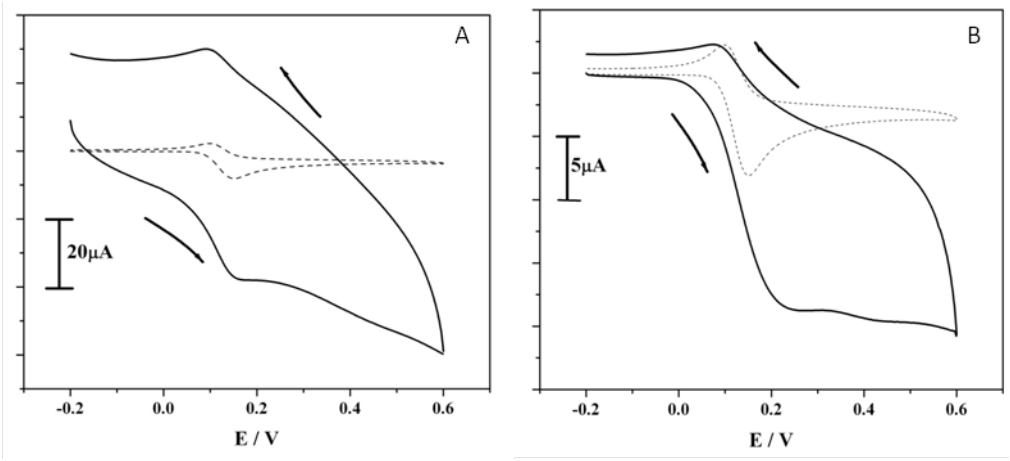


Fig. 4: It shows the Voltamograms of PAE (A) and PAA (B) at a scan rate 100 mVs^{-1}

Table 3: Evaluation of *in vivo* antioxidant markers

Assays	Catalase (units/mg protein)	SOD (units/mg protein)	Total peroxidase (units/mgprotein)	Lipid peroxidise (nmol/mgprotein)	GST (units/mg protein)	NO inhibition%
Control	90.11±10.07	2.275241±0.15*	0.2604±0.037**	1.167±0.010	0.2290±0.018	86.67±2.560*
CCl4	66.05±3.141	1.324421±0.018	0.1902±0.044	1.430±.0028	0.1840±0.0099	2.759±1.053
Silymarin	91.14±5.031*	1.876908±0.107	0.2723±0.065***	1.024±0.0071*	0.2186±0.024	85.06±3.386*
Group I	82.37±3.867	1.907492±0.022	0.1987±0.012	1.125±.012	0.2087±0.019	63.45±2.487
Group II	90.14±8.078	2.17973±0.0027*	0.2259±0.014	1.135±0.0060	0.2929±0.0029**	67.36±2.004*
Group III	94.50±2.500*	2.3605±0.0021**	0.2598±0.042**	1.012±0.0042*	0.2364±0.022	83.91±1.216*

Each value is expressed as mean ± SEM, n=6, *P<0.05, **P<0.01, ***P<0.001.

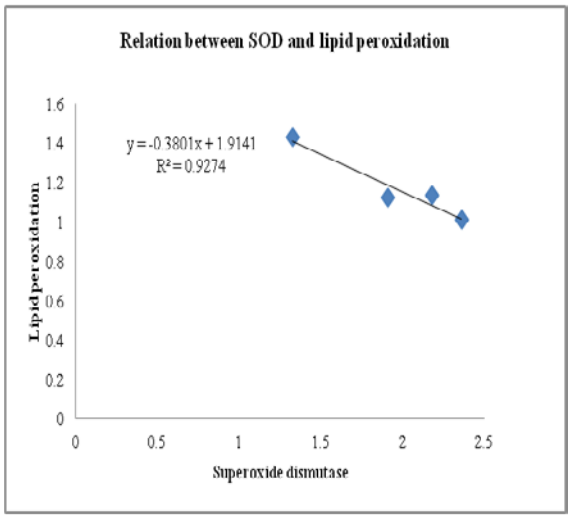


Fig. 5: It shows the negative correlation between SOD and Lipid peroxidation

Antioxidant response elements (ARE) is a sequence that mediates transcriptional activation of genes exposed to oxidative stress. Proteins that are encoded by the ARE include enzymes associated with glutathione biosynthesis, redox proteins with active sulphydryl moieties and drug metabolising enzymes [53]. In the present study, concentration of GST in CCl_4 stress induced rats was significantly low. The extract pretreated treated animals showed a rise in the level of GST almost restoring it to normal levels (Table 3). Phenolic antioxidants at lower concentrations activate MAPK pathways leading to the induction of phase II detoxifying enzymes like GST for cellular protection signalling [54]. The presence of significant antioxidant molecules in PAE could have attributed to the increased level of GST in treated animals.

CONCLUSION

The result of the investigation demonstrated that *P. acidus* has antioxidant and protective activity. The study has compiled ample evidence of *P. acidus* as an antioxidant. This provides a good pharmacological logic for using this plant in many colloquial treatments.

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