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Full Length Original Research Paper

Evaluation of Antioxidant activity of Ethanolic extract of *Sphaeranthus amaranthoides* Burm.f

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Abstract:

The ethanolic extract of the whole plant of *Sphaeranthus amaranthoides* was evaluated for antioxidant activity by various *in vitro* methods like DPPH decoloration assay, Nitric oxide scavenging assay, hydrogen peroxide, superoxide anion radical scavenging assay and reducing power assay. Ascorbic acid was used as a standard for comparison. The extract was found to scavenge the free radicals in a dose dependent manner. The IC_{50} was calculated for each in vitro antioxidant assays. The ethanolic extract exhibited poor scavenging effect against hydrogen peroxide radical but better scavenging effect against DPPH, nitric oxide and superoxide anion radical. The *in vivo* antioxidant activity was evaluated against CCl_4 – induced liver toxicity in rats. CCl_4 induced *in vivo* toxicity decreased the level of catalase, SOD, GSH, Peroxidase, protein and increased the MDA level in the liver tissue. Treatment with alcoholic extract significantly increased the antioxidant enzymes level and reduced the MDA level in the liver tissue. The present study indicates that the ethanolic extract of *Sphaeranthus amaranthoides* possesses strong antioxidant activity which supports that the whole plant can be used as an alternate source for the Ayurvedic drug, Munditika for which the accepted source is *Sphaeranthus indicus.*

Keywords: Antioxidant activity, *Sphaeranthus amaranthoides*, *In vitro* models, *In Vivo*, Carbon tetrachloride.

Introduction

Oxygen free radicals and its metabolites (ROS) play a major role in the pathogenesis of several degenerative diseases like atherosclerosis, neurodegenerative diseases like Parkinson's syndrome, cancer, diabetes, arthritis, aging, ischemia and liver disorders [1]. Oxygen free radicals are naturally generated in the biological systems as byproducts of metabolism and are capable of causing cell death and tissue damage [2].The major site of ROS generation are mitochondria, microsomes, endoplasmic reticulum, phagocytic cells, endothelial cells and nuclei [3]. However, both enzymatic and nonenzymatic antioxidants defense to minimize the cellular damage. Enzymatic antioxidants are SOD, glutathione peroxidase, glucose -6-phosphate dehydrogenase[4]. Non – enzymatic antioxidants are obtained from natural sources and include Vit – C, E, flavanoids, tannins and carotenoids. Plants are rich sources of antioxidant principles and several plants/drugs in traditional system of medicine have been investigated for the antioxidant property [5][6][7]

Sphaeranthus amaranthoides is an erect fragrant herb distributed in moist situations throughout the plains of India. *Sphaeranthus amaranthoides* is used as a substitute for the Ayurvedic drug, Munditika [8]. The accepted source of Munditika is *Sphaeranthus indicus*[9][10]. The whole plant of *Sphaeranthus amaranthoides* is used for the

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treatment of several diseases like jaundice, fever, epilepsy, gastric disorders, and painful swellings [11]. *In- vitro* and *in-vivo* antioxidant activity of the whole plant of *Sphaeranthus amaranthoides* has not been investigated so far, hence the present investigation was undertaken to substantiate its use as a substitute for the Ayurvedic drug, Munditika.

MATERIALS AND METHODS

Collection of plant material

The whole plant was collected from Chermadevi (paddy fields) region of Tirunelveli district, Tamil Nadu in the month of January 2011 .The collected plant material was identified and authenticated by Dr. S.N.Yoganarasimhan, Taxonomist and Research coordinator, M.S. Ramaiah College of Pharmacy, Bangalore. Taxonomic identification was carried out using available literature[12].A voucher herbarium specimen (Gowri 044) has been deposited in the Department of Pharmacognosy, M.S.Ramaiah College of pharmacy, Bangalore. The plant material was thoroughly washed with water to remove the adhering dirt and sandy material, cut into small pieces and dried in shade.

Chemicals and Instrument used

Chemicals: 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ellman's reagent (5,5-dithios-(2-nitrobenzoic acid) (DTNB), TBA (Thiobarbituric acid),NBT(nitro blue tetrazolium), Sulphanilic acid were procured from Hi-Media Lab Ltd, Mumbai, India. Ascorbic acid was obtained from S.D.Fine Chem Ltd, Biosar, India. Silymarin was obtained from Micro Labs as Gift sample.

Instrument: UV Spectrophotometer (Shimadzu: UV-1601)

Preparation of Alcoholic extract

Coarsely powdered whole plant material (100g) was extracted with 95% ethanol in soxhlet apparatus. The extract was collected and concentrated using rotary evapourator under reduced pressure. The concentrated extract was stored in a dessicator for further drying. The yield was found to be 5.25%w/w.

Preliminary phytochemical screening [13][14][15]

The alcoholic extract was screened for the presence of various secondary metabolites by adopting standard procedures.

In vitro antioxidant methods

DPPH decoloration assay [16]

Free radical scavenging potential of extracts was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. 1ml of 0.1mM methanolic DPPH solution was mixed with 1.5 ml of different concentrations (20 - 100 µg/ml) of extracts and standard ascorbic acid in methanol in capped test tubes and incubated in dark for 30 min at room temperature. After incubation, absorbance of mixtures was measured at 517 nm against the blank using UV spectrophotometer.

DPPH radical scavenging activity was calculated using the following formula

$$\% \text{ inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{Absorbance of Control}} \times 100$$

Nitric oxide scavenging activity [17]

4ml of 10mM sodium nitroprusside was mixed with 1 ml of phosphate buffer and 1 ml of various concentration of extracts or standard ascorbic acid in DMSO. The reaction mixture (6ml) was incubated for 150 min at room temperature. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed, 1 ml of sulphanilic acid reagent (0.33%v/v) was added, mixed well and allowed to stand for 5 min for

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completion of diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride (0.1%w/v) was added, mixed and allowed to stand for 30 min in diffused light. A pink colored chromophore formed was measured at 540 nm. The % inhibition was calculated using the formula

$$\% \text{ inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{Absorbance of Control}} \times 100$$

Scavenging of Superoxide Radical by Alkaline DMSO Method [18]

To the reaction mixture containing 0.1 ml of NBT (1 mg/ml solution in DMSO) and 0.3 ml of the various concentration of the extracts in DMSO, 1 ml of alkaline DMSO (1ml DMSO contain 5Mm NaOH) was added to give a final volume of 1.4 ml and the absorbance was measured at 560 nm. The same procedure was repeated for the standard ascorbic acid. Pure DMSO was used as blank instead of alkali DMSO.

$$\% \text{ inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{Absorbance of Control}} \times 100$$

Scavenging of hydrogen peroxide [19]

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). 1ml of various concentrations of the extracts or standards in methanol was added to 2ml of hydrogen peroxide solution in PBS. Then finally the absorbance was measured at 230nm after 10 minutes. All readings were performed in triplicates and the percentage inhibition was calculated using equation

$$\% \text{ inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{Absorbance of Control}} \times 100$$

Reducing power assay [20]

2.5 ml of various concentrations of extracts were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide (10mg/ml). The mixture was incubated at 50°C for 20 min, Cool and adds 2.5 ml of 10% trichloroacetic acid. The mixture was centrifuged at 6500 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml deionised water and 0.5 ml of 0.1% of ferric chloride and the absorbance was measured at 700 nm, increase in absorbance indicates higher reducing power ability of the extracts.

All the assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance against extract concentration. Ascorbic acid was used as standard.

In vivo antioxidant study

Animals used

Healthy albino rats of either sex (170 – 200g), Wistar strain were used for the study. The animals were bred and maintained in the animal house of M. S. Ramaiah College of Pharmacy. Animal house was well maintained under standard hygienic conditions, at room temperature and humidity (60 ± 10%) with 12 h day and night cycle. The animals were provided with standard pellets and water *ad libitum*. The study was approved by the Institutional Animal Ethical Committee, MSRCP. (IAEC certificate no.MSRCP/P-10/2010).

Acute toxicity studies [21]

Female Albino rats of Wistar strain in the weight range 170-200 g were used for acute toxicity studies. The animals were fasted overnight and administered with a single dose of 2000mg/kg body weight and observed for any behavioral and neurological changes for first 2 hrs and for any toxicity or mortality for 72hrs.

Experimental protocol

Carbon tetrachloride induced oxidative stress [22]

The animals were divided into 5 groups of 6 animals each.

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Group 1 served as normal control and was given 10% Tween 80 for 5 days.

Group II was maintained as positive control and was administered 10% Tween 80 solution for 5 days and a 1:1 mixture of CCl_4 and olive oil (1 ml/kg b.wt, s.c) on days 2 and 3.

Group III was administered with standard silymarin at a dose of 100 mg/kg body weight p.o for 5 days and a 1:1 mixture of CCl_4 and olive oil (1ml/kg b.wt, s.c) on days 2 and 3.

Group IV and V (test groups) animals were administered alcoholic extract of *Sphaeranthus amaranthoides* at doses 200 and 400 mg/kg respectively ,p.o for 5 days and a 1:1 mixture of CCl_4 and olive oil (1 ml/kg b.wt, s.c) on days 2 and 3.

On 6th day, all the animals were sacrificed under ether anesthesia; livers were removed and washed with ice cold saline. Livers were weighed and homogenates in buffers were prepared by using a Tissue homogenizer (REMI motors Ltd, Mumbai) and total proteins, peroxidase, malondialdehyde, catalase, super oxide dismutase and glutathione levels were estimated.

Determination of liver antioxidant enzymes

Preparation of liver homogenate

The isolated liver was used for the preparation of liver homogenates.

1.Liver homogenate in Potassium chloride (0.15 M): A 10% w/v liver homogenate was prepared in 0.15 M potassium chloride buffer and centrifuged at 8000 rpm for 10 minutes. The supernatant solution was used for estimation of peroxidase, malondialdehyde, catalase, and total proteins.

2. Liver homogenate in sucrose in phosphate buffer pH (7.4): A 10% w/v liver homogenate was prepared using 0.25 % sucrose in 5M phosphate buffer, centrifuged at 8000 rpm for 10 minutes. The supernatant solution was used for estimation of super oxide dismutase and reduced glutathione.

Estimation of Superoxide dismutase (SOD) [23][24]

The method is based on the reduction of NBT (Nitroblue tetrazolium) by the formation of nitrite in the presence of EDTA. To 100µl of 10 % w/v liver homogenate in sucrose with phosphate buffer, 1ml of sodium carbonate, 400 µl nitro blue tetrazolium and 200 µl EDTA were added, and zero minute reading was taken at 560 nm after the adding of 400 µl of hydroxylamine hydrochloride. The mixture was incubated for 5 minutes and the reduction of NBT was measured after 5 minute at 560 nm.

Estimation of reduced Glutathione (GSH) [23][25]

Glutathione was determined using Ellman's reagent(5,5-dithios-(2-nitrobenzoic acid) /DTNB).1ml of 10 % w/v liver homogenate in sucrose in phosphate buffer was treated with 1.8 ml of distilled water and 2 ml of phosphate buffer (pH 7) and mixed well. Five minutes after adding 200 µl of DTNB reagent to the reaction mixture, absorbance due to the colored complex formed by sulphydryl group of glutathione, was read at 412 nm. The amount of glutathione was determined using its molar extinction coefficient of 13,600 /M/cm and expressed in term of nm/100 mg of Wet tissue.

Estimation of Catalase (CAT) [23][26]

Catalase activity was measured by either decomposition of H_2O_2 or liberation of O_2.100µl of 10% liver homogenate in 0.15 M potassium chloride buffer was mixed with 1.9 ml of phosphate buffer (pH 7). To the mixture 1 ml of 10 mM hydrogen peroxide solution was added and zero minute reading was read at 240 nm against phosphate buffer as blank. The decrease in absorbance at the end of 1 min after the addition of 1ml of hydrogen peroxide was again read.

Catalase activity was calculated by using molar extinction coefficient of H_2O_2 and expressed in terms of unit per milligram of wet tissue.

Estimation of Peroxidase [23]

Peroxidase was estimated based on the formation of periodide by the decomposition of H_2O_2.Peroxidase was estimated based on periodide formation.500 µl liver homogenate in potassium chloride buffer was mixed with 1 ml of 10 mM potassium iodide solution and 1ml of sodium acetate (pH 5.25). To the above mixture 200 µl of 12mM hydrogen peroxide was added and absorbance was noted at 353 nm after 5 minutes.

Estimation of Lipid peroxidation (TBARS) [27][28]

The extent of lipid peroxidation was determined by the reaction of sample lipid peroxides (MDA) with TBA (Thiobarbituric acid) in acidic condition to form a pink color chromophore, which was measured at 532nm. 500 µl liver homogenate in potassium chloride buffer was added to 1 ml of TBA: TCA: HCl reagent (containing 0.38%TBA, 15% TCA & 0.25N HCl) and boiled for 15 minutes, cooled. The mixture was then centrifuged at 10,000 rpm for 5 minutes. Absorbance of the supernatant solution was measured at 532 nm against reagent blank. The malondialdehyde content was estimated as TBARS and was expressed in terms of nmoles/100 mg of tissue, using the molar extinction co-efficient, 1.56×10^5.

Estimation of Total proteins

Total protein content was determined by biuret method using Agappe Diagnostic's Total protein Kit and the absorbance was measured at 546 nm using UV-visible spectrophotometer against protein reagent as blank. The protein content was calculated by using the following formula and expressed as total protein in gm per deciliter.

$$\text{Total protein in gm/dl} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 6$$

Where 6 refers to the protein concentration in standard solution in gram per deciliter.

Results and Discussion

Preliminary Phytochemical studies

The phytochemical screening of alcoholic extract showed the presence of alkaloids, carbohydrates, proteins, saponins and phenolic compounds like tannins, flavonoids and absence of phytosterols, fixed oils, fats and mucilage.

In- vitro antioxidant activity

The antioxidant activity of ethanolic extract of *Sphaeranthus amaranthoides* was evaluated by 4 different *in vitro* models. Ascorbic acid was used as the standard antioxidant. All the assays were done in triplicate and expressed as mean ± SEM value. It was observed that the extracts scavenged the free radicals in a dose dependent manner. The results were expressed in Table 1 in terms of IC_{50}, which is the concentration of sample required to scavenge 50 % of free radical.

Table 1: In vitro antioxidant activity (IC_{50} values (µg/ml)

Sample	IC_{50} values (µg/ml)			
	DPPH assay	Nitric oxide scavenging assay	Hydrogen peroxide scavenging assay	Superoxide anion scavenging assay
Ethanolic extract	10.66±0.66	10.66±1.76	1102±0.25	160±0.01
Ascorbic acid	13.65±0.02	84.66±5.92	234±0.15	57±1.00

DPPH decoloration assay is a simple and reliable antioxidant assay. DPPH is a stable free radical that can easily accept a hydrogen donor to become a stable phenoxy radical. The assay is based on the conversion of purple colored DPPH free radical to yellow colored stable diamagnetic molecule by reaction with the hydrogen donating

scavenger. In this model, the ethanolic extract 400mg was found to scavenge the DPPH radical to a greater extent. The maximum percentage of inhibition of alcoholic extract was 76.33 at 100µg where as the standard ascorbic acid showed 73.19 % inhibition at 20 µg. The alcoholic extract showed better DPPH scavenging potential than the standard ascorbic acid. (Fig.1)

Nitric Oxide (NO), produced endogenously from L-Arginine by nitric oxide synthetase, plays an important role in many physiological processes including vascular regulation, immune responses, and neural communication [29]. NO is extremely unstable and can interact with molecular oxygen to form nitrite (NO_2-) and nitrate (NO_3-), which acts as free radical. This model is based on the inhibition of nitrite free radical generated from sodium nitroprusside in buffer solution. Nitric oxide generated in physiological systems can be determined using Griess Reagent which is a mixture of *N*-(1-naphthyl) ethylenediamine and sulfanilic acid. Sulfanilic acid is quantitatively converted to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to *N*-(1-naphthyl) ethylenediamine dichloride to form a purple colored azo dye, which is detected by measuring the absorbance at 546nm.In nitric acid assay, the highest activity was observed with alcoholic extract with a percentage inhibition of 79.84 (100µg). The IC_{50} values were found to be 10.66 µg, 84.66 µg for alcoholic and ascorbic acid respectively. The extract was found to have better scavenging effect of NO than the standard ascorbic acid. (Fig.2)

Superoxide anion assay was carried out by alkaline DMSO assay. Superoxide is generated by the addition of NaOH to air saturated dimethyl sulfoxide (DMSO).The generated superoxide remains stable in solution, which reduces Nitroblue tetrazolium in to formazan dye at room temperature [30]. The maximum percentage of inhibition at 1000 µg was found to be 70.76, 90.79 for alcoholic and ascorbic acid respectively. The IC_{50} value was found to be 160 µg, 57 µg respectively for alcoholic and ascorbic acid. (Fig.3).

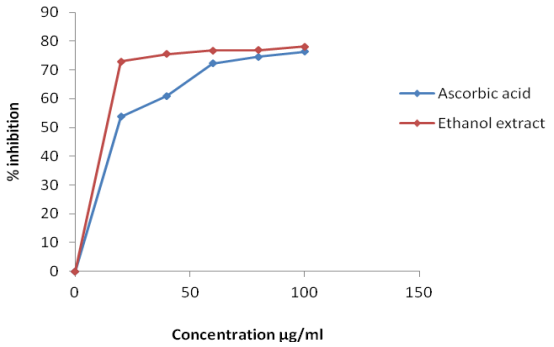


Fig.1. DPPH scavenging activity of ethanol extract of *Sphaeranthus amaranthoides*

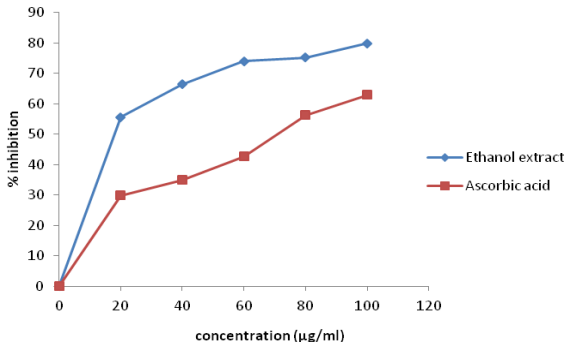


Fig.2. Nitric oxide scavenging activity of ethanol extract of *Sphaeranthus amaranthoides*.

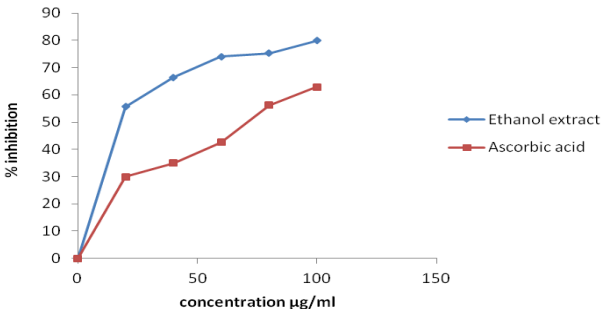


Fig.3. Superoxide anion radical scavenging activity of ethanol extract of *Sphaeranthus amaranthoides*.

The reductive ability of ethanolic extract is represented in Table 2.The ethanol extract

showed dose dependent effect .The increase in absorbance shown by the extract indicates the increase in reducing power.

Table 2: Reductive ability of *Sphaeranthus amaranthoides* ethanolic extract.

Concentration (µg/ml)	Absorbance at 700nm *Sphaeranthus amaranthoides* Ethanolic Extract
1000	0.526 ± 0.015
800	0.498 ± 0.321
600	0.450 ± 0.066
400	0.429 ± 0.032
200	0.182 ± 0.011

Absorbance due to ascorbic acid (20 µg) is 0.612
Values are mean ± SEM of triplicate determinations.

In-vivo antioxidant activity

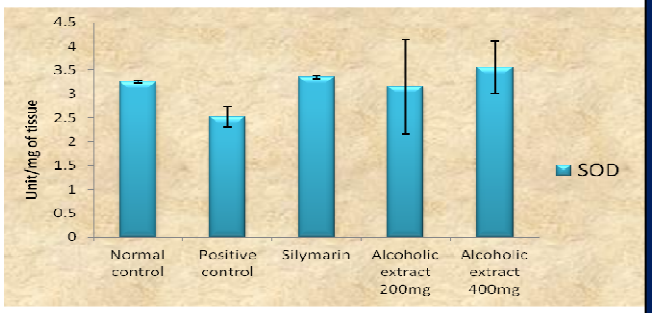
Acute toxicity studies of ethanolic extract did not show any mortality or toxic symptoms up to 2000 mg/kg b.wt.CCl_4 is a potent hepatotoxin is widely used in the evaluation of hepatoprotective effect of crude drugs. Hepatic damage caused by CCl_4 is predominantly due to lipid peroxidation which is caused by the free radicals derivatives of CCl_4 [31]. CCl_4 is metabolized to CCl_3 free radical by Cytochrome P450 in the liver, which reacts with oxygen to form highly reactive trichloromethyl peroxyl radical, $CCl_3OO^{\bullet}$ which affects the lipid and protein at the cellular level causing lipid peroxidation[32]. The antioxidant activity of ethanolic extract was shown in Table.3.

Table 3: Effect of ethanolic extract of *Sphaeranthus amaranthoides* on CCl_4 induced toxicity in rats

Parameters	Normal control	Positive control	Standard (Silymarin)	Ethanol extract (200mg)	Ethanol extract (400mg)
Liver weight (100gm/b.wt)	3.483±0.35	4.426±0.99	3.329± 0.13	4.211±0.13	3.675±0.3
Total protein (g/dl)	6.786±0.87	3.651±0.95	7.851±1.21	5.91±0.85	6.591±1.80
SOD (U/mg tissue)	3.25±0.02	2.52±0.21	3.351±0.04	3.15±0.99	3.55±0.05
CAT (U/mg tissue)	16.795±0.66	6.742±1.27	13.781±0.98	12.726±2.26	13.985±2.44
Peroxidase (nmol/100mg tissue)	24.315±1.98	14.341±0.84	25.295±0.40	22.005±1.00	23.501±2.25
MDA (nmol/100mg tissue)	0.288±0.09	1.1556±0.17	0.359±0.02	0.503±0.06	0.211±2.25
GSH (nmol/100mg tissue)	46.34±2.41	32.58±0.99	46.16±1.00	44.12±2.79	46.76±1.85

Statistical analysis

The data were expressed as Mean ± SEM and were tested with one way ANOVA followed by Tukey-Kramer multiple comparison test. Data compared against positive control group, p value is *** p< 0.001, ** p< 0.01,* p< 0.05.



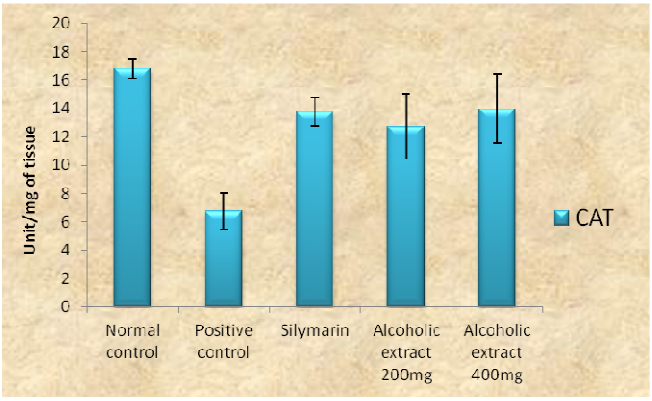


Fig 4. The SOD and Catalase level in the liver of CCl_4 intoxicted rats treated with silymarin and *Sphaeranthus amaranthoides* alcoholic extract.

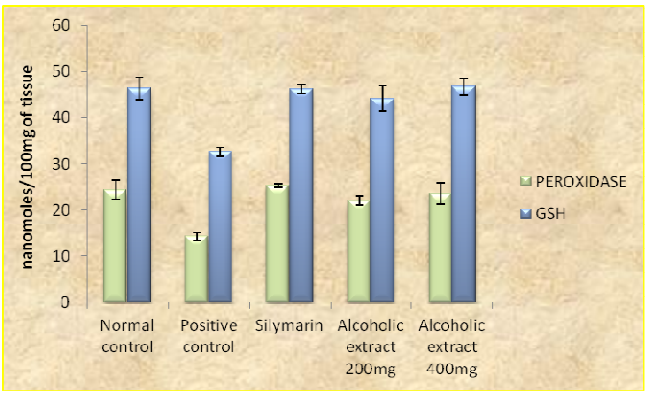


Fig 5. The Peroxidase and GSH level in the liver of CCl_4 intoxicted rats treated with silymarin and *Sphaeranthus amaranthoides* alcoholic extract.

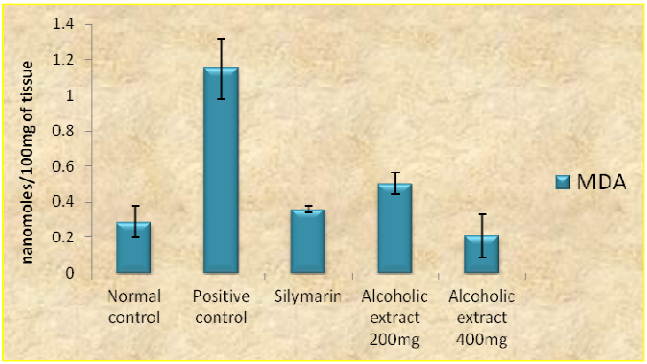


Fig 6. The MDA level in the liver of CCl_4 intoxicted rats treated with silymarin and *Sphaeranthus amaranthoides* alcoholic extract.

The reduction in the intracellular catalase, SOD, GSH concentration in the liver indicates the severe liver damage by CCl_4 while the groups treated with the ethanolic extract (200 & 400mg) increased the level of enzymatic antioxidants (P<0.001) and were comparable with the normal group(Fig.4 & 5). The ethanol extract showed dose dependent activity. Malondialdehyde is a secondary product of lipid peroxidation, which can be used as an index to measure the extent of lipid peroxidation. There was a significant increase in MDA level in CCl_4 induced group when compared to normal group, indicating the extent of lipid peroxidation .This was significantly (P<0.001) decreased in animals treated with 400mg of alcoholic extract indicating the protective role of ethanol extract against CCl_4 induced toxicity(Fig.6). Several studies have correlated the free radical scavenging activity with the presence of phenolic compounds [33][34].The phytochemical screening of the ethanolic extract showed the presence of phenolic compounds which may be responsible for the antioxidant property.

Conclusion

The result of the present study clearly indicates that the ethanolic extract of *Sphaeranthus amaranthoides* whole plant possesses antioxidant activity, which claims its traditional use as a substitute for *Sphaeranthus indicus*.

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