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Research Article

ANTIOXIDANT EFFECT OF ROOTS AND RHIZOMES OF *SMILAX ZEYLANICA* L. AN IN VIVO STUDY

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ABSTRACT

Aim of the study: To evaluate the antioxidant property of *Smilax zeylanica* L. using CCl_4 hepatotoxicity in rats.

Materials and methods: Hepatotoxicity was induced in Wistar albino rats by administration of CCl_4 (0.5ml/kg/day p.o. for 7days). Methanol extract of *S. zeylanica* roots and rhizomes (SZRM) was administered to the experimental animals at doses 200,400 and 600 mg/kg/day, p.o. for 7 days. Antioxidant effect was assessed by the estimation of hepatic levels of SOD, catalase, peroxidase, reduced glutathione, total proteins and malondialdehyde.

Results: In methanol extract-treated animals, the toxic effect of CCl_4 was controlled by restoration of the levels of hepatic antioxidant enzymes as compared to the positive control and standard drug silymarin-treated groups. The methanol extract at different doses exhibited significant increase in SOD, peroxidase, glutathione and proteins. The level of malondialdehyde was also significantly reduced.

Conclusion: The study revealed that the *S. zeylanica* possesses strong antioxidant property.

Key words: CCl_4 induced hepatotoxicity; hepatic antioxidant enzymes; in vivo antioxidant activity; *Smilax zeylanica*

INTRODUCTION

Human beings are exposed to free radicals in the environment through radiation and pollution. Free radicals are also produced naturally in the body through various metabolic reactions. These free radicals cause severe damage to cells, which can lead to degenerative diseases as well as premature ageing. Free radicals attack and damage cell membrane, cell organelles including DNA, leading to faulty translation of genetic material [1]. Their action is opposed by a balanced and co-ordinated system of antioxidant defenses which detoxify the reactive intermediates or easily repair the resulting damage. Antioxidants scavenge these free radicals and enable cells to rejuvenate or stabilize the process of life. Antioxidants scavenge free radicals by donating their own electrons to free radicals, thus preventing damage to cells. Upsetting this balance causes oxidative stress, which can lead to cell injury and death[2]. Variety of intrinsic antioxidants such as superoxide dismutase, catalase, reduced glutathione and peroxidase are present in organisms which protect them from oxidative stress, thereby forming first line of defense.

Ayurveda, an ancient Indian system of medicine is rapidly gaining global acceptability as a highly effective healthcare system. Many drugs in ayurveda derived from medicinal plants have been reported as rich sources of antioxidants and the use of such natural resources as diet supplements will help in reducing the incidence of many diseases related to oxidative stress[3].

The genus *Smilax* L. (Smilacaceae) consists of more than 300 species, distributed all over the world, out of which 24 are found in India[4]. Four species viz. *Smilax aspera* Linn., *Smilax perfoliata* Lour., *Smilax wightii* A.D.C. and *Smilax zeylanica* Linn. (Smilacaceae) occur in the forests and hills of South India[5,6].

Chopachinee is an important drug used in Ayurveda for the treatment of several diseases like diseases of the nervous system, epilepsy, psychosis[7,8], urinary disorders, polyuria, hemiplegia, Parkinson's disease, congenital diseases, leprosy, rejuvenator, blood purifier[9]. The accepted botanical source of Chopachinee is *Smilax china* L.[7]. *S. perfoliata* Lour, *S. glabra* Roxb., and *S. lanceifolia* Roxb. are used as substitutes of Chopachinee[7,8],

while *S. zeylanica* may be a potential alternate source[10]. The antiepileptic activity in *S. zeylanica*, which is one of the properties of the drug Chopachinee is established[11].

Species of *Smilax* are reported to contain phytoconstituents dioscin (spirostanol triglycoside), plant steroids such as smilagenin and sarsapogenin. The roots of S. zeylanica have a steroidal saponin glycoside diosgenin[8].

Species of *Smilax* such as *S. glyciphylla* Sm. (Australian sarsaparilla)[12] and *S. china* L. (European sarsaparilla)[13]have been screened for antioxidant properties whereas no systematic pharmacological work regarding antioxidant activity has been reported on *S. zeylanica*. Hence the present work is undertaken to ascertain the antioxidant potential of *S. zeylanica.* In this study the antioxidant property of roots and rhizomes of *Smilax zeylanica* L. has been evaluated by in vivo methods.

MATERIALS AND METHODS

Collection and identification of Plant material

The roots and rhizomes of *S. zeylanica* L. were collected from the vicinity of Kanyakumari District, Tamil Nadu, India, during June 2008. The plant material was identified and authenticated by Dr. S.N. Yoganarasimhan, Taxonomist and Research Coordinator at M. S. Ramaiah College of Pharmacy, Bangalore, Karnataka, India. The taxonomic identification was carried out following local flora[5], and authenticated with reference to the voucher herbarium specimen (No. 020), deposited at the herbarium of PG Department of Pharmacognosy, and crude sample in crude drug museum at M. S. Ramaiah College of Pharmacy.

Drugs and Chemicals

Silymarin was obtained as a gift sample from Microlabs, Bangalore, India. Nitro blue tetrazolium (NBT), was obtained from S.D. Fine Chem, Ltd., Biosar, India. Thiobarbituric acid and 5,5-dithiobis(2-nitrobenzoic acid)- Ellman reagent were procured from Hi-media Laboratories Ltd., Mumbai, India. Protein Estimation kits were procured from Agappe Diagnostics, Kerala, India.

Preparation of methanol extract

The roots and rhizomes of *S. zeylanica* were washed and dried at room temperature. After complete drying, it was powdered and passed through sieve no. 44-60 and stored in air tight container. The air-dried powdered plant material was successively extracted with solvents of increasing polarity. About 500 g was extracted in a soxhlet apparatus with different solvents of increasing polarity, starting from petroleum ether (60-80°C) followed by chloroform and methanol. Methanol extract (SZRM) was concentrated under reduced pressure (yield 3.96%w/w calculated in terms of air-dried weight of plant/ drug material). The concentrated extract was subjected to preliminary phytochemical tests for identification of various phytoconstituents[14].

HPTLC studies

HPTLC studies were performed using Camag HPTLC system equipped with Linomat V applicator, Camag TLC scanner 3 and WinCATS- 4 software for interpretation of data. An aluminum plate (10×10 cm) precoated with silica gel $60F_{254}$ (E Merck) was used as adsorbent. All the solvents used were of HPLC grade, obtained from MERCK. All the solvents used were of HPLC grade obtained from MERCK. Weighing was done on Precisa XB 12A digital balance[15].

Experimental animals

Albino Wistar strain rats in the weight range of 170-200 g were used for acute toxicity and antioxidant activity studies. The animals were bred and maintained in the animal house of M. S. Ramaiah College of Pharmacy, Bangalore, India. Animal house was maintained under standard hygienic conditions, with 12 h day and night cycle, at room temperature and humidity (60 ± 10%). Experimental animals were fed with standard rat pellets and water *ad libitum.* Animal maintenance was in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experimentation on Animals) guidelines. The study protocol was approved by the Institutional Animal Ethics Committee of M. S. Ramaiah College of Pharmacy (Protocol No. MSRCP/P-04/2008 Dt. 22.11.08).

Acute toxicity studies

Acute toxicity study of *S. zeylanica* was carried out according to OECD guidelines (www.iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECD_GL423.Organisation forEconomic Cooperation and Development (OECD) guidelines for testing of Chemical-423). Female rats of 8-12 weeks weighing around 170-200 g were used. Methanol extract (SZRM) at doses 300 and 2000 mg/kg were orally administered to separate groups of animals. The animals were observed continuously for 2 h for any symptoms of toxicity and /or death. They were under observation for further 2 weeks.

Antioxidant activity

Experimental protocol

The methanol extract of *S. zeylanica* roots and rhizomes (SZRM) was subjected to in vivo antioxidant activity studies. Albino Wistar rats of either sex weighing 170-200 g were used for the study. They were divided into 6 groups of 6 animals each.

Group I: Normal Control treated with vehicle (2% w/v acacia, 2 ml /kg, p.o.)

Group II: Positive hepatotoxic control (2% w/v acacia, 2 ml /kg, p.o + CCl_4 0.5 ml / kg, p.o.)

Group III: Silymarin 100mg/kg p.o + CCl_4 0.5 ml/kg, p.o.

Group IV: SZRM 200 mg/kg p.o. + CCl_4 0.5 ml/kg, p.o.

Group V: SZRM 400 mg/kg p.o. + CCl_4 0.5 ml/kg, p.o.

Group VI: SZRM 600 mg/kg p.o. + CCl_4 0.5 ml/kg, p.o.

The extract and standard drug were suspended in 2% w/v gum acacia in distilled water, as vehicle. They were administered orally once daily for 7 days. All groups except the normal control were administered CCl_4 0.5 ml/kg (1:1 in liquid paraffin), p.o. once daily for 7 days.

Sample collection

On the 8th day, 18 h after the last dose of CCl_4, all animals were sacrificed by excess anesthesia, for the isolation of liver. The isolated liver was perfused in ice cold saline,blotted dry and weighed. Liver was further divided in to 2 parts for preparation of liver homogenates. One part was used for the preparation of 10 %w/v homogenate in potassium chloride (0.15 M). It was centrifuged at 5724g for 10 mins and the supernatant obtained was used for estimation of total proteins, peroxidase, catalase, and malondialdehyde. The second part was used for preparation of 10 % w/v homogenate in 0.25 % w/v sucrose in phosphate buffer (5M, pH 7.4). This was also centrifuged at 5724g for 10 min. The supernatant obtained was used for estimation of super oxide dismutase and reduced glutathione[16,17.]

Biochemical estimations

Total proteins and antioxidant enzymes: Total proteins were estimated using commercial Protein estimation kits[18]. Peroxidase was determined from the concentration of periodide, the concentration of which was directly proportional to the amount of peroxidase in the reaction mixture containing appropriate amount of H_2O_2 and enzyme. Periodide was spectrophotometrically determined at its absorption maxima of 353 nm in a UV spectrophotometer (Shimadzu 1601)[19]. Catalase activity was measured by the decomposition of H_2O_2. The spectral region for hydrogen peroxide is 240 nm. The difference in absorbance per minute is a measure of catalase activity. Catalase activity was expressed in terms of units per milligram weight of wet tissue[20]. Superoxide dismutase assay was based on its ability to scavenge O_2* from reaction mixtures and thus inhibit reactions caused by O_2*. O_2* was generated by the auto-oxidation of hydroxylamine at pH 10.2 and the generated O_2* simultaneously reduces nitro blue tetrazolium (NBT) to form the corresponding nitrite, in the presence of EDTA. The reduction of NBT is inhibited by SOD in the test mixture and is measured spectrophotometrically at 560 nm[21].

Reduced Glutathione: GSH was estimated using Ellman Reagent i. e., 5-5'dithiobis (2-nitrobenzoic acid), which reacts with sulfhydryl compounds to give a relatively stable yellow color. The compound is water soluble

and is proportional to the amount of glutathione present in the sample. The amount of reduced glutathione was determined using its molar extinction coefficient of 13,600 M-1cm-1 and expressed in terms of nm/100 mg of tissue[22].

Malondialdehyde (MDA): In free radical induced hepatic damage, free radicals attack polyunsaturated acids, to form lipid radical. The lipid radicals readily react with molecular oxygen to produce peroxy radicals which initiate lipid per oxidation. This is considered as the major factor influencing breakdown and turnover of biomembranes. Increased level of lipid per oxidation is observed in CCl_4 intoxication. The chief secondary product of lipid per oxidation reaction is malondialdehyde (MDA). This reacts with thiobarbituric acid to form a chromogenic adduct with two molecule of TBA, which is a pink colored complex and is spectrophotometrically measured at 532nm. The malondialdehyde content was calculated as TBARS and expressed in terms of nm/100 mg of tissue, using the molar extinction co-efficient, 1.56×10^5 $M^{-1}cm^{-1}$ [23,24].

Statistical analysis

The data were expressed as Mean values ± S.E.M and were compared with that of hepatotoxic positive control (CCl_4 treated) and statistically analyzed by One Way ANOVA followed by Tukey-Kramer multiple comparison test.

RESULTS AND DISCUSSION

Oxidative stress is the result of imbalance between prooxidants and antioxidants and it is a major factor involved in the pathogenesis of many diseases such as heart diseases, hypertension, cancer, diabetes, inflammation, asthma, Alzheimer etc. Traditionally plant based antioxidant rich foods form a major part of our diet and plant- based antioxidants are supposed to play an important role in the maintenance of health in human beings[25].

Acute toxicity studies revealed that the lethal dose of *S. zeylanica* roots and rhizomes (SZRM) was found to be higher than 2000mg/kg bw. CCl_4 induced hepatotoxicity is characterized by an increase in liver mass which was

evident in the positive control group (Figure 1). CCl_4 intoxification significantly lowered total protein levels (by 66.95%) and at 400 and 600 mg/kg of SZRM, protein levels were significantly ($p<0.01$) increased (Table 1).

Figure-1: Effect of methanol extract of roots and rhizomes of *S. zeylanica* L. on liver weight

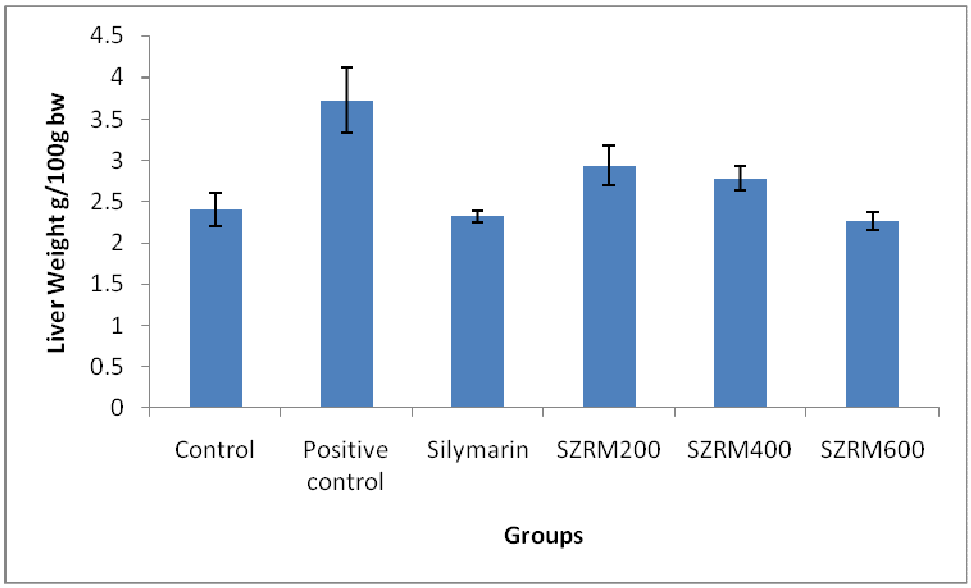


Table-1: Effect of methanol extract of *S. zeylanica* L. roots and rhizomes on in vivo antioxidant parameters.

Treatment	Total Proteins mg/dl	Catalase U/mg tissue	SOD U/mg tissue	Peroxidase nm/100mg tissue	Glutathione nm/100mg tissue	MDA nm/g protein
Normal Control	10.58 ±0.845	7.63 ±0.62	2.94± 0.12	24.74 ±0.49	53.65 ±0.33	2.95 ±1.8
Positive control	3.53 ±0.872	1.91± 0.46	1.70± 0.16	17.86±1.1	43.06± 0.36	27.41± 1.56
Silymarin 100 mg/kg	12.04 ±3.23**	1.98±0.31	3.67± 0.07**	25.09± 0.13**	53.49± 0.13**	3.12± 0.99***
SZRM 200 mg/kg	7.85 ±1.09	2.58±0.22	2.96± 0.33***	28.71± 0.06***	54.0± 0.34**	17.54± 1.5***
SZRM 400 mg/kg	10.91± 1.44*	2.7±0.31	3.69± 0.11***	27.88± 0.27***	55.76± 0.87***	7.79± 0.95***
SZRM 600 mg/kg	11.42± 1.65*	2.8±0.41	3.74± 0.06***	27.94± 0.30***	55.81± 1.1***	7.73± 0.66***

Values expressed as Mean ± SEM.

Tukey Kramer multiple comparison test *p<0.05, **p<0.01 and ***p<0.001, when compared against vehicle treated positive control.

Carbon tetrachloride produces significant reduction in catalase, GSH-Px, GSSGR, SOD and peroxidase with a significant elevation in MDA formation. Catalase levels decreased by 74.96%, SOD by 42.17% and peroxidase by 27.8% in the liver of positive control animals compared with that of normal control animals. Catalase levels did not increase to a significant extent with drug treatment. SOD and peroxidase levels increased significantly [$p<0.01$(silymarin) and $p<0.001$(SZRM)] in all the treated groups. The effect of SZRM was independent of dose (Table 1).

Glutathione (GSH) is one of the abundant tripeptide non-enzymatic biological antioxidants present in liver[26,27]. It acts as a substrate for the H_2O_2 removing enzyme glutathione peroxidase and for dehydroascorbate reductase[28]. CCl_4 administration markedly decreased liver glutathione levels in positive control animals when compared to the normal control animals. Treatment with silymarin and SZRM significantly increased the hepatic levels of glutathione ($p<0.01$ for silymarin and SZRM 200 mg/kg; $p<0.001$ for SZRM 400 and 600 mg/kg) (Table 1).

Lipid per oxidation is considered to be a destructive process which occurs in liver damage due to CCl_4 intoxication. Lipid peroxidation is an index of oxidative stress and is generally measured as malondialdehyde (MDA), which is a lipid peroxidative end product[29]. SZRM and silymarin showed significant ($p<0.001$) protective effects against lipid peroxidation, irrespective of the dose (Table 1).

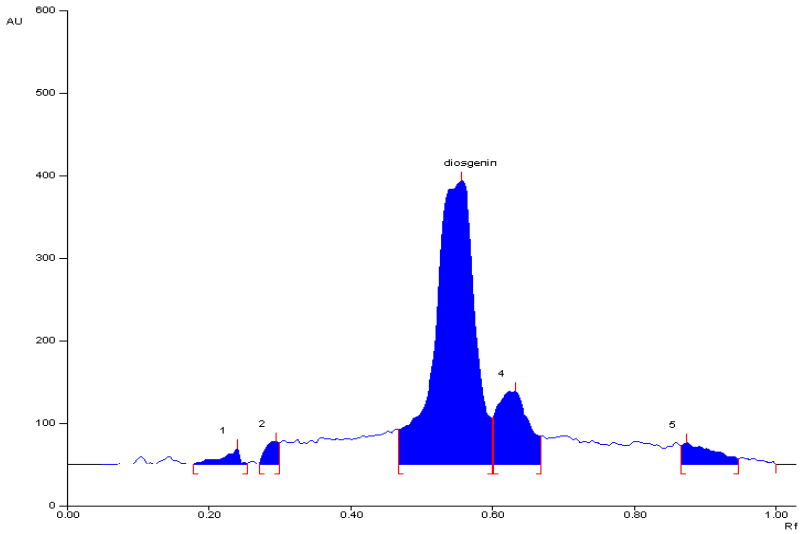
The antioxidant enzymes SOD, catalase and peroxidase constitute a mutually supportive team of defense against ROS. Catalase is an enzymatic haemoprotein localized in the peroxisomes and micro- peroxisomes**.** It decomposes hydrogen peroxide to water and oxygen, protects tissues from highly reactive hydroxyl radicals[30]. The reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide[31]. When the balance between ROS and antioxidant defences is lost, oxidative stress develops which through a series of events lead to the development of various diseases. Any compound natural or synthetic with antioxidant properties alleviates this damage partially or

totally. In this study, decline in the level of antioxidant enzymes in the positive control group is a clear indication of the development of oxidative stress and lipid peroxidation which has resulted in tissue damage.

The present study revealed that SZRM prevented excessive free radicals accumulation and protected the liver from CCl_4 intoxication, by inducing antioxidant enzymes which in turn detoxify the free radicals produced following carbon tetrachloride intoxication.

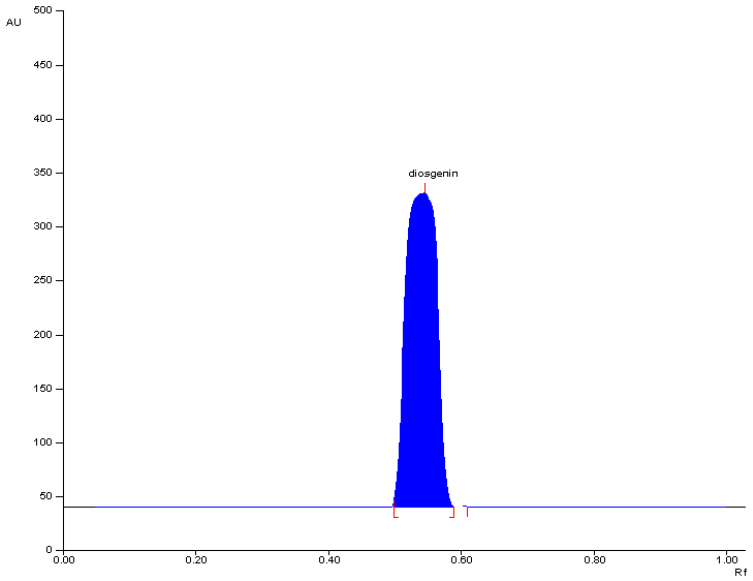
Preliminary phytochemical studies revealed the presence of glycosides, phytosterols, saponins, phenolic compounds and tannins. HPTLC analysis was carried out to confirm the presence of diosgenin in the methanol extract (Figures 2 and 3). Polyphenols and tannins are reported to possess antioxidant activity[32,33,34]. Similarily antioxidant potential of diosgenin[35,36,37], and β- sitosterol[38] have been experimentally proved. The presence of these phytoconstituents could have contributed to the antioxidant effect of *S. zeylanica.*

Figure 2: HPTLC fingerprinting of diosgenin in the extract of *S. zeylanica* L. roots and rhizomes at 254 nm.



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.18 Rf	0.1 AU	0.24 Rf	19.0 AU	3.77 %	0.25 Rf	1.3 AU	344.5 AU	1.73 %	unknown *
2	0.27 Rf	0.8 AU	0.29 Rf	27.8 AU	5.50 %	0.30 Rf	26.4 AU	425.1 AU	2.13 %	unknown *
3	0.47 Rf	42.2 AU	0.56 Rf	343.3 AU	68.06 %	0.60 Rf	56.3 AU	15141.5 AU	75.99 %	diosgenin
4	0.60 Rf	57.4 AU	0.63 Rf	88.2 AU	17.49 %	0.67 Rf	34.6 AU	3066.1 AU	15.39 %	unknown *
5	0.87 Rf	22.9 AU	0.87 Rf	26.1 AU	5.18 %	0.95 Rf	7.0 AU	947.8 AU	4.76 %	unknown *

Figure-3: HPTLC Chromatogram of standard diosgenin at 254 nm.



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.50 Rf	5.2 AU	0.55 Rf	290.2 AU	100.00 %	0.59 Rf	2.3 AU	10791.8 AU	100.00 %	diosgenin

CONCLUSION

Our study revealed that *S. zeylanica* exhibited strong antioxidant property. Moreover *S. zeylanica* extract was found to be relatively non toxic which renders potential therapeutic potential for the drug. Thus this study supports the fact that *S. zeylanica* can be considered as one of the potential alternate sources of the drug Chopachinee. Further work may be undertaken to isolate and evaluate the active principle(s) and elucidate exact mechanism of action.

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