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Research Article

IN VITRO ANTIOXIDANT ACTIVITY AND HPTLC STUDIES ON THE ROOTS AND RHIZOMES OF *SMILAX ZEYLANICA* L. (SMILACACEAE)

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ABSTRACT

The present study was conducted to investigate the antioxidant property of roots and rhizomes of *Smilax zeylanica* L. by *in vitro* methods. Methanol and aqueous extracts of the drug were evaluated for *in vitro* antioxidant activity. Methanol extract showed potential scavenging effect against DPPH, hydrogen peroxide and ABTS free radicals. HPTLC studies were also performed on both extracts. The study revealed that the extracts of *S. zeylanica* exhibited strong antioxidant effect in different *in vitro* systems

Keywords: ABTS assay; DPPH assay; hydrogen peroxide scavenging assay; *in vitro* antioxidant activity; nitric oxide radical scavenging; superoxide radical scavenging; *Smilax zeylanica*.

INTRODUCTION

A free radical is an atom or group of atoms that has at least one unpaired electron and is therefore unstable and highly reactive. Human beings are exposed to free radicals in the environment through radiation and pollution. Free radicals attack and damage cell membrane, cell organelles including DNA, leading to faulty translation of genetic material [1,2]. Their action is opposed by a balanced and co-ordinate 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. Upsetting this balance causes oxidative stress, which can lead to cell injury and death[3].

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[4].

The genus *Smilax* L. (Smilacaceae) consists of more than 300 species, distributed all over the world, out of which 24 are found in India[5]. Four species viz. *Smilax aspera* Linn., *Smilax perfoliata* Lour., *Smilax wightii* A.DC. and *Smilax zeylanica* L. (Smilacaceae) occur in the forests and hills of South India[6,7]. 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)[9] and *S. china* L.[10](European sarsaparilla) 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 HPTLC and *in vitro* antioxidant activities of *S. zeylanica* roots and rhizomes are presented.

MATERIALS AND METHODS

Chemicals and instrument

Chemicals: 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenz- thiazoline-6-sulfonic acid) diammonium salt (ABTS), were procured from Sigma-Aldrich, California, USA. Ascorbic acid and nitro blue tetrazolium (NBT) were obtained from S.D. Fine Chem, Ltd., Biosar, India. Sulphanilic acid was procured from Hi-media Laboratories Ltd., Mumbai, India.

Instrument: UV spectrophotometer (Shimadzu- UV-1601),

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[6], 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.

Preparation of extracts

The air-dried powdered plant material was successively extracted with solvents of increasing polarity, starting from petroleum ether (60-80°C) followed by chloroform and methanol. Finally, the marc was macerated with chloroform-water (0.25%v/v of chloroform in distilled water) for 24 h to obtain the aqueous extract. Methanol (SZRM) and aqueous (SZRA) extracts were concentrated under reduced pressure. The extracts were weighed and corresponding yields were 3.96%w/w for SZRM and 2.84%w/w for SZRA, calculated in terms of air-dried weight of plant/ drug material. The concentrated extracts were subjected to preliminary phytochemical tests for identification of various phytoconstituents[11].

Chromatographic studies

TLC and HPTLC studies were carried out on SZRM and SZRA following prescribed methods [12]. 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.

Table 1: Shows the preparation of mobile phases

Phytoconstituent	Solvent system	Solvent volume ratio
Glycosides	Ethyl acetate: Methanol: Water	100: 13.5: 10
Diosgenin	Toluene : Ethyl acetate	7:3
β- Sitosterol	Toluene: Diethyl ether	1:1
Polyphenolic compounds and tannins -	Ethyl acetate: Benzene: Formic acid	9:11:0.5
Saponins	Chloroform: Glacial acetic acid: Methanol: Water	60: 32: 12: 8
Sarsapogenin/ Smilagenin	Chloroform: Glacial acetic acid: Methanol: Water	60: 32: 12: 8

Post derivatisation reagent (Liebermann Burchard reagent) for diosgenin

Liebermann Burchard reagent was prepared by adding 10 ml each of acetic anhydride and conc. sulphuric acid to 100 ml of ethanol.

Aluminium sheet precoated with silica gel 60 $F_{254,}$ (5 X 10 cm) (MERCK) as adsorbent was used as stationary phase.

Procedure

The precoated silica gel 60 F_{254} TLC plates were activated by heating at 120^{o}C for about 30 min prior to use. 4 and 6 µl volumes of the extracts (5mg/ml) were applied with Linomat V applicator using a Hamilton syringe. The chambers were saturated overnight with the respective mobile phases. No prewashing of the plates was done. The TLC plates were developed, dried, (and post derivatised in case of diosgenin) and scanned successively at 254 nm, 366 nm and 425 nm, with slit dimension 6.00 x 3.00 nm, and scanning speed 20 nm/sec. The source of radiation was deuterium lamp, mercury lamp and tungsten lamp respectively. The R_f values and peak area were interpreted using the software. The developed plates were photo documented using Camag Reprostar 3 and photographs taken using 254 nm, 366 nm and visible light.

***In vitro* antioxidant activity**

The *in vitro* scavenging activities of the methanol and aqueous extracts of *S. zeylanica* roots and rhizomes against different free radicals were performed. The results are expressed in terms of IC_{50}, which is the concentration of the sample required to cause 50% inhibition of free radicals. Ascorbic acid was used as standard and the experiments were performed in triplicate.

DPPH free radical scavenging activity

To 200 µl of 100 µM DPPH solution, 10 µl of various concentrations SZRM, SZRA or the standard solution (both in DMSO), was added separately. They were incubated at 37 ^{o}C for 30 min. Absorbance was measured at 490 nm[13]. The ability to scavenge DPPH radical was calculated using the following equation.

$$\text{DPPH Scavenged (\%)} = \frac{\text{A (control)--A (test)}}{\text{A (control)}} \times 100$$

Scavenging of ABTS radical

To 50 ml of 2 mM ABTS in distilled water, potassium persulphate (17 mM, 0.3 ml) was added. It was left to stand at room temperature overnight in dark before usage. To 0.2 ml of various concentrations of SZRM, SZRA or standard in DMSO, 1.0 ml of DMSO and 0.16 ml of ABTS solution were added to make a final volume of 1.36 ml. Absorbance was measured after 20 min at 734 nm[14].

Scavenging of hydrogen peroxide

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of 1 ml of the SZRM, SZRA or standard in methanol were added to 2 ml of hydrogen peroxide solution. The absorbance was measured after 10 min at 230 nm[15].

Nitric oxide radical inhibition

The extracts and the standard ascorbic acid solutions were prepared in DMSO.

Preparation of reagents

1. Sodium nitroprusside: 10 mM solution of sodium nitroprusside was prepared in distilled water.
2. Naphthyl ethylene diamine dihydrochloride (NEDD, 0.1%): 0.1 g of NEDD was dissolved in 60 ml of 50% glacial acetic acid by heating and then diluted to 100 ml with distilled water.
3. Sulphanilic acid (0.33%) reagent: 0.33 g of sulphanilic acid was dissolved in 60 ml of 20% glacial acetic acid by heating and then diluted to 100 ml with distilled water.

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), PBS (pH 7.4, 1 ml) and SZRM, SZRA or standard solution (1 ml) were incubated at room temperature for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed, 1 ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 min for completion of diazotization. Then, 1 ml of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A pink colored chromophore was formed. The absorbance was measured at 540 nm[16].

Scavenging of superoxide radical by alkaline DMSO method

To the reaction mixture containing 1 ml of alkaline DMSO (1 ml of DMSO containing 5 mM NaOH in 0.1 ml of water) and 0.3 ml of SZRM, SZRA or standard (in DMSO), 0.1 ml of NBT (1 mg/ml) was added to give a final volume of 1.4 ml. The absorbance was measured at 560 nm[17].

RESULTS AND DISCUSSION

DPPH assay

DPPH is usually used as a substrate to evaluate antioxidant activity. DPPH assay is based on the measurement of the scavenging ability of antioxidant towards the stable DPPH radical[18]. The method is based on the reduction of purple colored methanol solution of DPPH in the presence of hydrogen donating antioxidants, by the formation of yellow colored non radical form of DPPH. Lower the absorbance higher the free radical scavenging activity. SZRM and SZRA were able to reduce purple colored DPPH to yellow colored picryl hydrazine. The methanol extract (SZRM) with IC_{50} value 7.6± 0.44µg/ml was more effective than the aqueous extract (SZRA) with IC_{50} value 125±0.13 µg/ml. the extracts showed an inhibition in free radical production with the methanol extract showing almost an equipotent effect with standard antioxidant ascorbic acid. The scavenging effect may be due to the hydrogen donating ability of *S. zeylanica*.

Scavenging of ABTS radical

The ABTS assay is based on the inhibition of the absorbance of radical cation, $ABTS^{+}$, which has a characteristic wavelength at 734 nm, by antioxidants. In this assay, the ABTS radical which is a blue green chromogen, is generated in a stable form using potassium persulphate. The formed colored radical is then mixed with antioxidant in the reaction medium and the colored radical is converted back to colorless ABTS[14]. In ABTS assay, SZRM exhibited potent scavenging effects against ABTS with an IC_{50} value 3.0± 0.033 µg/ml better than that of standard ascorbic acid with IC_{50} value 4.25± 0.29 µg/ml. The effect of aqueous extract (SZRA) was moderate (IC_{50} value 20.66±0.33 µg/ml).

Scavenging of hydrogen peroxide

Hydrogen peroxide is generated *in vivo* by several oxidase enzymes. In this method, when an antioxidant is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide is measured spectrophotometrically[15]. Hydrogen peroxide is a weak oxidizing agent which inactivates enzymes by oxidation of the essential thiol (SH-) groups. It rapidly transverses cell membranes and once inside the cell interior, interacts with Fe^{2+} and Cu^{2+} to form hydroxyl radicals, which is harmful to the cell[19]. The extracts showed good scavenging effects with IC_{50} values135±5.0 µg/ml and 127.5± 7.5µg/ml for SZRM and SZRA respectively.

Nitric oxide radical inhibition

Nitric oxide is implicated in diseases such as cancer and inflammation [20]. It also mediates smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated cytotoxicity[21]. Sodium nitroprusside spontaneously generates nitric oxide at physiological pH, in aqueous solutions. The nitric oxide, generated is converted into nitric and nitrous acids on contact with dissolved oxygen and water. The liberated nitrous acid was estimated using a modified Griess-Illosvoy method. Nitrous acid reacts with Griess reagent, to form a purple azo dye. In presence of antioxidants, the amount of nitrous acid will decrease and the degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. Methanol extract showed moderate nitric oxide scavenging effects with IC_{50} 176.66±46.75 µg/ml, whereas that of standard antioxidant

ascorbic acid was 93.0±0.61 µg/ml. Aqueous extract (SZRA) was comparatively less effective with an IC_{50} 350.0±0.79 µg/ml.

Scavenging of superoxide radical by alkaline DMSO method

The scavenging activity of extracts against superoxide radical generated in NAOH- alkaline DMSO- NBT system, resulting in the formation of the blue formazan was studied. In this method, superoxide radical is generated by addition of sodium hydroxide to air saturated dimethyl sulfoxide (DMSO). The generated superoxide remains stable in solution, which reduces nitro blue tetrazolium into formazan dye at room temperature. Superoxide scavenger capable of reacting inhibits the formation of a red dye formazan[22]. The extracts inhibited the formation of the formazan with SZRA (IC_{50} 62±0.22 µg/ml) showing more effect than SZRM (IC_{50} 125±0.28 µg/ml). Superoxide radical which is a precursor of many ROS, is shown to be harmful for various cellular components[3]. The studies showed that, the extracts of *S. zeylanica* roots and rhizomes exhibited significant effects against superoxide radical when compared with ascorbic acid. The results of in-vitro antioxidant studies are presented in Table2.

Table 2: Shows *in vitro* antioxidant studies on roots and rhizomes of *S. zeylanica*

Test	IC_{50} values (µg/ml) Mean ± SEM		
	SZRM	SZRA	Standard(ascorbic acid)
DPPH	7.6± 0.44	125±0.13	2.69± 0.14
ABTS	3.0± 0.033	20.66±0..33	4.25± 0.29
Hydrogen Peroxide	135±5.0	127.5± 7.5	197.5± 1.01
Nitric oxide	176.66±46.75	350.0±0.79	93.0± 0.61
Superoxide	125.0±0.28	62.0 ± 0.22	>1000.0

Each Value represents Mean ± SEM (n=3)

The phytochemical and HPTLC studies showed the presence of glycosides, phytosterols, polyphenols and tannins in methanol extract and saponins in the aqueous extract. HPTLC studies were carried out to confirm the presence of the reported phytoconstituents diosgenin, smilagenin and β- sitosterol (Yoganarasimhan, 2000**)** (Figures 1-4). The presence of these phytoconstituents could have contributed to the antioxidant effect of *S. zeylanica.*

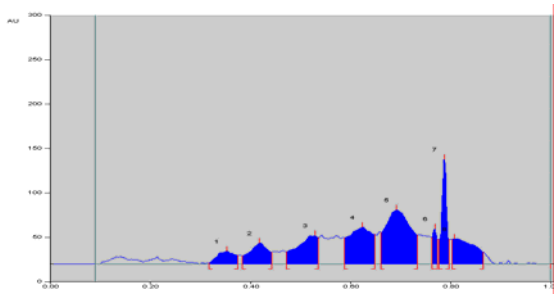

Fig. 1: HPTLC profile of Smilagenin in methanol extract of roots and rhizomes of *S. zeylanica* L. at 366nm

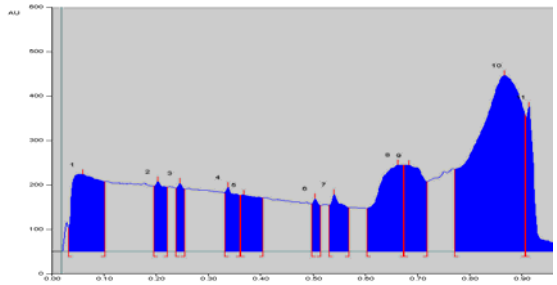

Fig. 2: HPTLC profile of saponins in aqueous extract of roots and rhizomes of *S. zeylanica* L. at 366nm

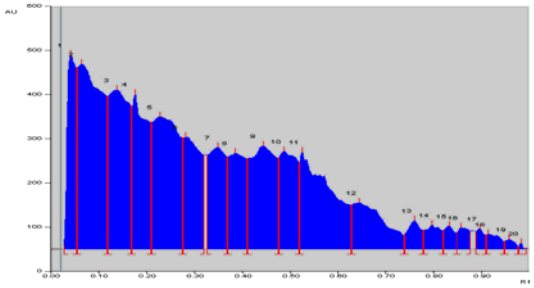

Fig. 3: HPTLC profile of β Sitosterol in methanol extract of roots and rhizomes of *S. zeylanica* L. at 366nm

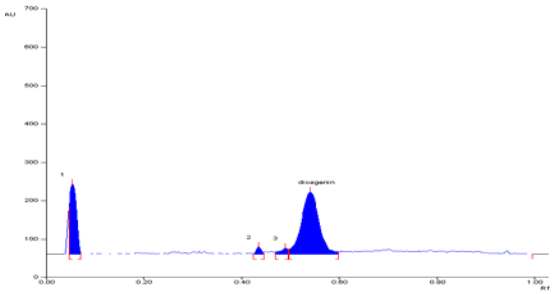

Fig. 4: HPTLC profile of diosgenin in aqueous extract of roots and rhizomes of *S. zeylanica* L. at 425 nm

CONCLUSION

Our study revealed that the extracts of *S. zeylanica* exhibited strong antioxidant and free radical scavenging effect in different *in vitro* systems. Hence further studies are needed to evaluate the *invivo* antioxidant potential of these extracts in various animal models.

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