Research Paper

Phytoconstituents Screening and Antioxidant Activity of *Syringodium isoetifolium* Leaf Extracts

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Kavitha *et al.*: Phytoconstituents and Antioxidant effect of Seagrass

This research aimed to explore the antioxidant potency of leaf extracts of *Syringodium isoetifolium* seagrass. In this study, seagrass leaves were collected from the local tribe's source, dried and extracted with water, alcohol, hydro alcohols, acetone and n-hexane. Phytochemical investigation on extracts was performed using chemical and gas chromatography-mass spectrometric techniques. It reveals that the extracts are enriched with pharmacologically active compounds such as phenol, polyphenol, alkaloid, glycoside, saponin, flavonoids, dicarboxylic acids and other constituents. Later, the crude extracts were estimated for antioxidant activity using different *in vitro* methods such as 2,2-diphenyl-1-picryl-hydrazyl-hydrate, total antioxidant, nitric oxide, superoxide anion and hydroxyl radical scavenging activities. The result demonstrated that the extracts exhibited higher radical scavenging activity as compared to the ascorbic acid. Among the different extracts, the hydroalcoholic extract had shown an excellent antioxidant activity due to the existence of more potent phytochemicals.

Key words: *Syringodium isoetifolium*, leaf extracts, phytochemical screening, antioxidant activity

The ecosystem is the structural and functional component of ecology where the living things interact with each other and the environment[1]. There are many ecosystems existing in the galaxy[2]. Marine ecosystems are the main ecosystems and exist in sea waters[3]. In which, seagrasses are a vital part of the marine ecosystem, found in shallow, sheltered, soft-bottomed marine waters[4]. They were found all along with the coastal areas of India[5,6]. These are belonging to the families of Cymodoceaceae, Zoseraceae, Hydrocharitaceae and Posidoniaceae[7]. Around the earth, 52 species of seagrasses were found so far in which 14 species were identified in the west and east coastal part of India[8]. The biomass of seagrass has been utilized frequently as food and drug by coastal indigenous people[9]. Seagrasses are well documented for the presence of potent diverse secondary metabolites. There is an immediate need to quantitatively survey the traditional knowledge of seagrasses in areas where they are abundant and serve as an important resource to coastal communities. In folk medicine, seagrasses have been employed for many therapeutic purposes such as skin diseases, fever, wounds, stomach problems, muscle pains and as a remedy against different kinds of rays[10]. They also provide different pharmacological activities like antioxidant[11], anti-microbial[12], anti-viral[13], against stomach problems[14], anti-diabetic[15], wounds[16], tranquillizer[17] and anticancer[18] activities etc. Human pollution has contributed most to seagrass declines around the world. There is a necessity to take some initiation to preserve the seagrass and ensure the existence of seagrass to the poor people in future. For many decades, herbal medicines have been used in developing countries as the primary source of medical treatments. The root of *Cymodocea* sp. is eaten as food commonly known as sea sugarcane. Some of *Cymodocea* sp. are used against malaria, cough and also used as tranquilizers for babies. *Halophila ovalis* was used by the fishing communities of Cuddalore and Nagapattinam districts of Tamil Nadu, South India as medicine to treat various skin diseases, burns and boils. *Cymodocea* sp. are being used as a tranquilizer for babies, as soothing helps during pregnancy and against cough and malaria. *Halophila stipulacea* (Forssk.) Asch., *Cymodocea* serrulata (R. Br.) Asch and Magnus and *Halodule pinifolia* (Miki) Hartog possessed effective antimicrobial effects against seven human pathogens[19,20]. From the past few decades, there has

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been an upsurge in the search for antioxidant drugs derived from plants.

In the review of the above observation and continuation of our interest to identify the therapeutic activity of *Syringodium isoetifolium* (*S. isoetifolium*), antioxidant activity was evaluated. The result suggested that all the extracts of *S. isoetifolium* were shown excellent activity like standard drugs and it is an important milestone in the development of a naturally derived antioxidant drug for various oxidative stress-related diseases.

MATERIALS AND METHODS

Collection of seagrass:

An excellent quality of *S. isoetifolium* from the family of Cymodoceaceae was collected from Devipattinam, Ramanathapuram District, Tamilnadu state, India. The collected materials of the plants were authenticated by Dr M. U. Sharief, Scientist E and head, Botanical Survey of India, Southern Regional Centre, T. N. A. U Campus, Coimbatore 641003, India. The Voucher specimen number of the *S. isoetifolium* is BSI/SRC/5/23/2021/Tech/372.

Preparation of seagrass extracts:

Collected seagrasses were washed thoroughly with sterile seawater to remove the extraneous dirt, dried in a shade, pulverized and prepared extracts with different solvents of increasing polarity such as distilled water, alcohol, hydro alcohol (30:70), acetone and n-hexane. After drying, the seagrass were sliced into small pieces and then mashed until it becomes a fine powder. The powdered seagrass was immersed in 450 ml of cold solvents in a closed container with gentle shaking for 24 h. These seagrass extracts were collected, filtered and the solvent was eliminated at 60° in the rotary evaporator (model: rotavapor R-210 from Buchi)[21,22]. The solid seagrass extracts have persevered in a dark container (fig. 1). The yield was calculated using the following formula

Percentage Yield=$(W_1 \times 100)/W_2$

Here W_1-Weight of extract after removing the solvent; W_2-Dry weight of the sample.

These plant extracts were utilized for the identification of different phytochemicals present in *S. isoetifolium*.

Quality control of *S. isoetifolium*:

Determination of ash value: 3 gm of *S. isoetifolium* powder was incinerated in a Silica crucible over the burner. The charred material was heated in muffle furnace for 6 h at 600°.The ash formed was white and free from carbon. It was cooled and weighed on the ash less filter paper[23].

Powder fineness and sieve size: Place 100 g of the powder being examined upon the appropriate sieve having a close fitting receiving pan and cover. Shake the sieve in a rotary horizontal direction and vertically by tapping on the hard surface for not less than 20 min. Weigh accurately the amount remaining on the sieve and in the receiving pan[24].

Phytochemical analysis:

The collected dried extracts of *S. isoetifolium* were analyzed for the presence of different phytochemicals by chemical and Gas Chromatography-Mass Spectrometry (GC-MS) techniques. In the chemical method, phytochemicals were identified through qualitative and quantitative ways using appropriate reagents. Whereas, GC-MS compounds were recognized through their mass value and distinctive mass fragmentation patterns[25].

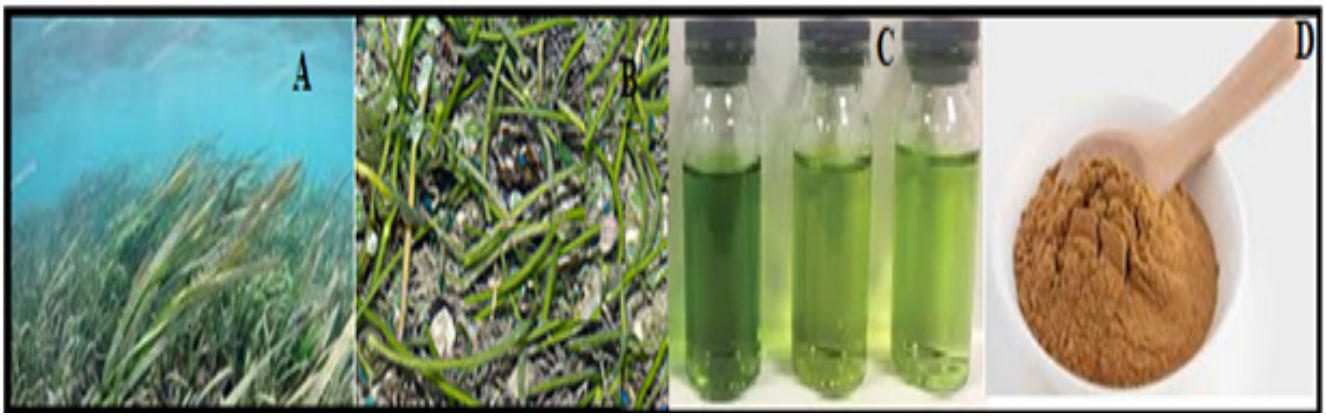

Fig. 1: Image of *S. isoetifolium* in shallow and (A): Deep water; (B): Shade dry; (C): Liquid extracts and (D): Dried extracts

Chemical method:

Chemicals: All chemical substances were purchased with the highest purity (≥98.0 %) and utilized for the identification of different phytochemicals present in the leaf extract of *S. isoetifolium*. The chemicals such as Ferric chloride ($FeCl_3$), Olive oil, Folin-Ciocalteu reagent, Aluminium chloride ($AlCl_3$), Ammonia, Sulfuric acid (H_2SO_4), Hydrochloric acid (HCl), Acetic anhydride, Chloroform, Mayer reagent, Ethanol, Ferricyanide, Glacial acetic acid, Sodium hydroxide (NaOH), Ammonium hydroxide (NH_4OH), Gallic acid, Sodium carbonate (Na_2CO_3), Ammonium chloride (NH_4Cl), Methanol, Potassium acetate (CH_3COOK), Folin-Denis reagent, 2,2-Diphenyl-1-Picryl-Hydrazyl-Hydrate (DPPH), Sodium phosphate, Ammonium molybdate, Sodium nitroprusside, Phosphate buffer, Sulfanilic acid reagent, 1,10-phenanthroline, Butylated Hydroxyl Toluene (BHT) etc., were all purchased from Merck, United States of America (USA).

Qualitative phytochemicals screening:

Chemical tests were performed with different reagents using standard procedures to know the phytochemicals present in the leaf extracts of *S. isoetifolium*[26].

Quantitative analysis of phytochemicals:

Total Phenolic Content (TPC): Approximately 1 ml of seagrass extract or Gallic acid standard phenolic compound was mixed to 9 ml of distilled water in a volumetric flask. About 1 ml of Folin-Ciocalteu's phenol reagent was added and blended gently. After a few minutes, nearly 10 ml of 7 % Na_2CO_3 solution was admixed into the above solution. The solution was diluted to 25 ml of purified water. The concoction was kept aside for 1.5 h at 23°, after which the absorbance was examined at 750 nm. The TPC was calculated from the extrapolation of calibration curve which was made from the different concentrations of seagrass/gallic acid solutions (20 to 80 µg/ml). The TPC was expressed as milligrams of Gallic acid equivalents per gram in a dried seagrass sample[27].

Total Flavonoids Content (TFC): The TFC was conducted according to Hossain *et al.*[28] using the colorimetric method. About 0.5 ml of seagrass extracts were mixed with 1.5 ml of methyl alcohol, 0.1 ml 10 % $AlCl_3$, 0.1 ml 1 M CH_3COOK and 2.8 ml of water. The above mixture remained at 20°-25° for 30 min and absorbance was measured at 510 nm. The calibration curve was made using quercetin solutions at the concentration of 20 to 80 µg/ml in methyl alcohol. TFC was disclosed as mg of QE/g of dry weight[28].

Total Saponins Content (TSC): The TSC in different concentrations of plant extracts were estimated by colorimetric methods. 0.25 ml of seagrass extract admixed with 1 ml of reagent mixture (H_2SO_4/glacial acetic acid 1:1 v/v). The content was heated at 60° for 30 min in a water bath and kept aside for some time. The absorbance of seagrass extract was calculated at a wavelength of 527 nm using a spectrophotometer. The TSC was expressed as 100 g^{-1} of oleanolic acid equivalents[29].

Total Tannin Content (TTC): The TTC was performed according to Medini *et al.*[30] method. 0.2 ml of seagrass extract admixed with 0.5 ml of Folin-Denis reagent, 1 ml of Na_2CO_3 solution and 7.5 ml of water. The content of the concoction was mixed well, kept aside for 30 min and the absorbance was measured at 760 nm. TTC was expressed as milligrams of tannic acid equivalents per gram of dried sample. The results were evaluated by correlation coefficient (R^2) and linear regression and using Microsoft excel[30].

GC-MS analysis:

GC-MS analysis of aqueous, ethanol and hydro alcoholic extracts of *S. isoetifolium* was done at SRM institute of science and technology, Kattankulathur, Chennai using Agilent 7890B GC connected to 5977A mass selective detector, furnished with Electron Ionization (EI) and a fused phenyl methyl silox column HP-5 (30 m×250 µm×0.25 µm film thickness) was used. The oven temperature was modified from -60°-325° for 55 min. Highly pure helium gas was used as carrier for this study. The flow rate of carrier gas was fixed 1 ml/min, for sample injection of 1 µl and the ionization voltage of MS-analysis was controlled by EI procedure at 70 eV[31].

Antioxidant activity:

Preparation of sample: *S. isoetifolium* leaves were air-dried at room temperature and pulverized into powder for extraction. The powder (20 g) was macerated in 1000 ml of hydro alcohol (30:70 %), shake vigorously for about 1 h using a rotatory shaker. The mixture was kept aside for 24 h and then the extracts were filtered with Whatman filter paper (No.1). The filtrate was dried using a water bath and prepared a range of concentrations (20, 40, 60 and 80 µg/ml) for *in vitro* antioxidant activity[31].

DPPH radical-scavenging activity:

The activity was done according to Gopi *et al.*,[32]. Take a 2 ml DPPH methyl alcohol solution (25 µg/ml) and mix with 0.5 ml seagrass extracts at various concentrations. The above content was stirred and allowed to rest at 30°-35° for 30 min. The absorbance was calculated at 517 nm.

Percentage radical scavenging activity=100-(A_c-A_s)/A_c×100

Where A_C=Absorbance in control; A_S=Absorbance of extracts.

Total Antioxidant Capacity (TAC):

The phosphomolybdate technique has been employed regularly to assess the TAC of seagrass extracts[33]. In the existence of seagrass extracts, a green-colored phosphomolybdenum V complex formed, which shows absorbance at 695 nm. 0.3 ml of seagrass extracts was mixed with 3 ml of reagent (28 M sodium phosphate, 0.6 M H_2SO_4 and 4 M ammonium molybdate) in a tube. It was incubated at 95° for 1.5 h. The absorbance was measured at 695 nm. Methyl alcohol (0.3 ml) is used as a blank[31]. The TAC was calculated according to the following equation Percentage inhibition=A_0-A_1/A_0×100

Where A_0=Absorbance of the control and A_1=Absorbance of the extracts.

Superoxide anion scavenging activity:

It was measured according to Pavithra *et al.*[34]. In this experiment, the superoxide anion was produced in 3 ml of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 ml of Nicotinamide Adenine Dinucleotide (NADH) (936 µM) solution, 0.75 ml of nitro blue tetrazolium chloride (300 µM) solution and 0.3 ml of various concentrations of the extracts. The reaction started with the addition of 0.75 ml of phenazine methosulfate (120 µM) to the mixture. After 5 min at room temperature, the absorbance was measured at 560 nm. The activity was calculated according to the following formula

Percentage inhibition=A_0-A_1/A_0×100

Where A_0=Absorbance of the control and A_1=Absorbance of the seagrass extracts.

Nitric Oxide (NO) scavenging activity:

The NO scavenging activity was determined according to Ali *et al.*,[35]. Sodium nitroprusside in an aqueous solution produces NO, which reacts with oxygen to generate Nitrite ions (NO_2^-). Take 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of seagrass extracts at different concentrations and the mixture was incubated at 25° for 2.5 h. After incubation, 0.5 ml was taken out and added into a 1.0 ml sulfanilic acid reagent (33 % of sulfanilic acid in 20 % glacial acetic acid) and incubated at 30°-35° for 5 min. Finally, 1.0 ml naphthyl ethylenediamine dihydrochloride (0.1 % w/v) was added and incubated 30°-35° for 30 min. The absorbance was measured at 540 nm using a spectrophotometer. The scavenging activity was measured by using the following equation

Percentage inhibition=A_0-A_1/A_0×100

Where A_0=Absorbance of the control and A_1=Absorbance of the extracts.

Hydroxyl radical scavenging activity:

Hydroxyl radicals scavenging activity was calculated by Fenton reaction according to Sowndhararajan *et al.*[36]. The hydrogen peroxide was added to the reaction mixture containing 90 µl of 1 mM 1, 10-phenanthroline, 60 µl of 1.0 mM $FeCl_3$, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2 and 1.5 ml of seagrass extracts at different concentrations. After incubation at 30°-35° for 5 min, the absorbance was measured at 560 nm using a spectrophotometer. The radical (OH.) scavenging activity was calculated using the following formula

Percentage inhibition=A_0-A_1/A_0×100

Where A_0=Absorbance of the control and A_1=Absorbance of the extracts.

Statistical method:

All the scavenging activities were done in triplicates. The quantity of seagrass required to inhibit radicals concentration by 50 % was graphically calculated by a linear regression method using GraphPad Instat software (version 3). The result is shown as graphically/mean±standard deviation.

RESULTS AND DISCUSSION

An excellent quality of leaf of *S. isoetifolium* from the family of Cymodoceaceae was collected from Devipattinam, Ramanathapuram District, Tamilnadu state, India. Collected seagrasses were washed thoroughly with sterile seawater to remove the extraneous dirt, dried in a shade, pulverized into fine

powder. It was used to study the quality control test such as ash value and degree of coarseness or fineness of the powder. The ash content of the seagrass was determined using a dry ashing method. It shows leaf of *S. isoetifolium* contained ash value of 29.42 %±0.35 %. The degree of coarseness or fineness of a powder is differentiated by nominal aperture size of the mesh of the sieve through which the powder is able to pass, expressed in µm. The particles of the powder which pass through a No. 355 sieve and not more than 40 % through a No.180 sieve. Therefore, the coarseness or fineness of the leaf powder is moderately fine. The results of this study are in line with the comparative proximate composition values for different species of the seagrasses. The presence of phytochemicals was identified through the chemical methods and GC-MS technique. The phytochemicals were confirmed in the chemical method through the qualitative and quantitative tests using different reagents (Table 1). Several phytochemicals such as tannin, saponin, steroids, terpenoids, triterpenoids, anthraquinone, flavonoids, polyphenol, glycosides, alkaloids and coumarins were present in Aqueous Extracts of *S. isoetifolium* (AESI), Ethanol Extracts of *S. isoetifolium* (EESI) and Hydro Alcohol Extracts of *S. isoetifolium* (HAESI), Acetone Extracts of *S. isoetifolium* (ACESI) and n-Hexane Extracts of *S. isoetifolium* (HESI) (Table 2). The phytochemical analysis of the *S. isoetifolium* was examined and summarized in Table 1-Table 8 and fig. 1-fig. 7. This was highly supported by the previously published studies on seagrass[37,38]. Among the different extracts, the hydroalcoholic extract had shown the highest yield of phytochemicals such as TPC (192.44±13.47 mg GAE/g), TFC (106.54±7.45 mg quercetin/g), TSC (52.61±3.68 mg Quillaja/g) and TTC (80.65±5.64 mg tannic acid/g). Moreover, the quantitative analysis data for *S. isoetifolium* in the present study was found to be consistent with those reported in the past[39-42].

In GC-MS, the existence of different phytochemicals was identified through their Molecular Weight (MW) and Molecular Formula (MF) etc. There were 63 compounds identified in this method from the HAESI extracts (fig. 2 and Table 3). The prevailing compounds found in the mentioned extracts are Hexadecanoic acid methyl ester, Hexadecanoic acid, Ethyl iso-allocholate etc., Phytochemicals present in the plant extract significantly contributes to biological activities. Therefore, it is necessary to identify the different phytochemicals of the plant extract. Earlier reports show that seagrasses have antioxidant activity due to the presence of polyphenols, flavonoids and other phytochemicals[43-45]. Flavonoids are naturally occurring biological compounds that can act as potent antioxidants and can prevent cardiovascular disease by preventing the oxidation of Low-Density Lipoprotein (LDL)[46]. In the review of the above observation and continuation of our interest to identify the therapeutic activity of *S. isoetifolium*, antioxidant activity was evaluated. Antioxidant activity is a complex procedure and is influenced by different mechanisms, which cannot be identified through a single method. Therefore, it is needed to perform different ways to measure antioxidant activity. Different aliquots of hydroalcoholic extract of *S. isoetifolium* and ascorbic acid were prepared for *in vitro* antioxidant activity such as DPPH, TAC, Superoxide radical scavenging activity, NO scavenging activity and Hydroxyl radical scavenging activities. Minimum Inhibitory Concentration (MIC) is also estimated by calculating the IC_{50} value. The DPPH radical scavenging activities increased with the increasing concentration (20, 40, 60 and 80 µg/ml) of HAESI extract. The radical scavenging activities of 23.64±1.65, 44.55±3.11, 62.73±4.39, 79.10±5.53 µg/ml respectively at various concentrations (20, 40, 60 and 80 µg/ml) was observed and IC_{50} was found to be 47.28 µg/ml (Table 4 and fig. 3). IC_{50} values of the scavenging assay were calculated by plotting the percentage of inhibition against the concentration HAESI extracts. Similarly, the radical scavenging activities of ascorbic acid were performed and the results were between 25.46±1.78 to 89.55±6.26 µg/ml with increasing concentrations. The IC50 value of the standard drug was found to be 40.84 µg/ml. The DPPH radical scavenging activities of HAESI extracts are competitive when compared to the standard at the given concentrations.

TABLE 1: QUANTITATIVE ANALYSIS OF PHENOL, FLAVONOIDS, SAPONIN AND TANNIN CONTENT PRESENT IN THE LEAF EXTRACTS OF *S. isoetifolium*

Name of sample	Total phenol (Milligrams GAE equivalents per gram)	Flavonoids (Milligrams of quercetin equivalents per gram)	Saponin (Milligrams of Quillaja saponin equivalents per gram)	Tannin (Milligrams of tannic acid equivalents per gram)
S. isoetifolium	192.44±13.47	106.54±7.45	52.61±3.68	80.65±5.64

TABLE 2: QUALITATIVE PHYTOCHEMICAL ANALYSIS OF LEAF EXTRACTS OF *S. isoetifolium*

S. No	Phytochemicals	Aqueous extract	Ethanol extract	Hydro-alcoholic extract (30:70 %)	Acetone	Chloroform
1	Tannin	++	++	++	-	+
2	Saponin	++	++	++	-	-
3	Flavonoids	+	++	++	-	+
4	Steroids	+	++	+	-	-
5	Terpenoids	+	+	+	+	-
6	Triterpenoids	+	+	+	+	-
7	Alkaloids	+	++	+	-	+
8	Anthroquinone	+	+	+	-	-
9	Polyphenol	++	++	++	-	-
10	Glycoside	+	+	+	+	-
11	Coumarins	+	+	+	+	-
12	Emodins	-	-	-	-	-
13	Anthocyanins	-	-	-	-	-
14	Carbohydrate	+	+	+	+	+
15	Carboxylic acid	+	+	+	+	+

Note: ("+" indicates the presence of the compounds; "-" indicates an absence of the compounds and "++" indicates the high concentration)

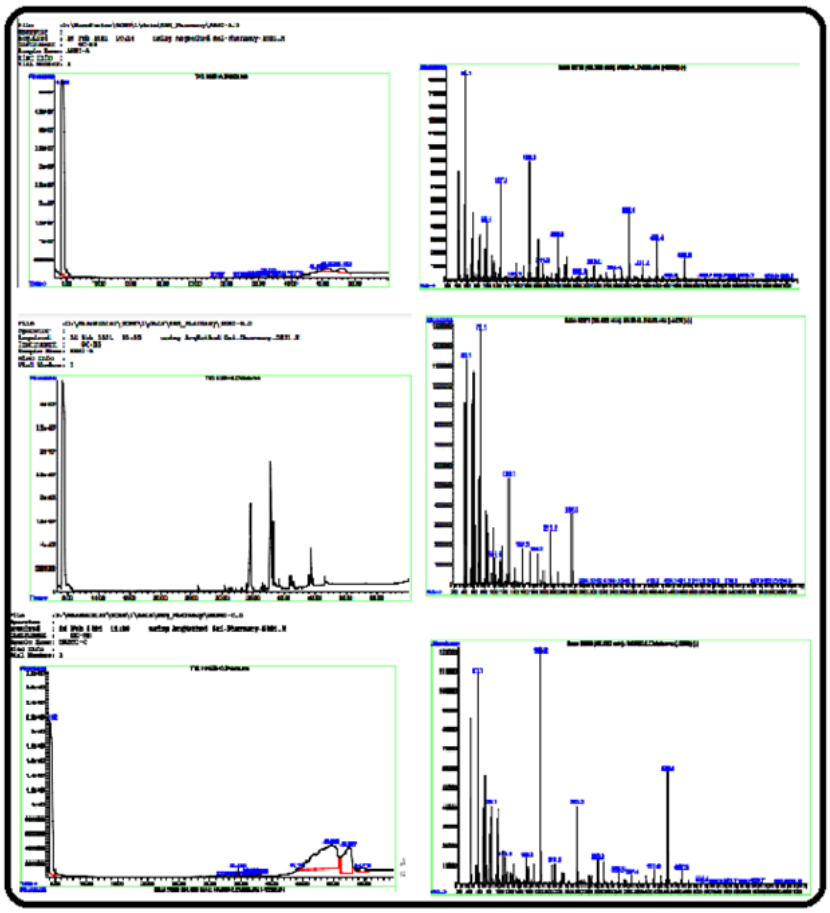

Fig. 2: Phytochemical constituents identified in the aqueous, ethanol and hydro alcohol leaf extracts of *S. Isoetifolium* using gas chromatography-mass spectrometry

TABLE 3: PHYTOCHEMICAL SCREENING LEAF EXTRACTS OF *S. isoetifolium* USING GC-MS TECHNIQUES

Peak	R. Time	Area %	Height %	MW	MF	Name of the compounds
AESI-A						
1	4.226	89.303	52862499	92	C_7H_8	Toluene
2	28.607	0.029	99898	296	$C_{21}H_{44}$	Heptadecane, 2,6,10,,15-tetramethyl phthalic acid
3	32.255	0.033	116198	306	$C_{18}H_{26}O_4$	Hex-3-yl isobutyl ester
4	33.05	0.016	83883	324	$C_{23}H_{48}$	Heptadecane, 9-hexyl-
5	33.243	0.045	134057	270	$C_{17}H_{34}O_2$	hexadeconic acid methyl ester
6	34.164	0.033	85822	278	$C_{16}H_{22}O_4$	Dibytylphthalate
7	34.58	0.157	141928	256	$C_{16}H_{32}O_2$	n-Hexadeconic acid
8	36.482	0.07	355706	294	$C_{19}H_{34}O_2$	11,14-Octadecadienoic acid methyl ester
9	36.578	0.225	873038	296	$C_{19}H_{36}O_2$	10-Octadecenoic acid methyl ester
10	37.039	0.026	112149	298	$C_{19}H_{38}O_2$	Heptadecanoic acid, 16-methyl-, methyl ester
11	38.183	0.278	118728	356	$C_{21}H_{40}O_4$	9-Octa decenoic acid (z)-2-hydroxy-1-(hydroxy methyl) ethyl ester
12	40.716	0.074	93685	456	$C_{27}H_{52}O_5$	Dodecanoic acid, 1-hydroxymethyl)-1,-2 ethanediyl ester
13	44.119	0.241	844372	390	$C_{24}H_{38}O_4$	Phthalic acid, di (2-propyl pentyl) ester
14	44.527	0.172	277878	456	$C_{27}H_{52}O_5$	Dodecanoic acid, 1-(hydroxy methyl)-1,2-ethanediyl ester
EESI-B						
1	4.137	56.942	44162888	92	C_7H_8	Toluene
2	5.021	0.719	876023	269	$C_{16}H_{28}ClN$	Benzyltri-n-propyl ammnonium chloride
3	5.482	0.483	732237	129	$C_7H_{15}NO$	N,N dimethyl pivalamide
4	26.104	0.448	787201	200	$C_{12}H_{24}O_2$	Dodecanoic acid
5	30.398	0.506	1002310	228	$C_{14}H_{28}O_2$	Tetra decanoic acid
6	31.111	0.192	385376	196	$C_{12}H_{20}O_2$	9,10-dimethyl tricyclo {4.2.1.1.(2,5)}decane-9-10-diol
7	31.386	0.041	350738	338	$C_{22}H_{42}O_2$	Phytol, acetate
8	31.579	0.058	381084	268	$C_{18}H_{36}O$	2-pentadecanone, 6,10,14-trimethyl
9	32.195	0.082	238835	278	$C_{16}H_{22}O_4$	Dibutyl phthalate
10	33.02	0.011	77071	366	$C_{26}H_{54}$	Octa decane, 3-ethyl-5-(2-ethyl butyl)-
11	33.117	0.046	329843	256	$C_{17}H_{36}O$	1-Hexadecanol,2-mehtyl-
12	33.183	0.066	490890	270	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester
13	34.067	0.79	1738912	254	$C_{16}H_{30}O_2$	Palmtoleic acid
14	34.543	9.237	18383784	256	$C_{16}H_{32}O_2$	n-Hexadecanoic acid
15	36.036	0.082	143713	268	$C_{17}H_{32}O_2$	Cis-10-heptadecenoic acid
16	36.289	0.065	237470	270	$C_{17}H_{34}O_2$	Heptadecanoic acid
17	36.43	0.068	505440	294	$C_{19}H_{34}O_2$	Methyl 9-cis, 11-trans-octa decadienoate
18	36.534	0.221	1492702	296	$C_{19}H_{36}O_2$	Trand 13-octa decenoic acid, methyl ester
19	36.831	0.189	1026914	296	$C_{20}H_{40}O$	Phytol

20	36.994	0.045	220832	298	$C_{19}H_{38}O_2$	Heptadecanoic acid, 16-methyl ester
21	37.871	19.654	27064423	282	$C_{18}H_{34}O_2$	Cis-vaccenic acid
22	38.168	2.877	13812305	284	$C_{18}H_{36}O_2$	Octadecanoic acid
23	38.502	0.64	1200659	280	$C_{18}H_{32}O_2$	9,12,Octadecadienoic acid
24	38.914	0.063	1491659	310	$C_{20}H_{38}O_2$	Ethanol z-(9,12-octa decadienyloxy)-(z,z)
25	39.193	0.711	1895472	280	$C_{18}H_{32}O_2$	9,12-octadeca dienoic acid (z,z)-
26	41.028	0.996	2585848	310	$C_{20}H_{38}O_2$	Cis-11-eicosenoic acid
27	41.4	0.419`	1535543	312	$C_{20}H_{40}O_2$	Eicosenoic acid
28	42.224	0.024	147908	356	$C_{21}H_{40}O_4$	9-octa decenoic acid, (z)-,2-hydroxy-1-(hydroxymethyl) ethyl ester
29	43.829	0.138	379780	330	$C_{19}H_{38}O_4$	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester
30	44.081	0.35	2336750	390	$C_{24}H_{38}O_4$	Phthalic acid, di(2-propyl pentyl) ester
31	44.26	2.605	8047321	338	$C_{22}H_{42}O_2$	Erucic acid
32	44.564	0.387	1530826	340	$C_{22}H_{44}O_2$	Docosanoic acid
33	46.555	0.74	1494775	356	$C_{21}H_{40}O_4$	9-octa decenoic acid (z)-,2-hydroxy-1-(hydroxy methyl ethyl ester)
34	47.662	0.086	229287	368	$C_{24}H_{48}O_4$	Tetra cosanoic acid
HAESI-C						
1	4.152	35.927	20959817	92	C_7H_8	Toluene
2	32.3	0.044	86703	320	$C_{19}H_{28}O_4$	Phthalic acid, 5-methyl hex-2-yl isobutyl ester
3	33.273	0.013	57102	270	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester
4	34.179	0.049	103427	376	$C_{23}H_{36}O_4$	Phthalic acid, butyl undecyl ester
5	34.432	0.764	1043999	256	$C_{16}H_{32}O_2$	N-hexadecanoic acid
6	36.519	0.026	132412	294	$C_{19}H_{34}O_2$	Methyl 10-trans, 12-cis-octadecadienoate
7	36.608	0.073	335007	296	$C_{19}H_{36}O_2$	Trans-13-octa decenoic acid, methyl ester
8	36.898	0.019	83247	296	$C_{20}H_{40}O$	Phytol
9	37.069	0.023	77335	298	$C_{19}H_{38}O_2$	Hepta decanoic acid, 16-methyl-,methyl ester
10	37.782	0.156	218002	282	$C_{18}H_{34}O_2$	Cis-vaccenic acid
11	38.116	0.139	210895	284	$C_{18}H_{36}O_2$	Octa decanoic acid
12	44.148	0.116	338489	390	$C_{24}H_{38}O_4$	Diisooctyl phthalate
13	49.646	43.343	3289601	456	$C_{27}H_{52}O_5$	Dodecanoic acid, 1-(hydroxy methyl)-1,2-ethane diyl ester
14	54.719	2.134	446409	436	$C_{26}H_{44}O_5$	Ethyl iso-allocholate

TABLE 4: DPPH RADICAL SCAVENGING ACTIVITY OF LEAF EXTRACTS OF *S. isoetifolium* AND ASCORBIC ACID AT DIFFERENT CONCENTRATIONS

Samples (percentage inhibition)	Concentrations (µg/ml)				IC_{50} Value (µg/ml)
	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	
S. isoetifolium	23.64±1.65	44.55±3.11	62.73±4.39	79.10±5.53	47.28
Ascorbic acid (Std)	25.46±1.78	47.28±3.30	78.64±5.50	89.55±6.26	40.84

Note: Values are expressed as Mean±SD for triplicate

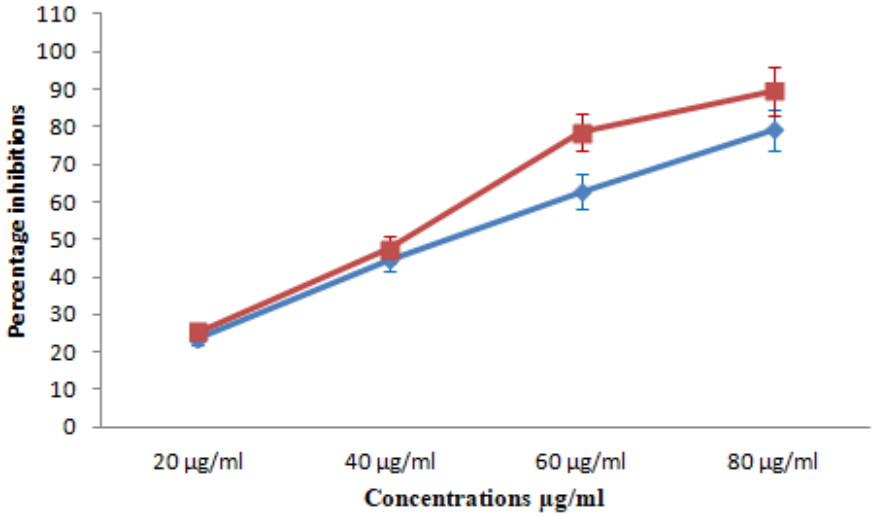

Fig. 3: DPPH radical scavenging activity of different concentrations of leaf extracts of *S. isoetifolium*
Note: Percentage of inhibition of hydro alcoholic extract (▬) and Percentage of inhibition of Ascorbic acid (●) at p<0.05

The hydroalcoholic extract showed the significant DPPH scavenging activity with the lowest IC_{50} value due to the presence of polyphenols compounds (Table 4). DPPH free radical scavenging activity reported in our study of *S. isoetifolium* is much higher than result reported from other seagrasses[47,48]. The TAC was increased with the increasing concentration (20, 40, 60 and 80 µg/ml) of HAESI extracts. The total antioxidant activities 22.50±1.57, 40.31±2.82, 65.62±4.59, 81.56±5.70 µg/ml respectively at different concentrations were observed and IC_{50} was found to be 47.53 µg/ml (Table 5 and fig. 4). Similarly, the total antioxidant radical scavenging activities of ascorbic acid were performed and the result was between 26.56±1.85 to 84.68±5.92 µg/ml with increasing concentration. The IC_{50} value of the standard drug was found to be 44.25 µg/ml. Superoxide radical is believed to be a chief source of reactive oxygen species in living things. The superoxide radical scavenging activity of different concentrations (20, 40 60 and 80 µg/ml) of HAESI extracts was evaluated with the same dose of ascorbic acid. The HAESI extract showed superoxide anion radical scavenging activities were 20.35±1.42, 45.71±3.19, 71.42±4.99, 89.28±6.24 µg/ml respectively at various concentrations and IC_{50} was found to be 44.24 µg/ml (Table 6 and fig. 5). In the same way, the superoxide radical scavenging activities of ascorbic acid was performed with the ascorbic acid and the result was between 28.57±1.99 to 92.14±6.44 with increasing concentrations. The IC_{50} value of the standard drug was found to be 37.94 µg/ml. In another method, NO scavenging activity of HAESI extracts was evaluated with the same doses of ascorbic acid ranging from 20-80 µg/ml. The HAESI extracts showed NO radical scavenging activities 20.47±1.43, 47.14±3.29, 69.04±4.83, 84.28±5.89 µg/ml respectively at different concentrations and IC_{50} was found to be 45.09 µg/ml (Table 7 and fig. 6). Similarly, the radical NO scavenging activity of ascorbic acid was performed and the result was between 23.33±1.63 to 91.42±6.39 with increasing concentrations. The IC_{50} value of the standard drug was found to be 41.67 µg/ml.

Athiperumalsami *et al.*[49] have reported highest antioxidant activity in the methanolic extract of *Halophila ovalis* than *Halodule pinifolia* tested by the NO scavenging method. At last, hydroxyl radical scavenging potential of HAESI extracts to inhibit hydroxyl-radicals (OH) was assessed at various concentrations 20-80 µg/ml. The HAESI extracts showed hydroxyl radical scavenging activities 21.66±1.51, 43.33±3.03, 68.75±4.81, 81.25±5.68 µg/ml respectively at different concentrations and IC_{50} was found to be 46.32 µg/ml (Table 8 and fig. 7). In the same way, the hydroxyl radical scavenging activity of ascorbic acid was performed and the result was between 25.41±1.77 to 93.33±6.53 µg/ml with increasing concentrations. The IC_{50} value of the standard drug was found to be 39.81 µg/ml. In agreement with previous studies there was a significant correlation between our antioxidant value and the values in the study of Duan *et al.*[50]. The result demonstrated that the hydroalcoholic extracts exhibited scavenging and/or reducing effects on DPPH, phosphomolybdate, superoxide, NO, hydroxyl radicals present in the reagents. The radical scavenging activity of hydroalcoholic extract of *S. isoetifolium* is due to the presence of polyphenols, flavonoids and other phytochemicals. At all concentrations of hydroalcoholic extract from 20 to 80 µg/ml, displayed

an excellent antioxidant effect. However, the lowest radical scavenging activity was observed in the lower concentrations of hydroalcoholic extracts. Whereas the high concentration of hydroalcoholic extract exhibits excellent antioxidant activity as similar to that of standard drug. The order of radical scavenging activity of hydroalcoholic extract of *S. isoetifolium* was found to be 80 µg/ml>60 µg/ml>40 µg/ml>20 µg/ml. Similar results were also obtained in studies conducted by other scientists who were involved in the determination of antioxidant activity in the seagrass at various concentrations[51-53]. All the extracts were less effective than the standard ascorbic acid radical scavenging activity and IC_{50} values. This present finding corroborate well with earlier reports in other species of seagrass.

In conclusion, the seagrass leaf extracts of *S. isoetifolium* were screened for phytochemicals by using chemical and GC-MS techniques. It is observed that the presence of phenol, polyphenol, alkaloid, glycoside, saponin, flavonoids, dicarboxylic acids and other constituents in the qualitative analysis. Whereas, quantitative determination shows the presence of TPC (192.44±13.47 mg GAE/g), TFC (106.54±7.45 mg quercetin/g), TSC (52.61±3.68 mg Quillaja/g) and TTC (80.65±5.64 mg tannic acid/g). The GC-MS analysis of the seagrass extract of hydro alcohol revealed the presence of 62 compounds that could contribute to the medicinal property of the plant leaves. Here, the compounds were used to identify the different phytochemicals present in the hydroalcoholic extract from the MF and MW of compounds. In addition to that, the HAESI extracts of *S. isoetifolium* proved to be the most effective radical scavenger activity in the DPPH, total antioxidant, NO, superoxide anion, hydroxyl radicals etc., the higher radical scavenging activity of HAESI extracts due to the presence of phenol, polyphenols and other compounds. They react with the various species of radicals such as hydroxyl, peroxide, superoxide and NO etc., and reduce their existence. Therefore, extracts of *S. isoetifolium* can be recommended for various oxidative stress-related diseases. The above result encourages us to isolate and confirm the specific phytoconstituent responsible for the observed antioxidant activity and their mode of action.

TABLE 5: TOTAL ANTIOXIDANT ACTIVITY OF LEAF EXTRACTS OF *S. isoetifolium* AND ASCORBIC ACID AT DIFFERENT CONCENTRATIONS

Samples (Percentage inhibition)	Concentrations (µg/ml)				IC_{50} value (µg/ml)
	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	
S. isoetifolium	22.50±1.57	40.31±2.82	65.62±4.59	81.56±5.70	47.53
Ascorbic acid (Std)	26.56±1.85	42.50±2.97	69.37±4.85	84.68±5.92	44.25

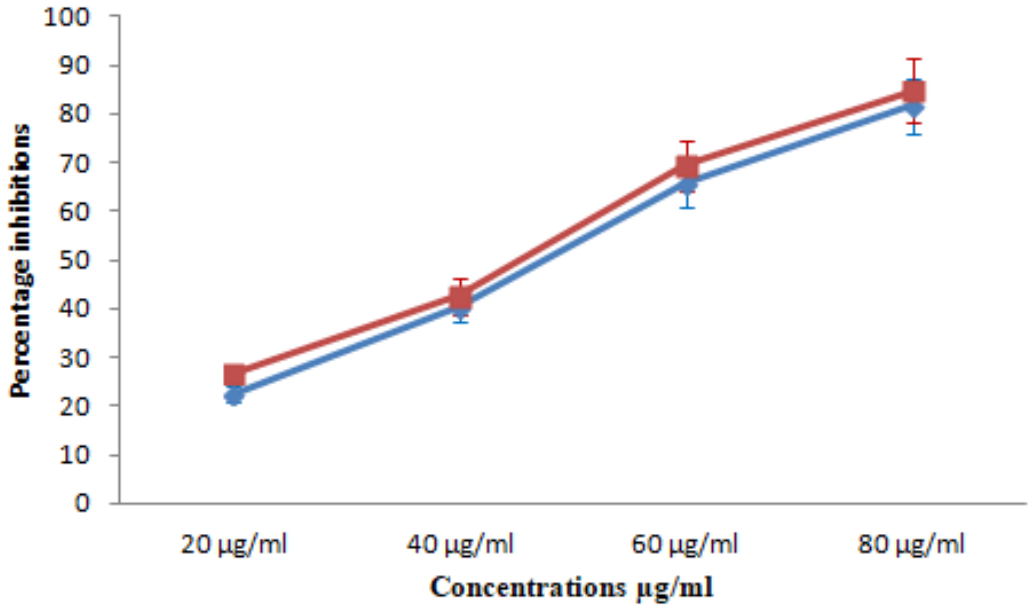

Fig. 4: Total antioxidant activity of different concentrations of hydroalcoholic extracts of *S. isoetifolium*
Note: Percentage of inhibition of sample (■) and Percentage of inhibition of Ascorbic acid (●) at p<0.05

TABLE 6: SUPEROXIDE ANION RADICAL SCAVENGING ACTIVITY OF *S. isoetifolium* AND ASCORBIC ACID AT DIFFERENT CONCENTRATIONS

Samples (Percentage inhibition)	Concentrations (µg/ml)				IC_{50} value (µg/ml)
	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	
S. isoetifolium	20.35±1.42	45.71±3.19	71.42±4.99	89.28±6.24	44.24
Ascorbic acid (Std)	28.57±1.99	54.64±3.82	75.71±5.29	92.14±6.44	37.94

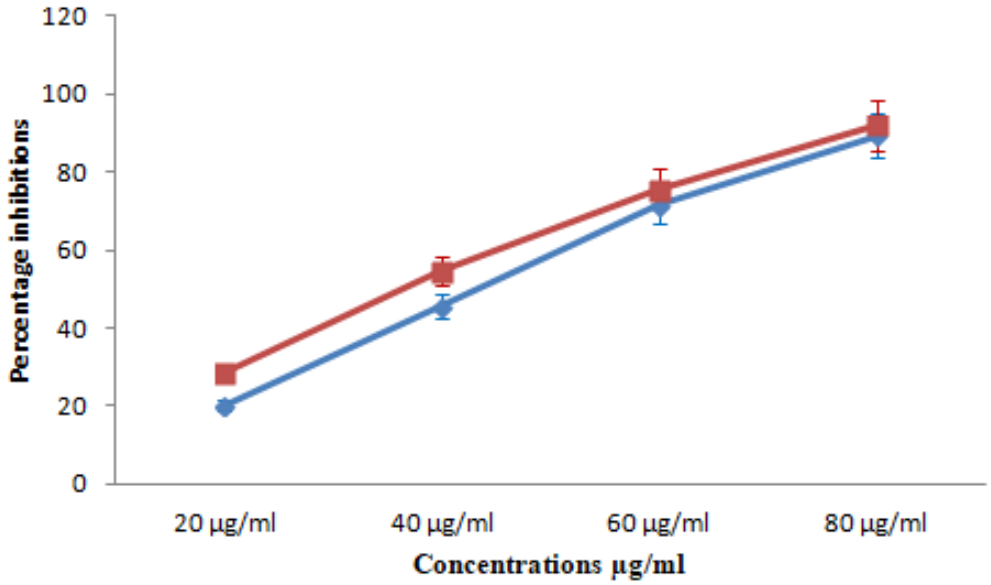

Fig. 5: Superoxide anion radical scavenging activity of different concentrations of hydroalcoholic extracts of *S. isoetifolium*
Note: Percentage of inhibition of hydroalcoholic extract (▬) and Percentage of inhibition of Ascorbic acid (▬) at p<0.05

TABLE 7: NITRIC OXIDE SCAVENGING ACTIVITY OF *S. isoetifolium* AND ASCORBIC ACID AT DIFFERENT CONCENTRATIONS

Samples (Percentage inhibition)	Concentrations (µg/ml)				IC_{50} value (µg/ml)
	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	
S. isoetifolium	20.47±1.43	47.14±3.29	69.04±4.83	84.28±5.89	45.09
Ascorbic acid (Std)	23.33±1.63	51.90±3.63	70.47±4.93	91.42±6.39	41.67

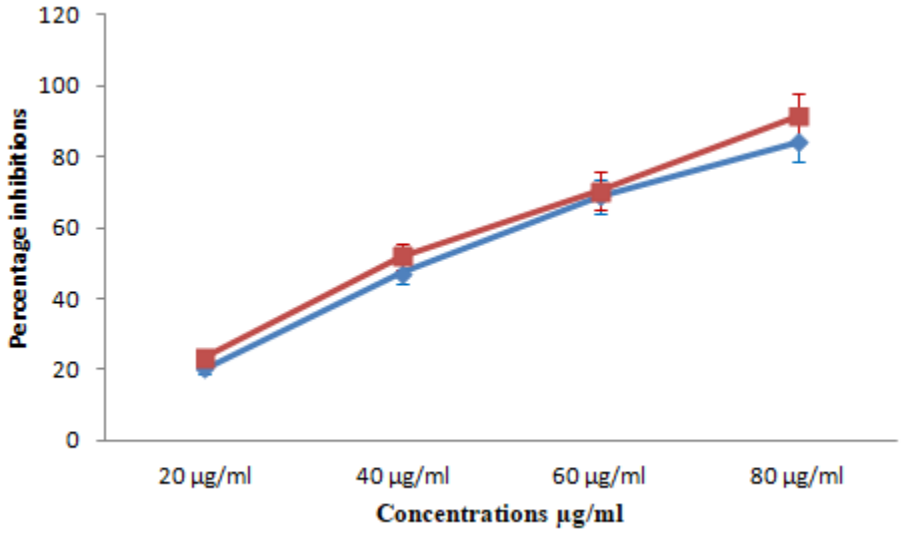

Fig. 6: Nitric oxide scavenging activity of different concentration of hydro alcoholic extracts of *S. isoetifolium*
Note: Percentage of inhibition of hydro alcoholic extract (▬) and Percentage of inhibition of Ascorbic acid (▬) at p<0.05

TABLE 8: HYDROXYL RADICAL SCAVENGING ACTIVITY OF *S. isoetifolium* AND ASCORBIC ACID AT DIFFERENT CONCENTRATIONS

Samples (Percentage inhibition)	Concentrations (µg/ml)				IC_{50} value (µg/ml)
	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	
S. isoetifolium	21.66±1.51	43.33±3.03	68.75±4.81	81.25±5.68	46.32
Ascorbic acid (Std)	25.41±1.77	53.75±3.76	72.91±5.10	93.33±6.53	39.81

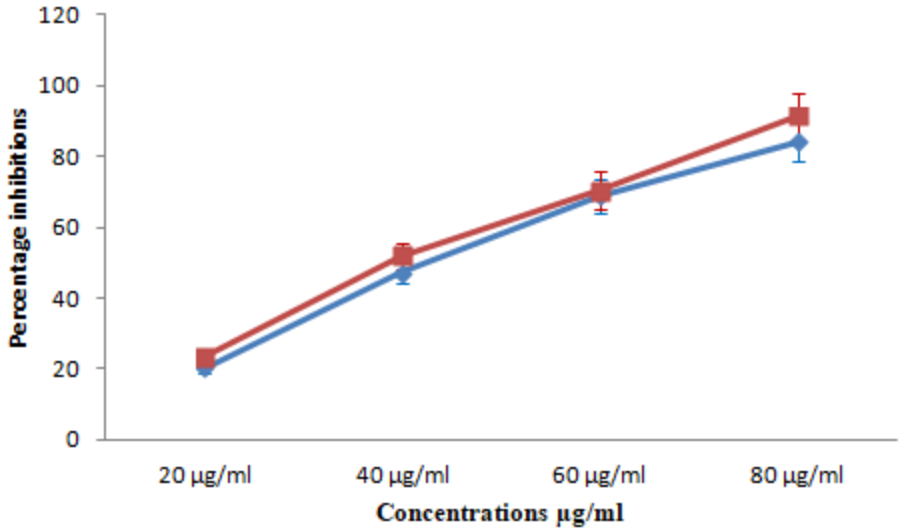

Fig. 7: Hydroxyl radical scavenging activity of different concentrations of hydro alcoholic extracts of *S. isoetifolium* Percentage of inhibition of hydro alcoholic extract ( ); Percentage of inhibition of Ascorbic acid ( ) at $p<0.05$

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Conflict of interests:

The authors declared no conflict of interest.

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