European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 07, Issue 03, 2020

Phytochemical Screening And In Vitro Antioxidant Activity Of Enicostemma Hyssopifolium

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Abstract: Medicinal plants and plant derived products have been part of the health care system since ancient human civilization. Traditional medicine uses plant extracts because of it's antioxidant activity. In contrast usage of synthetic antioxidants are being restricted due to several limitations. Thus, there is always demand for newer and alternative phytomedicines. The aim of the present study is to analyse phytochemical components and to explore the In vitro antioxidant potential of methanolic extract of Enicostemma hyssopifolium (MEEh). The plant extract was prepared by organic solvents. The MEEh was used for GC/MS analysis and for exploring the prospects of Enicostemma hyssopifolium. The radical scavenging bioassays such as DPPH and ABTS were carried out to study in vitro antioxidant potency. The lipid peroxidation assay was performed to analyse their inhibitory effects and ferric reducing power of the extract was also evaluated. GC/MS analysis revealed that the plant is a rich source of phytochemicals such as 3,5-Dimethoxyacetophenone, Ergost-5-en-3-ol which are known to have antioxidant potency. In the antioxidant assay of MEEh the IC_{50} was found to be 655.3>306.6 µg/ml. In lipid peroxidation assay, 33.45% of inhibition was found at 1000 µg/ml. The findings of the study revealed the potential of Enicostemma hyssopifolium as a source for natural antioxidants. It revealed that the plant could be a promising alternative agent in scavenging free radicals and may have ameliorative effect in complications associated with free radical damage.

Keywords: Enicostemma hyssopifolium, antioxidant, Bioassays, Phytochemicals, Screening tests, GC-MS.

1. INTRODUCTION

Antioxidants are natural or synthetic substances that may prevent or delay oxidative cell damage caused by physiological oxidants having distinctly positive reduction potentials, covering reactive oxygen species(ROS)/ reactive nitrogen species(RNS) and free radicals[1,2]. Role of antioxidants at cellular levels in humans are related to oxidative stress and free radicals and further to potential health effects in human. Excess production of ROS

leads to oxidative stress, which can cause number of diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neuro degenerative disorders [3], cardiovascular disease, Alzheimers disease, mild cognitive impairment, Parkinson disease and certain type of cancers. OS from ROS/RNS is important in the etiology of these diseases. Anti-oxidants play a crucial role in the maintenance of human health and prevention and treatment of these diseases because of their ability to reduce OS.

Phytochemicals are produced by plants, particularly the secondary metabolites, synthesized as a measure for self-defense against insects, pests, pathogens, herbivores. Phytochemicals including alkaloids, flavonoids, tannins, cyanogenic glycosides, phenolic compounds, saponins and lignins [4]. These phytochemicals show anti-carcinogenic and anti-mutagenic effects, which are major importance in the present scenario and proper understanding of phytochemicals is much required for drug discovery and for the development of novel therapeutic agents against major diseases[5].

Enicostemma hyssopifolium (chota chirayata) is a common weed of grassland and frequently found in open fields. It belongs to the family of Gentianaceae. The herb contains glycoside, which is used as laxative, stomachic, tonic and blood purifier[6]. The juice of whole plant is recommended by tribal and rural people of India to treat malarial fever. The present study focuses on the anti-oxidant activity of Enicostemma hyssopifolium plant extract. The plant was collected from the surrounding area of Chitradurga, Karnataka for the preliminary phytochemical analysis.

2. METHODS

Plant material:
Enicostemma hyssopifolium plant was collected during the rainy season from the surroundings of Chitradurga, Karnataka, India. The herbarium of specimen was vouched GSC/2015/Eh/1 and maintained for further reference. The plant material was washed thoroughly under running tap water and dried under shade. Then the plant material was kept in oven at 45^0C till it becomes moisture free. Later it was powdered and stored.
Preparation of extract: Aqueous extract and organic solvent (petroleum ether, chloroform and methanol) extracts were prepared separately and preserved aseptically at 5^oC in airtight container[7].

Chemicals:
Chemicals such as; Ferric chloride, HCl, Dragendorff's reagent, methanol, gallic acid, chloroform, H_2SO_4, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), α- Naphthol, ethanol, ascorbic acid (vitamin C), TCA, thio-barbituric acid, $FeSO_4$ are used. All chemicals are of A.R Grade (purity ≥ 99.0%) and purchased from Hi- Media and SD fine chemicals.

Phytochemical screening of Enicostemma hyssopifolium
The solvent extract of Enicostemma hyssopifolium was analysed for the presence of alkaloids, carbohydrates, tannins, saponins, flavonoids by the following tests.
Test for Alkaloids: Dragendoff's test: 0.2ml of sample was taken and 0.2ml of HCl was added. To this 2-3 drops of Dragendoff's reagent was added and the appearance of orange or red precipitate and turbid solution indicates the confirmation of alkaloids[8].
Test for Carbohydrates: Molisch's test: 0.2 ml of sample was mixed with few drops of Molisch's reagent (α- naphthol dissolved in alcohol).0.2 ml of H_2SO_4 was added along the walls of the test tube and observed for the appearance of a purple colour ring for positive result[8].

Test for Tannins: Braymer's test: 0.2 ml of plant extract was mixed with 2 ml water and heated on water bath for 10 minutes. The mixture was filtered and $FeCl_3$ was added to the filtrate and observed for dark green solution, which indicates the presence of tannin[9].
Test for Terpenoids: Salkowki's test: 0.2 ml of plant extract was taken in a test tube with 0.2 ml of $CHCl_3$. To this, concentrated sulphuric acid was added carefully to form a layer. The presence of reddish brown colour at the interface confirms the presence of terpenoids.
Test for Glycosides: 0.2 ml of sample was mixed with 0.2 ml of chloroform.0.2ml of acetic acid was added to this solution and the mixture was ice cooled. H_2SO_4 was added carefully and change from violet to blue and then to green colour confirms the steroidal nucleus (A glycone portion of glycoside).
Test for Steroids: Liebermann Burchard tests: 0.2 ml of sample was mixed with 0.2 ml of chloroform. To this 0.2ml of concentrated sulphuric acid was mixed. Appearance of red colour at the bottom of the chloroform confirms the steroids [10].
Test for Saponins: (Foam test) To 0.2 ml of extract 0.6ml of water was added in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins [11].
Test for Flavonoids: Alkaline reagent test: 0.2 ml of plant extract was taken in a test tube and mixed with dilute sodium hydroxide solution. To this diluted hydrochloric acid was added. Observation of yellow solution that turns colourless later would indicate the presence of flavonoids[9].

GC-MS analysis of Enicostemma hyssopifolium plant extract:
GC/MS analysis of the extract was performed using Thermo Scientific GC Trace 1310 Equipped with Thermo Scientific MS TSQ 8000. Grinded dry leaves were taken in 1:5 ratio of meoh and incubated for 4h at 50^0C. Methanolic extract was filtered using Whatmann No. 1. Filter-paper. Filtrate was evaporated to dry and extract (10mg/mL) was dissolved in 1ml of HPLC grade methanol. Agilent DB 5MS (30-meter X 0.25 mm) column was used for the GC/MS, column temperature was programmed from 60- 240^0C, with lower and upper temperatures being held for 2 min respectively. Analysis was done in the split-less mode. Helium was used as carrier gas (flow rate- 1ml/min). Test sample of 1 µl was injected for GC-MS analysis. Major compounds were identified by their retention time and mass fragmentation patterns with reference to the standard data from the National Institute of standards and technology (NIST), Wiley 9.0 library.

In vitro anti-oxidant studies:
DPPH and ABTS systems both have been widely used to measure the antioxidative status of various biological specimens. Both methods apply decolourization assays to monitor the decrease in their absorbance at characteristic a wavelength during the reaction. DPPH and ABTS radicals respectively absorb at 517 nm and 734 nm. When an antioxidant is added to the radicals, there is a degree of decolourization owing to the presence of the antioxidant. Change in absorbance by reducing DPPH was used to evaluate the ability of an isolated compound to act as a free radical scavenger.

DPPH: Scavenging assay is a simple chemical experiment for the primary evaluation of any compound for its free radical scavenging activity. To determine the antioxidant activity, DPPH **(**2,2-diphenyl-1-picryl-hydrazyl radical) was used as free radical. In brief, 75µl of DPPH solution; various concentration of test solution and quantity sufficient to 3ml with HPLC grade methanol. Various dilutions tested for reference standard were 0.5, 1.0, 1.5, 2.0, 2.5 µg/ml. Reaction mixture was mixed and incubated at 25°C for 15 minutes. Absorbance

was measured at 590nm using semi-auto-analyser. The measurements were also done for the solution containing only DPPH without any aqueous plant extract [12].

Lipid peroxidation assay: Occurrence of malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, is considered as a measure of general lipid peroxidation. A common method for measuring MDA, referred as the thio-barbituric acid-reactive substances (TBARS) assay [13] was used to measure the lipid peroxide formed. Here, Malondialdehyde is to react with thio-barbituric acid (TBA), egg-yolk homogenates as lipid-rich [14] media and record the absorbance maximum at 532 nm by yielding a pinkish red chromogen. Egg homogenate is mixed with the methanolic extract of whole plant, which is followed by the addition of ferrous sulphate and allowed to incubate for 30 minutes, to induce lipid peroxidation at room temperature. Later 20% of acetic acid and 0.08% of thiobarbituric acid (prepared in 1.1%SDS) and 20% of TCA was added with mixing. Reaction mixture is kept in hot water bath for 1 hour. After cooling to room temperature n-butanol was added and centrifuged at 3000rpm for 10 minutes. 50µl of deionised water was used as a blank.

ABTS radical scavenging activity: ABTS radical cations are produced by reacting ABTS and APS on incubating the mixture at room temperature in dark for 16 hours. Solution thus, obtained was further diluted with PBS to give an absorbance of 1.000. Different concentrations of the test sample and the reference standard (highest volume taken was 50µl) were added to 950µl of ABTS working solution to give a final volume of 1ml, made up by adding PBS. Absorbance was recorded immediately at 734nm. The Percentage of inhibition was calculated at different concentrations and the IC_{50} values were evaluated using Graph pad prism software [15].

Total Reducing Power assay: The Reducing power was determined by the method prescribed by Oyaizu et.al, [16] and is based on the substances, which have reduction potential, reacts with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferrous complex that has an absorption maximum at 700nm. The control was prepared by excluding samples. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicates the increase in reducing power. Sample in 400µl of methanol at various concentrations was mixed with a phosphate buffer (1ml, 0.2M, pH 6.6) and potassium ferricyanide (1ml, 1%), and the mixture was incubated at 50°C for 30min. Next, 1ml of trichloroacetic acid (10%) was added to the reaction mixture, which was then centrifuged at 3000rpm for 10min. Upper layer of the solution (1ml) was mixed with distilled water (1ml) and ferric chloride (170µl, 0.1%), and the mixture was incubated at 50°C for 30 min. Absorbance was measured at 700nm. Reducing property of test sample was standardized against quercetin and expressed as difference in optical density (OD) from control as well as test as 0.1 and is expressed as µg/ml, an intense absorbance confirms the increased reducing power.

Percentage of growth inhibition was calculated as follows;

$$\%\ Inhibition = \frac{(ODofControl - ODofSample)}{ODofControl} \times 100$$

3. RESULTS

Phytochemicals are natural bioactive chemicals of plant origin and naturally synthesized are basis for traditional herbal medicine practiced since years nonspecific. Table-1: Phytochemical analysis of Enicostemma hyssopifolium shows the presence of glycosides,

phenols, saponins, steroids, tannins, terpenoids, alkaloids, flavonoids and glycosides. In the phytochemical analysis, major compounds are found in methanolic extract. Therefore, for further studies, methanolic extract was used. In Table-2 and 3: GC-MS analysis, it is confirmed that Enicostemma hyssopifolium plant methanolic extract contains the compounds like flavonoids and alkaloids, which are related to the antioxidant or free radical scavenging activity and anti-inflammatory activity.

In Figure-2 and Table-4: DPPH assay is based on the ability of the stable free radical 2, 2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenolic. Bleaching of DPPH increases proportionate to the amount of extract in a given volume. Bleaching of the reagent colors by a test sample represents the capacity for hydrogen or electron donation by the test sample, and is closely correlated with the radical-scavenging activity. In DPPH assay, the sample showed significant level of dose-dependent DPPH radical scavenging activity with IC_{50} value of 655.3µg/ml when compared to standard Quercetin with IC_{50} value of 3.924µg/ml.

Lipid peroxidation involves the oxidative deterioration of lipids with unsaturation and is known as the initiation process, begins with the formation of conjugated dienes and trienes. Subsequently, a propagation process is carried out that consists of the reaction of the deprotonated species derived from the lipids with O_2, leading to the formation of peroxyl radicals (ROO•). This leads to the formation of hydro-peroxides that promote the formation of new R• radicals. The latter radicals react with each other to produce stable molecules of the R-R and ROOR type. To encourage the antioxidant activity of a chemical compound, it is necessary to inhibit the peroxidation of a fatty acid emulsion, where linoleic acid is used as a model. Hydro-peroxides derived from linoleic acid subsequently react with Fe^{2+}, causing the oxidation of this ion to produce Fe^{3+}. Fe^{3+}ions form a complex with thiocyanate (SCN^-), and this complex is used to measure the peroxide value. In Table-5: Lipid peroxidation assay, the sample showed highest 33.45% inhibition at highest conc. of 1000µg/ml.

In Figure-3 and Table-6: ABTS is soluble in both aqueous and organic solvents and consequently is useful for evaluating the antioxidant activity of samples in different media. ABTS assay measures the relative antioxidant ability of extract to scavenge the radical cation $ABTS^{+\cdot}$ produced by the oxidation of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate. Inhibition of ABTS•+ activity in an antioxidant sample has a strong correlation with the radical scavenging capacity DPPH• because both radicals have the capacity to accept electrons and H• from the antioxidant compounds present in the samples. In ABTS assay, sample showed significant level of dose-dependent ABTS radical scavenging activity with IC_{50} value of 306.6µg/ml when compared to standard Quercetin with IC_{50} value of 1.084 µg/ml.

In Figure-4 and Table-7: Reducing power of sample found to be 241.63mg Vitamin C/g. Overall, this result suggests that the tested sample have good antioxidant activity

4. DISCUSSION

In Indian folk medicine, many herbal medicinal plants are still identified and are in continuous use. They are used against diseases because of their medicinal values, which are due to the presence of phytochemicals in them.[17] The active components from plant are extracted with various solvent extracts procedures such as alcohols, chloroform, ether and water. Many of the metabolites are extracted in methanolic extract. Plants are the natural sources for the antioxidants that possess high quantity and quality of antioxidants, which can scavenge the free radicals. In the present investigation, the methanolic extract of Enicostemma hyssopifolium has shown potential antioxidant activity due to various phytochemicals present in plant. *Enicostemma hyssopifolium* plant methanolic extract was

separated by GC and the compounds were identified by the MS by the NIST and Wiley 9.0 libraries. GC–MS analysis revealed the presence of major biologically active compounds (2,6,10,14,18,22-Tetracosahexaene, 2-Hydroxy-gamma-butyrolactone, 2-Methoxy-4-vinylphenol, 2-Propanone, 3,4-Altrosan, 3,5-Dimethoxyacetophenone, Ergost-5-en-3-ol, Erythrocentaurin, Pentadecanoic acid, vitamin E) having medicinal importance as given in Table-3. There may be region wise differences in the presence of phytochemicals. The whole plant extract shows presence of phenolic compounds, tannins and flavonoids that play an important role as anticarcinogenic, which has major importance in the present scenario[18]. Higher absorbance value in reducing power assay indicates its potentiality of being strong antioxidant.

5. CONCLUSION

The increasing interest gained by antioxidants is due to the health benefits provided by natural resources. This consists in preventing the occurrence of oxidative stress related diseases, as a consequence of the attack of free radicals in different bio-components in human body. Our findings revealed the potential of Enicostemma hyssopifolium as a source for natural antioxidants and the plant could be a promising agent in scavenging free radicals and treating diseases related to free radical reactions and it could be potentially used in pharmaceutical industries. In conclusion, this study highlights for the first time that Enicostemma hyssopifolium has potential antioxidant power, which can be used in anticancer studies. Therefore, this study can be a guideline for further biological activities of investigation.

Conflict of Interest The authors declare that they have no conflict of interest.

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Table 1: phytochemical screening of plant extracts of Enicostemma hyssopifolium

Phytochemicals	**Distilled water**	**Chloroform**	**Petroleum ether**	**Methanol**
Alkaloids	-	-	-	+
Flavonoids	-	+	-	+
Glycosides	+	+	+	+
Phenols	-	-	-	+
Saponins	+	+	+	+
Steroids	-	-	+	+
Tannins	+	+	+	+
Terpenoids	+	+	+	+
Triterpenoids	+	+	+	+

Table:2: Details of GC/MS peaks

Peak	**Retention Time**	**Name of the Compound**	**Area%**
1	3.629	2-Propane , 1,3-dihydroxy	1.68
2	4.844	2-Hydroxy-gamma-butyrolactone	0.82
3	6.297	Glutaraldehyde	0.53
4	8.588	4-(6- Oxabicyclo[3.1.0]hex-1-yl)-but-3-yn-2-one	3.5
5	9.355	2-Methoxy-4-vinylphenol	3.55

6	10.51	2-Nonen-4-yne,	0.51
7	10.916	Sucrose	3.23
8	11.527	3,4-Altrosan	0.78
9	12.526	3,5-Dimethoxyacetophenone	0.86
10	12.857	3-Deoxy-d-mannoic lactone	1.71
11	13.765	4-methylphthalaldehyde	32.52
12	13.981	2(5H)-Furanone, 4-methyl-3-(2-methyl-2 propenyl)	1.15
13	14.412	Erythrocentaurin	4.94
14	14.624	Inositol, 1-deoxy	5.09
15	15.977	1,2-Epoxy-5,9,cyclododecadiene	0.58
16	16.319	αβ D-Glucopyranoside, 1- deoxy-1-undecylthio	1.15
17	16.374	3-Methyl-5-nitrosotropone	0.97
18	16.663	Pentadecanoic acid	2.36
19	18.132	Phytol	0.7
20	18.379	9,12-Octadecadienoic acid	0.84
21	23.827	2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23-hexamethyl (squalene)	7.36
22	24.766	Hexadeca-2,6,10,14-tetraen-1-ol,3,7,11,16-tetramethyl	0.78
23	25.781	1,4-Methanoazuklen-9-ol,decahydro-1,5,5,8a-tetramethyl/Longiborneol/Juniperol	0.65
24	26.407	vitamin E	0.87
25	27.681	Ergost-5-en-3-ol	2.67
26	28.051	Stigmasterol	2.82
27	28.795	γ-Sitosterol / Clionasterol	2.23
28	29.4	6a,14a-Methanopicene,perhydro-1,2,4a,6b,9,9,12a-heptamethyl-10-hydroxy	4.75
29	29.903	Lanosterol	0.8
30	30.152	5,16-Dinorlabdane,8,13:13,20-diepoxy	9.1
31	32.935	1,2-Bis(trimethylsilyl)benzene	0.52

Table: 3: Biological activity of compounds found in GC-MS

SL NO	**NAME**	**BIOLOGICAL FUNCTION**
1	2,6,10,14,18,22-Tetracosahexaene/2,6,10,15,19,23-hexamethyl (squalene)	Squalene is investigated as an adjunctive cancer therapy.
2	2-Hydroxy-gamma-butyrolactone	Has a role as a neurotoxin, Antithyroid and antivirus activities
3	2-Methoxy-4-vinylphenol	Anti-inflammatory activity
4	2-Propanone	Antifungal
5	3,4-Altrosan	Fungicide

6	3,5-Dimethoxyacetophenone	Antioxidant, analgesic, antipyretic and antiinflammatory.
7	3-Deoxy-d-mannoic lactone	Antifungal activity
8	Ergost-5-en-3-ol	Anti-oxidant in nature
9	Erythrocentaurin	Anti-inflammatory activity
10	Glutaraldehyde	Disinfectant, anti-oxidant
11	Inositol	Helps to improve ovarian function and reduce hyperandrogenism. It is also shown to reduce the risk of metabolic disease in people with PCOS.
12	Pentadecanoic acid	Anti-inflammatory activity
13	Phytol	Anti-inflammatory activity
14	Stigmasterol	A phyto sterol with potential anti-osteoarthritis
15	Sucrose	Antioxidant, Antiophthalmic, Flatugenic, Hypercholesterolemic, Preservative, Triglycerigenic, Uricogenic, Vulnerary
16	Vitamin E	Hypoglycemic, vasodilator, hepatoprotective, antioxidant, cancer preventive, antibronchitic, antiaging, anticataract, anticoronary, anti-inflammatory,anti-tumour,hypocholesterolemic, immunostimulant, antidermatitic, analgesic

Table:4: DPPH assay

Sample Name	**Conc. (µg/ml)**	**Absorbance**	**% Inhibition**	**IC50 µg/mL**
Control	0.00	0.631	0.000	
Quercetin	0.31	0.609	3.470	
	0.63	0.589	6.622	
	1.25	0.555	12.104	3.924
	2.50	0.421	33.286	
	5.00	0.297	52.883	
	10.00	0.232	63.229	
Methanolic extract of *Enicostemma hyssopifolium*	25	0.609	3.580	
	50	0.588	6.844	
	100	0.507	19.677	
	200	0.470	25.475	655.3
	400	0.424	32.842	
	800	0.283	55.196	

Table:5: Lipid Peroxidation assay

Sample Name	Conc. (µg/ml)	Absorbance	% Inhibition
Control	0	1.176	0.00
Methanolic extract of *Enicostemma hyssopifolium*	50	1.138	3.20
	100	1.104	6.14
	200	1.077	8.44
	400	1.036	11.87
	800	0.997	15.19
	1000	0.783	33.45

Table:6: ABTS radical scavenging activity				
Sample Name	**Conc. (µg/ml)**	**Absorbance**	**% Inhibition**	**IC50 ug/mL**
Control	0	0.7439	0.00	
Quercetin	0.35	0.6101	17.99	
	0.61	0.5101	31.43	
	1.25	0.3801	48.90	1.084
	2.5	0.2201	70.41	
	5	0.1001	86.54	
	10	0.0678	90.89	
Methanolic extract of *Enicostemma hyssopifolium*	12.5	0.620	16.72	
	25	0.530	28.82	
	50	0.510	31.51	
	100	0.487	34.52	306.6
	200	0.458	38.41	
	400	0.343	53.86	

Table 7(a): Total reducing power assay- Standard Vit C

sample name	Conc. µg/ml	Abs at 700nm
Control	0	0.1306
standard vit c	50	0.1784
	100	0.2128
	200	0.3028
	400	0.525

Table 7 (b): Vitamin C content in the sample

sample name	Conc. µg/ml	Absorbance	Conc. of vit-C Eq. (µg/800µg)	Conc. of vit-C (mg vit C Eq/g)
Methanolic extract of Enicostemma hyssopifolium	800	0.317	193.30	241.63

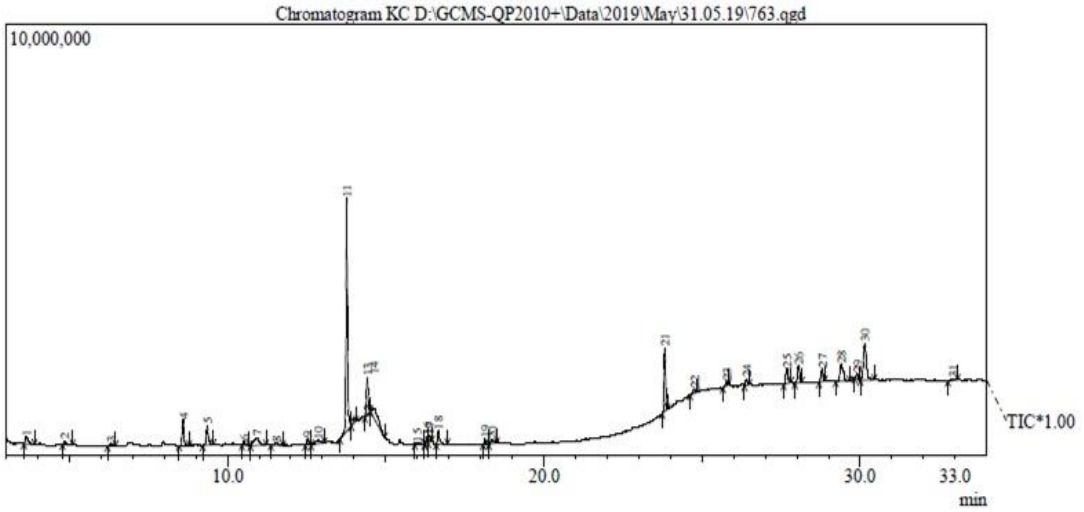


Figure-1: GC/MS analysis of methanolic extract of

Enicostemma hyssopifolium

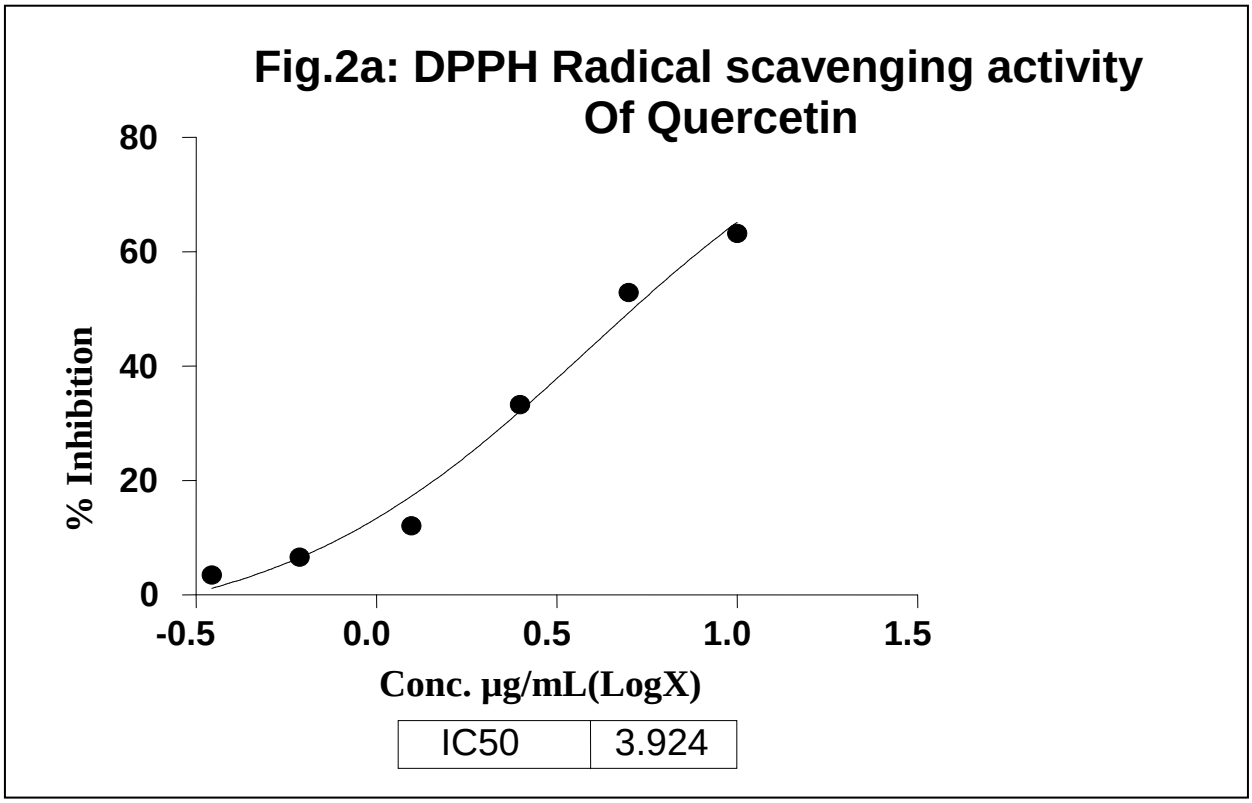
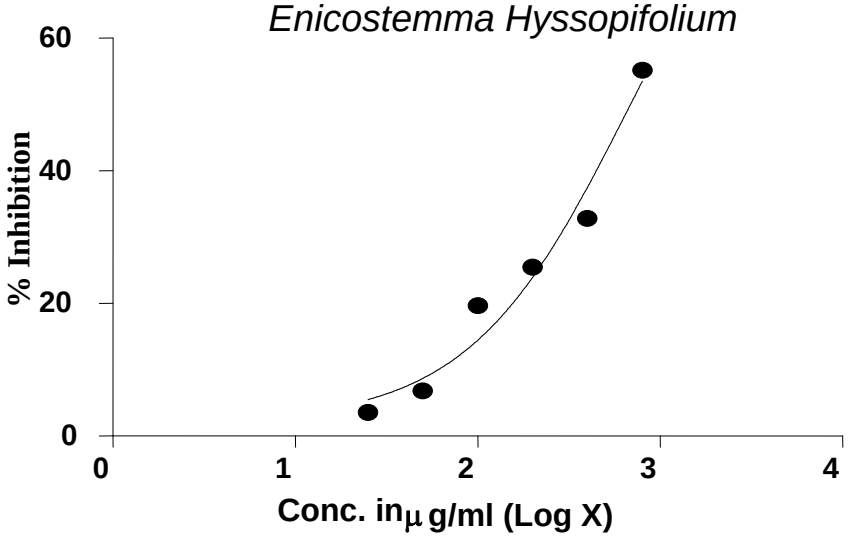
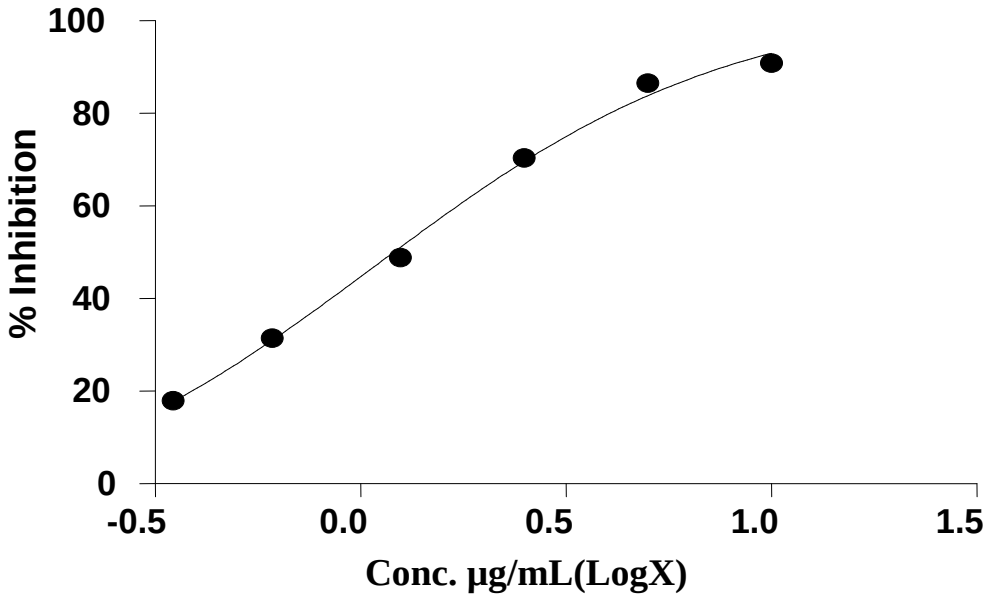


Fig. 2b: DPPH Radical scavenging activity of *Enicostemma Hyssopifolium*



IC50	655.3

Fig.3a: ABTS Radical scavenging activity of Quercetin



IC50	1.084

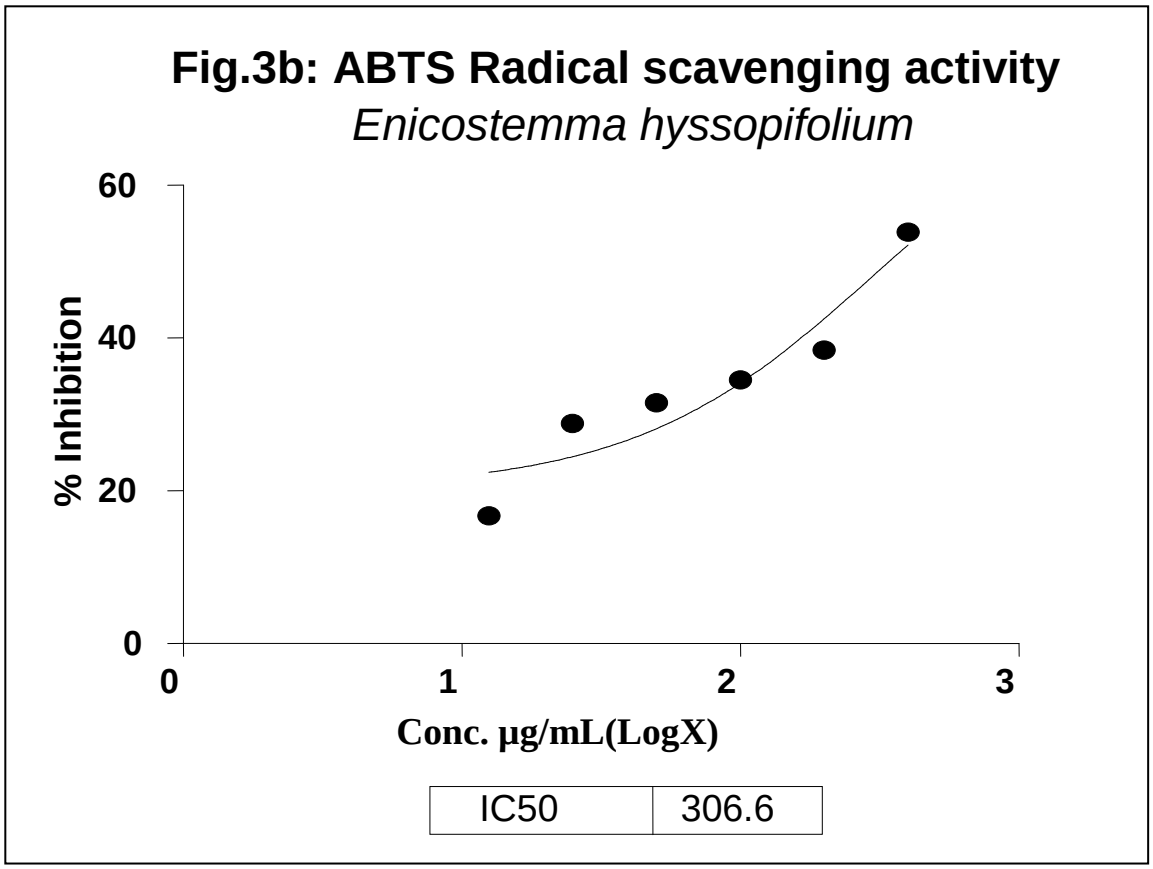
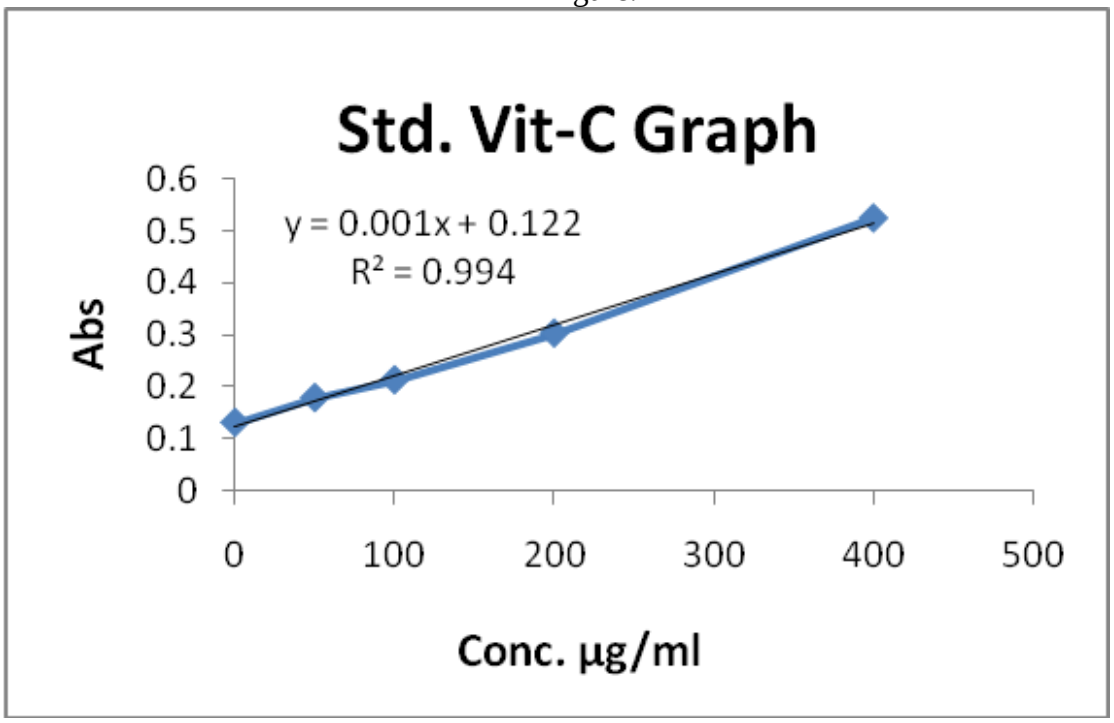


Fig.3b: ABTS Radical scavenging activity
Enicostemma hyssopifolium
% Inhibition
60
40
20
0
0
1
2
3
Conc. µg/mL(LogX)
IC50
306.6

Figure. 4


Std. Vit-C Graph
y = 0.001x + 0.122
R² = 0.994
Abs
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
500
Conc. µg/ml