

STUDIES ON PHYTOCHEMICALS, ANTIOXIDANT AND CYTOTOXICITY EFFECT OF HYBANTHUS ENNEASPERMUS

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ABSTRACT

Objectives: To determine the qualitative and quantitative phytochemical and evaluate radical scavenging, antioxidant and cytotoxicity effect of *Hybanthus enneaspermus*.

Methods: The phytochemical of *H. enneaspermus* was screened qualitative and quantitative with HPTLC and FT-IR. Radical scavenging effect of different concentrations of hydroethanolic extract of *H. enneaspermus* (HEEHE) was evaluated by DPPH, nitric oxide, hydroxyl and reducing power. The antioxidant potential was evaluated using Ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods and cytotoxicity was determined in Hep2 cell lines.

Results: Qualitative analysis of HEEHE showed the presence of alkaloids, flavonoids, carbohydrates, protein, phenol, steroid, tannin, glycosides and terpenoids. The results expressed higher amount of glycosides (45mg/g), tannins (42mg/g) and Iron (4.6mg/g) in HEEHE. 500µg/ml of HEEHE demonstrated significant radical scavenging and cytotoxicity effect. Lycopene exhibited significant amount (0.832g) among the different antioxidants. IR spectra specified the presence of asymmetric and aromatic compounds. HPTLC chromatogram showed the presence of flavonoid and glycosides in *H. enneaspermus*.

Conclusion: This study concludes therapeutic potential of *H. enneaspermus*, will be used for clinical trials..

Keywords: Antioxidant, Flavonoid, minerals, Lycopene, MTT assay.

INTRODUCTION

India is a rich heritage of medicinal plants contain a large amount of biologically active phenolic substances [1]. The formation of free radicals involved in the pathology of various diseases including diabetes, heart attacks, inflammation, neuro-degenerative diseases, cancers and ageing [2]. Antioxidants may offer resistance against oxidative stress by scavenging free radicals and they prevent diseases. Most of the authors co-related the antioxidant potential of the plant with their phenolic constituents [3,4]. Therefore, researchers focused their interest towards herbal medicines in the treatment of diseases because of their minimal side effects and availability [5]. Number of techniques and methods used to elucidate the active ingredients in the biological resources. Among these, the qualitative and quantitative analysis is very essential for identifying and quantification of active metabolites present in the medicinal plants which is important for pharmacological therapeutic action. Also, the HPTLC is considered as one of the most versatile tool in a recent year for the analysis of compounds with automated form of TLC. IR spectra also another technique used to determine the functional group of active constituents [6].

Hybanthus enneaspermus (L) F. Muell. (Family: Violaceae) is traditionally used to treat diuretic, demulcent properties, bowel complaints of Children, cooling liniment for head and cholera. Preliminary phytochemical investigations revealed, the considerable amount of alkaloids, flavonoids, carbohydrates, steroids, triterpenes, phenols and amino acids in aerials parts of the plant [7]. There are limited reports available on their antimicrobial, hepatoprotective, anti-ulcer, anti-secretory, analgesic activity, nephro protective, anti convulsant, anti-inflammatory and anti-diabetic activity [8, 9]. There was a lack of scientific data in the phytochemical and therapeutic quantity, total antioxidant and analytical analysis of plant metabolites in *Hybanthus enneaspermus*. Hence the present study aimed to evaluate the qualitative and quantitative analysis of phytochemicals and minerals; free radical scavenging, total antioxidant and reduction potential; FT-IR and HPTLC analysis of hydroethanolic extract of *Hybanthus enneaspermus* under *in vitro* conditions.

MATERIALS AND METHODS

Preparation and Extraction

Three kg leaves of *H. enneaspermus* were shade dried and pulverized. The coarse powder was used to prepare extract. 1kg powdered material of *H. enneaspermus* was cold macerated with 3000ml of hydro ethanol, aqueous, chloroform and acetone for three days. After that, the suspension was filtered and the residues were removed. The filtrate of each extract was evaporated and dried at 40°C under reduced pressure to separate the extract. The final residual extract was used for further experiments.

Qualitative Phytochemicals analysis

The crude hydroethanolic, aqueous, chloroform and acetone extract of *H. enneaspermus* was used to determine the preliminary phytochemicals [10].

Quantitative Phytochemical Analysis

0.5 to 1ml of HEEHE extract were evaluated for tannins, total free amino acids, Proline, flavonoids and glycosides [11].

Estimation of Minerals

Briefly, 5g of leaf powder was took in a silica crucible and heated in a muffle till there is no evolution of smoke. The crucible should be taken out, cooled at room temperature by keeping it in a desiccators and the ash moistened with concentrated sulphuric acid. The crucible with sulphated ash heated in a muffle furnace at 60°C till weight of the content is constant (2-3h). Sulphate ash obtained above should then be dissolved in 100ml of 5% hydrochloric acid solution. The solution should be stored in tightly capped plastic bottles and directly used for the determination of iron, calcium and magnesium by atomic absorption spectrophotometer.

Radical scavenging Activity of HEEHE

Different concentration of HEEHE (100, 200, 300, 400 and 500 µg/ml) was evaluated for DPPH, superoxide [12], nitric oxide, hydroxyl radical scavenging activity [13] and reduction potential [14].

Total Antioxidant Assay**Ferric Thiocyanate Method (FTC)**

Four mg of HEEHE mixed with 4ml of absolute ethanol, 4ml of 2.52% linolenic acid, 8.0ml of 0.05M phosphate buffer (pH7.0) and 3.9ml of water placed in a vial at 40°C over dark condition. 0.1ml of this solution was added 9.7ml of 75% ethanol and 0.1ml of 30% ammonium thiocyanate. Then 0.1ml of 0.02M ferrous chloride was added to 3.5% HCl to the reaction mixture. The absorbance was measured at 500nm every 24h until one day after the absorbance of control reached maximum. Ascorbic acid was used as positive control [15].

Thiobarbituric Acid assay (TBARS)

Two ml of 20% trichloroacetic acid and 2ml of 0.67% of 2-thiobarbituric acid were added to 1ml of HEEHE. The mixture was kept in a water bath. After cooling the mixture, was centrifuged at 3000×g for 20min. The absorbance was measured at 552nm [16].

MTT Assay

Cell viability was evaluated by the MTT colorimetric technique with slight modification [17]. Briefly, in each well 200 µl of MTT (3-(4, 5-dimethylthiazol-2)-2, 5 diphenyl tetrazolium bromide) without phenol red (5mg/ml in PBS), was added to each well contained different concentrations of HEEHE. The plates were incubated for 6-7h in 5% CO₂ incubator. MTT was reduced by active metabolic cells and to generate NADH and NADPH. The resulting purple intracellular formation stabilizes the MTT crystals and quantified by spectrophotometric mean.

Infra Red Spectroscopy

A thin disc is prepared under anhydrous conditions from a powder containing about 1mg of HEEHE and 100mg potassium bromide, using a mould and press. The spectral range of measurement is taken from 4000-667cm⁻¹ for 3min and recorded [6].

Table 1: Qualitative Phytochemical Screening of *H. enneaspermus*

Name of Phytochemical	Hydro Ethanolic Extract	Water Extract	Chloroform Extract	Acetone Extract
Alkaloids				
a) Dragendorff's test	++	+	-	-
b) Wagner's test	++	+	-	-
c) Mayers Test	++	+	-	-
Flavonoids				
a) Alkaline reagent	+++	++	-	-
b) Shinodas test	+++	++	-	-
Saponins	-	-	-	-
Carbohydrates				
a) Fehlings test	+	+	-	-
b) Benedicts test	++	++	-	-
c) Molischs test	+++	-	-	-
Alurone grains	-	-	-	-
Proteins				
a) Millons test	+++	+	-	-
b) Biurets test	++	+	-	-
c) Ninhydrin	+	++	-	-
Phenols				
a) Ferric chloride test	+	-	-	-
b) Lead acetate test	-	-	-	-
c) Libbermanns test	-	-	-	-
Steroids				
a) Libbermanns burchards test	++	+	+	+
b) Salkowski test	+	+	-	-
Tannins				
a) Ferric chloride test	++	++	-	-
b) Lead acetate test	+++	++	-	-
Phlobatannins	-	++	-	-
Glycosides				
a) Legal's test	+++	-	-	-
b) Keller-killiani test	+++	-	-	-
Terpenoids	++	++	-	-
Lignins	-	++	-	+
Acidic Compounds	+	-	-	-
Napthoquinones	-	-	-	-
Inulin	++	-	-	-
Suberin	-	-	-	-

(+++ = High; ++ = Moderate; + = Low; - = Absent)

Table 2: Quantitative Analysis of Phytochemicals and Minerals in the *H. enneaspermus*

Parameters	Quantity(mg/g)	Minerals	Quantity(mg/g)
Tannin	42	Calcium	2 mg
Free amino acids	14	Magnesium	1.5 mg
Proline	30	Iron	4.6 mg
Flavonoids	20	Phosphorus	3.4 mg
Glycosides	45		

Table 3: Free Radical Scavenging Activity of HEEHE

Concentration (µg/mL)	Percentage % of Inhibition DPPH	Superoxide Radical	Nitric Oxide	Hydroxyl Radical	Reducing Power
100	63.70	28.17	40.72	44.42	21.70
200	68.88	39.39	47.50	56.35	42.28
300	73.21	52.92	52.60	65.72	53.14
400	82.63	65.81	55.72	73.21	73.21
500	89.88	80.88	63.12	82.88	89.85
IC ₅₀	78.49	254.51	210.52	112.56	236.51

HPTLC analysis

100mg of the HEEHC was dissolved in 1ml of ethanol water (1:1). The solution was centrifuged at 3000×g for 5 min and used for HPTLC analysis. 3µl of the above test solution was loaded at 6mm band length in the 5x10 Silica gel 60F₂₅₄ TLC plate (Hamilton syringe and CAMAG LINOMAT5). The sample loaded plate was kept in TLC twin through developing chamber with respective mobile phase (Ethyl acetate: Butanone: Formic acid: Water 5: 3: 1:1) and the band was developed in the respective mobile phase up to 90mm. The developed band was dried. The plate was kept in Photo documentation chamber (CAMAG REPROSTAR 3) and captured the images at White light, UV 254nm and UV366nm. The developed band was sprayed with 1% ethanolic aluminum chloride reagent and dried at 120° C. Finally the plate was fixed in the scanner and scanning was done at a254nm.

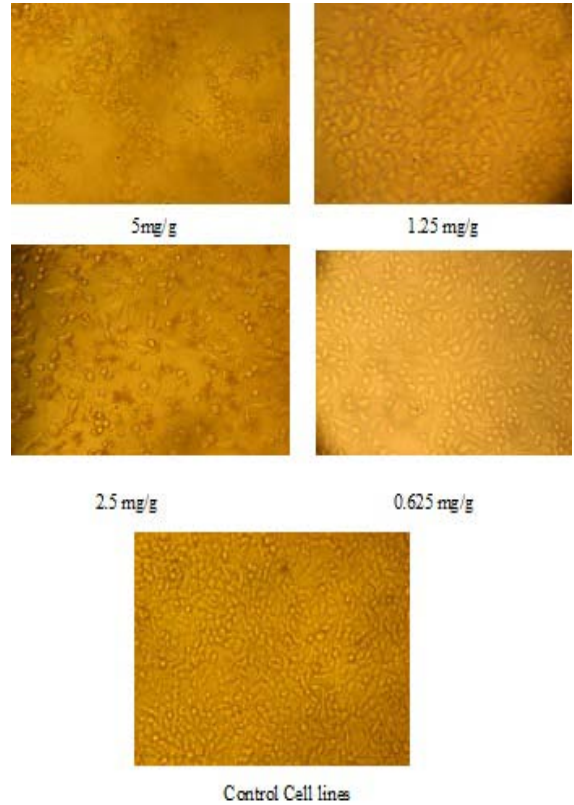


Fig. 3: Cytotoxicity of HEEHE on Hep2 Cell Line

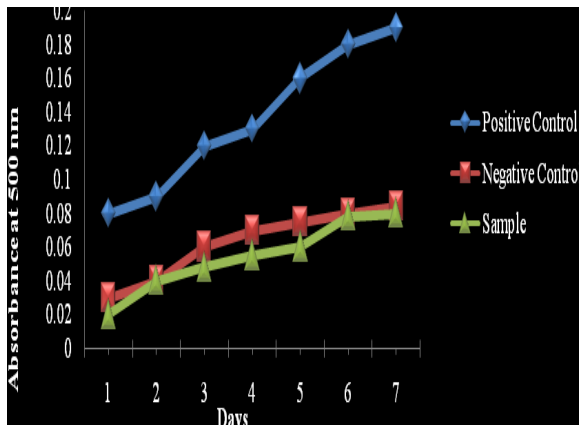


Fig.1: Estimation of total antioxidant in hydroethanolic extract of *H. enneaspermus* by Ferric Thiocyanate method

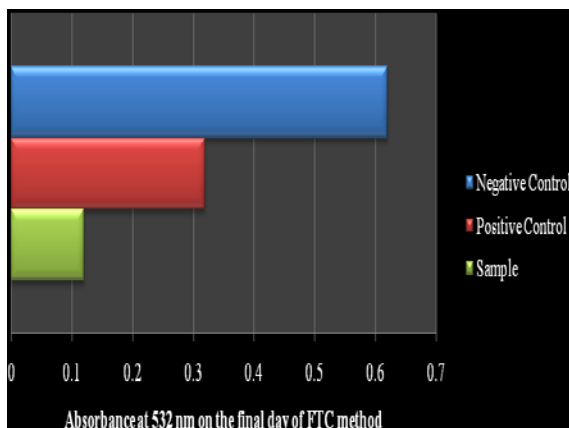


Fig.2: Estimation of total antioxidant in hydroethanolic extract of *H. enneaspermus* by Thiobarbituric acid method

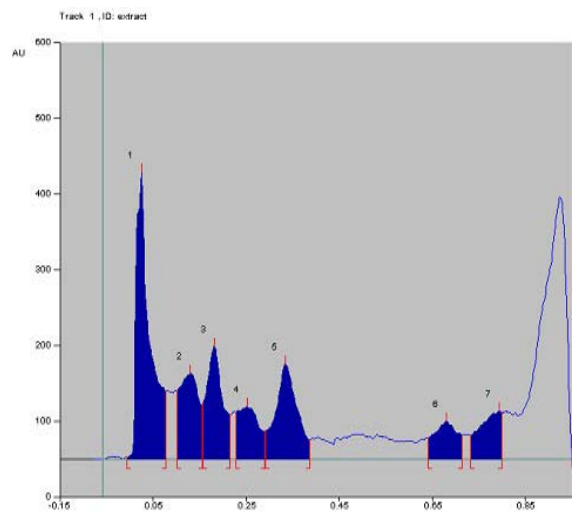


Fig. 4: Peak Densitogram of Flavonoids in HEEHE by HPTLC. Rf value (0.09) represents flavonoid, Rf value (0.11, 0.10, 0.10 and 0.12) represents unknown compounds

Table 4: Quantitative Analysis of Antioxidants in HEEHE

Parameters	Quantity(g)
Carotenoids	0.047
Lycopene	0.832
Ascorbic acid	0.821
Tocopherol	0.301
Reduced Glutathione	0.543
Total phenol	0.710

Table 5: Cell viability of HEEHE on Hep2 Cell Line Studies

S. No.	Concentration (mg/ml)	Dilutions	Absorbance	Cell viability
1	5	Neat	0.05	9.43
2	2.5	1:1	0.12	22.64
3	1.25	1:2	0.18	33.96
4	0.625	1:4	0.24	45.28
5	0.3125	1:8	0.27	50.94
6	0.156	1:16	0.32	58.49
7	0.07812	1:32	0.36	67.92
8	0.03906	1:64	0.42	79.24
9	0.01953	1:128	0.48	90.56
10	Cell control	-	0.53	100

Table 6: Characteristic IR-spectral frequencies of Phytochemicals

Type	Absorption frequency(cm ⁻¹)	Intensity	Remarks and assignment
Cycloalkanes	3380 ± 10	M	Asymmetric stretching
Alkanes	2928 ± 10	S	Asymmetric stretching
Aromatic	1630 ± 5	M -> S	C=C skeletal stretching
Acetates	1380-1365	S	Asymmetric stretching with methyl

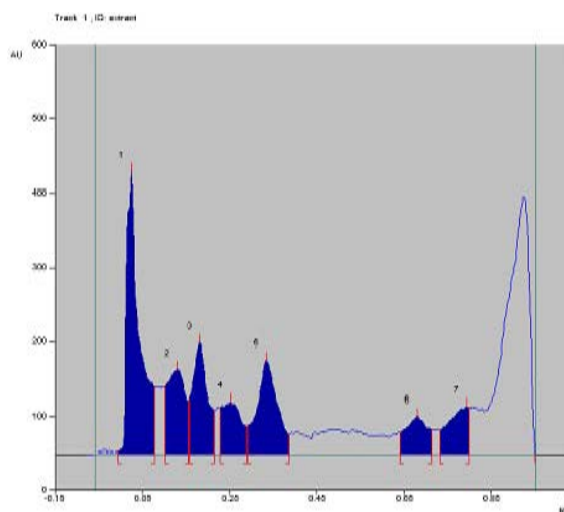


Fig. 5: Peak Densitogram of Glycosides in HEEHE BY HPTLC. Rf value (0.78) represent glycosides, Rf value (0.03, 0.13, 0.19, 0.26, 0.34 and 0.68) represents unknown compounds

RESULTS

Phytochemical studies

The results of qualitative phytochemical analysis revealed the presence of various phytochemicals in the HEEHE was summarized in Table 1. Then compared to water, chloroform and acetone, the 50% of HEEHE contained huge levels of phytochemicals, therefore used for the quantitative and antioxidant study. This might be due to high solubility of plant metabolites in the aqueous ethanolic medium. The quantitative estimation of HEEHE metabolites and minerals was represented in Table 2. Then compared to other, glycosides, tannin and Iron were present in huge amount.

Radical Scavenging activity

The free radical scavenging activity of HEEHE was depicted in Table 3 with IC₅₀ values. DPPH free radical scavenging of HEEHE was analyzed by photometric assay. The HEEHE have better DPPH activity by photometric assay. Scavenging activity towards the superoxide radical (O₂⁻) is measured in terms of inhibition of O₂⁻. DPPH, superoxide, nitric oxide and hydroxyl radical scavenging activity of HEEHE was increased with the percentage of inhibition at the concentration of 500µg/ml (80.88, 63.12 and 82.88%). Reduction potential was compared with ascorbate determined by Fe²⁺ to Fe³⁺ colour transformation.

Antioxidant assay

Total antioxidant assay of HEEHE was determined by FTC and TBA methods (Fig 1 and 2). It showed low absorbance values, which indicated a high level of antioxidant activity of the ethanolic extract of study plant. The amount of carotenoids, lycopene, vitamin C, vitamin E, reduced glutathione and phenol in HEEHE was indicated in Table 4. Lycopene was present in a higher amount (0.832/g) than compared to other antioxidants.

Cell line Studies

The MTT assay is a rapid and highly accurate colorimetric approach that widely used to determine cell growth and cell cytotoxicity, particularly in the development of new drug. It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction on the reduction of MTT to formazan. The toxicity and cell viability of HEEHE had been shown in Fig 3. The result expressed that the cell viability was considerably decreased (9.43%) in a dose dependent manner (Table 5).

Analytical Studies

The FT-IR characteristic peaks of HEEHE at 3380, 2928, 1630 and 1072cm⁻¹ which indicates the presence of alkenes, cycloalkane and aromatic group (Table 6). High performance liquid chromatography (HPTLC) method is a very desirable method for phenolic constituent's estimation from plant. HPTLC analysis of HEEHE

flavonoids was compared with standard quercetin. The respective yellow and yellow green fluorescence zone of UV 366nm was observed from the chromatogram, which confirmed the presence of flavonoids (Fig 4). The peak area and densitogram were recorded after scanning. The glycosides present in HEEHE were analyzed using degoxin as a standard. The fluorescence zone of UV 366nm was confirmed the presence of glycoside (Fig 5). The peak area and densitogram were recorded after scanning.

DISCUSSION

Generally, secondary metabolites and nutrients confirmed the pharmacological and ethno medicinal values of the plants [18]. Most bounded polyphenols are condensed tannins and comprising up to 50% of the dry weight of the leaves. Which are easily bound to proteins and carbohydrates resulting in reduction in digestibility of these macromolecules [19]. In the present study, the significant amount of tannins (42mg/g) and glycosides were observed which might be responsible for scavenging activity. Amutha et al., [20] pointed out the methanolic extract of *Hybanthus enneaspermus* showed significant *in vitro* antioxidant property. In this concern, the HEEHE exhibited antioxidant potential with the involvement of Lycopene (0.832g) than vitamin C and E. Harborne [21] stated that the phenolic compounds are majorly involved in antioxidant and anti-tumour activity. As well as, the quantitative determination of flavonoids and glycosides played a vital role in free radical scavenging and cytotoxicity effect.

Majorly, Iron performed a wide range of biological functions connected with oxidation reduction. In accordance with the present results, iron exhibited the most abundant mineral (42mg/g) in HEEHE followed by Phosphorous (3.4mg/g), calcium and magnesium. Reactive oxygen species can be important mediators of damage to cell structures, nucleic acid, lipids and proteins [22]. The DPPH radical scavenging activity of HEEHE might reduce the radical to the corresponding hydrazine when it reacts with hydrogen donors in antioxidant principle.

The HEEHE have better hydroxyl radical scavenging ability (IC₅₀ 112.56µg/ml). It might be due to quick initiation of lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids [23]. Nitric oxide radical generated by endothelial cells and macrophages, nearly 63% free radicals were scavenged by HEEHE [24]. The HEEHE contain 8.2mg/g of Vitamin-C and 3mg/g of Vitamin E. Vitamin C is one of the most extensively studied antioxidant and detected in the majority of plant cell types [25]. It readily scavenges ROS and acts as co antioxidant by regenerating vitamin-E and reduced glutathione. Similarly, the Vitamin-E are lipophilic antioxidants synthesized by all plants scavenges lipid peroxy radicals through the concerted action of other antioxidants [26]. Phenols, the aromatic compounds with hydroxyl groups they are highly capable of scavenging free radicals [27]. We observed lesser quantity of vitamin E (0.301mg/g) and moderate level of phenol (7.1mg/g). Liu et al., [28] reported the subfraction of ethanolic extract (EA6) of *W.chinensis* was cytotoxic to nasopharyngeal carcinoma (NPC) CNE-1 cells. In the present study, the microscopic observation clearly showed the cytotoxic nature of HEEHE. Earlier reports depicted the ethanolic extracts of *F.deltoidea* showed a significant reduction in the number of human ovarian carcinoma cell lines [29]. It clearly indicated the potential of ethanolic derived Phytoconstituents of the study plant. Krishnan and Maru [30] suggested that the peaks at 1620-1636 cm⁻¹ represented the carbonyl groups from polyphenols such as catechin gallate, epicatechin gallate and theaflavin. The presence of aromatic compounds with phenyl nucleus was recorded at 1630 cm⁻¹ in the HEEHE. The most common aglycones in flavonols are kaempferol, quercetin and myricetin. Among these, the flavonols and their glycosides were detected at 270, 365 and 370nm [31], in the case of the present study, flavonoid from HEEHE detected at 366nm.

CONCLUSION

We concluded the *in vitro* biological activity of *H. enneaspermus* might be the action of an active constituent like glycosides, tannin, flavonoid and minerals such as iron.

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CONFLICT OF INTEREST

The authors declared there is no conflict of interest

REFERENCES

- Dixon RA, Paiva NL. Stress-induced phenylpropanoid metabolism. *Plant Cell* 1995;7:1085-1097.
- Finkel T, Holbrook NJ. Oxidants oxidative stress and the biology of ageing. *Nature* 2000;408:239-247.
- Satyavani K, Gurudeeban S, Ramanathan T, Balasubramanian T. Radical Scavenging Effect and GCMS identification of alkaloid fractions from *E. agallocha* L. *Inventi Rapid:Ethnopharmacol* 2013;1:1-4.
- Gurudeeban S, Satyavani K, Ramanathan T. Bitter apple (*C.colocynthis*)-An overview of chemical composition and biomedical potentials. *Asian J Plant Sci* 2010;9:394-401.
- Savithramma N, Linga Rao M, Ankanna S. Screening of traditional medicinal plants for secondary metabolites. *Int J Res Pharma Sci* 2011;2:643-647.
- Harborne JB, A. *Phytochemical Methods:to Modern Techniques of plant Analysis*. Chapman and Hall Ltd London p 279 1973.
- Patel DK, Kumar R, Sairam K, Hemalatha S. *H.enneaspermus* (L.) F. Muell:a concise report on its phyto-pharmacological aspects. *Chinese J Natural Med* 2013;11:199-206.
- Raveendra Ratnam K, John De Britto A. Antimicrobial activity of a medicinal plant *H. enneaspermus* (L.) F. Muell. *Natural Prod Rad* 2007;6:366-368.
- Vuda M, DSouza R, Upadhy S, Kumar V, Rao N, Kumar V et al. Hepatoprotective and antioxidant activity of aqueous extract of *H.enneaspermus* against CCl₄-induced liver injury in rats. *Exp Toxicol Pathol* 2012;64:855-859.
- Evans WC. Trease and Evans *Pharmacognosy*, 14th Ed, Harcourt Brace and Company. Asia Pvt. Ltd, Singapore:1997. p.227.
- Mallick CP, Singh MB. *Plant enzymology and Histoenzymology* (Ed), Kalyani publishers, New Delhi:1980. p.286.
- Sanchez MC. Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci Technol Int* 2002;8:21-137.
- Kunchandy E, Rao MNA. Oxygen radical scavenging activity of curcumin. *Int J Pharm* 1990;58:237-240.
- Jayaprakash GK, Singh RP, Sakariah KK. Antioxidant activity of grape seed extracts on peroxidation models in-vitro. *J Agric Food Chem* 2001;55:1018-1022.
- Kikuzaki H, Nakatani N. Antioxidant effects of some ginger constituents. *J Food Sci* 1993;58:1407-1410.
- Ottolenghi A. Interaction of ascorbic acid and mitochondrial lipids. *Arch Biochem Biophys* 1959;79:355-358.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival:application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
- Sathish Kumar T, Rajasekaran P, Shanmugam S. Phytochemical constituents and antibacterial activities of *Elacocarpus ganitrus* Roxb. and *Canthium parviflorum* L. Leaves. *Advanced biotech* 2007;23-25.
- Nwogu LA, Igwe CU, Emejulu AA. Effects of *Landolphia owariensis* leaf extract on the liver function profile and haemoglobin concentration of albino rats. *Afr J Biotechnol* 2008;2:240-242.
- Amutha Priya D, Ranganayaki S, Suganya Devi P. Phytochemical screening and antioxidant potential of *H. enneaspermus*:A rare ethano botanical herb. *J Pharm Res* 2011;4:1497-1502.
- Harborne JB. *Phytochemical Methods. A guide to modern technique of plant analysis*, Chapman, New York:1984. p 89.
- Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico Biol Interac* 2006;160:1-40.
- Kannan H, Nakatani N. Antioxidant effects of ginger constituents. *Food sci* 2004;58:1407-1410.

24. Lata BC. Myocardial infarction in young vs old male rats: Pathophysiologic changes. *Am Heart J* 2000;96:70-80.
25. Borland A, Elliott S, Patterson S. Are the metabolic components of crassulacean acid metabolism up-regulated in response to an increase in oxidative burden?. *Exp Bot* 2006;57:319-328.
26. Igamberdiev AU, Hill RD. Nitrate, NO and haemoglobin in plant adaptation to hypoxia: An alternative to classic fermentation pathways. *J Exp Bot* 2004;55:2473-2482.
27. Alkawah A, Gandhi C, Balaraman R. Effect of green tea and vitamin E combination in isoproterenol induced myocardial infarction in rats. *Plant Foods Hum Nut* 2004;64:75-80.
28. Liu M, Wang W, Li X, Shi D, Mei H, Jin X et al. *Wedelia chinensis* inhibits nasopharyngeal carcinoma CNE-1 cell growth by inducing G2/M arrest in a Chk1-dependent pathway. *Am J Chin Med* 2013;41:1153-68.
29. Akhir NAM, Lee SC, Abdul Majid FA, Sarmidi MR. Cytotoxicity of Aqueous and Ethanolic Extracts of *Ficus deltoidea* on Human Ovarian Carcinoma Cell Line. *Br J Med Med Res* 2011;1:397-409.
30. Krishnan R, Maru GB. Isolation and analysis of polymeric polyphenol fractions from black tea. *Food Chem* 2006;94:331-340.
31. Kim D, Lee CY. Reversed-phase HPLC analysis of polyphenolics separated into nonanthocyanin and anthocyanin fractions. *Curr Protocol Food Anal Chem* 2002;11.3.1-11.3.16.