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In-vitro antioxidant potential of *Heliotropium zeylanicum*

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Abstract
Antioxidant activity *of crude extracts of Heliotropium zeylanicum* whole plant was investigated for its free radical scavenging activity by determining the DPPH, FRAP, SOD, ABTS and H_2O_2 radical scavenging activity. Maximum antioxidant potential was exhibited on ethanol extract.

Key Words: Free radicals, ROS, Crude extracts, DPPH, FRAP.

Introduction

Free radicals or reactive oxygen species (ROS) are formed by exogenous chemicals or endogenous metabolic processes in the human body. These are capable of oxidizing biomolecules viz., nucleic acids, proteins, lipids and DNA and can initiate different degenerative diseases like neurological disorders, cancer, emphysema, cirrhosis, atherosclerosis, arthritis etc. In recent years there is an upsurge in the areas related to newer developments in prevention of disease especially the role of free radicals and antioxidants.

Heliotropium zeylanicum is an important medicinal plant belongs to the family Boranginaceae. In Tamil Nadu this plant is widlely found in and around Narthamalai, Pudukottai District. The plant is found to possess high valued medicinal properties. It is used as diuretic, astringent, emollient, vulneary and also used to local application for fever. Root part is used to cure cough and promote menstruation (Kokwaro 1993 and Martin 1990). The present study was

under taken to screen the antioxidant potential of *Heliotropium zeylanicum* a wild herbaceous plant is very common in all tropical countries, including India.

Materials and methods

DPPH Radical Scavenging Activity

DPPH radical scavenging activity was determined by the method of (Blios 1958). All sample extracts at different aliquots of 50 – 250 µL were dissolved and made upto 1000 µL with methanol. Later it was mixed with 5 ml of DPPH solution (0.1 mM) and allowed to stand for 20 min at 27°C.Decolourization of purple to yellow in all the tubes were measured spectrophoto metrically at 517 nm. A blank was prepared using methanol solvent without adding plant extract which served as control. Radical scavenging activity was expressed and percentage of inhibitions was calculated using the following formula. % Inhibition = [(A0 – A1)/A0] X 100.
Where, A0 is the absorbance of the control, A1 is the absorbance of extract/standard.

ABTS Assay

ABTS method This assay was based on the ability of different substances to scavenge 2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid (ABTS.+) radical cation. The radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and whole plant the mixture for 4-16 h until the reaction was complete and the absorbance was stable. The ABTS.+ solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm for measurements. The photometric assay was conducted on 0.9 mL of ABTS. +Solution and 0.1 mL of tested samples (100 and 200 µg/mL) and mixed for 45 sec; measurements were taken immediately at 734 nm after 15 min. The antioxidative activity of the tested samples was calculated by determining the decrease in absorbance at different concentrations by using the following equation: E= ((Ac-At)/ Ac) × 100, where: At and Ac are the respective absorbance of tested samples and ABTS .+, was expressed as µ mol 15. Extraction and determination of water soluble pigments (phycobiliprotein) The water soluble phycobiliproteins pigments including allophycocyanin (APC), phycocyain (C-PC) and Cphycoerytherine (C-PE) were determined according to Bryant *et al.*,1979 The absorbance (A) of the solution was recorded at the following wave lengths: 650 nm; 620 nm; 565 nm.

Ferric Reducing Antioxidant Power Assay

Ferric reducing antioxidant power assay was performed according to the method followed by (Pulido *et al.*, 2000). The ferric reducing antioxidant power (FRAP) reagent was prepared by adding 2.5 mL of 20 mmol/L TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mmol/L HCL and 2.5 mL of 20 mmol/L $FeCl3 \cdot 6H2O$ and 25 mL of 0.3 mol/L acetate buffer (pH 3.6). 90 µL of plant extracts and standard was added with 270 µl of distilled water. 2.7 mL of FRAP reagent was mixed and incubated at 37°C in water bath, 30 µl of methanol for the blank reagent was prepared similarly. After incubation, the absorbance was read immediately at 593 nm. The FRAP value is expressed as mmol Fe (II) equivalent/mg extract.

Scavenging assay (SOD)

The activity of SOD in tissue was assayed using the method of Kakkar *et al.*, 1984. The assay mixture con-tained sodium pyrophosphate buffer 1.2 mL (pH 8.3,0.025 mol/L), phenazine methosulphate 0.1 mL (186 mmol/L),NBT 0.3 mL (300 mmol/l), NADH 0.2 mL (780 mmol/L) and appropriately diluted enzyme preparation and water in a total volume of 3 mL. After incubation

at 30°C for 90 s, the reaction was terminated by the addition of 1.0 mL of glacial aceticacid. The reaction mixture was stirred vigorously and shaken with 4.0 mL of n-butanol. The color intensity of the chro-mogen in the butanol layer was measured at 560 nm againstn-butanol.

Hydrogen peroxide (H2O2) assay

Hydrogen peroxide assay was carried out according to the modified method of (Ruch *et al.,* 1989). A solution of hydrogen peroxide (2mol/L) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined by spectrometric absorbance at 230nm. Extracts were prepared at the concentration of 100µg/ml and added to the hydrogen peroxide solution (0.6mL). Negative reaction mixture was also prepared in the same method without add plant extracts while blank solution containing phosphate buffer without hydrogen peroxide was prepared simultaneously. The % of inhibition activity was calculated from the formula % of inhibition activity= [(A0 – A1)/A0] X 100. Where, A0 is the absorbance of the control, A1 is the absorbance of extract/standard.

Results and Discussion

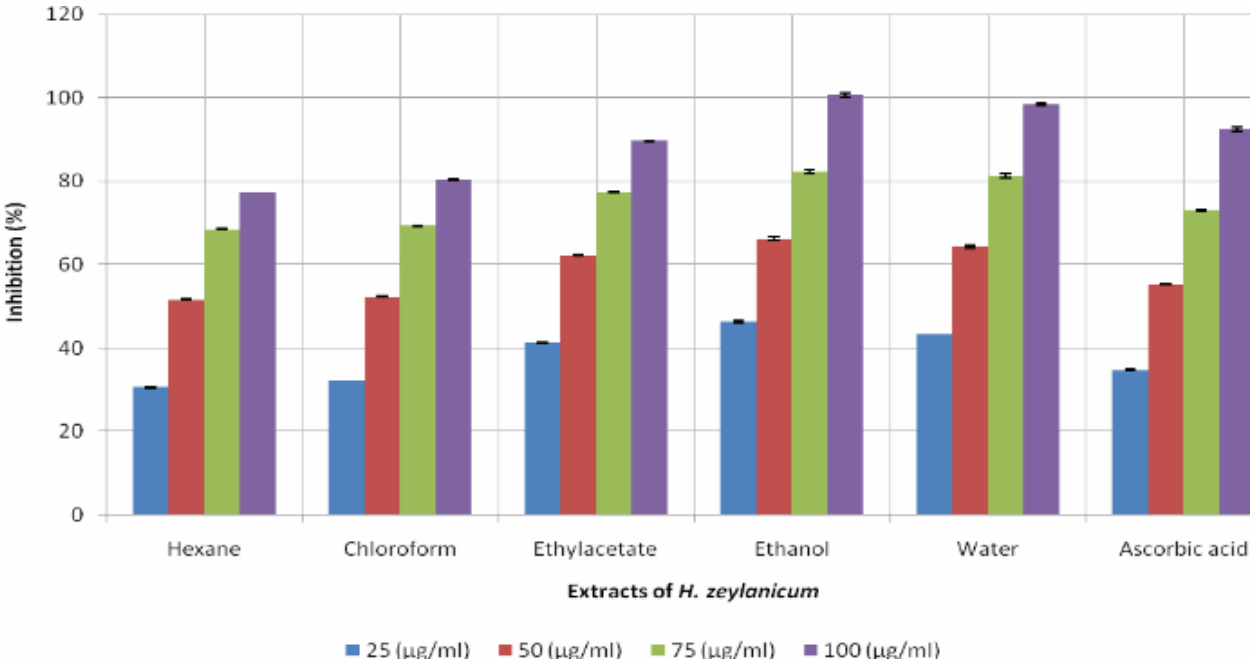
DPPH free radical scavenging

DPPH radical scavenging ability is widely used as an index to evaluate the antioxidant potential of medicinal plants. *In vitro* antioxidant studies of the five extracts of *H. zeylanicum* showed the extent of DPPH radical scavenging at different concentrations (25, 50, 75 and 100 µg/ml) of *H.zeylanicum*. This was measured, with ascorbic acid as the standard. The antioxidants could reduce the stable radical DPPH to the positively yellow coloured diphenyl-picryl hydrazine, in the DPPH assay. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction. (Table 1 and Fig. 1) illustrate an effective increase in the inhibition of concentration of DPPH radical due to the scavenging ability of extracts of *H. zeylanicum* which is also dependent on its concentration. The scavenging effect of ethanol extract of (100.65±0.59%) *H. zeylanicum* was higher than that of other extracts such as water (98.37±0.20%), ethylacetate (89.65±0.04%), chloroform (80.37±0.14%), hexane (77.32±0.05%) and ascorbic acid (92.55±0.51%). IC50 value of different extracts was found against DDPH scavenging activity using MS-Excel. The results revealed that the ethanol extract from whole plant extract of the plant might exhibit the highest free radical scavenging activity against DPPH, by the reduction in the concentration of DPPH. Similarly, Sharma *et al*., (2009) stated that ethanol extract of the stem bark of *Albizia procera* showed potent antioxidant activity in DPPH scavenging model when compared with ascorbic acid. Manoj and Kumar Narashimhan *et al*. (2015) also stated that the ethanolic extract of *Hippophaer hamnoides* showed significant higher antioxidant activity and correlated with total phenol content in comparison with other solvent extracts. Stewart *et al.* (2013) reported a high correlation between DPPH radical scavenging potential and total phenolic content. Sarah *et al*. (2014) also revealed that two of the compounds from *E. angustifolia* had significant antioxidant effect against DPPH. The results of various extracts of *H. zeylanicum* indicate that the ethanol extracts with high total phenolic contents presented high radical scavenging activities which could be related to the inherent nature of phenolic compounds, thus contributing to their electron transfer or hydrogen donating ability. It has also been reported that the compounds with different polarity revealing various rates of antioxidant potential, is solidly based on the types of solvent used (Kumaran, 2007).

Table 1: Effect of *H. zeylanicum* on the percentage of DPPH activity

H. zeylanicum (µg/mL)	DPPH activity (%) (Mean±SE)					
	Hexane	Chloroform	Ethyl acetate	Ethanol	Water	Ascorbic Acid
25	30.65±0.08	32.2±0.05	41.32±0.13	46.32±0.16	43.32±0.06	34.82±0.08
50	51.65±0.06	52.32±0.11	62.32±0.12	66.32±0.33	64.34±0.48	55.33±0.07
75	68.45±0.10	69.32±0.05	77.43±0.09	82.32±0.58	81.32±0.58	73.02±0.18
100	77.32±0.05	80.37±0.14	89.65±0.04	100.65±0.59	98.37±0.20	92.55±0.51
IC50(µg/mL)	51.32	49.26	34.89	29.11	32.53	44.26

Fig.1: Effect of *H. zeylanicum* on the percentage of DPPH activity



ABTS free radical scavenging

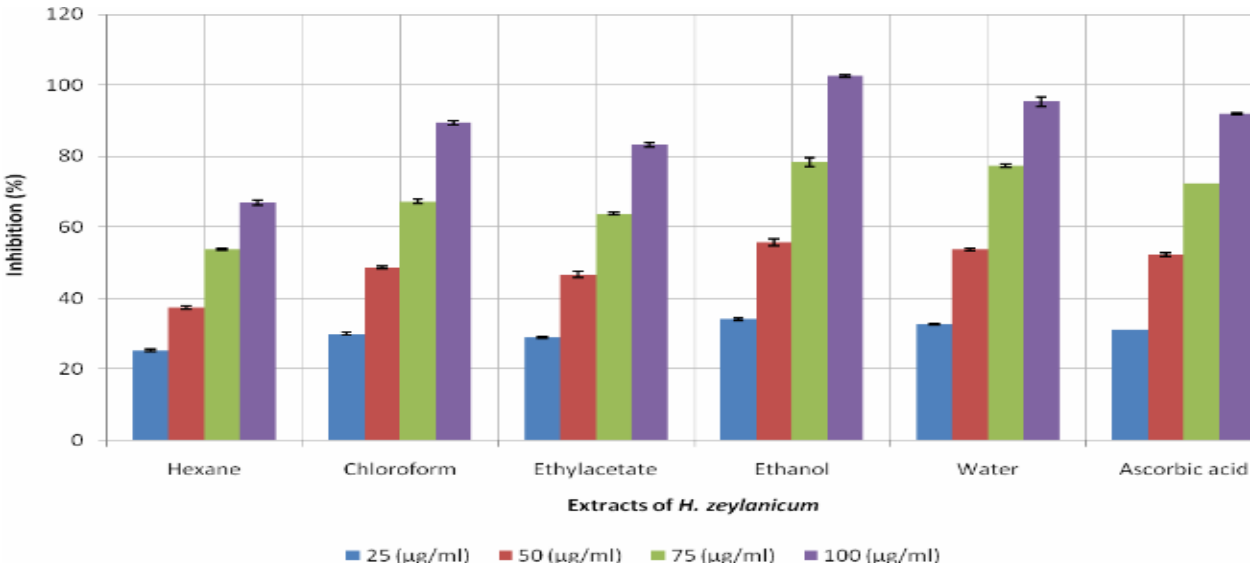
The extract efficiently scavenged ABTS (2, 2'-azinobis-3- ethylbenzothiozoline- 6-sulphonic acid) radical based on the scavenging ability of light by ABTS radicals. From (Table 2 and Fig. 2) the high activity was found to be 102.55± 0.33% in ethanol extract and followed by water extract (95.35± 1.27%), chloroform extract (89.4± 0.52), ethyl acetae extract (83.26± 0.60%), and hexane extract (66.95± 0.69%). The control (ascorbic acid) has activity of 92.01±0.18% and IC50 values were calculated by MS-Excel. for five extracts of *H.zeylanicum* and reported in Table 2 and Fig 2. A previous study reported that alcoholic extract of *Rotula aquatica* and *Ancistrocla dusheyneanus* (Jamuna and Ravishankar, 2013) and *Aristolochia krysagathra* (Jegadeeswari *et al*., 2014) also showed the high ABTS scavenging activity. The whole plant methanol and ethanol extracts of *Aristolochia krysagathra* were fast and effective scavengers of

ABTS radical and this activity was higher than that of standard. ABTS a protonated radical has characteristic absorbance maxima at 734nm which decreases with the scavenging of the proton radicals (Adeolu *et al*., 2008). Therefore, the ABTS radical scavenging activity of ethanol extract of leaves of *H. zeylanicum* indicates its ability to scavenge free radicals, there by lipid oxidation via chain breaking reaction.

Table 2: Effect of *H. zeylanicum* extracts on the percentage of ABTS activity

***H. zeylanicum* (µg/mL)**	**ABTS activity (%) (Mean±SE)**					
	Hexane	**Chloroform**	**Ethyl acetate**	**Ethanol**	**Water**	**Ascorbic Acid**
25	25.4±0.45	30.04±0.42	28.95± 0.14	34.11±0.34	32.76±0.28	31.21±0.03
50	37.43± 0.59	48.67± 0.29	46.69± 0.86	55.72± 0.99	53.7±0.32	52.22±0.50
75	53.83± 0.29	67.25± 0.61	63.84± 0.31	78.37± 1.25	77.34± 0.59	72.37±0.04
100	66.95± 0.69	89.4± 0.52	83.26± 0.60	102.55±0.33	95.35± 1.27	92.01±0.18
IC50(µg/mL)	69.76	51.26	54.61	43.10	45.02	47.75

Fig.2: Effect of *H. zeylanicum* extracts on the percentage of ABTS activity



FRAP Activity

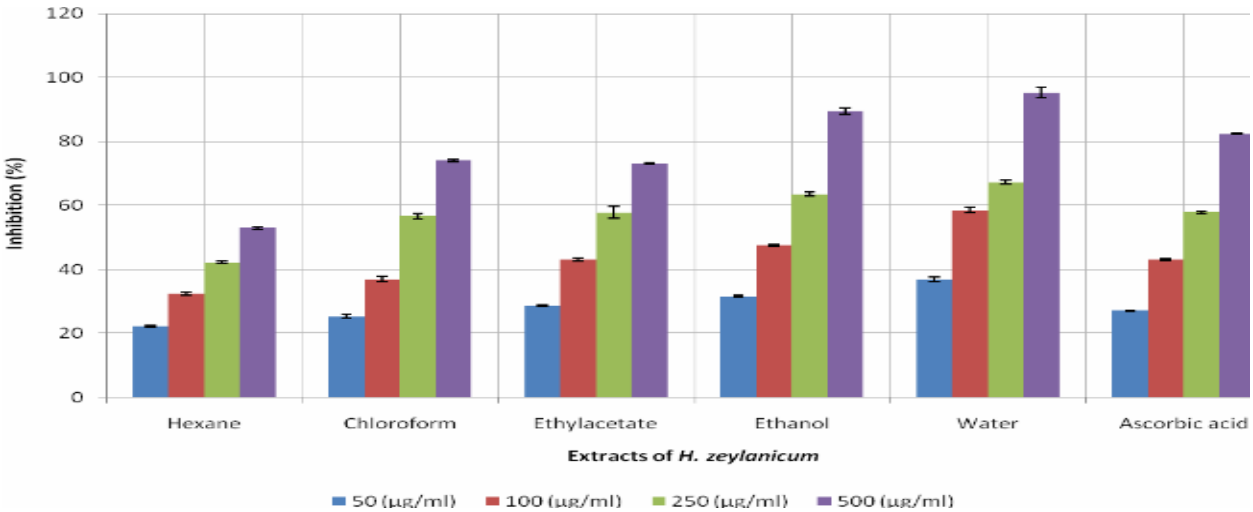
The increased concentration of various extracts showed the increased absorbance that indicates an increase in reductive ability. *In vitro* antioxidant studies of the five extracts of *H. zeylanicum* showed the extent of FRAP activity at different concentrations (25, 50, 75 and

100µg/mL) of *H. zeylanicum*. From the (Table 3 and Fig. 3), the FRAP of water extract was found to be 95.28±1.56% at maximum concentration (100µg/mL), followed by the ethanol extract (89.51±1.06%), chloroform (74.02±0.35%), ethyl acetate (73.11±0.28%) and hexane extract (52.96±0.32%). The control (Ascorbic acid) was found to be 82.37± 0.09 % of activity and IC50 values were determined by MS-Excel for five extracts of *H. zeylanicum* and reportedin (Table 3). The results of the ferric reducing assay indicated that water extract had stronger reducing power than all other extracts. This signifies consistent reduction of Fe3+ to Fe2+, indicating the reduction potential of the plant. It suggests that aqueous leaf extract of *H. zeylanicum* has increase in the FRAP activity and this might be due to the high concentrations in the total phenolics and total flavonoids (Barku *et al*., 2013). An interesting finding is that the FRAP activity showed more correlation with total phenols and total flavonoids. Our results confirm the importance of flavonoids and phenolics as the antioxidant agents in aqueous leaf extract of *H. zeylanicum* that significantly contribute to the total antioxidant capacity. However, the ability of reducing power of all the solvent extracts of the whole plant extract *H. zeylanicum* was significantly higher than the synthetic antioxidant, ascorbic acid. This could be due to the presence of more reactive concentration of bioactive constituents (especially phenols and flavonoids) in water than all other extracts. On par with the present study, the aqueous extracts of *Moringa oleifera* showed best results than other alcoholic solvents (Dolly *et al*., 2013).

Table 3: Effect of *H. zeylanicum* extracts on the percentage of FRAP activity

***H. zeylanicum* (µg/mL)**	**FRAP activity (%) (Mean±SE)**					
	Hexane	**Chloroform**	**Ethyl acetate**	**Ethanol**	**Water**	**Ascorbic Acid**
25	22.22±0.19	25.37±0.52	28.75±0.21	31.61±0.25	36.86±0.80	27.16± 0.11
50	32.45±0.60	37.03±0.75	43.19±0.49	47.62±0.31	58.62±0.85	43.15± 0.26
75	42.25±0.44	56.7±0.89	57.84±1.85	63.56±0.67	67.28±0.58	57.91± 0.38
100	52.96±0.32	74.02±0.35	73.11±0.28	89.51±1.06	95.28±1.56	82.37± 0.09
IC50(µg/mL)	93.20	65.09	61.28	51.85	42.78	58.83

Fig.3: Effect of *H. zeylanicum* extracts on the percentage of FRAP activity



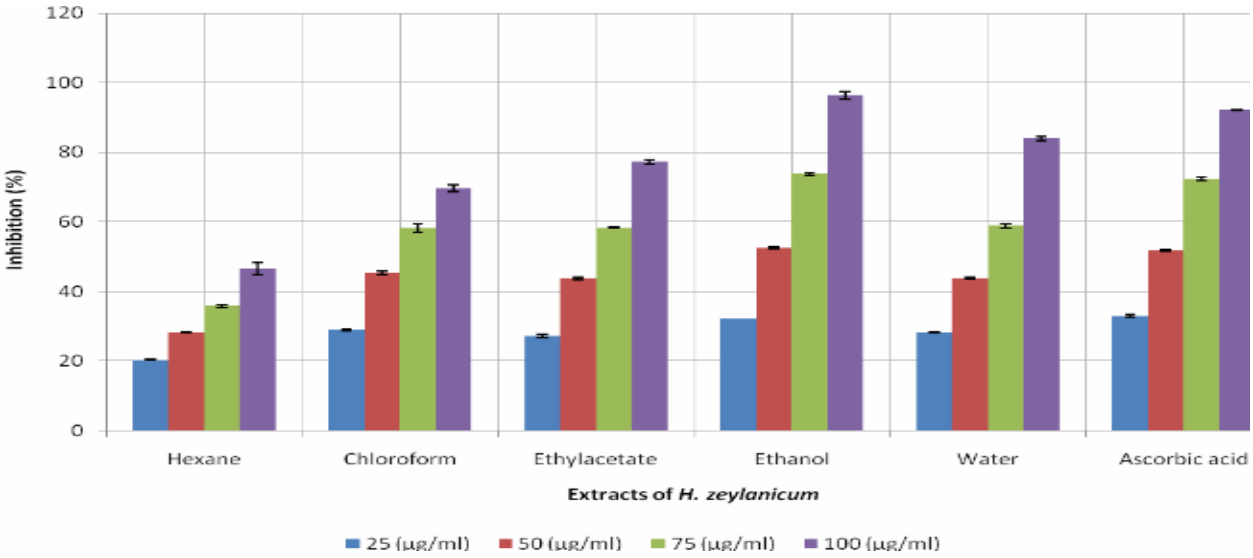
SOD Activity

Results of superoxide anion scavenging activities of various extracts of *H. zeylanicum* are shown in (Table 4 and Fig. 4). The different extracts exhibit good superoxide anion radical scavenging activity from 25 to 100µg/mL. The SOD scavenging activities were found to be 96.35±1.21, 83.96±0.63, 77.25±0.64, 69.67±0.88 and 46.67±1.76% for ethanol, water, ethyl acetate, chloroform and hexane, respectively at 100µg/ml. IC50 values were determined by MS-Excel for five extracts of *H. zeylanicum* and reported in (Table 4). On the other hand, at the same concentration, the standard (ascorbic acid) showed inhibition of 92.14±0.10%. The study on Triphala (equal proportion of alcoholic extract of *Terminalia chebula,Terminalia belerica*, *Emblica officinalis*) reported that it has high superoxide scavenging activity Sabu and Ramadasan Kuttan, (2002). Similar to our study, Subhrajyoti *et al*., (2013) evaluated 70% alchoholic extract of *Diplaziumes culentum* for superoxide free radicals and efficient free radical scavenging properties. This could be due to the presence of reactive concentration of bioactive constituents and mixture of other nutrients in the extract.

Table 4*:* Effect of *H. zeylanicum* extracts on the percentage of SOD activity

H. zeylanicum (µg/mL)	SOD activity (%) (Mean±SE)					
	Hexane	Chloroform	Ethyl acetate	Ethanol	Water	Ascorbic Acid
25	20.34±0.16	29.01± 0.26	27.22± 0.57	32.17±0.08	28.34± 0.12	32.96±0.38
50	28.33±0.07	45.41± 0.48	43.81± 0.41	52.56±0.33	43.83± 0.34	51.81±0.22
75	35.88±0.34	58.28±1.19	58.35± 0.09	73.85±0.36	58.91± 0.65	72.36±0.47
100	46.67±1.76	69.67±0.88	77.25± 0.64	96.35±1.21	83.96± 0.63	92.14±0.10I
IC50(µg/mL)	112.16	61.40	59.98	46.45	57.33	46.95

Fig.4: Effect of *H. zeylanicum* extracts on the percentage of SOD activity



H_2O_2 Activity

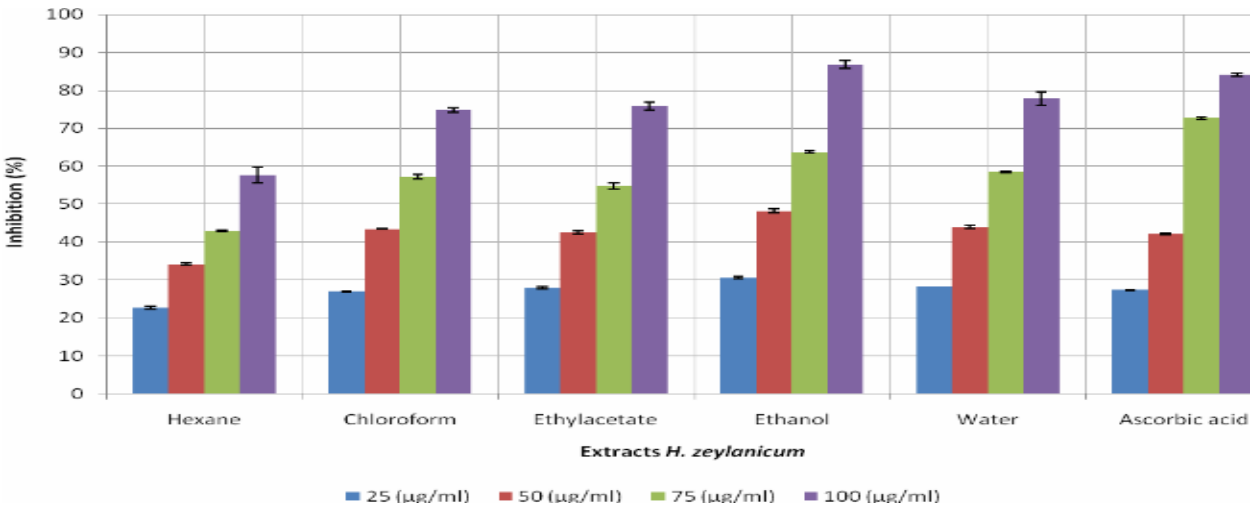
Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. H_2O_2 is very important due to its capability to diffuse biological membranes. The (Table 5 and Fig. 5) showed the results of H_2O_2 scavenging activity of various extracts of *H. zeylanicum* and the highest percentage of activity was found with ethanol (86.77±0.96%), followed by water (77.83±1.77%), ethylacetate (75.83±1.09%), chloroform (74.83±0.60%) and hexane (57.67±2.03%) at highest concentrations (100µg/mL). IC50 values were determined by MS-Excel for five extracts of *H. zeylanicum* and reported in Table 5. The ascorbic acid was found to be 84.07±0.42% of activity. Similarly, a study on the ethanolic

extract of leaves of *Phyllanthus wightianus* revealed that it exhibits a high a potential hydrogen peroxide antioxidant activity (Joseph *et al*., 2014). Subhrajyoti *et al*., (2013) also
reported that 70% alcoholic extract of *Diplazium esculentum* exhibited the good activity against hydrogen peroxide and superoxide ions. The acidic methanol and water extracts of six herbaceous plants (*Biden salba, Lycium chinense, Mentha arvensis, Plantago asiatica, Houttuynia cordata, and Centella asiatica*) containing antioxidants can protect against DNA damage in human lymphocytes induced by hydrogen peroxide (Kuan-Hung Lin . *et al*., 2013). Scavenging of OH- is an important antioxidant activity because of its very high reactivity, which can easily cross the cell membranes at specific sites, react with most biomolecules and furthermore cause tissue damage and cell death. In the present study, all the extracts of *H. zeylanicum* at 100µg/mL scavenged $H_2O_2$and this may be attributed to the presence of phenolics, which could donate electrons to $H_2O_2$and thus
neutralize it to water (Mathew and Abraham, 2006).

Table 5: Effect of *H. zeylanicum* extract on the percentage of H2O2 activity

***H. zeylanicum* (µg/mL)**	**H2O2activity (%) (Mean±SE)**					
	Hexane	**Chloroform**	**Ethyl acetate**	**Ethanol**	**Water**	**Ascorbic Acid**
25	22.75±0.41	26.98±0.13	27.92±0.35	30.64±0.37	28.31±0.06	27.39±0.12
50	34.16±0.30	43.4± 0.10	42.5± 0.37	48.18±0.48	44.02±0.41	42.08±0.23
75	42.89±0.20	57.24±0.56	54.67±0.80	63.73±.32	58.47±0.20	72.7±0.32
100	57.67±2.03	74.83±0.60	75.83±1.09	86.77± 0.96	77.83± 1.77	84.07±0.42
IC50(µg/mL)	85.91	61.53	62.14	52.53 .	59.19	54.33

Fig 5: Effect of *H. zeylanicum* extract on the percentage of H2O2 activity



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