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Evaluation of Antioxidant Activity and Radical Scavenging Capacities of Different Fractions of *Nepeta hindostana*

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The aim of this research is to study the *in vitro* antioxidant and radical scavenging activity of *Nepeta hindostana* components which are effective against different disorders like cardiovascular and kidney diseases. These are the traditionally used herbs against degenerative diseases so it is an important task to evaluate their antioxidant activity. The herb was extracted in aqueous as well as in different organic solvents. Antioxidant potential and radical scavenging activity of the seven fractions were investigated by using different antioxidant assays such as {2,2´-azino*bis*(3-ethylbenzothiazoline-6-sulphonic acid)}, ferric reducing antioxidant power, total phenolic contents and diphenyl-1-picrylhydrazyl radical scavenging assay. Trolox equivalent antioxidant activity of the aqueous and organic fraction of these herbs was determined by calculating the % inhibition of the coloured radical solution after reaction with sample and standard antioxidants by comparing with the standard curve formed by Trolox as standard antioxidant. Total phenolic contents and ferric reducing antioxidant power decolourization assays, showed a wide range of antioxidant activity. Using total phenolic content assay the amount of total phenolics components ranged from 0.294 to 7.872 μM and Ferric reducing antioxidant power ranged from 1.032 to 7.906 μM which showed the presence of many phenolic components in its composition. On the basis of such antidisease activity of these herbs attributes the presence of these antioxidant components.

Key Words: *Nepeta hindostana*, 2,2′-Azino*bis*(3-ethylbenzothiazoline-6-sulpohonic acid), Radical cation, Trolox equivalent antioxidant capacity, Ferric reducing antioxidant power.

INTRODUCTION

In living systems, normal metabolic processes are responsible for the generation of free radicals. The free radical chain reactions are usually produced in the mitochondrial respiratory chain[1], liver mixed function oxidases, through xanthine oxidase activity, atmospheric pollutants, transition metal catalysts, drugs and xenobiotics. In addition, chemical mobilization of fat stores under various conditions such as lactation, exercise, fever, infection and even fasting, can result increased radical activity and damage. Free radicals or oxidative injury now appears the fundamental mechanism underlying a number of human neurologic and other disorders[2]. For instance, in diabetes, increased oxidative stress which co-exists with reduction in the antioxidant status has been postulated[3]. Currently, the possible toxicity of synthetic antioxidants has been criticized. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea and herbs may contribute to shift the balance toward an adequate antioxidant status. This interest in natural antioxidant, especially of plant origin, has greatly increased in recent years[4].

Nepeta hindostana is generally called as Cal Mint and is Vernacular name is Billi Lotin. It belongs to Lamiaceae family. It is a medium sized annual herb, 15-40 cm high, branches are present at the base. These are erect or ascending, quadrangular, ridged, softly pubescent and often floriferous throughout their length. Leaves are 1.3-5.0 by 1.3-8.0 cm, petioles 8-12 mm long, flowers are pedicellare, 6 mm, blue or purple, slightly dotted, one sided stalked and branched, clusters at intervals along softy hairy spikes. The leaves are also chewed to relieve toothache[5,6]. Nutlets are mucilaginous when moistened, broadly oblong, brown with white spots. Fresh herb has a strong smell like that of mint[7-11].

Adjunct to cancer therapy, short-term benefits were noted in the treatment of arrhythmia, especially in case of coronary disease and particularly in premature beats, treatment of cervix erosion, in the treatment of jaundice, dysentery, diarrhea and urinary infection, pruitis, ringworm, leprosy and itching allergic reactions. Extract of the plant is used for the treatment of fever and pain, including earache and toothache[12]. It is also administered as a remedy for cold[13], chest cavity[14], distended stomach or children's rickets[15].

Although some preliminary studies on antioxidative potential of the plant extracts and its herbomineral formulations have been carried out[16,17] but no comprehensive study on the radical scavenging and antioxidant capacity has been undertaken as yet. The objective of the present study is to evaluate radical scavenging and antioxidant potential of different organic and aqueous extracts of *Nepeta hindostana* by using Trolox equivalent antioxidant capacity (TEAC) assay, ferric reducing antioxidant power (FRAP) assay, total phenolic contents (TPC) assay, DMPD free radical scavenging assay and ABTS decolourization essay.

EXPERIMENTAL

Nepeta hindostana was purchased from a local market, Paparr Mandi, Lahore, in January 2010 and identified by Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore.

Extraction of antioxidant components: Socked 10 g of finely ground herb in 100 % methanol (4 × 200 mL) at room temperature with mild shaking (2 × 24 h). The extract was filtered out and the residue was extracted again to ensure complete extraction. From 100 % of the filtrate, methanol was evaporated under reduced pressure to obtain a crude residue. The residue was re-suspended in distilled water (200 mL).The aqueous solution was successively partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol (4 × 25 mL for each extraction). The non-aqueous layer was separated and stored at 4 ºC until used for further analysis.

Standard antioxidant such as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxlic acid), N,N -dimethyl-*p*-phenylene diamine (DMPD) ICN Biomedical I, *n*-hexane, acetone, methanol, acetic acid, ethyl acetate, ethyl alcohol, ferric chloride, sodium chloride, sodium acetate, dichloromethane, potassium per sulfate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, all of them are E.Merk, deionized water, (ABTS) 2,2-Azino-*bis*-3-ethylbenzothiazoline-6-sulphonic acid, I-diammonium salt, Aldrich chemical Co., TPTZ (2,4,6-tripyridal-s-triazine), hydrochloric acid. Follin-Ciocalteau's reagent and potassium persulfate (di-potassium peroxdisulfate) were purchased from Fluka (UK). HPLC grade ethanol was purchased from Rathburn Chemicals Ltd. (Walkerburn, Peebleshire, Scotland). Spectrophotometric measurements were made on UV-1700 PharmaSpec. UV-visible spectrophotometer, Shimadzu, Japan equipped with temperature control device. All the solutions were made in triplicate and experiments were performed three times. The results obtained were averaged.

$ABTS^{\bullet+}$ radical cation decolourization assay: (in buffer media): ABTS radical scavenging assay depends upon the scavenging activity of ABTS radical cation generated from a reaction between ABTS and 3.49 mL of (10 mM) potassium persulfate and making the total volume to 14.28 mL by adding 0.780 mL of deionized water in it. The antioxidant components reduce bluish green ABTS radical to colourless native ABTS depending upon their nature and quantity on a time-dependant scale. Trolox is generally used as a standard antioxidant for comparison purposes. For the determination of trolox equivalent antioxidant capacity of the extracts, $ABTS^{\bullet+}$ decolourization assay was followed[21]. ABTS radical cation was produced by a reaction between ABTS and potassium persulfate (7 mM and 2.45 mM final concentrations, respectively) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use and intense bluish green coloured stable radical cation ($ABTS^{+}$) was generated. To study the antioxidant activity of standard antioxidant and indigenous medicinal herb, the ABTS stock solution was diluted with PBS buffer (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 ºC.

Then 3.49 mL of diluted $ABTS^{\bullet+}$ solution (A 734 nm = 0.70 + 0.020) was transferred into the cuvette and note down the absorbance as Å. Then added 10 µL of sample solution, the absorbance reading was taken as 25 ºC exactly 1 min after initial mixing and upto 6 min. Appropriate blank were run in each case. All determinations were carried out at least three times in succession and in triplicate at each separate concentration level of the standards. The percentage inhibition of absorbance was calculated by the following formula.

$$\text{Inhibition (at 734 nm) (\%)} = (1- I_f/I_o) \times 100$$

where I_o is the absorbance of radical cation solution before addition of sample/standard antioxidants and I_f is the absorbance after addition of the sample/standard antioxidants. $ABTS^{\bullet+}$ radical cation also prepared by using methanol. The resultant data was plotted between concentration of antioxidants and that of Trolox for the standard reference curve.

Total phenolic contents assay (TPC): Total phenolic contents of the extracts were determined by a reported method[18]. Stock solution of gallic acid was made by dissolving 0.500 g gallic acid in 10 mL of C_2H_5OH in a 100 mL conical flask and diluted it to volume with double distilled water. Sodium carbonate solution was prepared by dissolving 200 g of anhydrous sodium carbonate in 800 mL of distilled water. After boiling and subsequent cooling of the solution, a few crystals of sodium carbonate were added. The solution was stand for 24 h, filtered and volume was raised to 1 L with distilled water. To prepare a calibration curve, 0, 1, 2, 3, 5 and 10 mL of stock solution of phenol were added into 100 mL conical flask separately and then diluted to volume with distilled water. The final solutions contained concentrations of 0, 50, 100, 150, 250 and 500 mg/L gallic acid,the effective range of assay. From each calibration solution and sample or blank, 40 µL were pipetted into separate cuvettes and to each 3.16 mL of distilled water was added. Folin-Ciocalteu's reagent (200 µL) was added and mixed well. After 8 min, 600 µL of Na_2CO_3 solution was mixed thoroughly in the solution. The solution was allowed to stand at 20 ºC for 2 h and absorbance of each solution was noted at 765 nm against the blank. A concentration *versus* absorbance linear plot was thus obtained. Alternately, they can be left at 40 ºC for 0.5 h before reading the absorbance and noted the absorbance at 765 nm. Create a calibration curve with standard and determine the level in sample.

Ferric ion reducing antioxidant power assay (FRAP): The ferric ion reducing capacity of plant extract was measured according to the reported method[19]. Freshly prepared FRAP solution contained 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution

in 40 mM hydrochloric acid solution and 2.5 mL of 20 mM ferric chloride ($FeCl_3$) solution. The mixture was incubated at 37 °C throughout the reaction period. 3 mL of FRAP reagent was mixed with 100 µL of sample and 300 µL of distilled water. Absorbance readings were taken at 593 nm after every minute for 6 min. Results were compared with standard curve of ferrous sulphate.

2,2′-Diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH): 2,2-Diphenyl-1-picrylhydrazyl free radical scavenging potential was obtained by reported method[20]. 2,2-Diphenyl-1-picrylhydrazyl is one of the few stable and commercially available organic nitrogen radicals and has a UV-vis absorption maximum at 515 nm. Upon reaction, solution colour fades, the reaction progress is monitored by a spectrophotometer.

Briefly, DPPH solution (3 mL, 25 mg/L) in methanol was mixed with appropriate volumes of neat or diluted sample solutions (0.1 mL). The reaction progress of the mixture was monitored at 517 nm over a time period of 40 min until the absorbance becomes stable. Upon appropriate reduction, the purple colour of the solution changed to yellow diphenylpicrylhydrazine. The percentage of the DPPH remaining ($DPPH_{rem}$ %) was calculated as:

$$(\%)\ DPPH_{rem} = 100 \times [DPPH]t=t/[DPPH]t=0$$

where [DPPH]t= 0 is the concentration of DPPH radical before reaction with antioxidant samples and while $DPPH_{rem}$ is proportional to the antioxidant concentration and [DPPH]t=t is the concentration of DPPH radical after reaction with antioxidant sample at time t. A kinetic curve showing the scavenging of DPPH radical in terms of decrease in absorbance at 517 nm as a function of time (min) was plotted for each fraction of the samples. EC_{50} value, which is the concentration of a substance that reduces the amount of DPPH radical to half of the original concentration under experimental conditions, was also determined for each fraction.

RESULTS AND DISCUSSION

$ABTS^{\bullet+}$ decolourization assay: The ABTS decolourization assay was applied to evaluate *in vitro* radical scavenging potential of different fractions of *N. hindostana*. The reduction potential of the ABTS radical cation is comparable to that of hydroxyl radical produced during metabolic reactions *in vivo*. Trolox equivalent antioxidant capacity (TEAC) values were obtained by comparing the % inhibition values of fractions samples with the standard Trolox curve. Column graphs are plotted for the TEAC values of each fraction of the sample (Fig. 1). Trolox equivalent antioxidant capacity values ranged from 0.970-4.185 µM of trolox equivalents. Amongst different fractions ethyl acetate, aqueous extract before partitioning, aqueous extract after partitioning and methanol fractions showed higher TEAC values than those of *n*-hexane, chloroform and *n*-butanol. With few exceptions a general trend of increase in the TEAC value with increasing polarity of extractive solvent may be seen in Fig. 1. The fractions with less polar extractive solvent showed small TEAC values indicating low solubility of phenolic and other antioxidant components in non-polar medium. Two media were used for the generation of ABTS free radical, one was methanloic and other was buffer media, but buffer media shows more % age inhibition. In methanolic medium maximum peak value (3.493) is given by 1-butanol, which indicates that it has maximum radical scavenging activity and minimum in *n*-hexane. In buffer medium more radical scavenging capacities as compared to methanolic medium. Order of radical scavenging activity of different fractions in buffer medium as ethyl acetate > aq. (after partitioning) > metahnol > aq. (before partitioning) > *n*-hexane > chloroform > 1-butanol (Fig. 1A-C).

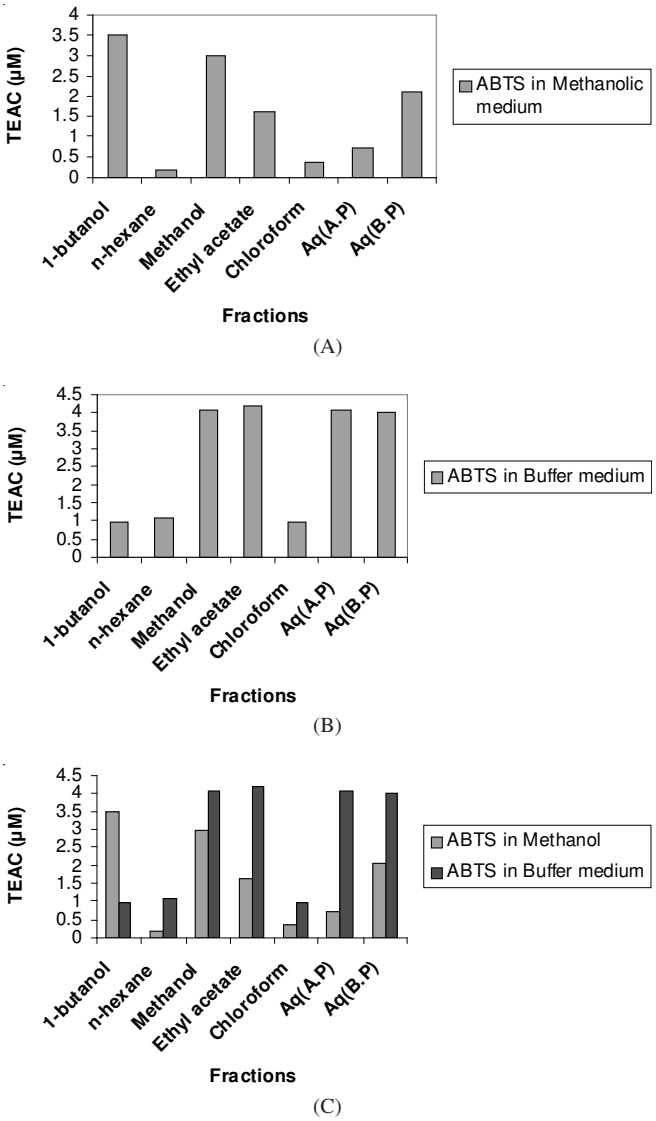

Fig. 1. Trolox equivalent antioxidant capacity values of extracts of by ABTS assay (A) In methanolic medium (B) In buffer medium, (C) Comparison between methanolic and buffer medium

Total phenolic contents: Follin-Ciocalteu's reagent (FC reagent) is usually used in the laboratories for the determination of phenolic components in plants/ herbal extracts and other fractions. Phenolic compounds react Follin-Ciocalteu's reagent only under basic conditions at pH 10. Hydroxyl moieties of phenolic compounds have the ability to reduce yellow coloured Follin-Ciocalteu's reagent to blue colour. The change in the colour is monitored spectrophotometrically at 765 nm. All the fractions of *Nepeta hindostana* showed high values of total phenolic contents. Total phenolic content values ranged from

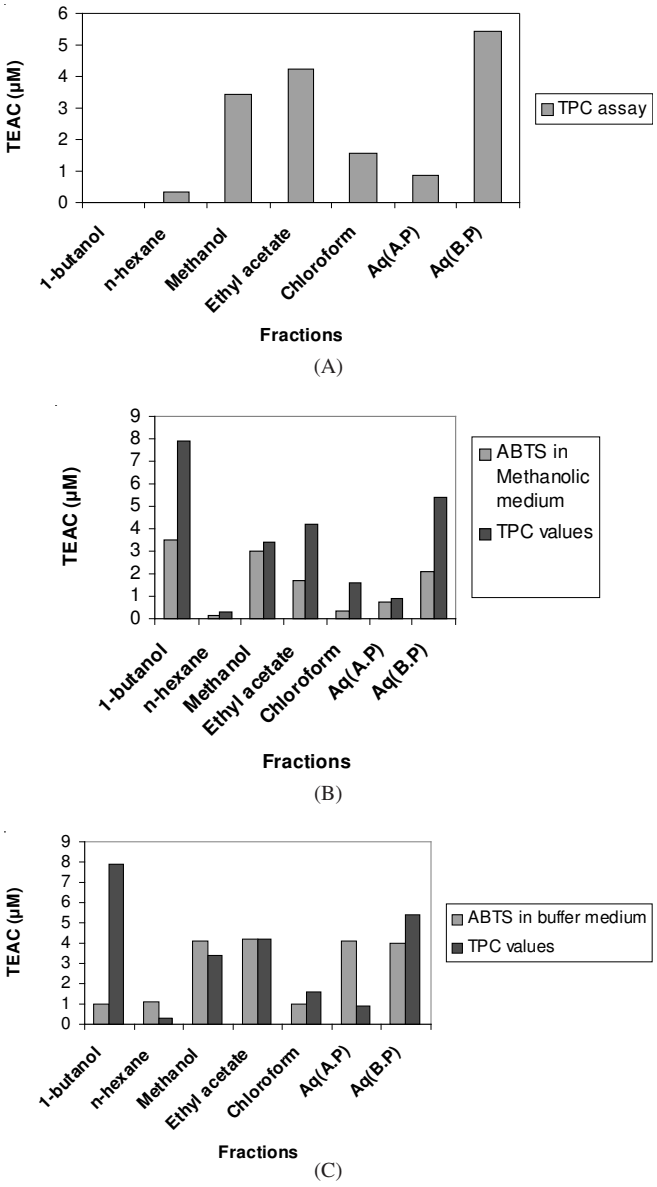

Fig. 2. (A) Trolox equivalent antioxidant capacity values of *Nepeta hindostana* by using TPC assay (B) Comparison between TEAC values of ABTS in methanilc medium and TPC values (C) Comparison between TEAC values of ABTS in buffer medium and TPC values

0.292 to 5.415 µM of GAE/100 g of dry weight (Fig. 2). Employing TPC assay, the order of antioxidant activity of different fractions of *Nepeta hindostana* was found to be aq. extract (before partitioning) > ethyl acetate > methanol > chloroform > aq. extract (after partitioning) > *n*-hexane.

Ferric reducing antioxidant power (FRAP): The FRAP assay is employed to assess antioxidant power by knowing the ability of the sample to reduce ferric to ferrous ion at low pH. The FRAP assay as developed by Benzie and Strain which involves a single electron reduction of the $Fe(TPTZ)_2(III)$ complex (pale yellow) to the $Fe(TPTZ)_2(II)$ complex (blue) by sample antioxidants at acidic pH. Any antioxidant species with lower reduction potential than that of Fe(III)TPTZ salt (0.7 V) can reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ contributing to ferric reducing antioxidant power value[22]. This reduction is progressed spectrophotometrically at 593 nm. Appearance of intense blue colouration show the presence of reducing components in the sample. The original method of Benzie and Strain uses a 4 min interval but we noted that the reaction/ colour change is in progress even after 4 min interval. Absorbance readings, therefore, were taken at a 6 min interval after addition of sample to TPTZ reagent allowing the reaction to reach a steady state. Ferric reducing antioxidant power values for different fraction ranged from 1.032 to 7.906 µM (Fig. 3). Higher TAC values for aq. extract (befire partitioning), *n*-butanol, aq. extract (after partitioning) and ethyl acetate.

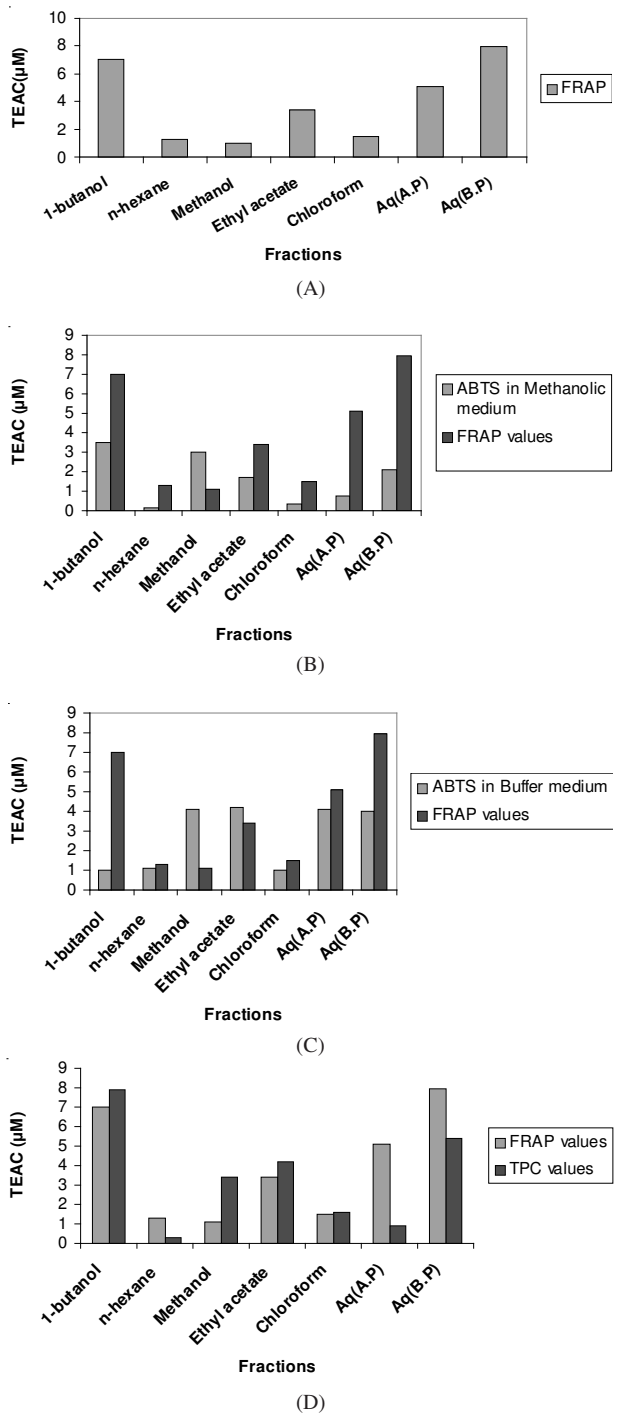

Fig. 3. (A) Trolox equivalent antioxidant capacity values of the fractions of *Nepeta hindostana* by FRAP assay (B) comparison between TEAC values of ABTS in methanolic medium and FRAP values (C) comparison between TEAC values of ABTS in buffer medium and FRAP values (D) Comparison between FRAP values and TPC values

2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH): DPPH is a preformed stable radical used to measure radical scavenging activity of antioxidant samples and has a UV. Visible absorption maximum at 515 nm. Upon reaction the solution colour fades. The progress of the reaction is monitored by a spectrophotometer. 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay was performed by taking parameters, the absorbance of antioxidant and the time for completion of the reaction of antioxidants, into consideration. Kinetic curves obtained by plotting absorbance against time showed that all the fractions of *Nepeta hindostana* contained high levels of DPPH radical scavenging agents (Fig. 4). It is clear from the figure that the curves obtained from ethyl acetate is much steeper in the first 15 min, showing fast reaction of antioxidant components with DPPH radical and all other fractions show the curve almost parallel at its origin, indicates the completion of the antioxidants.

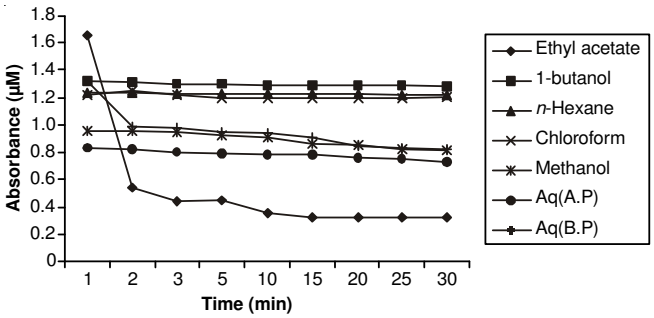

Fig. 4. Absorbance values of different fractions of *Nepeta hindostana* by DPPH assay

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