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ANTIOXIDANT ACTIVITY AND FT-IR ANALYSIS OF METHANOLIC EXTRACT OF *ADHATODA BEDDOMEI*

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ABSTRACT: Antioxidants play an important role in inhibiting and scavenging the free radicals, thereby protecting the humans from degenerative diseases and infection. The methanolic extract of *Adhatoda beddomei* (*A.beddomei*) was screened for antioxidant activity using 1,1-diphenyl-2-picryl hydroxyl (DPPH) q*u*enching assay, 2,2'-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) decolorization test, scavenging activity towards superoxide anion, hydrogen peroxide (H_2O_2) radical, hydroxy radical, reducing power and ferric reducing antioxidant power (FRAP) Assay. In the present study, reducing power, FRAP and the free radical scavenging activity were increased with increasing concentrations of the extracts. The antioxidant activity of the samples was comparable with that of the standard antioxidant butylated hydroxyl toluene (BHT). The results indicate that both the stem and leaf extracts of *A. beddomei* displayed antioxidant activity, it might be related with high quantity of phenols and flavonoids present in the extracts. Maximum free radical scavenging activity was exerted by the leaf extract of *A. beddomei* then the stem extracts and this plant can be established as a good source of natural antioxidant.

KEYWORDS: *Adhatoda beddomei*, DPPH, Antioxidant activity, Butylated hydroxyl toluene, FRAP.

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1. INTRODUCTION

Free radicals are produced continuously in all the cells during the normal cellular function. Excessive production of free radical from the endogenous or exogenous sources plays a vital role in

many diseases. The production of excessive free radicals stimulates the oxidative damage and plays a decisive role in the development of various diseases in humans including atherosclerosis, coronary heart disease, cancer and neurodegenerative disorder [1]. Antioxidants prevent free radical mediated tissue damage by scavenging them or by preventing its formation. Antioxidants are synthetic or natural and the limitations in the utilization of synthetic antioxidants have been enforced due to their toxic effect [2]. Hence, there is a increasing interest in replacing synthetic antioxidants with natural plant-based alternatives. Many plants have antioxidant compounds and these compounds protect the cells from the cellular damage mediated by the free radicals including hydroxyl radicals, peroxyl radicals, and singlet oxygen, thereby preventing the development of diseases. These components are responsible for the colour, flavours, smell and parts of a plant which naturally give disease resistance for the plant and the consumer. The researcher has discovered new effective drugs from plants are based on the use of crucial oils and plant extracts to treat various diseases. *A. beddomei* is a medicinal plant and exhibiting various medicinal properties including antiemetic and antibechic activity. *A. beddomei* belonging to family Acanthaceae, are traditional Indian medicinal shrubs commonly used in the treatment of several ailments such as cough, bronchitis [3,4] asthma [5], inflammation, haemorrhage, haemorrhoids, diarrhoea, and eye diseases [6]. Moreover, there is no information pertaining to the antioxidant potential of *A. beddomei* stems and leaves [7]. Based on the traditional information of medicinal system, the present study was carried out to investigate the antioxidant activity of methanolic extracts of stems and leaves of *A. beddomei.* Free radical scavenging is the widely used procedure for screening the antioxidant activity of various extracts and compounds. Free radical scavenging ability is generally governed by the assay of DPPH, +, FRAP, total antioxidant activity, metal chelating activity, superoxide anion, hydroxyl radical and H_2O_2 scavenging activity. The DPPH assay is very common and proved as the best way for the assay of antioxidant activity.

2. MATERIALS AND METHODS

Plant material

A. Beddomi leaves were collected in and around Ullannoor, Pathanamthitta District, Kerala, in the month of January-February. Authentication of the plant was done by the Department of Botany, Annamalai University, Chidambaram.

Reagents

DPPH was purchased from Sigma-Aldrich Chemical Co., St. Louis. Methanol, Chloroform, Ethylacetate, BHT, Potassium persulphate, ABTS (2, 2'–azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), Hydrogen peroxide, sodium nitroprusside and all other chemicals used were of analytical grade purchased from E. Merck, Mumbai, India.

Preparation of plant extracts

The shade dried leaves of plant (at 20º) were powdered in mechanical grinder. 20 grams of leaf and stem powder was weighed, 150 ml of solvent was added and kept for 3 days. The extract was filtered using Whaatman No.1 filter paper and the supernatant was collected. The residue was again extracted two times (with 3 days of interval for each extraction) and supernatants were collected. The supernatants were pooled and evaporated (at room temperature, 28 ±1C) until the volume was reduced to 150 ml. Extracts of the leaves and stems were stored in the freezer till the use.

Assessment of antioxidant activity

DPPH radical scavenging activity

The radical scavenging activity of *A. Beddomi* against DPPH• was determined spectrophotometrically in a dark room by the method of Brand Williams *et al.,* (1995) [8]. DPPH• is a stable free radical and accepts an electron, or hydrogen radical to become a stable diamagnetic molecule. DPPH• reacts with an antioxidant compound that can donate hydrogen and gets reduced. The change in colour (from deep violet to blue) was measured. The intensity of the colour developed was depends on the amount and nature of radical scavenger present in the sample. 1ml of various concentrations of *A. Beddomi* stems and leaves extract was taken, 1ml of DPPH was added and this was made up to 3ml with water. The blue colour developed was read at 517nm and BHT was used as a standard. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula:

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2,2'-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) Assay

The total antioxidant activity of the samples was measured by [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] ABTS radical cation decolorization assay according to the method of Re *et al.* (1999) [9]. Percentage of inhibition was calculated as described earlier.

Ferric reducing antioxidant power (FRAP) assay

The FRAP (Ferric reducing antioxidant power assay) procedure described by Benzie and Strain (1999) [10]. The principle of this method is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants.

Superoxide anion radical scavenging assay

Superoxide anion scavenging activity was measured based on the method described by Liu *et al.,* (1997) [11]. 0.1ml of sample solution was mixed with 1ml of NBT and 1ml of NADH solution. This mixture was incubated at 25ºC for 5 minutes. A control was performed with reagent mixture without the sample. Absorbance was measured spectrophotometrically at 560 nm. BHT was used as reference standard. Percentage of inhibition was calculated as described earlier.

Hydroxyl radical scavenging activity

The scavenging activity of the extract on hydroxyl radical was measured according to the method of Klein *et al.,* (1991) [12]. Various concentrations of extracts were added with 1.0ml of Ferrous ammonium sulphate - EDTA solution, 0.5ml of EDTA solution (0.018%), and 1.0ml of dimethyl sulphoxide (DMSO) .The reaction was initiated by adding 0.5ml of ascorbic acid and incubated at 80–90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of1.0 ml of ice - cold TCA. 3 ml of Nash reagent was added and left at room temperature for 15min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectrophotometrically at 412 nm against reagent blank. BHT was used as reference standard. Percentage of inhibition was calculated as described earlier.

Scavenging activity against hydrogen peroxide

The scavenging capacity of extracts on hydrogen peroxide was determined according to the method of Nabavi *et al.,* (2009) [13]. Test samples were prepared with 2.0 ml of various concentrations of extracts (25 to 200 µg/ml) and a solution of H_2O_2 (1.2 ml, 40 mM) in phosphate buffer (pH 7.4). A blank solution was prepared in the same way without H_2O_2. After 10 min incubation of the mixture, the absorbance was recorded at 230 nm. BHT was used as reference standard. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and the percentage of inhibition was calculated as described earlier.

Reducing power assay

The reducing power of the extracts was determined by the method of Oyaizu 1986 [14]. Substances, which have reductions potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+),which then reacts with ferric chloride to form ferric ferrous complex that as an absorption maximum at 700nm. Various concentrations of samples (25-200 µg/ml) were mixed with of phosphate buffer and 5 mL of 0.2 M phosphate buffer at pH 6.6. To this, 5 mL of 1% potassium ferricyanide solution was added. The mixture was incubated at 50°C in water bath for 20 min. After cooling, 5 mL of 10% TCA was added and the content was centrifuged at 1,000 rpm for 10 min. The upper layer of the supernatant (5 mL) was mixed with 5 mL of distilled water. To this, 1 mL of ferric chloride (0.1%) was added and vortexed. Then, the absorbance of the reaction mixture was read spectrophotometrically at 700 nm against water blank. BHT used as a standard.

Metal ion chelating activity

The chelation of ferrous ions by extracts was estimated by method of Dinis *et al.,* 1994 [15]. The samples at different concentrations were added to 0.05 ml of 2mM $Fecl_2$. The reaction was initiated by the addition of 5mM ferrozine (0.2mL) and the mixture was mixed well and stands for 10 min at room temperature. The absorbance was measured at 562 nm spectrophotometrically. The Fe^{2+}chelating activity (%) was calculated as described earlier

Phosphomolybdenum assay

The total antioxidant activity of the extracts was evaluated by the phosphomolybdenum assay [16]. This assay is based on the reduction of M_0 (VI)–M_0 (V) by the antioxidants and subsequent formation of a green phosphate/M0 (V) complex at acidic pH. 0.3 ml of BCA sample is taken in a tube and mixed with 3 ml of reagent containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and it was incubated at 95°C for 90 min. The absorbance of the mixture was measured at 695 nm with methanol blank. The percentage of inhibition was calculated as described earlier.

Fourier Transform Infrared Spectrophotometer (FT-IR):

Fourier Transform Infrared Spectrophotometer (FT-IR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in the compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. Dried powder of the stems and leaves methanolic extracts were used for FT-IR analysis [17]. 10 mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered sample of each plant specimen was loaded in FT-IR spectroscope, with a scan range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

Statistical Analysis

All the above experimental assays were performed and carried out in triplicate. Experimental assays results are expressed as mean ± standard deviation. The results were analyzed using one way analysis of variance and the group means were compared using Duncan's multiple range tests using SPSS version 17.

3. RESULTS AND DISCUSSION

Effect of methanolic extracts of *A.beddomei* leaves and stems on DPPH assay

DPPH assay is one of the most commonly used methods for screening antioxidant activity of plant extracts. Methanol extracts of *A.Beddomi* leaves and stems displayed the antioxidant activity in a concentration dependent manner by the removals of the stable radical DPPH at 25, 50, 75 and 200µg/ml (Figure.1). The maximum scavenging activity was occurred at the maximum dose (200µg/ml) of methanolic extract of *A.Beddomi* leaves and stems were found to be 50.55% and 55.65% respectively, whereas BHT was found to be 60.33 % at 200 µg/ml. The scavenging activity of the extract might be due to the reduction of DPPH by accepting a hydrogen atom from the extracts. The overall activity of the extracts was lower than that of commercial antioxidant BHT.

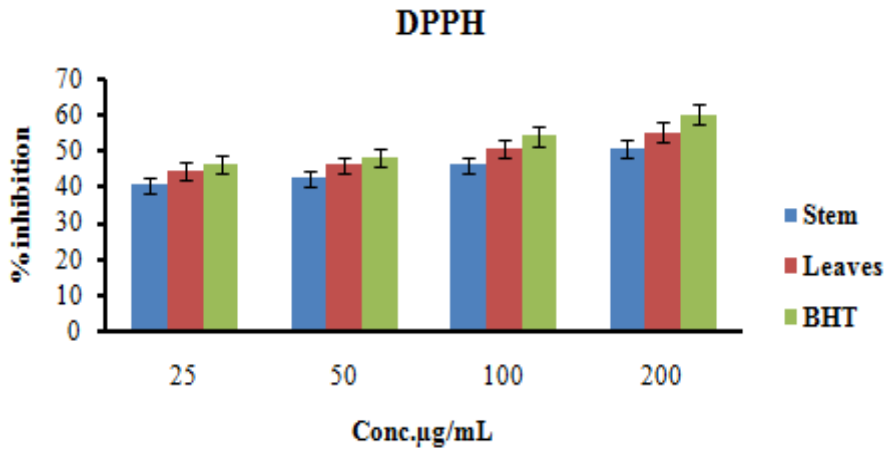

Figure 1. DPPH scavenging activity of *A.Beddomei* leaves and stems extracts

The result of this study has proved that *A.Beddomi extracts* is a powerful DDPH inhibitors or scavenger, stated by the lowest percentage of DPPH.

Effect of methanolic extracts of *A.Beddomei* leaves and stems on ABTS assay

ABTS radical is a more drastic radical and is normally used for the screening of antioxidants in the mixtures including the biological fluids and plant extracts. Antioxidant activity of the *A. Beddomi* extracts are evaluated by the discoloration of the ABTS and the increased discoloration is expressed as a percentage inhibition of ABTS [18].

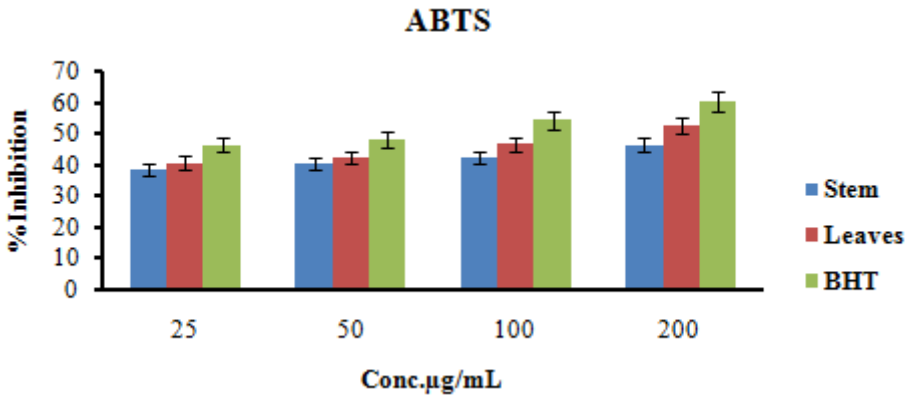

Figure 2. ABTS scavenging activity of *A. Beddomei* leaves and stems extracts.

The results of the ABTS scavenging activity of *A.Beddomei* extracts are demonstrated in Figure. 2**.** The percentage inhibition of stems and leaves of *A.Beddomei* was 46.33 and 52.55 % at 200µg/ml and BHT was found to be 60.33 % at 200µg/ml. All concentrations of the extracts revealed effective ABTS scavenging activities in dose dependent manner. The results of the ABTS scavenging activity of *A.Beddomei* extracts are demonstrated and it is calibrated against BHT, reference standard.

Effect of methanolic extracts of *A. Beddomei* leaves and stems on FRAP assay

FRAP assay is based on the capacity of an antioxidant to reduce Fe3+ to Fe2+ ions, forming an

intense blue Fe-TPTZ complex in the presence of TPTZ (2,4,6-tris-(2-pyridyl)-s-triazine). Figure.3. shows the ferric reducing ability of the methanolic extracts of leaves and stems of *A.Beddomei* and was found to be 53.75 % and 48.44 % respectively at 200µg/ml. BHT was used as a standard antioxidant with 60.33 %. All concentrations of samples were exhibited potent FRAP activities in a concentration dependent manner and the highest activity was noted for methanol extract of leaves (53.75 %) followed by the methanol extract of stems (48.44 %) of *A.Beddomei*. The absorbance of *A.Beddomei* leaves and stems extracts were increased in increased concentration, due to the Fe2+-TPTZ complex formation by reducing Fe3+ to Fe2+ ions. The reducing properties of *A.Beddomei* extracts are may be due to the presence of phenolic compounds, which donating a hydrogen atom to Fe^{3+}.

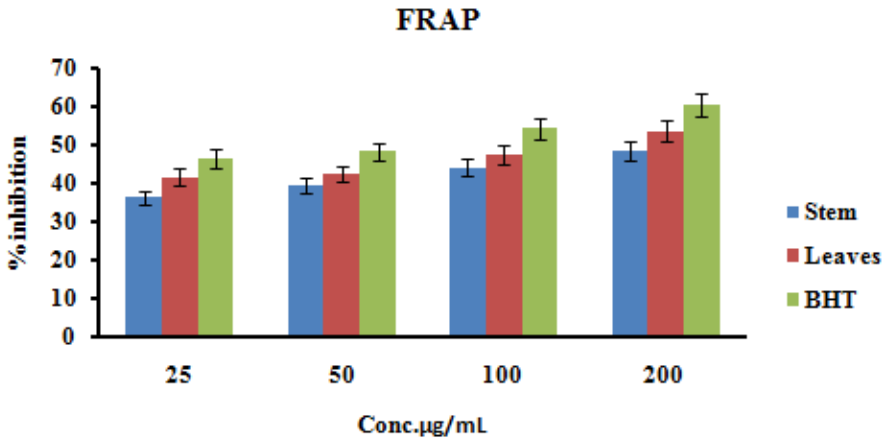

Figure 3. *A. Beddomei* leaves and stems extracts on FRAP.

Effect of methanolic extracts of *A.Beddomei* leaves and stems on superoxide anion

Superoxide is the primary oxygen free radical produced in mitochondria, which damage the biomolecules and initiate the lipid peroxidation. Superoxide anion radical can be produced at pH 7.4 by xanthine/xanthine oxidase. This superoxide anion radical reduces nitroblue tetrazolium into blue formazan.

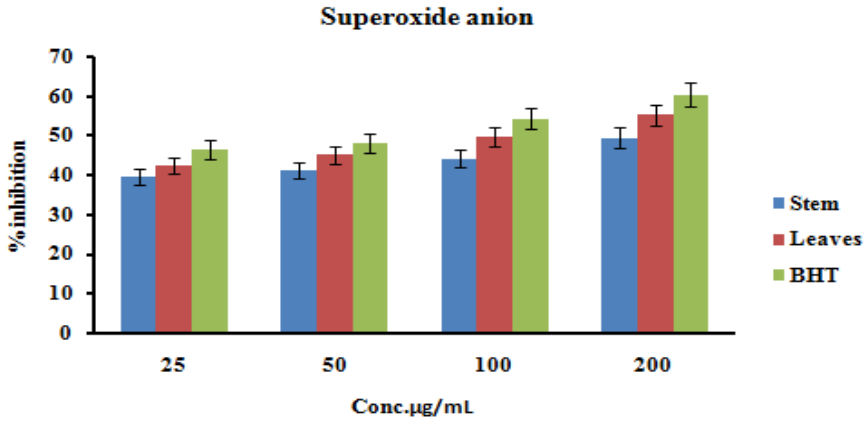

Figure 4. Leaves and Stem extracts of *A. Beddomei* on superoxide radicals.

The superoxide anion scavenging ability of methanolic extracts of leaves and stems at maximum concentration was 55.26 and 49.56 % respectively (Figure.4). Percentage inhibition of BHT on superoxide anion was 60.33 %. Both the stems and leaves extracts exhibited strong antioxidant activities in a dose dependent manner.

Effect of methanol extracts of *A.Beddomei* leaves and stems on hydroxyl radical

Hydroxyl radicals are the most important active oxygen species causing lipid peroxidation and damaging the biological molecules [19, 20]. Hydroxyl radical can abstracting hydrogen atoms from membrane lipids and brings about the peroxidic reaction of lipids [21]. Thus, removing $OH^{\bullet}$ is essential for the protection of living systems. In the present study, the percentage inhibition of methanol extracts of leaves and stems on hydroxyl radicals were maximum exhibited at highest concentration (48.42 and 55.55 %). BHT was used as standard antioxidant with 60.33 %. All concentrations of the samples exhibited efficient scavenging activities in a concentration dependent manner (Figure.5).

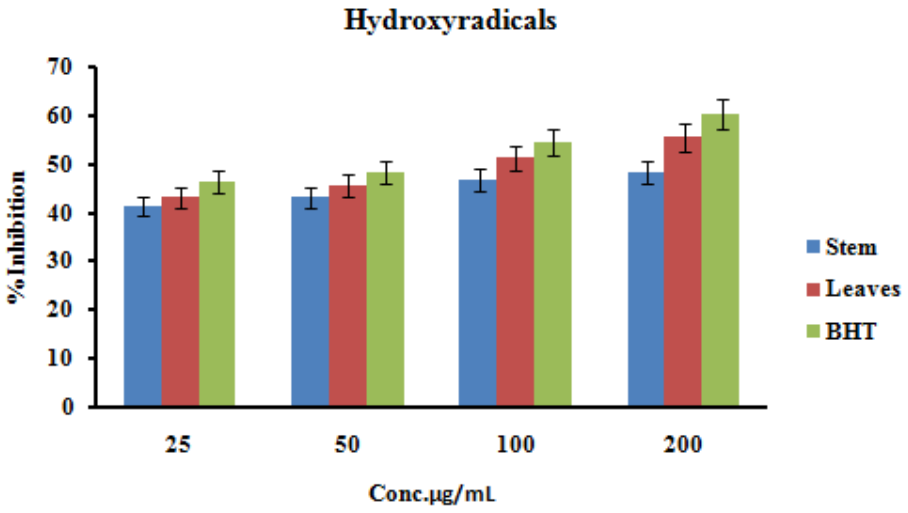

Figure 5. *A. Beddomei* leaves and stems extracts on hydroxyl radicals.

Effect of Methanol extracts of *A.Beddomei* leaves and stems on H_2O_2

Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly by the oxidation of thiol (-SH) groups. H_2O_2 can cross cell membranes rapidly and it can react with Cu^{2+} and Fe2+ ions to form hydroxyl radicals and it may be the reason for many of its toxic effects [22]. Figure.6. shows the H_2O_2 radicals scavenging activity of methonalic extract of leaves and stems of *A.Beddomei.* The maximum radicals scavenging activity of leaves and stems of *A.Beddomei* was 52.53 and 49.66 % where as BHT showed H_2O_2 radicals scavenging activity of 55.26 %. The concentration from 25 to 200 µg/ml of extracts exhibited powerful scavenging activities in a concentration dependent manner. Among the concentrations, 200µg/ml exhibited strong activity then all other doses.

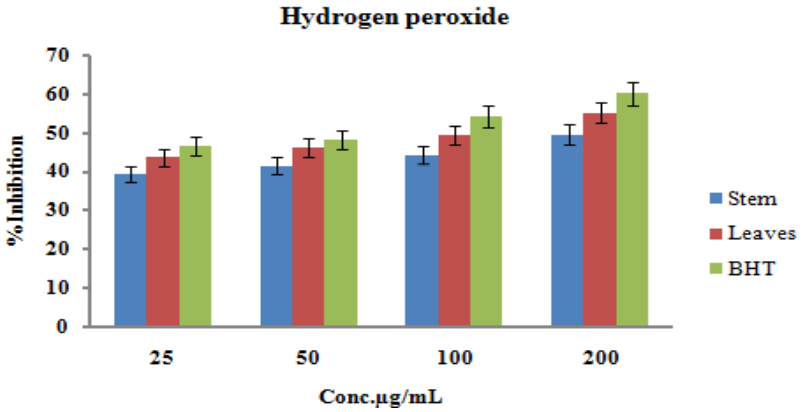

Figure 6. Activity of leaves and stem extracts of *A. Beddomei* on H_2O_2

Effect of methanol extracts of *A.Beddomei* leaves and stems on reducing power

Reduction power is widely used to assess the antioxidant activity of plants. The reduction power of the extracts was determined by the potassium hexacyanoferrate (III) reduction method. The reducing power of the extracts was augmented in a concentration dependent manner (Figure.7). The reducing power of the extracts might be due to the conversion of Fe+3/Hexacyanoferrate (III) complex to Fe+2/Hexacyanoferrate (III) complex by the reductans present in the extracts. In particular, the leaf extracts had the highest reducing power among the stems extracts tested, this result shows that leaf extracts consists of more polyphenolic compounds that cause the greater reducing power. The percentage inhibition of methanol extracts of leaves and stems were exhibited maximum at 200µg/ml (52.32 % and 57.45). The standard antioxidants BHT was also exhibited maximum scavenging activity at 200µg/ml (60.33 %).

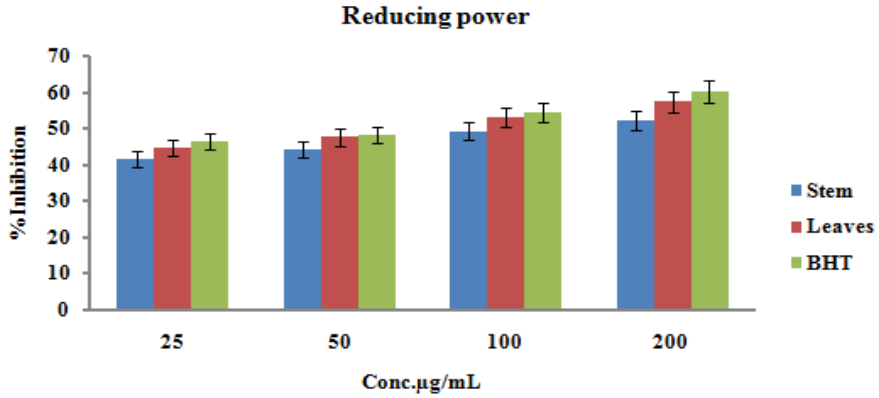

Figure 7. Reducing power of *A. Beddomei* extracts of leaves and stems

Metal chelating activities of *A. Beddomei extracts*

Iron is essential for the activity of many enzymes, transport of oxygen and respiration. However, excess free iron implicated in the induction and development of free radicals and causes oxidative changes in proteins, lipids and other cellular components. Ferrozine can make complexes with ferrous ions, chelating agents present in the samples interrupted in the formation of the red coloured complex as a result the decreased red color formation [23]. Thus, the chelating effect of the extracts can be determined by measuring the rate of color reduction.

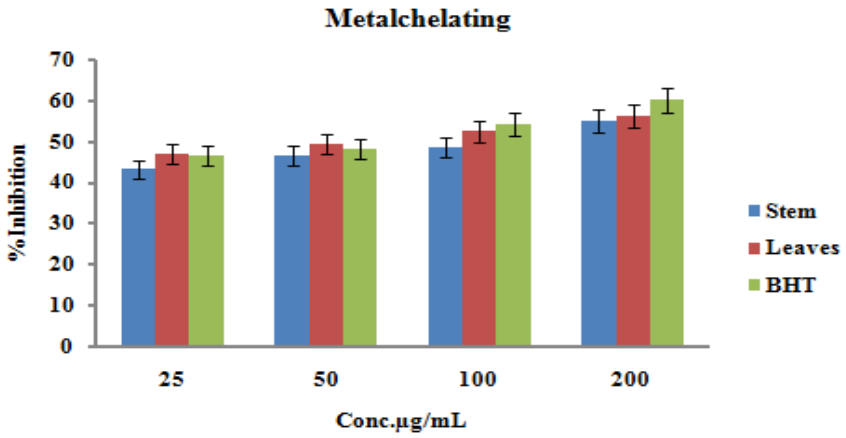

Figure 8. Metal chelating activities of plant extracts at different concentration of leaves and stems of *A. Beddomei*

A. Beddomei plants extracts were analysed in the concentration range of 25-200 µg/ml, all the concentrations demonstrated strong metal chelating activities in concentration dependent manner (Figure.8). Chelating agents present in the extracts form bonds with a metal thereby stabilize the oxidized form of the metal ion. Methanolic extracts of *A. Beddomei* leaves & stems demonstrated an ability to chelate metal ions in all concentrations and were found be maximum at 200µg/ml. The chelating activity of methanolic extracts of leaves and stems at 200µg/ml were 52.62 % and 48.55 % respectively, which is lower than the positive standard BHT (60.33 %).

Effect of methanol extracts of *A.Beddomei* leaves and stem on phosphomolybdenum assay

The total antioxidant capacity of *A.Beddomi* plant extract of leaves and stems were estimated from their ability to reduce the Mo (VI) to Mo (V), leads to the formation of green phosphate/Mo (V) complex [24,25]. Increased green phosphate formation indicated increased total antioxidant potential. Thus, the total antioxidant potential of *A.Beddomi* can be determined by evaluating the formation of green phosphate.

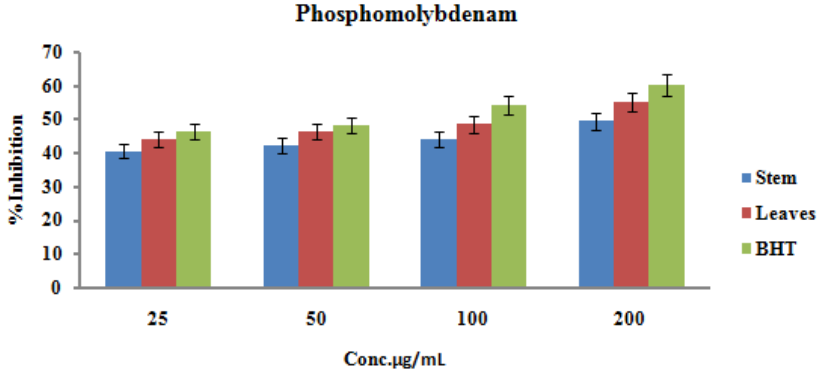

Figure 9. Shows phosphomolybdenum antioxidant capacitity of leaves and stems extracts *A.Beddomei*

In this study, both the aqueous extracts of *A.Beddomei* at 200µg/ml showed the higher phosphomolybdenum reduction (44.26 and 49.62 %) (Figure.9). This result is consistent with the

highest anti radicals' effect of the extracts. The leaf extracts of the *A.Beddomei* was the best antioxidant as demonstrated by the maximum phosphomolybdenum reduction observed in the leaves extracts than the stems extract.

Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectrum was used to identify the functional groups of the active components present in extracts based on the peaks values in the region of IR radiation [26]. The results of FTIR analysis of *A.Beddomei* extracts confirmed the presence of polymeric hydroxyl group (3277cm-1), alkanes (2919.626 cm-1), ketones (2851.971 cm-1), Aliphatic amines (1603.052 cm-1), and Carboxylic acid (1021.705cm-1) (Figure.10). The polymeric hydroxyl groups, alkanes, phenols, ketones and carboxylic acid peak confirm the presence of polyphenols in *A.Beddomei,* attributed to their antioxidant effect.

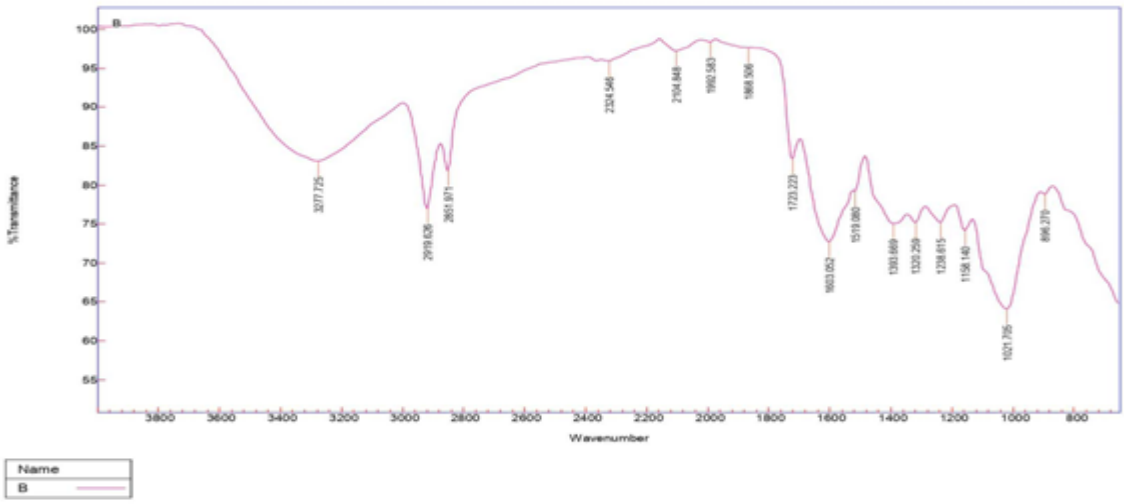

Figure 10: Fourier Transform Infrared Spectrophotometer (FT-IR) analysis of methanolic extract of *A.beddomei* leaves.

Table: 1 FT-IR peak values of methanolic extract of *A. beddomei* leaves

S.No	Peak values	Functional groups
1	3277.725	-OH group and C-H group
2	2919.626	C-H stretching and Alkanes
3	2851.971	C=O group
4	1603.052	Aliphatic amines
5	1021.705	Carboxylic acid

4. CONCLUSION

The results obtained in the present study indicate that methonalic extract of *A.beddomei* leaves exhibit free radical scavenging activity, reducing power and metal chelating activity. The tested plant extracts have moderate to potent antioxidant activity in dose dependent manner. Among the

different concentrations of extracts, 200µg/ml showed maximum antioxidant activity. The antioxidant activity of methonalic extracts of *A.beddomei* might be due to the presence of polyphenolic compounds. The findings of the current study propose that *A.beddomei* might act as a potential source of natural antioxidants and further detailed studies on *in vivo* assays are necessary to characterize them as biological antioxidant.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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