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In vitro antioxidant activity of *Anaphalis lawii* (Hook. f) Gamble and *Helichrysum buddleioides* DC - a comparative study

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ABSTRACT

The present study was conducted to determine antioxidant activity of two Asteraceae plants namely Anaphalis lawii (Hook. f) Gamble and Helichrysum buddleioides DC. The entire plants were shade dried and extracted by maceration process using methanol. Assays namely DPPH radical scavenging, ABTS radical scavenging, Nitric oxide scavenging, Ferric reducing and Iron chelating activity were performed to determine in vitro antioxidant activity of extract. In all assays, extract of H. buddleioides displayed marked activity when compared to extract of A. lawii. Folin-Ciocalteau reagent method and Aluminium chloride colorimetric estimation method were used to estimate the content of total phenolics and flavonoids respectively. Extract of H. buddleioides contained high phenolics and flavonoids when compared to A. lawii which might have accounted for marked antioxidant activity. The plants can be used against oxidative stress.

Key Words: *Anaphalis lawii, Helichrysum buddleioides, Total phenolics and Total flavonoids*

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I. Introduction

Oxidation processes that occur in cells of living organisms are essential for energy generation. Reactive oxygen species and reactive nitrogen species such as superoxide radical, hydroxyl radical, singlet oxygen, hydrogen peroxide, peroxyl radical, peroxynitrite anion and nitric oxide are produced during normal metabolism. These reactive species are known to damage biomolecules such as proteins, lipids and nucleic acid. Excess production of these reactive species is implicated in several diseases or disorders such as ageing, cancer, diabetes and cardiovascular, inflammatory and

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neurodegenerative diseases. Cells possess enzymatic (for example, superoxide dismutase, glutathione oxidase and catalase) and non-enzymatic (for example, vitamin C and E) antioxidant defense system. A balance exists between the production of these reactive species and antioxidant defense of the cell. However, in pathological conditions there is an extra need for antioxidants. Strong restrictions have been placed on synthetic antioxidants such as BHA, BHT and PG due to their suspected carcinogenicity. Antioxidants from natural sources are considered as one among the important alternatives. Extracts and purified compounds from plants are shown to exhibit antioxidant property. Polyphenolic compounds including flavonoids are the major antioxidant principles in plants and their antioxidant property is due to their redox properties which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers and metal chelating agents (Rizzo *et al*., 2010; Awah and Verla, 2010; Venkatachalam and Muthukrishnan, 2012; Rakesh *et al*., 2013; Boora *et al*., 2014; Sasikumar and Kalaisezhiyen, 2014; Okoh *et al*., 2014; Thiruvengadam and Sankar, 2015). Two Asteraceae members namely *Anaphalis lawii* (Hook.f.) Gamble and *Helichrysum buddleioides* DC were selected for this study. *A. lawii* is an erect, densely woolly herb reaching up to 30cm in height. The plant is distributed in Western Ghats, Coorg, Bababudan hills of Karnataka, Brahmagiris, hills of Coimbatore, N. Nilgiris, Anamalais, Pulneys and hills of Tinnevelly, at 5000-7000ft (Gamble, 1993; Pullaiah *et al*., 2007). The whole plant is air-dried, powdered and consumed with food as Kayakalpa by the Malasars of the Velliangiri hills in the Western Ghats of Nilgiri Biosphere Reserve, India (Raghupathy *et al*., 2008). The plant is reported to exhibit bioactivities such as antimicrobial, antioxidant and wound healing activity (Kekuda *et al*., 2012; Dileep *et al*., 2013; Yamini *et al*., 2014; Kekuda *et al*., 2016). *H. buddleioides* is an undershrub reaching up to 1 meter in height. It is distributed in Western Ghats, Mysore, Bababooduns, Anamalais and Ceylon (Fyson, 1974; Pullaiah *et al*., 2007). The plant juice is used to treat cut and wounds for rapid healing by Badaga population in the Nilgiri district of Tamilnadu (Manikandan, 2008). Plant is reported to exhibit antifungal activity against *Bipolaris sorokiniana* (Kekuda *et al*., 2016). In this study, antioxidant potential of extract of *A. lawii* and *H. buddleioides was examined*.

II. Materials and Methods

Collection and extraction of plant material: The plants were collected at Chikmagaluru during November 2015 and authenticated by referring standard flora (Fyson, 1974; Gamble, 1993; Pullaiah *et al*., 2007). The whole plants (leaves, inflorescence, roots and stems) were shade dried, powdered and extracted by maceration process using methanol. 20g of each of the plant was mixed with 100ml of methanol in clean conical flask. The flasks were shaken well, sealed and left for 48 hours during which the flasks were stirred occasionally. The content of each flask was filtered through filter paper (Whatman No. 1) and the filtrates were evaporated to dryness at 50^{o}C (Kekuda *et al*., 2016).

DPPH [1,1-diphenyl-2-picrylhydrazyl] radical scavenging assay: In clean and labeled tubes, 1ml of various concentrations (12.50-200µg/ml) of extract of *A. lawii* and *H. buddleoides* in methanol was mixed with 3ml of DPPH radical solution (0.004% in methanol) followed by incubating the tubes in dark for 30 minutes. The absorbance of reaction mixture in each of the tubes was measured spectrophotometrically (at 517nm). Ascorbic acid was used as reference standard and methanol replacing extract served as control. Scavenging of DPPH radicals was calculated using the formula:

Percent scavenging of DPPH radicals = (Ac – At / Ac) x 100, where 'Ac' refers to absorbance of DPPH control and 'At' refers to and absorbance of DPPH in presence of extract/ascorbic acid. The IC_{50} value was calculated by origin 6.0 software. IC_{50} denotes the concentration of extract required to scavenge 50% of DPPH radicals (Rakesh *et al*., 2013).

ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate)] radical scavenging assay:
Generation of ABTS radical solution was carried out by mixing ABTS (7mM) stock solution with potassium persulfate (2.45mM) and incubating the mixture in dark for 16 hours at room temperature. The resulting radical solution was diluted to an absorbance of 0.7 at 730nm using distilled water. In clean and labeled test tubes, 1ml of different concentrations of extract of *A. lawii* and *H. buddleoides* (12.50-200µg/ml) was mixed with 4ml of ABTS radical solution. The tubes were left at room temperature for 30 minutes and the absorbance of each of the tubes was measured spectrophotometrically at 730nm. Ascorbic acid was used as reference standard. The scavenging of ABTS radicals was calculated using the formula:

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Percent scavenging of ABTS radicals = (Ac – At / Ac) x 100, where 'Ac' refers to absorbance of ABTS control and 'At' refers to and absorbance of ABTS in presence of extract/ascorbic acid. The IC_{50} value was calculated by origin 6.0 software. IC_{50} denotes the concentration of extract required to scavenge 50% of ABTS radicals (Rakesh *et al*., 2013).

Nitric oxide radical scavenging assay: Sodium nitroprusside (10 mM) in phosphate buffer saline (pH 7.4) was mixed with various concentrations of extract of *A. lawii* and *H. buddleioides* (12.50-200µg/ml). The mixture was then incubated at 30°C for two hours and 0.5 ml of the incubated solution was mixed with 0.5 ml of Griess reagent. The absorbance of chromophore was measured at 546 nm in a spectrophotometer. Ascorbic acid was used as reference standard. The nitric oxide radical inhibition (%) was calculated using the formula:

% inhibition = (A0 - A1 / A0) x 100, where A0 and A1 represents the absorbance before reaction and the absorbance after reaction has taken place with Griess reagent respectively (Alam *et al*., 2013). The IC_{50} value of the extract was calculated by origin 6.0 software.

Ferric reducing assay: Various concentrations of extracts (12.50-200µg/ml) were prepared in methanol. 1ml of methanol containing extract was mixed with 2.5ml of phosphate buffer (200 mM, pH 6.6) and 2.5ml of potassium ferricyanide (1%). The tubes were stirred well and placed in water bath maintained at 50°C for 20 minutes. The tubes were cooled and 2.5ml of trichloroacetic acid (10%) and 0.5ml of Ferric chloride (0.1%) were added to each of the tubes. After 10 minutes, the amount of iron (II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700nm in spectrophotometer. Ascorbic acid was used as standard. An increase in absorbance with increase in extract concentration indicates reducing potential (Kekuda *et al*., 2012).

Iron chelating activity: The chelating of ferrous ions by different concentrations (12.50-200µg/ml) of extracts and EDTA (standard) was determined. The reaction mixture contained 1.0 ml of various concentrations of the extracts and 0.05ml of $FeCl_3$ (2mM). The reaction was started by adding 0.2ml of ferrozine (5mM). The tubes were shaken vigorously and left for 10 minutes at room temperature. The absorbance of the reaction mixture was measured at 562 nm against a reagent blank. A lower absorbance of the reaction mixture indicates chelating potential. The control contained all the reagents except extract/standard. Ascorbic acid was used as standard. Metal chelating activity was determined using the formula:

Metal chelating activity (%) = (Ac – At / Ac) x 100, where 'Ac' refers to absorbance of control and 'At' refers to and absorbance of test (Kekuda *et al*., 2012). The IC_{50} value was calculated by origin 6.0 software.

Total phenolic content (TPC) of extracts: The TPC of extracts was estimated by Folin-Ciocalteau reagent (FCR) method. In brief, 0.5ml of extract was mixed with 0.5ml of FC reagent (1:10 diluted) and 2ml of sodium carbonate (2%). The tubes were left to stand for 30 minutes at room temperature. The absorbance was read at 765nm in a spectrophotometer. A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000 µg/ml). The TPC of extracts was expressed as mg Gallic acid equivalents (GAE) from the graph (Kekuda *et al*., 2012).

Total flavonoid content of extracts: Aluminium chloride colorimetric method was employed to estimate total flavonoid content (TFC) of extract of *A. lawii* and *H. buddleoides* (Kekuda *et al*., 2012). A dilute concentration of extract (0.5ml) was mixed with methanol (0.5ml), water (4ml), $NaNO_2$ (5%, 0.3ml) and incubated for 5 minutes at room temperature. After incubation, $AlCl_3$ (10%, 0.3ml) was added and incubated at room temperature for 6 minutes. 1M NaOH (2ml) and distilled water (2.4ml) were added to tubes and the absorbance of reaction mixture was measured against blank (without extract) at 510nm in a spectrophotometer. A calibration curve was constructed using different concentrations of Catechol (standard, 0-120µg/ml) and the TFC of extracts was expressed as mg Catechol equivalents (CE)/g of extract from the graph.

III. Results and Discussion

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DPPH radical scavenging activity of extracts

Various in vitro assays are available to evaluate radical scavenging effect of various kinds of samples including plant extracts. DPPH assay which is based on quenching of stable free radicals is one of the widely used assays. This in vitro assay utilizes a stable, nitrogen centered and commercially available free radical DPPH which is soluble in methanol and displays a maximum absorption at 517nm. This stable radical becomes a stable diamagnetic molecule when it accepts an electron or hydrogen atom from donor species (antioxidant substances). The antioxidants reduce the purple colored DPPH radical to a yellow colored non-radical compound, diphenylpicrylhydrazine and the extent of reaction is dependent on the hydrogen donating ability of compounds. The assay is popular due to its simpler protocol, cost effectiveness and the results obtained are reproducible. Besides, the radical is stable and need not to be generated (Chung *et al.*, 2006; Magalhaes *et al.*, 2008; Kekuda *et al.*, 2012; Nagmoti *et al.*, 2012; Padmanabhan and Jangle, 2012; Venkatachalam and Muthukrishnan, 2012; Sasikumar and Kalaisezhiyen, 2014; Okoh *et al.*, 2014; Thiruvengadam and Sankar, 2015). We evaluated radical scavenging effect of extracts of *A. lawii* and *H. buddleoides* by DPPH assay. Both extracts scavenged radicals in a concentration dependent manner (Figure 01). Among extracts, *H. buddleoides* (IC_{50} value 42.56µg/ml) exhibited stronger scavenging potential when compared to *A. lawii* (IC_{50} value 90.76µg/ml). At concentration 100µg/ml, both extracts scavenged DPPH radicals to >50%. A scavenging of 68% and 79% of radicals was observed at an extract concentration of 200µg/ml by *A. lawii* and *H. buddleoides* respectively. Reference antioxidant ascorbic acid scavenged DPPH radicals more efficiently when compared to both extracts (IC_{50} value 6.10µg/ml). Although both extracts displayed low scavenging activity when compared to ascorbic acid, it is evident that the extracts possess hydrogen donating ability and hence extracts can serve as free radical scavengers and inhibitors, acting possibly as primary antioxidants (Chung *et al.*, 2006). In a previous study, Kekuda *et al.* (2012) reported marked scavenging of DPPH radicals by flower and leaf extract of *A. lawii*.

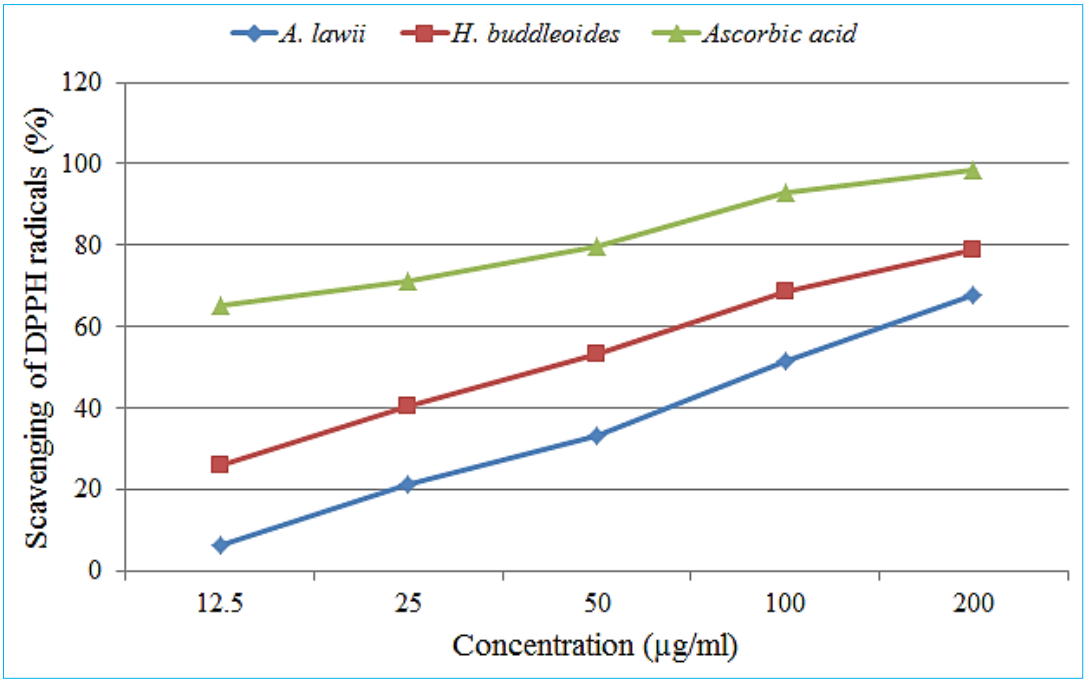

Figure 01. Scavenging of DPPH radicals by extracts and ascorbic acid.

ABTS radical scavenging activity of extracts

The ABTS radical cation decolorization assay measures the relative ability of compounds to scavenge the radical ABTS and is one among the widely used in vitro assays for determining the antioxidant capacity of various kinds of samples including plant extracts. When compared to DPPH radical, the ABTS radical has to be generated prior to adding samples containing antioxidants. The assay involving scavenging of ABTS radical cations is used in determining the antioxidant capacity of both lipophilic and hydrophilic antioxidants. A compound capable of donating electrons will reduce the blue-green ABTS radical solution to colorless neutral form. This reduction process is indicated by the suppression of characteristic long wavelength absorption spectrum of radical (Re *et al.*, 1999; Magalhaes *et al.*,

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2008; Wangcharoen and Morasuk, 2007; Venkatachalam and Muthukrishnan, 2012; Kekuda *et al*., 2015). The result of scavenging of ABTS radicals by extracts of *A. lawii* and *H. buddleioides* is shown in Figure 02. Extract of *H. buddleoides* and *A. lawii* scavenged ABTS radicals dose dependently. *H. buddleoides* exhibited high scavenging potential (IC_{50} value 19.21µg/ml) when compared to *A. lawii* (IC_{50} value 144.13µg/ml). At concentration 25µg/ml and higher, extract of *H. buddleoides* scavenged ABTS radicals to >50% whereas *A. lawii* showed a scavenging of >50% only at the higher extract concentration. A scavenging of 64.81% and 98.14% of radicals was observed at an extract concentration of 200µg/ml by *A. lawii* and *H. buddleoides* respectively. When compared to both extracts, the reference antioxidant ascorbic acid scavenged ABTS radicals more efficiently with an IC_{50} value of 5.6µg/ml. Although the observed results are lower than reference standard, it is evident that extracts possess electron donating potential.

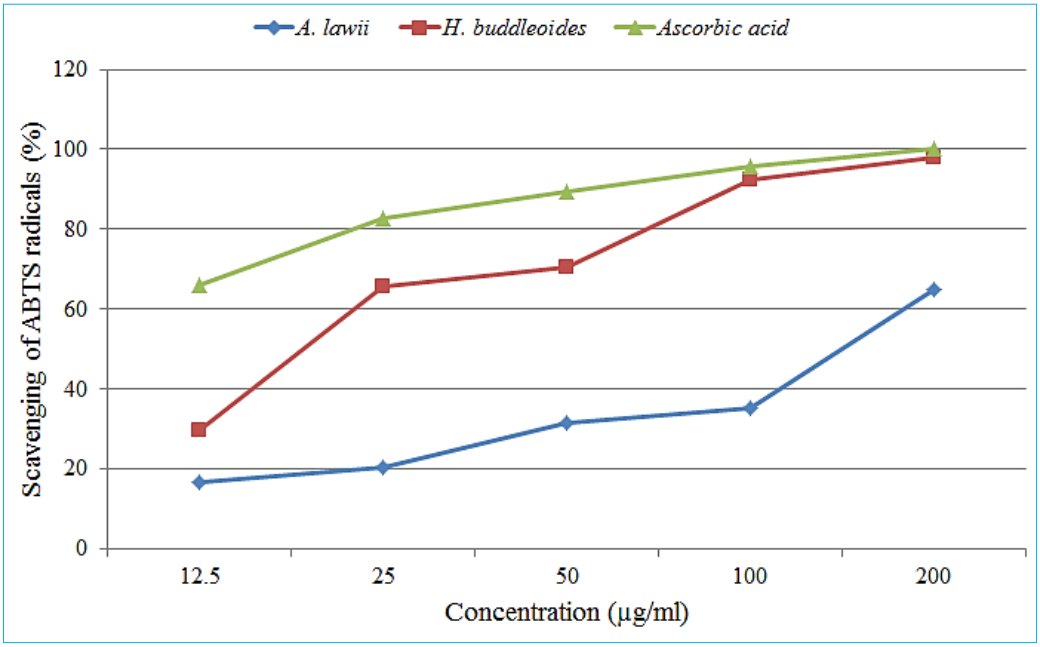

Figure 02. Scavenging of ABTS radicals by extracts and ascorbic acid.

Nitric oxide scavenging activity of extracts

Nitric oxide (NO), a diffusible free radical (due to its unpaired electron), is a potent pleiotropic mediator of several physiological processes in the body. NO is generated by endothelial cells, macrophages and neurons and plays several roles as an effectors molecule in diverse biological systems. NO displays reactivity with certain types of proteins and other free radicals. The toxicity exhibited by NO becomes adverse when NO reacts with superoxide radical and forms highly reactive peroxynitrite anion. Chronic exposure to NO is associated with various ailments such as carcinoma and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis, and ulcerative colitis. NO scavenging activity is one of the widely used in vitro assays to determine scavenging potential of several types of samples including plant extracts (Magalhaes *et al*., 2008; Awah and Verla, 2010; Nagmoti *et al*., 2012; Venkatachalam and Muthukrishnan, 2012; Boora *et al*., 2014; Sasikumar and Kalaisezhiyen, 2014; Okoh *et al*., 2014; Thiruvengadam and Sankar, 2015). In the present study, we screened the NO scavenging effect of extract of *A. lawii* and *H. buddleioides*. Both the extracts were effective in scavenging NO radicals in a dose dependent manner (Figure 03). At concentration 100µg/ml and higher, both extracts displayed >50% scavenging of NO radicals. Among extracts, marked scavenging efficacy was observed in case of *H. buddleoides* (IC_{50} value 40.41µg/ml) when compared to *A. lawii* (IC_{50} value 64.26µg/ml). Scavenging potential of reference standard (IC_{50} value 19.29µg/ml) was higher than that of extracts.

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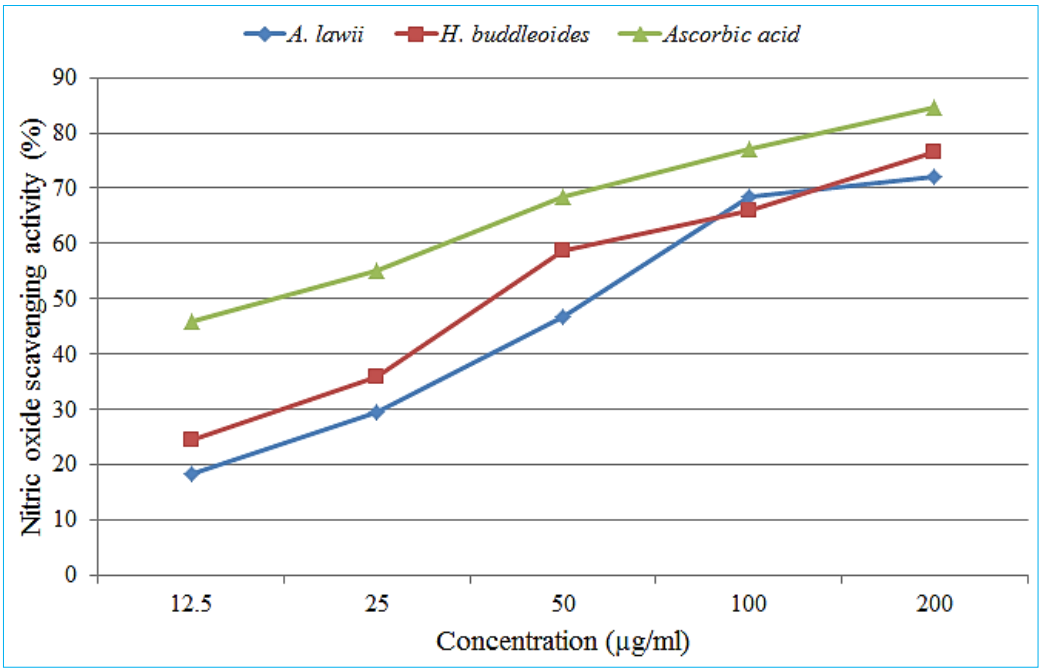

Figure 03. Scavenging of nitric oxide radicals by extracts and ascorbic acid.

Ferric reducing activity of extracts

The antioxidant potential of plant extracts can be related to their reducing activity. In the present study, the reducing potential of extracts of *A. lawii* and *H. buddleioides* was evaluated by ferric reducing assay. The reducing potency of compounds is associated with the presence of substances called reductones, which exert antioxidant action by breaking the free radical chains through hydrogen donation. Reductones are also reported to prevent peroxide formation by reacting with certain precursors of peroxides. In this assay, the presence of reductants in the samples causes reduction of Fe^{+3} to Fe^{+2} by donating electron. The amount of Fe^{+2} complex that is formed can be measured by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance indicates an increase in reductive ability (Chung *et al*., 2006; Kekuda *et al*., 2012; Nagmoti *et al*., 2012; Padmanabhan and Jangle, 2012; Sasikumar and Kalaisezhiyen, 2014). The result of ferric reducing potential of extracts and ascorbic acid is shown in Figure 04. The absorbance of reaction mixture was found to increase with increase in concentration of extracts/standard. This indicated reducing potential of extracts. Among extracts, *H. buddleoides* exhibited stronger reducing power when compared to *A. lawii*.

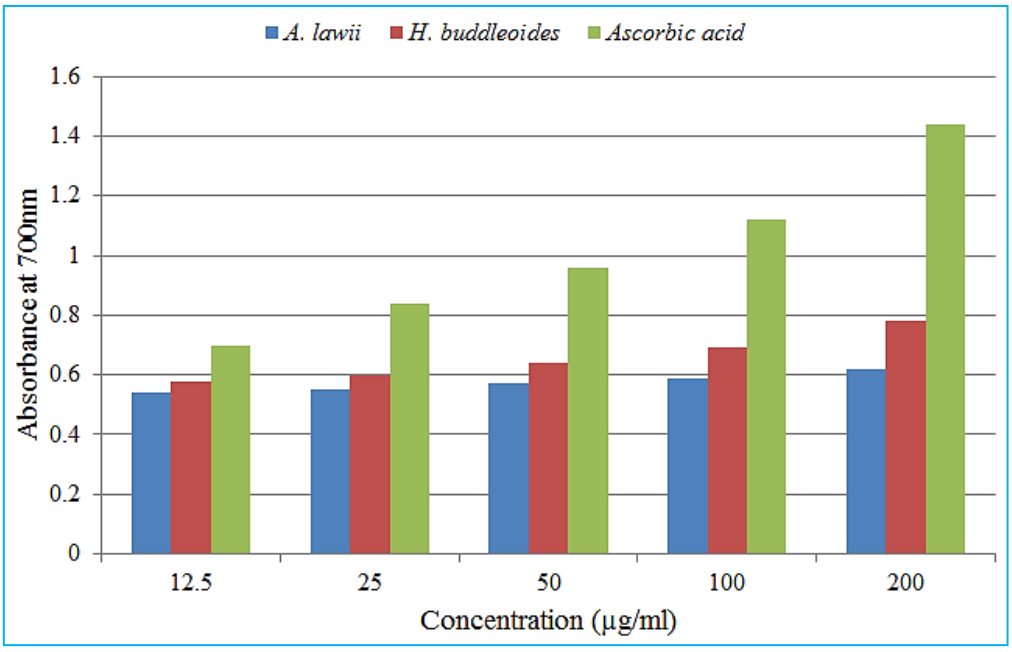

Figure 04. Ferric reducing activity of extracts and ascorbic acid.

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Iron chelating activity of extracts

Iron is crucial for all organisms as it is needed for oxygen transport, respiration and for activity of several enzymes. Several radical reactions are formed from ferrous iron (Fe^{2+}). The transition metal ion Fe^{2+} is able to perpetuate the formation of free radicals by gain or loss of electrons. Hence, chelation of iron seems to be an effective therapeutic approach in terms of reduction of the formation of ROS. Iron chelators are involved in mobilizing iron from tissues by forming soluble and stable complexes. Iron-related complications can be minimized by chelation therapy and by this quality of life and overall persistence can be improved. The available chelators have some drawbacks such as poor oral bioavailability, short plasma half life and side effects. Hence, iron (II) chelating activity of plant extracts is of great significance (Ebrahimzadeh *et al*., 2008; Mirzaei and Khatami, 2013; Sudan *et al*., 2014; Sasikumar and Kalaisezhiyen, 2014; Adjimani and Asare, 2015). In the present study, the iron chelation assay was carried out to assess the chelation capacity of the extract of *A. lawii* and *H. buddleioides*. Ferrozine can quantitatively form complexes with ferrous iron resulting in the formation of a red colour. In the present study, the extracts and standard interfered with the formation of ferrous and ferrozine complex, suggesting that they exhibited chelating activity and captured ferrous ion before ferrozine (Kekuda *et al*., 2011; Adjimani and Asare, 2015). In the present study, in the presence of extracts/EDTA, the color complex (ferrozine-Fe^{2+}) formation was disrupted and was indicated by reduction of the red color of the complex. Both extracts displayed dose dependent chelation of iron (Figure 05). Among extracts, *H. buddleoides* exhibited stronger chelating ability with an IC_{50} value of 21.79µg/ml when compared to *A. lawii* (IC_{50} value 40.56µg/ml). EDTA exhibited stronger chelating effect with an IC_{50} value of 3.40µg/ml when compared to extracts.

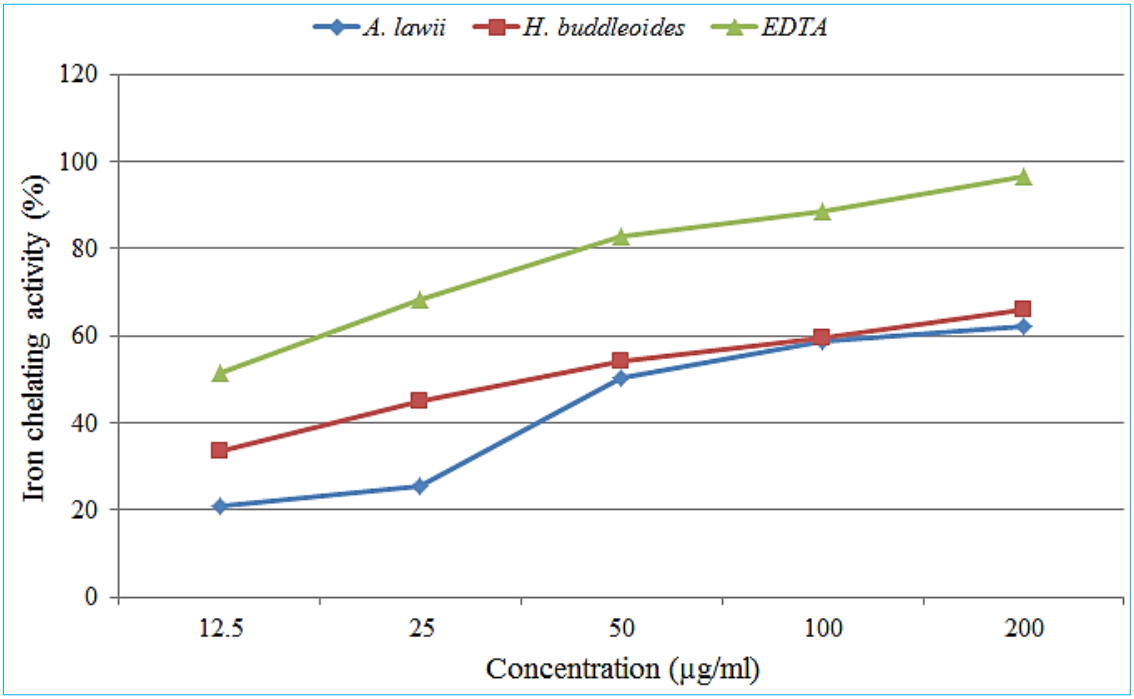

Figure 05. Iron chelating activity of extracts and EDTA.

Total phenolic and flavonoid content of extracts

Polyphenolic compounds including flavonoids are the major phytocompounds having potent antioxidant activity. Phenolic compounds are distributed in various parts of the plants such as leaves, roots and flowers and are a class of natural antioxidants capable of acting as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators and thus are effective free radical scavengers and inhibitors of lipid peroxidation. Dietary intake of phenolic compounds is strongly associated with longer life expectancy, reduced risk of developing chronic diseases, cancer, diabetes, obesity and reduced blood pressure. Phenolic contents of plants have been extensively studied for their contribution to antioxidant activity of plants. The total phenolic and flavonoid content in plants usually correlates highly with the free radical scavenging activity (Venkatachalam and Muthukrishnan, 2012; Kekuda *et al*., 2012; Padmanabhan and Jangle, 2012; Nagmoti *et al*., 2012; Rakesh *et al*., 2013; Farasat *et al*., 2014). We estimated TPC and TFC of extracts by FCR and Aluminium chloride colorimetric estimation method respectively. The content of both phenolics and flavonoids was higher

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in *H. buddleioides* when compared to *A. lawii* (Table 01). A positive correlation between TPC and TFC and antioxidant activity of extracts was observed, i.e., extract of *H. buddleioides* containing high TPC and TFC displayed marked antioxidant activity when compared to *A. lawii* which was found to contain lesser TPC and TFC. Similar positive correlations between TPC and antioxidant activity of plants were observed in studies of Piluzza and Bullitta (2011), Venkatachalam and Muthukrishnan (2012), Kekuda *et al*. (2012) and Sasikumar and Kalaisezhiyen (2014).

Table 01. Content of total phenolics and flavonoids in extracts

Contents	*A. lawii*	*H. buddleioides*
Total phenolic (mg GAE/g)	45.62	68.33
Total flavonoid (mg CE/g)	16.56	32.64

IV. Conclusion

Antioxidant activity of *A. lawii* and *H. buddleioides* was evaluated. Results obtained indicated promising antioxidant activity of plants. Presence of phenolics and flavonoids in the plants might have accounted for the observed antioxidant potential. Positive correlation between the antioxidant activity and phenolics and flavonoid contents suggests that these polyphenolic compounds are the main contributors of antioxidant activity of both plants. Further studies on recovery of bioactive components form the extracts and their antioxidant activity determinations are to be carried out.

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