INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 12, No. 5, p. 219-232, 2018

RESEARCH PAPER OPEN ACCESS

Phytochemical screening and *in vitro* anti-oxidant activities of *Ehretia serrata* Roxb. and *Ehretia obtusifolia* Hochst. Ex A. DC (Family: Boraginaceae)

Zill-e-Huma[1], Muhammad Ibrar[1], Fazal Hadi[*1], Barkatullah[2], Memrez Khushal gigyani[3]

[1]*Department of Botany, University of Peshawar, Pakistan*

[2]*Department of Botany, Islamia College University, Peshawar, Pakistan*

[3]*Veterinary Research Institute, Peshawar, KP, Pakistan*

Key words: *Ehretia serrata*, *Ehretia obtusifolia*, Alkaloids, Phenolic compounds, Ascorbate, Anti-oxidant

http://dx.doi.org/10.12692/ijb/12.5.219-232 Article published on May 30, 2018

Abstract

The objective of current study was to investigate the presence of qualitative and quantitative phytochemical compounds and anti-oxidant potential of methanolic extracts of leaf, fruit and stem bark of *Ehretia serrata* Roxb. and *Ehretia obtusifolia* Hochst. Ex A.DC. Results of qualitative phytochemical screenings of examined parts of these plants shown the presence of phenol compounds, starch, protein, reducing sugar, steroids, tarpenoids, fats, aldehydes, anthraquinone, glycosides, amino acids, saponine, alkaloids, tannins and phlobatannin. The quantitative analysis of phenolic compounds and flavonoids showed that *E. serrata* leaf and *E. obtusifolia* stem bark have significant amount (43.6±0.34 and 38.35±0.44; 38.00±1.33, 34.11±0.39) of both compounds, which showed relatedness of these compounds with significant scavenging activity. Based on IC_{50} (µg/ml), leaf extract of *E. serrata* showed highly significant ($p>0.01$) results (IC_{50} µg/ml; 0.12) after 30 min compared to other parts. In *E. obtusifolia* highly significant value was observed in stem bark (IC_{50} µg/ml; 0.03) followed by fruits and leaf. Highly significant ($p>0.01$) % DPPH inhibition was observed in *Ehretia serrata* stem bark (94.1%) after 60 min and in fruit (85%) of *Ehretia obtusifolia* after 30 min. The present study revealed that all investigated parts of both plants possesses high antioxidant potency due to the presence of high amount of bioactive compounds, this may also justify their use in ethno-medicine as an anti-oxidant agent.

* **Corresponding Author:** Fazal Hadi ✉ hadibotany@uop.edu.pk

Introduction

Medicinal plants are diverse from each other and specified for a particular action by the presence of chemical ingredient's specific for curing a specific disease. Medicinal plants are vital resources of important bioactive chemical compounds such as alkaloids, tannins, flavonoids and phenolic compounds which lead and clues for modern drug design and formation (Doss, 2009; Acharya, 2012; Bharathamma & Sudarsanam, 2015; Begum *et al.*, 2015; Singh *et al.* 2015; Acharya *et al.* 2012; Singh, 2012 and Hakemi *et al.* 2012). Many medicinally important plants are rich in many bioactive compounds important for curing many diseases (Soares *et al.*, 2003; Yadav & Agarwala, 2011; Hegde & Jayaraj, 2016). To treat chronic disease and production of drugs with precise activity, relationship between the phytochemical compounds and the bioactivity of medicinal plant is very much important (Okwu & Josiah, 2006; Pandey *et al.*, 2013; Hegde *et al.*, 2015;). Evaluation of crude drug is important for discovery of new sources of low price homoeopathic ingredients which then lead to the synthesis of modern chemical compound for treating severe diseases (Badugu, 2012). Phytochemical screening gives us hints and evidences to the discovery of new valuable Drugs (Khan *et al.*, 2011).

Family Boraginaceae is commonly called 'forget me not family'. Many species of this family are famous for curing of many diseases in all over the world due to the presence of important of bio active compounds. Some of the species are tested for isolation and characterization of various chemical constituents such as pyrrolizidine alkaloids, naphthoquinones, flavonoids, terpenoids, triterpenoids and phenols. Many biological and physiological actions of living organism are dependent on organic compounds such as tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids (Kavanagh., 1963; Arunkumar & Muthuselvam., 2009). Current study was done for the first time to investigate qualitative and quantitative phytochemicals in investigated species. The identified phytochemicals from medicinal plant have important physiological effects on body showing analgesic, expectorant, emulsifying and antibiotic and anti-oxidant effects (Savithramma *et al.*, 2011; Gupta *et al.*, 2012).

here are natural enzymatic and non-enzymatic defensive system in human body to neutralize free radicals and oxidants which are responsible for producing cancer (Kaarteenaho-Wiik & Kinnula, 2004), cardiovascular disease (Singh and Jialal, 2006), neural disorders (Sas *et al.*, 2007), Alzheimer's disease (Smith *et al.*, 2000), mild cognitive impairment (Guidi *et al.*, 2006), Parkinson's disease (Bolton *et al.*, 2000), alcohol induced liver disease (Arteel, 2003), ulcerative colitis (Ramakrishna *et al.*, 1997), aging (Hyun *et al.*, 2016) and atherosclerosis (Upston *et al.*, 2003). Dietary intake antioxidants are important from protection against oxidant and free radicals. Significant evidence proved that any plants having natural antioxidant nutrients and phytochemicals may be of chief importance in disease prevention (Hyun *et al.*, 2016). Some major researches suggested that mixture of antioxidants, rather than single antioxidant, are more effective over the long term (Ramakrishna *et al.*, 1997). Antioxidants are effective with countless benefits in improving life quality by inhibiting or delaying the beginning of degenerative diseases (Upston *et al.*, 2003; Khettaf *et al.*, 2016).Few medicinal uses of *E.serrata* e.g juice of the bark of *Ehretia serrata* is used in the treatment of fevers as well as green and unripe fresh fruit is used in a form of pickle which give us clue for the presence of many important nutritive, biologically active compounds and anti-oxidant agents in experimented plant (Ajaib & Zaheer-UD-Din, 2014; Haq *et al.*, 2012). Ethnobotanical importance of *E.obtusifolia*, such as usage of its leaves as an infusion to treat sore throat and teething pain, as well as usage of roots to treat painful menstruation and infertility in women and painkiller, specifically against abdominal pain in Zimbabwe and in Tanzania, gives us hints for the presence of biologically active compounds in it (Coates, 1983).

E. serrata and *E. obtusifolia* has not been the subject of any phytochemical or pharmacological studies before than that to our knowledge. In this study, we are interested in studying for the first time the chemical composition and anti-oxidant activity of the methanolic extract of leaf, fruit and stem bark of both species. It should be noted that to date, no studies on the chemical composition and biological activity of both plants species has been made.

Materials and methods

Extracts of different parts of the examined plants and all reagents used were of analytical grade. They include: Methanol, benedict reagent, Indane 1, 2, 3 trione hydrate, Potassium mercuric iodide solution, $FeCl_3$, lead acetate solution, HCl, Choloroform, H_2SO_4, ferric chloride, Fehling solution, Diethyle Ether, NaCl, Folin-Denis reagent, tannic acid, 20% Na_2CO_3, distilled water, tannic acid, spectronic 20D, potassium acetate, Aluminium chloride, 1, 1-diphenyl-2-picryl hydrazyl (DDPH), Extracts of different parts of the plants Ascorbic acid, Test tubes, beakers etc.

Sample collection

Leaves, fruits and stem bark of *Ehretia serrata* and *Ehretia obtusifolia* were collected in March, 2015 from Peshawar, KP, Pakistan. Verification was done by curator, department of Botany, University of Peshawar and a voucher specimen of *Ehretia serrata* and *Ehretia obtusifolia* with No. 9887 and No. 9888 deposited in the herbarium of the Department of Botany University of Peshawar, KP, Pakistan.

Preliminary qualitative phytochemical screening

Phytochemical screening tests were carried out for detection of various bioactive compounds in all the extracts individually. Some of conducted test are given below.

Carbohydrates detection test

Benedict test: (Alkaline solution containing cupric complex) few drops of benedict reagent were added in methanolic extract and boiled it on water bath. Appearance of reddish brown precipitation will confirm presence of carbohydrates (Evans, 2009).

Proteins and amino acids detection test

Ninhydrin method: (Indane 1, 2, 3 trione hydrate) few drops of 0.2% Ninhydrin solution was mixed in extract and boiled in test tube. Formations of violet colour precipitates indicate presence of amino acid and protein (Edeoga *et al.*, 2005).

Alkaloids detection test

Mayer's test (Potassium mercuric iodide solution): Addition of Mayer's reagent in 30 ml methanolic extracts solution drop wise, creamy white precipitate formation show presence of alkaloids (Deore *et al.*, 2015).

Phenol detection test

Ferric Chloride Test: To 10ml extract solution, few drops of $FeCl_3$ solution were added. Appearance of bluish black or green colour will show presence phenols (Chavre, 2015).

Fixed oil detection test

Each powder of leaf, stem bark and fruit root of both *Ehretia serrata* and *Ehretia obtusifolia* plants were kept in filter paper and pressed. Permanent greasy spots appearence on filter paper will be the sign of presence of fixed oil (Onocha *et al.*, 2011; Hegde *et al.*, 2015).

Flavonoids detection test

Lead Acetate test: Few drops of lead acetate solution were mixed with plant extract solution. Yellow colour precipitate appearance in solution indicates the presence of flavonoids (Onocha *et al.*, 2011).

Saponin detection test

Hydrochloric acid test: In this test few drops of HCl were added in extract solution, appearance of pink colour indicates the presence of saponin. If addition of ammonia solution with HCl in extract solution change colour of extract solution into deep violet, will show presence of saponin (Tiwari *et al.*, 2011).

Terpenoids detection test

Salkowski's test: Mixing of 2ml of extract solution with several drops of chloroform and H_2SO_4, and the formation of red colour precipitate in bottom of test tube will show the presence of terpenoids (Harborne, 1998).

Tannins detection test

Ferric chloride test: Mixing of 5ml of ferric chloride solution in 10ml of extract solution, formation of bluish black precipitate will show presence of tannin (Somkuwar and Kamble, 2013).

Anthraquinones detection test

Borntrager's Test: Mixture of 10% $FeCl_2$ solution and 10 ml extract solution were heated and 2ml of pure HCl and was filtered. After cooling the filtrate mixture were quickly shaken with diethyl ether followed by addition of concentrated ammonia solution. Formation of pink or deep red colour aqueous layer will indicate the presence of anthraquinone (Niratker & Sailsaja., 2014).

Glycosides detection test

Borntrager's test: To the extract solution (1mL), 5% H_2SO_4 (1 mL) was added. The mixture was boiled in a water bath and then filtered. Filtrate was then shaken with equal volume of chloroform and kept to stand for 5 min. Then lower layer of chloroform was shaken with half of its volume with dilute ammonia. The formation of rose pink to red colour of the ammoniacal layer gives indication of anthraquinone glycosides (Joshi *et al*., 2013).

Phytosterols detection test

Salkowski's Test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes (Evans, 2009 & Ozcan *et al*., 2011)

Reducing sugar detection test

Fehling's test: Few drops of Fehling's solution was added and then heated on a water bath for 2 minutes. Appearance of a reddish-brown precipitate of cuprous oxide indicates the presence of combined reducing sugar (Somkuwar & Kamble, 2013).

Starch detection test

1 mL of aqueous extract was added 10 mL of NaCl saturated solution. After heating, starch reagent was added a blue-purplish colour is a positive test for the presence of starch. Plant powder sample was mixed with distilled water in a test tube, and then was shake it well, and filtered to take plant extract. Then to each plant extract, 1% aqueous hydrochloric acid was added and each plant sample was then boiled with the help of Hot plate stirrer. Formation of red coloured precipitate confirmed a positive result (Joshi *et al*., 2013).

Quantitative phytochemical screening

Total phenols determination

The percentage of phenol in methanolic extract leaf, fruit and stem bark of *Ehretia serrata* and *Ehretia obtusifolia* was determined by following Mc Donald *et al.* (2001). 2 gram of each experimented part extract of two plants treated individually with 20 ml of Folin-Denis reagent followed by 30ml of 20 % Na_2CO_3 and then diluted all the samples by a factor 100 with distilled water. All the Mixtures were kept for 30 min at room temperature and then filtered. The absorbance was measured With Spectronic 20D (Milton Roy), at 770nm against the tannic acid. The determination of phenolic compounds of each sample was done by contrasting absorbance curve with standard curve using as blank.

Total flavonoids determination

Determination of total flavonoids contents in all experimented parts (leaf, fruit and stem bark) of *Ehretia serrata* and *Ehretia obtusifolia* were done by following aluminium chloride, colorimetric method of Chang *et al.* (2002). Solutions of individual extract and methanol prepared by mixing 1 gram of each extract in 10ml of methanol separately.

From each stock solution 0.5 ml were mixed with 1.5 ml of methanol, 0.1ml of 1M potassium acetate, 0.1ml of 10% Aluminium chloride and 2.8ml of distilled water. All the Mixtures were kept for 30min at room temperature followed by measurement of absorbance of each reaction mixture at 415nm.The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to100µg/ml in methanol.

Antioxidant activity

Free radicals formation due to different nutritional and medicinal deterioration is controlled by number of systems called antioxidant. The antioxidant potency of experimented parts of *Ehretia serrata* and *Ehretia obtusifolia* were determined following procedure of Lalitha *et al.* (2013).

Procedure

The DPPH (1, 1-diphenyl-2-picryl hydrazyl) was used to detect the free radical scavenging potency of different experimented parts of the two plants. DPPH solution of 0.1mm was prepared in ethanol. Different concentrations (10, 100 and 1000µg/ml) of extracts were formed. A mixture of 1ml DPPH and 3 ml of each extract were formed. Mixtures were shake thoroughly, and before incubation were kept at room temperature in dark for 30min.

Spectrophotometer (UV-VIS) was set on 517nm and absorbance of these prepared solution were checked after 30 mints, 45 mints and 60 mints at the set wavelength. Ascorbic acid was used as a standard. The % age of antioxidant potency of extracts were calculated by using following formula.

% Antioxidant potential=

$$\frac{\text{Absorbance of control (nm)} - \text{Absorbance of test nm}}{\text{Absorbance of control}} \times 100$$

Finally, the IC_{50} value defined as the concentration was calculated from the separate linear regression plots of the mean percentage of the antioxidant activity against concentration of the test extract (µg/ml).

Statistical Analysis

Statistical analysis of data was done through one way ANOVA trough SPSS (22) computer software, Means ± SEM were calculated through Microsoft excel, Dunnet test were used for comparison between control and tested treatments. The probability $P<0.05$ were considered significant. Half maximal inhibitory concentration (IC_{50}) was calculated from the % inhibition versus extract concentration non-linear regression curve of each extract.

Results

Phytochemical screening

Phytochemical screening of Ehretia serrata leaf

In present work phytochemical screening of *Ehretia serrara* leaf was carried out to find out the phytoconstituents in its methanolic extract. Phenol compounds, protein, reducing sugar, steroids, terpenoids, fats, glycosides, amino acids, saponin, alkaloids, tannins, phlobatannin and starch grains were detected in leaf of *Ehretia serrata* (Table 1). Aldehydes and anthraquinone were not detected in the leaves.

Phytochemical screening of Ehretia serrata fruit

Several active constituents of pharmacological importance may be available in a single plant (Rai *et al.*, 2013).

In the present investigation fruit of *Ehretia serrata* was found to contain phenol compounds, protein, reducing sugar, steroids, terpenoids, fats, glycosides, amino acids, saponin, alkaloids, phlobatannin, starch. Aldehydes, anthraquinone and tannins were not detected in the fruit (Table 1).

Phytochemical screening of Ehretia serrata stem bark

Similar to the leaves and fruit, stem bark also contained phenol compounds, protein, reducing sugar, steroids, terpenoids, glycosides, amino acids, saponin, alkaloids, tannins and starch. Fats, aldehydes, anthraquinone and phlobatannin were not detected (Table 1).

Phytochemical screening of Ehretia obtusifolia leaf

Leaf of *Ehretia obtusifolia* was investigated for detection of various bioactive compounds. The leaf contained phenol compounds, Protein, reducing sugar, steroids, terpenoids, fats, glycosides, amino acids, saponin, alkaloids, tannins, starch and phlobatannin. Aldehydes and anthraquinone were not detected in leaf (Table 2).

Phytochemical screening of Ehretia obtusifolia fruit

Fruit of *Ehretia obtusifolia* was also investigated for phytochemical screening and found to contain important secondary bioactive constituents such as phenols, protein, reducing sugar, steroids, terpenoids, fats, glycosides, amino acids, starch, saponin and tannins but aldehydes, anthraquinone, alkaloids and phlobatannin were not detected (Table 2).

Phytochemical screening of Ehretia obtusifolia stem bark

In contrast to leaf, and fruit, the stem bark showed the presence of all screened compounds e.g phenol compounds, protein, reducing sugar, steroids, terpenoids, glycosides, amino acids, saponin, starch, alkaloids, tannins and phlobatannin. Most of the important active compounds such as Fats, Aldehydes and Anthraquinone were absent (Table 2).

Table 1. List of Phyto-constituents found in different parts of *Ehretia serrata*.

S.No	Name of functional group	Leaves	Fruit	Stem bark
1	Phenol compounds	+	+	+
2	Starch	+	+	+
3	Protein	+	+	+
4	Reducing sugar	Light green	Red	Reddish brown
5	Steroids	+	+	+
6	Terpenoids	+	+	+
7	Fix oil	+	+	-
8	Aldehydes	-	-	-
9	Anthraquinone	-	-	-
10	Glycosides	+	+	+
11	Amino acids	+	+	+
12	Saponine	+	+	+
13	Alkaloids	+	+	+
14	Tannins	+	-	+
15	Phlobatannin	+	+	-

Table 2. List of Phyto-constituents found in different parts of *Ehretia obtusifolia*.

S.No	Name of functional group	Leaves	Fruit	Stem bark
1	Phenol compounds	+	+	+
2	Starch	+	+	+
3	Protein	+	+	+
4	Reducing sugar	Greenish brown	Red	Greenish orange
5	Steroids	+	+	+
6	Tarpenoids	+	+	+
7	Fats	+	+	-
8	Aldehydes	-	-	-
9	Anthraquinone	-	-	-
10	Glycosides	+	+	+
11	Amino acids	+	+	+
12	Saponine	+	+	+
13	Alkaloids	+	-	+
14	Tannins	+	+	+
15	Phlobatannin	+	-	+

b. Total phenolic and flavonoids

Total phenolic and flavonoids in leaf, fruit and stem bark of Ehretia serrata.

Total phenolic compounds and flavonoids are scientifically proves to be correlated with the antioxidant activity. All parts of *Ehretia serrta* showed high amounts of these tow phytochemical compounds in which highest amount of phenolic compounds observed in leaf (43.6±0.34) followed by fruit (35.5±0.67) and stem bark (25.0±0.66). Flavonoids are considered one of the most abundant and important natural bioactive constituent found in plants. Flavonoids were found almost in same quantity in leaf (38.35±0.44) and fruit (37.97±1.33) of *Ehretia serrata* followed by stem bark (23.09±1.39) (Table 3).

Total phenolic and flavonoids in leaf, fruit and stem bark of Ehretia obtusifolia.

Total phenolic compounds and flavonoids were also observed in leaf, fruit and stem bark of *Ehretia obtusifolia* and found significant amount pf both compounds in all three parts extracts. Highest amount of phenolic compounds (38.00±1.33) and flavonoids (38.00±1.33) were observed in stem bark followed by fruits with 35.81±0.98 and 26.37±0.48mg/g respectively. Low amount of phenolic compounds and flavonoids (34.13±0.43; 24.41±0.78) compared with other two parts were seen in leaf of *Ehretia obtusifolia* (Table 3).

Table 3. Total phenol compounds and flavonoids in leaf, fruit and stem bark of *Ehretia serrata* and *Ehretia obtusifolia*.

S. No	Phenolic content (mg/g)	Flavonoids (mg/g)
Ehretia serrta leaf	43.6+0.34	38.35+0.44
Ehretia serrata fruit	35.5+0.67	37.97+1.33
Ehretia serrata stem bark	25.0+0.66	23.09+1.39
Ehretia obtusifolia leaf	34.13±0.43	24.41±0.78
Ehretia obtusifolia fruit	35.81±0.98	26.37±0.48
Ehretia obtusifolia stem bark	38.00±1.33	34.11±0.39

Anti-oxidant activity

Anti-oxidant activity of Ehretia serrata leaf

Leaf extract of *Ehretia serrata* showed highly significant (P<0.01) antioxidant activity at 10µg/ml dose after 30, 60 and 90min, and showing percent inhibition of 72.9±0.62, 70.0±0.57 and 70.2±0.78 respectively, compared to Ascorbate (showed percent inhibition of 75.2±0.31 after 30min, 71.3±0.32 after 60min and 70.9±0.35 after 90min). With increasing dose, the anti-oxidant potency of leaf extract of *Ehretia serrata* becomes significantly (P<0.01) increased, and percent inhibition were 80.5±1.50, 75.03±1.10 and 77.4±0.60 at 100µg/ml dose, after 30, 60 and 90 min respectively, compared to Ascorbate, which showed percent inhibition of 85.2±1.12, 80.9±0.32 and 84.1±0.40 after 30min, 60 min and 90min respectively. At 1000µg/ml dose the leaf extract of *Ehretia serrata* showed significant (P<0.05) result after 30min (81.94±0.83), 60 min (70.9±0.92) and 90min (80.1±0.21), compared to Ascorbate which showed percent inhibition, 95.2±0.81, 92.2±1.10 and 97.1±0.34 after 30min, 60min and 90min respectively. Low IC_{50} value is correlated with high savenging activity. All results based on IC_{50}, showed significancy at all time duartions e.g after 30min (IC_{50}=0.12µg/ml), 60min (IC_{50}=1.12µg/ml) and 90 min (IC_{50}=1.1µg/ml) compared to ascorbate with IC_{50}=0.41µg/ml, IC_{50}= 0.54µg/ml and IC_{50}=1.96µg/ml respectively (Table 4; Fig 1).

Table 4. Percent anti-oxidant activity by leaf, fruit and stem bark of *Ehretia serrata*.

µg/ml	%DPPH inhibition by ESL			%DPPH inhibition by ESF			%DPPH inhibition by ESS			%DPPH inhibition by Ascorbate		
	30 min	60 min	90 min	30 min	60 min	90 min	30 min	60 min	90 min	30 min	60 min	90 min
10	72.9 **±0.62	70.0**±0.51	70.2 **±0.78	70.0** ±1.10	45.1 ±0.35	45.3 ±0.15	70.2 **±0.5	71.0 **±0.62	27.1 ±0.10	75.2 ±0.31	71.3 ±0.32	70.9 ±0.35
100	80.5 **±1.50	75.0 *±1.10	77.4 **±0.60	80.9** ±0.57	61.6 ±0.40	51.9 ±0.48	74.1** ±0.4	74.9** ±0.31	40.0 ±0.32	85.2 ±1.12	80.9 ±0.75	84.1 ±0.40
1000	81.9* ±0.83	70.9 ±0.92	80.1 ±0.21	90.5**±0.35	69.2 ±0.12	73.2* ±0.81	93.2** ±1.12	94.1**±0.18	28.4 ±0.71	95.9 ±0.81	92.2 ±1.10	97.1 ±0.34
IC_{50} µg/ml	0.12	1.12	1.1	3.37	63.6	34.8	2.2	0.34	879.7	0.41	0.54	1.96

Values are reported as mean ± SEM of five replicates. The data was analyzed by one way ANOVA followed by Dunnett's test. * Significant at P < 0.05, ** highly significant at P<0.01 and "no symbol" non-significant at P>0.05.

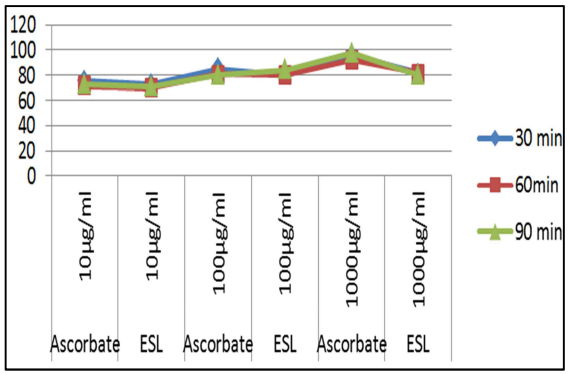

Fig. 1, % DPPH inhibition by *Ehretia serrata* leaf.

Anti-oxidant activity of Ehretia serrata fruit

Fruit extract of *Ehretia serrata* at 10µg/ml dose showed highly significant (P<0.01) percent DPPH inhibition after 30 min (70.0 ±1.10), which gradually decreased with passage of time, become non-significant (P>0.05) percent inhibition (45.1±0.15) after 60 min and (45.3±0.15) after 90 min, compared to ascorbate (percent DPPH inhibition of 75.2±0.31 after 30min, 71.3±0.32 after 60min and 70.9±0.35 after 90min). Effectivity was significantly (P<0.01) increased with incensement of dose e.g at 100µg/ml, percent inhibition was 80.9±0.57 (30 min), and then decreased with passage of time, 61.6±0.40 after 60min and 51.9±0.48 after 90min, compared to ascorbate which shown percent inhibition of 85.2±1.12, 80.9±0.32 and 84.1±0.40 after 30min, 60min and 90min respectively.

At dose level 1000µg/ml high significancy (P<0.01) was observed, after 30 min (90.5±0.35), the anti-oxidant activity was gradually decreased with time, and after 60min and 90min it was (69.2±0.12) and (73.2±0.81), compared to ascorbate with percent inhibition of 95.2±0.81, 92.2±1.10 and 97.1±0.34 after 30min, 60min and 90min respectively. Significancy based on IC_{50} showed that high effectivity seen after 30min (IC_{50}= 3.37µg/ml) compared to ascorbate at this time duration (IC_{50}=0.41µg/ml) than rest of two time durations, e.g 60min (IC_{50}=63.6µg/ml) and 90 min (IC_{50}=34.8µg/ml) compared to ascorbate (IC_{50}=0.54µg/ml after 60min, and IC_{50}= 1.96µg/ml after 90min) (Table 4; Fig. 2).

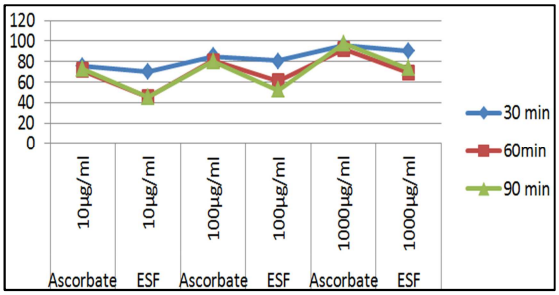

Fig. 2. % DPPH inhibition by *Ehretia serrata* fruit.

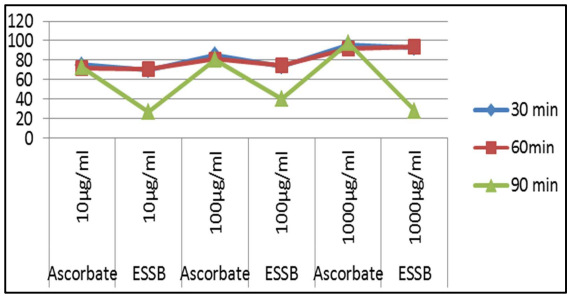

Fig. 3. % DPPH inhibition by *Ehretia serrata* stem bark.

Anti-oxidant activity of Ehretia serrata stem bark

The anti-oxidant effect of stem bark methanolic extract of *Ehretia serrata* at 10µg/ml was highly significant (P<0.01) after 30min and 60 min, and showed percent DPPH inhibition of 70.2±0.52 and 71.0±0.62, compared to control (ascorbate) with 75.2±0.31 and 71.3±0.32 respectively, as well as the percent inhibition was decreased after 90min (27.1±0.10), compared to ascorbate (70.9±0.35). At dose 100µg/ml and 1000µg/ml results were highly significant (P<0.01) after 30min (74.1±0.48 and 93.2±1.12), and after 60 min (74.9±0.31 and 94.1±0.81), compared to control with values 85.2±1.12; 95.9±0.81 after 30min and 80.9±0.32; 92.2±0.75 after 60min respectively. The anti-oxidant activity showed by methanolic extract of stem bark after 90min was non-significant, and percent inhibition was shown as 40.0±0.32 and 28.4±0.71, compared to control (ascorbate) which had 84.1±0.40 and 97.1±0.34 respectively (Table 4; Fig 3). Significancy based on IC_{50} showed that stem bark extract showed high inhibition after 60 min (IC_{50}= 0.34µg/ml) compared to ascorbate at this time duration (IC_{50}=0.54µg/ml) followed by 30min (IC_{50}=2.2 µg/ml) and then 90min (IC_{50}=897.7µg/ml) compared to ascorbate (IC_{50}=2.2µg/ml after 30 min, and IC_{50}= 1.96µg/ml after 90 min) (Table 4).

Anti-oxidant activity of Ehretia obtusifolia leaf

Methanolic extract of *Ehretia obtusifolia* leaf showed highly significant (P<0.01) anti-oxidant potency with percent inhibition of 76.50±0.98 at 10µg/ml, 70.4±0.23 at 100µg/ml and (80.9±0.12 at 1000µg/ml after 30min, compared to control (78.8±0.34 at 10µg/ml, 72.1±1.12 at 100µg/ml and 92.9±0.18 at 1000µg/ml after 30min). After 60min the percent inhibition decreased and non-significant (P>0.05) at 10µg/ml (23.23±0.15) and 100µg/ml (47.01±0.23) but slightly increase (60.0±1.1) at 1000µg/ml compared to control (70.9±0.39; 80.1±0.46 and 93.1±0.16 at all three doses after 60 min respectively). After 90min the scavenging activity percentages of leaf extract of *Ehretia obtusifolia* were tremendously decrease and non-significant (P>0.05) 22.33±0.15 at 10µg/ml, 31.08±0.12 at 100µg/ml and 40.00±0.35 at 1000 µg/ml, compared to ascorbate (71.5±0.46; 86.0±0.35 and 98.6±0.78 percent inhibition at all three doses after 90min) (Table 5; Fig 4).

After comparion of IC_{50}, Leaf extract showed significant results (IC_{50}=2.5µg/ml) compared to ascorbate (IC_{50}=2.31 µg/ml) after 30min. while rest of the two results (IC_{50}=235.6µg/ml after 60 min and IC_{50}= 9090.4µg/ml after 90 min) were non-significant, compared to ascorbate (IC_{50}=0.86 µg/ml and IC_{50}= 0.68µg/ml) (Table 5).

Table 5. Percent anti-oxidant activity showed by leaf, fruit and stem bark of *Ehretia obtusifolia*.

µg/ml	%DPPH inhibition by EOL			%DPPH inhibition by EOF			%DPPH inhibition by EOSB			% DPPH inhibition by Ascorbate		
	30 min	60 min	90 min	30 min	60 min	90 min	30 min	60 min	90 min	30 min	60 min	90 min
10	76.5**±0.98	23.2±0.12	22.3±0.15	70.0**±0.87	70.0**±0.35	50.7±0.45	58.0±0.81	46.6±0.78	55.0±1.40	78.8±0.34	70.9±0.39	71.5±0.46
100	78.3**±0.23	47.0±0.23	31.0±0.12	71.0**±0.89	77.0**±0.67	65.9±0.68	55.1±0.51	55.6±0.78	60.8±0.57	72.1±1.12	80.1±0.46	86.0±0.35
1000	80.1*±0.12	60.0±1.1	40.0±0.35	85.3**±0.13	80.1**±1.11	75.0±0.12	63.0±0.91	70.0±1.12	65.6±0.69	92.9±0.18	93.1±0.16	98.6±0.78
IC_{50} µg/ml	2.5	235.6	990.4	0.83	1.7	9.02	0.03	0.12	1.08	2.31	0.86	0.68

Values are reported as mean ± SEM of five replicates. The data was analysed by one way ANOVA followed by Dunnett's test. * Significant at P < 0.05, ** highly significant at P<0.01 and "no symbol" non-significant at P>0.05.

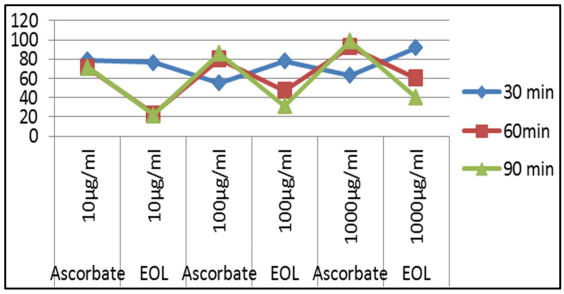

Fig. 4. % DPPH inhibition by *Ehretia obtusifolia* leaf.

Anti-oxidant activity of Ehretia obtusifolia fruit

The Fruit extract showed highly significant (P<0.01) results (70.01±0.87) at 10µg/ml dose, 71.01±0.89 at dose level 100µg/ml and 85.33±0.13 at dose level 1000µg/ml after 30min, compared to control with values of 78.8±0.34 at 10µg/ml, 72.1±1.12 at level 100µg/ml and 92.2±0.18 at 1000µg/ml after 30min. After 60min percent anti-oxidant potency of fruit extract was (70.0±0.35, 77.0±0.67 and 80.1±1.11) highly significant (P<0.01) at three doses (10µg/ml , 100 µg/ml and 1000µg/ml), compared to ascorbate (70.9±0.39, 80.1±0.46 and 93.1 ±0.16 respectively at all three doses). After 90min the anti-oxidant potency of fruit extract were non-significant and showed percent scavenging activity of 50.7±0 at dose 10µg/ml, 65.9±0.68 at 100µg/ml and 75.00±0.12 at 1000µg/ml, compared to ascorbate (71.5±0.46, 86.0±0.35 and 98.6±0.78). After comparing IC_{50} values, *Ehretia serrata* fruit extract showed significancy (P<0.01) (IC_{50}=1.7µg/ml and IC_{50}=0.01µg/ml), compared to ascorbate (IC_{50}=2.31µg/ml and IC_{50}=0.86µg/ml) but non-significant (IC_{50}= 9.02µg/ml), compared to control (IC_{50}=0.68µg/ml) (Table 5; Fig 5).

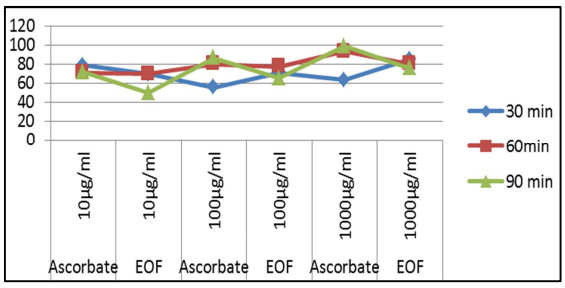

Fig. 5. % DPPH inhibition by *Ehretia obtusifolia* fruit.

Anti-oxidant activity of Ehretia obtusifolia stem bark

Stem bark of *Ehretia obtusifolia* showed less significant scavenging activity at dose level of 10µg/ml (58.0±0.81), which is decreasing with increasing dose and at 100µg/ml it was (55.0±0.51) and at 1000µg/ml it was significant (P<0.05) (63.0±0.91), (IC_{50}=0.03µg/ml) after 30min, compared to ascorbate with percent DPPH inhibition of 78.8±0.34 at 10µg/ml, 72.1±1.12 at level 100µg/ml and 92.2±0.18 at 1000µg/ml (IC_{50}=2.31µg/ml). After 60min stem bark extract showed non-significant (P>0.05) percent DPPH inhibition (46.6±0.78, 55.6±0.78 and 70.0±1.12) at 10µg/ml, 100 µg/ml and 1000µg/ml dose, with IC_{50} value 25.9µg/ml, compared to ascorbate (70.9±0.39, 80.1±0.46 and 93.1±0.16 with IC_{50}=0.86µg/ml). After 90 min scavenging activity was non-significant at dose level 1000µg/ml (65.5±0.69) as compared to control (98.6±0.78), low effectiveness was observed at dose level 10µg/ml (55.0±1.40) and 100µg/ml (60.8±0.57), compared to control (71.5±0.45 and 86.0±0.35) with IC_{50} value 1.08µg/ml, compared to ascorbate (control) with value of IC_{50}=0.68µg/ml (Table 5; Fig 6).

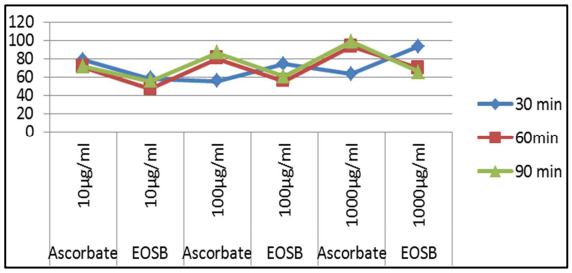

Fig 6. % DPPH inhibition by *Ehretia obtusifolia* stem bark.

Discussion

The curative potency of medicinal plants mainly dependent on presence of secondary bioactive compounds e.g flavonoids, alkaloids, anthraquinone, terpenoids and phenolic compounds. In the present work phytochemical screening was carried out for finding the nature of secondary metabolites in these plants having pharmaceutical potentials for pharmaceuticals exploration and public health benefits (Rai *et al.*, 2013). Alkaloids and saponin are effective against many diseases and gives protection from pathogens (Fluck, 1973; Sodipo *et al.*, 1991). All parts of *Ehretia serrata* and *Ehretia obtusifolia* showed presence of many bio active compounds such as phenol compounds, starch, protein, reducing sugar, steroids, tarpenoids, fats, aldehydes,

anthraquinone, glycosides, amino acids, saponine, alkaloids, tannins and phlobatannin. Due to presence of alkaloids and saponin the plant can be used to cure various diseases. Similarly tannins play a major curative role in treatments of some human disorders. Similar phytochemical work was done by (Patra *et al.* (2009) on leaves of *Hygrophila spinosa*.

Shrivastava & Leelavathi (2010) describe phytochemical screening test of *Catunaregum spinose* which supported present work. Phenolic compounds are considered helpful in curing various disorders . Havsteen, (2002), Thomas *et al.* (2008) and Kumar *et al.* (2011) screened alkaloids, flavonoids, carbohydrate glycosides, tannins, terpeniods, phenol, steroids and saponin from different plant extracts by using multiple biochemical tests. Our present work is in line with these reports.

Presence of High amount of total phenols and total flavonoids in leaf, fruit and stem bark of *Ehretia serrata* and *Ehretia obtusifolia* might be related to anti-oxidant property of all experimented parts. According to some scientist extraction of biochemical active constituents are important to categorized plants as medicinal plants (Sugumaran & Vetrichelvan, 2008). Various other researchers such as Harborne, (1998) and Kokate, (1994) got similar results by investigated ethanolic extract of root and leaf of different medicinal plants for detecting these phytochemicals using standard methods. Many other workers e.g Bharathamma & Sudarsanam, (2015); Goswami, (2015); Singh *et al.* (2015); Acharya *et al.* (2012); Singh, (2012) and Hakemi *et al.* (2012) carried out similar supported the importance of qualitative phytochemical screening of crude drugs, which help in field of phytochemistry and pharmacology of medicinal plants. Thomas *et al.*, (2008) proved the relationship of bioactive compounds with many pharmaceutical during the study of fruit of *Averrhoa carambola*. Qualitative and quantitative phytochemical screening results revealed the scavenging effectivity of both plants strongly due to the presence of high amount of these compounds. Many disorders like neurodegenerative diseases, cancer and AIDS are due to involvement of free radicals.

Antioxidants through their scavenging power are useful for the management of those diseases. DPPH stable free radical method is a facile, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts. (Koleva *et al.*, 2002; Roleira *et al.*, 2015). The highest scavenging activity of *Ehretia serrata* was seen after 30min based on IC_{50}, leaf (IC_{50}=0.12µg/ml,), fruit (IC_{50}= 3.37µg/ml) and in stem bark after 60min (IC_{50}= 0.34µg/ml) could be due to the presence of flavonoids and tannins, further proved by Sobrinho *et al.*, 2016 who studied anti-oxidant activity of the essential oil from *Eupatorium ballotifolium* Kunth (Asteraceae) and give the same reason.

These results might be correlated with presence of certain volatile compounds and phenols which having high antioxidant properties (Sacchetti *et al.*, 2005). The highest anti-oxidant activity showed by different parts e.g leaf (IC_{50}=2.5µg/ml), fruit (IC_{50}=0.01µg/ml) and stem bark (IC_{50}=0.03µg/ml) of Ehretia obtusifolia after 30min might be correlated with the presence of phenols and flavonoids, which are mostly of the plant origin (Pietta, 2000; Kolemva *et al.*, 2002; Aguiar *et al.*, 2015). Plant secondary metabolites are considered as important resources for biological applications such as anti-oxidant activities, analgesic and anti-bacterial activities and their identification may contribute in the potential applications on several areas of science (Silva *et al.*, 2016). Ahmad *et al.* (2011) carried out scavenging activity of *Euphorbia prostrata*. By increasing concentration of extract the anti-oxidant activity increased which support the present work in which plants parts showed highest ($p>0.01$) scavenging activity at higher dose level**.**

Conclusion

It is concluded from the above results that leaf, fruit, and stem bark of both *Ehretia serrata* and *Ehretia obtusifolia* have important bio-active and anti-oxidants compounds, which shows their importance in pharmaceutical industry, and both plants are recommended for furfure research and drug formation against free radicals.

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