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Comparative Study of Antioxidant Activity from some Egyptian Plants and Phytochemical Composition

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ABSTRACT

This study evaluated antioxidant potential of methanol extract of three Egyptian plants, *Bauhinia alba* (MEBA), *Solanum rantonnetii* (MESR) and *Derris rubosta* (MEDR) and also investigated their main phytoconstituents. The extracts leaves of the of the three plants were subjected to antioxidant assays, namely total antioxidant activity, reducing power, metal chelating, hydrogen peroxide, radical scavenging activities were studied according to *in vitro* methods at the different concentrations (25-100 μg mL^{-1}) and each extract was subjected to phytochemical analysis. The results proved that MEBA had strong inhibition (%) of peroxide levels 84.88±0.83 when compared with the standards, such as BHA, TRO and RUT which have 82.03±1.04, 80.56±0.60, 78.50±0.41, respectively. The MEBA demonstrated effective reducing power, hydrogen peroxide scavenging activity and metal chelating activities. On the other hand, no correlation was found between antioxidant activity and total phenol and flavonoid contents. Chromatographic separation of MEBA proved the identification of betulin, betulinic acid, ursolic acid, kaempferol, isorhamnetin, kaempferol 3-O-α-rhamnoside, isorhamnetin 7-O-α-rhamnoside and rutin. Hence, it was concluded that *Bauhinia alba* has antioxidant activity and can be further employed for medicinal applications.

Key words: Antioxidant activity, *Bauhinia alba*, leaves, flavonoid, triterpenes, free radical scavenging activity

INTRODUCTION

Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (El-Hela and Abdullah, 2010). Antioxidant can be defined as a molecule that is capable of slowing or preventing the oxidation of other molecules. Antioxidants are often reducing agents, such as thiols or polyphenols. They are believed to play an important role in preventing the development of such chronic diseases as cancer, heart disease, stroke, Alzheimer's disease, Rheumatoid arthritis and cataracts (Devare *et al.,* 2012). Natural antioxidants that are present in the food increase the resistance toward oxidative damages and they may have an essential impact on human health. Therefore, consumption of food that contain phytochemical with potential antioxidant properties can decrease the risk of onset of several diseases (Ajaib *et al*., 2011). One of the most targets of medicinal plants are their good therapeutic performance and low

toxicity. In recent years, studies on antioxidant activity of medicinal plants have increased remarkably due to increased interest in their potential of being used as a rich and natural source of antioxidant compounds (Liu and Ng, 2000). *Bauhinia alba* also known white orchid semi-tropical tree, is a tree from *Caesalpiniaceae* family with smooth or slightly ridged grey bark grows in moist rich soil in mild climates. The large fragrant blooms ranges in color from snowy to creamy white. In traditional medicine, *B. alba* was used for treating skin diseases, asthma, diarrhea and it was used as a blood purifier and tonic (Kirtikar and Basu, 1991). Few reports about *B. alba* biological activities and bio-active phytoconstituents, where antioxidant activity of different extracts from the flowers of *B. alba* was determined by Uddin *et al.* (2012). In this study, antioxidant activity and phytochemical contents of methanolic extracts, were investigated. Our findings suggested that MEBA could be used as a possible food supplement and for treatment of some diseases.

MATERIALS AND METHODS

UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240 (NRC, Egypt). ^{1}H-NMR and ^{13}C-NMR (Varian Unity Inova). MS (Finnigan MAT SSQ 7000, 70 ev). (Silica gel (0.063-0.200 mm for column chromatography) and Sephadex LH-20 (Pharmacia Fine Chemicals). Thin Layer Chromatography (TLC) F_{254} plates. Solvent mixtures, BAW (n-butanol: acetic acid: water 4:1:5 upper phase, 15% acetic acid: water: glacial acetic acid: 85:15). Paper Chromatography (PC) Whatman No.1 (Whatman Led. Maid Stone, Kent, England) sheets for qualitative detection of flavonoids and sugars.

Chemicals: Ammonium thiocyanate, ferrous chloride, potassium hexacyanoferrate (III), ferric chloride, sodium carbonate, aluminum chloride, gallic acid and potassium acetate were purchased from E. Merck. Linoleic acid, anhydrous ethanol, 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^{\cdot}$), N,N-dimethyl-p-phenylenediamine (DMPD$^{\cdot+}$), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS$^{\cdot+}$), Folin-Ciocalteu's phenol reagent, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) butylated hydroxyanisole (BHA), hydrogen peroxide, HCl, catechin, rutin hydrate, trolox, trichloraceticacid (TCA) and polyoxyethylene sorbitan monolaurate (Tween-20) were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals were of analytical grade and obtained from either Sigma-Aldrich or Merck.

Plants identification and collection: Leaves of *Bauhinia alba*, *Solanum rantonnetii* and *Derris rubosta* were collected from Al-Zohiriya garden, Giza, Egypt in May, 2012. All the plants were identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC) and by Mrs. Tereeza Labib consultant of plant taxonomy at the ministry of agriculture and director of Orman botanical garden, Giza, Egypt. All voucher specimens were deposited in the herbarium of Al-Zohiriya garden, Giza, Egypt.

Preparation of plants extracts: Air dried leaves of *B. alba* (600 g), *S. rantonnetii* (340 g) and *D. rubosta* (380 g) were extracted with methanol (70%) several times at room temperature by maceration method. *Bauhinia alba*, *S. rantonnetii* and *D. rubosta* extracts were concentrated under reduced pressure to give 30, 24 and 27.5 g, respectively. Each extract was phytochemically screened according to the methods described by Yadav and Agarwala (2011).

General method for acid hydrolysis: The 5 mg of compounds 6, 7 and 8 in 5 mL 10% HCl was heated for 5 h. The aglycones were extracted with ethyl acetate and identified by co-TLC with

authentic standards. The sugars in the aqueous layer were identified by co-paper chromatography (co-PC) with authentic markers on Whatman No. 1 sheets in solvent system (n-BuOH-AcOH-H_2O 4:1:5 upper layer).

Determination of antioxidant properties

Total antioxidant activity: Total antioxidant activities were determined by using ferric thiocyanate (FTC) method (Mitsuda *et al*., 1996). Stock solutions of the extracts and standards were prepared in DMSO (1000 μg mL^{-1}). Then, they were diluted using Phosphate Buffer Solution (PBS) (2.5 mL, 0.04 M, pH 7.0). The extracts and standards (2.5 mL) were added to linoleic acid emulsion (2.5 mL) in PBS (0.04 M, pH 7.0). The linoleic acid emulsion was prepared by homogenizing linoleic acid (15.5 μL), Tween-20 (17.5 mg) as emulsifier and PBS (5.0 mL, 0.04 M, pH 7.0). Linoleic acid emulsion (2.5 mL) and PBS (2.5 mL, 0.04 M, pH 7.0) were used for the control reaction. The reaction mixtures (5.0 mL) were incubated at 37°C in polyethylene flasks. The peroxides which were newly formed during linoleic acid peroxidation should oxidize Fe^{+2} to Fe^{3+}; subsequently cause a complex formation with thiocyanate that has a maximum absorbance at 500 nm (OPTIZEN POP UV/Vis Single Beam Spectrophotometer). The assay steps were repeated every 10 h until reaching a maximum (60 h). Inhibition values were calculated through the following equation:

$$\text{Peroxide inhibition levels (\%)} = [1-(A_s/A_c)]\times 100$$

Here, A_c is the absorbance of control reaction, which contains only linoleic acid emulsion and phosphate buffer and A_s is the absorbance of the extracts or standards.

Ferric Ions (Fe^{3+}) reducing antioxidant power assay (FRAP): The ferric ions reducing powers of the extracts and standards were determined by the colorimetric method (Oyaizu, 1986). The extracts and standards (2.5 mL, 25-100 μg mL^{-1}) were mixed with PBS (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1.0%) and incubated at 50°C during 20 min. Trichloroacetic acid solution (2.5 mL, 10%) was added to obtain the final reagent. Then, 2.5 mL of it was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). The Fe^{3+}/Fe^{2+} transformation was investigated in the presence of the extract or standard and the absorbance values were measured at 700 nm.

$$Fe^{+3}\text{ reducing power (\%)} = (A_s/A_c)\times 100$$

where, A_c is the absorbance of control, A_s is the absorbance of the extracts or standards.

Hydrogen peroxide scavenging activity: The hydrogen peroxide scavenging activity was carried out by the previous procedure described by Ruch *et al.* (1989). In this assay, a solution of H_2O_2 (40 mM) was prepared in PBS (0.1 M, pH 7.4). extract solutions (3.4 mL) (in PBS) were added to H_2O_2 solution (0.6 mL). Absorbance of the reaction mixture was recorded at 230 nm and the results were expressed as inhibition (%). Also, the results were expressed as SC_{50} by linear regression analysis and represent mean of the data:

$$H_2O_2\text{ scavenging activity (\%)} = [1 - (A_s/A_c)]\times 100$$

Here, A_c is the absorbance of the control (contains H_2O_2 in PBS) and A_s is the absorbance in the presence of the extracts or standards.

DPPH free radical scavenging activity: The DPPH radical scavenging activities were studied by following a previous report (Blois, 1958). Serially diluted extracts (3.0 mL) at the different concentrations (25-100 μg mL^{-1}) were added to $DPPH^{\cdot}$ solutions (1.0 mL, 0.2 mM) in ethanol. The mixtures were shaken forcefully and allowed to sit at room temperature for 30 min. Then, absorbance was recorded at 517 nm and the results were expressed as SC_{50} by linear regression analysis using solutions of samples and standards at the different concentrations.

$DMPD^{\cdot+}$ radical scavenging activity assay: The DMPD radical scavenging activity assay was carried out according to the method of Fogliano *et al*. (1999). The $DMPD^{\cdot+}$ solutions (100 mM) was prepared by using deionized water. The 1 mL of this solution was added to acetate buffer (100 mL, 0.1 M, pH 5.25) and the colored radical cation ($DMPD^{\cdot+}$) was obtained by adding 0.2 mL of a of ferric chloride solution (0.05 M). This solution (225 μL) was transferred directly to the tube and absorbance values were measured at 505 nm (absorbance of control tube). The extracts and standards (15 μL) and $DMPD^{\cdot+}$ (210 μL) were added to all tubes. After all tubes were stirred and left to stand for 10 min, a decrease in absorbance was measured at 505 nm using a spectrophotometer. The acetate buffer solution was used as a blank sample. The results were expressed as SC_{50} by linear regression analysis and represent mean of the data.

$ABTS^{\cdot+}$ radical scavenging activity assay: The ABTS radical cation scavenging activity was performed by using a previous method described by Re *et al.* (1999) with slight modification. For this experiment, $ABTS^{\cdot+}$ was generated by the reaction of 2.0 mmol L^{-1} ABTS with 2.45 mmol L^{-1} potassium persulfate. The reaction mixture was allowed to stand in the dark for 16 h at room temperature and used within 2 days. At first, the $ABTS^{\cdot+}$ solution was diluted with a PBS (0.1 M pH 7.4) to give an absorbance of 0.750±0.020 at 734 nm. Then, the diluted $ABTS^{\cdot+}$ solution (1.0 mL) was added to extracts or standards (3.0 mL) solution in PBS. The percentage inhibition of $ABTS^{\cdot+}$ was calculated for each concentration relative to a blank absorbance. The results were expressed as SC_{50} by linear regression analysis and represent mean of the data.

Ferrous metal ions chelating activity: Following the method of Dinis *et al.* (1994), ferrous ion (Fe^{2+}) chelating activity was measured via the inhibition of the Fe^{2+}-ferrozine complex. Firstly, extract and standard solutions (0.4 mL) in ethanol were added to $FeCl_2$ solution (0.1 mL, 2.0 mM). In order to initiate the reaction, ferrozine solution (0.1 mL, 5.0 mM) in ethanol was added. Then, the mixture was shaken vigorously and kept at room temperature during 10 min. The absorbance of the complex was measured with using a spectrophotometer at 562 nm. The percent inhibition of Fe^{2+}-ferrozine complex formation was calculated by using the formula given below:

$$\text{Ferrous ions chelating effect (\%)} = [1-(A_s/A_c)]\times 100$$

where, A_c is the absorbance of the control (contains only $FeCl_2$ and ferrozine), A_s is the absorbance of the extracts and standards.

Determination of total phenolic and flavonoid contents: The total phenolic contents were analyzed by the colorimetric method (Singleton *et al.*, 1999) with minor modifications. 0.5 mL of sample (1000 μg mL^{-1}) was mixed with distilled water (7.0 mL) and subsequently with

Folin-Ciocalteu's phenol reagent (FC reagent) (0.5 mL). After 6 min, Na_2CO_3 solution (2.0 mL, 2.0%) was added into the mixture. The observed color absorbance was measured by a spectrophotometer at 760 nm after 90 min. The total phenolic contents were expressed as microgram of gallic acid equivalent by using an equation that was obtained from standard gallic acid graph (0-400 μg mL^{-1}) (R^2 = 0.9998).

The total flavonoid contents were determined by using a modified colorimetric method described previously (Dewanto *et al*., 2002). The 1.0 mL of the extract was added to test tubes containing DMSO (5.0 mL) and subsequently $NaNO_2$ solution (1.0 mL, 5.0%) was added to each of them. After 6 min, $AlCl_3$ solution (1.0 mL, 10%) was added to the tubes and the mixtures were allowed to stand for 5 min before further addition of NaOH solution (2.0 mL, 1.0 M). The absorbance values were read at 510 nm. Total flavonoid contents were expressed as microgram of catechin equivalent that was obtained from standard graph (0-200 μg mL^{-1}) (R^2 = 0.9977).

Isolation of the components of methanol extract of *Bauhinia alba*: Methanol extract of *Bauhinia alba* leaves (30 g) was subjected to silica gel column chromatography eluting with n-hexane, dichloromethane, ethyl acetate and methanol gradually. One hundred and fifty fractions of 100 mL conical flask were collected. The fractions that showed similar Paper Chromatography (PC) in two solvent systems Butanol-acetic Acid-Water (BAW) 4:1:5 and 15% acetic acid were combined to give three main fractions.

Fraction 1 (5.28 g) was subjected to sub-column of silica gel eluted with n-hexane: Dichloromethane where elution with n-hexane-dichloromethane (60:40) yielded compound 1 and further elution with n-hexane-dichloromethane (80:20) gave compound 2 while elution with n-hexane-dichloromethane (90:10) afforded compound 3.

Fraction 2 (4.35 g) was subjected to sub-column of silica gel eluted with dichloromethane: Ethyl acetate, Elution with dichloromethane; ethyl acetate (80:20) gave compound 4 and further elution with ethyl acetate yielded compound 5.

Fraction 3 (7.45 g) was subjected to sub-column of silica gel eluted with dichloromethane: Methanol, Elution with dichloromethane; methanol (80:20) afforded compound 6 and further elution with dichloromethane, methanol (60:40) gave compound 7 and further elution with methanol gave compound 8. The isolated compounds were purified on sephadex LH-20 column using methanol, ethyl alcohol and different systems of methanol and distilled water.

Statistical analysis: The data was analyzed using the statistical software program SPSS 15.0 for Windows. All results were given as Mean±Standard Deviation (SD) of triplicates. The differences at $p<0.05$ were "considered as significant".

RESULTS AND DISCUSSION

This study evaluated antioxidant activity of the methanolic extracts of *B. alba*, *S. rantonnetii* and *D. rubosta* and phytochemical analysis was performed. Methanol extract of *B. alba* (MEBA) was the active one as antioxidant agent (Table 1 and 2) and (Fig. 1, 2 and 3). Phytochemical analysis of the three extracts showed that MEBA contained triterpenes, flavonoids, tannins, carbohydrates, alkaloids and coumarins. The MESR had triterpenes, flavonoids, tannins

and carbohydrates, while MEDR provided the presence of triterpenes, tannins, flavonoids and carbohydrates (Table 3). Chromatographic separation of MEBA revealed the identification of

Table 1: SC_{50} values (μg mL^{-1}) of the extracts and standards on the $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and $DMPD^{\cdot+}$ radicals

Parameters	MEBA	MESR	MEDR	BHA	RUT	TRO
$DPPH^{\cdot}$	74.32±0.59	162.87±1.11	138.04±0.96	8.70±0.09	17.74±0.14	27.03±0.20
$DMPD^{\cdot+}$	29.73±0.48	89.73±1.02	130.37±1.63	14.62±0.43	11.27±0.19	28.49±0.20
$ABTS^{\cdot+}$	25.29±0.61	239.12±1.34	294.80±1.59	8.63±0.29	17.68±0.28	4.41±0.06

MEBA: Methanol Extract of *Bauhinia alba*, MESR: Methanol Extract of *Solanum rantonnetii*, MEDR: Methanol Extract of *Derris robusta*, BHA (butylated hydroxyanisole), RUT: rutin, TRO: trolox

Table 2: Total phenol (TPC) and flavonoid (TFC) of the plant extracts

Parameters	MEBA	MESR	MEDR
TPC[a]	164.88±1.06	159.17±1.40	80.58±1.08
TFC[b]	3.92±0.22	62.29±1.28	22.21±0.99

MEBA: Methanol Extract of *Bauhinia alba*, MESR: Methanol Extract of *Solanum rantonnetii*, MEDR: Methanol Extract of *Derris robusta*. [a]μg gallic acid equivalent mg^{-1}, [b]μg catechin equivalent mg^{-1}

Table 3: Phytochemical analysis of the three extracts

Phytoconstituents	MEBA	MESR	MEDR
Triterpenes and/or sterols	+	+	+
Carbohydrates and/or glycosides	+	+	+
Flavonoids	+	+	+
Coumarins	+	-	-
Alkaloids and/or nitrogenous compounds	+	-	-
Tannins	+	+	+
Saponins	-	-	-

(+) Presence of the constituents, (-) the absence of the constituents MEBA: Methanol Extract of *Bauhinia alba*, MESR: Methanol Extract of *Solanum rantonnetii*, MEDR: Methanol Extract of Derris robusta

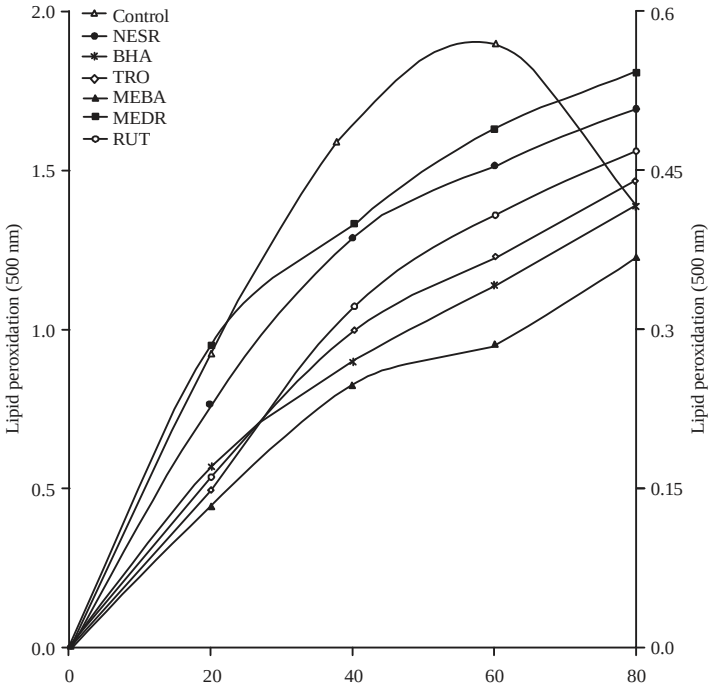

Fig. 1: Total antioxidant activities of plant extracts and standards in the linoleic acid emulsion system at the same concentration (100 μg mL^{-1}). MEBA: Methanol Extract of *Bauhinia alba*, MESR: Methanol Extract of *Solanum rantonnetii*, MEDR: Methanol Extract of *Derris robusta*, BHA (butylated hydroxyanisole), RUT: Rutin, TRO: Trolox. Note: Left axis for control and right axis for samples and standards

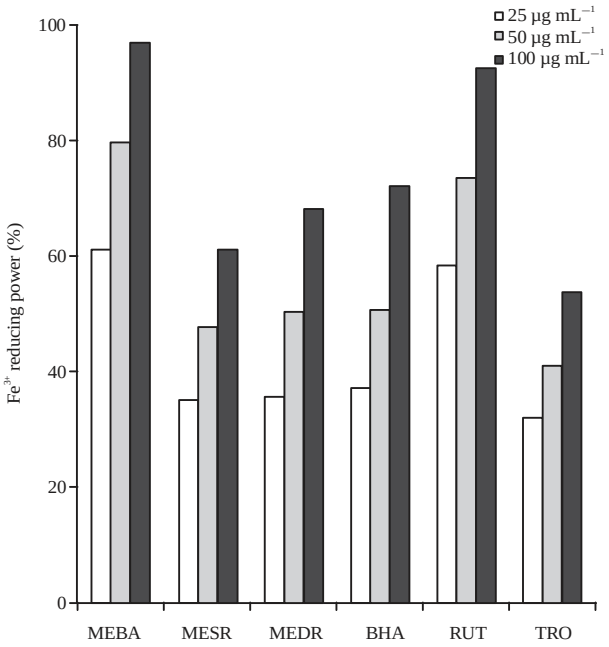

Fig. 2: Ferrous ions reducing powers (%) of plant extracts and standards at the different concentrations. MEBA: Methanol Extract of *Bauhinia alba*, MESR: Methanol Extract of *Solanum rantonnetii*, MEDR: Methanol Extract of *Derris robusta*, BHA (butylated hydroxyanisole), RUT: Rutin, TRO: Trolox

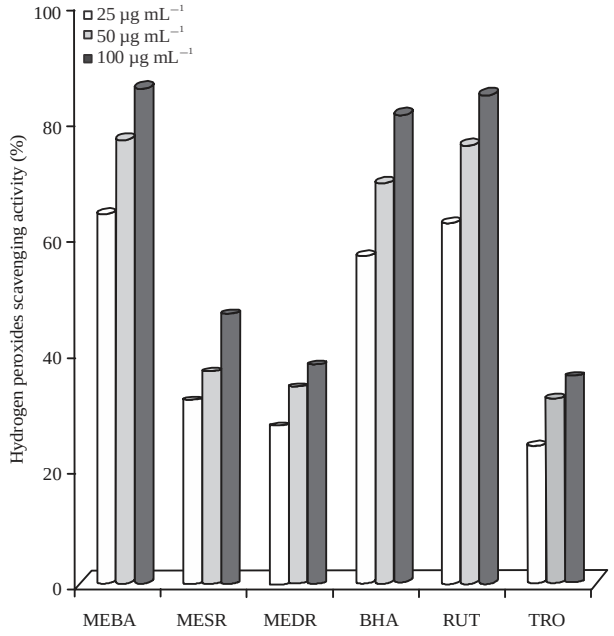

Fig. 3: Hydrogen peroxide scavenging activities (%) of plant extracts and standards at different concentrations. MEBA: Methanol Extract of *Bauhinia alba*, MESR: Methanol Extract of *Solanum rantonnetii*, MEDR: Methanol Extract of *Derris robusta*, BHA (butylated hydroxyanisole), RUT: Rutin, TRO: Trolox

betulin, betulinic acid, ursolic acid, kaempferol, isorhamnetin, kaempferol 3-O-α-rhamnoside, isorhamnetin 7-O-α-rhamnoside and rutin.

Total antioxidant activity: The total antioxidant activity of the extracts was determined by ferric thiocyanate (FTC) method. Peroxide level, which is produced because linoleic acid gets oxidized by air during the experiment, is measured indirectly. Released peroxides cause oxidation of Fe^{+2} ions to Fe^{+3}, which latter forms a complex with SCN^- that has a maximum absorbance at 500 nm (Inatani *et al.,* 1983). In the presence of antioxidants, Fe^{3+} ion formation becomes more difficult and therefore, the absorbance of SCN^- complex is expected to be lower. The results of antioxidant activity assays of the extracts after 60 h incubation with linoleic acid emulsion were summarized in Fig. 1. The oxidation of linoleic acid inhibited by the extracts compared to the controls. At 100 μg mL^{-1}, the inhibition (%) the extracts and standards in order of MEBA (84.88±0.83)>BHA (82.03±1.04)>TRO (80.56±0.60)>RUT (78.50±0.41)>MESR (76.03±0.08)>MEDR (74.18±0.72) ($p<0.05$).

Fe^{3+} reducing power capacity: The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. In this assay depending on the reducing power of antioxidants, the yellow color of the test solution changes to various shades of green and blue owing to formation of Perl's Prussian Blue ($Fe_4[Fe(CN)_6]_3$), which has a maximum absorbance at 700 nm (Chung *et al*., 2002; Guder and Korkmaz, 2012a). Figure 2 shows the reducing capability of the extracts at the different concentrations compared to BHA, rutin and trolox. The reducing power of the extracts increased with increasing amount of the extract. Even though, MEBA has the highest reductive activity at the same concentration (100 μg mL^{-1}), all extracts demonstrated more efficient reducing power than trolox ($p<0.05$). The reducing capacity (%) decreased in the following order: MEBA (96.88±0.28)>RUT (92.78±0.92)>BHA (72.05±0.02)>MEDR (68.21±0.90)>MESR (61.07±0.11) >TRO (53.89±0.57).

Hydrogen peroxide scavenging activity: Hydrogen peroxide itself is not very reactive but sometimes, it can be toxic to cells by producing hydroxyl radicals ($^{\cdot}OH$). The H_2O_2 can cross membranes and slowly oxidize a number of biomolecules and compounds (Halliwell, 1991). Addition of H_2O_2 to cells in culture can lead to formation of transition metal ion-dependent hydroxyl radical, mediating oxidative DNA damage. Therefore, removing hydrogen peroxide as well as superoxide anion radical is very important for protecting pharmaceuticals and food systems (Gulcin, 2010). The H_2O_2 scavenging abilities of the extracts were shown in Fig. 3 in comparison with standards ($p<0.05$). SC_{50} (μg mL^{-1}) values of the extracts and standards decreased in the order of MEBA (9.38±0.08)<RUT (10.79±0.32)<BHA (18.47±0.68)<MESR (116.85±0.64)<MEDR (189.06±1.20)<TRO (192.96±0.73).

Free radical scavenging activity: Antioxidant properties, especially radical-scavenging activity, are very important due to the deleterious role of free radicals in foods and in biological systems. Excessive formation of free radicals accelerates the oxidation of lipids in foods and decreases food quality and consumer acceptance (Akoh and Min, 2002). As a chemical $DPPH^{\cdot}$, $DMPD^{\bullet+}$ and $ABTS^{\cdot+}$ are very important stable radicals for determination of radical scavenging activities of natural and synthetic materials. For this reason, we determined the radical scavenging activity (Ancerewicz *et al.,* 1998). Table 1 showed that, the SC_{50} values (μg mL^{-1}) of the extracts and standards on the $DPPH^{\cdot}$ increased in that order: The BHA (8.70±0.09)>RUT (17.74±0.14)>TRO (27.03±0.20)>MEBA (74.32±0.59)>MEDR (138.04±0.96)>MESR (162.87±1.11)

($p<0.05$). In a similar way, MEBA has an effective $DMPD^{\cdot+}$ radical scavenger as well as standards (Table 1). The SC_{50} values (μg mL^{-1}) of standards were found as 11.27±0.19, 14.62±0.43 and 28.49±0.20 for RUT, BHA and trolox, respectively ($p<0.05$). As is seen in Table 1, $ABTS^{\cdot+}$ radical scavenging activities of the extracts and standards were determined as TRO (4.41±0.06)>BHA (8.63±0.29)>RUT (17.68±0.28) >MEBA (25.29±0.61)>MESR (239.12±1.34)>MEDR (294.80±1.59) ($p<0.05$).

Ferrous ion chelating activity: Ferrous ion is known as the most important lipid pro-oxidant due to its high reactivity among other transition metal ions. For example, ferrous ion (Fe^{2+}) chelation is one of the most important antioxidant effects by retarding metal-catalyzed oxidation. The effective ferrous ion chelators may also provide protection against oxidative damage to cells (Halliwell and Gutteridge, 1984). Ferrozine can quantitatively form from complexes with Fe^{2+} in this method. Since, the intensity of the red color of this complex decreases in the presence of other chelating agents, one can assume that the complex formation is disrupted as a result of chelating. Therefore, monitoring the intensity of the color allowed us to estimate the metal chelating activity of the coexisting chelators (Guder and Korkmaz, 2012b). In this assay, the extracts containing Fe^{2+}-ferrozine complex were treated with the extracts which have chelating activity and therefore capable of capturing ferrous ion from this complex. The formation of the Fe^{2+}-ferrozine complex is not complete in the presence of the extracts, indicating all of the extracts chelate with the Fe^{+2}. At the 50 μg mL^{-1}, the metal chelating activity of all extracts and standards decreased ($p>0.05$) in the following order, MEBA (83.11±0.86)>MESR (79.22±1.27)>MEDR (77.61±0.13)>RUT (76.81±0.29) >BHA (74.66±0.02)>TRO (62.73±1.05).

Total phenolic content: Phenolic compounds have antioxidant properties due to their ability of scavenging free radicals and active oxygen species such as singlet oxygen (Hall and Cuppett, 1997). According to results obtained (Table 2), the gallic acid equivalents of total phenolic contents of extracts ranged from 73.44±0.94 to 164.88±1.06 μg in 1 mg of Dried Extracts (DE) ($p>0.05$). These phenolic compounds may directly contribute to the antioxidative activity. On the other hand, some researchers found no such relationship between antioxidant activity and total phenolic content since other compounds can be responsible for the antioxidant activity (Yang *et al.,* 2002). Likewise, our findings also suggest that there is no correlation between antioxidant activity and total phenolic contents. Flavonoids are not only responsible for giving fruits and vegetables various red, blue, or violet colors but also are related to a group of bioactive compounds called stilbenes (Hui, 2006). Flavonoids are very important plant constituents because of having active hydroxyl groups and their antioxidant activity (Kumar *et al.,* 2008). In this work, the total flavonoid contents were expressed as μg of catechin equivalents/mg of the DE. As shown in Table 2, the highest flavonoid contents of 62.29±1.28 μg of catechin equivalents/mg of dried extracts was observed in the MESR and the lowest flavonoid contents was observed in the MEBA i.e., 3.92±0.22 μg of catechin equivalents/mg of dried extracts.

Characterization of the compounds from methanol extract of *B. alba*

Structure elucidation of the isolated compounds from MEBA

Betulin (1): The 24 mg, white powder. ^1H NMR ($CDCl_3$, 500 MHz) δ 4.75 (1H, d, 1.4 Hz, H-29b), 4.62 (1H,d, 1.4 Hz, H-29a), 3.58 (1H, d, J =11, H-28b), 3.82 (1H, d, J = 11, H-28a), 3.16 (1H, dd, J = 15.8, 5.4, H-3), 1.69 (3H, s, H-30), 0.98 (3H, s, H-27), 0.97 (3H, s, H-26), 0.94 (3H, s, H-23),

0.82 (3H, s, H-25), 0.74 (3H, s, H-24). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 150.64 (C-20), 109.86 (C-29), 78.9 (C-3), 60.65 (C-28), 56.24 (C-5), 50.58 (C-9), 49.45 (C-19), 48.28 (C-17), 47.94 (C-18), 43.6 (C-14), 41.28 (C-8), 38.96 (C-1), 38.88 (C-4), 37.44 (C-10), 37.27 (C-13), 35.26 (C-7), 34.18 (C-22), 29.85 (C-21), 29.25 (C-16), 28.18 (C-23), 27.54 (C-2), 27.12 (C-15) 25.34 (C-12), 20.96 (C-11), 19.27 (C-30), 18.46 (C-6), 16.24 (C-25), 16.12 (C-26), 15.48 (C-24), 14.86 (C-27). EI-MS: m/z 442. It was monitored by TLC and detected by heating the plate at 110°C after spraying with p-anisaldehyde-sulfuric acid. The spectral data was in agreement with literature (Tijjani *et al*., 2012).

Betulinic acid (2): The 24 mg, white needles. ^{1}H NMR ($CDCl_3$, 500 MHz): 4.78 (1H, d, J = 1.7 Hz, H-29b); 4.64 (1H,d, J = 1.7 Hz, H-29a), 3.24 (1H, dd, J = 16.2, 5.4 Hz, H-3), 2.95 (1H, ddd, J = 16.8, 10.8, 6.2 Hz, H-19b), 2.26 (1H, m, H-19a), 1.94 (1H, m, H-21b), 1.72 (3H, s, H-30), 1.42 (1H, m, H-21a), 0.96 (3H, s, H-27), 0.94 (3H, s, H-26), 0.92 (3H, s, H-23), 0.84 (3H, s, H-25), 0.76 (3H, s, H-24), 0.66 (1H, d, J = 9.2 Hz, H-5). ^{13}C-NMR ($CDCl_3$, 100 MHz,): δ ppm 180.58 (C-28), 109.72 (C-29), 150.34 (C-20), 79.28 (C-3), 56.34 (C-17), 55.84 (C-5), 50.54 (C-9), 49.28 (C-19), 46.86 (C-18), 42.74 (C-14), 40.78 (C-8), 39.46 (C-4), 38.74 (C-1), 38.48 (C-13), 37.28 (C-22), 37.25 (C-10), 34.64 (C-7), 32.18 (C-16), 30.52 (C-15), 29.78 (C-21), 27.95 (C-23), 27.48 (C-2), 25.54 (C-12), 20.82 (C-11), 19.54 (C-30), 18.34 (C-6), 16.14 (C-26), 15.98 (C-25), 15.34 (C-24), 14.78 (C-27). EI-MS m/z: 456. It was monitored by TLC and detected by heating the plate at 110°C after spraying with p-anisaldehyde-sulfuric acid. The spectral data was in agreement with literature (Baek *et al*., 2010).

Ursolic acid (3): The 18 mg, White powder. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 5.42 (1H, t, J = 3.8 Hz, H-12), 3.28 (1H, dd, J = 10.4, 4.5 Hz, H-3), 2.14 (1H, d, J =11.5 Hz, H-18), 1.16 (1H, m, Ha-22), 2.24 (1H, dd, J = 13.2, 4.2 Hz, Hb-22), 1.28 (3H, s, Me-23), 1.24 (3H, s, Me-27), 1.18 (3H, s, Me-26), 0.98 (3H, s, Me-24), 0.94 (3H, d, J = 6.5 Hz, Me-29), 0.92 (3H, d, J = 6 Hz, Me-30), 0.79 (3H, s, Me-25). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 180.48 (C-28), 138.82 (C-13), 125.82 (C-12), 78.58 (C-3), 55.64 (C-5), 53.28 (C-18), 47.86 (C-9), 47.78 (C-17), 41.84 (C-14), 39.64 (C-8), 39.28 (C-20), 39.24 (C-1), 39.22 (C-19), 38.94 (C-4), 36.98 (C-22), 36.86 (C-10), 33.26 (C-7), 30.48 (C-21), 29.78 (C-15), 28.26 (C-23), 27.54 (C-2), 24.24 (C-16), 23.16 (C-27), 22.94 (C-29), 22.86 (C-30), 18.36 (C-6), 16.64 (C-11), 16.46 (C-26), 15.28 (C-24), 14.82 (C-25). EI-MS: m/z 456. It was monitored by TLC and detected by heating the plate at 110°C after spraying with p-anisaldehyde-sulfuric acid. The spectral data was in agreement with literature (Seebacher *et al*., 2003).

Kaempferol (4): The 8 mg, yellow powder. UV λmax (nm) (MeOH): 268, 294 sh, 368; (NaOMe): 276, 322 sh, 412; ($AlCl_3$): 270, 306, 352, 424; ($AlCl_3$/HCl): 268, 306, 352, 425; (NaOAc): 275, 306, 380; (NaOAc/H_3BO_3): 267, 296, 320, 369. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 8.12 (2H, d, J = 8 Hz, H-2', 6'), 6.96 (2H, d, J = 8 Hz, H-3', 5'), 6.47 (1H, d, J = 2 Hz, H-8), 6.19 (1H, d, J= 2 Hz, H-6). It gave a yellow colour under UV light. On exposure to ammonia or spraying with $AlCl_3$ reagent, respectively, it afforded florescent yellowish green colour. Its spectral data was in agreement with kaempferol (Said *et al*., 2010).

Isorhamnetin (5): The 12 mg, yellow powder. UV λmax (nm): (MeOH): 268, 295, 336, 378 (NaOMe):284, 324, 442, ($AlCl_3$): 265, 300, 426, ($AlCl_3$/HCl): 264, 300, 350sh,428 (NaOAc): 278, 334, 388, (NaOAc/H_3BO_3): 258, 269, 298, 372. EI-MS: m/z 316.

It was isolated as a yellow spot under UV light and on exposure to ammonia or spraying with $AlCl_3$ reagent, respectively, it gave a lemon yellow colour. Its spectral signals proved the structure of isorhamnetin (Amani *et al*., 2006).

Kaempferol 3-O-α-rhamnoside (6): The 15 mg, yellow powder. ^{1}H-NMR (CD_3OD, 400 MHz): δ 7.78 (2H,d, J = 8.2 Hz, H-2',6,). 6.94 (2H,d, J = 8.2 Hz, H-3',5'). 6.44 (1H, d, J = 2.2 Hz, H-8), 6.26 (1H, d, J = 2.2 Hz, H-6). 5.36 (1H, d, J = 2.2 Hz, H-1''), 0.92 (CH_3, d, J =6.2 Hz), 3.1-4 (rest of sugars protons). ^{13}C-NMR (CD_3OD, 100 MHz): δ ppm 179.85 (C-4), 166.26 (C-7), 161.82 (C-5), 159.58 (C-4'), 158.24 (C-2), 136.46 (C-9), 132.26 (C-3),122.94 (C-6'), 116.82 (C-2'), 116.26 (C-3'), 106.15 (C-1'), 103.78 (C-5'), 104.75 (C-10) , 100.14 (C-1''), 95.16 (C-8), 94.98 (C-6), 73.28 (C-5''), 72.46 (C-3''), 72.32 (C-2''), 72.26 (C-4''), 17.94 (CH_3-rhamnosyl).

It was isolated as a deep purple spot under UV light and on exposure to ammonia or spraying with $AlCl_3$ reagent, respectively, it gave a florescent yellow colour, acidic hydrolysis of afforded kaempferol aglycone and rhamnose sugar and NMR spectral data was in accordance with those of kaempferol 3-O-α-rhamnoside (Granja-Perez *et al*., 1999).

Isorhamnetin 7-O-α-rhamnoside (7): The 18 mg, yellow crystals. UV λmax (MeOH): 270, 298, 318, 364, (NaOMe): 272, 298, 358, 424, ($AlCl_3$): 269, 300, 322, 422 ($AlCl_3$/HCl): 269, 298, 324, 422, (NaOAc): 269, 372, (NaOAc/H_3BO_3): 268, 364. ^{1}H-NMR (DMSO-d_6, 300 MHz,) δ ppm 7.82 (1H, d, J=2.2 Hz, H–2`), 7.68 (1H, dd, J = 8.5, 2.2 Hz,, H-6`), 6.94 (1H, d, J = 8.5 Hz, H-5'), 6.78 (1H, d, J = 2.2 Hz, H-8), 6.42 (1H, d, J = 2.2 Hz, H-6), 5.54 (1H, d, J = 2.2, H–1''), 3.78 (3H, s, OCH_3), 1.12 (3H, d, J = 6.2 Hz, CH_3-rhamnose), 3.15-4 (rest of sugar protons). This compound afforded a yellow spot under UV light and changed to lemon yellow when subjected to ammonia and $AlCl_3$ and complete acid hydrolysis yielded isorhamnetin as an aglycone and rhamnose as sugar moiety and its spectral data was very similar to that described by Manguro *et al*. (2005).

Quercetin 3-O-rutinoside, (Rutin) (8) (Fig. 4): The 20 mg, yellow powder: UV λmax (MeOH): 258, 269, 361; (NaOMe): 276, 322, 416; ($AlCl_3$): 232, 276, 302, 366; ($AlCl_3$/HCl): 232, 276, 302, 366; (NaOAc): 284, 306, 381; (NaOAc/H_3BO_3): 261, 312, 376. ^{1}H-NMR (400 MHz, DMSO-d6): δ ppm 7.54 (2H, m, H-2', 6'), 6.85 (1H, d, J = 9 Hz, H-5'), 6.38 (1H, d, J = 2.5 Hz, H-8), 6.19 (1H, J = 2.5 Hz,H-6), 5.35 (1H, d, J = 7.5 Hz, H-1''), 4.39 (1H, s, H-1'''), 3.90-3.20 (m, remaining sugar protons),

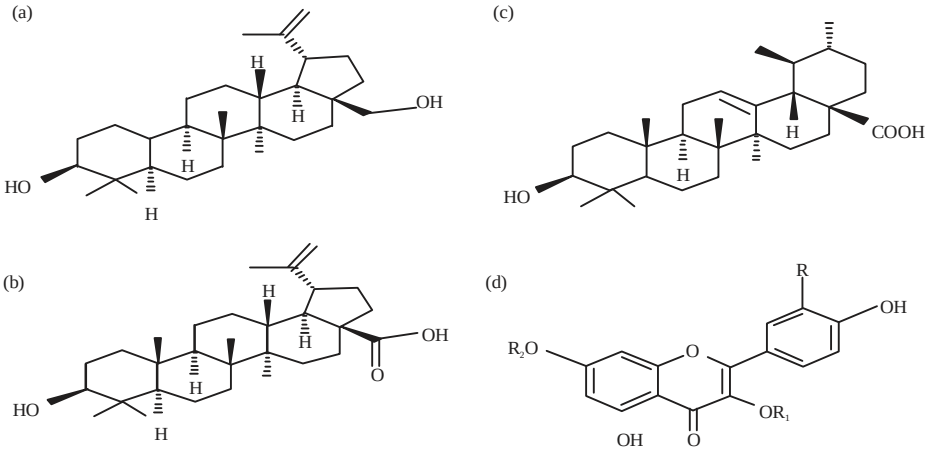

Fig. 4(a-d): Chemical structures of the compounds isolated from *Bauhinia alba* leaves methanol extract, (a) Betulin, (b) Betulinic acid, (c) Ursolic acid and (d) 4: Kaempferol (R, R1, R2 = H), Isorhamnetin (R = OCH_3, R1, R2 = H), Kaempferol 3-O-α-rhamnoside (R, R2 = H, R1 = rhamnose), Isorhamnetin 7-O-α-rhamnoside ((R=OCH_3, R1=H, R2 = rhamnose), Rutin (R = OH, R1=rutinose, R2 = H)

0.99 (3H, d, J = 6 Hz, H-6'''). ^{13}C NMR (DMSO-d_6, 100 MHz): δ ppm 177.85 (C-4), 164.70 (C-7), 161.68 (C-5), 157.14 (C-2), 156.95 (C-9), 148.92 (C-4'), 145.25 (C-3'), 133.76 (C-3), 122.12 (C-6'), 121.66 (C-1'), 116.73 (C-2'), 115.72 (C-5'), 104.41 (C-10), 101.66 (C-1'''), 101.23 (C-1''), 99.24 (C-6), 94.16 (C-8), 74.58 (C-3''), 72.33 (C-5''), 72.2 (C-4'''), 71.05 (C-2''), 70.8 (C-2'''), 70.87 (C-3'''), 70.49 (C-4''), 63.74 (C-6''), 18.19 (C-6'''). This compound yielded a deep purple spot under UV light and changed to yellow when subjected to ammonia and $AlCl_3$ and complete acid hydrolysis gave quercetin as an aglycone and glucose and rhamnose as sugar moieties and its spectral data was very similar to that described by Sintayehu *et al*. (2012). All the chemical structures of the isolated compounds are shown in Fig. 3. The isolated compounds from MEBA proved antioxidant activity. Betulinic acid, which is a naturally occurring pentacyclic triterpenoid exhibited a variety of biological activities including inhibition of Human Immunodeficiency Virus (HIV), antibacterial, antimalarial, anti-inflammatory, anthelmintic and antioxidant properties (Yogeeswari and Sriram, 2005). Ursolic acid isolated from ethanol extract of aerial parts of *Sambucus australis* showed significant antioxidant activity by inhibiting DPPH. Ursolic acid strongly scavenged DPPH radical, with IC_{50} value of $5.97\times10^{-2}\pm1\times10^{-3}$ mg mL^{-1} (Do Nascimento *et al*., 2014). Kaempferol isolated EtOAc soluble fraction of *Sonchus oleraceus* exhibited stronger antioxidant activity (13 μg mL^{-1}), comparable to α-tocopherol and curcumin, which were used as positive controls (Yin *et al*., 2008). The antioxidant properties of the pure isorhamnetin were evaluated by the scavenging of the diphenylpicrylhydrazyl radical (DPPH), iron (III) to iron (II)-reducing and iron-chelating assays. Antioxidant assays showed that isorhamnetin performed significantly compared with ascorbic acid and BHT and the linear correlations were good in these assays. Isorhamnetin may have potential as a natural antioxidant and can be used as food additive due to its antioxidant activity (Pengfei *et al*., 2009). The antioxidant activity of rutin was measured against stable, non-biological radicals such as 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) using a spectrophotometric assay, the results prove a potential capacity of rutin as antioxidant agent (Zielinska *et al*., 2010).

CONCLUSION

Based on our results, MEBA could be used as the antioxidant agent by inhibiting the peroxide levels and the scavenging the hydrogen peroxide and free radical. MEBA extract has very significant chelating activity on ferrous ions and this can be explained by the presence of the isolated bio-active compounds, therefore it can be used as natural therapeutic food for several disorders. On the other hand, MESR and MEDR have higher total phenol and flavonoid contents, so they can be used as a possible supplement in food, pharmaceutical and medical industry.

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