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In vitro Assessment of Antioxidant Activity of *Anisomeles indica*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MA and MM designed the study and wrote the protocol. Authors KMK and SP supervised the work. Authors MU and MAI carried out all laboratories work and performed the statistical analysis. Author SS managed the analyses of the study and wrote the first draft of the manuscript. Author US managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The present research carried out to estimate the antioxidant activity of *Anisomeles indica* (L.) O. Kuntze leaves by employing some *in vitro* methods *i.e.* free radical scavenging activity, reducing ability and chelating ability. The leaves extract showed an excellent activity in all three mechanisms. TEAC (Trolox equivalent antioxidant capacity) values of *A. indica* range from 11.17 ±

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0.239 to 0.75 ± 0.658 mMol. While the FRAP (ferric reducing antioxidant power) value and % age bound value ranges from 118.4 ± 0.495 to 14.44 ± 0.339 mM and 72.91 ± 0.770 to 56.61 ± 0.843 % respectively. The major phyto-constituents *i.e*. total phenolic and flavonoid contents ranges from 1145.5 ± 0.593 to 198.5 ± 0.395 mg/L of GAE and 3123.7 ± 0.395 to 1154.5 ± 0.376 mg/L of Quercetin equivalent. The results divulge that leaves of *A. indica* are potential source of natural antioxidants showing metal chelating, reducing and free radical scavenging abilities.

Keywords: Antioxidants; ABTS; flavonoids; total phenolic contents; reducing ability.

1. INTRODUCTION

Antioxidant research is an important and interesting topic in the field of medicine as well as in the food industry. In recent years research on important biologically active compounds in plants and herbs has proved the use of naturally occurring antioxidants more reliable than the synthetic compounds. Plants are basic source of natural antioxidants [1]. These are plentiful in fruit and vegetables and other foods involving nuts, grains and meats. Beta-carotene is used mostly in foods of orange color like potatoes, carrots, apricots, pumpkin, squash, and mangoes. It is also very rich in some green leafy vegetables like spinach, collard greens, and kale [2].

These are involved in chain breaking reactions which combine with lipid radicals and change them into stable products. Natural antioxidants are mostly phenolic and comprise the followings [3]. Fundamentally two standard reactions have been possible for antioxidants. Firstly the chain- breaking reaction during which the naturally antioxidants transfer electrons to the free radicals in the system, like radicals of lipid. While the other reaction involved elimination of ROS and RNS initiator by initiator catalyst [4].

Plants possess various antioxidant constituents having the ability to neutralize free radicals as well their vicious effects. The antioxidants play its role by employing its capacity to scavenge reactive species i.e. superoxide, hydroxyl, peroxyl radical or by reducing or chelating mechanisms [5]. Such chemical compounds that can hinder the oxidation of lipids or other molecules and also check the damages of the body's cells done by oxygen. They perform the following mechanisms: reducing action, free radical-scavenging, complexing of pro-oxidant metals and quenching of singlet oxygen. Modern biological researches have clearly showing that numerous phyto-nutrients of fruit and vegetables save the human body against smash up by ROS. The use of synthetic antioxidants like phyto-chemicals was reported to have realistic health benefits [6]. The medicinal plants have been broadly used for the healing of many degenerative diseases in the Sub-continent. Such herbal material is used in food enrichment as well as unambiguously the cure of several diseases [7].

The use of natural antioxidants has been forestalling because of their harmful effects. That's why research is again turned towards the field of bioresources to find more natural and probably economic and helpful antioxidants to change the artificial ones [8].

Anisomeles indica belongs to the family *Lamiaceae*. The morphological characteristics of leaves of *A. indica* are acute apex, crenate margin, asymmetric base, reticulate venation and hairy to soft pubescent shape. The color of leaves is green to yellowish green and taste is slightly astringent with characteristics odor [9]. *A. indica* has been widely used to treat dysfunction, hypertension, anti-oxidant and anti-inflammatory in various countries as medicine. It has carminative, astringent and toxic properties [10]. It is used in traditional medicinal formulations.

A. indica has been widely used to treat dysfunction, hypertension and anti-inflammatory. Ethnobotanically the plant material (fresh or dried) can be used as washing purpose for external exflictions, eczema, snakebites and skin problems. The leaves are chewed for toothaches. It is also used in cold, fever, intermittent fever, abdominal pain dyspepsia. When the plant is burned, it acts as mosquito repellent. Its aerial parts are also used as an analgesic [11].

In this study, we determine the antioxidant potential of leaves of *A. indica* by mainly focusing on its free radical scavenging, reducing and metal chelating mechanisms in addition to its phyto-constituents determination.

2. MATERIALS AND METHODS

2.1 Plant Sample and Extraction Procedure

The plant of *Anisomeles indica* was collected from District Kotli, Azad Jammu & Kashmir and at from Dr. Sulatan Ahmad Herbarium, Department of Botany,. GC University Lahore with a voucher No.GC.Herb.Bot.2101. The plant material was dried under shade at room temperature for two months and the leaves of the plant were carefully separated and grinded to a fine powder of 1 mm mesh size [12]. The powdered plant material (100 g) was extracted successfully with appropriate quantity of methanol by using Soxhlet extractor for 72 hours at a temperature not more than the boiling point of the solvent [13]. The extraction was filtered by using Whatman No.1 filter paper and concentrated in vacuum at 40℃ by means of Stuart-RE300 rotary apparatus and considered the production of extract. Dissolution the residues in correct volume of doubly distilled water and acceptable it to extracts using solvents (30 x 3 mL) of different polarity (*n*-hexane, Ethyl acetate, chloroform, *n*-Butanol), as shown in Fig. 2. Such different organic fractions and the H_2O fraction were evaporating under vacuum pump by Stuart-RE300 rotary apparatus, upto the boiling point of the solvents. After that the value of each residue was determined. To find out the antioxidant potential residues mixed with respective solvent to form the stock solutions (3 mg/mL) for further proceeding.

2.2 Chemicals

All the chemicals and solvents (methanol, chloroform, *n*–hexane, *n*-butanol, ethyl acetate, double distilled water and ethanol) were of analytical grade. 2,2`-azinobis-(3-ethylbenzo-thiazoline-6-sulphonic) diammonium salt (ABTS), 2,4,6-tris-2-tripyridyl-s-triazine, ferrozine were obtained from Fluka chemicals, Switzerland. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma Aldrich Chemical Co., Gillingham, Dorset, UK). Folin-Ciocalteu reagent was purchased from Merck chemicals, Germany. Ferrous sulphate ($FeSO_4$), Ferrous chloride, Na_2EDTA, aluminum chloride were obtained from Fluka Chemicals, Switzerland. Experiments were performed on UV-1700 Pharma- Spec UV-Visible Spectrophotometer, Shimadzu, Japan equipped with CPS controller. All samples were analyzed thrice and results obtained were averaged.

2.3 Methodology

2.3.1 Determination of total phenolic contents

The phenolic components were found by using Folin Ciocalteu reagent [14]. Each plant extract or gallic acid was added to F.C reagent (5 mL 1:10 diluted with double distilled H_2O) and 4 mL of 1 M solution of sodium carbonate. It hangs about for 25 min at 37℃ and absorbance value was calculated at 765 nm. The typical curve was arranged using 0, 50, 100, 150, 200, 250 mg/L solutions of standard phenolic compound (Gallic acid) in CH_3OH:H_2O (50:50, v/v).Total phenolic contents of fractions were represent as (GAE) mg/L. All samples were analyzed thrice.

2.3.2 Total flavonoids determination

The total flavonoids substance was found out with aluminum chloride ($AlCl_3$) according to a well-known method using quercetin as a standard [15]. Now 250 μL of sample fraction or quercetin standard solutions was dissolve with 1.25 mL of doubly distilled H_2O in test tube and further mixing of 75 μL of 5% $NaNO_2$ solution. After 7 min 150 μL of 10% $AlCl_3.6H_2O$ solution was dissolve and remains as such for 7 min. After this 1 M NaOH added to 500 μL and solution volume was upto to 2.5 mL with doubly distilled H_2O and assorted good. The absorption of given sample was calculated directly with the blank sample at 510 nm to find curve and correlation coefficient (R2), slope and cut off of the regression equation were determined by the technique of least square. The quantity of total Flavanoids was expressed as Quercetin equivalents (QE mg/L).

2.3.3 ABTS assay

Free radical decolorization of ABTS by little variation was determined by following [16]. In short, firstly ABTS free radical cation was formed by combining with ABTS solution (7 mM) with 2.45 mM potassium persulfate. The blend was remaining as such for 13-16 h in shade at room temperature before use. Now sample was mixed with buffer of pH 7 to get the absorption value of 0.7 ± 0.2 units at 734 nm.

To evaluate the antioxidant potential, further mixed with 12 μl of sample to 3.0 mL of diluted solution of radical monocation (A = 0.7 ± 0.2) and monitor the variation in absorption after 1 min gap for 7 min. Suitable blank solvent was running to comparable. All samples were run in three times and mean values of absorption were

considered. The measured value of Trolox was ready by intrigues between absorbance at 734 nm and percentage inhibition. The formula was given as;

Percentage inhibition (at 734 nm) = (1-Af /A0) x 100

2.3.4 FRAP assay

This assay is commonly used reducing control of different extracts (leaves stem etc.) was investigated by [17] with alteration. In brief, this reagent consist of 2.6 mL of 10 mM TPTZ solution in 40 mM HCl with 2.6 mL of 20 mM $FeCl_3$ as well as 30 mL of 0.3 M acetate buffer of pH 3.6. The new solution was primed by mixing 25 mL acetate buffers, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3$. $6H_2O$ solution and heated at 37℃ prior to using. Then 4 ml of freshly FRAP reagent added 300 µl of double distilled H_2O and 100 µl of *Anisomeles indica* extract and noted its absorption at 593 nm for 4 minutes.

2.3.5 Metal chelation method

The chelating activity of ferrous ions by *Anisomeles indica* fraction was predicted by the technique of [18]. To 50 µl of 5 mM solution of $FeSO_4.7H_2O$, added 200 µl of 2.0 mM solution of Ferrozine and its level raised upto 5 mL with two times distilled C_2H_5OH. Allowed this solution to incubate at 36℃ for 10 minutes and absorption value was considered at 562 nm. A control sample was also run in parallel without the addition of test sample. Iron (II) chelating ability was calculated as

% Chelating activity = [(Acontrol-Asample) / Asample] X 100

3. RESULTS AND DISCUSSION

Over the years, the study of medicinal plants unveils the mechanism of action and justifies the claims of conventional healers. A perspective of this research has been the study of bioactive components and to find their efficiency in different mechanisms of antioxidant action.

3.1 Total Phenolic Content Assay

The Folin-Ciocalteu reagent has maximum absorbance at 765 nm [19]. The utmost phenolic contents were observed for methanol extract (1265.5 ± 0.569 mg/L of GAE), water fraction (1145.4 ± 0.593 mg/L of GAE), butanol fraction (1142.5 ± 0.0569 mg/L of GAE). While the lowest phenolic contents were found for ethyl acetate fraction (465.4 mg/L of GAE), chloroform fraction (457.3 ± 0.295 mg/L of GAE), and for hexane fraction (198.9 ± 0.395 mg/L of GAE) (Table 1) (Fig. 1). The results are depicting a link among the poly-phenolic contents and the antioxidant potential of the relevant extract, which is in conformity with previous results concerning different plants as indicated by Kapoor [20].

3.2 Total Flavonoid Contents Determination

The contents of total flavonoid were determined by aluminum chloride colorimetric method and represent in terms of quercetin equivalent (mg/L). These results obtained from analysis of flavonoids showed that water sample had highest value (3123.7±0.395 mg/mL of QE), methanol fraction had (2395.45±0.258 mg/L of QE), while ethyl acetate and butanol fractions showed closed values (1395.45±0.964 mg/L of QE) and (1311.81±0.680 mg/L of QE). Similarly *n*-hexane and chloroform fractions also showed much closed values of total flavonoids contents which were 1154.54 ± 0.376 and 1165.45 ± 0.356 mg/L of QE respectively (Fig. 2). Similar results were also obtained by Ajaib et al. [21] while working with some medicinal grasses of Lahore, Pakistan. Hence it is observed that water fraction has the crown value of TFC. The priority order of TFC of different fractions of *A. indica* as follow:

Water fraction > methanol > ethyl acetate > butanol > hexane > chloroform (Table 1).

3.3 FRAP Assay

All fractions had reducing power but to a different echelon. The results denoted that hexane fraction of *Anisomeles indica* leaves showed the highest reducing property. While the chloroform fraction had 85.68 ± 0.183 mMol and butanol extracts showed FRAP values of 43.12 ± 0.758 mMol, the remaining extractions of methanol and ethyl acetate showed FRAP values 40.53 ± 0.550 mMol and 14.44 ± 0.339 mMol respectively (Table 1) (Fig. 3). The least reducing power gives rise from the ethyl acetate fraction. From this analysis, it is established that hexane extract of *Anisomeles indica* leaves comprise the effective antioxidant components possessing an efficient reducing mechanism capable of ceasing oxidation chain reactions these findings are also similar with Siddiqui et al. [22] during investigation of antioxidants in *Pyrus pashia*.

Table 1. Antioxidant activities of *Anisomeles indica*

Ser. no.	Sample	TEAC value (mMol)	FRAP value (mMol)	% age bound iron	TPC (mg/L of GAE)	TFC (mg/L of QE)
1	MW	9.17 ± 0.879	46.48 ± 0.194	68.92 ± 0.357	1145.4 ± 0.593	3123.7 ± 0.395
2	ME	2.41 ± 0.236	14.44 ± 0.339	63.21 ± 0.725	465.4 ± 0.695	1395.45 ± 0.964
3	MH	0.75 ± 0.658	118.4 ± 0.495	63.59 ± 0.490	198.9 ± 0.395	1154.54 ± 0.376
4	MB	6.5 ± 0.892	43.12 ± 0.758	56.66 ± 0.843	1142.5 ± 0.0569	1311.81 ± 0.680
5	MC	1.77 ± 0.326	85.68 ± 0.183	72.91 ± 0.770	457.3 ± 0.295	1165.45 ± 0.356
6	MM	11.17 ± 0.239	40.53 ± 0.550	66.13 ± 0.263	1262.5 ± 0.569	2395.45 ± 0.258

3.4 Metal Chelating Activity

The major plan to prevent ROS production that is linked with simultaneously oxidation-reduction metal catalysis including chelation of the metal ions. *Anisomeles indica* fractions hinder with the development of ferrous and ferrozine complex, signifying that it had chelating action, and hold on ferrous ion prior to ferrozine. The analysis observed that chloroform had highest MCA 72 ± 0.770%; water fraction showed 68.92 ± 0.357%, while methanol fraction had 66.13 ± 0.263%, very closed values of hexane and ethyl acetate 63.59 ± 0.490 & 63.21 ± 0.725% respectively (Fig. 4). The lower value of MCA was reported for butanol fraction which was given in Table (4) 56.66 ± 0.843% (Table 1).

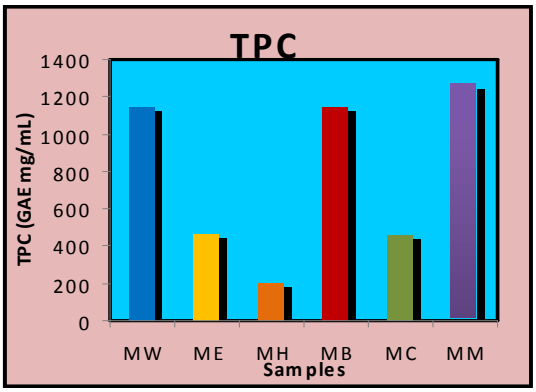

Fig. 1. Graphical representation of phenolic contents

3.5 Trolox Equivalent Antioxidant Capacity (TEAC) Assay

This procedure was adopted to evaluate the free radical scavenging ability of the leaves fractions with the help of ABTS•+ radical cation. ABTS•+ is basically nitrogen centered colored radical cation which on reaction with antioxidant becomes colorless and allowed us to study the direct interaction of antioxidant with a radical cation having redox potential equivalent to peroxyl radicals generated within the body [23].

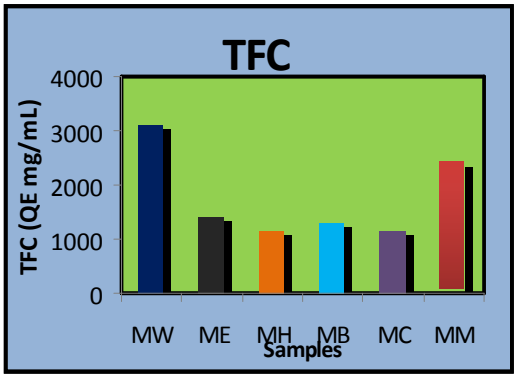

Fig. 2. Graphical representation of total flavonoid contents

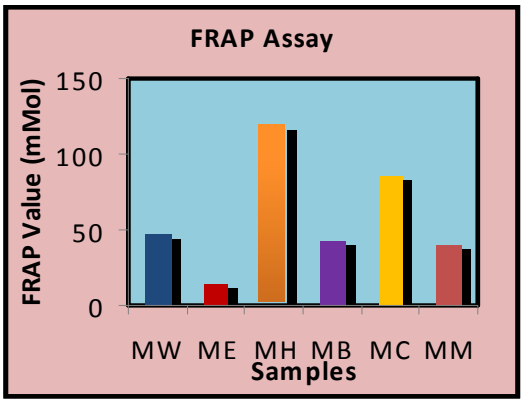

Fig. 3. Graphical representation of FRAP assay activity

The outcomes of ABTS assay are described as TEAC values. The values of the tested fractions are acquired after comparison the % age inhibition with that of Trolox standard. The crown TEAC values were obtained for methanol, water, butanol extracts having the values 11.17 ± 0.239 mMol, 9.17 ± 0.879 mMol and 6.5 ± 0.892 mMol respectively. While the remaining extracts

showed a relatively low TEAC values as shown in Table 1.

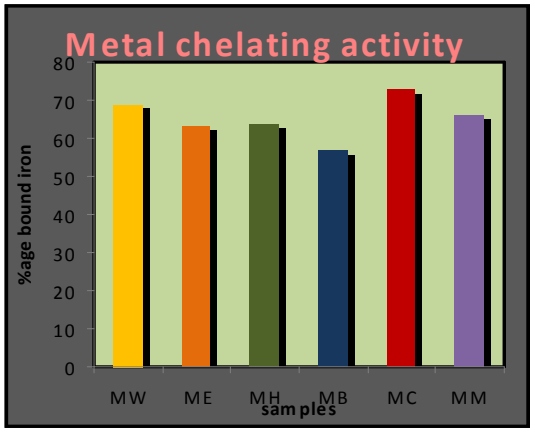

Fig. 4. Graphical representation of metal chelating

4. CONCLUSION

The study considers that the leaves extract of *A. indica* possesses antioxidant potential, which may be supportive to protect the advancement of different oxidative stress linked diseases. As all the leaves extracts show its activity in terms of all proposed mechanisms of antioxidant action. Moreover research on the separation and recognition of antioxidant components in the leaf may go ahead to chemical compounds having potential uses for medicinal as well as for food stuff.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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