

Original Research Article

In Vitro Assessment of Antioxidant Activity of *Anisomeles Indica*

Summary: The present research 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 ± 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.

Key words: Antioxidants, ABTS, Flavonoids, total phenolic contents, reducing ability.

Introduction

Antioxidant research is an important 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. Some leafy green vegetables also very rich source of antioxidant like collard greens, spinach, and kale [2]. Natural antioxidants are mostly phenolic compounds and are involved reactions of chain breaking which combine with radicals of lipid and convert them into stable products [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 reactive oxygen species (ROS) and reactive nitrogen species (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, peroxy radical or by reducing or chelating mechanisms [5]. Antioxidant constituents can hinder the oxidation of lipids or other molecules and also check the damages of the body's cells done by oxygen. They perform different mechanisms such as free radical-scavenging, reducing action, quenching of singlet oxygen and complexing of pro-oxidant metals. Modern biological researches clearly showed that abundant phyto-nutrients of vegetables and fruit save the human body against smash up by reactive oxygen species (ROS). The use of synthetic antioxidants like phyto-chemicals was reported to have realistic health benefits [6-8]. 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 [9-11].

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 [12]

A. 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 [13]. *A. indica* has been widely used to treat dysfunction, hypertension and anti-inflammatory in various countries as medicine. It has carminative, astringent and toxic properties [14]. Ethno botanically 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 [15].

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 determin

Experimental

Plant sample and extraction procedure

The plant of *A. indica* was collected from District Kotli, and Azad Jammu Kashmir and identified by Dr. Sultan 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 1mm mesh size. The powdered plant material (100 gm) 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. The extraction was concentrated in vacuum at 40° C by rotary apparatus. 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, ethylacetate, chloroform and *n*-butanol), these fractions were evaporated by rotary apparatus. 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.

Determination of Total phenolic contents

The phenolic components were found by using Folin Ciocalteu (FC) [16]. Each plant extract or gallic acid was added to F.C reagent (5 ml 1:10 diluted with double distilled H₂O) and 4 ml of 1M solution of Sodium Carbonate. It hangs about for 25 min at 37°C and absorbance value was calculated at 765 nm. The typical curve was arranged by using 0, 50,100, 150, 200, 250 mg/L solutions of standard phenolic compound (GA) in CH₃OH:H₂O (50:50, v/v). Total phenolic contents of fractions were represent as (GAE) mg/L. All samples were analyzed thrice.

Total flavonoids determination:

The total flavonoids substance was find out with aluminum chloride (AlCl₃) according to a well-known method using quercetin as a standard [17]. Now sample fraction or quercetin (standard solutions) of 250 µL was dissolve in doubly distilled H₂O of 1.25 mL in test tube and then add 75 µL solution of 5 % NaNO₂. After 7 min 150 µL solution of 10 % AlCl₃ .6H₂O was dissolve and remains as such for 7 min. After this 1M NaOH added to 500 µL and solution volume was upto to 2.5 mL with doubly distilled H₂O 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).

ABTS assay:

Re *et al.* developed a method of free radical decolorization of ABTS (2,2'-azinobis(3-ethylbenzothiazoline)-6-sulphonic acid) by little variation [18]. In short, firstly ABTS free radical cation was formed by combining with 7 mM solution of ABTS with potassium persulfate of 2.45 mM. 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 μ l of sample to 3.0ml of diluted solution of radical monocation ($A = 0.7 \pm 0.2$) 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 734nm and percentage inhibition. The formula was given as;

$$\text{Percentage inhibition (at 734nm)} = (1 - A_f / A_0) \times 100$$

FRAP Assay

This assay is commonly used reducing control of different extracts (leaves stem etc.) was investigated by Benzie *et al.* with alteration [19]. 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, 2.5mL TPTZ solution, and 2.5mL $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution and heated at 37°C prior to using. Then 4 ml of freshly FRAP reagent added 300 μ l of double distilled H_2O and 100 μ l of *A. indica* extract and noted its absorption at 593 nm for 4 minutes.

Metal chelation Method:

The chelating activity of ferrous ions by *A. indica* fraction was predicted by the technique of Dinis *et al.* [20]. To 50 μ l of 5 mM solution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, added 200 μ l of 2.0 mM solution of Ferrozine and its level raised upto 5 ml with two times distilled $\text{C}_2\text{H}_5\text{OH}$. Allowed this solution to incubate at 36°C 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

$$\% \text{ Chelating activity} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{sample}}] \times 100$$

ation.

Result 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 (table 1).

TPC (Total phenolic content) was measured as GAE (gallic acid equivalent) mg/ml. The highest phenolic contents were observed by methanol extract (1265.5 ± 0.569 mg/L of GAE), water extract (1145.4 ± 0.593 mg/L of GAE) and butanol extract (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).

The contents of total flavonoid were determined by aluminum chloride colorimetric method and represent in terms of Quercetin equivalent (mg/L). Results showed that water sample showed 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. Hence it is observed that water fraction has the crown value of TFC.

All fractions had reducing power but to a different level. The results denoted that hexane fraction of *A. 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 mM, the remaining extractions of methanol and ethyl acetate showed FRAP values 40.53 ± 0.550 mMol and 14.44 ± 0.339 mMol respectively. The least reducing power gives rise from the ethyl acetate fraction. From this analysis, it is established that hexane extract of *A. indica* leaves comprise the

effective antioxidant components possessing an efficient reducing mechanism capable of ceasing oxidation chain reactions.

The major plan to prevent ROS production that is linked with simultaneously oxidation-reduction metal catalysis including chelation of the metal ions. *A. 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 metal chelating activity (MCA) $72 \pm 0.770 \%$; water fraction showed $68.92 \pm 0.357 \%$, while methanol fraction had $66.13 \pm 0.263 \%$, very closed values of hexane and ethyl acetate 63.59 ± 0.490 & $63.21 \pm 0.725\%$ respectively. The lower value of MCA was reported for butanol fraction $56.66 \pm 0.843\%$.

TEAC (Trolox equivalent antioxidant capacity) assay was adopted to evaluate the free radical scavenging ability of the leaves fractions with the help of ABTS $\bullet^+$ radical cation. ABTS $\bullet^+$ 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 peroxy radicals generated within the body [21].

The outcomes of ABTS (2,2'-azinobis(3-ethylbenzothiazoline)-6-sulphonic acid) 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.

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 foodstuff.

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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

Table No 1: Antioxidant Activities of *Anisomeles indica*

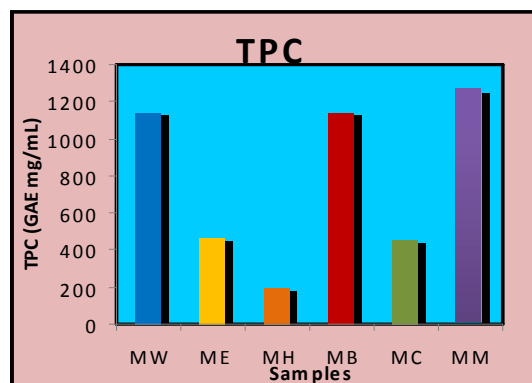


Fig. 1: Graphical representation of Phenolic Contents

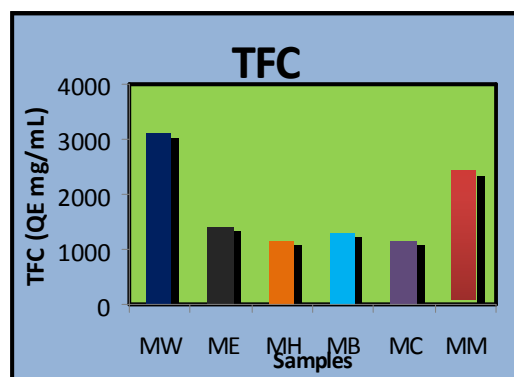


Fig. 2: Graphical representation of total flavonoid contents

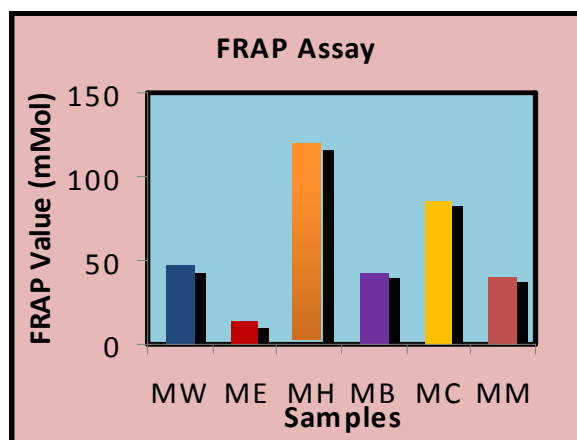


Fig. 3: Graphical representation of FRAP Assay

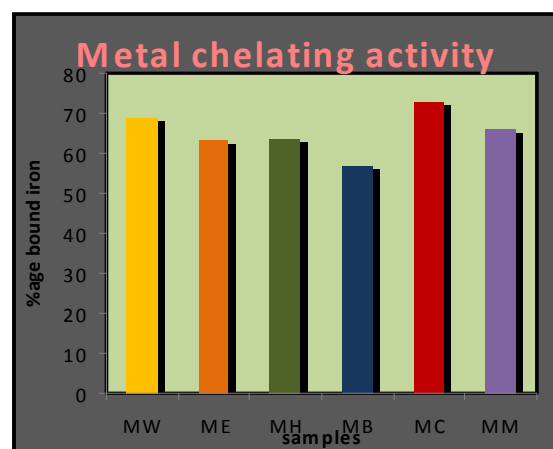


Fig.4: Graphical representation of Metal Chelating Activity