Original Article

# Antioxidant activity measured in different solvent fractions obtained from *Mentha spicata* Linn.: An analysis by ABTS$^{\cdot+}$ decolorization assay

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Antioxidant compounds are abundantly available in plants and play an important role in scavenging free radicals, thus providing protection to humans against oxidative DNA damage. *Mentha spicata Linn*., commonly called spearmint, belongs to the family lamiaceae. It was selected in the present study because Mentha extracts have antioxidant properties due to the presence of eugenol, caffeic acid, rosmarinic acid and α-tocopherol. Four solvent fractions (hexane, chloroform, ethyl acetate and water) of ethanolic extract of dried leaves powder of *M. spicata* were analyzed for total antioxidant activity (TAA) and relative antioxidant activity (RAA) and compared with standard antioxidants such as Quercetin, β-carotene, L-ascorbic acid and glutathione using ABTS$^{\cdot+}$ decolorization assay (ABTS / Potassium persulphate). The antioxidant activity was assumed to be from the total phenolic content of the ethanolic extract. Total phenolics are found to be highest in ethyl acetate fraction (54 mg/g) and least in hexane fraction (13 mg/g) and more or less similar in water and chloroform fractions (30-32 mg/g). TAA is found to be less in hexane and chloroform fractions (<53 % at 50 μg/ml) and highest in ethyl acetate (95% at 20μg/ml) and water (84% at 30μg / ml) fractions. The RAA of ethyl acetate fraction is 1.1 compared to quercetin (at 5μM/ml), but greater when compared to β-carotene (15 μM/ml), L-ascorbic acid (15μM/ml) and glutathione (15μM/ml). The RAAs with these antioxidants are in the range of 1.31 – 1.6. The values of RAAs for water fraction also show similar trend and are in the range of 1.0 – 1.4. The antioxidant activities of the solvent factions are closely related to the content of total phenolics present in them.

**Key Words: ABTS radical cation, Antioxidant activity, Relative antioxidant activity, Phenolics, solvent fraction, *Mentha spicata.***

## Introduction

*Mentha spicata Linn*. commonly called spearmin[1] belongs to the family lamiaceae. The genus *Mentha* consist of more than 25 species and are well known for monoterpenes like menthol, menthone, carvone and pulegone. They are widely used by food and pharmaceutical industries as a flavor or in fragrance formulations.[2] *M. spicata* synonymous of *M. viridis Linn*. is a herbaceous perennial, with a pungent smell. It is cultivated all over India for culinary purposes. *M. spicata* leaves are generally given for fever and bronchitis and its decoction is used as lotion in aphthae.[1] The mint family is characterized by their volatile oil. However, the essential oil of *M. spicata* has been shown to have strong toxic effects on the insect *Acanthoscelides obtectus* and exhibited repellent and inhibitory activity.[3] This aromatic herb is mostly produced and being consumed in Morocco.[4] The herb is considered as stimulant, carminatives, antispasmodic, stomachic and diuretic, and it is also used for gas pain, rheumatism, toothache, muscle pain and mouth wash.[5] *Mentha* species like *M. arvensis* and *M. citrata* Ehrh. had been shown to consist of high percentage of Menthol and essential oils.[6-7] The ethyl acetate fraction of methanol extract of *Mentha spicata* var. *crispa* showed inhibitory activity on exocytosis in antigen-stimulated rat basophils.[8] *Mentha* extract has been found to have antioxidant and antiperoxidant properties due to the presence of eugenol, caffeic acid, rosmarinic acid and α-tocopherol[9] and it could enhance error-free repair for DNA damage and hence could be antimutagenic.[10] Aqueous extract provides protection against radiation induced chromosomal damage in bone marrow of mice by decreasing serum acid phosphatase and increasing serum alkaline phosphatase. [11]

We have estimated the total antioxidant activity in dried leaves of *M.spicata* in different fractions (hexane, chloroform, ethyl acetate and water) of ethanolic extract, using ABTS$^{\cdot+}$ (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) decoloration method. Additionaly the total phenolic compounds are also estimated in these ethanolic fractions.

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ABTS with potassium per sulfate generates bule/green $ABTS^{\cdot+}$. The radical shows maximum absorbance at 645 nm, 734nm and 815nm, as previously reported.[12] This method can be used for both pure compounds and biological samples.[13] Antioxidants transfer an hydrogen atom to radical cation and causes discoloration of the solution.[14]

## Materials and methods

*Mentha spicata Linn*., was commercially purchased and identified from Center for advance studies in Botany, University of Madras (Herbarium voucher number-855). β-Carotene, L-Ascorbic acid, Quercetin, 2,2′-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) and Potassium persulfate were obtained from Sigma–Aldrich, USA. All solvents used in this study are analytical grade.

### *Extraction of solvent fractions*

The shadow dried leaf powder of *M. spicata* (150g) was immersed in 500ml of 95% ethanol and the filtrate was collected for three times with constant stirring of the mixture at every 24 hrs interval of a 72 hrs total collection period (all the time 95% ethanol was used for obtaining the filtrate) and the final filtrate volume was found to be 1.2 litre. The extract was then concentrated under reduced pressure at 40°C using vacuum rotary evaporator. The yield of ethanol extract was 10.35g. Ethanol extract was partitioned between hexane and water (6:1). The aqueous layer further fractionated with chloroform ($CHCl_3$) and ethyl acetate.[15] Three solvent fractions (Hexane, Chloroform and Ethyl acetate) were collected and concentrated with vacuum rotary evaporator. The yields of these fractions constituted 26.8%, 21% and 10.8% of the ethanolic extract respectively. The aqueous fraction was lyophilized (Flexi-Dry μp at 50 MT and -85°C) and constituted 8% of ethanolic fraction.

### *Estimation of total antioxidant activity (TAA)*

$ABTS^{\cdot+}$ stock solution was prepared by mixing 5ml of 7mM ABTS (2,2'-azino-bis- (3-ethylbenzothiazoline –6-sulfonic acid) and 88 μl of 140 mM $K_2S_2O_8$. The stock solution was diluted with ethanol and PBS (pH 7.4) to give absorbance of 0.75 (± 0.02) at 734 nm. The radical decoloration assay was performed in a volume of 1ml of diluted $ABTS^{\cdot+}$ solution and appropriate volume of extract or pure compound.[16] The standard stock solution of β-carotene, quercetin, L-ascorbic acid and glutathione was prepared in acetone[13] ethanol and water[17] respectively. The standard antioxidants (final concentrations 0-15μM) were added to the diluted $ABTS^{\cdot+}$ solution in ethanol or PBS and the absorbance reading was taken at room temperature. Appropriate solvents blank were run in each assay and all the experiments were carried out in five readings. The total antioxidant activity (TAAs) and relative antioxidant activity (RAAs) was calculated using the following equations.[13]

$$\text{Total antioxidant activity (TAA \%)} = \frac{\text{O.D } ABTS^{\cdot+} - \text{O.D Sample}}{\text{O.D } ABTS^{\cdot+}} \times 100$$

$$\text{Relative antioxidant activity (RAA)} = \frac{\text{TAA test compound}}{\text{TAA Standard compound}}$$

### *Estimation of total phenolics*

Total phenolics were estimated by Price and Butler (1977) method.[18] Test tube containing 25ml of deionised water and 250μl of ethanolic fractions (Hexane, Chloroform, Ethyl acetate and Water) were mixed with 3ml of ferric chloride (0.1M solution of $FeCl_3$ in 0.1M of HCl). After 3min 3ml of potassium ferricyanide reagent (0.008M $K_3Fe(CN)_6$) was added and allowed to stand for 10-15min at room temperature and the reaction mixture was read at 720 nm. Quercetin was used as standard for estimates and the value are expressed as mg/g of solvent fractions.

## Results

The absorbance spectrum of $ABTS^{\cdot+}$ at different concentrations reveals the maximum absorbance at 734nm[12] (Fig. 1).

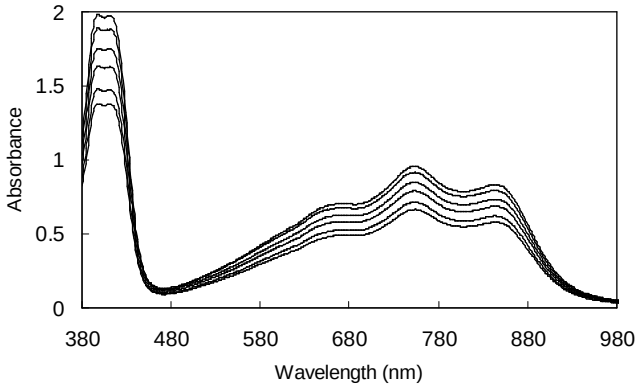


**Figure 1**. Absorbance spectrum of ABTS radical cation.

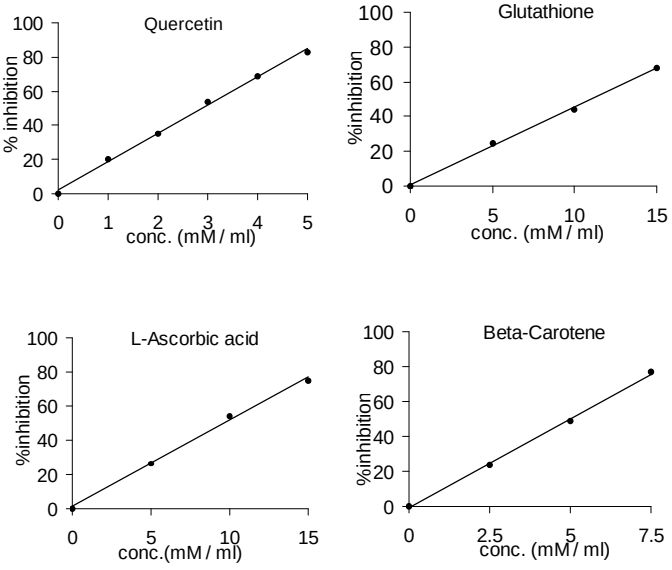


**Figure 2.** Percentage inhibition $ABTS^{\cdot+}$ with respect to increasing concentration of standard antioxidants.

The different concentrations of each standard were read at 1min interval up to 30min. The results showed that all were essentially completed in 1-3 minutes[17] therefore, time points 1 and 5 min selected for reading the absorbance after initial mixing of the reactants. The standards quercetin, β-carotene, L-ascorbic acid and glutathione showed 50% of inhibition at 3,5,10 and 12 μM concentration respectively (Fig. 2).

TAA of solvent fractions were determined at several concentrations (10-50μg/ml). TAA of hexane fraction was found to be gradually enhanced with increasing concentration and then the activity was saturated above 50μg/ml. The radical quenching activity of chloroform fraction was also found to be similar to the hexane fraction. TAA of ethyl acetate fraction was found to be maximum at 20 μg/ml concentration. The water fraction showed highest ABTS radical quenching activity at 30μg/ml. The relative antioxidant activities (RAAs) were computed by comparing the TAAs of the test compounds with those of the standard compounds. RAAs are higher than 1 for ethyl acetate and water fractions but lower for the hexane and chloroform fractions. The RAAs of ethyl acetate and water fractions are higher when compared to the hexane and chloroform fractions at the corresponding concentration (Table 1).

The TAAs of ethanolic fractions were expressed as equivalent of the standard compounds (mM ± SD). The TAAs of lipophilic fractions showed higher radical scavenging activity (291.26±4.54) at lesser concentration than the hydrophilic fraction (515.67±6.72). The maximum TAAs of Quercetin equivalent was found to be at lower concentration when compared to the TAA of β-carotene equivalent. The quercetin and β-carotene equivalent TAAs of lipophilic fraction such as hexane, chloroform, and ethyl acetate showed little difference between 1 and 5 min. Thus the lipophilic antioxidant activity essentially completed the reaction after one min and residual reaction is negligible (Table 2).

**Table 1.** TAA and RAA with known antioxidants of solvent fractions of *Mentha spicata* at various concentrations.

| Solvent Fractions | Fraction Con. (μg /ml) | TAA (%) | RAA | | | |
|---|---|---|---|---|---|---|
| | | | Quercetin | Beta - Carotene | L. Ascorbic acid | Glutathione |
| Hexane | 10 | 8 | 0.092 | 0.109 | 0.111 | 0.131 |
| | 20 | 18 | 0.206 | 0.234 | 0.249 | 0.295 |
| | 30 | 24 | 0.276 | 0.312 | 0.332 | 0.393 |
| | 40 | 32 | 0.367 | 0.416 | 0.443 | 0.524 |
| | 50 | 41 | 0.471 | 0.533 | 0.567 | 0.672 |
| Chloroform | 10 | 9 | 0.103 | 0.117 | 0.124 | 0.147 |
| | 20 | 22 | 0.252 | 0.286 | 0.304 | 0.360 |
| | 30 | 32 | 0.367 | 0.416 | 0.443 | 0.525 |
| | 40 | 37 | 0.425 | 0.481 | 0.512 | 0.606 |
| | 50 | 53 | 0.609 | 0.688 | 0.734 | 0.869 |
| Ethyl acetate | 5 | 25 | 0.287 | 0.325 | 0.346 | 0.410 |
| | 10 | 57 | 0.655 | 0.740 | 0.789 | 0.934 |
| | 15 | 79 | 0.908 | 1.026 | 1.094 | 1.295 |
| | 20 | 95 | 1.091 | 1.234 | 1.315 | 1.557 |
| Water | 10 | 36 | 0.413 | 0.468 | 0.498 | 0.590 |
| | 20 | 68 | 0.781 | 0.883 | 0.941 | 1.144 |
| | 30 | 84 | 0.965 | 1.091 | 1.163 | 1.377 |

TAA: Total Antioxidant Activity expressed as %inhibition of $ABTS^{\cdot}$; TAA of known compounds are based on concentrations at which maximum inhibition ($ABTS^{\cdot+}$) occurs (Quercetin (5 μM / ml)**:** 87%; β-Carotene (7.5μM/ml)**:** 77%) L. Ascorbic acid (15μM/ml) : 72.2%; Glutathione (15μM/ml)**:** 61%; RAA = Relative antioxidant activity.

**Table 2.** Total phenolics and its antioxidant activity of different solvent fractions of *M. spicata*.

| Solvent Fractions | TAA - 1min | | TAA - 5min | | Total Phenolics |
|---|---|---|---|---|---|
| | *Quercetin* | *β-Carotene* | *Quercetin* | *β-carotene* | |
| Hexane | 40.09 ± 0.67 | 69.48 ± 1.17 | 41.28 ± 1.25 | 77.12 ± 1.43 | 14.00 ± 1.58 |
| Chloroform | 46.77 ± 0.84 | 81.82 ± 2.42 | 47.57 ± 1.09 | 87.11 ± 1.47 | 30.00 ± 2.28 |
| Ethyl acetate | 291.26 ± 4.54 | 504.86 ± 7.86 | 309.54 ± 4.48 | 560.74 ± 7.47 | 54.00 ± 4.74 |
| | *Ascorbic acid* | *Glutathione* | *Ascorbic acid* | *Glutathione* | |
| Water | 515.67 ± 6.72 | 651.10 ± 8.49 | 608.01 ± 7.08 | 749.78 ± 8.79 | 32.00 ± 3.16 |

Mean ± Standard Deviation ($N$ = 5); TAA: Total antioxidant activity; expressed as standard (quercetin, β-carotene, L-ascorbic acid and glutathione) equivalents (mM/g of solvent fractions of *Mentha spicata*); Total phenolics ($N$ = 3); expressed: mg/g of solvent fractions of *Mentha spicata*

The total phenolic content was also found to be higher in ethyl acetate (54.00±4.74) and water fraction (32.00 ± 3.16) when compared to that of hexane and chloroform fractions (Table 2).

**Discussion**

In the present investigation, antioxidant activity of hexane, chloroform, ethyl acetate and water fractions of ethanolic extract of *M. spicata* was determined using $ABTS^{\cdot+}$ decolorization method. Among the standard compounds, quercetin had three folds higher TAA compared to the L- ascorbic acid and glutathione due to its number of hydroxyl group with double bonds in the C ring at 2 and 3 positions and the 4-oxo groups (Fig. 2). The structural advantage of quercetin can be judged by its TEAC (Trolox equivalent antioxidant capacity) value to 4.70 ± 0.1 mM.[19] β-Carotene also has two fold TAA than that of ascorbic acid and glutathione[20] which is also observed in the present study. Antioxidant reactions involve multiple steps initiation, propagation, branching, and termination. Antioxidants fall into two mechanistic groups: those that inhibit or retard the formation of free radicals from their unstable precursors (initiation) and those that interrupt the radical chain reaction (propagation and branching). The former are called as preventive antioxidant and the latter as chain-breaking antioxidants.[21]

The radical scavenge activity was found to be maximum for ethyl acetate fraction (95%) which was fourfolds higher than that of hexane (18%) and chloroform (22%) fractions at corresponding concentrations. In the case of the water fraction, the TAA (84%) seemed to be maximum at its highest concentration (30µg/ml) and it is 3.5 fold higher than hexane (24%) and 2.5 fold higher than the chloroform fraction (32%) at the corresponding concentration (Table1). Aqueous extracts have greater antioxidant activity than the organic extracts because most of the active compounds in the leafy part of the vegetable may be dissolved in the water.[22] According to Winston (1999),[23] the leafy part of the vegetables contain the active component which consist of the flavonoid, terpenoid, lignan, sulphide, polyphenol, carotenoid, caumarin, saponin, curcumin and sterol. While comparing the TAAs of lipophilic fractions (hexane, chloroform and ethyl acetate) with quercetin and β-carotene standards, ethyl acetate fraction had the highest activity at lower concentration 291.26 ± 4.54 for 1 min and 309.54 ± 4.48 for 5min. However, antioxidant activity of the water fraction in terms of glutathione equivalent activity is greater when compared to the ascorbic acid equivalent activity (Table 2).

Since the TAAs are higher for ethyl acetate and water fractions compared to hexane and chloroform fractions, their RAAs are also found to be higher. This is mainly because of its hydrophilic antioxidant properties with an activity similar to Trolox.[19] The radical scavenging activity of ethyl acetate fraction is mainly due to the presence of higher amounts of phenolic compounds.[24] The presence of free OH group in phenolic compounds are mainly responsible for antioxidant activity.[25] The ethyl acetate and water fractions have highest total phenolics than the hexane and chloroform fractions (Table 2). Cakir *et al.*, (2003)[24] reported that ethyl acetate fraction of *Hypericum hyssopifolium* showed highest DPPH radical scavenging activity because of the total phenolic compounds. Additionally, this fraction contains some prooxidants. They isolated six phenolic compounds and they can be used in oil-water emulsion systems. In the present study, total phenolics and TAAs of the solvent fractions of the ethanolic extract of *M. spicata* were found to be positively correlated.

This study indicates that the ethyl acetate fraction of ethanolic extract of *M.spicata* has high antioxidant activity against $ABTS^{\cdot+}$ than the hexane and chloroform fraction. It is due to the presence of high content of phenolics, which could be the most effective in protecting the body against various oxidative stressors. The structure of phenolic compounds showing antioxidant activity in the ethyl acetate fraction need to be identified.

**Abbreviations**

ABTS: 2,2′-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid)
TAA: Total antioxidant activity
RAA: Relative antioxidant activity
PBS: Phosphate saline buffer
TEAC: Trolox equivalent antioxidant capacity
DPPH: 1,1,3,3-tetramethoxypropane and 1,1- diphenyl –2 – picryl – hydrazyl

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Original Article

# Antioxidant activity measured in different solvent fractions obtained from *Mentha spicata* Linn.: An analysis by $ABTS^{\cdot+}$ decolorization assay

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## 不同溶剂分部分离的留兰香（*Mentha spicata* Linn.）提取物的抗氧化活性：$ABTS^{+}$脱色分析

抗氧化物质大量存在于植物中，这些物质具有清除自由基的重要作用，保护人类 DNA 不受氧化损伤。留兰香，俗称荷兰薄荷，属于薄荷科，其提取物中含有丁子香酚、咖啡酸、迷跌香酸和 α-抗生育酚而具有抗氧化特性，因而被选研究对象。通过 $ABTS^{+}$脱色法分析了四种溶剂（正几烷、氯仿、乙酸乙酯、水）分部分离的留兰香叶干粉的乙醇提取物的总抗氧化活性（TAA），相对抗氧化活性（RAA）并与标准抗氧化剂如斛皮素、β-胡萝卜素、L-抗坏血酸、谷胱甘肽的抗氧化活性对照。留兰香的抗氧化活性被认为是来自其乙醇萃取物中的总酚含量。总酚含量最高的是乙酸乙酯分部分离物(54mg/g)，最低的是正己烷分部分离物(13mg/g)，水和氯仿分部分离物中的含量相近(30-32mg/g)。总抗氧化活性最高的是乙酸乙酯分部（95％，20μg/ml），其次是水分部(84%，30μg/ml)，正几烷和氯仿分部则较低(<53%，50μg/ml)。乙酸乙酯分部的 RAA 以斛皮素(5μM/ml)作对照是 1.1，以β-胡萝卜素(15μM/ml)、L-抗坏血酸(15μM/ml)、谷胱甘肽(15μM/ml)作对照则较高，在 1.31－1.6 之间。水分部的 RAA 表现出与乙酸乙酯分部相似的变化规律，其值在 1.0－1.4 之间。各溶剂分部分离物的抗氧化活性与其总酚含量密切相关。

**关键词：**ABTS 阳离子、抗氧化活性、相对抗氧化活性、酚、溶剂分部、留兰香