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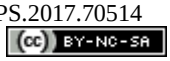
ISSN 2231-3354 

Iron Chelation and Iron Reducing Activity of Tissue Cultured and Tissue Culture Derived *Mentha Spp*.

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ABSTRACT

The present study was carried out to study the effect of maturity of tissue cultured and tissue cultured derived *Mentha* spp on iron chelation and iron reducing potential. *Mentha sps.* selected for the study were *Mentha piperita* (PPR 611), *Mentha arvensis* (SH) and *Mentha spicata* (SPR-8). Methanolic extracts of selected *Mentha* spp were prepared and total phenolic and flavonoid contents were determined in tissue cultured(TC) and tissue culture derived plantlets (TC_o and TC_1). Furthermore, a comparison of chelation of ferrous ions and iron reducing power of extracts was made in the three species. Total phenol content increased in time dependent manner and was found to be more in tissue culture derived plantlets ($TC_0$and TC_1) in all the three species compared to tissue cultured plants (TC). The phenols were found to be highest in SPR- 8 plants followed by PPR 611.On the contrary, flavonoid content decreased over growth period in all *Mentha* spp. Also PPR 611 showed higher content of flavonoids compared to other species. The difference in production of these secondary metabolites may be due to culture induced variations in biosynthetic pathways. The iron reducing potential of tissue culture derived plants also increased with increase in development period and SPR-8 showed more promising results. Tissue cultured raised and tissue culture derived plantlets of SPR-8 had better iron chelation property and the activity decreased in tissue cultured derived plantlets with an increase in development period.

INTRODUCTION

Herbs have been identified as source of various phytochemicals, many of which possess important antioxidant activity. *Mentha* is a genus of aromatic perennial herbs belonging to the family *Lamiaceae*, distributed mostly in temperate and sub-temperate regions of the world. Most of the commercially important mints are hybrids or amphiploids. The *Lamiaceae* family (Labiatae) is one of the largest and most distinctive families of flowering plants, with about 220 genera and almost 4000 species worldwide. It is one of the most diverse and widespread plant families in terms of ethnomedicine (Naghibi *et al.*, 2005).

Members of this genus are the most important sources of essential oil production in the world. It has been estimated that the annual production of oils from the three *Mentha* spp, namely, peppermint (*M. piperita* L.), cornmint (*M. arvensis* L.) and spearmint (*M. spicata*), is in excess of 23,000 metric tons with a value exceeding $400 million. This makes them one of the most economically important essential oils producers (Lawerence, 2006). The antioxidant activity of plant extracts is widely used in the food industry as potential inhibitors of lipid per oxidation, which become important and interesting object of research because of the increasing usage of natural antioxidants (Scherer and Godoy, 2009; Grul'ova *et al.*, 2012).

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Phenolic compounds, also known as polyphenol antioxidants, which include flavonoids and phenolic acids, are found naturally in spearmint plants. Phenolic compounds inhibit lipid peroxidation, scavenge free radicals, iron and copper ions. Phenolic compounds exhibit a range of biological activities, including anticancer, antibacterial, antioxidant and anti-inflammatory properties (Lu Y and Foo, 2002).

Mentha is known to be endowed with a variety of biological and pharmacological properties due to the high content of secondary metabolites (Choudhury *et al.,* 2006). Previously antiallergic (Yaamamura *et al.,* 1998), anti-oxidant (Kannat *et al.,* 2007), anti-platelet (Togolini *et al.,* 2006), anti-proliferative (Manasroi *et al.,* 2006), chemo-preventive (Saleem *et al.,* 2000), H_2O_2 scavenging (Kumar and Chattopadhyay 2007) and anti-mutagenic (Yu *et al.,* 2004) activity of this plant had been reported. This plant also possesses anti-Candida and also radio protective activity against gamma radiation (Marta *et al.,* 2005; Ganesh and Manjeshwar, 2002).

Because of the possible toxicities of the synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), increasing attention has been directed toward natural antioxidants (Namiki 1990), especially plant phytochemicals. The antioxidant potential of different cultivars of tissue cultured *Mentha* has been shown in a number of *in vitro* studies (Akashdeep *et al.,* 2011, 2014). They are capable of direct chain-breaking antioxidant action by radical scavenging. In addition to being capable of scavenging non-physiological radicals such as $DPPH^{\cdot}$ and $ABTS^{\cdot}$ (Payet *et al.,* 2005; Cai *et al.,* 2006), they are capable of scavenging a variety of reactive species such as superoxide, hydroxyl, peroxyl radicals and hypochlorous acid (Halliwell *et al.,* 2005). They can also suppress the formation of reactive oxygen speciesby chelating transition metal ions capable of catalyzing oxidative reactions (Mira *et al.,* 2002).

The application of plant tissue culture has gained major industrial importance in three main areas such as breeding and genetics (Julia and Claudia 2012), as model systems for plant biochemistry and pathology and production of secondary metabolites (Tisserat and Vaughn 2008). Therefore, the application of the tissue culture technique will be very useful in order to conserve and future augmentation of the metabolites present in these very useful medicinal plants. Moreover, antioxidant potential of herbs varies greatly with varietal differences, cultivation conditions and environment (Dragland *et al.,* 2003).

Therefore, the study was planned to study the effect of maturity of tissue cultured (TC) and tissue culture derived (TC_0 andTC_1) *Mentha* spp on phenolic content, flavonoid content, iron chelation and iron reducing potential.

MATERIAL AND METHODS

Plant Material

The *Mentha* spp *viz*. *Mentha piperita* (PPR 611), *Mentha arvensis* (SH), *Mentha spicata* (SPR-8), selected for present studies, were obtained from the School of Agricultural Biotechnology, PAU, Ludhiana.

Micropropagation of *Mentha species*

Preparation of culture medium, Inoculation (culturing) and propagation

Leaf segments from healthy plants were used in present study. The explants were thoroughly washed in running tap water and surface sterilized with mercuric chloride solution ($HgCl_2$) (0.1%) for five minutes, followed by washing with sterile double distilled water to remove traces of $HgCl_2$. The explants of *Mentha* were inoculated aseptically into culture vessel containing Murashige and Skoog medium (1962). Nutrient medium was homogenized by boiling and by continuous stirring before adding agar. The pH of the medium was adjusted as 5.8 prior to addition of agar by using 0.1N NaOH and 0.1N HCl. After adding, 2,4 dichlorophenoxyacetic acid(2,4 D) (1mg/l) in combination with Benzylamino purine (BAP) (0.5mg/l), about 15 - 20 ml of media was dispensed into each culture tube. After autoclaving, the culture vials were kept inside the inoculation chamber. The explants(leaves) of *Mentha* spp were inoculated aseptically into culture vessel. These cultures were incubated at a temperature of 25±2°C with relative humidity (60%-80%).Cultures were given florescent light (3000-5000 Lux), 16 h light/8 h dark regimes.

After one month of culturing, tissue cultured samples (TC) of all the three *Mentha* spp were shifted to soil in glass house for further development and were named as TC_0. After 30 days, cutting (3 cm) of few plants of TC_0 was done and these cuttings were replanted in soil in glass house and were called TC_1. Both these generations of tissue cultured derived plants (TC_0 and TC_1) were grown for the three months. Samples were collected from tissue culture (TC) and tissue culture derived plants (TC_0 and TC1) for further analysis.

Preparation of plant extract

The plant material (leaves) 2 g was crushed with methanol (25 ml) and shaken overnight at 30 °C. Extracts were filtered and residues were re-extracted twice under same conditions using same solvent. Solvents were removed from the combined filtrates under vacuum at 45°C in Buchi rotary evaporator and extracts were stored at -20 °C until further use.

Estimation of phenols and flavonoids

The extracts were analyzed for total phenols and flavonoids and the results were expressed as mg/g dry weight of leaves. Total phenolic compound contents were determined by the Folin-Ciocalteau assay (Ebrahimzadeh *et al.,* 2008 a, b). The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2N Folin-Ciocalteau reagent (Sigma–Aldrich) for 5 min and 2.0 ml of 75 g/l sodium carbonate were then added. The absorbance of reaction was measured at 760 nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA) after 2 h of incubation at room temperature. The samples were prepared in triplicates and mean value of absorbance was obtained. The standard curve was

prepared using 50 to 250 mg/ml solutions of gallic acid in methanol-water (1:1, v/v). Total phenol values are expressed in terms of mg gallic acid equivalents per g dry weight (mg GAE/g DW), which is a common reference phenolic compound.

Flavonoid content of tissue cultured and tissue culture derived plant extracts were determined by the method of Chang *et al.*, 2002. 0.5 mL solution of each plant extracts (at 10% w/v) in methanol were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 mL of distilled water, and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA). Catechin was used as standard to make calibration curve. Total flavonoid content of mint plants was expressed as milligrams catechin equivalentsper gdry weight (mg CEE/gDW).

Evaluation of antioxidant potential using *in vitro* models

Metal chelating activity

The chelating effect on ferrous ions was determined according to the method of Dinis (1994) with some modifications. The method is based upon the ability of the extract to compete with ferrozine for ferrous ions. The various extracts (0.25 ml) were mixed with 1.75 ml of methanol and 0.25 ml of 250 mM $FeCl_2$. This was followed by the addition of 0.25 ml of 2 mM ferrozine, and was allowed to equilibrate for 10 min at room temperature. The purple coloration formed was read at 562 nm. The control contained 80% methanol instead ofthe extract. The chelating activity was calculated according to the equation given below.

Metal chelating activity (%) = ((A0- As)/ As) × 100

Iron reducing activity

Iron reducing power of extract was determined by the method of Oyaizu (1986). Different concentrations of various extracts in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide ($K_3Fe(CN)_6$) (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min and 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 1000 g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The amount of iron(II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussion blue at 700 nm after 10 minutes. Increase in absorbance of the reaction mixture indicated the reducing power. Ascorbic acid was used as standard.

Statistical analysis

All the data was reported as mean ± standard deviation of three replicates.

RESULTS AND DISCUSSION

Phenolics and flavonoid content of *Mentha* spp

Phytochemical constituents in the plant samples are known to be biologically active compounds and they are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer (Hossain and Nagooru, 2011; Suresh and Nagarajan, 2009). Phenolics have antioxidative, antidiabetic, anticarcinogenic, antimicrobial, antiallergic, antimutageneic, and antiinflammatory activities (Saidu *et al.*, 2012; Sasikumar *et al.*, 2010).

Flavonoids also have antioxidant property as they inhibit oxidative and hydrolytic enzymes, have impact on radical scavenging, anti-inflammatory and anti-cancerous activity (Liu *et al.*, 2008; Alsabri *et al.*, 2013). Total phenolic content of methanolic extracts of mint plants was evaluated using Folin Ciocalteau reagent and results were expressed as mg Gallic Acid (GA) equivalents per g dry weight. It is evident from the result that total phenol content of TC plantlets was highest in SPR-8 (3.20 mg/GAE) followed by SH (1.16 mg/GAE) and PPR 611 (0.67 mg/GAE). Tissue culture derived plants (TC_0 and TC_1) had substantially higher content of total phenols as compared to TC plantlets.

The total phenol content increased over the growth period of tissue culture derived (TC_0 and TC_1) plants in all the three species and was found to be highest in TC_1 SPR-8 plants (7.3 mg/GAE) at 3 months of development period followed by PPR 611(mg/GAE). Phenols are very important plant constituents; they show high scavenging ability of free radicals due to their hydroxyl group. Therefore, the phenolic content of plants may contribute directly to their antioxidant action (Tosun *et al.*, 2009). The high correlation between the values of phenol concentration in plant extracts and antioxidant activity is a well-documented study (Borneo *et al.*, 2008; Katalinic *et al.*, 2004).

The secondary plant metabolites flavonoids show significant antioxidant and chelating properties, which arebased on the structure and substitution pattern of hydroxyl groups (Sharififar, 2008). Tissue culture samples of all the three species of *Mentha* had higher content of flavonoids as well (1.85, 0.71 and 3.8 mg CEE for PPR 611, SH and SPR 8 respectively) in comparison to TC_0 and TC_1 samples at either of the growth period. Further, there was gradual decrease in flavonoid content in TC derived plants (TC_0 and TC_1) over a growth period of three months in all the *Mentha* spp. Like total phenols, the flavonoid content of SPR-8 was found to be higher than other species.

However, in TC derived plants (TC_0 and TC_1), PPR 611 had higher content of flavonoids as compared to that of SPR-8 and SH. Therefore, it can be concluded that tissue culture derived plants may vary from the parent plant in the content of secondary metabolites.

An increase in total phenols and flavonoids in TC derived plantlets points out the suitability of plant tissue culture in enhancing the yield of particular phytochemicals. A number of factors like biochemical, physiological and environmental may affect production of secondary metabolites, therefore, a marked difference in secondary metabolites may be due to culture induced variations in different biosynthetic pathways.

Table 1: Total phenol(mg GAE/g DW) and flavonoids(mg CEE/g DW) content of Menthaspp at different development periods.

Stage	Development period (month)	*Mentha piperita* PPR 611	*Menthaarvensis* SH	*Mentha spicata* SPR 8
(Total phenol mg GAE/g DW)				
TC		0.67±0.03	1.16±0.03	3.20±0.02
TC_0	1	3.70±0.03	1.50±0.03	4.36±0.02
	2	4.60±0.03	5.20±0.06	4.70±0.03
	3	6.50±0.06	5.58±0.07	6.80±0.08
TC_1	1	2.70±0.06	2.20±0.05	3.90±0.02
	2	4.60±0.01	5.10±0.06	6.30±0.04
	3	6.15±0.07	5.50±0.06	7.30±0.08
Flavonoids (mg CEE/gDW)				
TC		1.85±0.01	0.71±0.04	3.80±0.04
TC_0	1	1.70±0.01	0.69±0.03	1.68±0.02
	2	1.50±0.01	0.78±0.04	1.12±0.05
	3	1.30±0.01	0.68±0.03	0.97±0.06
TC_1	1	1.49±0.04	0.68±0.02	1.30±0.02
	2	1.40±0.07	0.61±0.06	0.87±0.06
	3	1.20±0.04	0.60±0.05	0.86±0.02

Values are mean ± SD, n=3

Table 2: Reducing power of Mentha spp at different development periods.

Stage	Development period (month)	*Mentha piperita* PPR 611	*Menthaarvensis* SH	*Mentha spicata* SPR 8
TC		1.7±0.03	1.1±0.02	2.4±0.02
TC_0	1	1.6±0.02	2.4±0.03	0.8±0.01
	2	2.3±0.02	1.2±0.01	2.4±0.02
	3	2.2±0.06	1.6±0.02	2.4±0.03
TC_1	1	0.9±0.01	0.7±0.01	1.0±0.02
	2	1.5±0.01	1.4±0.02	1.6±0.03
	3	1.8±0.02	2.3±0.03	2.4±0.05

Values are mean ± SD, n=3

Concentration of ascorbic acid for Δ1 OD = 1100 (µg/ml)

**RRP = Relative reduction potential

$$= \frac{\text{concentration of ascorbicacidfor } \Delta 1 \text{OD } (\mu g/ml)}{\text{concentratiion of extractfor } \Delta \text{ OD}(\mu g/ml)}$$

Table 3: Iron chelation properties of Mentha spp at different development periods.

Stage	Development period (month)	% Fe-Chelation		
		Mentha piperita PPR 611	*Mentha Arvensis* SH	*Menthas spicata* SPR 8
TC		28.96±2.21	25.96±2.28	43.60±3.26
TC_0	1	28.84±3.32	23.83±2.21	42.76±3.57
	2	19.77±1.16	16.41±1.19	40.16±3.98
	3	17.61±1.19	10.09±1.06	36.29±2.71
TC_1	1	18.65±2.23	22.11±2.26	41.58±2.53
	2	8.44±0.98	14.29±1.06	33.2±2.73
	3	6.93±0.03	8.67±1.02	23.16±2.94

Values are mean ± SD, n=3.

Iron reducing power assay (Fe (III) to Fe (II))

Different studies have indicated that the antioxidant effect is related to the presence of reductones (Yen and Duh 1993). Reductones are reported to be terminators of free radical chain reactions (Gordon 1990), thus, the antioxidant activity of a methanolic extractmay be related to its reductive activity. This was done by determining the reduction of ferricyanide to ferrocyanide by the extract. The ferrocyanide so formed was measured by converting it to ferri-ferrocyanide- a blue colored complex. All the TC extracts of different species of *Mentha* possessed the ability to reduce iron (III) and do so in a linear concentrationdependent fashion. Table 2 showed that of all the three species, SPR-8 was the most effective in this regard. This matches well with the reported antioxidant potential of *Mentha* spp especially with respect to hydroxyl and nitric oxide radical scavenging potential (Akashdeep *et al.*, 2011). Further, it is evident from the results that iron reducing potential of tissue culture derived plants (TC_0 and TC_1) increased with developmental period. The increase in iron reducing activity over developmental period matched well with the increase in total phenols and flavonoids.

Iron chelation

Iron is the most common cofactor within the oxygen handling biological machinery and, specifically, lipid peroxidation of biological membranes is the main pathogenic mechanism of iron overload induced tissue damage (Bonkovsky 1991). An effective therapeutic approach can play a double role in reducing the rate of oxidation - one by sequestering and chelating cellular iron stores (Rothman *et al.*, 1992) and other as radical trap (i.e., antioxidant activity). Since plants has shown antioxidant and free radical scavenging activity (Bridges and Hoffman 1986), the present study, primarily incorporates the *in vitro* iron chelation

potency of *Mentha*. Free iron ions are responsible for the generation of free radicals. Thus iron chelation can be useful property to prevent the formation of free radical. Iron chelation properties of *Mentha* extracts at a concentration of 500 µg/ml was determined by its ability compete with ferrozine for iron ions in free solution. For this the extracts were incubated with ferrous ions and ferrozine. Un-chelated ferrous ions would react with ferrozine to give an intense purple-blue colour. From the absorbance of Fe^{2+}-ferrozine complex formed in the presence and absence of extract, the percent chelation was determined. It is evident from the results (Table 3) that tissue culture raised *Mentha* plantlets of SPR 8 or tissue cultured derived plants found to have better iron chelation property in comparison to other two species. Moreover, TC PPR 611, SH and SPR 8 *Mentha* extracts were better in iron chelation in comparison to TC derived plants of respective *species*. Iron chelation activity of TC derived plant samples (TC_0 and TC_1) decreased over the growth period. Moreover, antioxidant can prevent the activity of free radicals either by scavenging or by inhibiting the free radicals. So a plant having both the iron chelating and iron reducing activityis more preferable a plant having one of these activities. The mentha plant has both these activities (Akbar *et al.*, 2014). Iron is known to generate free radicals through the Fenton and Haber-Weiss reaction (Halliwell and Gutteridge 1990). It is an extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components (Smith *et al.*, 1992). Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent by oxidative damage. Likewise, it is reported that the metal ion chelating capacity of the phenols and polyphenols plays a significant role in reducing the concentration of the catalyzing transition metal in lipid peroxidation and stabilizing the oxidized form of the metal ion (Duh *et al.*, 1999, Gordon 1990). Thus the higher degree of iron chelation by methanolic extracts of SPR 8 *Mentha* spp, proves its usefulness.

CONCLUSION

Based on the above mentioned results, it was concluded that the variation in total phenolic and flavonoid content in tissue cultured and tissue culture derived plantlets can be due to culture induced variations in biosynthetic pathways. Also, the iron reducing and iron chelation property was found to be better in *Mentha Spicata* sps. Iron reducing property increased with increase in growth period, however the iron chelation decreased with increase in development period for three months. Also the application of tissue culture techniques in conserving and augmentation of secondary metabolites in *Mentha* spp was evident from the results.

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