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Antioxidant potential of tissue cultured *Mentha spicata*

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ABSTRACT

Environmental pollutants and food contaminants are posing an oxidative stress in humans. The body's endogenous defenses may not be adequate to prevent the damages completely. So, it is pertinent to look for some exogenous source of natural antioxidants that can be incorporated in the diet to modulate body's antioxidant defense mechanisms. Herbs can be important sources of natural antioxidants. Keeping this in view, present study had been carried out to evaluate antioxygenic potential of tissue culture raised *Mentha spicata* (SPR 8). This mentha species was found to be rich in phenolics. Various *in vitro* models viz. hydroxyl, nitric oxide,super oxide radical scavenging potential, iron reducing power, iron chelation and inhibition of linoleic acid peroxidation revealed *Mentha spicata* (SPR 8) to be a potent source of antioxidants. Usefulness of *Mentha spicata* was further proved using *ex vivo* models involving human erythrocytes. Being rich in iron and PUFA erythrocytes are vulnerable to oxidative damage when exposed to molecular oxygen. Moreover, human erythrocytes when incubated at low temperature tend to lose their endogenous antioxidants. The rate of loss of antioxidant potential in the presence of an extract can therefore be an index of antioxidant potential. The *Mentha spicata* (SPR 8) extract protected erythrocytes against induced LPO of erythrocytes as well as osmotic fragility over the incubation period of 72 h. Loss of activities of antioxidant enzymes over the incubation period was prevented to a good extent using *Mentha* extracts. Thus, tissue culture raised *Mentha spicata* (SPR 8) can be a potent source of antioxidants.

Key words: Antioxidants, Erythrocytes ,Herbs, *Mentha spicata* ,Oxidative stress

INTRODUCTION

Antioxidants are molecules that slow or prevent the oxidation of other molecules by scavenging free radicals, which play a major role in the pathogenesis of many age related diseases. In biological systems, reactive oxygen species are produced as by-products or intermediates during normal metabolism [1, 2]. Some of these reactive oxygen species, such as superoxide anion, hydrogen peroxide and nitric oxide are physiologically useful and, infact necessary for life, but can also be harmful if present in excess or in inappropriate amounts. All these oxidants can react with various components of living cell such as proteins, DNA or lipids and cause damage by changing the chemical structure of these components. Their increased concentration in human body causes many pathological conditions such as inflammatory, neurological and psychiatric diseases, and carcinogenesis, etc [3]

Biological systems have developed several protective mechanisms to prevent ROS formation or their detoxification. These protective mechanisms include antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and non-enzymatic antioxidants that repair oxidative cellular damage [4]. The state which is characterized by a disturbance in the balance between ROS production on one hand and ROS removal and repair of damaged complex molecules on the other is called oxidative stress [5]. The endogenous defenses may not be adequate to prevent the damages completely, so it is pertinent to look for some exogenous sources of antioxidants that can be incorporated in the diet to modulate body's antioxidant defense mechanism. Further these efforts may prevent onset or help treat the diseases caused and/or fostered due to free radicals. Use of synthetic antioxidants has been restricted because of their suspected safety concerns [6]. Therefore, identification and development of safer natural antioxidants/compounds is more beneficial and need of present day. Increasing intake of dietary antioxidants may help maintain an adequate antioxidant status and therefore, the normal physiological functions of living system [7].

Recently, herbs have also been identified as source of various phytochemicals, many of which possess important antioxidant activity. Many medicinal plants contain large amounts of antioxidants other than vit C, vit E and carotenoids. Many herb species, especially those of *Lamiaceae* family, such as sage, orenago, thyme, mentha, show strong antioxidant activity [8]. 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 spearmint, *Mentha spicata*, is a hybrid of *Mentha longifolia* and *Mentha rotundifolia* [9]. Members of the genus are characterized by their volatile oils which are of great economic importance, being used by the flavor, fragrance, and pharmaceutical industries[10].

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Thus ,in the present study , the antioxidant potential of tissue cultured *Mentha spicata*(SPR 8) was evaluated using various *in vitro* and *ex vivo* models.

MATERIALS AND METHODS

Plant material and prepration of extracts

The mentha species *Mentha spicata* (SPR-8) was raised through tissue culture in a liquid culture using MS medium, in tissue culture laboratory of School of Agricultural Biotechnology, PAU, Ludhiana,Punjab. Samples were collected after one month of culturing of Mentha SPR 8.

The plant material 2 g was crushed with methano(25ml) and shaken for 12 h at room temperature. After filtration, the residues were re-extracted twice under same conditions. Solvents were removed from the combined filtrates under vaccum at 45°C in Buchi rotary evaporator. The yield of crude extracts was determined gravimetrically. This extract was used for evaluation of *in vitro* and *ex vivo* antioxidant potential. Total phenols[11], ortho- dihydroxyl phenols[12]and flavonols[13] were also determined.

In vitro evaluation :

For *in vitro* antioxidant potential, the extract was evaluated for its hydroxyl [14], nitricoxide [15], superoxide [16] radical scavenging potentials ,inhibition of lipid peroxidation[17], iron reducing power[18] and iron chelation activity[19].

Ex vivo evaluation:

Human blood used for *ex vivo* evaluation was obtained from a disease-free healthy human being with the help of hospital faculty, Punjab Agricultural University, Ludhiana, Punjab. A small volume of blood was used to determine the packed cell volume (PCV) using a hematocrit centrifuge. The blood samples collected were centrifuged at 1000 x g for 10 min at 4°C to obtain erythrocytes. The erythrocytes were washed thrice with phosphate buffered saline (PBS, pH 7.4) and PCV was adjusted to 5% with PBS using its hematocrit value. Erythrocytes preparation was mixed with mentha extract to give a desired concentration of 100 or 200 µg/ml extract and incubated at 10°C for 72 hours. The samples were drawn at 0, 24, 48, 72 h of incubation. A control was run without extract side by side. The samples were then analyzed for antioxygenic potential in terms of their resistance to induced LPO, osmotic fragility and antioxidants enzymes. A portion of erythrocytes prepration was used to determine the Haemoglobin content [20].

Lipid peroxidation (LPO) in Erythrocytes

The LPO in erythrocytes was determined by measuring the malonyldialdehyde (MDA) produced on exposure of erythrocytes to H_2O_2, using thiobarbituric acid (TBA) [21].

Osmotic fragility of Erythrocytes

Osmotic fragility of erythrocytes was estimated by determining per cent hemolysis of erythrocytes in solutions of different NaCl concentration (0.0-0.9%). The percent hemolysis in different saline solutions was calculated assuming 0% hemolysis in normal saline and 100% in 0% saline. Percent hemolysis was plotted against the salt concentration. From the curve salt concentration required for 50% hemolysis was determined [22].

Antioxidant Enzymes

The erythrocyte samples were lysed by diluting sample with distilled water. The lysates were used to determined the activities of Catalase [23], superoxide dismutase[23], glutathione peroxidase[24] and glutathione reductase [25].

RESULTS AND DISCUSSION

The methanolic extract of *Mentha spicata* (SPR 8) was prepared and analysed for total phenol, ortho-dihydroxy phenol and flavonols. Total phenol, Ortho-dihydroxy phenol and flavonol contents of *Mentha spicata* (SPR 8) were found to be 3.20mg/g, 1.46 mg/g and 3.8 mg/g respectively .

In vitro Studies

The methanolic extract of mentha species were analysed for antioxidant potential in terms of their ability to scavenge free radicals produced in *in vitro* systems.

Among the several free radicals, hydroxyl radical ($OH^{\cdot}$) is the most potent oxidant, produced during radiation exposure as well as Fenton raction [26, 27]. In order to evaluate hydroxyl radical scavenging potential of various extracts, the inhibition of deoxyribose degradation by hydroxyl radical generated from Fe^{2+}-ascorbate - EDTA - H_2O_2 system was determined. Concentrations of *Mentha spicata* (SPR 8) extract needed for 50% inhibition (IC_{50}), was found to be 40 µg/ml and was much lower than that of ascorbic acid (118 µg/ml) indicating the potential of *Mentha spicata* (SPR 8) as an important source of antioxidants.

In order to evaluate nitric oxide radical scavenging potential of extract, the inhibition of chromophore formation with Gries reagent by nitric oxide radical spontaneously generated from sodium nitroprusside was determined. The concentrations needed for 50% inhibition (IC_{50}) , was 200 µg/ml for *Mentha spicata* (SPR 8), whereas, IC_{50} value of ascorbic acid was found to be much higher i.e. 2700 µg/ml . Nitric oxide reacts with other radicals such as O_2- to form peroxynitrite ($ONOO^-$), a powerful oxidant and this reaction is widely believed to represent a major pathway for generating reactive nitrogen species *in vivo* [28]. Hence, the excess production of NO can induce oxidation of proteins, lipids and DNA and is associated with several diseases [29,30]. The inhibition of development of chromophore observed may be due to the antioxidant principles in the extract which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite [31]. Thus, to avoid or delay this proxidation process, addition of antioxidants from *Mentha spicata* (SPR 8) to foods can be one of most extensive methods and hence inclusion of mentha as part of regular diet can be highly useful.

Superoxide radical scavenging potential was determined by the inhibition of NBT reduction by superoxide radicals generated from photo-

reduction of riboflavin. Superoxide anion is the first reduction product of oxygen which is measured in terms of inhibition of generation of O_2-. The results clearly depict that methanol extracts of *Mentha spicata* (SPR 8) scavenge the superoxide radicals generated by photoreduction of riboflavin in a concentration dependent manner to a good extent. The concentrations needed for 50% inhibition (IC_{50}) was 255.0 µg/ml for *Mentha spicata* (SPR 8). Ascorbic acid did not show any significant superoxide radical scavenging activity probably because of the oxidation of ascorbic acid by riboflavin in presence of light to ascorbyl semiquinone radicals. Photoexcited riboflavin can perform one electron oxidation of ascorbic acid generating a riboflavin radical [32].

The methanolic leaf extracts of *Piper sarmentosum* (Kodak) and *Morinda elliptica* (Mengkudu) had been also reported to possess high superoxide radical scavenging activity[33]. The aqueous extracts of coriander inhibited superoxide anion in *in vitro* system and 50% scavenging of superoxide radical occurred at concentration of 370 µg/ml of coriander[34].

Thus based upon hydroxyl radical, nitric oxide and super oxide radical scavenging properties of the extracts, it appears that *Mentha spicata* (SPR 8) has potent antioxidant properties.

As discussed earlier *Mentha spicata* (SPR 8) holds good antioxidative properties which could be due to its high content of phenolics. Phenolic compounds are considered as a major group of compounds that contribute to the antioxidant activities of botanical materials because of their scavenging ability on free radicals due to their hydroxyl groups [35,36]. Therefore, it can be concluded that plants rich in phenolic compounds have high antioxidant properties. Plants belonging to the *labiatae* family are rich in polypehnolic compounds and a large number of them are well known for their antioxidant properties [37,38]. Methanolic extracts of *Salvia sp*. were also reported to be most effective DPPH (1, 1, Diphenyl-2-Picryl-Hydrazyl) radical scavenger [39]. *Polygonium hyrcanium* which contains the highest amount of total phenols and may contribute to its potential to scavenge the free radicals [40]. Antioxidant activity of brahma rasayana by its ability to scavenge the hydroxyl, superoxide and nitric oxide radicals has also been reported [41].

Table 1 :Inhibition (%) of linolenic acid peroxidation by *Mentha spicata* (SPR 8)

Concentration (µg/ml)	Time (h) 0.5	1.0	1.5	2.0	3.0	4.0
TC SPR-8						
50	25.00	25.92	44.44	26.82	18.60	6.97
100	40.00	40.74	50.00	51.21	20.93	18.60
200	40.00	40.74	55.55	56.09	39.53	27.90
500	50.00	55.55	66.66	60.77	53.48	30.23
Ascorbic acid (positive control)						
50	5.00	14.81	22.22	13.00	4.65	0.00
100	5.00	18.52	30.56	19.51	9.30	0.00
200	15.00	25.93	33.33	19.51	11.63	0.00
500	25.00	37.03	36.11	24.39	16.28	2.32

The methanolic extracts of mentha species also exhibited inhibition of the peroxidation of linoleic acid in a concentration dependent manner. Methanolic extract of *Mentha spicata* (SPR-8)was found to be more effective in preventing linoleic acid peroxidation, as compared to standard antioxidant ascorbic acid (Table 1). Ascorbic acid showed maximum inhibition of 37.03% at a concentration of 500 µg/ml upto 1 h of incubation. Where as *Mentha spicata*(SPR 8) extract found was more effective and showed maximum inhibition of 66.66% upto 1.5 h of incubation. Being hydrophilic, ascorbic acid may prove less effective antioxidant in oil in water emulsion system. By moving to water phase, the hydrophilic antioxidants become too diluted to adequately protect the oil at oil-water interface. Thus, a poor antioxidant potential of ascorbic acid observed in this system is obvious. On the other hand, the herbal extracts used for in the present study possess some unique structural elements which help them act at an oil-water interface and thus show better activity in linoleic acid system.

The inhibitory activity of clary sage extract (250 µg/ml) towards the peroxidation of linoleic acid was reported to be higher than that of á-tocopherol [42]. In this context, the presence of various flavonoids in herbal extracts might have been involved in inhibition of peroxidation [43]. Different studies have indicated that the antioxidant effect is related to the presence of reductones [44]. Reductones are reported to be terminators of free radical chain reactions [45], thus, the antioxidant activity of an methanolic extract may be related to its reductive activity. This was determined 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. The concentration of ascorbic acid and extract required for increase of 1OD was determined from the graphs and found to be 1100 and 450 µg/ml respectively indicating the higher potency of extract as a reductant.

Free iron ions are responsible for the generation of free radicals. Thus iron chelation can be another 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 pres-

Table :2 Antioxidant activity of tissue cultured *Mentha spicata* (SPR 8) :Effect on induced *in vitro* lipid peroxidation of erythrocytes

Incubation period (h)	MDA (µ moles/g Hb) Control	Concentration of mentha extract 100 µg/ml	200 µg/ml
0	466.82	219.70	164.76
24	521.78	439.40	357.00
48	576.70	466.86	384.46
72	700.30	521.52	439.40

Table :3 Protective role of tissue cultured *Mentha spicata* (SPR 8) on osmotic fragility of erythrocytes

Incubation period (h)	Control	Concentration of mentha extract	
		100 µg/ml	200 µg/ml
SC_{50} value*			
0	0.38	0.30	0.26
24	0.40	0.35	0.33
48	0.48	0.38	0.35
72	0.53	0.50	0.48

**Saline concentrations needed for 50% hemolysis of erythrocytes*

Table :4 Protective role of tissue cultured *Mentha spicata*(SPR 8) :Effect on antioxidant enzymes of erythrocytes

Incubation period (h)	Control	Concentration of mentha extract	
		100 µg/ml	200 µg/ml
Glutathione peroxidase activity (U/mg Hb)			
0	7.25	11.47	11.48
24	4.51	9.19	11.76
48	4.28	8.85	11.64
72	3.26	5.39	8.37
Glutathione reductase activity (mU/mg Hb)			
0	123.8	123.8	123.8
24	85.3	101.8	112.8
48	79.8	82.5	93.5
72	49.5	52.3	60.5
Super oxide dismutase activity (U/mg Hb)			
0	7.33	7.63	7.63
24	4.89	6.21	7.05
48	3.46	5.21	6.23
72	1.58	3.42	4.86
Catalase activity (U/mg Hb)			
0	24.5	24.4	24.5
24	19.5	19.1	21.6
48	15.0	16.7	20.2
72	7.4	10.2	12.6

ence and absence of extract, the percent chelation was determined. It is evident from the results that tissue culture raised mentha extract at a concentration of 500 µg/ml resulted in 43.60% of iron chelation. These results indicate a good chelation property of *Mentha spicata* (SPR 8).

***Ex vivo* studies**

Mentha spicata (SPR 8) has been shown to have a high degree of antioxidant potential as determined by a number of *in vitro* model such as hydroxyl radical, nitric radical, superoxide radical, scavenging potential, inhibition of linoleic acid peroxidation, iron reducing power, iron chelation activity etc. As none of these methods simulate *in vivo* situations, so there is need to develop models which are more close to *in vivo* system and as such *ex vivo* model may be useful. Being rich in metal ions and PUFA, erythrocytes are vulnerable to oxidative damage when exposed to molecular oxygen directly. Moreover they do not have ability to repair membrane damages and regenerate antioxidants [46]. So, the human erythrocytes if preserved in phosphate buffered saline would tend to lose the natural antioxidant potential and thus may become more prone to induced lipid peroxidation and loss of activities of antioxidant enzymes. Thus, in order to evaluate the true antioxidant properties one can look for loss of endogenous antioxidants as a function of preservation period both in the presence and absence of extracts to be analyzed.

The most commonly used indicator of damage is LPO. Increased LPO of erythrocytes could be due to generation of oxygen free radicals and loss endogenous antioxidants [47], which in turn may also lead to increased osmotic fragility of erythrocytes as well. Erythrocytes samples drawn at 0, 24, 48 and 72 h of incubation were analysed for their resistance to induced LPO and activities of antioxidant enzymes such as SOD, CAT, GPX and GR. Increase in TBARS would suggest an increase in oxygen radicals that could be either due to their increased production and/or decreased destruction [48] The results revealed a significantly lower degree of LPO in test erythrocyes as compared to control, indicating increased protection of erythrocytes against H_2O_2 , the effect being better at higher concentration (Table 2). With the increasing incubation period, the antioxidant potential of erythrocytes decreased substantially as evidenced by higher degree of H_2O_2 induced LPO. The loss of antioxidant potential over the incubation period of 72 h was lower in the test groups indicating that mentha extract has a potential to protect antioxidant potential of erythrocytes. The decreased sensitivity of red blood cells to oxidative damage in the presence of mentha extract observed, can be an index of antioxidant potential. Susceptibility of erythrocytes to auto-oxidation pertaining to high oxidative stress in therapeutic situations or in alternate environments can therefore be accepted as a useful and convenient cell system via *ex vivo* incubation of RBC and to evaluate the effects of augmented antioxidative potential through supplementation of herbal extracts.

Lipid peroxidation which is an autocatalytic free radical process had been reported to be inhibited by green tea polyphenols like epigallocatechin gallate that can act as an antioxidant by trapping proxyl radicals [49]. Several constituents in these herbal extract may have counteracted the free radicals through effective scavenging and decreased MDA formation. Inhibition of auto-oxidation of PUFA observed *ex vivo* in the present study is indicative of efficient protective mechanism enhanced by additional antioxidants.

Flavonoids have been shown to possess various biological properties related to antioxidant activity[50]. Flavonoids are very effective scavengers of peroxyl radicals and they are also chelators of metals and inhibit the Fenton and Haber – Weiss reactions, which are important sources of oxygen free radicals. Potential role for Dietary intake of flavonoid containing foods had been reported to lower the

risk of certain pathophysiology that have been related with free radical mediated events[52]. Flavonoids such as isoquercitin and rutin known to be present in coriander [53] may be responsible for antioxidant activity of methanolic extracts. Thus, the observed antioxidant activity of mentha extract may be due to the presence of flavonoids in them.

Protection of erythrocyte membrane integrity and antioxidant enzymes

Free radicals had been reported to induce changes in erythrocytes that results in severe deterioration of membrane structure and function causing increased osmotic fragility, accelerated cell aging and premature cell death ultimately [54]. The reports from several studies had produced clear evidence that there exists a good correlation between type and severity of disease and antioxidant level in the blood[55].

Thus, the oxidative damage to erythrocytes was also determined by determining their osmotic fragility. For this, erythrocyte samples were subjected to osmotic shock with varying concentrations of saline. Percent hemolysis was plotted against saline concentration. Percent hemolysis of erythrocytes in 0.9% and 0% saline were assumed to be 0% and 100% respectively. From the plots, the concentration of saline needed for 50% hemolysis (SC_{50})was determined. Table 3 present the SC_{50} values in the absence and the presence of extracts mentha at the concentrations of 100 and 200 µg/ml for 24 to 72 h. At 0 h, 50% hemolysis (SC_{50}) occurred at 0.38% of saline in control and 0.30 and 0.26% in the test groups indicating the reduced susceptibility to osmotic shock. Moreover, with the increase in incubation period, erythrocyte fragility increased in its magnitude in both test as well as in control groups but the increase recorded was of lower degree in test groups.

These results support the effectiveness of mentha extract in protecting the integrity of erythrocytes and thus establishing protectiveness of mentha extract towards osmotic fragility. In fact, this observation is also supported by the observed lower degree of induced lipid peroxidation. Preincubation of erythrocytes with *Nigella sativa* and *Allium sativum* has been reported to protect erythrocytes against protein degradation, loss of deformability and increased osmotic fragility caused by H_2O_2 [56].

Protection of antioxidant enzymes of erythrocytes

Various enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase constitute a mutually supportive team of defense against ROS. These enzymes react with reactive oxygen species and neutralize them, before they inflict damage in vital cell components. While SOD lowers the steady-state level of O_2ÿ• , catalase and peroxidase do the same for H_2O_2 [57]. Activities of antioxidant enzymes decreased as a function of incubation period for both the control and test (Table 4). Activities of antioxidant enzymes Viz. SOD, CAT, GPX and GR at 0 h time were almost comparable in both the test samples (100 and 200 µg/ml) and control. With the incubation period, the activities of these enzymes decreased and the decrease was relatively slower in the test erythrocytes as compared to the control ones. . Therefore, it can be concluded that incubation of erythrocytes with mentha extract slowed down the loss of antioxidant enzymes. Thus the herbal extract adds to the antioxidant defense system of erythrocytes. This supports the observed increase in resistance of erythrocytes to peroxidative damages.

Tissue cultured *Mentha spicata* (SPR 8) extract , thus, protect the erythrocytes integrity by their reducing vunerability of cells to induced LPO and prevent loss of activity of their antioxidant enzymes . Hence, *Mentha spicata* can be a good source of antioxidants that can be subsequently used in development of functional foods/nutraceuticals .

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