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Pharmacological investigation of methanol extract of *Mentha piperita* L. roots on the basis of antimicrobial, antioxidant and anti-inflammatory properties

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ABSTRACT

Mentha piperita L. (Lamiaceae) leaves have been traditionally implemented in the treatment of minor sore throat and minor mouth or throat irritation by the indigenous people of India, although the compounds responsible for the medicinal properties have not been identified. The plant has been used in the Ayurvedic system of medicine for centuries. The methanol root extract of the plant was evaluated for antioxidant, antimicrobial and anti-inflammatory properties. The extract was found to possess maximum potency against infectious pathogens. Besides the antimicrobial effect maximum antioxidant capacity was observed in methanol extracts. The methanol extract of the roots of the plant also possessed maximum anti-inflammatory activity in carrageenan induced animal model in dose dependent manner at a dose of 50 mg/kg. The values $p<0.05$ were found to be significant. Different other extracts showed no potency in comparison to other solvent extracts of the plant. These active crude methanol extracts were also assayed for cellular toxicity to fresh sheep erythrocytes and found to have no cellular toxicity.

Keywords: *Mentha piperita* root, antimicrobial activity, antioxidant activity, anti-inflammatory activity, methanol extract.

INTRODUCTION

Mentha piperita L. (common name: peppermint) member of the large mint family Lamiaceae, is a fast-growing, perennial herb which can reach up to 1.5m high in favorable conditions. *M. piperita* is an extremely variable species with a widespread distribution in Europe, Mediterranean region, and eastwards into Asia. In Indian folk medicine, the leaves are used for relief of minor sore throat and minor mouth or throat irritation. It is also used in treatments for

minor aches and sprains, and in nasal decongestants in addition to its antiparasitic, carminative, antiseptic and stimulant properties [1]. Menthol ($C_{10}H_{20}O$) is a terpenoid, found in the essential oils of the mint family (Mentha sp.). It is a waxy, crystalline substance, clear or white in color, which is solid at room temperature. Several isomers of menthol exist, some with a menthol smell, others without. In nature it only occurs as (-) menthol, which has the strongest smell and its formal name is (1R, 2S, 5R)-isopropyl 5methylcyclohexanol. The other isomers are known as isomenthol, neomenthol and neoisomenthol (-) menthol can be described as fresh, sweet, minty, cooling, refreshing. The (+) isomer is similar, but less minty, more herby, with musty, bitter, phenolic and herbaceous notes, and is less refreshing. (-) menthol has also got about four times the cooling power of the (+) isomer [2]. Antimicrobial, antioxidant and anti-inflammatory properties of the plants were reported [3-7].

MATERIALS AND METHODS

Experimental Section

All the chemicals and reagents used were from C.D.H and Ranchem. Glass wares used were from Borosil. The media and broth used for microbial culture were from Hi-Media Pvt. Limited, Bombay, India.

Plant material

The authenticated sample was collected from local gardens of Dehradun (U.K), India and was further confirmed in Botanical Survey of India (BSI), Dehradun. Voucher specimens have been deposited in BSI, Dehradun, India.

Preparation of plant extracts

The method [8] was adopted for preparation of plant extracts with little modifications. Briefly four 20 g portions of the powdered plant material were soaked separately in 100 ml of water, hexane, methanol and petroleum ether for 72 h. Each mixture was stirred after every 24 h using a sterile glass rod. At the end of extraction, each extract was passed through Whatmann filter paper no1 (Whatmann, England). The filtrate obtained were concentrated in vacuo using rotary evaporator at 30^{o}C.

Determination of Antibacterial and Antifungal activity

Culture Media

The media used for antibacterial test was soyabean casein digest agar/broth and Sabouraud's dextrose agar/broth of Hi media Pvt. Bombay, India.

Inoculum

The bacteria were inoculated into soyabean casein digest agar/broth and incubated at 37^{0} C for 4 h and the suspension were checked to provide approximately 10^{5} CFU/ml. Similar procedure is done for fungal strains by inoculating in Sabouraud's dextrose broth for 6 h.

Microorganisms used

The test organisms (*Bacillus subtilis* ATCC6051, *Proteus vulgaris* ATCC 6380, *Salmonella typhimurium* ATCC 23564, *Pseudomonas aeruginosa* ATCC 25619, *Escherichia coli* K-12, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus faecalis*, *Lactobacillus acidophilus*

were the bacterial strains obtained from institute of Microbial Technology (IMTECH) Chandigarh, India. The fungal test organisms used for study are *Candida albicans, Aspergillus niger, Saccharomyces cerevisae* and *Penicillium notatum* obtained from pure lab cultures of Roorkee Research & Analytical Laboratories, Roorkee (U.K), India.

Determination of antimicrobial activity

The agar well diffusion method [9] was modified. Soyabean casein digest agar (SCDA) was used for bacterial cultures. The culture medium was inoculated with the microorganism separately suspended in soyabean casein digest broth. Sabouraud's dextrose agar (SDA) was used for fungal cultures. The culture medium was inoculated with the fungal strains separately suspended in Sabourauds dextrose broth. A total of 8 mm diameter wells were punched into the agar and filled with plant extracts and solvent blanks (distilled water, hexane, methanol and petroleum ether as the case may be). Standard antibiotic (Amphotericin, concentration 1mg/ml) was simultaneously used as positive control. The bacterial plates were then incubated at 37^{o}C for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. The same procedure was done for determining antifungal activity but in this case standard antibiotic (Streptomycin, concentration 1 mg/ml) was used as positive control and fungal plates were incubated at 37^{o}C for 72 h. Here also the diameter of zone of inhibition observed was measured.

Determination of MIC and MBC

The antibacterial and antifungal plant extracts were then after evaluated to determine MIC and MBC values. The serial dilution technique by using N-saline for diluting the plant extract was adopted and serially diluted plant extract tubes were incubated for 48 h. The minimum dilution of the plant extract that kills the bacterial and fungal growth was taken as MLC (Minimum lethal count) while the minimum dilution of plant extract that inhibits the growth of the organism was taken as MIC.

Determination of cellular toxicity using sheep erythrocytes

The method [10] was employed to study cellular toxicity. Briefly 10 fold serial dilution of the extract were made in phosphate buffered saline. A total volume of 0.8ml for each dilution was placed in an ependroff tube. A negative control tube (containing saline only) and a positive control tube (containing tap water) were also included in the analysis. Fresh sheep erythrocytes were added to each tube, to give a final volume of 1 ml. Solutions were incubated at 37^{o}C for 30 minutes and all tubes were centrifuged for 5 minutes and then observed for hemolysis.

Determination of *in vitro* antioxidant activity

Determination of Antioxidant Activity by DPPH Radical Scavenging Method

The extract solution for the DPPH test [11] was prepared by re-dissolving 0.2 g of each dried extract in 10 ml methanol. The concentration of DPPH solution was 0.025 g in 1000 ml of methanol. Two ml of the DPPH solution was mixed with 10, 20 and 40 µl of the plant extract/methanol solution and transferred to a cuvette. The reaction solution was monitored at 515 nm, after an incubation period of 30 minutes at room temperature, using a UV-Visible Systronics spectrophotometer.

The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

Inhibition%= (Abst=0 min -Abst=30 min)/ Abst=0 min ×100

Where Abst=0 min was the absorbance of DPPH at zero time and Abst=30 min was the absorbance of DPPH after 30 minutes of incubation. Ascorbic acid (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of plant extract solution to the Ascorbic acid equivalent. IC50 is the concentration of the sample required to scavenge 50% of DPPH free radicals.

Determination of Superoxide Anion Radical Scavenging Activity

Superoxide Anion Radical scavenging Activity was measured [12] with some modifications. The various fractions of root extracts of the plant were mixed with 3 ml of reaction buffer solution (pH 7.4) containing 1.3 µM riboflavin, 0.02 M methionine and 5.1 µM NBT. The reaction solution was illuminated by exposure to 30W fluorescent lamps for 20 minutes and the absorbance was measured at 560 nm using Systronics UV-VIS spectrophotometer. Ascorbic acid (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of plant extract solution to the Ascorbic acid equivalent. The reaction mixture without any sample was used as negative control.

The Superoxide anion radical scavenging activity (%) was calculated as:

$$\frac{Ao - As}{Ao} \times 100$$

Where, Ao = absorbance of positive control
As = absorbance of sample

Determination of *in vivo* anti-inflammatory activity

Animals

Male albino rats (180–200 g) were used taking into account international principles and local regulations concerning the care and use of laboratory animals [13]. The animals had free access to a standard commercial diet and water *ad libitum* and were kept in rooms maintained at 22 ± 1ºC with a 12-h light/dark cycle. The institutional animal ethical committee has approved the protocol of the study.

Carrageenan-induced edema in rats

6 Groups of five animals each were used. Paw swelling was induced by sub-plantar injection of 0.1 ml 1% sterile carrageenan in saline into the right hind paw. The solvent extracts of *M.piperita* at dose of 50 mg/kg were administered orally 60 minutes before carrageenan injection. Aspirin (10 mg/kg) was used as reference drug. Control group received the vehicle only (10 ml/kg). The inflammation was quantified by measuring the volume displaced by the paw, using a plethysmometer at time 0, 1, 2, 3, and 4 h after carrageenan injection.

Statistical analysis

The results were expressed as mean ± S.D. Statistical significance was determined by analysis of variance and subsequently followed by Turkey's tests. P values less than 0.05 were considered as indicative of significance. The analysis was performed using INSTAT statistical software.

RESULTS AND DISCUSSION

Determination of antimicrobial activity

The antimicrobial activity was determined by measuring the diameter of zone of inhibition recorded. Methanol extracts was found to be most potent antimicrobial agent in comparison to other extracts. Hexane and petroleum ether extracts showed similar antimicrobial activity but less significant in comparison to methanol extracts (results of antimicrobial effect of other solvent extracts are not mentioned). The results are in accordance with the previous studies done on this aspect. The initial screening of antimicrobial activity of extracts were assayed *in vitro* by the agar diffusion method using active against all tested bacterial and fungal strains (Table 1). The highest inhibitory effect was observed against *S. mutans* (zone of inhibition: 25.3 mm) while the weakest activity was demonstrated against *S. aureus* (zone of inhibition: 15.0 mm). In view of the results obtained by the well diffusion method, the minimal inhibitory concentration MIC of menthol was determined by broth microdilution assay (Table 2). The highest MIC value (15.6 µg/ml) was observed against *P.aeruginosa, S. pyogenes, B.subtilis, E.coli K-12, S. mutans* and *Salmonella typhimurium* while *S. faecalis, S. aureus* and *L. acidophilus* ranked next (MIC 31.2 µg/ml). Moreover, methanol extract observed good antifungal activity against the fungal strains i.e. *Candida albicans*, *Saccharomyces cerevisae* and *Penicillium notatum* (zone of inhibition range: 15.0–18.5 mm; MIC: 125.0). The least antifungal activity was observed against *Aspergillus niger* (zone of inhibition: 15.0 mm).The standard drug streptomycin was active against all reference bacteria (zone of inhibition range: 17.9–26.2mm; MIC range: 15.6–7.8 µg/ml). In addition, amphotericin B demonstrated good antifungal activity against all the fungal strains (zone of inhibition range: 17.6 -18.5mm; MIC range: 17.4-7.8 µg/ml). *In vitro* studies in this work showed that methanol extract inhibited the growth of all tested bacteria except *S.aureus* and observed good antifungal activity against all the fungal strains except *A.niger*. The zones of inhibition ranged from 15.0–25.3 mm and 15.0–18.5 mm in diameter against bacterial and fungal strains respectively using the well diffusion method. Furthermore, MIC values ranged from 15.6–31.2 µg/ml against tested bacteria except *S.aureus* and 125.0 µg/ml against all fungal strains except *A.niger*. The experiments were performed in triplicates and the mean of diameter of zone of inhibition were observed. Further screening of the methanol extract confirmed the presence of menthol as the active constituent responsible for antimicrobial activity (results are not mentioned). Menthol ($C_{10}H_{20}O$) is a terpenoid, found in the essential oils of the mint family (Mentha sp.). Terpenes or terpenoids have been previously reported to be active against bacteria [14, 15] fungi [16, 17], viruses [18, 19] and protozoa [20, 21]. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds.

Table 1: Diameter of zone of inhibition (mm) of methanol extract of root from *Mentha piperita* against pathogenic microorganisms Diameter of zone of inhibition (mm)

Microorganisms	Methanol extract (200 µg/ml)	Control	
		S	A
Staphylococcus aureus	15.0	25.6	-
Streptococcus mutans	25.3	20.2	-
Streptococcus faecalis	22.3	18.6	-
Streptococcus pyogenes	23.5	19.8	-
Lactobacillus acidophilus	20.0	21.7	-
Pseudomonas aeruginosa	24.2	19.6	-
E.coli K-12	24.3	26.2	-
Bacillus subtilis	23.4	25.5	-
Salmonella typhii	24.2	24.6	-
Candida albicans	18.5	18.6	17.6
Aspergillus niger	15.0	19.5	18.5
Penicillium notatum	17.3	18.7	18.5
S.cerevisae	17.5	17.9	17.8

S, Streptomycin; A, Amphotericin (1mg ml^{-1}); -, Not tested

Table 2: Minimum Inhibitory Concentration (MIC) of methanol extract of root from *Mentha piperita* against pathogenic microorganisms

	Minimum Inhibitory Concentration (MIC)		
Microorganisms	Methanol extract (200 µg/ml)	Control	
		S	A
Staphylococcus aureus	31.2	7.8	-
Streptococcus mutans	15.6	15.6	-
Streptococcus faecalis	31.2	15.6	-
Streptococcus pyogenes	15.6	15.6	-
Lactobacillus acidophilus	31.2	15.6	-
Pseudomonas aeruginosa	15.6	15.6	-
E.coli K-12	15.6	7.8	-
Bacillus subtilis	15.6	7.9	-
Salmonella typhii	15.6	7.9	-
Candida albicans	125	15.6	17.4
Aspergillus niger	125	15.6	7.8
Penicillium notatum	125	15.6	7.8
S.cerevisae	125	15.6	17.4

S, Streptomycin; A, Amphotericin (1mg ml^{-1}); -, Not tested

Determination of Antioxidant Activity by DPPH Radical Scavenging Method

DPPH Radical scavenging activity was determined of each of the fractions of the plants extracts. The experiments were performed in triplicates and mean values of Antioxidant activity of each of the fraction of the plant extract were determined. The four extracts of *Mentha piperita* (root) tested for antioxidant activity using DPPH radical scavenging method were determined, the methanol and aqueous successive extracts showed the maximum antioxidant activity with IC_{50} values of 36.15 µg/ml and 32.10µg/ml, respectively. The petroleum ether and hexane extracts also showed antioxidant activity with IC_{50} values of 45.10 and 42.57 µg/ml. The known antioxidant ascorbic acid exhibited IC_{50} value of 78.7 µg/ml as shown in Table 3.

Table 3: Antioxidant Activity by DPPH Radical Scavenging Method

Plant	DPPH-Radical Scavenging Method (IC_{50}); µg/ ml			
	M	W	P	H
Mentha piperita	36.15	32.10	45.10	42.57

Ascorbic acid = 78.17 µg/ml

The results are the average of three determinations for extracts.

Determination of Antioxidant activity by Superoxide Anion Radical Scavenging Activity

Superoxide anion radical scavenging was determined of each of the fractions of the plant extracts. The experiments were performed in triplicates and mean values of antioxidant activity of each of the fraction of the plant extract were determined. The values of antioxidant activity determination by Superoxide Anion Radical Scavenging method follow the same order as that of DPPH assay. Among the four extracts of *Mentha piperita* (root) tested for antioxidant activity using Superoxide Anion radical scavenging method, the methanol and aqueous successive extracts showed the maximum antioxidant activity with 65.68 % and 62.34 % inhibition. The petroleum ether and hexane extracts also showed antioxidant activity with 56.67% and 54.18% inhibition. The known antioxidant ascorbic acid exhibited 87.8% inhibition as shown in **Table 4.**

Table 4: Antioxidant Activity by Superoxide Anion Radical Scavenging Method

Plant	Superoxide Anion Radical Scavenging Method (%)			
	M	W	P	H
Mentha piperita	65.68	62.34	56.67	54.18

Ascorbic acid = 87.80%

The results are the average of three determinations for each of the extracts

Determination of Anti-inflammatory activity

Carrageenan-induced edema in rats

The anti-inflammatory effects of the solvent extracts of roots of *Mentha piperita* on carrageenan-induced edema in rat's hind paws are presented in Table 5. The anti-inflammatory activities of extracts were found to have effect in dose-dependent manner. There was a gradual increase in edema paw volume of rats in the control group. However, in the test groups, methanol extract and aqueous fractions (100 mg/kg) showed a significant reduction in the edema paw volume. There was no reduction in inflammation found in case of rats treated with petroleum ether and hexane extracts. The results showed that methanol fractions of the roots causes significant reduction in inflammation i.e 95 % (50 mg/kg) followed by crude aqueous extract i.e 80 % (50 mg/kg) compared to standard anti-inflammatory drug aspirin i.e 68.62% (25 mg/kg). The values of reduction in paw volume, 0.10 ± 0.002, 0.14 ± 0.002 and 0.16 ± 0.002 were found significantly of methanol extract, aqueous extract and aspirin, respectively at 4 h after carrageenan administration. The present study provides evidence that the methanol fraction and aqueous extract of *Mentha piperita* acts as potent anti-inflammatory agent in rats in acute inflammation model.

Table 5: Anti-inflammatory activities of different extracts of *Mentha piperita*
Paw volume (ml) ± SD

Experiment	Control	Aspirin (25mg/kg orally)	Methanol extract (50 mg/kg)	Aqueous extract (50mg/kg)	Petroleum ether (50mg/kg)	Hexane (50 mg/kg)
1h after treatment	0.25±0.003	0.21±0.003	0.23± 0.003	0.28±0.003	0.20±0.003	0.34±0.003
2h after treatment	0.25±0.003	0.18±0.003	0.20± 0.003	0.24±0.003	0.15±0.003	0.34±0.003
4h after treatment	0.25±0.003	0.16±0.002	0.10± 0.002	0.14±0.002	0.30±0.002	0.34±0.002

±, S.D, Standard Deviation

CONCLUSION

A number of EO components have been registered as flavorings in foodstuffs. The flavorings registered are considered to present no risk to the health of the consumer and include amongst others carvacrol, carvone, cinnamaldehyde, citral, p-cymene, eugenol, limonene, menthol and thymol [22]. In the present study, the active compound present in methanol extract of the roots of the plant demonstrated promising antimicrobial activities against the most prevalent microorganisms in oral infections. Simultaneously the extract showed prominent antioxidant and anti-inflammatory activity. The use of this plant in the treatment of sore throat, mouth or throat irritation as well as an antioxidant and potent anti-inflammatory agent is validated, scientifically supported by the results obtained in this work.

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