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Evaluation of γ-irradiation treatment on the antibacterial activities of *Mentha piperita* L. essential oils *in vitro* and *in vivo* systems (CLP inflammatory model)

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► ***Short report***

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ABSTRACT

Background: *Mentha piperita L.* essential oils have different antibacterial activity. In the present study, we investigated the effect of γ–irradiation on the antibacterial activities of *Mentha piperita* L. essential oils *in vitro* and *in vivo* systems. ***Materials and Methods***: The aerial parts of peppermint were irradiated in a cobalt^{60} source with 0, 10 and 25 kGy absorbed doses. Then, the plants were subjected to Clevenger extraction to obtain essential oils. The peppermint oils were evaluated for the potential activity against four pathogenic bacteria: *Escherichia coli*, *Pseudomonas aeruginosa, Bacillus cereus* and *Staphylococcus aureus in vitro* system. The antibacterial assays were determined by using agar well diffusion and disk diffusion methods. In addition, a broth macrodilution method was utilized to evaluate the minimum inhibitory concentrations (MIC's) of the peppermint essential oils. For evaluation the *in vivo* antibacterial activity, a surgical procedure by Cecal Ligation and Puncture (CLP) in rats was used. The blood samples were taken from the rats for blood colony forming units (CFU) determination. ***Results:*** The results indicated that the oil was effective in reducing CFU caused by sepsis-induced CLP operation. Also, the peppermint oil was effective against all Gram positive and Gram negative organisms tested *in vitro* system. Irradiated peppermint had no significant antibacterial effects compared with non irradiated one. ***Conclusion***: This study indicated the antibacterial activities of peppermint oils both *in vivo* and *in vitro* systems which is sustained even after gamma-irradiation treatment.

Keywords: *Mentha piperita L, essential oils, γ-irradiation, antibacterial activity, sepsis, CLP.*

INTRODUCTION

One of the most reliable and clinically relevant rodent models to mimic human polymicrobial sepsis in the animal model (murine) is cecal ligation and puncture (CLP) [1]. To treat the sepsis, medicinal plants are an important therapeutic aid for various ailments [2]. *Mentha piperita L.* (common name: peppermint) member of the large mint family Lamiaceae is also used topically as an anti-inflammatory [3], antiviral [4] antifungal [5] and antibacterial activity against some Gram positive and Gram negative bacteria [6]. In this study, for the first time, the *in vitro* antibacterial activities of peppermint essential oil were examined against different species of Gram negative and Gram positive bacteria in compare to its antibacterial effects *in vivo* sepsis model such as CLP.

On the other hand, one of the most problem using medicinal plants is their microbial contamination. Therefore, decontamination of aerial parts of peppermint by a safe method

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(γ-irradiation) can be an effective way to treat various problems in food supply pollution [7]. So, in this study, for the first time, the antibacterial activities of irradiated peppermint oil at two radiation doses of 10 kGy (the maximum permissible dose for radiation in food) and 25 kGy (sterile dose that all biological agents contaminating peppermint die) were studied to consider whether the γ-irradiation preserved the *in vivo* and *in vitro* antibacterial activities [8].

MATERIALS AND METHODS

γ-irradiation treatments and oil extraction

Plant samples were purchased in May-June from Isfahan, Iran. Fresh Iranian cultured peppermints (50 g) were packed in heat-sealed polyethylene pouches. They were passed by a Co60 source for irradiation at two doses (10 and 25 kGy) using a high dose rate research irradiator (Co^{60} Gamma cell 220 [AECL1]; Canada). The temperature and dose rate for all the samples were 22–23°C and 0.37 Gy/s, respectively. The dose range within the samples was 20% of the actual dose. The control and irradiated samples were stored in plastic containers at room temperature (28–30°C) under similar conditions [9].

The oils of irradiated and non-irradiated samples were isolated by the hydrodistillation method with a Clevenger-type apparatus for 2 hours. The extracted oils were stored in colored bottles in a freezer until further use. The isolation yields for the oil derived from non-irradiated and irradiated peppermint were 0.9% (w/w) [9].

Microbial strains and growth media

Escherichia coli (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus cereus*(ATCC 9634) and *Staphylococcus aureus* (ATCC 25923) were purchased from the Microbial Culture Collection Bank, Qom University of Medical Sciences. All bacteria were cultured on Mueller Hinton Agar (MHA) (Merck) at 37°C for 18–24 h. The bacterial suspensions were made in sterile saline to a concentration of approximately 1.5×10^8 CFU/mL (0.5 Macfarland standards).

Agar diffusion tests

Screening of antibacterial activity was performed by well diffusion and disc diffusion techniques. The MHA plates were seeded with 0.1 ml of the standardized inoculum of each test organism. For agar well technique, sterile Pasteur pipette of 6 mm diameter was used to cut uniform wells on the surface of the inoculated MHA plates. 25 µl of pure oil and Gentamicin 0.1mg ((Mast Co., UK) as positive control) added to each well. For disc diffusion technique, filter paper discs (6 mm in diameter) were impregnated with 25 µl of the pure oil. Discs of infusion and antibiotic discs included ciprofloxacin (5µg), chloramphenicol (30µg) and gentamicin (10µg) (Mast Co., UK) were placed on the surface of the inoculated MHA plates**.** The inoculated plates were incubated at 35-37° C for 24 h. After incubation, inhibition zone diameters were measured to the nearest millimeter [10-11]. All tests were performed in triplicate.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The culture tube containing 1 ml sterilized Mueller-Hinton Broth (MHB) was inoculated with 25 µl of peppermint oils and 975 µl Dimethyl sulfoxide (DMSO) as solvent. 100µl of bacterial suspension was added uniformly to each test tube and incubated at (37±1)˚c in 24-48 h. The tubes contain MHB and bacterial suspension was used as positive growth control. After 48 h of incubation, the tubes showing no increase in turbidity at each time interval (24-48 h) were streaked onto MHA plates to check the growth. All tests were performed in triplicate.

Animal treatment: Cecal Ligation and Puncture (CLP) model

Male Wistar rats (260±20g), were purchased from the Animal Housing Rooms (AHRs), Azad University, Qom, Iran. Polymicrobial sepsis was induced by CLP model. First, animals were anesthetized with an i.p. injection of a mixture of xylazine and ketamine (mg/kg b.w). After the initial incision, the cecum was ligated distal of the ileocecal valve, without causing intestinal

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obstruction and was punctured (20-gauge needle) twice in the midline. The incision was closed with a 3-0 suture wire. Then, the scruff of each rat was injected normal saline (37 °C; 5 ml per 100 g b.w) subcutaneously for fluid resuscitation.

The animals were divided in to 8 groups (n=5/ group). In Sham-operated group (SOP), rats undergone laparotomy and received DMSO as vehicle. In CLP group, animals received vehicle alone after operation. In CLP treated groups, oils (100 or 200 mg/kg b.w) were injected intraperitoneally (i.p) immediately after CLP. Peppermint oils were diluted in DMSO and injected immediately after CLP. 24 h after CLP operation, blood samples were collected for CFU count.

Blood culture method

Blood bacterial counts were estimated by the pour plate method. After blood sampling, 10-fold serial dilutions of blood were made in saline normal. 1ml of each dilution was transferred rapidly to Brain Heart Infusion (BHI) agar plates and was incubated for 48 h at 37°C. Plates that contained between 30 and 300 colonies were counted by colony counter (colony forming units=CFU).

Statistical analysis

Data are presented as means ± standard error. The results were subjected to one-way analysis of variance followed by Tukey's honestly significant differences using SPSS 13.0 software. The significance was considered as $P < 0.05$.

RESULTS

Agar diffusion methods

Table 1 shows antibacterial activity (zone of inhibition in millimeters) of the peppermint oil. All strains except *P. aeruginosa* were sensitive to the oil and the most sensitive strain was *B. cereus* (P<0.05). *S. aureus* and *E. coli* (P>0.05) showed less sensitivity as compared with *B. cereus*. Irradiated peppermint with gamma radiation (10 and 25 kGy) had no effect on the antibacterial activity of essential oils (P>0.05).

As shown in table 2, peppermint oil showed maximum activity against *S. aureus* and *B. cereus* producing the maximum zone of inhibition (31mm) (30mm) respectively (P>0.05), followed by *E. coli* (5^3 mm) (P<0.05). *P. aeruginosa* was resistant to the oils (P>0.05). Comparison between peppermint oil and the antibiotics studied showed more inhibition zone of the oil on the *S. aureus* (P<0.05). All strains were found to be sensitive to all the antibiotics (P<0.05) while *P. aeruginosa* was found sensitive to all the antibiotics except chloramphenicol (P>0.05) (table 2). Irradiated peppermint (10 and 25 kGy) had no effect on the antibacterial activity of the essential oils (P>0.05).

MIC and MBC method

The results of the MIC and MBC determinations are summarized in table 3. A progressive effect of the antibacterial activity with increase in the concentration of the oil was observed. Oil examined exhibited antibacterial activity, which generally increased in the following order: *P. aeruginosa* < *E. coli* < *B. cereus* < *S. aureus* (P<0.05). There is no significant antibacterial activity between *P. aeruginosa* and *E. coli* as Gram negative bacteria (P>0.05), and *B. cereus* and *S. aureus* as Gram positive bacteria in MIC (P>0.05).

MBC of the strains were equal to the amount of their MIC values. Irradiated peppermint with gamma radiation (10 and 25 kGy) had no effect on the antibacterial activity of essential oils in both MIC and MBC (P>0.05).

Blood culture

Total bacterial blood counts are presented in table 4. According to our finding, no colony forming units (CFUs) was observed in blood culture in SOP group, whereas in CLP group 100% cultures were positive. According to the results, bacterial load in the groups treated with the essential oils significantly reduced in compared to CLP group (P<0.05). There was no significant differentiation between irradiated and non-irradiated peppermint oil (P>0.05) meaning that the irradiated essential oils sustained the antibacterial activities.

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Table 1. Diameter of microbial inhibition zone (mm) of peppermint essential oils determined by Agar well method.

Microorganisms / Dose (kGy)	*Escherichia coli* (ATCC 25922)	*Pseudomonas aeroginosa* (ATCC 27853)	*Bacillus cereus* (ATCC 9634)	*Staphylococcus aureus* (ATCC 25923
0 (control)	13.66± 0.33[b]	nz	28.83± 0.16[a]	12 ± 0[b]
10	13.83± 0.16[b]	nz	28.66± 0.16[a]	11.33 ± 0.33[b]
25	13.66± 0.16[b]	nz	28.5± 0.28[a]	11.5 ± 0.28[b]

Data are mean ± SEM of three analyses. nz, no zone. [a] P<0.05 is considered as the most sensitive as compared to S. aureus and gram-negatives. [b] P<0.05 is considered as the least sensitive as compared to B. cereus (P>0.05).

Table 2. Diameter of microbial inhibition zone (mm) of peppermint essential oils determined by Disk diffusion method.

Microorganisms / Dose (kGy)	*Escherichia coli*	*Pseudomonas aeruginosa* (ATCC 27853)	*Bacillus cereus* (ATCC 9634)	*Staphylococcus aureus*
0 (control)	19.83 ± 0.16[b]	nz	29.5 ± 0.28[a]	30.5 ± 0.28[c,a]
10	18.16 ± 0. 6[b]	nz	30 ± 0.28[a]	31 ± 0.57[a,c]
25	18.16 ± 0.44[b]	nz	29.66 ± 0.33[a]	30.66 ± 0.33[a,c]
Antibiotics				
Ciprofloxacin	23.83 ± 0.16	32.66 ± 0.33	35.66 ± 0.33	26.66± 0.33
Chloramphenicol	22 ± 0.000	nz	34.83 ± 0.1	20.83 ± 0.16
Gentamicin	21.66 ± 0. 33	24.66 ± 0.33	33.83 ± 0.16	22 ± 0.000

Data are mean ± SEM of three analyses. nz, no zone. [a] P<0.05 is considered as the most sensitive as compared to gram-negatives (P>0.05). [b] P<0.05 is considered as the least sensitive as compared to B. cereus and S. aureus. (P>0.05). [c] P<0.05 is considered significantly different from tests antibiotics for each bacterium, within each column.

Table 3. MIC and MBC determinations of the peppermint essential oils.

Doses (kGy)		0		10		25	
		MIC	MBC	MIC	MBC	MIC	MBC
		EO con.	EO con.	EO con.	EO con.	EO con.	EO con.
Microorganisms	***Bacillus cereus***	5.2 ± 1.04	5.2 ± 1.04	6.25 ± 0	6.25 ± 0	6.25 ± 0	6.25 ± 0
	Staphylococcus aureus	2.08± 0.52[a]	2.6 ± 0.52[a]	1.04 ± 0.26[a]	1.04 ± 0.26[a]	1.04± 0.26[a]	1.04± 0.26[a]
	Escherichia coli	6.25 ± 0	6.25 ± 0	5.2 ± 1.04	5.2 ± 1.04	6.25 ± 0	6.25 ± 0
	Pseudomonas aeroginosa	6.25 ± 0	12.5 ± 0	8.33 ± 2.08	10.41 ± 2.08	4.16 ± 1.04	6.25 ± 0

Data are mean ± SEM of three analyses. EO con., essential oil concentration (mg/mL); MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration. [a] P<0.05 is considered as the most sensitive as compared to P. aeroginosa, B. cereus and E. coli (P>0.05).

Table 4. Blood bacterial number (Cfu/ml) in sepsis rats treated with Peppermint essential oils before and after ϒ- irradiation.

Groups	Dillution 10^{-2}
Laparotomy (DMSO)	0
CLP (DMSO)	22172 ± 3.8[*]
CLP+non-irradiated E.O (100 mg/kg b.w)	680 ± 4.9[**]
CLP+Irridiated E.O (10 kGy) (100 mg/kg b.w)	7788 ± 4.8[**]
CLP+Irridiated E.O (25 kGy) (100 mg/kg b.w)	2580 ± 1.6[**]
CLP+non-irradiated E.O (200 mg/kg b.w)	8220 ± 4.8[**]
CLP+Irridiated E.O (10 kGy) (200 mg/kg b.w)	63 ± 0.34[**]
CLP+Irridiated E.O (25 kGy) (200 mg/kg b.w)	40 ± 4[**]

In Sham- operated group (SOP),rats undergone laparotomy and received DMSO as vehicle; In CLP group, animals received vehicle alone; CLP+ E.O groups, E.Os (100mg/kg and 200mg/kg) was injected (i.p) immediately after CLP. Values of mean ± S.E.M. obtained from five rats.
**P<0.05 is considered significantly different from laparatomy group within each group.*
*** P<0.05 is considered significantly different from CLP group within each group.*

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DISCUSSION

Present study reveals the antimicrobial susceptibility of peppermint oils (irradiated and non irradiated) *in vitro* and *in vivo* system. The *in vitro* antibacterial affects of essential oils were tested against *E. coli*, *S. aureus, B. cereus* and *P. aeruginosa.* The results indicated that the peppermint oil was effective against organisms tested (tables 1-3). Inefficient penetration of oil in agar medium as compared with broth medium was required a higher concentration of oil to inhibit the growth of pathogenic bacteria in agar well method. [12]. In 2010, Sharafi *et al.*, showed the antibacterial effect of *peppermint* against some bacteria by agar diffusion method [13]. Organisms tested were sensitive to the oil with the sensitivity order of *E.coli*> *S. aureus* > *Pseudomonas aeruginosa*. The results of MIC and MBC observed in the present study are similar with the studies of Sokovic *et al.* [14] who reported that peppermint oil had antibacterial activity against *S. aureus, E. coli* and *P. aeroginosa*. In another study [6], antimicrobial effect of mint essence against 21 pathogen microorganisms was showed that this essence strongly inhibits growth of pathogen microorganisms. Similar results are also reported in peppermint oil against Gram positive and Gram negative bacteria [12].

Fourteen compounds are identified in the oils from Persian peppermint with the main chemical components such as menthol (50.9%), menthone (14.9%), α-gurjunene (8.7%), neo-menthol (6.5%) and 1, 8-cineole (3.7%) [9], which may exert these antibacterial activities. Menthol has a broad antibacterial effects against both Gram negative and Gram positive bacteria [15]. On the other hand, in Gram positive bacteria the lack of lipopolysaccharide (LPS) layer can prevent the entry of bio-molecules (such as oil) into cells [16].

In our bacteriologic studies of septic rat following CLP, injection administration of peppermint oil showed a significant reduction in the number of CFUs from the bloodstream which was increased significantly after CLP surgery (table 4). Similar results were obtained by Kim *et al.* [17] who showed the administration of myrrh (0.1 or 0.2 mg/kg) led to a marked increase in survival and an increased bacterial clearance.

In this surgery, due to intestinal damage, leakage of stool into the peritoneal space and persistence of microorganisms or their toxins in the bloodstream can be changed over time within the dimension of ligation, diameter of the perforation and other parameters like pH, nutrient supply, redox potential and flushing action of defecation [18-19].

On the other hand, our results indicated that γ -irradiation treatment used as a safe technique for decontamination of herbal drugs did not changed the antibacterial activities of the oils both *in vivo* and *in vitro* systems (tables 1-4). According to previous studies, GC analysis showed that the peppermint and *zataria multiflora* oil compounds have not considerably changed after irradiation with doses of 10 and 25 kGy [9-10]. Fatemi *et al.* [7] also confirmed that antibacterial activity of caraway seeds essential oils was retained after -γirradiation at 10 and 25 kGy. Artichoke and artichoke irradiated up to a doses of 15 kGy showed the same antibacterial activity [20].

CONCLUSION

In conclusion, the results showed that the peppermint oils has potential antibacterial effects on both *in vitro* and *in vivo* systems sustained even after γ-irradiation treatment. Finally, it can be concluded that the active chemical compounds present in peppermint should certainly find place in treatment of various bacterial infections and indicate this herb should be studied more extensively to explore its potential in the treatment of infectious diseases as well.

Conflicts of interest: *none to declare.*

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