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Antimicrobial Activity and Fingerprint Patterns of Some Pathogenic Bacterial Species against Extracted Oils from Genetically Modified *Mentha piperita* L.

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Abstract: ***Gamma rays was used in the irradiation process with 2,4,6,8 and 10 K-Rad dose for Mentha piperita L. seeds, Then Essential oils were obtained by hydro-distillation method and chemical composition was analyzed using gas chromatography (Gc) and gas chromatography mass spectrometry (GC/MS).Twelve components were identified throughout the extract and the major represented components were Menthol, Menthone and 1,8-cineol. A study of antibacterial activity for the extracted essential oils was done on a six pathogenic human bac GC/MS terial species using agar well diffusion method. The minimum inhibitory concentration (MIC) was also done for the six bacterial species. Random Amplified Polymorphic DNA (RAPD) fingerprinting was used to illustrate the antibacterial activity within the bacterial species. The most antibacterial effect of the extracted oils from the irradiated seeds was expressed in dose 10 KR at concentration of 1:1 for Proteus mirabilis and Klebsiella pneumonia.***

Keyword: *Mentha piperita* L., GC/MS, MIC and RAPD

1. Introduction

Medicinal plants have been used for centuries in traditional medicine because of their therapeutic value. Mint species have been exploited by man for more than two thousand years. Peppermint itself has been used for more than 250 years [1]. Mint plants belong to the *Lamiaceae* family and consist of 23 species, not including the numerous varieties obtained by spontaneous hybridization [2] [3].Peppermint oils is one of the most popular and widely used essential oils in food products, cosmetics, pharmaceuticals, dental preparations, mouth washes, soaps, chewing, gums, candies, confectionery and alcoholic liquors [4]. One of the mutagens which have successfully used to cause beneficial induce mutation in crops was gamma rays. Recently gamma rays are common used in plant breeding programmers for improving vegetative growth and seed quality, because there are known for their simple application, good penetration, reproducibility, high mutation frequency and less disposal problems meanwhile by inducing variability in the genetic constitution [5]. *Mentha piperita* L. (peppermint) is a perennial, glabrous and strongly scented herb belonging to family *Lamiaceae* . *Mentha piperita* oils posses a number of pharmacological properties such as antioxidant and antiperoxidants properties [6], [7].

Peppermint oils showed good antimicrobial activity against *Escherichia coli* and *Pseudomonas aeruginosa* [8]. The antibacterial activity of peppermint leave's juice against Gram negative bacilli was higher than that of its stem juice [9]. The antimicrobial activity of peppermint oils against Escherichia coli was higher than that of Staphylococcus aureus. This oil had good antioxidant activity in two systems of 1,1- Diphenyl-2- Picryl -Hydrazyl (DPPH) free radical scavenging and β- carotene/linoleic acid systems [10]. Because of difference in chemical composition of peppermint essential oils from different regions of world, we analyzed the chemical composition of peppermint oils for local *Mentha piperita* and its antimicrobial effect against a large number of microorganisms.

Among whole genome fingerprinting PCR methods, random amplified polymorphic DNA (RAPD) was used for demonstrating differences between bacteria[10] .RAPD can used for typing of organisms without pervious knowledge of DNA sequences. RAPD has a received considerable attention in recent years as a molecular typing method due to its simplicity, sensitivity, flexibility and relatively to low cost [11], [12] and [13].

The aim of this study was to investigate the antibacterial activities of the extracted essential oils from the modified Egyptian *Mentha piperita* L. RAPD typing method was used to determine the antibacterial effects and to apply for characterization of six bacterial species as follow *Staphylococcus aureus* and *Streptococcus pyogenes* as a gram +ve bacteria and *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Klebsiella pneumonia* as a gram -ve bacteria.

2. Materials and Methods

Seed Materials

Seeds of *Mentha piperita* were kindly provided by Medicinal and Aromatic Plants Department, Horticulture Research Institute, Agriculture Research Center ,Dokki, Giza, Egypt. Seeds were irradiated with 0.0 (control), 2, 4, 6, 8 and 10 k-rad gamma ray doses at the National Center for Radiation Research and Technology, Nasr City, Cairo, Egypt. A field experiment was conducted, where irradiated seeds were sown in pots in complete randomized block design with three replicates during the successful season 2011 and 2012 at the experimental research farm of Agriculture Research Center ,Dokki, Giza, Egypt. Essential oils of fresh herbs were obtained by hydro-distillation. All of this work was done by [14].

Bacterial Strains

The obtained extracted crude oils was screened against sensitivity of six pathogenic bacterial species ATTC as follow: Streptococcus pyogenes (ATTC 7002), Staphylococcus aureus (ATTC 29737) as gram positive, and Escherichia coli (ATTC 10536), Pseudomonas aeruginosa (ATTC 25619), Klebsiella pneumonia (ATTC 10031) and Proteus mirabilis (ATTC 19615) as gram negative bacteria. The microorganisms used were obtained from microbiological laboratory at National Organization of Drug Control and Research (NODCAR).

Antibacterial activity

The agar well diffusion method was used to determine the antibacterial activity of the extracted oils [15] and [16]. For bioassay a bacterial suspension in sterile normal saline was prepared (equally 0.5 McFarland). Six millimeter diameter wells were punched into the Muller Hinton agar test agar by a sterile cork borer. Approximately 0.1 ml of the extracted oils from seeds which irradiated by different doses of gamma-rays, (0, 2, 4, 6, 8, 10) KR., then different concentrations was done as (1:1, 1:2, 1:5and 1:10 v/v) and the plates were incubated at 37°C for 24hrs.After incubation, bioactivity was determined by measuring diameter of inhibition zones (DIZ) in mm. all samples were tested in duplicate. Controls containing sterile (DMSO) without oils were also employed, as a negative control [17].

Microbial content assay

According to [18] microbial content of the Mentha oil samples was evaluated through total plat count (TPC) of the microbial content for bacteria, yeasts, and fungi. The estimation was done by taking 10 g of *Mentha* oil and applying 90 ml of sterilized physiological substance (saline) to obtain a dilution of 1/10.The required dilution was prepared and the agar media culture was prepared as following: agar (15g), Tryptone (5g), dextrose as glucose (1g) and yeast extract (2.5g). The pH value was 7±0.2. The agar Media are placed in Petri dishes which have been prepared in advance, then sterilized and incubated at 35ºC for 48 hours. Five replicates after each test analysis was made and the total count was calculated for each (1 g) of the samples of irradiated and control (non-irradiated *Mentha* oil).

Determination of Minimum inhibitory Concentrations (MIC)

MIC was determined by micro-dilution method using serially diluted plant extracts according to [19] the National Committee for clinical laboratory standards (NCCLS) (National Committee for Clinical laboratory Standards, 2000). MIC of extract was determined by dilution of concentration of 0.0-100mg/ml. equal volume of oils extract and nutrient broth were mixed in a test tube.

Specifically 0.1ml of standardized inoculums was added in each tube. The tubes were incubated at 37°C for 24h.Two control tubes, tube containing the growth medium, saline and the inoculums were maintained for each test batch. Lowest concentration (highest dilution) of the extract and no turbidity (no visible microbial growth) are regarded as MIC when compared with the control tubes.

Bacterial growth and DNA isolation

Staphylococcus aureus and *Streptococcus pyogenes* as a gram +ve bacteria and *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Klebsiella pneumonia* as a gram -ve bacteria were cultivated in Luria-Bertani (LB) broth at 37°C. Bacterial cells were centrifugated and the weight of each cell pellet was determined. Genomic DNA was isolated using the QIA DNA Mini Kit cat.No#51304(QIAGEN,Germany). The bacterial pellets were suspended in 200 µl phosphate buffered salin.15 µl lysozyme was added and incubated for 15 min. at 37°C.Subsequently 200 µl binding buffer and 40 µl proteinase K were added, mixed immediately and incubate for 10 min. at 72°C, then 100 µl isopropanol was added to precipitate DNA. The filter tubes and the collection tubes were combined and samples were pipette, and then centrifuged for 1 min. at 6000 xg. Then the upper reservoir was washed twice with 500 µl washing buffer and centrifuged for 1 min. at 6000xg. 200 µl of pre-warmed (70°C) elution buffer was added and tubes were centrifuged for 1 min at 500 xg. **DNA quality**

Using TE as the blank control and optical absorbance values at wavelengths of (A230, A260, and A280nm respectively) and DNA concentration for each bacterial sample was determined using a spectrophotometer (HP). The A260/A280 and A260/A230 ratios were calculated accordingly.DNA samples were further analyzed by 0.7% Agarose gel (Sub_Cell GT Serial No# 63S9830 Bio-Rad,USA) electrophoresis using ethidium bromide stain to determine the DNA size and to assess RNA contamination.

Random Amplified Polymorphic DNA (RAPD) Fingerprinting

RAPD was carried out with some modification. The PCR mixture was composed of 10XPCR buffer: 100 mMTris-HCL, 500 mMKCL, 15 mMMg Cl2, 0.01%(w/v)gelatin ,PH8.3.2 mM of each dATP,dGTP,dCTP and dTTP was added. Taq DNA polymerase :Taq DNA polymerase (5U/ µl:Sigma).Template DNA :10 to 25 ng/ µl.Stock solution containing good quality, protein free, DNA can be resuspended in high-quality sterial, deionized water or TE buffer (Tris-Edita)PH8.0 .RNase (20ng per 1 ng of DNA).The following primer 5`-TCGCCGCAAA -3`was used in this study as 25 pmol/ µl. The amplifications were done in Thermal Cycler (Biometera) programmed for 40 cycles, each consisting of one denaturation step (94°C for 15 sec), one annealing step (35°C for 30 sec), and one extension step (72°C for one min). After the 40th cycle one extra extension step was performed for 7 min at 72°C. Amplification products were then separated on 1.2% agarose gels containing ethidium bromide, visualized under UV light and photographed using Polaroid film type 667.

3. Results and Discussion

Essential oils and GC analysis

Data presented in Table (1) demonstrated the effect of gamma rays on peppermint (*Mintha piperita*). The dose of 2 k-Rad had a positive effect which increased some oil such as: ferpinine, As- limonene , Menthone, Menthol Transanethol, Decanol , Juniperin and b- caryophyllene meanwhile negative effect which reduce the percentage of

α- terpinene, camphin, 1,8 – cineol and sabinene.The dose of 6 k-Rad was equal in its effect with the dose of 8 k-Rad gamma rays when the increased the above mentioned constituents but the dose 6 k=Rad resulted in increased juniperin comparing with control. Also, the dose of 4 k-Rad and 8 k-Rad had a negative effect on the concentration of transanethol, decanol and juniperin. Furthermore, the leathal dose 10 k-Rad had a positive effect on the concentration of menthol,menthone and b- caryophyllene comparing with control. The high concentration of menthol was obtained by dose of 8 k-Rad and 10 k-Rad (48.04, 48.00%) respectively. In addition the high concentration of menthone was obtained by dose of 6 k-Rad (21.98%).Generally the dose 10k-Rad had positive effect values of most components of the active constituents. The similar results reported by [14], [20] and [21].

Table 1: Chemical profile of leaves peppermint (*Mentha piperita*) oils extract which irradiated by gamma rays.

No. peaks	R.T	Components	Amount (%)					
			0 KR	2 KR	4KR	6 KR	8KR	10KR
1	4.2	α- terpinene	3.50	3.40	3.60	3.60	3.80	3.30
2	4.9	camphin	4.47	3.49	3.20	2.05	2.58	2.83
3	5.6	Sabinene	2.38	2.39	2.30	2.50	2.57	2.35
4	6.5	1,8 – cineol	4.58	3.82	4.01	3.40	5.01	5.87
5	6.9	γ - ferpinine	2.08	3.10	3.08	4.14	2.16	2.82
6	7.2	As- limonene	3.60	3.80	2.44	2.11	2.1	2.30
7	8.2	Menthone	13.60	13.80	15.04	21.98	14.70	16.00
8	8.5	Menthol	40.32	42.03	45.98	44.55	48.04	48.00
9	9.2	Transanethol	3.05	4.07	2.53	2.09	2.17	2.86
10	9.5	Decanol	3.38	3.62	2.44	3.08	2.54	2.08
11	10.1	Juniperin	3.53	4.62	3.11	4.54	1.08	2.03
12	10.7	b- caryophyllene	2.41	2.50	2.27	1.81	2.17	2.80
		Others (< 100)	13.1	9	9.2	4	11	7.2
		Total	100	99.64	99.2	99.85	99.92	100.44

Antibacterial activity

Table (2) revealed that increasing in concentration of oils, and increasing in dose of γ irradiation is associated with increasing in inhibition zone diameter (antimicrobial activity). In this respect, gamma radiation changes the contents of total phenols and tannins, showing higher concentrations in a dose of 10 k- Rad [22].

Also, Antimicrobial activity mainly was due to the phenolic compounds, especially tannins [23]. This action because of tannins can inhibit bacterial enzymes and/or complexion with the enzyme substrates, or its action on the bacteria end cell membranes, or maybe due to its chelation, reducing availability of essential oils for microbial metabolism which agree with [24].

Among the six bacterial species which represented in Table(2) *Proteus mirabilis* and *Klebsiella pneumonia* showing the highest inhibition zone diameter with irradiation dose of 10 KR at a concentration of 1:1 representing in 42mm and 33mm respectively but there was no significant difference shown in case of *Pseudomonas aeruginosa* and *Streptococcus pyogenes* at the same irradiation dose and concentration. Among the gram positive bacteria *Streptococcus pyogenes* showed the highest Inhibition zone represented in 28mm.By doing ANOVA test it was confirmed our current results in Table (2).

Table 2: Antibacterial activity Inhibition zone (mm) of extracted oil from modified Egyptian *Mentha piperita* L.

Oil extract								
Bacterial strains		Concentration (mg/ml)	Control	2.0KR	4.0KR	6.0KR	8.0KR	10.0KR
Gram -ve bacteria	*Klebsiella pneumonia*	1:01	+	16	17	20	23	33
		1:02	+	14	12	15	21	30
		1:05	+	11	10	11	15	24
		1:10	+	3.7	4.5	6	9	11
	Proteus mirabilis	1:01	+	18	20	26	29.6	42
		1:02	+	14	19	22	25	38
		1:05	+	10.3	13	17	21	31
		1:10	+	4.9	6.3	7.2	9	16
	Pseudomonas aeruginosa	1:01	+	11	14	17	21	29
		1:02	+	9	11.9	13.8	17	25
		1:05	+	6.7	8.2	9.9	11	19
		1:10	+	3.4	4.3	6	8.7	9
	Escherichia coli	1:01	+	26	14	26	28	19
		1:02	+	27	12	25	24	14
		1:05	+	22.3	7	19	29	18
		1:10	+	5.91	8	9	20.2	10
Gram +ve bacteria	*Staphylococcus aureus*	1:01	+	10	11.4	15.3	18	24
		1:02	+	9	7	12	15.9	20

		1:05	+	6	5	11	13.2	17
		1:10	+	3.7	4	6.3	9.4	10.6
	Streptococcus pyogenes	1:01	+	13	15.9	18	23	28
		1:02	+	11.2	13.4	16	20.3	23.1
		1:05	+	9.4	11	12	17	19
		1:10	+	5.9	8	9	10.2	16

'-' microorganism Grow without inhibition zone.

Table (3) revealed that, the difference between the extracted oils from the irradiated seeds and non-irradiated one (control). The Table indicate that, the microbial counts were higher for non-irradiated samples (control) and lowest for 10.0KR irradiated one. Indicate that the use of irradiation treatment might affect the microbial counts. The reduction of microbial count is parallel to Menthol concentration where it considers the main cause of total bacterial count reduction. . In this respect, when Menthol concentration percentage were 40.32%, 42.03%, 45.98%, 44.55%, 48.04% and 48.00% ; respectively the total bacterial count were $3.7x10^7$, $8.6x10^4$, $9.5x10^3$, <10, <10 and <10 ; respectively. In this respect, phenolic compounds like menthol and carboxylic acids only can cross the microbial cell membrane. Finally, the higher reduction in total bacterial counts of menthe extract samples might be due increase menthol percentage which increase with increase irradiation dose till 8KR. this agree with [25]. On other hand increase of menthol is due to *Mentha piperita* L. seed irradiations where genetic mutation takes place. This agrees with [26] who stated that the effects may be due to the structural rearrangements in DNA caused by different types of DNA damages.

Table 3: Total bacterial count of extracted oil from irradiated seeds of *Mentha piperita* L. with various doses of γ-rays (Cfu/ml)

Radiation Dose KR	*Total Aerobic count*
Control	$3.7x10^7$
2	$8.6x10^4$
4	$9.5x10^3$
6	<10
8	<10
10	<10

The Minimum inhibitory concentration (MIC) of essential extracted oil from the Egyptian *Mentha piperita L.* seeds which were irradiated by gamma rays was done against the six pathogenic bacteria represented (mg/ml). The result show that, the range of MIC was between 0.4 and 0.7 v/v for the six bacterial species. This is due to amount of menthol in the sample. From the result of MIC and zone of inhibition value and by the comparison with slandered, it is evident that the extract possesses antimicrobial activity [27].

RAPD Analysis

RAPD-PCR analysis revealed that six pathogenic bacterial species *Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus mirabilis ,Escherichia coli*, *Staphylococcus Aureus and Streptococcus pyogenes* had a different RAPD pattern which showed in Fig (1). RAPD technique allowed the amplification of many bands in all isolated bacteria. There were differences in intensity of bands within the same pattern or between the different patterns. All of six pathogenic bacterial species isolates were amplification twenty seven bands ranging in size from 500 bp to about 4300 bp occurred. All isolates were found to have about of 500 bp, 1000 bp and about 3300 bp. All of them have antibacterial testing which was 2500 bp bands in *Klebsiella pneumonia, Pseudomonas aeruginosa ,Escherichia coli* and *Staphylococcus aureus* but in *Proteus mirabilis and Streptococcus pyogenes* have a lot of addition bands in 3500 bp and 4200 bp Fig(1).These results confirmed with results in Table(2).it is clear that *Proteus mirabilis* as (-ve) showed the highest inhibition zone diameter with irradiation dose of 10 k-Rad at concentration of 1:1 representing 42 mm. On the other hand *Streptococcus pyogenes* as (+ve) have the same bands and give the highest inhibition zone diameter with irradiation dose of 10 k-Rad at concentration of 1:1 representing 28. Any structural changes in DNA lead to functional changes, which are the result of DNA damage mostly after exposure to ionizing radiations. Variation in phenotype of an organism is the result of damage in the DNA molecule that causes altered gene expression. DNA modification ranges from changes in single base, substitution, and deletion, to epigenetic modification [28].

In Fig (2) twenty four bands ranging in size from 500 bp to about 330 bp was amplified in all bacterial species of minimum inhibitory concentrations (MIC).There was a common bands in all the isolates 500 bp,100 bp and 2500 bp. The patterns of all bacterial species resulting from antibacterial testing which was 2500 bp bands. These results confirmed with results of MIC which indicated that the result of zone of inhibition value and by the comparison with slandered, it is evident that the extract possesses antimicrobial activity. The simplicity and wide applicability of the RAPD method dependent on the use of short nucleotide primers, This primers are designed within constraints including 1- a length of not less than nine nucleotide 2- a GC content of ≥ 50 % 3- a lack of palindromic sequences [28].RAPD can be used for typing of organisms without previous knowledge of DNA sequences. The use of a single primer leads to amplification of several DNA fragments randomly distributed throughout the genome [29].

The antimicrobial action of essential oil components, the lipophilic character of their hydrocarbon skeleton and the hydrophilic character of their functional groups are of the main importance. The activity rank of essential oil components is as follows: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons. The highest activity was reported for phenols – thymol, carvacrol and eugenol, which is explained by the acidic nature of the hydroxyl group, forming a hydrogen bond with an enzyme active center. Therefore, essential oils with phenols as main compounds express the highest activity against microorganisms in 2500 bp bands [30].

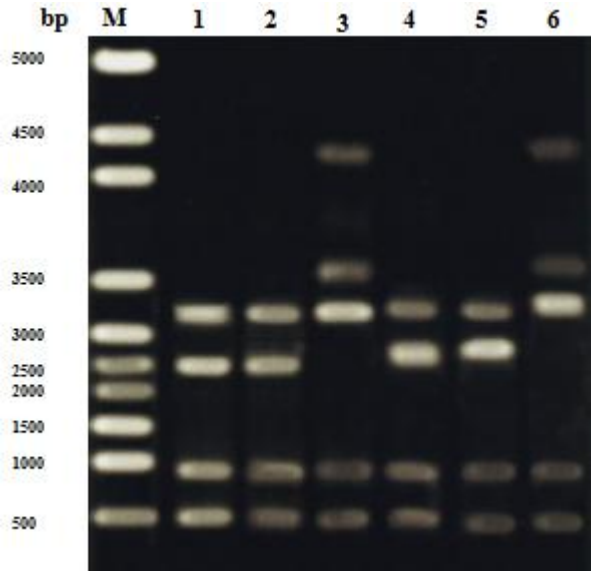

Figure 1: Polymerase Chain Reaction-Amplified of six pathogenic bacterial species(1=*Klebsiella pneumonia,2=Pseudomonas aeruginosa,* 3= *Proteus mirabilis* 4=*Escherichia coli*,5= *Staphylococcus Aureus and 6= Streptococcus pyogenes* a random amplification of polymorphic DNA assay fingerprints, separated by gel electrophoresis and detected by ethidium bromide stain. M=DNA molecular weigh marker.XVII (500bp Ladder) cat, No # 1855646 Roch Diagnostics GmbH Inc,*Germany*.

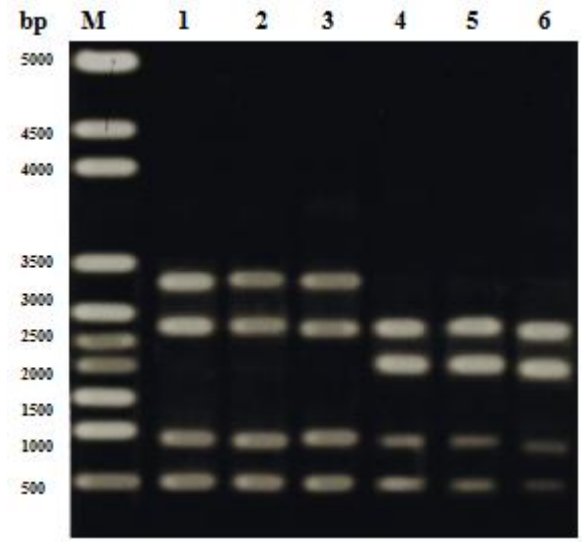

Figure 2: Polymerase Chain Reaction-Amplified of six pathogenic bacterial species of minimum inhibitory concentrations (MIC) 1=*Klebsiella pneumonia,2=Pseudomonas aeruginosa,* 3= *Proteus mirabilis* 4=*Escherichia coli*,5= *Staphylococcus aureus and 6= Streptococcus pyogenes* a random amplification of polymorphic DNA assay fingerprints, separated by gel electrophoresis and detected by ethidium bromide stain. M=DNA molecular weigh marker.XVII (500bp Ladder) cat, No # 1855646 Roch Diagnostics GmbH Inc,*Germany*

4. Conclusion

Increasing in concentration of oil, and increasing in dose of γ irradiation is associated with increasing in inhibition zone diameter (antimicrobial activity). Gram –ve bacteria had a higher response than Gm +ve bacteria against the irradiated oil extract. The most antibacterial effect of the oils extracted from the irradiated seeds was expressed in dose 10KR at concentration 1:1 for *Proteus mirabilis* and *Klebsiella pneumonia* showing the highest inhibition zone diameter of 42mm and 33mm respectively among all the tested species. The results of RAPD showed that any structural changes in DNA lead to functional changes, which are the result of DNA damage mostly after exposure to ionizing radiations

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