ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Effect of Ethanole and Methanole Extraction of Mentha piperitaL. leaves on some Pathogenic Bacteria, Cellular Immune Response and Phagocytosis in Rabbits

Asaad Mohammed Kadhim[1], Rana Adnan Faaz[2], Layla Adnan Abd-Al-Jabbar[3], Layla Adnan Abd-Al-Jabbar[4] and **Hazim Saad Jabbar Al-Maliki[5]**

1. Department of Surgery, College of Dentistry, University of Basrah-Iraq.
2. Department of Microbiology, College of Veterinary Medicine, University of Basrah-Iraq.
3. Department of Physiology, Pharmacology andChemistry, College of VeterinaryMedicine, University of Basrah-Iraq.
4. Department of Basic Sciences, College of Dentistry, University of Basrah-Iraq.

Manuscript Info

Manuscript History:

Received: 12 October 2015
Final Accepted: 25 November 2015
Published Online: December 2015

Key words:
Mentha piperita ; *Salmonella spp.* ; phagocytosis ;Skin test ;cellular immune

Corresponding Author

Dr.ASAAD MOHAMMED KADHIM

Abstract

This experiment was conducted to evaluate the effect of ethanol and methanol extraction of (Mentha piperita) on some pathogenic bacteria , cellular immune responses and phagocytosis in rabbits . Eight pathogenic bacteria used in this study.

Antimicrobial activity of M. piperita was evaluated by disk diffusion methods. The inhibition zone was found ranging from 10.00 to 18mm in ethanolic extraction. The inhibition zone was found ranging from 10.00 to 24mm in methanolic extraction and the highest zone of inhibition produced ethanol and methanolic extract against salmonella species .Twelve rabbits were used , and the cellular immune response done by Skin test about (1ml) of soluble antigen of salmonella spp. was injected in the of groups , this experiment showed that group (A , B) : after 24 hours show , redness area and thickness of skin at sit of injection . The thickness measured by vernier caliper after 24 hours about (2.10±0.01mm), (2.95±0.130 mm) respectively , and the thickness of group (A , B) after 48 hours (1.70±0.06 mm) , (1.87±0.1.40 mm) respectively . The phagocytosis in presented Mentha piperita extraction decreased the count of bacteria in group A(with ethanol extraction) about 86.75% and reached to 25.75% , in group B (with methanol extraction) about 73.25% and reach to 18.5% .

Introduction

Mentha piperita L. (common name: peppermint) member of the large mint family Lamiaceae, 1

The principal components of the oil are menthol (35-55%), menthone (20-30%) and menthyl acetate (3-10%) 2

M. piperita was also used as an analgesic and to treat headache 3

Also has antibacterial properties antiviral, antifungal and, antinematodal . It also found to be strongly effective against *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Enterococcus faecium, Klebsiella pneumoniae, Escherichia coli* 4.

Peppermint oils relaxing action acts as counter-irritant and analgesic with the ability to reduce pain and improve blood flow to the affected area. Peppermint oil and menthol have moderate antibacterial effects . 5

An antiallergenic activity among the flavonoid glycosides including eriocitrin, narirutin, hesperidin, luteolin-7-Orutinoside, isorhoifolin, diosmin, rosmarinic acid and 5,7-dihydroxycromone-7-O-rutinoside . Of the compounds

tested, only luteolin-7-O-rutinoside showed a potent inhibitory effect on histamine release induced by compound 48/80 and an antigen–antibody reaction. 6

M. piperita effected on humoral and cellular immunity was expressed as cytotoxicity towards target cells by activation of cytotoxic or "killer" T-cells. The action of the cytotoxic T-cell is also inhibited by adrenocorticosteroids. But the humoral arm of the immune response was responsible for the production of the antibodies; this was carried out by cells derived from the bone marrow (β-cell). 7

In cell-mediated immunity (CMI), T lymphocytes function to eliminate microbes that have been ingested into vesicles by phagocytes or that have infected host cells and are living in the cytoplasm. 8

Phagocytosis the process by which foreign particles including bacteria are ingested by certain endothelial cells of body 9

Some papers have reported the beneficial effect of *Mentha pipperita* on performance broiler chickens . But evidence about the effect of mentha extract and artichoke leaves meal on blood parameters and immune systems 10,11

The aim of study to investigated that the effect of two type of extraction of *Mentha pipperita* leaves on some pathogenic bacteria ,and to improvement the cellular immune response and stimulanted phagocytosis in rabbits.

Material and methods:-

Extraction of *Mentha piperita* :

Leaves *Mentha piperita* were washed and crushed by pestle and mortal. The 10 g of each leaves was dissolved in 100 ml of methanol as solvents . extract were prepared by maceration. The crushed macerated parts of these medicinal plants were placed at magnetic stirrer and were equally mixed in organic solvents by using magnet. The conical flasks of these extracts were covered by cotton plugs to avoid the solvent evaporation. The extracts were placed in shaking incubator at for 48 hours. After shaking, they were filtered with muslin cloth. The filtered extracts of the medicinal plant were centrifuged at 6000 rpm for 20 minutes. The supernatants were collected in sterile flask.The filtered extracts were stored at 4° C. Ethanol extraction of *Mentha piperita* as the same .

Anti-bacterial activity

A total of 8 bacterial species isolated from clinical specimens of stool, urine, milk ,cheese , in microbiology laboratory and central research laboratory of veterinary collage of Basrah university .

Media: Mueller-Hinton agar (MHA) was used as base medium for screening of antibacterial activity and Mueller-Hinton broth (MHB) for preparation of inoculum.

Preparation of MacFarland Nephelometer standard: McFarland tube number 0.5 was prepared by mixing 9.95 ml 1% Suplhuric acid in MHB and 0.05 ml 1% Barium chloride in distilled water in order to estimate bacterial density The tube was sealed and used for comparison of bacterial suspension with standard whenever required 12.

Preparation and standardization of inoculum: Four to five colonies from pure growth of each test organism were transferred to 5 ml of MHB. The broth was incubated at 35- 37^{o}C for 18-24 hours. The turbidity of the culture was compared to 0.5 McFarland Nephelometer standard to get 1.5 x 10^{8} CFU/ml.

Disk Diffusion Method

For the detection of antibacterial activity, the disc-diffusion assay was used 13 .

The pure bacterial suspension in normal saline was prepared and the turbidity was adjusted by comparison with a 0.5 McFarland turbidity standard. The sterile Muller-Hinton agar plates (each containing 20ml) were inoculated with sterile, non-toxic swab which was dipped into the standardized pure bacterial suspension in order to obtain a uniform inoculation. The three discs(sterile Whatman no.1 filter paper discs 6mm in diameter) of extraction at concentration (1.0µg/ml) were placed and gently pressed on the agar surface and each of the three discs was impregnated . Antibiotics drugs Gentamycin (10mg) used as control . All the plates were then incubated at 37ºC for 18-24 hours. the diameters of the zones of inhibitions in all the plates were measured across the center of the disc millimeter using a vernia caliper

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The estimation of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were carried out by the broth dilution method 14.

Dilutions of extraction from (0.02- 15) µg/ml were used. Test bacteria culture was used at the concentration of 10^5 CFU/ml. MIC values were taken as the lowest extraction concentration that prevents visible bacterial growth after 24 h of incubation at 37°C, and MBC as the lowest concentration that completely inhibited bacterial growth .The optical density was determined using a UV-VIS spectrophotometer at 630 nm. . Gentamycine was used as reference and appropriate controls with extraction were used.

Preparation of soluble *salmonella Spp.* antigen

The antigen that prepared for skin test (delayed type hypersensitivity) according to 15 .

Bacterial suspension of *salmonella spp.* obtain from over night brain heart in fusion agar culture was sonicated for 50 minuts at interval in a water cool sonication and the homogenated was centrifugation twice by cool centrifuge at 8000 rpm .for 30 minuts each time to remove cellular debris , the supernatants were passed through a 0.22 µm Millipore filter and stored at (-20 C^0) until used. Protein content was determined according to 13

preparation of laboratory animals :

The laboratory animals were twelve mature male rabbits at weight (1200- 1400) Kg . and at age two month , assigned into three group each one contain four animals.

Detection of cellular immunity :

The cellular immunity done by Skin test (16), about (1ml) of soluble bacterial antigen was injected in the of groups:

First group: four rabbits injected intradermal with *sallmonella spp.* antigen in the fore arm

Second group : as the same

Third group (control group):

rabbits were injected intradermally with (1 ml) of sterile PBS (pH=7.2).

The thickness of the fore arm was measured by vernier caliper before and 24, 48 hours after Injection.

Injection of ethanolic and methanolic *Mentha piperita L*. extraction in the rabbits :

After 24 hours of skin test , the animal injected of *Mentha piperita L*. extraction about (1ml) at concentration 1.0µg/ml .

First group A: four rabbits injected intradermally with ethanolic extraction in the fore arm of rabbits **.**

Second group B : four rabbits injected intradermally with methanolic extraction in the fore arm of rabbits **.**

Third groupC : (control group):

rabbits were injected intradermally by (1 ml) of sterile PBS (pH=7.2). Rabbits were inspected daily for the development of macroscopic skin lesions , and the thickness of the fore arm was measured by vernier caliper .

phagocytosis test or Phagocytosis activity:

The phagocytosis test was carried out to the two type of extraction ethanolic and methanolic *Mentha piperita L*. extraction. according to method of (17) and (18) .

The blood were collected from two group of rabbits that injected with two type of extraction . The blood collected from cardiac venues in to test tube containing anticoagulant . Culture of clinical isolated of *Staphylococcus* growing at 37^o C was diluted by PBS to obtain the concentration of bacteria about 1×10^3 CFL/ml . The blood samples from rabbits that injected by extraction ethanolic was diluted by PBS and divided into four test tubes , each one contained 0.5 ml of blood suspension (blood +PBS) added to each one 0.5 ml of bacteria suspension and then each tube putted in the routed incubator at 37°C. One test tubes incubated at zero time, second tube at 30 min. , third test tube at an hour and the fourth tube at two hours. After that 0.1 ml from each test tube were taken and cultured on Muller Hinton agar and incubated for 24hrs. in 37°C and then the bacterial number were counted for each plate, and as the same in the other animal injected with methanolic extraction of *Mentha piperita.*

Results and discussion

The results of antibacterial activity of ethanol extraction of *Mentha piperita*

are presented in **Tables 1** . disc-diffusion method showed bacteriostatic activity in concentration of (1.0 µg/ml) .

Good inhibition zones were obtained of ethanol extraction of *M. piperita* against bacteria *E.coli , Salmonella Spp.,Bacillus cerus at (15mm,18mm and 15mm)* respectively, MIC (1.3) µg/ ml for *E.coli* and *Bacillus cereus* , and (1.0) for *Salmonella Spp* , MBC (1.5 µg/ml) for *E.coli* and *Bacillus cereus* ,(1.3 µg/ml) for *Salmonella Spp*

(1) The inhibition zone (mm) , Minimum Inhibitory Concentration (MIC) , Minimum bacterial Concentration (MBC) of ethanol extraction of *Mentha piperita* against pathogenic bacteria

Best inhibition zones were obtained of methanol extraction of *M. Piperita* are presented in Tables 2 .
The inhibition zone (15mm) in *E.coli*., *Psedomonas Spp* and *Klebsella Spp***.** and (18mm) for *ProteusSpp and*

Bacteria	**1.0µg/ml**	**MIC**	**MBC**	**Gentamycine (10mg)**
E.coli	**15mm**	**1.3**	**1.5**	**20mm**
Staphylococcus aureus	**10mm**	**2.0**	**2.5**	**20mm**
Klebsella. Spp.	**10mm**	**2.0**	**2.5**	**20mm**
Bacillus cerus	**15mm**	**1.3**	**1.5**	**20mm**
Psedomonas Spp.	**10mm**	**2.0**	**2.3**	**15mm**
Streptococcus Spp	**10mm**	**2.0**	**2.3**	**22mm**
Salmonella Spp.	**18mm**	**1.0**	**1.3**	**20mm**
Proteus Spp.	**10mm**	**2.0**	**2.5**	**20mm**

Bacillus cerus .,(24mm) for Salmonella Spp . MIC (1.5 µg/) ,MBC(2.0) for *Ecoli*., *Psed Spp* and *Klebsella spp.*, MIC(1.3 µg/ml) and MBC(1.5) , for *ProteusSpp and Bacillus cerus* and MIC(1.0) , MBC (1.3) for *Salmonella Spp* .

(2) The inhibition zone (mm) , Minimum Inhibitory Concentration (MIC) , Minimum bacterial Concentration (MBC) of methanol extraction of *Mentha piperita* against pathogenic bacteria

The present study showed the presence the phytochemicals such as tannins and flavanoids and the free radical scavenging activity and antibacterial activity 19 .
Also the leaf contains many potent compounds such as menthol, menthone, menthyl acetate, menthofuran, and limnone 20 .

Bacteria	**1.0µg/ml**	**MIC**	**MBC**	**Gentamycine (10mg)**
E.coli	**15mm**	**1.5**	**2.0**	**20mm**
Staphylococcus aureus	**10mm**	**2.3**	**2.5**	**20mm**
Klebsella. Spp.	**15mm**	**1.5**	**2.0**	**20mm**
Bacillus cerus	**18mm**	**1.3**	**1.5**	**20mm**
Psedomonas Spp.	**15mm**	**1.5**	**2.0**	**15mm**
Streptococcus Spp.	**12mm**	**2.0**	**2.3**	**22mm**
Salmonella Spp.	**24mm**	**1.0**	**1.3**	**20mm**
Proteus Spp.	**18mm**	**1.3**	**1.5**	**20mm**

The plant had active substances were solubl e in organic solvents so plant extraction obtained more activity than commercial antibiotics , results of this study showed that the potential usefulness *M. piperita* in the treatment of various pathogenic diseases or infection as it may help in the innovation of new chemical classes of antibiotics or drugs. Antimicrobial agents now days available in the market are inadequate due to their low effectiveness, toxicity, and prove costly in case of long-drawn-out treatment. The discovery of a potent medication from plant origin will be a great development in microbial infection therapies. Therefore, there is needed to develop new antimicrobial agents which can satisfy the current demand 21.

Also the study showed the circular redness area and thickness of skin at sit of injection appeared after 24 and 48 hours . In group (A , B):the thickness that measured by vernier caliper after 24 hours about (2.10±0.01mm), (2.95±0.130 mm) respectively , and the thickness of group (A , B) after 48 hours (1.70±0.06mm) , (1.87±0.1.40 mm) , the control group (C) does not show any reaction, after injection of the soluble antigen of *Salmonella species* ,. these due to reaction by perivascular accumulation of lymphocytes and macrophage migration to the central layer of injection ,heterophillic infiltration was observed mostly at early hours of the reaction 22, table (3).

Table (3): Thickness of the skin of the fore arm of rabbits after injection of soluble *salmonella* antigen

Time of injecting antigen	**Group A**	**Group B**	**Group C**
0- time	0.00	0.00	0.00
24- time	2.10±0.01	2.95±0.130	0.00
48- time	1.70±0.06	1.87±0.1.40	0.00

In each group of animal Post-injected of *salmonella* antigen immediate treatment was performed through intradermal injection with (1 ml) of series injected the ethanolic and methanolic extraction using insulin syringes. (23)

show decreased in the thickness of skin group A at 48 times about for group A (0.535± 0.03) , group B(0.232 ±0.02) respectively also the healthy of the animals began improved table 4.

Table (4): Thickness of the skin of the fore arm of rabbits after injection of ethanol and methanol of *Mentha piperita* extraction .

Time of injecting antigen	**Group A**	**Group B**	**Group C**
0- time	0.00	0.00	0.00
24- time	0.412± 0.05	1.524 ±0.03	0.00
48- time	0.535± 0.03	0.232 ±0.02	0.00

The auther 24 mentioned that ***Mentha piperita*** **extraction** maintains the structural integrity of immune cells due to its strong antioxidant action which protects cell membrane from free radical oxidants, there by resulting in an improved immune response.

Phagocytes (macrophages) worked an important role in resistance to infection. They are part of the nonspecific first line of defense because of their ability to engulf and degrade invading microorganisms table (5).

Table (5) : The count of bacteria *salmonella spp.* in present of ethanol and methanol of *Mentha piperita* extraction with phagocytosis

Animal grouping	Zero time	Half hour time	One hour time	Two hour time
Group A(with ethanol extraction)	86.75%	51.25%	32.34%	25.75%
Group B(with methanol extraction)	73.25%	41.74%	37.00%	18.5%

Phagocytosis was done targeted to microbial function , microbes were initially engulfed into a plasma membrane – drives vacuole , the phagosome, which proceeds to acquired derivative properties by complex process termed maturation (25).

In the present study the count of bacteria of *salmonella spp.* in present of ethanol extraction of zero time about (86.75%) and was reach to percentage (25.75%) at two hour time . The count of bacteria *salmonella spp* in present of methanol extraction about (73.25%) at zero time and was reach to the percentage(18.5%) after two hours' time .

The auther 26 , 27 reported that macrophages perform a variety of functions other than phagocytosis; they act as secretory cells, produce nitric oxide that kills intracellular microorganisms and also secrete many different proteins such as lysosome enzymes and cytokines that play a key role in regulating immunity. 28,29 reported that had antimicrobial effects against wide range of bacteria which improves the general healthy conditions of animal that may be reflected in increased immune response.

References

1. Al-Rawi, H.L. Chakravarty; 1988. Medicinal plants of Iraq, Ministry of Agriculture and Irrigation, State Board for Agricultural and Water Resources Research, National Herbarium of Iraq, Baghdad,
2. Escop,. (2003)Thymi herba monographs on the medicinal uses of plant drugs. Exeter, u.k: European in diets for broiler chickens. J. Medicinal Plants scientific cooperative on phytotherapy.
3. Tunon, H., Thorsell, W., and Bohlin, L. (1993) .Mosquito repelling Activity of Compounds Occurringin achilleamillefolium. Economic Botany, 48, pp. 111-120.
4. Sartoratto A, A.L.M. Machado, C. Delarmelina, G.M. Figueira, M.C.T. Duarte and V.L.G. Rehder. (2004). Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. Braz. J. Microbiol. Vol. 34(4): 1517-1520.
5. Diaz R, Quevedo-Sarmiento J, Ramos-Cormenzana A, Cabo P and Cabo J. (1988). Photochemical and antibacterial screening of some species of Spanish Lamiaceae. Fitoterapia. Vol. 59: 330-333.
6. Inoue T, Sugimoto Y, Masuda H, Kamei C. 2002. Antiallergic a .effect of flavonoid glycosides obtained from Mentha piperita b .L. Biol Pharm Bull 25: 256–259.
7. CK Katiyar, NB Brindavanam, P Tiwari, DBA Narayana. (1997). Immunomodulation. (Eds.). Narosa Publishing House, New Delhi., 163-187 .
8. Abbas Abul K., and Andrew H. Lichtman.(2002) Effectore mechanism of Cell mediat immunity . *Basic Immunology: Functions and Disorders of the Immune System*, -- Chapter 6
9. Mitov, I.; Denchev, V. and Linde, K. (1992). Humoral and cell-mediated immunity in mice after immunization with live oral vaccines of S. typhimurium :auxotrophic mutants with two attenuating markers. Vaccine,volume 10. issue 1, 61-66 .
10. Galib, M., M.W. Al-kassi and A. Noor, 2010 A comparative study on diet supplementation with mixture of herbal plants and dandelion as a source of probiotics on the performance of broilers. Pakistan. J.

. Nutrition, 9(1): 67-71 .

11. Hosseini mansoub, N., 2011. The evaluation different leaves of mentha pulagum on performance and blood parameters of broilers. J. American. Sci., 7(8): 1214-1218.
12. Baron EJ, Peterson LR, Finegold SM. 1994.Methods for testing antimicrobial effectiveness.In: Bailey and Scott's diagnostic microbiology, 9th ed. (pp 168-193). St. Louis:Mosby-Year Book, Inc.,
13. Lennette, E.H. (1985). *Manual of clinical Microbiology.* Fourth Edition. American Association of Microbiology, Washington DC p.1149.
14. Vanden Berghe DA, Vlietinck AJ, Van Hoof L (1986) Plant products as potential antiviral agents. Bulletin de I´Institut Pasteur 84, 101-147.
15. Mitov, I.; Denchev, V. and Linde, K. (1992). Humoral and cell-mediated immunity in mice after immunization with live oral vaccines of S. typhimurium :auxotrophic mutants with two attenuating markers. Vaccine,volume 10. issue 1, 61-66 .
16. Hudson , L. and Hay , F.C. (1980). "Practical Immunology " 3rd ed Scientif .
17. Hand and King .L.N.(1978). Serum opsonization activation of Salmonella in sickle cell disease.Am.J.Med.64.388.
18. Lopez ,P.C.Sanchez ,R. Batlle and C.Nerin(2005). Solid and vafpour phase antimicrobial activity six essential oil susceptibility of selected food bacterial .J. Agric Food chem.,53(17):6939-6946.
19. Kaur J, Xavier R, Marimuthu, Leng KM, Rajasekaran A, Kathiresan S, Sreeramanan S (2010). Preliminary investigation on the antibacterial activity of mango (*Mangifera indica* L: Anacardiaceae) seed kernel. Asian Pacific J. Trop. Med., 3(9): 707-710.
20. Fleming T, 1998: PDR for Herbal medicines: Medical Economic Company, JNC
21. HJ. Boer, A. Kool, A. Broberg, WR. Mziray, (2005), "Antifungal and antibacterial activity of some herbal remedies from Tanzania" *J. of Ethnopharma*, 96, PP-461-9.
22. Lopez ,P.C.Sanchez ,R. Batlle and C.Nerin(2005). Solid and vafpour phase antimicrobial activity six essential oil susceptibility of selected food bacterial .J. Agric Food chem.,53(17):6939-6946 .
23. Godin, B. E.; Touitou, E.; Rubinstein, A; and Athamna, M. (2005). A New approach for treatment of deep skin infections by an ethosomal antibiotic preparation: An in vivo study. J. Antimicrob. Chemother., 55: 989-994.
24 . Martin ,L.B.,P. Han, J.Lewittes, J.R.Kuhlman, K.C. klasing and M.Wikelski, (2006). Phytohemagglutinin inducing skin swelling in bird : histology support for a classic immunoecological technique .Funct.Ecol..20:290-299.
25 . Nickels CHF, (1996) . Antioxidants improve cattle immunity following stress. Anim Feed Sci Tech, 62: 59-68.
26 . Warren L. Lee ,Rene E. Harrison ,Sergio Grintein (2003). phagocytosis by neutrophils ,microbes and infection Curr. Pharm. Des,51299-1306.
27 . Tizard I, 1996. An Introduction to Veterinary immunology. 5th Ed, Saunders Publishers , Philadelphia, USA, pp: 30-38
28 . Stafford JL, NF Neumann and M Belosevic, 2002. Macrophage-mediated innate host defense against protozoan parasites. Crit Rev Microbiol, 28: 187-248.
29 . Iscan. G., N. Kirimer, M. Kurkeuglu, K.H. Baser and F. Demirci (2002). Antimicrobial screening of *Mentha piperita* essential oils. Journal of Agricutural and Food Chemestry, 50:3943-3946.
.