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Antibacterial activity of ethanolic extract of wild Mint leaves against different pathogenic bacteria isolated from burn wound infections

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Burn wounds is one of the most important infections in hospitals that being colonized and infected with Gram positive and gram negative bacteria. *Mentha arvensis* is one of the plants that possess antimicrobial properties including different parts like flower, bark, stem, leaves. Antibacterial activity of ethanolic extract of *Menthaarvensis*leaves was investigated against different pathogenic bacteria isolated from burn wound infections by well diffusion methods. Gram negative bacteria *pseudomonas aeruginosa* , *klebsiellapneumonae* and gram positive *staph aureus* were isolated from burn wound infections .Different concentration of *menthaarvensis* leaves ethanolic extract (50,150,200) mg/ml were used. Results revealed that *Menthaarvensis* leaves ethanolic extract at high concentrations(150,200) mg/ml have strong antibacterial activity against pathogenic bacteria (*Staph aureus ,Pseudomonas aeruginosa*) isolated from burn wound infections in which the diameter of zone of inhibition were respectively increased with increased the concentration of ethanolic extract of *Menthaarvensis* leaves while *klebsiellapneumonae* was resistant to ethanolic extract of *menthaarvensis* at all concentration

Keywords: Mentha arvensis ,ethanolic extract, antibacterial activity

INTRODUCTION

Wild Mint is one of the important medicinal plant that belongs to the Lamiaceae family. Its a perennial herbaceousplant that reach to 10–60 cm heightend.The plant leaves are placed in pairs, simple ,hairy, long about 1–2 cm width with a serrated coarsely margin .The flowers of it are pale pink or white on the stem at the bases of the leaves (Thawkar et al., 2016).

Typical composition of Wild Mint are menthone ,menthol , isomenthone , , methyl acetate, cineole , limonene , methofuran (Mustafa et al., 2018).25% of the active substances in medicines that produced pharmacologically are obtained from plants. Also, active substances of most artificially produced medicines are structurally similar to the chemicals which were firstly isolated from plants (Mimica et al .,2003).

Mentha species have a significant role that used for various purposes including medical, nourishing aims and as spice. The whole plant is aromatic, anaesthetic, antispasmodic, antiseptic, also has agents that fight against inflammation, remove gas from the digestive system, induce sweating, assist or promote the flow of menstrual fluid, induction of milk secretion , relieve fever and thirst, give strength and tone to the stomach (Mahboubi, 2017). A tea made from the leaves of Menthaarvensis has traditionally been used in the treatment of fevers, headaches, digestive disorders. The extract of leaves has been applied to areas of swelling and pain, also used for treatment of rheumatism and arthritis (Biswas et al., 2014). The essential oil of leaves is act as

antiseptic, Analgesic, Antimicrobial, Antioxidant, Antibacterial, Sedative, Anti-inflammatory, Cytotoxic, Anticancer, Anti-allergic (Freires et al., 2015).

Burn injuries are considered as one of the major health problem throughout the world. Also its considers as one of the major types of injury that effects about 1% of peoples in the world and its responsible for 1% of total major disease in hospital. Burn injuries (>95%) occurs in developing countries that lead to significant morbidity and mortality (Barret and Herndon, 2003).

Skin is considers as first barrier against foreign bodies. Serious thermal injury lead to a total loss in the skin surface over large body areas. Burn wound infection occurs when microbial entery,then spread in the tissue causes tissue invasion, and systemic infection that correlates with the size of the burn injury. The body aids to maintain homeostasis by initiating a process of contraction and coagulation of blood vessels immediately after a burn injury (Norbury et al., 2016).

Burn wound considers as a favorable area for opportunistic colonization of microorganisms with exogenous and endogenous origin.Burn wound sepsis is occurs due to imbalance in the normal equilibrium between bacteria and host immune defense that lead to numerical increase in bacteria (Keen et al , 2010).

MATERIALS AND METHODS

Isolation of bacteria

(50) samples were collected from patients suffering from burn wound from (2) hospital in Baghdad., a sterile swaps was used for taken samples, then culture on nutrient agar , MacConkey& Blood agar and incubated at 37°C for 24 hrs.

For diagnosis of *klebsiellapneumoae* in samples, lactose fermenting colonies were transferred from MacConkey agar and sub-cultured on EMB agar plates while non- lactose fermented colonies were transferred and streaked on king A, king B medium for isolation of *pseudomonas aeruginosa* .Gram positive *staph spp* were isolated on blood agar, then subcultured on Mannitol salt agar and staph 110 and incubated at 37°Cfor 24 hrs.

Diagnosis of bacteria

Isolates of bacterial were diagnosed by many steps include direct microscopic observation, Gramstaining ,certain Biochemical tests, Catalase ,coagulase tests & Api20E system.

API 20E system for Identification of gram negative bacteria

Gram negative bacteria were identified by this system that contain 20 performance standard biochemical tests. Tap water 5ml of were put in the incubation tray to provide a humid atmosphere during incubation. Many pure isolated colonies were transferred from MacConkey agar and dilute in a sterilized saline until reach to turbidity of ($1x10^8$) McFarland tube no 5 ,then inoculate it in API 20E microtubes system by a sterile Pasteur pipette according to the manufactures instruction in which filled to end of both the tubes and couple section of CIT, VP and GEL microtubes while add oil to H2S ,ADH, LDC, URE , and ODC micro tubes ,other tests are filled to the end of the tubes,then put in incubator at 37°C for 24 hrs. Recorded all the reactions that dont need addition of re agents, while One drop of Kavoc's reagent, ferric chloride and Voges-Proskauer reagent were added to the corresponding microtubes**.** Identification of the isolates by using the analytical profile index supplied by the manufacturer.

Staph aureus identification

From mannitol salt agar, selected isolated colonies were used for identification of *staph aureus* that were subjected to further tests:

Catalase test

A few isolated colonies is transferred from mannitol salt agar to a slide then few drops of 3%$H_2O_2$is added. Bacteria give positive results for enzyme if gas bubbles appeared.

Coagulase tube test

pure culture of *S. aureus* were inoculated in nutrient broth and incubated at 37^{o}C for 24 hours, two tube that contain citrated rabbit plasma 0.4 ml were used ,then culture 0.1 ml was put in first tube while 0.1ml of normal saline were added to second tube . Tubes were incubated at 37^{o}C for 24 hours, then read the results.

Antibiotic sensitivity Test

This test was done according to NCCLS to ensure an accurate results .Selected colonies from test bacteria (*pseudomonas aeruginosa, staph aureus* and *Klebsiellapneumonae)* were cultured in a nutrient broth and incubated at 37°Cfor 24 hrs ,broth cultures were centrifuged at 80000 rpm for 10 mint, then bacterial precipitate was diluted to match a 10^5McFarland turbidity

.Mueller-Hinton media was used in this test. The pH of the media was adjusted to be 7.2 ,then with a sterileswab streaked each bacteria on mullerhinton agar plate to form a bacterial lawn, streaking was repeated in different directions, then allow to dry for 5 minutes, a discs containing specific antibiotics were gently pressed on the agar surface by a sterile forceps ,the Plates was incubated 24hrs at 37°C.

Preparation of ethanolic extract of *Menthaarvensis* leaves

The plant was obtained dry from market .50 gm of the plant were soaked in 250 ml of ethanol (95 %) and used for hot extraction for 3 h using a Soxhlet extraction apparatus. The extract was concentrated under reduced pressure in a rotary evaporator at 45 C.*Menthaarvensis*ethanolic extract was used to prepare different concentration of it (50,150,200) mg/ml, filtrated through (0.45), then by 0.22 µm Milipore filters**.**

Antibacterial Susceptibility test for ethanolic extract of *Menthaarvensis* leaves

Antibacterial activity of ethanolic extract of menthaarvensis leaves was tested by a well diffusion method using a sterile Muller Hinton agar plates . with Pasteur pipette 4 well was done to put the menthaarvensisethanolic extract , the last one was used to put the negative control (normal saline).Bacterial suspension 0.2ml at concentration 10^8Macfarland turbidity was spread by a sterile cotton swab over the agar surface, then allowed plates to dry for 5 minutes. 0.1 ml of different concentrations of menthaarvensis leaves ethanolic extracts (50,150,200) mg/ml were loaded in wells, while of normal saline 0.1 ml were put in the last well. Plates were incubated for 24hrs at 37°C for appearance of zones of inhibition around the wells .Anti-bacterial activity was evaluated by measuring the diameter of inhibition zones estimated in millimeters.

RESULTSAND DISCUSSION

A total of (50) samples were collected from patients with burn infections from (2) hospitals in Baghdad, during the period from 1/7/2017 to 1/9/2017.

Results showed that among the total of (50) samples that were collected, only (19) isolate (38%) were gave typical morphological characteristics and biochemical test that related to *Pseudomonas aeruginosa* while the (10) isolates (20%) were gave typical morphological characteristics and biochemical test that related to *staph aureus* and (5) isolates (10%) related to*klebsielapneumonae* while the rest (16) isolates related to different genera.

Isolation and laboratory identification of *Klebsiellapneumonae* is depends upon streaked the bacterium on MacConkey and EMB agar and incubated at 37°C for 24 hrs. On MacConkey agar, larg pink mucoidness colonies are produced, due to fermentation of lactose. Growth on EMB agar produces dark colonies with a greenish-black metallic sheen. For isolation of *Pseudomonas aeruginosa* , samples streaked on maCconcky agar , non-fermented colonies appeared also isolates produce pyocyanine pigment on king A medium and pyoveridine pigment on king B medium .Alsoon blood agar its appears beta hemolytic on it .Results in this study has been agreed with (Warren *etal*.,2000).*staph aureus*colonies appeared beta haemolytic on blood agar and produce opaque yellow colonies on Mannitol-Salt agar due to fermentation of mannitol (Hanselman*etal*.,2009) .

Microscopic examination

After stained with gram stain. *staphaureus* appears as gram positive grape like clusters. *pseudomonasaeruginosa* and *klebsiellapneumonae* were appeared as gram-negative rods, Slender or shaped in pairs,non spore forming.

Identification of gram negative bacteria by API 20E system

pseudomonasaeruginosa colonies were identified by API 20E system. It give positive results in Arginine Dihydrolase, Citrate utilization , Arabinose fermentation .It give variable results in Gelatin liqifaction. Strains were gave negative results in B-lactamase test , Lysine Decarboxylase , ornithine Decarboxylase , H2S production , urease production, Indole production ,Tryptophane Deaminase ,Vogesproskauer , Manitol , inositol , Sorbitol , Rhaminose ,sucrose, Melibiose, Amygdaline , Glucose Fermentation. The results mentioned above were in agreement with those mentioned by Warren et al., (2000) .while *klebsiellapneumonae* give positive results in Citrate utilization, Lysine Decarboxylase , ornithine Decarboxylase, urease production and sugar fermentation.

Identification of *staph aureus*

Staph aureus were gave positive results for catalase and coagulase tests.

Antibiotic susceptibility test

Results of Antibiotic susceptibility test for

staph aureus isolates reveals that 100% of them were sensitive to chloramphenicol, CFM ciprofloxacin ,nitrofurantion, and Erythromycine . Results also reveals that 100% of isolates were resistant to ampicillin, bacitracin, amoxicillin and as shown as shown in figure (1.1)

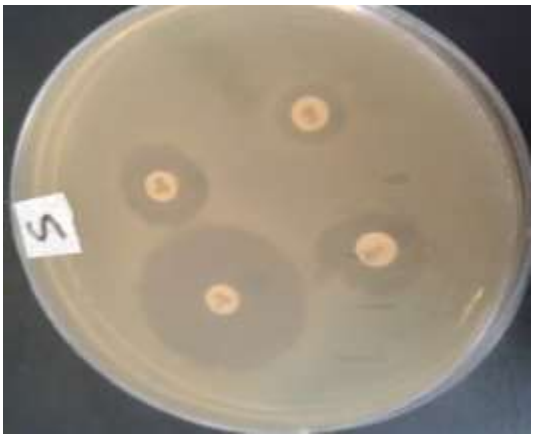

Figure 1.1: Antibiotic susceptibility test for *staph aureus* isolate.

Results of Antibiotic susceptibility test for pathogenic bacteria isolated from burn infections reveals that *klebsiellapneumonae* isolates were sensitive 100% to chloromphenicol, ciprofloxacin and nitrofurantin,also have intermediate sensitivity to erthromycine ,amoxicillin but it was resistant to ampicillin, a bacitracin and CFM.

Results of Antibiotic susceptibility test for *pseudomonas aeruginosa* isolates reveals that 100% of them were sensitive to ciprofloxacin ,Erythromycine ,amoxicillin ,chloramphenicol, ampicillin, nitrofurantion, .Results also reveals that 100% of isolates were resistant to bacitracin as shown in table (1.1).

Gene responsible for antibiotic resistance may pass it to other species of bacteria, such as *Staphylococcus aureus*, through a process called horizontal gene transfer. Also plasmids code for multiple drug-resistance is present in gram negative bacteria that often ready to transfer those plasmids to other species (Wibbenmeyer*etal.*,2010). *P. aeruginosa* may develops resistance either by mutation in chromosomally-encoded genes or by the horizontal gene transfer of antibiotic resistance determinants (Ravichandraetal.,2012) .

outbreaks of Carbapenem-resistant Enterobacteriaceae have been estimated in burn units . A study in single-center from 2008 to 2012 reported that multidrug resistance bacteria causing burn infections as defined by the Centers for Disease Control and Prevention is Pseudomonas spp., A. baumannii, S. maltophilia, and S. aureus, at rates 90.8%, 33.8% ,21.1%, and 82% respectively (Van etal.,2014).

Burn center length of stay is also a major risk factor for infection with MDR bacteria, that out of 125 patients, 10% of bacterial species isolated during the first 7 days were MDR as compared with 44% after 28 days of hospitalization (Rochell and Paul, 2016).

Furthermore, in other study of >5000 burn patients, the rates of MDR Gram-negative bacteria increased sharply during hospitalization. From the first week of admission to week 4 or later, rate of multi drug resistance Enterobacteriaceae per 1000 patient-days is increased from 0.26 to 0.46 for extended-spectrum β-lactamase-producing Enterobacteriaceae, and 0.52 to 2.61 for fluoroquinolone-resistant Enterobacteriaceae .The rate of MDR Pseudomonas spp. is increased from 0.04 per 1000 patient-days in the first week to 1.85 per 1000 patient-days in the 4th week and later of admission (Tissotetal .,2006).

Detection of antibacterial activity of Mentha arvensis leaves ethanolic extract against different pathogenic bacteria isolated from burn infection .

Detection antibacterial activity of *Menthaarvensis* leaves ethanolic extract against different pathogenic bacteria isolated from patients with burn infection were done by use well diffusion methods and different concentration of mentha arvensis leaves ethanolic extract (50,150,200) mg/ml were used as shown in table (1.2). Results reveals that *Menthaarvensis* leaves ethanolic extract at high concentrations(150,200) mg/ml have strong antibacterial activity against pathogenic bacteria (*Staph aureus* ,*Pseudomonas aeruginosa*) isolated from burn infections in which the diameter of zone of inhibition were respectively increased with increased the concentration of extract of *Menthaarvensis* leaves as shown in figure (1.2).

Table (1.1): Antimicrobial susceptibility test for *Klebsiellapneumoniae, pseudomonas aeruginosa, Staph aureus* isolates.

NO Of bacteria	CIP 5 mg/ml	C 30 mg/ml	E 15 mg/ml	AM 10 mg/ml	F 100 mg/ml	AX 25 mg/ml	B 10 mg/ml	CFM 5 mg/ml
K1	S	S	I	R	S	I	R	R
K2	S	S	I	R	I	I	R	R
K3	S	I	I	R	I	I	R	R
PS1	S	S	S	S	S	S	R	S
PS2	S	I	I	S	I	I	R	I
PS3	I	S	S	I	I	S	R	S
SA1	S	S	I	R	I	R	R	S
SA2	S	I	I	R	S	R	R	S
SA3	I	S	S	R	I	R	R	S

NO. number;PS*. Pseudomonasaeruginosa* ;SA. *Staph aureus* ;K. *klebsiellapneumonae ,* S. sensitive ,R .resistant ,I .intermediate.

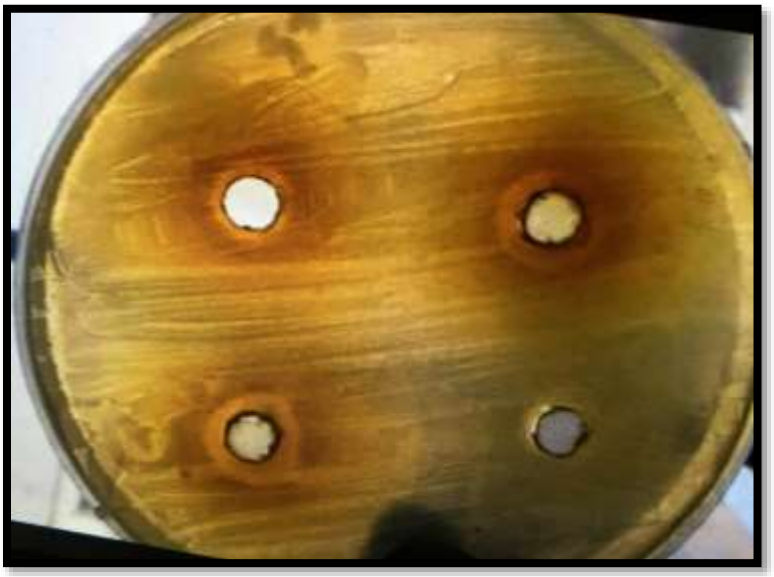

Figure 1.2: Antibacterial activity of *MenthaArvensis* leaves ethanolic extract against *Staph aureus* isolates

Table1.2: Antimicrobial activity of *Menthaarvensis* leaves ethanolic extract on the growth of *pseudomonas aeruginosa, Staph aureus* and *klebsiellapneumonae* isolates.

	Concentration of *Menthaarvensis* extract (mg/ml) Zone of inhibition (mm)			
Bacteria *Spp*	50 Mg/ml	150 Mg/ml	200 Mg/ml	PBS Mg/ml
SA1	12	19	20	0
SA2	11	20	21	0
SA3	12	20	21	0
PS1	14	20	22	0
PS2	12	20	21	0
PS3	11	21	23	0
K1	5	4	5	0
K2	5	2	3	0
K3	4	3	4	0

SA: *Staph aureus*; PS: *Pseudomonas aeruginosa* ;K: *klebsiellapneumoniae*

Results also reveals that *klebsiellapneumonae* isolates were resistant to

antibacterial activity of *Menthaarvensis* leaves ethanolic extract at all concentration as shown in table (1.2).

A lot of studies was conducted on antimicrobial activities of plant extracts and callus extracts. The investigation was carried out on inter-nodal and leaves extracts of *M.arvensis* in order to determine the anti-bacterial efficacy of chloroform, ethanol, ethyl acetate and water extracts against *Salmonella typhi, Streptococcus pyogenes , Proteus vulgaris and Bacillus subtilis*. The antibacterial activity of the extract could possibly be due to presence of phenolic and terpenoid compounds that induced membrane damage (Malik et al.,2012).

The leaves of *M. arvensis*, is a common edible aromatic herb has been known to possess various pharmacological properties including antimicrobial properties. In a study, potent antibacterial properties of the ethanolic extract of *M. arvensis* leaves against *A. baumannii* bacterial strains. The antibacterial effect of the extract could be due to induction of reactive oxygen species (ROS) generation, protein leakage and membrane damage (Johnson et al., 2011).

The antimicrobial activity of the ethanolic extract of *Mentha species* was assessed using both well diffusion and microdilution method . Mint extract was investigated for its antibacterial activity against seven selected pathogenic bacteria: *Bacillus fastidiosus, Staphylococcus aureus, Proteus mirabilis, Proteus vulgaris,Salmonella choleraesuis, Escherichia coli, Pseudomonas aeruginosa, Klebsiellapneumoniae* and *Serratiaodorifera*. Mentha extract at different concentrations (1:1, 1:5, 1:10, and 1:20) was active against all tested bacteria except for *S.aureus*, and the highest inhibitory effect was observed against *S. mutans*using the well diffusion method (Deepak etal., 2017).

The antibacterial activity of the *Ocimum sanctum, O. basilicum, Menthaarvensis*were assessed using the disc diffusion method at different concentration. The effect of different concentrations of the crude leaf and stem extracts. Both aqueous as well as ethanolic extract of leaves and stem were successful in inhibiting the bacterial growth. All the test plants showed significant inhibitory activity in ethanol extracts when compared to aqueous extracts. Possibly because some active substances were present in water extracts but in low concentrations also active substances were soluble in organic solvents and therefore not present in water extract (Suresh et al., 2012).

In another study it was found that the plant ethanolic extracts worked in dose dependent manner against pathogenic bacteria and. showed maximum activity at highest concentration (150mg/ml). This is in accordance with the results reported by surva et al.,(2011) who found the inhibitory effects of *Mentha*leaf ethanolic extract against bacterial strains increased with an increase in concentration, however, degree of toxicity of different concentration of the plant extract may differ from one microorganism to another. It may be because the leaves are rich in bioactive molecules which are known to show medicinal activities as exhibiting physiological and antimicrobial activities (Petretto et al .,2014).

The effects of leaves and stem derived calli extracts on *Proteusspp*. showed that the plants can be used in the treatment of urinary tract infection caused by *Proteus* sp. Through the bacterial efficacy studies, its confirmed that the *in vitro* raised calli tissue was more effective compared to *in vivo* tissue. (Johnson et al., 2011). In another study the crude extract of *Menthaarvensis*in different solvent 50% and 10% ethanol, ethyl acetate, chloroform and was tested against human pathogens *Streptococcus mutans, Streptococcus sangunis, Staphylococcus aurues, Lactobacillus casei* isolated from patients having dental disease. Water and ethanolic extract showed a broad spectrum of very significant antibacterial activity of producing a clear zone of inhibition against tested bacteria (Sharma et al., 2013).

The extracts of *Menthaarvensis*contain significant amounts of phytochemicals with antioxidative properties which could serve antimicrobial property of the *Menthaarvensis*.The secondary metabolites commonly present in the leaves are Alkaloids, Tannins, Flavonols, Steroids, Xantones and glycosides, The GCMS analysis of revealed, the presence of Eucalyptol, Isomethone, Linalool, methyl esters that regarded as a potential source for plant-based pharmaceutical products (Horvath et al.,2017).

CONCLUSION

menthaarvensis leaves ethanolic extract at high concentration(150,200) mg/ml have strong antibacterial activity against pathogenic bacteria (*Staph aureus ,Pseudomonas aeruginosa*) isolated from burn wound infections due to the presence of active compounds like phenolic and terpenoid compounds that induced membrane damage.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

BHS designed and performleed the experiments and also wrote the manuscript. RN, and MA make diagnosis of bacteria, API20E and antibiotic sensitivity. BHS make antibacterial activity of the extract. All authors read and approved the final version.

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