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Determination of Minimal Inhibition Concentration and Minimal Bactericidal Concentration of *Hirudo medicinalis* oil on pathogenic bacteria

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ABSTRACT

Fatty acids are important constituents and commonly possess antimicrobial activities. The analysis of essential oil extract of *Hirudo medicinalis* by gas chromatography revealed one peak, leinoleic acid as unsaturated fatty acids. The discussion of the results of antibacterial activity of leeches oil against gram positive bacteria such as *Staphylococcus aureus, Streptococcus pyogenes and* MRSA *Staphylococcus aureus* revealed that these bacteria were sensitive to the concentrations of 100% which was ranged 21mm, and allow inhibition zone in 25% concentration ranged 13.8 mm by *Streptococcus pyogenes.* While in gram negative bacteria showed that *Enterococcus faecalis and Pseudomonas aeruginosa* more sensitive to the oil extracts at 100% concentration, however at 25% showed a decrease in inhibition zone to 3.3mm by *Klebsiella pneumonia.* The essential oil extracts possessed antibacterial activities against a total of 7 bacteria (3 Gram positive and 4 Gram negative), the mean zone of inhibition ranged between 2.6 and 22 mm. Oil leeches demonstrated MIC values of 512mg/ml against *Pseudomonas aeuroginosa* and showed the lowest MIC and MBC values (128mg/ml) against *Enterococcus faecalis*. The MIC and MBC values for leeches oils against MRSA *Staphylococcus aureus* were considerably higher (1024 mg/ml) than the other bacteria tested.

Keywords: essential oil, *Hirudo medicinalis,* antibacterial activity.

1. INTRODUCTION

Leeches are segmented worms belong to the phylum Annelida ,leeches share a Clitellum and are hermaphrodites, the majority of leeches lives in fresh water environments, while some species can be found in terrestrial and marine environments (1,2). The medical leech, such as *Hirudo medicinalis*, feeding on vertebrate blood and invertebrate hemolymph, leeches have been historically used in medicine to remove blood from patients, into the 18th and 19th centuries in both Europe and North America .In modern times ,leeches are used medically in procedures such as the reattachment of body parts and reconstructive and plastic surgeries (3,4). Medicinal leeches have been found to secrete saliva containing about 60 different proteins (5,6). These achieve a wide variety of goals useful to the leech as it feeds, helping to keep the blood in liquid form and increasing blood flow in the affected area ,several of these secreted proteins serve as anticoagulants (such as hirudin), platelet aggregation inhibitors (most notably apyrase, collagenase, and calin), vasodilators, and proteinase inhibitors (7). It is also thought that the saliva contains an anesthetic, as leech bites are generally not painful (4). When leeches bite, they create a tunnel to the lymphatic system remove toxins from the lymph system. This can be a profoundly effective means of systemic detoxification. The creatures also deposit their saliva which contains a range of biologically active – healing – substances (8, 9).

Antibiotic resistance is the biggest challenge in the treatment of infectious diseases.. The problem of

microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, action must be taken to reduce this problem; for example, to continue the studies to develop new antimicrobial agents especially from traditionally used medicinal leeches. The leech has a long history of use in folk medicine (1,4). In traditional medicine, it has been used for the treatment various infectious diseases. Fatty acids and lipids are constituents of most organisms cells, where they function as membrane compounds, storage products, metabolites and as source of energy which may mediate the chemical defense against microorganisms (9,10).

So, the present study was aimed to find out the fatty acid composition and antibacterial efficacy of the oil extract from the whole leech of *Hirudo medicinalis* against human pathogenic microorganisms, and determination of MIC and MBC of leeches oil.

2. MATERIALS AND METHODS

2.1 Source and rearing of leeches

Local leech used in the study was provided by AL-Sbatin Center for Complementary Medicine and Cupping Healing in Baghdad city.

2.2 Leech Preparation

In this study, forty *Hirudo medicinalis* leeches with 50-70 mm length were used, In order to dispose bacteria and fungi , the leeches were left for 2 days at room temperature 25^0Cin an aqueous solution of antibiotics and fungicides (Penicillin , Nystatin).

Leeches were cut into segments were pounded in a homogenizer with the addition of phosphate buffer (pH 7.4), until a homogenous suspension was obtained, then centrifuged for 20 min at 3000 g , the liquid was filtered by using Millipore filter 0.45 mm and the crude extract was stored at 4^oC until the oil extraction was studied.

2.3 Oil extraction

The oil extraction was obtained from forty leeches by steam distillation using Clevenger system, during 3 hour for each run was contained 20 g of leeches with 300 ml distilled water, The aqueous phase was separated by separation funnel using diethyl either, with vigorously shaken. In separation phase each run repeated for 3 times, and in each time the separation funnel containing 2 ml leeches oil and 2 ml diethyl ether and shake vigorously for 15 min. Then the oil was obtained by using vacuum rotary evaporated apparatus, until all the diethyl ether was completely evaporated, leaving the absolute essential oil and stored in a glass flask at 22^oC until analysis by GC-MS. The concentration, 100%, 75%, 50% and 25% were made by diluting the concentrated extract with the required volume of dimethyl sulphoxide (DMSO).

2.4 GC-MS Analysis

Analysis of the oil was carried out by using gas chromatography at Ibn Sena center in Baghdad University , gas chromatography analysis was carried out on by Shimadzn GC-14A gas chromatography with FID detector and SE-30 column (length and inner diameter) ,the operating conditions were as the follow: carrier gas was He^+(30ml/min constant flow) the oven temperatures for the first 2 min was 100^oC and then increased at a rate of 10^0c /min until 300^0c hold for 2 min ,injector and detector temperature were set at 250^oC and 350^0c respectively .

2.5 Procurement of bacteria

The pathogenic bacteria used in this study were 7 isolates included Gram-positive bacteria, *Staphylococcus aureus* , MRSA *Staphylococcus aureus, Streptococcus pyogenes* and Gram negative bacteria, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli and Klebsiella pneumonia* were obtained from the laboratories of bacteriology at Baghdad teaching hospital .Colonies from an overnight culture were taken with a sterile loop and inoculated into sterile test tube containing 5 ml Muller Hinton ,the turbid of this bacterial suspension was adjusted to reach 0.5 McFarland that is equal to 1×10^8 bacterial cell/ ml (11).

2.6 Determining MIC and MBC

The growth inhibitions were determined by broth microdilution method according to the protocols of the Clinical and Laboratory Standards Institute. The minimal inhibitory concentration (MIC) of an antibacterial agent is the lowest concentration that inhibits bacterial isolate, while the minimal bactericidal concentration (MBC) is the lowest concentration required to kill bacterium (12,13). Serial dilution of leeches oil extract (32-1024mg/ml) were prepared done using dimethyl sulfoxide (DMSO) in the wells of microtiter plate starting with stock solution of primary concentration of 0.01mg/ml, and the wells were inoculated with bacteria in each well of the microplate. After incubation in 24h at 37^0C, the growth inhibition was measured by reading the absorbance at a wavelength of 630 nm, using a multi-well scanning spectrophotometer (LKB). Growth inhibitions were determined for samples that inhibited the growth of the microorganism in the microplate well test.

After determination the MIC, the MBC was determined by taking 50µl from each well and subculture it on agar plate. These experiments were carried out in triplicates and control cultures were prepared for all bacteria.

2.7 Evaluating the antibacterial activity of leeches oil

The disk –diffusion method was used to assess the antibacterial effect of leeches oil at different concentration (25%, 50%, 75%, 100%) by using dimethyl sulfoxide (DMSO) against selected bacteria *Staphylococcus aureus*, MRSA *Staphylococcus aureus, , Streptococcus pyogenes* and Gram-negative bacteria,

Enterococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumonia and Escherichia coli.

One hundred µl of each bacteria were taken separately and expelled onto separated petri dishes containing Muller Hinton media ,Filter paper disks (5 mm) were prepared and sterilized with autoclave ,then disk was impregnated with 15 µl of each oil concentration 25%, 50, 75 and 100%, one of the disk was soaked with DMSO as a control after inoculation with bacteria 5 disks were placed on the Muller Hinton on each petri dish, control include DMSO then incubate for 24 hr, at 37^{o}C. The zone of inhibition were measured by digital venire.

3. RESULTS AND DISCUSSION

The wide range of occurrence of antibiotic resistance suggests that, in principal, any organism could develop resistance to any antibiotics (14). The antibiotic resistance to antibiotic drugs increases the cost of treatment and often results in treatment failure. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain (15). Therefore, action must be taken to reduce this problem; for example, to continue the studies to develop new antimicrobial agents especially from traditionally used medicinal leeches. *Hirudo medicinalis* (also known as medical leeches).

The objective of the present work paper is to investigate gas chromatography (GC) is a technique which allows the separation of volatile compounds from complex mixtures as well as their quantification has become one of the most important tools in the analysis of essential oil ,now days, the development of efficient capillary columns allows the individualization of several hundreds of components (16) . Gas chromatography has several advantage as being fast analysis, efficient and sensitive.

The analysis of essential oil extract of *Hirudo medicinalis* by gas chromatography revealed one peak appear of linoleic acid as unsaturated fatty acids due to messing other standards in Ibn Sena center (Fig.1).

The essential oil extracts possessed antibacterial activities against a total of 7 bacteria (3 gram positive and 4 gram negative, the mean zone of inhibition ranged between 2.6 and 22 mm (table 1).

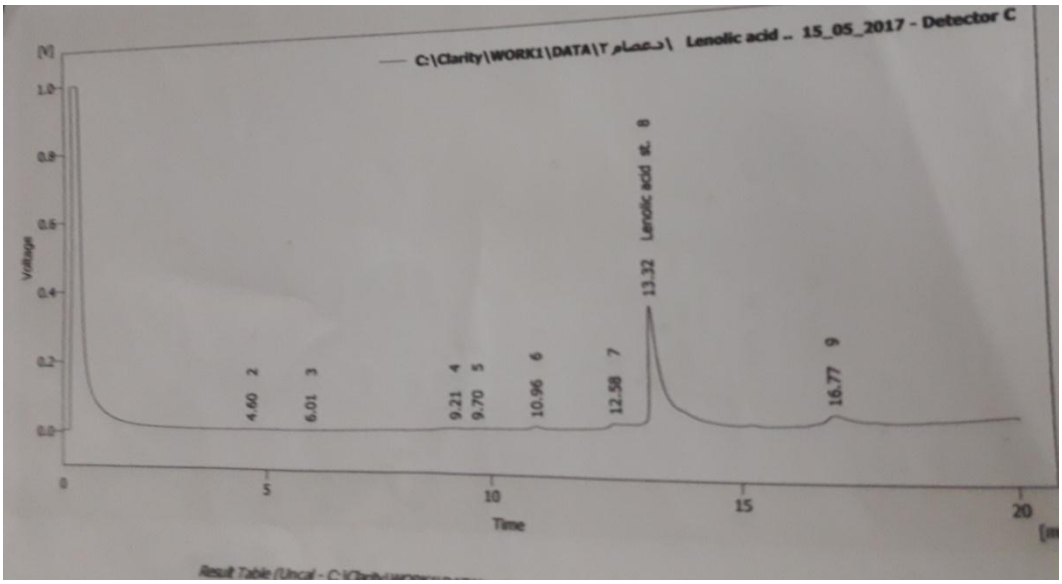

Figure 1: Gas Chromatogram of fatty acid methyl esters from the *Hirudo medicinalis*

Table 1: Activity of leeches oil against bacterial isolates.

Bacterial isolates	100%	75%	50%	25%
Staphylococcus aureus	22.0	19.1	16.1	14.4
Streptococcus pyogenes	21.0	18.3	15.0	13.8
MRSA *Staphylococcus aureus*	20.0	17.3	15.1	15.0
Enterococcus faecalis	21.0	18.0	15.2	13.4
Pseudomonas aeruginosa	20.0	18.0	17.2	15.1
Escherichia coli	10.6	5.3	3.7	2.6
Klebsiella pneumonia	9.9	5.4	4.2	3.3

The discussion of the results of antibacterial activity of leeches oil against *gram positive bacteria such as Staphylococcus aureus, Streptococcus pyogenes and* MRSA *Staphylococcus aureus* revealed that these bacteria were sensitive to the concentrations of 100% which was ranged 21mm, and allow inhibition zone in 25% concentration ranged 13.8 mm by *Streptococcus pyogenes.*

While in Gram negative bacteria showed that *Enterococcus faecalisand* and *Pseudomonas aeruginosa more* sensitive to the oil extracts at 100% concentration compared with other Gram negative bacteria (Table1), however at 25% showed a decrease in inhibition zone to 3.3mm by *Klebsiella pneumonia.*

Our finding revealed that differences could be due to the nature and level of antibacterial agents present in the extracts, and this mode of action on different test microorganisms may be contributed to the presence of a reaction between the functional group of lenoleic acid and bacterial cell wall, so the acid will lead to inhibition of bacterial growth and this acid had a good biological activity. Essential oil derived from leeches are known to possess biological activity against prokaryotic and eukaryotic organism (21) ,the number and position of double bonds is more important to fatty acids longer than 12 carbons than for fatty acids with fewer carbons (24).

The results of this study has shown that gram negative bacteria are more resistant to leeches than gram positive bacteria ,this is probably because of the structural differences in the outer membrane of these bacteria ,gram negative bacteria have a thicker layer of lipopolysaccharide outer membrane, that cover the cell wall, which block the penetration of antibacterial compounds making them more resistant compared to the gram positive bacteria (22).

The oil extract showed good antibacterial activity against all the test bacteria at 100% concentration which was assessed qualitatively and quantitatively by calculating MIC and MBC which were tested by broth microdilution method (table 2).

Our results showed MIC and MBC values of 512mg/ml against *Pseudomonas aerogenosa* indicating that this oil possess bactericidal properties, while the lowest MIC and MBC values (128mg/ml) against *Enterococcus faecalis* since both values similar ,the oil must be considered to possess strong bactericidal activity.

The MIC and MBC values for leeches oils against MRSA *Staphylococcus aureus* were considerably higher (1024 mg/ml) than the other bacteria tested suggestion that this oil possess much weaker bactericidal activities.

Table 2: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) data obtained by the broth microdilution method.

Bacterial isolates	MIC_{50} (mg/ml)	MBC_{50}(mg/ml)
Staphylococcus aureus	512	512
Streptococcus pyogenes	256	512
MRSA*Staphylococcus aureus*	1024	1024
Enterococcus faecalis	128	128
Pseudomonas aeruginosa	512	512
Escherichia coli	512	608
Klebsiella pneumonia	320	512

The present finding also support author study which concluded that possibility of the therapeutic use of linolenic acid as an antibacterial agent should be explored (17). The past twenty years have been increasingly rapid advances in field of α-Linolenic acid was generally considered to have low toxicity, so it may potentially be administered to patients infected with MRSA as a dietary treatment (18). Linolenic acid, although present in human skin (19) may well be an important naturally occurring antibacterial agent, and its presence could explain why pathogenic staphylococci are rarely found on intact skin (20). In 1954 Nieman (17) demonstrated that some natural defense systems of higher organisms, such as the self-disinfection of the skin, are due at least partly to the presence of antibacterial fatty acids *in situ*, however, the potential antibacterial activity of fatty acids *in vivo* may be neutralized by adsorption on proteins in the bloodstream.

There was evidence of degree of susceptibility to the all being reflected in the gram reaction of the organisms, gram positive and gram negative bacteria were both susceptible to the leeches extract (oil) at different ratio, the inhibition zones became smaller with decreasing concentration of oil. Nieman C (17) demonstrated the antibacterial activity of α-linolenic acid against methicillin-resistant *S.aureus* (MRSA).

The MIC value of leeches oil against different bacteria used in this study ranged from 128-521 mg/ml while the MBC ranged from 256-1024 mg/ml.

In a different study by Knapp and Melly (25), investigation led that Gram-positive bacteria are more affected by slightly longer chain lengths, while Gram-negative organisms are affected by very short. The stereochemistry of unsaturated compounds has an important role as *cis*-isomers are more active than *trans*-isomers.

However, another study showed that linoleic acid inhibited bacterial enoyl-acyl carrier protein reductase (FabI), an essential component of bacterial fatty acid synthesis, which has served as a promising target for antibacterial drugs. These FabI-inhibitory activities of various fatty acids and their derivatives very well correlated with the inhibition of fatty acid biosynthesis using [(14)C] acetate incorporation assay, and importantly, also correlated with antibacterial activity (18).

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