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Ultrasonic Assisted Extraction and Antimicrobial Activity of local Moringaoleifera leaves

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ABSTRACT
Background: Medicinal plants are attracting increasing interest due to their application in ethnomedicine to treat common ailments such as colds and fevers, where their therapeutic claims have been substantiated by strong scientific data. Investigation of medicinal plants has begun with extraction processes that significantly affect the extraction results (e.g., yield and phytochemical content) and subsequent experiments conducted. Ultrasound-assisted extraction (UAE) is a frequently used extraction method due to its efficiency in reducing extraction time and solvent usage. However, application of ultrasound energy above 20 kHz may affect the active phytochemicals by generating free radicals.
Method: The leaves of Moringaolifera plant were dried under shade and then weighed. Ultrasound Assisted Extraction (UAE) method was carried out to extract the active compounds. Two samples each weight 15 g of M. oleifera dried leaves was extracted using 500 mL of both (Methanol: water 50:50) and petroleum ether (40–60) °C. The sonication conditions were UP400St ultrasonic processor for 10 min at room temperature. Two different polar aqueous methanolic extract and nonpolar organic petrulium ether extract weredried and subjected to phytochemical screening tests. GC-MS technique was utilised to characterize the nonpolar petrulium ether extract.After that, the antibacterial activity was examined against both gram positive (G +ve) and gram negative (G -ve) strains.
Results: The phytochemical tests revealed the abundance of flavonoids, saponins, tannins, terpenes and alkaloids in the M. olifera extracts. Compounds like Fatty acids, aldehydes, Vitamin E and steroids as identified by GC-MS method. The current study, both aqueous methanolic and petroleum ether extracts in concentrations of 100, 200, 400, 800 µg/ml were verified against Gram positive G+ve and Gram negative G-ve bacteria. The results showed that methanol/water extract has been moderate to good effective in inhibition of G+ve bacteria S. aureus in tested concentration 200, 400, 800 µg\ml in comparison with gentamycin antibiotic while no effect was showed on G-ve bacteria E. coli. There was no antibacterial activity of petroleum ether extract on G+ve bacteria S. aureus in all tested concentration while it has a moderate effectiveness in G-ve bacteria E. coli on concentration 200 µg\ml this is suggested that it needs a particular concentration in order to give its activity.
Conclusion: Moringaolifera is a promising plant in treating bacterial infection as it contains many important phytochemicals that can be extracted and isolated from it.

Keywords: Moringaolifera, Ultrasound Assisted Extraction (UAE), Methanol, petroleum ether, GC-MS.

INTRODUCTION

Due to their abundance of secondary metabolites, plants are a significant source of bioactive principles.(1)New natural antioxidants are being investigated for application in the food industry and in pharmaceuticals in order to avoid the unfavourable side effects of synthetic antioxidants.(2),(3)Moringaoleifera is regarded as a food plant with a number of therapeutic uses in industrial settings.(1),(4)Although the tree is native to India, it has been brought to many parts of the world.(5)M. oleifera has been grown for the first time in Tunisia in recent years in an effort to enhance the nation's forest resources and create new, highly valuable products. Many ailments,

including cancer, "tired blood" (anaemia), asthma, diabetes,constipation, heart problems, hypertention, and bacterial, viral, and parasite infections, have been treated with M. oleifera in traditional medicine. Researchers are paying more attention to this plant because of its potential applications in a variety of sectors. Indeed, numerous investigations have demonstrated the potential antioxidant(6), antibacterial (7), antiulcer, anticancer(8), anti-inflammatory(9), antispasmodic(10), and antihypertensive (11)properties of M. oleifera leaves. An essential step in the manufacture of plant extracts is the extraction technique and solvent utilised. Indeed, a number of investigations found that when extraction was carried out using various solvents, quantities of the bioactive chemicals and the associated antioxidant power were found.(12) In order to collect chemicals and subsequently assess their biological qualities, it is crucial to determine the impact of the extraction techniques using the suitable solvents. (13),(14)Ultrasound frequencies between 20 kHz to 2000 kHz are used in UAE.(15)The ultrasonic acoustic cavitation's mechanical action improves the permeability of cell walls and the surface contact between solvents and samples. The materials' physical and chemical characteristics are changed by ultrasound, which also breaks down the plant cell wall, allowing chemicals to be released and improving the solvents' mass transfer into the plant cells. (16)The aim of the current study is to extract the bioactive compounds such as phenolic, vitamins, flavonoid and other compounds of M. olifera leaves plant via the best modern method the ultrasound assisted extraction (UAE) by using two different polarities, the efficient solvents the polar aqueous methanol and non-polar petroleum ether. Phytochemical analysis was verified by the chemical tests as well as Gas-Chromatography-Mass Spectroscopy (GC-MS) technique and finally the biological potency against gram-negative bacteria E. coli and Gram-positive bacteria S. aureus was examined for both aqueous methanolic and petroleum ether extract.

Phytochemical study

Plant material:

About (100g) of Moringaoleifera leaves, one year old, were Collected from Baghdad city, Iraq, in summer morning, July 2023. The Plant materials were dried under shade in a dry and good ventilated place for two weeks and they were stored in dark and dry place until their use.

Extraction procedure:(17)

Aqueous- methanol solvent:

The extraction was carried out by the using of Ultrasonic Assisted Extraction method. At first, 15 g of dried M. oleifera leaves was extracted using 500 mL of 50% Methanol: water solvents in a sonication UP400St ultrasonic processor for 10 min at room temperature. Then, the extract was filtered to remove solid materials. The filtrate was dried at room temperature.

Petroleum ether solvent:

With a same extraction method, about 15g of M. oleifera dried leaves was extracted using 500 mL of Petroleum Ether (40–60) °C solvent in a sonication UP400St ultrasonic processor for 10 min at room temperature. Then, the samples were filtered to remove solid materials. Then the extract was dried at room temperature.

Phytochemical tests:

Some of the important chemical tests were done to identify the active constituents in plant extract as follows:

Flavonoid test:

The reaction was carried out by adding 1 ml of dilute sodium hydroxide solution to 1.5 ml of methanolic extract. The mixture was evaluated for the development of a yellow color, which was considered a positive result.

Saponin test:

Froth test:After mixing 2 grams of plant material with 20 ml of water, it is cooked for three minutes. When the mixture is heated, it is filtered. Then ten millilitres of the filtered mixture are mixed with five millilitres of water and shaken. The formation of foam indicates a successful result.

Tannin's test:

Lead acetate test: Some dropsof 1% fresh lead acetate solution were combined with1 milliliter of the extract. The yellow precipitate indicates the presence of tannins.

Alkaloid's test:

Mayer's test: A few drops of ethanol, two drops of diluted 10% HCl, and two drops of Mayer's reagent were combined with one milliliter of the extract. A positive outcome is indicated by yellow precipitate.

Terpenoids test:

Petroleum ether extract 3 ml was gradually mixed with $CHCl_3$1ml and concentrated $H_2SO_4$2 ml. The formation of a reddish-brown color in the solution indicated the presence of terpenes. (18), (19)

Gas-Chromatography-Mass Spectroscopy (GC-MS):

Shimadzu Japan gas chromatography QP2010PLUS was used to analyse the petroleum ether leaf extract of M. olifera using a fused GC column (2010) and polymethyl silicon (0.25 nm 50 m) coated with the following parameters: field ionisation detector (FID) temperature 300 °C, injection temperature 220 °C, nitrogen at a flow rate of 1 ml/min, split ratio 1:75, and temperature program from 80 to 200 C held at 80° C for 1 minute, rate 5 °C/min, and at 200° C for 20 minutes. The column has a diameter of 0.25 mm, a length of 30 m, and a flow rate

of 50 ml/min. A mass spectrometer was used to empty the elute, and the sampling rate and detector voltage were set at 0.2 s and 1.5 kv, respectively. (20)

Antibacterial activity
METHODOLOGY
To assess the prepared samples methanol and petroleum ether (40-60°C) extracts antimicrobial properties. Two to three colonies of bacteria with identical phenotypic traits that were growing on blood agar medium were transferred to tubes holding normal saline solution to create bacterial suspension. With the use of VITEK Densities (bioMérieux), the bacterial suspension's turbidity was adjusted to meet the McFarland 0.5 criterion in 0.45% sodium chloride.Gram-negative bacteria E. coli and Gram-positive bacteria S. aureus were cultivated on Mueller Hinton agar using the disc diffusion method to investigate the inhibitory efficacy of the produced compound at doses of 100, 200, 400, and 800 µg/ml.Whatman filter paper No. 3 was used to prepare the filter paper discs, and holes with a diameter of around 6 mm were punched using a standard office hole punching machine. After that, the discs were autoclaved for 30 minutes at 15 pounds of pressure. Sterile discs were submerged in a succession of prepared compound dilutions in petri dishes. The discs were then left to dry for four hours at 37°C in a sterile incubator without the petri dishes being covered. (21)In this investigation, the antibiotic was interpreted in accordance with CLSI by utilizing the antibiotic gentamycin as a control. (22)The biological study was performed in the laboratories of college of pharmacy / University of Kerbala.

RESULTS
Phytochemical study:
methanol solvent:
After the extraction of Moringa leaves, the extracts were tested to identify the presence of alkaloid, flavonoid, saponin and tanninsas presented in table 1 and 2 Moringaolifera extracts rich in the most important phytochemicals especially flavonoids in aqueous/ methanol extract and alkaloids most abundant in petroleum ether one.

Table 1: phytochemicals in Aqueous-methanolic extract

Test	Results
flavanoids	+++
Saponin	++
Tannins	++
Alkaloids	+

Table 2: phytochemicals in petroleum ether extracts

Test	Results
Alkaloids	+++
Terpenes	+++

Gas-Chromatography-Mass Spectroscopy (GC-MS)
Petroleum ether leaves extract of Moringaolifera has a very important phytochemicals like fatty acids, aldehydes, Vitamin E and steroids as identified by GC-MS method. These compounds are Hexadecanoic acid, methyl ester, Pentadecanoic acid, 11-Octadecenoic acid, methyl ester, 9-Octadecenoic acid, methyl ester, (E)-, Methyl stearate, Octadecanoic acid, 9-Octadecenamide, (Z)-, Vitamin E, Campesterol, Stigmasterol, beta.-Sitosterol. All the results presented in table 3 and fig. 1.

Table 3: Gas Chromatography Mass Spectroscopy results of M. olifera petroleum ether leaves extract.

Compound	Retention time (Rt) (min.)	Similarity index (SI) %
Hexadecanoic acid, methyl ester	22.103	95
Pentadecanoic acid	22.656	72
11-Octadecenoic acid, methyl ester	24.214	99
9-Octadecenoic acid, methyl ester, (E)-	24.327	99
Methyl stearate	24.526	99
Octadecanoic acid	25.028	89
9-Octadecenamide, (Z)-	27.235	89
Vitamin E	27.945	99
Campesterol	30.490	95
Stigmasterol	31.147	91
beta.-Sitosterol	31.433	95

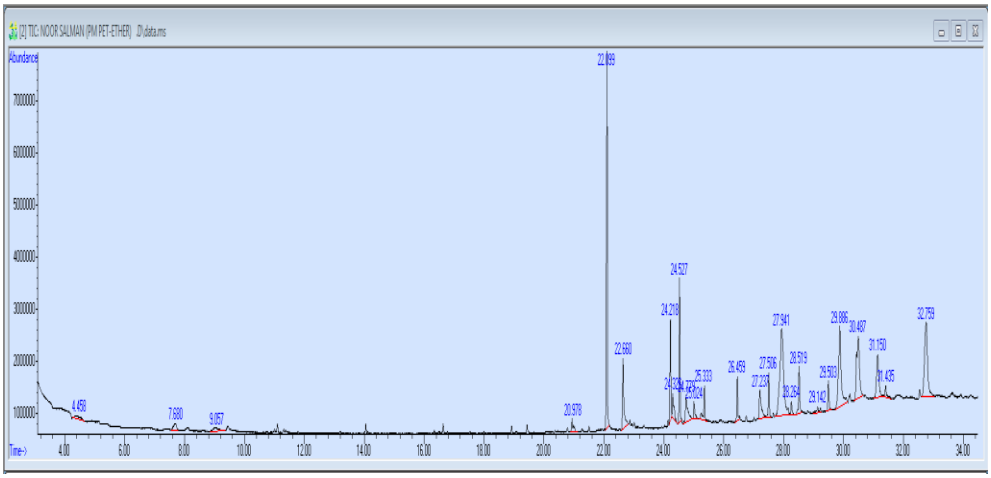

Figure 1: Gas Chromatography Mass Spectroscopy chromatogram of M. olifera petroleum ether leaves extract.

Antibacterial assay

Both crude extracts of moringa leaves were submitted to investigate their antibacterial activity and the results were reported as in Table 3a and 3b.

Table 3a: Antibacterial activity of the (Methanol: Water 50:50) extract

Methanol: Water 50:50 concentration µg\ml	**S. aureus (G+ve bacteria) Inhibition zone(mm)**	**E. coli (G-ve bacteria)**
100	0(R)	0(R)
200	17(S)	0(R)
400	15(S)	0(R)
800	21(S)	0(R)
Gentamycin disk (10 u)	24(S)	15(S)

S=Sensitive; R=Resistance

Table 3b: Antibacterial activity of the Petroleum ether extract.

(Petroleum ether extract) concentration µg\ml	**S. aureus (G+ve bacteria) Inhibition zone(mm)**	**E. coli (G-ve bacteria)**
100	0(R)	8(R)
200	0(R)	13(I)
400	0(R)	0(R)
800	12(R)	0(R)
Gentamycin disk (10 µg)	20(S)	17(S)

S=Sensitive; R=Resistance

DISCUSSION

Moringaoleifera is a versatile plant due to its micro- and macronutrients which are phenols, vitamins, alkaloids, polysaccharides, sterols, saponins, tannins, quercetin, and flavonoids. These components are all abundant in Moringaoleifera. Because of these chemical components, Moringaoleifera is a multipurpose plant, and its leaves are rich in about fifty-nine Flavonoids and phenolic acid derivatives were the most common, with uncertain characterization. In addition, thirty of these compounds were initially discovered.(23)

In the current study the ultrasound assisted extraction technique and two extraction solvent were used to extract the hydrophilic and hydrophobic compounds found in the dried Moringaolifera leaves collected in Baghdad/Iraq The drying prosses was done at room temperature and not by oven in order to preserve the active compounds that may be destroyed by heat. After investigating the UAE technique found that using methanol: water (50:50, v/v) resulted in a M. oleifera which contains the highest concentration of flavonoids and phenolic compounds. The resultant extract employing petroleum ether (40–60) °C solvent decreasing polarity has been examined by GC-MS technology shown in table (3). Research on plant-derived organic compounds and their biological activities is increasing, primarily due to their potential as a source of new drugs. Gas chromatography-mass spectrometry (GC-MS) is an effective method for reliably identifying biologically active components in plant research.[19]As well as, many of the compounds that extracted are similar to what identified by another study,

like hexadecenoic acid and pentadecanoicacid .(24)According to another study M. olifera ethanol leaves extract showed antibacterial activity against many bacterial strains like Staphylococcus epidermidis,Staphylococcus aureus, Bacillus subtilus, Streptococcus mutans, Escherichia Coli, Proteus vulgaris, Pseudomonas aeruginosa. (25)So the current study rely on the comparison between the two extracts from this medicinal plant which are (methanol:water 50:50) and petroleum ether by a modern extraction method UAE against two bacterial strains which were Gram negative (E. coli) and Gram-positiveStaphylococcus aureusbacteria. As the Aqueous-methanol plant extract contains most important medicinal compounds such as flavonoids, alkaloids, Tannins and saponin, and each compound known for it is medicinal importance. For instance, Alkaloids are naturally occurring chemicals that contain essential nitrogen atoms. They are used as medicines for recreational purposes and often have pharmacological effect. Flavonoids act as antioxidants and enhance the benefits of vitamin C. In addition, they have been shown to have biological activity against viruses, cancers, liver toxins, and other microorganisms. (26)Tannins exhibit potential antiviral, antibacterial and antiparasitic effects. Saponins induce hemolysis of red blood cells.(27)Antibacterial activity has been evaluated for its important medicinal properties against pathogenic organisms. As a result, in this study the (methanol:water 50:50) extract is rich in polar bioactive compounds such as flavonoids and phenolic compounds presented a good antibacterial activity against Gram positive bacteria in the tested concentration as compared with the reference gentamycin antibiotic. However, Aqueous methanol exhibited no effect and petroleum ether extracts showed intermediate activity at concentration (200) µg/ml against Gram negative bacteria may be due to this bacterial strain resistant to the extracted components. In addition, petroleum ether extract showed no activity against Gram positive bacteria probably as a result of this bacteria had a resistance to the compounds extracted by this solvent or it may need a greater concentration to be sensitive.

CONCLUSION

Moringaoliferais a rich plant with a very important phytochemicals that's make it has a biological activity against G +ve, S.aureus bacteria, this result after using a modern method of extraction which is Ultrasound Assisted Extraction (UAE) Method.

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