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Evaluation of Bioactivity of Maximal Inhibitory Concentration of Alkaloids Isolated from Mentha spicata Leaves Against Some Pathogenic Fungi

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Abstract:
Alkaloids were isolated and identified from Mentha spicata leaves. Qualitative analysis, thin layer chromatography(TLC), functional groups test and infra-red spectrum were carried out for identification of these alkaloids. TLC results showed presence of two alkaloidic compounds in alkaloids extract. The Bioactivity and biochemical effect of alkaloids, was investigated towards four pathogenic fungi. The maximal inhibitory concentration was measured to be (250 mg/ml) and the Bioactivity of this concentration was recorded against pathogenic fungi represented by Aspergillus fumigatus, Candida albicans, penicillum crysosporm and Aspergillus niger with inhibition zone diameters equal to (23, 15, 15 and 11 mm) respectively. Therefore, the alkaloidic compounds isolated can be used as an herbal theuraputic substituent to the different diseases caused by these fungi but this work demands further clinical and pharmaceutical studies.

Keywords. Mentha spicata leaves, Alkaloids, Maximal inhibitory concentration, Bioactivity, pathogenic fungi.

Introduction:

Plants have been a potential source of medicine, though in a crude form, have been used from time immemorial to heal various aliments. A variety of biochemical active compounds that are present in different parts of a plant has spurred a renewed interest in developing an alternate therapy. Plants are an essential part of human society since the civilization started. Medicinal plants are the boon of the nature to cure a number of diseases of human beings. In many parts of the world medicinal plants are used against fungal, bacterial and viral infections (1,2). Many recent studies have ratified the belief that the environment or nature has for long been

a very important source of medicines. A good number of our modern drugs have been isolated from plants, based on their use in traditional medicine, the reason for this case is presence of many chemical compounds in the medicinal plants leading to treatment of various disease (3, 4). Mentha spicata is commonly called spearmint belongs to the family lamiaceae. It is an herbaceous rhizomatous perennial plant growing 30-100 cm tall, with variably hairless to hairy stems and foliage and a wide-spreading freshly underground rhizome.

The leaves are 5-9 cm long and 1.5-3 cm broad. Mentha spicata (mint) leaves are extensively used as herbal medicines all over the world. This medicinal plant is considered as stimulant, carminative, antispasmodic, stomachic and diuretic, also it is used in the treatment of gas pain, rheumatism, toothache and muscle pain. Mint possesses antioxidant properties due to the presence of active chemical compounds such as menthone, menthol, rosmarinic acid and carvone (5, 6). Mentha spicta contains many active chemical compounds such as alkaloids, volatile oils, tannins, flavonoids, steroids, resins and coumarins. The principle constituent is carvone along with some other monoturpenic constituents like limonene, phellendrene, dihydrocarveol, cineol, alph pinene and linalool. It possesses antiemetic properties also used as analgesic, stimulant, expectorant and carminative (7, 8). The antifungal activity of spearmint was studied, where its essential oil was found to has some antifungal effects. Also mint extracts were used as refrigerant, aphrodisiac, anti-inflammatory, antiseptic, antispasmodic and diuretic (9).

Alkaloids are basic nitrogenous compounds containing heterocyclic ring and the alkaloid molecule must contain nitrogen atom connected at least two carbon atoms and have at least one ring and they derived from plants sources, also they have physiological effects on human or animal. Most alkaloids in plants are biosynthesized from various amino acids as their direct precursors and they are found in 15-30 % of all flowering plants. The more dramatic actions of alkaloids are analgesics, mydriatics, miotics, antimicrobial and antileukemic (10,11). Therefore, this study aimed to evaluate the medicinal activity of maximal inhibitory concentration of alkaloids isolated from Menthe Spicata against some pathogenic fungi.

Materials and Methods:

Collection and taxonomic of plant; Mentha Spicata plant was purchased from the local market of Abu Al-khaseeb region at Basrah governorate in Iraq. The leaves were dried well in dark, grinded and kept in glass bottles in biochemistry laboratory. The plant was taxonomied by a botanist in the biology department at education college for pure sciences in university of basrah.

Chemicals:

All chemicals were of analytical grade and were supplied as the following:

Ethanol, acetic acid, α-naphthol, sulphuric acid, ferric chloride, bismuth sub-nitrate, potassium hydroxide, ninhydrin, potassium iodide, ammonium hydroxide, chloroform, benzene, mercuric chloride, lead acetate, copper sulphate, sodium tri-citrate, distilled water.

Culture medium:

Potato dextrose agar (PDA) and saboured dextrose agar (SDA) media were prepared according to information determining by manufacturing company.

Pathogenic Fungi:

Several pathogens fungi represented by Aspergillus Fumigalus, Candida albicans, Penicillum crysosporm and Aspergillus niger were gotten from clinical isolates are used in this work. Also these pathogenic fungi were kept well in lab until the day of use.

Isolation of Alkaloids from Mentha spicata:

Forty grams of Mentha spicata leaves were mixed with 250 ml of 10 %(v/v) ethanol acetic acid and the mixture was stirred on magnetic stirrer for 24hr and it was filtered by Buchner funnel. The filtrate was concentrated to quarter of its volume by rotary evaporator and acidified with 5 ml of concentrated sulphuric

acid then the acidic fraction was basified by ammonium hydroxide to pH equal to 9. The extraction process was carried out by using separation funnel by adding (3*20 ml) of chloroform then alkaloids were isolated from organic layer and dried (12) with yield to 3.82 gm.

Preliminary qualitative tests:

The alkaloids isolated were underwent several detections represented by the fellowing:

1- Alkaloids detection: was carried out by using Dragenrooff and Mayer reagents (13).

2- Carbohydrates detection: was achieved by using Molish reagent (12).

3- Phenols detection: was done by using (1%) ferric chloride (14).

4-Tannins test; was achieved by using lead acetate (1%).

5-Flavonoids test; was carried out by using alcoholic potassium hydroxide (5N).

6-Glycosides detection: was carried out by using Benedict reagent (12).

7-Saponin detection: was made by using (5%) mercuric chloride (14).

8-Amino acids detection: was achieved by using (1%) ninhydrin (15).

Thin Layer Chromatography (TLC):

TLC technique was carried out for separation of alkaloidic compounds abuntant in alkaloids extract and determination of their purity. One hundred microlitres of alkaloids were toulerenced on silica gel plate (2*10 cm) and was put in glass jar, then ethanol – distilled water – acetic acid solvents were used as eluent system with ratios equal to (10: 20: 20) for 40 minute. The glass plate was dried and the components were developed by UV-lamp and 233 nm, iodine vapour and Dragendroff reagent (16).

Infra-red (IR) spectroscopy:

Infra-red (IR) spectrum was recorded for alkaloids isolated by using (IR-8400s-Japan) spectrophotometer. The alkaloidic sample was fixed on sodium chloride disc and the spectral range was measured at (600-4000 cm-1).

Functional groups detection (12).

1- Double bond test: was achieved by using potassium permanganate reagent.

2- Alkaloids test: was carried out by using Mayer reagent

3- Aldehyde and keton groups test: was examined by using 2,4-dinitrophenyl hydrazine

Bioactivity of Maximal inhibitory concentration:

Different concentrations of alkaloids isolated, were used to determine the maximal inhibitony concentration. This concentration was tested by using diffusion method against pathogenic fungi isolates represented by Aspergillus fumigatus, candida albicans, penicillum crysosporm and Aspergillus niger by using potato dextrose agar (PDA) and sabourad dextrose agar (SDA) as culture media. The pathogenic fungi were cultured in petri dishes containing these culture media which were sterilized by autoclare at

121 oC for 15 min and 1 atm pressure. The chloromaphenicol as an anti-biotic was added to growth fungi and prevent growth of bacteria. The dishes were incubated in the incubator at 25 oC for seven days. 5ml of distilled water was added into fungi, then 0.1ml of fungal colloidal was taken and added into each petri dish, then the concentration of (250 mg/ml) of alkaoids, was added as disc to all dishes after that the petri dishes were incubated at 25 oC for 3-7 days, finally the inhibition zone diameters were measured (17).

Results and Discussion:

In the current study, alkaloids were isolated and purified from Mentha spicata leaves with extraction percentage equal to 9.55%, this percentage is concedered to be very good because most alkaloidic compounds in plants, are presnt in small amounts. Also this amount of alkaloids indicates presence of little alkaloidic compounds with low concentrations. It is known that alkaloids occur in various medicinal plants and they are biochemically synthesized from different amino acids. Table (1) shows the chemical preliminary qualitative analysis results of alkaloids extract isolated from Mentha spicata leaves. It is noticed that alkaloidic extract gave a positive test with dragendroff reagent where orange precipitate was formed, this ensures presence of alkaloids but carbohydrates, glycosides, phenols, tannins, flavonoids, saponins and amino acids were not present because they showed negative tests with Molish, Benedict, Ferric chloride (1%), lead acetate (1%), alcoholic potassium hydroxide (5N), mercuric chloride (5%) and ninhydrin (1%) reagents respectively. Different studies ensure presence of alkaloids in the medicinal plants including Mentha spicata, also various researches indicated the medicinal and biochemical role of alkaloids exsisting in many medicinal plants such as Anisomeles malabarica L., Hyptis sauveolens L., Ocimum gratissimum L., Ocimum sanctum L., Leucas aspera, Coleus aromaticus, Ocimum bascillicum L. and Mentha spicata (18,19). The plants of Lamiaceae including Mentha spicata are important for their medicinal properties among plants. The chemical extracts of this plant family have different dramatic physiological effects such as diuretic, sedative, digestive, antiparasitic, carminative, appetizer, anticonvelsant, anti-inflammatory and stimulant. They are also used to treat fever, cough, headaches, stomachaches, wound healing, heart diseases and dysmenorrheal (20).

Table (1) Preliminary qualitative analysis tests of alkaloids isolated from *Mentha spicata* leaves.

Reagent	Test result	Notes	Conclusion
Dragenroff	+	Formation of orange precipitate	Alkaloids are present
Mayer	+	Formation of a precipitate with turbidity	Alkaloids are present
Molish	-	Violet ring is absent	No carbohydrates
Bendict	-	Red precipitate is absent	No glycosides
$FeCl_3$ (1%)	-	Bluish-green colour is absent	No phenols
Pb(Ac)2	-	Light brown precipitate is absent	No tannins
Alcoholic KOH(5N)	-	Yellow precipitate is absent	No flavonoids
$HgCl_2$ (5%)	-	White precipitate is absent	No saponins
Ninhydrin(1%)	-	Violet colours is absent	No amino acids

Thin layer chromatography technique is a useful analytical tool for the isolation and identification of organic compounds including alkaloids. The
data of TLC technique results are shown in table (2). It was observed that two spots were separated from alkaloids extracts, have rate of flow equal to 0.81 and 0.97.

Table (2) Thin layer chromatography result of alkaloids isolated from Mentha spicata leaves

Eluent system	Reagents	Result	Rate of flow (Rf) values	Conclusion
Ethanol – dissIled Water-acetic acid (10: 20: 20)	UV-lamp	Ligth orange	0.81, 097	Pure compounds are present
	Iapour-2	Brown	0.81., 0.97	Nitrogenous organic compounds are present
	Dragendroff	orange	0.81, 0.97	Alkaloids are present

By using eluent system above with different ratios depending on UV-lamp, iodine vapour and Dragendroff reagents, the two components were noticed this ensure presence of two alkaloidic compounds in alkaloids extract. The results of quantitative separation of secondary metabolite represented by alkaloids from the leaves of *Mentha*spicata by thin layer chromatography are always given by the obtained R_f values, also fby spicatathe data of these values are useful to establish the purity and identity of alkaloidic compounds abundant in medicinal herbs including Mentha spicata. Therefore, TLC technique is a simple, robust, reproducible method for separation of photochemicals which were reported in different Lamiaceae plants including mint. Also it was proved that the colors of the separated spots in thin layer chromatography and their position relative to standard substances are important characteristics for the identification of chemical extracts including alkaloids (21,22).

Infra-red(IR) spectrum results of alkaloids isolated from Mentha spicata are shown in figure (1) and table (3). From this spectrum, it was recorded several different bands belongs to structural and/or functional groups are present in the chemical structure of belongs to stretching 1-f a broad band at 3450 cm^{-1} Appearance o isolated alkaloids. Appearance of vibration of hydroxyl group abundant in alkaloids, and presence of a medium band at H) group, while the medium -indicates stretching vibration of aromatic (C 1-cm 2920nce of H) group. Appeara-represents bending vibration of aliphatic (C 1-band at 2835 cmshows the stretching vibration of amino 1-number 3230 cma sharp band at the wavegroup (N-H) existing in chemical structure of alkaloidic compounds, also the sharp band at 1650 cm indicates the stretching vibration of imine group (C=N) which 1-cm represent the major group abundant in alkaloids. Presence of a sharp band at 1620indicates stretching vibration of aromatic (C=C) group, whereas the presence of a e bending vibration of aromatic (C=C) group, also shows th 1-sharp band at 1400 cmbelongs to stretching vibration 1-number 1735 cmpearance of a weak band at waveapindicates the stretching vibration of 1-of keton group (C=O). The weak band at 725 cmbenzene ring containing aromatic substitution (ph-x) (23).

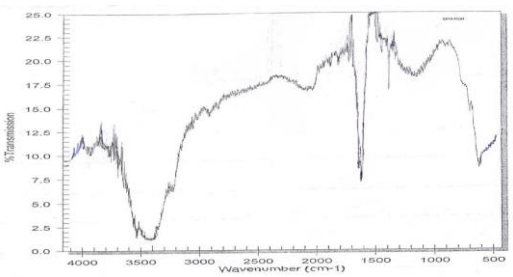

Figure (1). Infra-red (IR) spectrum of alkaloidic compounds isolated from Menth spicata leaves.

Table (3) Absorption bands and their related structural and functional groups in IR-spectrum of alkaloides isolated from Mentha spicata leaves.

Band frequency(cm)	Band shape	Band type	Assignment of band	Structural and functional group
3450	broad	O-H	stretching	Hydroxyl group of alkaloids
2920	medium	C-H	stretching	aromatic
2835	medium	C-H	bending	aliphatic
3230	medium	N-H	stretching	Amino group
1650	sharp	C=N	stretching	Alkaloids or amines
1620	sharp	C=N	stretching	aromatic
1400	sharp	C=N	bending	aromatic
1735	weak	C=N	stretching	Keton group
725	weak	Ph-X	stretching	Substituted benzene ring

Infra-red spectrometry is considered as a tool for identification of the chemical structure approximately for organic compounds including alkaloids

depending on characterization of assignment and active chemical groups existing in the structure of alkaloidic compounds which are responsible for bioactivity of these compounds. The functional groups tests result of alkaloids isolated from *Mentha spicata* leaves are shown in the table (4). It was observed that alkaloidic extract contains double bond system, alkaloidic groups and carbonyl (keton or aldehyde) groups. From these data the alkaloids isolated have double bonds represented by (C=C) and (C=N) bonds also the presence of (C=N) group is considered to be a correlation between double bond test and the active nitrogenous group of alkaloids. The indications of functional group tests are disappearing of potassium permanganate color, formation of a precipitate with turbidity and formation of an orange precipitate

Table (4) Results of functional groups tests of alkaloids isolated from Mentha spicata leaves.

Reagent	Test result	Indications	Conclusions
KMnO4	+	Disappearance of potassium permanganate colour	Double bond is present
		Formation of a precipitate with	Alkaloidic
Mayer	+	turbidity	groups are present
Di-nitrophenyl hydrazine (DNPH)	+	Formation of yellowish-orange precipitate	Carbonyl groups are present

The bioactivity of maximal inhibitory concentration of alkaloidic extract was evaluated against four pathogenic fungi. The 250 mg/ml was recorded to give the greater activity among all used concentrations as in table (5), therefore, it was considered to be the maximal inhibitory concentration. The inhibition zone diameters which were recorded of this concentration against various pathogenic fungi, were equal to (23, 15, 15 and 11mm) towards *Aspergillus fumigatus, Candida albicans, Penicillum crysosporm and*

Aspergillus niger fungi respectively. Also from the table (5), it was noticed that *Aspergillus niger* fungus had more biochemical resistance towards the maximal
inhibitory concentration but *Aspergillus fumigatus* fungus was less biochemical
resistance towards the same concentration. Many various studies ensured and proved the high medicinal activity of alkaloidic compounds isolated from the medicinal plants against pathogenic microbes (bacteria and fungi) because the alkaloids have physiological effect and dramatic therapy properties (24, 25). The metabolic mechanism of alkaloidic compounds activity is represented by chemical bonding of these compounds with nucleic acids (DNA & RNA) then inhibition of metabolism of these acids (26). Also the medicinal and biochemical activities of alkaloids, result from inhibition of various enzymes action which are responsible for metabolic pathways in living cell of microorganisms including pathogenic fungi. In addition to, alkaloidic compounds extracted from medicinal herbs inhibit protein biosynthesis pathway leading to lack in cell proteins which are responsible for biochemical synthesis of whole living cell (27). The functional which has the ability) (mine group group abundant in alkaloid is ichemical to decompose and destruct the living cell of pathogenic fungi (10, 14). It is known that these fungi cause several various infections diseases such as skin infections and inflammatory of respiratory and digestive systems N C

Table (5) Bioactivity of maximal inhibitory concentration of alkaloids isolated from Menthe spicata leaves against pathogenic fungi.

Maximal inhibitory concentration (mg/ml)	Pathogenic fungi	Inhibition zone diameters (mm)
250	Aspergillus fumigatus	23
250	Candida albicans	15
250	Penicillum crysosporm	15
250	Aspergillus niger	11

Conclusions:

The current research evaluated and proved the great bioactivity of the maximal inhibitory concentration of alkaloids isolated from leaves against some pathogenic fungi represented by *Aspergillus fumigatus*, *Candida albicans*, *Penicillum crysosporm and Aspergillus niger*. These fungi cause different diseases for human and animals, therefore it is recommended to use *Mentha spicata* alkaloids as a herbal medicinal substituent for treatment of diseases caused by these pathogenic fungi. Also, the bioactivity of alkaloids comes from presence of various functional and active groups in the chemical structure of these compounds leading to inhibit the growth of pathogenic fungi.

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