Phytochemical Investigation of An Iraqi Manna Lichen, Lecanora esculenta

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***Abstract*:**

This investigation provides new data on chemical characterization of aqueous & lipid extracts of the Iraqi Manna lichen, Lecanora esculenta. Preliminary chemical detection of some medicinal active ingredients of the crud manna lichen powder, & the acidity of the water extract, were performed using different reagents. The total lipid was extracted by chloroform-methanol 2:1 (V/V), & separated by salicylic acid column into neutral & polar lipid. Fractions of these lipids were further analyzed by gas/liquid chromatography (GLC) to detect of various saturated and non-saturated fatty acids. The polar lipid fraction, the phospholipids was performed by thin-layer chromatography analysis (TLC), whereas analysis of the trimethyl silylate (TMS) derivative of the unsaponifiable lipid fraction for detection of sterols was performed by GLC analysis.

The results revealed neutral pH, and absent of the medical active ingredients; glucosides, tannins, alkaloids, resins, saponins, and coumarin. The approximate percentage of aqueous extract and total lipid extract of lichen respectively were 6%, & 1%. Neutral and polar lipid analyzed by GLC were both found to contain different percentages of various saturated and non-saturated free fatty acids in ratio respectively of about, 1:1.5 and 1:2. The detected saturated fatty acids both in neutral and polar lipids were; caprylic, capric, lauric, pentadecanoic, myristic, palmitic, stearic, and archidic acids, with hendecanaic acid which was detected only in neutral lipid, whereas isopalmitic and isostearic acids were detected only in polar lipid. In the unsaturated ones in both lipid moieties were; myristoleic, palmitoleic, oleic, linoleic, linolenic, and eicosenoic acids, with hexadecadinoic acid detected only in polar fraction. TLC analysis of the polar fraction revealed presence of the phosphatides; Lecithin, Sphingomyline, Phosphotidylserine, Phosphotidylinositol, Lysophosphatidyl ethanolaminem, Phospha-tidyl ethanolamine, whereas, GLC analysis of the TMS derivative of the unsaponifiable lipid fraction, revealed presence of Beta-Sitosterol, Oestradiol, and Pregnanestriol, though before silylation, the fraction showed presence of Campesterol, Oestrone, and Oestriol. The results suggested presence of a various compounds of anabolic & nutritional values.

***Index Terms*— Gas/liquid chromatography, Lecanora esculenta, Manna lichen, Phospholipid, Sterols.**

I. INTRODUCTION

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Lichen is a dual non-flowering, slow-growing plant formed of a symbiosis between fungi and a photosynthetic organism (algae and/or cyanobacteria), in which the fungi providing the partner with water and minerals, while the partner supplies the fungi with carbohydrates [Shuttle worth & Zim, 1969]. Lichens can grow on mountainous, limestone rocks, marble, bone rocks, and even barren lands [MacDonald,1951], and thrives in deserts, forests, where the air is clean, cold and dry atmosphere [Shuttle worth & Zim, 1969]. Lecanora esculenta is the scientific name for lichen known in Arabic as Manna lichens. It consists of only a body called thallus in which the root is not distinguished from the stem or leaves. Its external form is either papery (foliose) or a hard, wrinkled scale with few small shield-like scales in a whitish-pink color [Hale, 1961]. It is spread in the western desert of Iraq, specifically in the Al-Fikara and Al-Sweibat regions, where they call it locally "Krikshah" [Rashid & Mursi, 1986], and in the Sinai Peninsula. It may be the manna that the Torah mentioned that the children of Israel scavenged on in the wilderness, and whose mention was confirmed by the Almighty's saying in Surat Al-A'raf 160:7 (And We sent down manna and quails to you, eat of the good things that We have provided for you). Although, Schimtischek [1980] believes that the manna that the Israelis subsisted on in the Sinai desert, was made by the coccids on the tamarisk tree.

Lichens is a source of chemical compounds, founds to contain several aromatic acids and aliphatic acids. Of the aromatic acids, usnic acid [Hesse, 1907], atranoric acid, barbutnic acid, variadric acid, soluzinic acid, pheodielic acid [Shibata & Husach-Chin, 1965], gangabonidin, sekkikaic acid, obtusatic acid , stictic acids, and others [Huneck and Follmann, 1965; Thun and Solberg, 1980], whereas of the aliphatic acids,

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isomuronic acid and nuropogolic acid, as well as phenolic and flavonoid compounds, and compounds of the depsid structure of lecanoric acid, obtusic acid, and atranorin, were also isolated [Bodo & Molho, 1980]. Lichens have also been found to contain several types of lipids of the two main groups: neutral lipids like; free fatty acids, sterols, steryl esters, acylglycerols, and wax, and polar lipids like; phospholipids, glycolipids and betaine lipids, which play different roles. Triacylglycerols act as a compact, easily metabolized and non-hydrated energy store. Waxes are commonly extracellular components such as the surface covering, which function both to reduce water loss and to protect plants from noxious environmental conditions. Betaine lipids are a family of glycerolipids (nonphosphorous, zwitterionic, polar head group and an ether bond connecting the head group with a diacylglycerol "DAG" backbone), widely distributed in lower plants and algae as well as in some non-photosynthetic micro-organisms. Polar lipids and sterols are important structural components of all cell membranes. There are many examples of what could be termed biologically active lipids e.g. inositol lipids, sphingolipids, and oxidation products [Bychek-Guschina, Kotlova, & Heipieper,1999; Hiroki, et al, 2018].

As a source of chemicals with valuable biological properties, the complex sugars of some lichens has been used since long time ago as antineoplastic against sarcoma type cancer [Shibata & Husach-Chin, 1965], especially the complex sugar, glucans type pastulan (GE-3) and type alichenan (VR-1-1), which isolated respectively from the lichen Gyrophore esculeta and Uanea rubescens, whereas GE-3 derivative, lauroyl, has also been found to have a strong effect on sarcoma type, but not on cancer type carcinoma [Nishikawa et al, 1979]. Recently, one of the studies, indicated that lichen-derived compounds and extracts exert significant cytotoxicity against GBM cells, inhibit the kynurenine pathway enzymes, and have anti-inflammatory properties, antioxidant & anti-cholinesterase properties. These results also demonstrate that lichen-derived extracts and compounds, especially (−)-usnic acid, can be regarded as prototypes of pharmacologically active compounds within the CNS, especially suitable for the treatment of GBM [Studzinska-Sroka, et al, 2021].

Manna has an anti-growth effect on germs that grow on cress seeds. The fungal part of some lichens was also used as an antibiotic to stop the growth of bacteria (Bacteriostatic) of a type gram positive [Ramme-Schmidt, 1966], whereas its compounds such as usnic acid and atranoric acid were used as antibiotics when performing surgeries and during parturition. It has also been found that usnic acid present in some lichens has a strong effect in preventing the growth of bacteria gram positive species, mycobacterium species, and penicillin-resistant, Staphylococcus aureus [Hale, 1974]. Furthermore, an extract of some Indian lichens found to have anti-bacterial activity of both gram positive and gram negative. In addition to the effect of lichens on bacteria, they have an inhibitory effect on the growth of fungi (fungistatic) of the type Trichosporum and some viruses such as Tobacco mosaic virus, whereas some others have a fatal effect on some types of plants (phytocidal) and fungi As for humans, its side effects have been proven, such as allergy and photosentitization, caused by atranortin and other aromatic acids [Thun & Solberg, 1980]. In industries, lichens had multiple uses, as in the manufacture of Harristweeds dye, and sunflower paper (Litmus paper), which is widely used in some medical examinations [Shuttle worth & Zim, 1969]. Recent studies showed the extracts of lichen to possess cytotoxic/cytostatic activity on HeLa, LS174, and A549 cells [Tomovic, 2019], and that bioactive compounds like phenol and flavonoid content in the lichen can be used as natural antioxidant agents [Tomovic, 2019; Pc, 2022], whereas in other it has been found to have on cytotoxic and antimicrobial activities [Kocovic et al, 2022]. A recent molecular docking studies evaluate the antioxidant capacity and enzyme inhibitory activities of 14 lichen extracts, revealed that Dactylina arctica stands out for its higher antioxidant capacities, and Asahinea scholanderi and Cetraria cucullata extracts were the best inhibitors of AChE and BuChE, whereas the major secondary metabolites identified by HPLC were alectoronic acid and α-collatolic acid for Asahinea scholanderi, usnic acid and protolichesterinic acid for Cetraria cucullata, and concluded that these species could be effective in the treatment of neurodegenerative diseases [Urena-Vacas, et al, 2022]. Usnic acid with a dibenzofuran structure were also identified in the extracts by HPLC [Tomovic, 2019; Pc, 2022; Kocovic et al, 2022]. The major secondary metabolites in Asahinea scholanderi identified in recent molecular study by HPLC were alectoronic acid and α-collatolic acid and in Cetraria cucullata were usnic acid and protolichesterinic acid [Urena-Vacas, et al, 2022].

The present study is intended to investigate the lipid constituents of an Iraqi lichen analyzed by methods of lipid analysis that are available for many laboratories [Guschina, 2002], and have been used by the author for extraction, separation and quantification of basic lipids.

II. MATERIALS AND METHODS

Manna lichen was collected from Al-Fikara and Al-Sweibat areas in the Western Desert of Al-Rutba district/Iraq, and identified with the help of the botanical herbarium of the Botany Directorate in Abu Ghraib, during the spring season. These lichens were thoroughly cleaned of dust and impurities, then ground in a metal mortar until they became small granules, which were kept in closed glass containers until use.

The phytochemical investigation involved, preliminary chemical detection of some medicinal active ingredients of the crud manna lichen powder, before preparation from the crud, the aqueous extract and the total lipid extract, for subsequent separation of the total lipid into neutral lipids and polar lipids by a silicic acid column. Furthermore, for detection of the most important active ingredients in the lipid fraction, the fatty acids in neutral lipid, and the fatty acids in polar lipid, as well as the sterols in the non-saponifible part of total lipid, were determined and analyzed by gas/liquid chromatography (GLC),

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whereas determination of some types of phospholipids was done by thin-layer chromatography method (TLC).

In the preliminary chemical detection, the reagents of Fehling, Benedict, Dragendorff, Wagner, Mayer, and solutions of ferric chloride, lead acetate, mercuric chloride, and ammonical silver nitrate, were used. Reagents and solutions were prepared according to the method mentioned by Stahl [Stahl, 1969], and developed by Shihata [1951]. The tests involved mixing of 10 grams of lichen powder with 50 ml of distilled water, filtering the solution and testing the filtrate for detection of its reaction with sunflower leaves, and a pH meter-7020 (Pye Unicum U.K.) to measure the acidity. For detection of tannins, the lichen filtrate was left to cool, and divided into two parts, one added to a 1% lead acetate solution, and the other added to a 1% ferric chloride solution, to indicate presence of gallstones by the appearance of a precipitate of gelatinous metabolism, and a bluish-green color respectively. For detection of glucosides before analysis, the aqueous filtrate were either mixed in equal part with Fehling's reagent, or 1.0ml of it added to 5ml of Benedict's reagent, and left in a boiling water bath for 10 minutes, to infer the presence of free sugars as a red precipitate. For detection of glucoside after analysis, a few drops of dilute HCL was added to 5 ml of the filtrate, lifted in a boiling water bath for 20 minutes, and the acidity was normalized by sodium hydroxide (NaOH). Presence of sugars was detected by means of the Fehling's reagent, and result was compared to that observed before the analysis. For detection of saponins, the lichen filtrate was shake vigorously in a test tube, to infer the presence of soapy substances from the appearance of thick foam. For the same purpose, 5ml of ammoniated silver nitrate solution, or of mercuric chloride solution, were added to 5 ml of the filtrate in a test tube left for 10 minutes without stirring in a water bath at a temperature of 100 °C before they left to cool. The presence of soapy substances, were indicated by the appearance of a silver mirror on the wall of the test tube, or the appearance of a white precipitate respectively. For detection of the alkaloids, 10 grams of powdered lichen boiled in 50 ml of distilled water was diluted with 4% HCl, and filtered after it cooled and tested by using 0.5 ml of the filtrate on a watch glass with all of the following reagents; Wackner's reagent, Meyer's reagent, Drakendrov's reagent, or picric acid, to indicate the presence of alkaloids by the appearance of a brown precipitate, a white precipitate, an orange precipitate, or a yellow precipitate respectively. It is worth mentioning that some alklocosides give a positive reaction with the general test for the alkaloids mentioned above [Fahmy, 1933]. For detection of resins, 50 ml of ethyl alcohol, concentration of 95%, was added to 5 gm of lichen powder, left in a boiling water bath for one minute, filtered, and 100 ml of acidified distilled water was added to the filtrate, where the presence of resinous substances is indicated by the appearance of turbidity or white saliva (Turbidity). Lastly, for detection of coumarins, a small amount of lichen filtrate was dissolved in alcohol in a test tube covered with filter paper moistened with a dilute sodium hydroxide solution, placed in a boiling water bath for a few minutes, and exposed then to ultraviolet light using a device (UV Source DESA, German), to infer the appearance of yellow-green fluorescent coloration on coumarin [Geissman, 1962].

Preparation of total lipid and aqueous extracts of manna lichen:

Total lipid and aqueous extracts of manna lichen were prepared according to the method of Folch, & Stanley [1957]. Preparation of the total lipid extract in breve was by homogenized one part of the lichen with three parts of a compound solution of chlorophyll-methyl alcohol at a ratio of 1:2 (vol/vol). The top layer of the mixture that contains the total lipid was withdrawn by using the siphoning method, which was then filtered with Fat-Free filter paper that allows the fat particles to pass through, in a process repeated by adding a new volume of the compound solution to obtain the remaining amount of lipid substances in the mixture. This lipid crude extract, was then washed by mixing with one-fifth of its volume distilled water and the upper layer was siphoned off in a process repeated twice, and mixed with chloroform-methyl alcohol-water in a separatory funnel, in the following proportions 3:4:8 respectively, to obtain total fat from the top layer. For preparation of the aqueous extract of manna lichen, the same method of extraction of the total lipid was followed, where the distilled water used as a solvent instead of chloroform-methyl alcohol. The lipid extract or aqueous extract then concentrated to obtain a dry powder of active ingredients, by evaporation of the solvents by means of a rotary vacuum evaporator (Buchi, Switzerland company), and preservation of the extract after passing nitrogen gas over it in tightly closed glass bottles away from sunlight and stored at a temperature of -10 °C.

Separation of neutral lipids from polar lipids:

The total lipid were separated into neutral and polar lipids according to the McCarthy and Duthie method mentioned by Al-Shibibi [1967], using chromatographic separation on activated salicylic acid column.

Activation of salicylic acid:

A 100gm of salicylic acid (mesh 100, Fluka, Switzerland), mixed well in 1L flask with 400ml ethyl alcohol 95%. The top layer that carries the fine particles withdrawn by siphon, left for 5 minutes to settle down before washed in a process repeated twice using the same volume of acetone first, and anhydrous diethyl ether then after. The salicylic acid now ready to place in a flat container to dry at room temperature before by oven at a temperature of 105°C overnight to be ready for use.

Salicylic acid column preparation:

A column (1.5 x 20 cm) was prepared by placing filter paper type (Wattman No. 450) on top of the glass wool on the bottom side of the column, and 5g of activated salicylic acid were taken and mixed with 20 ml of chloroform, then transferred to the column and the sides of the column were washed with chloroform, where it was used about 25 ml, keeping in mind that the level of chloroform should be kept above the surface of the salicylic acid by about half a centimeter to prevent drying of the surface.

Extraction of neutral lipids from the total lipid extract:

Four grams of total lipid extract were dissolved in 20 ml of chloroform and the mixture was transferred quietly to the surface of the column, then the sides of the column were washed

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well, and after the majority of the solvent passed through the salicylic acid column, the column was washed with 150 ml of chloroform in six equal batches, each of 25 ml. The chloroform and its contents were collected in a glass flask, and the solvent was evaporated using a rotary vacuum evaporator at a temperature not exceeding 45 °C to obtain the neutral lipids.

Extraction of the remaining polar lipids in the salicylic acid column:

This was done after the neutral lipids descended through the salicylic acid column, by washing the column with 150ml of chloroform in six equal batches, each of 25ml, and before the level of the last batch of chloroform reached the level of salicylic acid packed in the column, the solvent was changed to alcohol Absolute methanol to remove the polar lipids, then change the receiving flask to collect an amount of 150ml of ethyl alcohol that was evaporated by the rotary vacuum evaporator at a temperature of 45°C. The polar lipids were collected in glass bottles after they were emptied with a stream of nitrogen, and they were tightly closed and kept at a temperature not exceeding -10 °C.

Detection of fatty acids in neutral lipids by GLC:

For preparation of fatty acid esters in neutral lipids, the method of DeMan [1964] was used to prepare the methyl esters of the neutralizing fatty acids by using approximately 0.1 g of the neutralizing lipids with 0.1 ml of sodium methylate (0.2N) dissolved in absolute methyl alcohol, and placed in a vial of 3 ml volume, mixed well and sealed using a heat source, and placed in an oven at a temperature of 60-70 °C, for one hour, after which the sample would be ready for diagnosis in a GLC (5710, Hewlett Packard, USA) with a procedure modified to get rid of methyl alcohol, as increasing its concentration causes the methyl ester of butyrate to be hidden. The methyl alcohol was eliminated by adding to 10 microliters of the prepared ester, a drop of 0.15% concentration slain, and 3 ml ethyl chloride, and the mixture was mixed well and left for one minute, then the top layer was taken and evaporated at room temperature until dryness, which then mixed well with 0.1 ml carbon disulfide (CS2). One µl of the mixture then was taken for injection into the device for the diagnosis of fatty acids.

Detection of fatty acids in polar lipids by GLC:

For preparation of fatty acid esters in polar lipids, the Morrison and Smith [Morrison & Smith, 1964] method was used, by taking a small amount of polar lipids after drying them from methyl alcohol, and adding 3 ml of a solution of boron trifluoride in 14% methyl alcohol (BDH, UK) and 1 ml of absolute ether in closed glass tubes (100 x 13). These tubes are exposed to a stream of pure nitrogen before being tightly closed, and placed in a boiling water bath for 35 minutes. The closed tubes are then transferred to an ice bath and an amount of water is added to the extent that the liquid level is 1.5 inches from the mouth of the tube. The test tubes were re-immersed in the ice bath, and then extracted with 2 mM ethyl chloride. The top layer is withdrawn by siphon method, and the solvent is dried over 0.5 g of anhydrous sodium sulfate in a small bale, which is dipped in an ice bath, then the ethyl chloride is evaporated after being transferred to a new bale at room temperature under a light stream of nitrogen.

Conditions used in the diagnosis of fatty acids:

In the identification of fatty acids of neutral lipids and polar lipids, a metal column 6 inches long and 4 mm in diameter was used as a liquid phase (Diethylene glycol succinate) at 10% weight of solid chromosorb WA/W DMCS with a diameter of 80-100 mesh. The initial oven temperature is 100 °C for two minutes, the final temperature is 190 °C until the separation is completed, the average temperature per minute is 8 °C. The temperature of the injection area and the detector is 250 °C. The inert gas was nitrogen with flow rate of 25 ml/ minute, whereas hydrogen flow rate is 30ml/minute, and the air flow rate is 300 ml/min. The individual components of the fatty acids were identified by comparison with standard methyl esters (Applied science, USA). After analyzing 0.2 µl of a concentration of 10% (vol/vol) of the sample in CS2 in the device, the percentage of the curve (% Area under peak) and the % of the volumetric molecular weight (% mole) were calculated as follows:

$$\text{Area \%} = \text{Area } i / \sum 1\ i = 1 \quad X \quad 100$$

$$\text{Mole \%} = \text{Area } i / \text{M.wt. } i \quad / \quad \sum n\ i=1 / \text{average M.wt.} \quad X\ 100$$

Single component of any acid = i; Total number of components = n

Determination of some types of phospholipids by thin-layer chromatography:

The phospholipids in polar lipids were separated into their types. Glass sheets covered with a layer of silica gel (Silica Gel, Merck,W. Germany) with a thickness of 0.25mm and dimensions of 20x20 cm were used. The standard phospholipids used for comparison with those shown in the model were obtained from Supelco, USA. The plates were activated for 1hour before use in an oven at 105 °C.

Separation method:

Skipski, et al. [Skipski, Peterson, & Barclay, 1964] method in which a mixture of chloroform-ethyl alcohol-acetic acid-water in a ratio of 2:4:15:25 (vol/vol) respectively was used as a developing solvent system, and a small amount of polar lipids were added by capillary tubes in the form of spots, 2 cm away from the bottom edge of the paper. The plates then were placed in a chromatographic jar containing exposure solutions at a depth of 1.5 cm, and its inner sides were surrounded by filter paper, to help make parallel and quick equilibrium. The plates are left inside the chromatographic jar until the exposure solutions reach a distance of 15 cm from the point of placement of the sample to the top, and then the plates are removed from the chromatographic jar, and left to dry for 20-30 minutes in the air before the process of detecting the materials contained in the sample.

Detection method:

The general method reported by Al-Shibibi [Alshibibi, 1967] was used to detect the separation spots by burning the plates in an electric oven at a temperature of 120 °C for 20 minutes, after spraying them with a solution of 50% sulfuric acid saturated with potassium dichromate. It should be mentioned that there are special methods for detecting separated spots, such as that used to determine phosphatides like phosphatidyl

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ethanolamine, and the method of using special reagents to determine the presence of choline in phosphatides. Bial's reagent was also used to detect glycolipids [Randerath, 1964].

Detection of sterols in the unsaponified portion of the total lipid of manna lichen:

Saponification process:

The saponification process was carried out to isolate the saponified from the non-saponified part before GLC according to Burch field and Storrs [Burchfield & Storrs, 1962] method. By taking 10ml of total lipid and adding 30ml of alcoholic potassium hydroxide solution to it, where it was subjected to reflex heating for 60 minutes at a temperature of 60°C, then left to cool and then transferred to a separation funnel and equal parts of distilled water and absolute ether are added to it. The ether layer that contains the unsaponified part is separated from the water layer that contains the soaped part. By evaporating the ether, the unsaponified part was obtained.

Extraction of sterols from the unsaponified fraction by Urea Clathrate method:

Sterols from other compounds in the unsaponified fraction was purified by the method of Von Rudloff [1961] and Downing, Kranz, & Murray [1960] in which 250 mg of the unsaponified fraction was placed in a beaker with 5.5 parts urea and 75 volumes ethanol. The mixture was heat to reversible temperature, then leave to cool. The insoluble precipitates were removed by filtration and washing with cold ethyl alcohol. Then, 4 gm of urea was added to the filtrate, heated to the reversible temperature, and left to cool, to form a new group of crystals. The process was repeated to obtain a filtrate of the unsaponified fatty fraction that did not contain alcohols and aliphatic hydrocarbons with non-abandent branches.

Identification of sterols by GLC:

After dissolving it with carbon disulfide, 0.2 µl of unsaponified substance was injected into the device, in order to diagnose sterols and compare them with standard animal and plant sterols (Supleco, USA).

Preparation of the Trimethylsilyl (TMS) Derivative of Sterols:

5 mg of the unsoaped substance was add to 1ml of "z" Tri-Sil. Solution (1.5meq/ml N-tri methylsilylimidazole in silylation grade pyridine; Pierce company, USA), and placed in the special tube for preparing of the derivative (Sillivial), and left for 30 minutes in the oven at a temperature of 65 °C, then 0.2 µl were taken from it and injected into the device to diagnose sterols. In the same way, a derivative (TMS) of the standard sterols was prepared.

Conditions used in the diagnosis of sterols and their (TMS) derivatives:

Sterols and their (TMS) derivatives, and standard sterols and their derivatives were identified under the same conditions. Where a metal column 3 feet long and 4 mm in diameter used as a liquid phase, OV, in proportions of 3% of the solid material Chromosorb WA/W DMCS with a diameter of 80-100 mesh, was left in oven with initial temperature of 200 °C for two minutes, and the final temperature of 300 °C until complete of the separation. The average temperature of the device per minute was 8°C, the temperature of the injecting region was 250 °C, and the temperature of the detector was 300 °C, while the inert gas was nitrogen, and its flow rate was 25 ml/min.

III. RESULTS

Preliminary detection of some active medicinal components of lichen:

The preliminary detection of some active medicinal components of lichen were carried out using the previously mentioned chemical tests. The results of these tests showed that there were no glucosides, tannins, alkaloids, resins, saponins, and coumarins in the water extract. The acidity of the water extract also showed that it was almost neutral, as indicated in Table (1).

Determination of the characteristics of the total lipid and aqueous extracts of the lichen:

It was found that the percentage of total lipid in the lichen, after washing and purifying it, was about 1%, with a dark green color and a distinctive aromatic odor. It was found that the percentage of the aqueous extract, after obtaining the total lipid extract, and after drying it in the form of a yellow powder with a special smell, was 6%.

Determination of some basic components in the total lipid of lichen by chromatographic analysis:

Analysis of fatty acids in neutral fats by gas/liquid chromatography:

From the results of the analysis, it was found that the neutral lipids in the lichen contain the following fatty acids: caprylic, capric, hendecanoic, lauric, myristic, myristoleic, pantadecanoic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, archidic, and eicosenoate, but the latter one in table (2) is unknown to us.

It is clear from table (2) that palmitic acid constitutes the highest percentage among the present fatty acids, with a percentage of 23.441%, followed by linoleic, myristic, oleic, caprylic, eicosinoate, lauric, capric, stearic, palmistoleic, arecidic, linolenic, pentadecanoic, henidecanoic, and finally myristolic in the following percentages 20.110, 11.014, 8.014, 7.370, 6.441, 11.507, 4.007, 3.756, 3.506, 2.278, 1.652, 0.801, 0.670, 0.885 respectively.

The percentage of unsaturated fatty acids (myristoleic, palmitoleic, oleic, linoleic, linolenic, and eicosinoic) is 40.097%, while the percentage of saturated fatty acids (caprylic, capric, hindcanoic, lauric, myristic, pentadecanoic, palmitic, stearic, and aredicanoic) is 59.903%. That is, the ratio of unsaturated to saturated fatty acids is 1.49:1. This is also illustrated by the curves in Fig. (1-A and B), obtained from the gas/liquid chromatography analysis of fatty acids in neutral fats.

Gas/liquid chromatography for the identification of fatty acids in polar lipids:

From analysis of the results, it was found that the part of the polar lipis in the lichen contains the following fatty acids: caprylic, capric, lauric, myristolic, pentadecanoic, isopalmitic, palmitic, palmitoleic, hexadcadionic, isostearic, citric, oleic, linoleic, linolenic, orchidic, and finally eicosinoids, as shown in table (3). Linoleic acid constitutes the highest percentage among the fatty acids found in the polar fatty fraction, with a

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percentage of 37.126%, followed by: linolenic, palmitic, eicosinoate, lauric, myristic, arecedic, caprylic, capric, stearic, palmitoleic, isopalmitic, myristoleic, hexadecadanoic, Isostearic, then pentadecanoic acid according to the following percentages, respectively, 9.934%, 8.940%, 8.685%, 7.749%, 7.238%, 4.683%, 4.683%, 2.583%, 2.086%, 1.589%, 1.362%, 0.340%, 0.2 12% , 0.170%, 0.151%. The percentage of unsaturated fatty acids (myristolic, palmitoleic, oleic, linoleic, linolenic, and eicosinoate) is 65.890%, while the percentage of saturated fatty acids (caprylic, capric, lauric, myristic, pentadecanoic, isopalmitic, palmitic, isosteric, stearic, and aredic) is 34.11 0 %, that is, the ratio of saturated to unsaturated fatty acids is 1.93:1, which is the opposite of the relationship between unsaturated fatty acids and their saturated counterparts in neutral lipids to fatty acids.

Thin-Layer Chromatography (TLC) process for the identification of phospholipids in polar lipids:

Analysis of the results showed that the polar lipids in the lichen contain the following phosphotides: sphengomyline, lecithine, phosphotidylserine, hosphotidylinositol, lysophosphotidyl ethanolamine, and finally phosphotidyl ethanolamine, and as shown in table (4) and Figure (3).

Gas/liquid chromatography process for identification of sterols in the unsaponified fraction of total lipid:

It was revealed from the analysis of the unsaponified portion of the total lipid of the manna lichen that it contains plant sterols: campesterol, beta-sitosterol, and other sterols, Beta-oestradiol, pregnanetriol, osterol oestriol, pregnanediol, and oestrone. The presence of a cholesterol compound was not diagnosed, as is evident from Figures (4A) and (4B) and table (5).

Table (5) and figure (4B) shows that the following compounds: beta-sitosterol; Pregnanetriol; Pregnanediol; oestriol; and oestrone has the same retention time as peak No. (5) in figure (5A). And after performing the process of preparing the (TMS) derivative of the unsaponified part shown in figure (5A) by injecting it into the (GLC) device, and after the process of preparing the (TMS) derivative of the following standard sterols: estradiol; androstenedione; pregnanetriol, pregnanediol, and the standardized plant sterols, beta-sitosterol. It was found that pregnanetriol and beta-sitosterol in figure No. (5B) possess the same retention time as curve No. (9) in figure No. (5A), and the presence of beta-sitosterol (fig. 5B) was confirmed because it has the same retention time as curve No. (13 and 14) in figure (5A). The presence of pregnanediol and androstenedione derivatives was not proven according to figure (5A and B). These results are summarized in table (6).

Table (1): Shows results of the preliminary chemical analysis for some of the medically active components in Manna lichen.

	Kind of Analysis	Result
1	Reaction	Almost neutral (pH 6.9)
2	Glucosides	(-)
3	Tannins	(-)
4	Coumarin	(-)
5	Alkaloids	(-)
6	Resins	(-)
7	Saponids	(-)

Table (2): shows the type, percentage, and molecular concentration of fatty acids in the neutral lipid fraction of manna lichen

Peak & No. of Carbon atom of fatty acids	Fatty acids%	Fatty acid Name	Fatty acid Partial Concentrat. %
1 C8	7.137	Caprylic acid	12.074
2 C10	4.007	Capric acid	5.675
3 C11*	0.676	Hendecanoic acid	0.885
4 C12	4.507	Lauric acid	5.490
5 C14	1 1.019	Myristic acid	11.773
6 C14=1	0.404	Myristoleic	1.511
7 C15*	0.801	Pantadecanoic	0.806
8 C16	2 3.441	Palmitic acid	22.302
9 C16=1	3.506	Palmitoleic acid	3.362
10 C18	3.756	Stearic acid	3.222
11 C18=1	8.014	Oleic acid	6.922
12 C18=2	2 0.110	Lenoleic acid	17.492
13 C18=3	1.652	Lenolenic acid	1.448
14 C20	2.278	Archidic acid	1.779
15 C20=1	6.411	Eicosenoate	5.037
16Unknown	1.981	-	-

* Tentative Identification
C = 1 Represent one double bonds
C = 2 Represent two double bonds
C = 3 Represent three double bonds

Table (3): shows the type, percentage, and molecular concentration of fatty acids in the polar lipid fraction of manna lichen.

Peak No.	No. of carbon aton of fatty acic	Fatty acids%	Fatty acid Name	Fatty acid Partial conc-entration %
1.	C8	2.838	Caprylic acid	4.939
2.	C10	2.583	Capric acid	3.763
3.	C12	7.238	Lauric acid	0.069
4.	C14	4.683	Myristic aci	5.147
5.	C14=1	0.340	Myristoleic	0.377
6.	C15*	0.151	Pantadecanc	0.146
7.	C16**	1.362	Isopalmitic	1.333
8.	C16	8.685	Palmitic acid	8.502
9.	C16=1	1.589	Palmitoleic acid	1.568
10.	C16=2	0.212	Hexadecadinoic	0.211
11.	C18br	0.170	Isostearic acid	0.150
12.	C18	2.086	Stearic acid	1.840
13.	C18=1	8.940	Oleic acid	7.945
14.	C18=2	37.126	Lenoleic acid	33.955
15.	C18=3	9.934	Lenolenic acid	8.955
16.	C20	4.314	Archidic acid	3.465
17.	C20=1	7.749	Eicosenoate	6.264

* Tentative Identification;
** Br branched of the fatty acid
C = 1 Represent one double bonds
C = 2 Represent two double bonds
C = 3 Represent three double bonds

Table (4): shows the phospholipids of the fraction of the polar fatty acids of manna lichen in comparison with standard phospholipids by thin-layer chromatography.

Types of standard phospholipids	Presence in polar lipids of Manna lichen
Phosphotidyl choline	(-)
Lysolecithine	(-)
Phosphotydil ethanolamine	(+)
Phosphotidyl inositol	(+)
Phosphotydil serine	(+)
Sphengomyline	(+)
Lecithin	(+)
Lysophosphatidyl ethanolamine	(+)

(-) Represent negative result;
(+) Represent positive results

Table (5): Shows types of the sterols in the unsaponified fraction of the lichen analyzed by gas/liquid chromatography in comparison with standard sterols.

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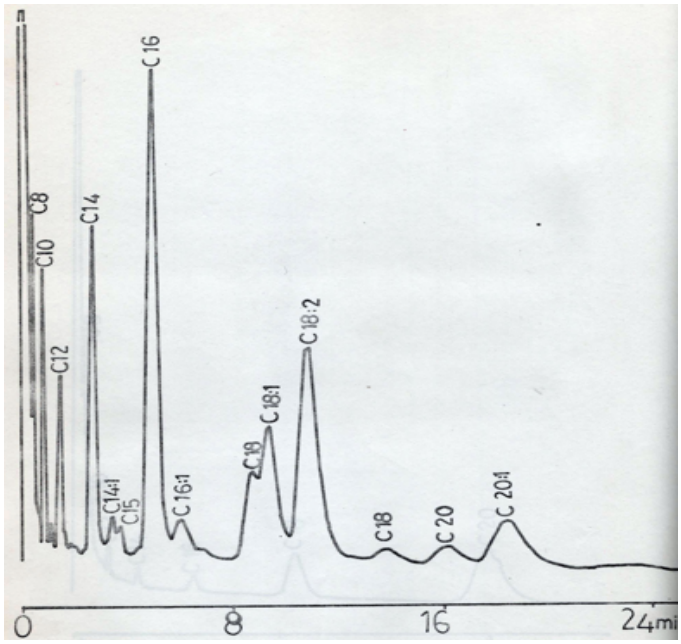
No.	Standard Sterols	Presence/ absence in lichen	Corresponding retention time in the model in minutes
1.	Beta-oestradiol	(+)	5.0
2.	Beta-sitosterol	(+)	10.5
3.	Cholesterol	(-)	-
4.	Campsterol	(+)	9.5
5.	Oestriol	(+)	5.0
6.	Oestrone	(+)	5.0
7.	Pregnanediol	(+)	5.0
8.	Pregnanetriol	(+)	5.0

(-) negative result
(+) positive result

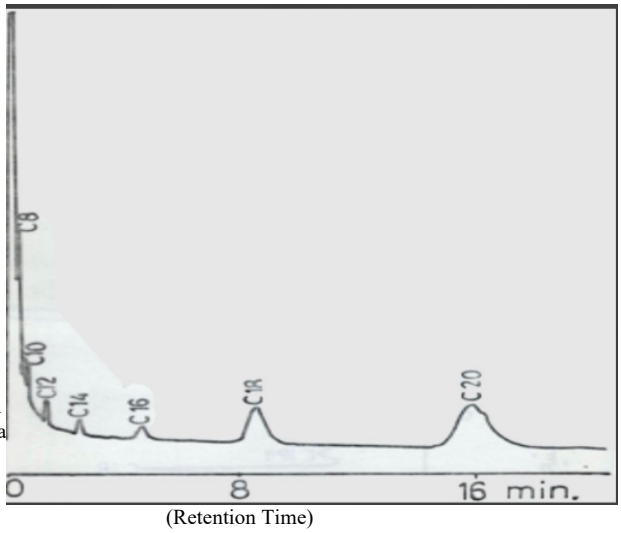
Table (6): Shows types of the sterols in the derivative (TMS) unsaponified fraction the manna lichen analyzed by gas/liquid chromatography in comparison with standa sterols.

No.	Standard Sterols	Presence/ absence in Lichen	Corresponding retention time in the model in minutes
1.	Androstenedione	(-)	
2.	Beta-sitosterol	(+)	11
3.	Oestradiol	(+)	8.25
4.	Pregnanediol	(-)	-
5.	Pregnanetriol	(+)	8.25

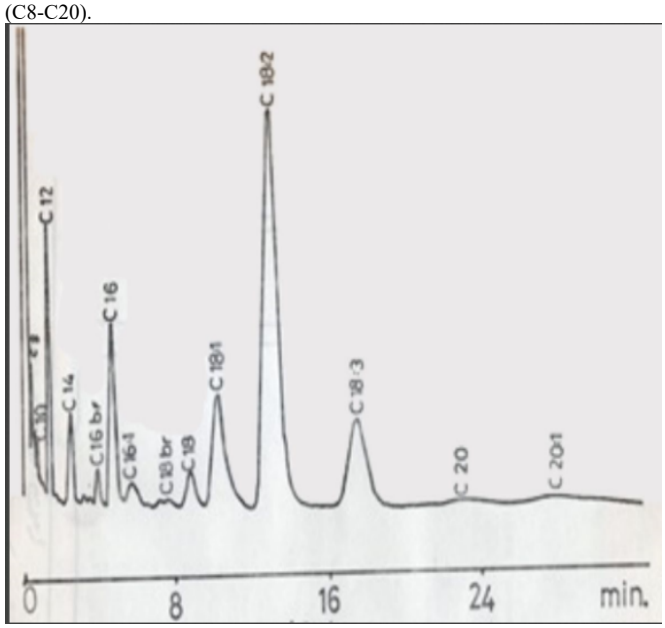
(-) negative result
(+) positive result.



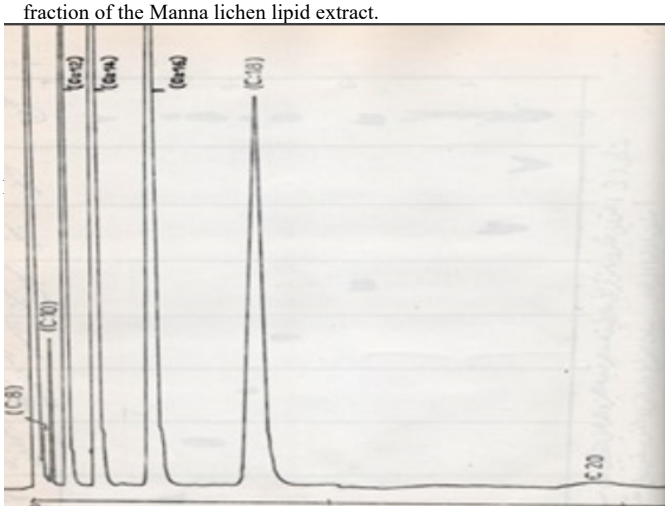
(Retention Time)
Figure (1-A): Gas/liquid chromatography analysis for the fatty acids in neutral fraction of the Manna lichen lipid extract.



(Retention Time)
Figure (1-B): Gas/liquid chromatography analysis for the standard fatty acids (C8-C20).



(Retention Time)
Figure (2-A): Gas/liquid chromatography analysis for the fatty acids in polar lipid fraction of the Manna lichen lipid extract.



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(Retention Time) 24min
Figure (2-B): Gas/liquid chromatography analysis for the standard fatty acids (C8-C20).

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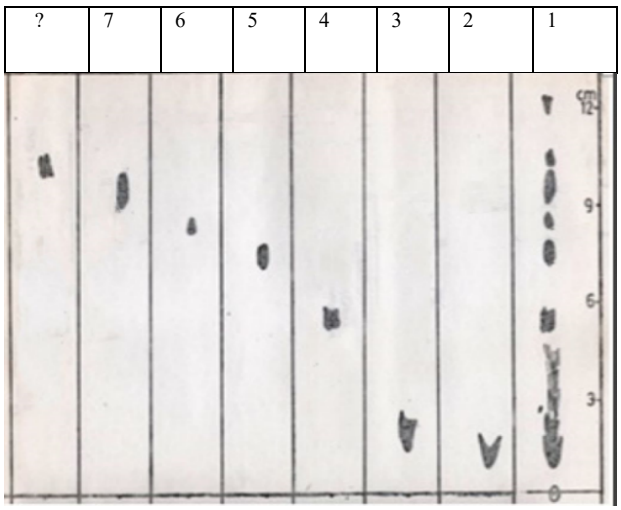
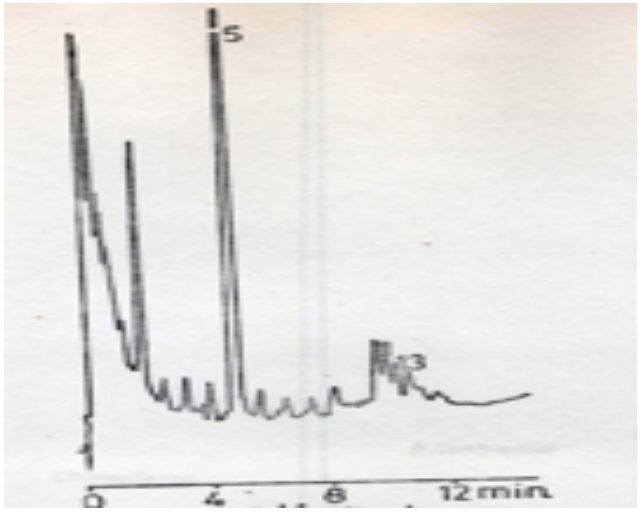
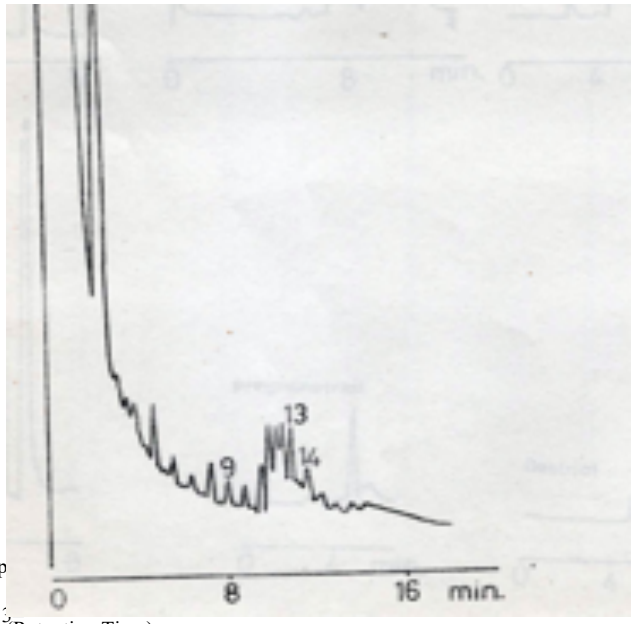


Figure (3): Thin-layer chromatography analysis for the fatty acids in polar lip fraction of the manna lichen lipid extract, demonstrate types of phospholipid comparison with their standard counterparts. 1= Sample; 2= Sphengomyline; Lecithine; 4= Phosphotidyl serine; 5= Lysophosphotidyl ethanolamine; 6= Phosphotidyl ethanolamine.



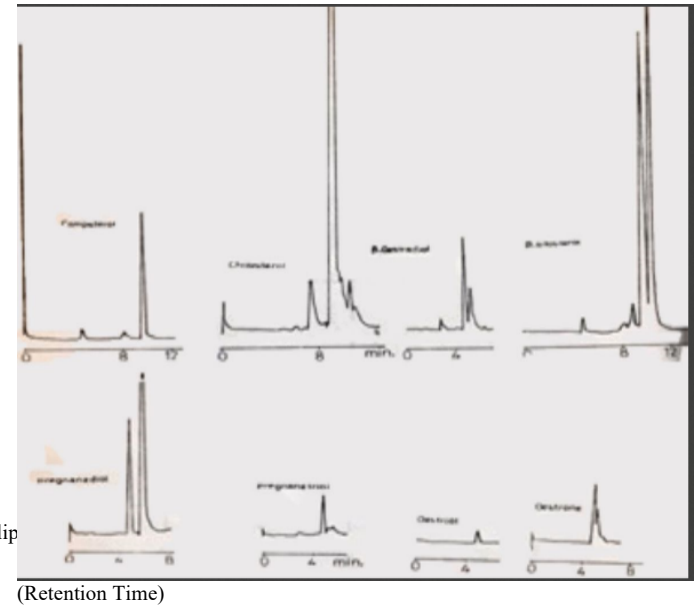
(Retention Time)

Figure (4-A): Gas/liquid chromatography analysis or the sterols in unsaponified lip fraction of the Manna lichen lipid extract.



(Retention Time)

Figure (5-A): Gas/liquid chromatography analysis for the derivative (TMS) in unsaponified lipid fraction of the Manna lichen lipid extract.



(Retention Time)

Figure (4-B): Gas/liquid chromatography analysis for the standard sterols.

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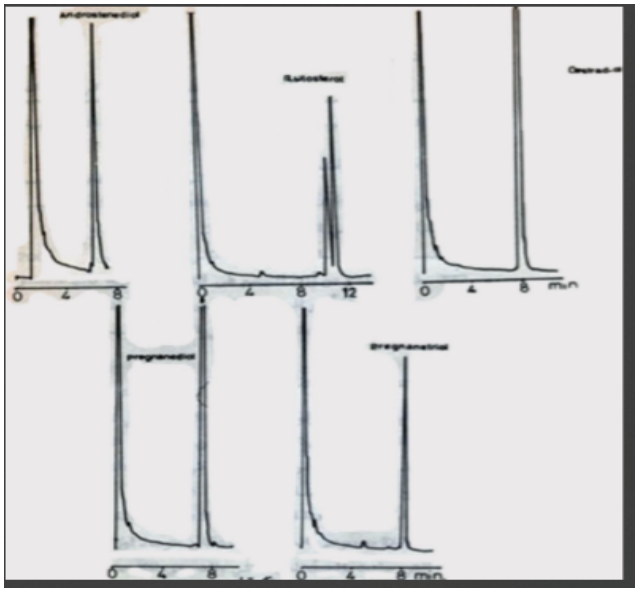

(Retention Time)

Figure (5-B): Gas/liquid chromatography analysis for the derivative (TMS) of standard sterols.

IV. DISCUSSION

Preliminary chemical analyzes conducted in this study with the aim of identifying some of the basic medicinal components of manna lichen led to preliminary conclusions indicating the absence of glucosides, tannins, alkaloids, resins, saponins, and coumarins, which are components that plants contain as a whole sometimes or some of them at other times in the many parts of them such as roots, stems, leaves, fruits, flowers and seeds, which makes the pH of the aqueous extract in these plants tend to move away from neutrality, while it was shown by measuring the acidity of the aqueous lichen extract that it is almost neutral. The failure to find the above effective medicinal ingredients in the preliminary examination may indicate a relative difference in the components of lichen from the nature of known wild plants, which may explain the possibility of its use as food for humans and animals, as indicated by [Rashid & Mursi, 1986; Hale, 1974], and this reasoning was reinforced by measuring the acidity of its semi-neutral aqueous extract, which may have made its taste palatable and acceptable to animals.

It was found, through the lipid and aqueous extract of lichen, that the percentage of the total lipid extract was approximately 1%, with a dark green and a special smell, while the percentage of the aqueous extract was about 6%, with a yellow color and a special smell as well. The special aromatic smell of both of the lichen extracts may be due to their containing, as in other lichens, on different types of aromatic acids such as atranoric acid [Tomovic, 2019; Pc, 2022; Kocovic et al, 2022]. The skin sensitivity and redness at the injection sites of the aqueous extract in rabbits and rats may be due to the presence of these acids that mentioned by Thune and Solberg [Thun & Solberg, 1980], to be the pathophysiology of photo-sensitization. As for the dark green color of the total lipid extract of the lichen, it is not excluded that it is due to the chlorophyll pigment that Beltman, et al. [1980] found lichens to contain, or that this pigment may be a result of the combination of the lichen with some chemical compounds.

This analytical investigation in part probably is the first in Iraq studying the manna lichen that is present in its western desert, therefore can be considered a working guide for future studies dealing with analytical aspects using different methods. Gas/liquid chromate-graphy analyzed free fatty acids in the neutral lipid part, as they are expressed by the number of its carbon atoms: C8: C10: C11: C12: C14:C14=1: C15: C16: C16=1: C18: C18=1: C18=2: C18=3: C20: C20=1, and the fatty acids in the polar fatty acids part were also identified according to their number of carbon atom: C8: C10: C12: C14: C14=1: C15: C16br: C16: C16=1: C16=2: C18br: C18: C18=1: C18=2: C18=3: C20: C20=1. It also turns out that the hendecanoic acid (C11) is found only in the neutral fatty fraction, which did not contain the following acids: isobalmetic (C16br), hexadecadecanoic acid (C16=2), and isosteric (C18br), while these acids were present in the polar fatty fraction.

Furthermore, the results also turned out that the ratio of unsaturated to saturated fatty acids in neutral lipids was 1.49:1, while the relationship was the opposite in polar lipids, where the ratio of saturated to unsaturated fatty acids was 1.93:1, and these identifying data may consider a pioneer quantitative and qualitative study on the saturated and unsaturated fatty acids of neutral and polar lipids component of the Iraqi manna lichen, Lecanora esculenta. The presence of fatty acids in other lichens has also been indicated by Beltman, et al, [1980] during his study to compare the amount of chlorophyll in these lichens and the contents of fatty acids, in relationship to air pollution, where they indicated that there is a direct relationship between the percentage of unsaturated fatty acids and air pollution in the area where lichen grows. Very recently, scientists have started to realize that lipid metabolism is a key factor in the adaptation mechanisms of many organisms to environmental and anthropogenic stress. In lichens, the importance of their lipids in the response and adaptation to environmental factors such as temperature, elevation, light, high levels of radiation, and Sulphur have been studied [Bychek-Guschina, Kotlova, & Heipieper, 1999; Piervittory et al., 1995].

The interest in the proportions of fatty acids in the lichen which shown in tables 1 and 2, is due to its essentiality as a macromolecule playing thousands of functions within the human body [Hiroki, et al., 2018]. One of these, is the necessity in the process of emulsification of fats and in the digestion of fatty substances in the small intestine of animals [Wilson, Gisvol, & doerge, 1971], and the special importance of fatty substances in the cell existence and the signaling, and in the function of the skin cells, as their deficiency in the diet causes the onset of the eczema, dryness and cracking of the skin and some other pathological conditions, hair loss and increased skin permeability, besides a roles in weight loss, reducing inflammation (chronic obstructive pulmonary disease), absorbing vitamins, drug delivery, make up the majority of neural tissue and the backbone of very important hormones, as well as in determining the quality of human and animal body fat, whose softness increases as the amount of unsaturated fatty

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acids increases in the diet, with the consequent secondary results of the components of milk fat [Carla, de Carvalho & Maria, 2018; Drescher & Van Hoogevest, 2020; Kotlyarov & Kotlyarova, 2021]. It has been proposed that a deficiency of essential fatty acids causes sterility in males, especially during the growth period, in addition to that a deficiency of linoleic acid in mice caused death of fetuses. It has also been found that arachidonic acid (C20 = 4) is the source of prostaglandin type of PGF2α and PGE2, which are known for their physiological effects in addition to their role in fertilization [Karim, 1975]. Goodman and Gilman [1975] mentioned that some unsaturated fatty acids are medicinal sources of important in reducing serum cholesterol by helping transport and dispose of cholesterol in the form of esters of arachidonic acid, which formed of linoleic and linolenic acid, as in the event of a deficiency of these unsaturated fatty acids, abnormal esters are formed and deposited in the tissues. The unsaturated fatty acids form alpha-lipoprotein instead of beta-lipoprotein and also help in the excretion of bile acids. The results of the polar lipid fraction analyzed by thin-layer chromatography (TLC) showed for the first time that manna lichen contains the following phospholipids: sphingomyelin, lecithin, phosphatidylserine, phosphatidyl inositol, lysophosphatidyl ethanolamine, & phosphatidyl-ethanolamine. These important compounds enter in large quantities in the structure of body tissues, especially the nervous ones, such as the brain and nerves [Viadimiroy and Ponteleeva, 1965; Carla, de Carvalho & Maria, 2018]. Sphingomyelin is present mainly in brain tissue, spleen, and lungs, and lecithin in large quantities also in brain tissue, adrenal glands, and red blood cells, while phosphatidylserine inositol enters many animal, plant, and bacterial tissues, mainly brain, liver, and lung tissue [Drescher & Van Hoogevest, 2020; Kotlyarov & Kotlyarova, 2021]. The results of the unsaponified portion of the total lipids analyzed by gas/liquid chromatography, after preparation of the (TMS) derivative for both of the model and the standard sterols, indicated that manna lichen contains beta-sitosterol, pregnanetriol, and oestradiol. Campsterol and oestrone were also identified in the model before it used for the preparation of the derivative (TMS). Preparation of the (TMS) derivative of the model and the standard sterols were done due to the equal retention time for each of oestriol, oestrone, pregnanediol, pregnanetriol and beta-oestradiol, and their appearance in one curve before the process of preparing of the derivatives as illustrated by curve [Schimitischek, 1980] in figure (4a). The preparation of a derivative (TMS) of the standard sterols includes oestradiol, androstendione, pregnanetriol, pregnanediol and beta-sitosterol, for availability. The methyl esters prepared in this study will not need further purification because they do not contain materials that interfere with the analysis of long chains of ethyl esters, such as some derivatives of sterols, by GLC.

The presence of beta-sitosterol in the lichen corresponds to the referring by Huneck and Follmann [1965] for containing of the lichen Lecanora dispersa on both the beta-sitosterol and the asterol compound. The confirmation of the absence of pregnanediol after preparing a derivative (TMS) in this study, can be explained by the absence of the hormone progesterone in quantities that can be measured by gas / liquid chromatography, or by measuring the biological effect which is similar to that of progesterone hormonal of the lipid and/or aqueous extracts of lichen, on the endometrium of rabbits, given that pregnanediol is one of the progesterone metabolites. Pregnanetriol is one of the metabolites of the hormone corticosterone, which is secreted by the adrenal gland with six other products, and the precursor of these seven products is the pregnenolone, which faces a group of changes that go through progesterone leading to the formation of corticosterone, which has been indicated to be present in a number of microorganisms such as filamentous fungi, some bacteria and protozoa, and some plants [Haftman, 1970]. Indeed, the results of an independent study [Hesse, 1907] on the pharmacological effects of the lichen, Lecanora esculenta, came to reinforce the results of absence of pregnanediol after preparing a derivative (TMS) analyzed by the gas/liquid chromatography, by finding of a biological effect similar to the estrogen hormone (but not the progesterone or the androgen hormones) effects when the biological effects of aqueous and lipid extracts of the lichen, were tested in adult ovariectomized females mice by the vaginal smears examination macroscopically, on the uterine weight of female rabbits, injected with the lichen extracts.

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