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Research article

Metabolomic profiling of Iraqi propolis Samples Collected from Al-Diwanyiah city

Ali Mohammed Ghazi[1] Mohanad A.Al-Bayati[2]
[1-]*Unit of zoonotic disease research, College of Veterinary Medicine, University of Al-Qadisiyah, Iraq*
[2-]*Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Baghdad-Iraq*
Corresponding Author Email:ali.ghazi@qu.edu.iq
Co-Author Email: aumrmumu@covm.uobaghdad.edu.iq

Abstract:

Propolis is a complex compound and important natural bee product that belongs to the great family of bee products with variety in color, chemical composition as well as in valuable biological and pharmacological effective. To date, No data about the Iraqi specified chemical constituents of Al-Diwanyiah propolis have been reported. The present data was investigated the phytochemical components of the ethanolic extract of collected propolis samples from varies honey bee colonies via distinguished fraction employing GC-MS technique. The chemical compounds were determine and identified by comparison with mass spectra library of the GC-Mass data system and with literature mass spectra. The results exhibited the total extract yields ranged from 38.6-54.4 % with mean percentage equal 45.7%. A fifty one different phytochemical metabolomic compounds were tentatively identified in local propolis samples belong to the at least twelve phytochemical categories. The major phytochemical categories were flavonoids, alcoholic compounds, fatty acids and their esters, terpenes, aromatic acids, aldehyde compounds, aliphatic hydrocarbons, aromatic hydrocarbons and other categories were esters, sugar and metabolic derivatives, dicarboxylic acid. The predominant flavonoids identified in propolis under test were Pinocembrin and tectochrysin. The present study showed local propolis is a rich source of natural bee active substances for identified profiling metabolomic markers biological and finalized therapeutic remedied applications in different pharmaceutics as well as GC-Mass is a good technique to provide an overall view of propolis composition.

Keywords: Iraqi propolis, GC-Mass, metabolomics, phytochemical

Introduction:

Propolis known commonly as bee glue is resinous or some time wax like substance, dark colored hive product composed of variety of botanical exudates collected from different plant sources as poplar, birch, horse chestnut, alder, beech, and conifer trees and employed by honey bees as protective hive barriers against different contaminating pathogens (1, 2).The color of propolis vary from green, red to dark brown based mainly upon the plant exudates of bees selective from the flowers buds, leaf buds and the tree barks (3, 4) It has a characteristic smell of popular bunds, honey wax and vanilla and show a sell of aromatic resins of great value, the aroma is altered according to the phyto-geographical zones characteristics of beehives surroundings and seasonal time of collection (5,6). Different propolis extracts have been documented to share a diverse array of broad bioactivities as antibacterial, antifungal, anti-parasitic, anti-inflammatory, anti-proliferative, free radical scavenging activities and due to the broad bioactive metabolites ascribed to propolis , it

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has long been used in the traditional medicine (7) In the last four decade and with development of modern separation and purification analytical technique as HPLC, TLC, GC as well as identification techniques as MS, NMR, GC-MS a large number of chemical studies on different propolis samples have been published. These studies have revealed that more than 300 different constituents present in the propolis samples including aliphatic acids, esters, aromatic acids, fatty acids, carbohydrates, aldehydes, amino acids, ketones, chalcones, dihydrochalcones, terpenoids, vitamins, and inorganic substances (8) its compositions varies mainly with various geographical locations, bee species and seasons, as well as their extracts but in general propolis collected from beehives, also known as crude propolis, is typically composed of about 50% balsam (Cream) resin (polyphenolic fraction) , 30% waxes, 10% essential and aromatic oils, 5% pollen, and 5% various organic substances, including wood fragments (9). This research, therefore aimed to investigate the chemical composition, characterization as well as relative concentration of vital compounds present in the ethanolic extract of local propolis samples using gas chromatography mass spectrometry to gain insight into determine identity of local metabolomic of propolis and its probability possible pharmacological and therapeutic effectiveness of studied propolis.

Material and Methods:

Propolis collection and extraction:

- **Crude propolis collection and certification**

Raw local propolis samples 500 gm were collected by transparent glass slide plaques directly from honey bee colonies located in the Al-Diwanyiahh city during the December 2018 and certified in the honey division / department of plant protection / directorate of agriculture / ministry of agriculture in Al-Diwanyiahh city , No. 2446, dated 10/2/2019 after that conserved in dark and closed containers at 4°C to prevent excessive oxidation until transported to the laboratory and for processing.

- **Crude propolis extraction**

The procedure described by Dziedzic and his colleagues (10) with some modification was used for the extraction process. The extraction process was beginning by frozen the propolis samples under -20 °C for 24 h. then they were crushed by electrical grinder to obtain powder, after that 50 gram of the obtained powder was soaked in the 500 ml of 70% ethanol solution (1/10 W/V) in a dark glass container and incubated at 37° C for 14 days. The solution was shaken twice a day for short time throughout the incubation period. After 14 time period, the obtained extract was filtered by Whatman filter paper No. 4. To remove waxes and insoluble soluble substances, the suspension was subsequently frozen at -20°C for 24 hours, and then filtered with Whatman NO.4 filter paper. The freezing filtration cycle was repeated three times. The final filtration led to represent the balsam (tincture) of propolis and is referred to as ethanolic extract of propolis. The solutions were evaporated via rotary vacuum evaporator under reduced pressure at 40°C. The remaining extract was incubated at 37°C for two weeks till the remainder of the ethanol was evaporated and the resulting sticky like substance were weighed and kept at -20°C until use.

- **Yield of extraction :**

The yield percent of propolis extract was estimated from the proportion of dry weight of extracted propolis to crude propolis as following equation.(11).

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$$\text{Propolis yield} = \frac{\text{Weight of extracted propolis}}{\text{Weight of crude propolis}} \times 100$$

GC-MS analysis of propolis.

- **Sample preparation for GC-MS.**

One milligram of dry propolis extract was reacted with 50 µl pyridine and 100 µl N-Methyl-N-tri-methyl-silyl-trifluoroacetamide (MSTFA) including in a sealed glass tube for 30 min at 60°C to prepare samples for gas chromatography. Sample volume of 1 µl were injected and analyzed by GC-MS.

- **GC-MS conditions :**

Gas chromatography- mass spectrometry was achieved on an automated pyrolysis Gas chromatography – mass spectrometry (py-GCMS) brand of type shimadzu GCMS-QP 2010 plus under electron impact ionization (70 eV) to determine different compounds that presented therein. Approximately 1 µg of propolis extract prepared for GM was injected by using 10 µl syringe into the quartz chamber in the pyrolysis unit then heated in an oxygen free environment at a temperature of 400° C. The temperature injector was 280° C and the temperature of the interface was 230 ° C and the MS scan range was 35 to 450 atomic mass units (AMU) . The chromatographic column used for analysis was a capillary column of the type RTX-5MS with a length of 60 m, internal diameter of 0.25 mm film thick 0.25µm, containing 5% diphenyl and 95% methyl poly-siloxane. The temperature program of the oven was set at a 50° C early for the first 6 minutes, after that elevated to a 280° C for 21 minutes. The carrier gas used was helium at a flow rate of 20 ml/minute. Mass spectrometry was set with temperature Ion source 200° C, under electron impact ionization 70 eV and setting Mass Range (BM) between 40 up to 600 m/z.

- **Identification of compounds :**

The compounds of local propolis were identified by comparison with the mass spectra library of the GC-Mass data system and with literature mass spectra. Identified Peaks in GC-MS were also confirmed by comparing the acquired mass spectra with those in the commercial reference libraries through computer search. Spectral matches for some compounds could be found in the Wiley and National Bureau of Standards (NBS) mass spectral library (12).

Results:

Propolis extracts yield percentage and characteristics:

The yield percentage of local propolis ethanolic extraction process 70% for ten patches was 45.7±1.56 % for each 50 gm of crude powder material table (1). The characteristics of final product of propolis after complete dryness was sticky in consistency and glossy brownish to dark brown in color with determined odor figure (1).

Table (1) yield percent of ethanolic extract of local propolis

Raw propolis weight (gm)	Yield weight (gm)	Min-Max yield weight (gm)	Yield %	Min-Max yield %
50	22.85±0.78	19.3-27.2	45.7±0.78	38.6-54.4

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- the values expressed the means ± SEM of the ten patches for each 50 gm of crude propolis

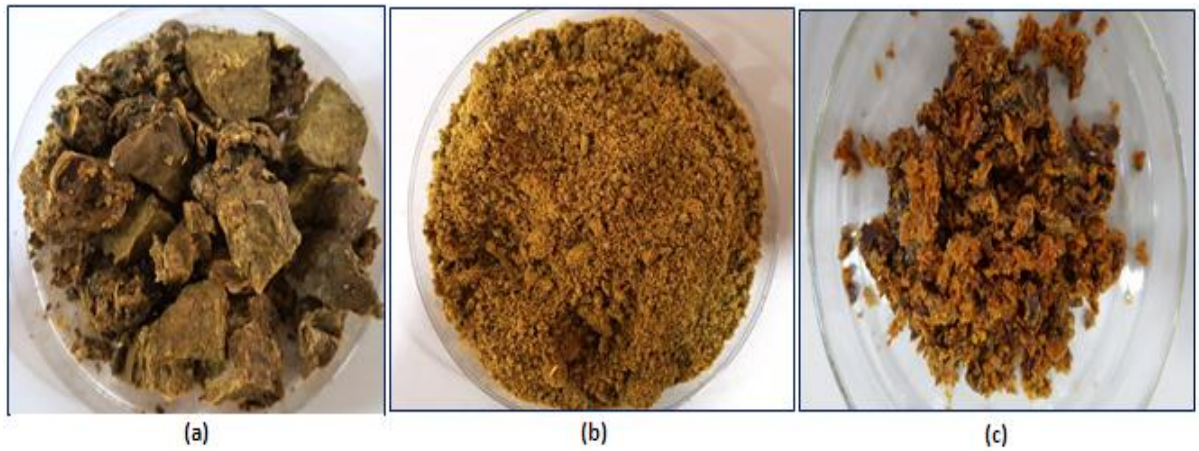

Figure (1) local propolis sample (a) crude material (b) powder material (c) propolis extract.

GC-Mass of analysis profiles.

The chemical composition of local propolis samples after silylation process and GC-Mass analysis was exhibited 51 major chemical compounds have been tentatively identified listed in the table (2) figure (2) including the chemical compound name, molecular formula, molecular weight, retention time (RT), area for each peak as well as percentage peak area. The results elicited that propolis samples contain seven chemical constituents were belonged to the category of flavonoids represent 42.44% from total component of propolis extract include pinostrobin 17.92%, tectochrysin 10.45%, Piocembrin 4.72%, Chrysin 4.69%, Norizalpinin 3.47%, apigenin 0.72% and quercetin 0.47%. Nine compounds were belonged to the category alcoholic compounds represent 13.66% including phenethylalcohol 6.17%, tetracosanol 2.34%, 1-octacosanol 2.02, cinnamic acid 1.33%, hydroquinone 0.7%, ethyl palmitate 0.33%, cinnamyl alcohol 0.23%, 1-heptatriacotanol 0.16%. eight compounds belonged to the category of the fatty acids and their esters including palmitic acid 5.07%, 1,19 eicosadiene 1.58%, linoleic acid 1.26%, 9-tetradecynoic acid, methyl ester 1.15%, stearic acid 0.64%, myristic acid 0.45%, cis-5,8,11,14,14 eicosapentaenoic acid0.20%, 17-laurostearic acid 0.18%. Ten compounds were belonged to the category of terpenes 4.46% including 0.79% Limonene, 0.78% a-bisaolene, 0.65% 2-naphthalenemethanol1,2,3,4,4a,5,6,7 octahydro a,a,4a,8 tetramethyl, 0.51% Gamma-eudesmol, 0.47% trans-a-bergamotol, 0.42% 4-methyl-m-dioxane, 0.30% a-acetoxybetulenol, 0.25% 7-a-acorenol, 0.19% β-pinene bicycloheptarne, and 0.1% geraniol. Three compounds were belonged to the aromatic acids 10.15% including 2.18% ferulic acid, 1.12% vanillic acid, and 0.98% 2,5-dimethoxycinammic acid. Two compounds described as aldehyde category including 2-hydroxy-5-methylbenzaldehyde 8.48%, 3-methylbenzaldehyde 1.67%. Four compounds were belonged to the aliphatic hydrocarbons (5.18%) including n-heptacosane 3.80%, 1,5,5,trimethyl-6-methylene-cyclohexene 0.57%, chloro-eicosane 0.56%, and a-octadecene 0.25%. two compounds were belonged to the aromatic hydrocarbons 3.31% including 2.88% 2-methyoxy-4-vinylphenol, 0.43% 2,6,10,14,18 pentamethyl 2,6,10,14,18 eicosapentaene. In addition to the Dicarboxylic acids (malonic acid) 3.31%. Less amount of phytochemical categories that detected in local propolis sample were aliphatic acids 0.42%

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esters 0.21%, sugar and sugar derivatives 0.54%.

Table (2) Chemical composition of ethanolic extract of local propolis and its percentage

Peak No.	compounds	Retention time	CAS	formula	Mol. Weight g/mol.	area	Area %
1	7-α-acorenol	2.017	28400-11-5	C15H26O	222.37	103199	0.25
2	β-pinene bicycloheptanre	7.3	127-91-3	C10H16	136.23	76292	0.19
3	Glycerol(prppanetriol)	9.883	56-81-5	C3H8O3	92	222618	0.54
4	Phenethyl alcohol	10.800	60-12-8	C8H10O	122.16	2542157	6.17
5	4-Methyl-m-dioxane	11.750	1120-97-4	C5H10O2	102.13	173737	0.42
6	α-octadecene	12.850	112-88-9	C18H36	252.48	104288	0.25
7	hydroquinone	13.867	123-31-9	C6H6O2	110.112	288049	0.7
8	3-methylbenzaldehyde	14.158	620-23-5	C8H8O	120.15	689107	1.67
9	Geraniol	14.950	106-24-1	C10H18O	154.25	42764	0.1
10	Cinnamyl alcohol	16.492	104-54-1	C9H10O	134.17	93991	0.23
11	2-methoxy-4-vinylphenol	16.658	7786-61-0	C9H10O2	150.177	1186432	2.88
12	Benzenepropionic acid	17.508	501-52-0	C9H12O2	152.19	86456	0.21
13	Nonanoic acid	18.300	112-05-0	C9H18O2	158.24	51212	0.12
14	2-hydroxy-5-methylbenzaldehyde or 5-methylsalicylaldehyde	20.525	18362-36-2	C8H8O2	136.15	3496434	8.48
15	N-(2,4-Dimethylphenyl)formamide	21.425	60397-77-5	C9H11NO	149.19	84528	0.21
16	cis-5,8,11,14,17-Eicosapentaenoic acid	22.717	10417-94-4	C20H30O2	302.45	83335	0.20
17	Laurostearic acid	23.333	143-07-7	C12H24O2	200.32	73772	0.18
18	1,4-Methanoazulen-7(1H)-one,octahydro-4,8,8,9-tetramethyl	24.408	77-53-2	C15H27O	222.12	155944	0.38
19	Gamma-Eudesmol	25.108	1209-71-8	C15H29O	282.26	210278	0.51
20	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,7-octahydro-.α.,.α.,4a,8-tetramethyl,	25.817	88034-74-6	C15H24O	220.35	269722	0.65
21	A-acetoxybetulenol	26.008	26680-54-6	C12H18O3	210.27	124751	0.30
22	trans-α- -Bergamotol	27.458	13474-59-4	C15H24	204.35	193829	0.47
23	Myristic acid	28.800	544-63-8	C14H28O2	228.37	185807	0.45
24	Octadecenoic acid	29.183	63891-61-2	C18H34O2	222.36	125631	0.30
25	1,5,5-Trimethyl-6-methylene-cyclohexene	30.058	514-95-4	C10H16O	136.23	235302	0.57
26	α- bisaolene	30.483	17627-44-0	C10H16	204.35	322701	0.78
27	1-heptatriacotanol	30.842	105794-58-9	C37H76O	536.01	66118	0.16
28	Chloro-eicosane	31.375	42217-02-7	C20H41Cl	316.99	229946	0.56
29	2,5-dimethoxycinnamic acid	32.092	10538-51-9	C11H12O4	208	403039	0.98
30	Palmitic acid	32.475	57-10-3	C16H32O2	256.43	2090196	5.07
31	Ethyl palmitate	32.808	628-97-7	C18H36O2	284.48	136042	0.33
32	Limonene	32.875	138-86-3	C10H16O2	136.23	325084	0.79
33	9-Tetradecynoic acid, methyl ester	34.058	55538-60-8	C15H26O2	238.37	472337	1.15
34	Linoleic acid	34.242	60-33-33	C18H32O2	280.45	518313	1.26
35	Malonic acid, 6-heptynyl	34.725	141-82-2	C3H4O2	104.06	1897696	4.60
36	Stearic acid	35.067	57-11-4	C18H36O2	284.48	264096	0.64
37	Ferulic acid	35.550	1135-24-6	C10H10O4	194.18	897619	2.18
38	Cinnamic acid	36.142	140-10-3	C9H8O2	148.16	546395	1.33
39	Quercetin	36.283	117-39-5	C15H10O7	302.23	192866	0.47
40	Apigenin	36.825	520-36-5	C15H10O5	270.24	298482	0.72

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41	Pinostrobin	37.550	18956-15-5	C16H14O4	270.28	1947018	4.72
42	pinocembrin	38.483	480-39-7	C15H12O4	256.25	7385552	17.92
43	tectochrysin	39.608	520-28-5	C16H12O4	268.26	4307884	10.45
44	Chrysin	41.158	480-40-0	C15H10O4	254.24	1931859	4.69
45	norizalpinin	41.992	548-83-4	C15H10O5	270.24	1429493	3.47
46	n-heptacosane	43.885	593-49-7	C27H56	380.74	1567990	3.80
47	tetracosanol	46.231	506-51-4	C24H50O	354.66	962936	2.34
48	Vanillic acid	47.019	1696-60-2	C16H16N2O4	300	462357	1.12
49	2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene	48.375	75581-03-2	C25H42	342.16	177372	0.43
50	1,19 eicosadiene	51.177	14811-95-1	C20H38	278.52	650109	1.58
51	1-octacosanol	51.863	557-61-9	C28H58O	410.77	832256	2.02
total						41215391	100

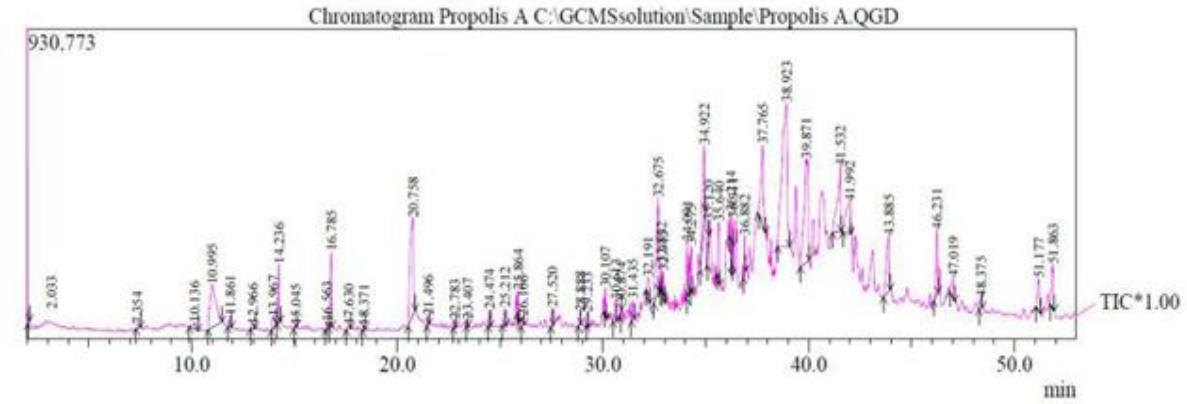

Figure (2): GC-Mass chromatogram of the ethanolic extract of local propolis sample.

Discussion:

The range of total extract yields of the local propolis was 38.6-54.4 %, an average percentage 45.7% resulting from ten patches of each 50 gm of raw propolis used in each extraction process (table 1). The propolis product was sticky consistency, glossy dark brown color and distinguish aroma odor. The color and odor of propolis samples are dependent largely on its botanical source (13). Principal botanical origin of the propolis in the non-tropical zones of Asia and north America is poplar, in addition to the willow, linden, pine, cherry and other trees, whereas in the tropical zones there are no poplars and honey bees employ other sources for propolis production (4). Although the botanical origin of local propolis is not yet identified, but on the basis of the Iraqi environment, the predominant plants for propolis source probably return to *Salix acmophylla L* and *Eucalyptus camaldulensis*, sider, orange trees which the local flora regions rich in these plants where honey bees can visit it and collect propolis (14). The difference in propolis yield and composition may be attributed to the composition and characteristics of raw propolis samples (15) furthermore, technique of collection, processing of crude propolis, as cleaning, sieving and removing of the wax and debris also can affected (11) It is worth mentioning that type of solvent and extraction procedure could play a key role in the extraction yield and the isolation of bioactive compounds from the propolis samples (16). The yield of propolis extract is apparently higher with organic solvents especially ethanol

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(ethyl alcohol) which are dissolve 50-70% of propolis constituents compared with 10% of its weight (17). another factor, the amount of extraction solvent (the ratio of propolis / solvent) used for extraction process also can be affected on the yield extraction, therefore employ large volume of solvent in extraction process might play important role in increasing of yield where the increase in the solvent volume increase the rate of dissolving with an obvious proportionality. (11). Although there are many solvent with different polarity can be used successfully in the propolis extraction as ethanol, water, chloroform, methanol, Dichloromethane, ethyl acetate, acetone and hexane (18) In biological assays, the most often used solvent is ethanol alcohol which containing different percentage of water, 70% ethanol was recorded to extract most of the biologically active components of propolis but not wax (19;20). Ethanol is excellent solvent for extraction of the phenolic compounds as flavonoids compared with other organic and inorganic solvents (16). Propolis consists of a mixture of beeswax and plant exudate. (21) Definitely, the phytochemical compositions of propolis samples vary among different samples (22) this depends mainly on the flora in the areas where it is collected (23). As well as collecting season, bee selective behaviors or species or races of the bees collecting it (13). Therefore, the propolis samples are potentially a very variable product (24). Analysis of GC-Mass chromatograms of ethanolic extract of local propolis showed a total of 51 peaks within the range of 0.1-17.92% represented the presence of fifty one phytochemical constituents belong to at least nine phytochemical category. These compounds were identified by comparison with the chromatographic retention characteristics and mass spectra of authentic standards, literature mass spectra and the mass spectral library of the GC–MS data system. The heights of the peak indicate the relative concentrations of the constituent present in the sample. The compounds of local propolis samples are listed in the table (2) the peak numbers are demonstrated in the figure (1). The GS-Mass technique is reported to be a diagnostic tool for the correct identification of active ingredients in different plants (25). GC-Mass providing an overall view of propolis composition, efficiently identifying flavonoids, phenolic acids derivatives acids, sugars and other compounds (21) Therefore GS-Mass fingerprinting analysis can be used to give a quantitative information about the major active phyto-constituents in a plant extract and make it useful for evaluate the quality of extract. (26). The results revealed that, the local propolis samples was characterized by the presence of Flavonoids (42.44%), alcoholic compounds (13.66%), fatty acids and their esters (10.53), aldehyde compounds (10.15%) aliphatic hydrocarbons (5.18%) were the main categories of phytochemical compounds recorded in the local propolis ethanolic extract and all of them with concentrations more than 5%. Flavonoids are the major group of phenolic compounds and utilized as criterion to evaluate the quality of temperate propolis (27) these lipophilic compounds are readily extracted by ethanol alcohol (28). The quantity of these compounds related essentially with vegetation where the bees collect propolis. (29)The reported flavonoids in the present study were pinocembrin 17.92%, tectochrysin 10.45%, pinostrobin 4.72%, norizalpinin 3.47% and others represented 1.19 including apigenin and quercetin. Propolis form Europe and China had high flavonoid and phenolic contents (23). On the other hand, propolis from Brazil showed high terpenoid and cumaric acid derivative (30). Flavonoids and other phenolic compounds found to be responsible for antioxidant and antimicrobial action beside other pharmacological activities (31). Among the flavonoids compounds, pinocembrin is one of the main components found in the tested

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propolis and represent 17.92% from total chromatogram area and 42.22% of total flavonoids extracted from local propolis. The propolis from Argentina, Italy and Spain show a great amount of pinocembrin (approximately 49%, 48% and 39% of the total identified flavonoids, respectively. Fontana and his colleagues (32) found pinocembrin, chrysin and galangin to be the flavonoids at the highest concentration, which is in good agreement with the results of the present study. In another study, Bankova and his colleagues (19) also found that pinocembrin, galangin and chrysin are the main flavonoids in other propolis samples. The alcoholic category was the second abundant group determined in the tested local propolis component. The identified alcolholic compounds were phenethyl-alcohol which present in concentrations higher than 5%. Other alcoholic compounds include tetracosanol, 1-octacosanol, cinnamic acid, hydroquinone, ethyl palmitate, cinnamyl alcohol, 1-heptatriacotanol were present in concentrations 2.34, 2.02, 1.33, 0.7, 0.38, 0.33, 0.23, 0.16% respectively. Phenethyl-alcohol is important part of the odor of propolis and probably the most prestigious aroma chemical in the world of perfumery, medically have significantly antifungal activity.(33). Other important phytochemical category of compounds identified in the propolis samples was terpenoids, which are account for the characteristic resinous odor and contribute to the pharmacological effects of propolis as they exhibit antimicrobial, antioxidant, antitumor and other biological activities. (34,35). Triterpenoids have been reported to occur in diverse plant species as resin or gum constituents (12) they are founds in the plants leaves, barks and resins and their concentrations vary dependent on the plant species.(27). Sesquiterpenes are the most abundant chemical components in propolis. According to the number of the rings, sesquiterpenes fall into four categories: acyclic, monocyclic, dicyclic and tricyclic. (34) In the present study three Sesquiterpenes were identified include 7-α- acorenol, Gamma-eudesmol, α-bisolen on the other hand, Mono-terpes as β-pinene and limonene which presented in the tested propolis samples were recorded as major components of volatile oil of Brazilian origin and are responsible for biological effects of essential oils of propolis, and these compounds are already known to play role in inhibitory effect on bacteria.(36). Fatty acids and their esters are the base components of propolis. (37). The total concentration of fatty acid and their esters in the tested propolis samples was 10.53. In a previous research, the total fatty acid concentration of Yemeni propolis samples was ranging from 0.25 to 20.78% (12), In other report the fatty acids composition of eight propolis samples collected from 6 regions of Algeria was identifying by GC-FID techniques led to identification of over 34 compounds belongs to the various groups of fatty acid such as Saturated Fatty Acids; Monounsaturated Fatty Acids; Polyunsaturated Fatty Acids; Omega-3and Omega-6 (37) various *in vitro* studies have reported that fatty acids can be used successfully in the treatment of prostate enlargement mainly through inhibition 5-a-reductase enzyme as lauric and myristic acid (38)other fatty acids, Ferulic acid is one of the aromatic acids category identified in local propolis and represent about 2.18% of total propolis content, the main source of propolis being always poplar buds and have significant antioxidant activity and anticancer activity(39,40). Analysis of the present work revealed the presence of aliphatic hydrocarbons 5.18% and aromatic hydrocarbon 2.88% which considered as base components of crude propolis from any botanical source (39).Only one Sugar compound reported in the local propolis samples was glycerol in the percentage 0.54% of total propolis content.On the basis of the

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above results, its concluded that GC-Mass under the experimental conditions represents a valuable technique for the qualitative assay of the relevant compounds in propolis extracts. In the same time, Iraqian propolis samples appeared to be rich in the flavonoids compounds especially Pinostrobin and tectochrysin in addition to alcoholic compounds and fatty acids. Thus, local propolis being a rich source of the variety of biological active chemical constituents provide a basis to make propolis as a promising natural medicine to prevention and treatment of various diseases and can be used for different therapeutic applications as anticancer, antimicrobials, anti-proliferative, anti-inflammation….etc.

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