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GC-Mass spectrometry based metabolomic profiling of three different propolis extracts

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Abstract

Propolis is a natural substance rich in medically active chemical compounds. The presence of these compounds depends on a variety of factors, including the method of extraction and the solvent used for the extraction process. The current work aimed to investigate the effect of extracting propolis using different solvents on its chemical composition. The propolis extraction ratio were 34.53, 43.4 and 38.68% for watery, ethanolic, and ethyl acetate propolis extract respectively.

The chemical composition of three extracts was analyzed using GC-Mass analysis which identified 16, 26, and 24 chemical components in watery, ethanolic, and ethyl acetate extracts respectively. The chemical composition of three extracts was varied greatly but all extracts were rich in phenolic compounds. 5-hydroxy-7-methoxyflavanone and 2-isopropenyl dihydrofuran were predominant in the watery extract whereas 1-methylethyl)-8-oxo-1,2-dihydrofurano, Malonic acid, 6-heptynyl, and 1,4-Naphthalenedione, 2-1-buten were predominant in ethanolic extract. 2H-1-Benzopyran-2-one-7-methoxy, 9-Tetradecynoicacid, methyl ester, and 9-Octadecenoic acid were predominant in the ethyl acetate extract.

Key words: Propolis, Extraction, GS-Mass, Metabolomic

Introduction

Natural compounds are found in plants, and in addition to providing food for people and other animals, insects also use plants as a source of raw materials to make a variety of highly valued natural goods for human use. Melliferous plants and honeybees are the main subjects of this statement. In order to make propolis, bee pollen, honey, and beebread, bees use plant raw resources found outside of their hive, such as nectar, pollen, and resins (1). In recent times, bee products have garnered significant interest from medical professionals and academics because to its dual benefits of high nutritional content and diverse biological and therapeutic properties. Honey, beeswax, royal jelly, pollen, bee venom, and propolis are some of these products. Propolis (bee glue), the third plant-derived bee product, is not a food item like honey and pollen. It serves as a protective substance and building material for bees (2). Bees make propolis, honey, bee pollen, beebread, and other products from different plant materials found around the hive, such as nectar, pollen, and resins. The primary byproduct of bees and their source of energy is honey; bee pollen and beebread are significant sources of different dietary molecules as protein, amino acids, lipids, fatty acids, carbohydrates, vitamins, and minerals for the bee family (1). Due to the presence of active chemical groups such phenols, terpenes, and flavonoids, the propolis is regarded as one of the most significant bee products in terms of medicine and therapy (3). Propolis is an animal product, but the majority of its ingredients-especially the active ones - come from plants (4). Normally, propolis has a very intricate and diverse composition, demonstrating the existence of beeswax, resin, essential oils, and pollen. Bees produce the wax, but plants are the source of resin and oils, which are often extracted via secretions or by chopping pieces of

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vegetative tissues (5). Propolis's broad application can be attributed to its complex chemical makeup and this natural substance has both volatile and fixed components; according to some writers, it contains over 500 compounds (6). Propolis content varies depending on the botanical and geographical origin of propolis (7). The propolis as ultimate product is derived from three sources: plant resins that the bees gather; chemicals released by their metabolism (wax); and other elements that the bees add throughout the propolis-elaboration process (1). The plant species from which the propolis is derived, bee species, environmental elements like climate features, the time and method of harvest, as well as the extraction methodology and solvent utilized, all affect the chemical makeup and biological activity of raw propolis (8; 9). The bioactive components of bee glue are extracted with the intention of removing the wax, which is always present in propolis (up to 20%), and dissolving the significant plant-derived chemicals (10). More than 300 chemicals have so far been identified, and its chemical components are quite complicated (11) So the present work was aimed to comparison the chemical constituents extracted by three different solvents with different polarity.

Materials and methods

Ethical of approval

This research complies with the ethics rules of the University of Al-Qadisiyah, Iraq's Faculty of Veterinary Medicine.

Propolis collection and preparation.

The raw propolis materials used in this study was collected from various sites located in Al-Diwaniya city at the end of the spring to early summer.

To get rid of undesirable items, the propolis samples were carefully cleaned with fresh water. Following cleaning, the samples were first allowed to air dry before being further dried for two weeks at room temperature in the shade. Following a 24-hour freezing period, the dried propolis samples were processed, sliced into little pieces, and ground in a grinder to a fine powder. The powdered ingredients were stored in an airtight container at room temperature.

Propolis extraction

The process of propolis extraction was performed as method reported by (12) with little modifications as shown in figure 1 The watery extract of propolis (WPE), the ethanolic extract of propolis (EEP), and Ethyl acetate extract of propolis (EAPE) were prepared by cold maceration of propolis samples in water, absolute ethanol, and absolute ethyl acetate solvents respectively via placed in the dark glass containers at room temperature for 15 days with strong shaker for 10 minutes daily, Following filtration using Whatman filter paper No. 1 (185 mm in diameter) to exclude waxes and insoluble components, the liquids are evaporated to dryness at 60° C with reduced pressure.

Propolis extraction yields

By calculating the ratio of the weight of extracted propolis to the weight of raw propolis, the propolis extract yield was calculated using formula (13).

propolis yield %= (weight of extract)/ (weight of crude propolis) ×100

Propolis preparation for GC-Mass

An automated pyrolysis gas chromatography system connected to a mass spectrometer was used to accomplish GC-MS analysis for metabolomics under the ideal circumstances.

In a reactive sealed glass tube, 1 milligram of propolis extract, 50 µl pyridine, and 100 µl N-methyl-N-tri-methyl-silyl-trifluoroacetamide were combined, and the mixture was heated to 80° C for 30 minutes. GC-MS was used to examine a sample volume of 1 µl that was injected into a quartz chamber in the pyrolysis unit using a 10 µl syringe (15).

Compound identification

The compounds of various propolis extracts were identified by comparing the sample spectra with the mass spectra library of the GC-Mass data system and with mass spectra from the literature. The detected peaks in GC-MS were confirmed by comparing the acquired mass spectra with the commercial reference libraries using a computer search. High rated spectral matches for compounds were found in the Wiley and the National Bureau of Standards (NBS) mass spectral library (16).

Results

Propolis yield

The yield of propolis extract was calculated as weight/ weight percent yield of 50 gm of propolis. Based on Table 2 the ethanolic propolis extract showed the highest extractive yield percentage compared to the other propolis extract with final yield percentage 43.4±1.67%, followed by 38.68±0.66% for the ethyl acetate propolis extract and 34.53% for watery propolis extract. The average percentages of the obtained extracts were significantly (P<0.05) difference in the extraction ratio among different propolis extracts.

Table 1 yield of extraction and percentage yield of primary and final propolis extract.

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Type of extract	Raw amount	Final yield recovery (gm)	Final yield %
Watery propolis extract	50 gm	17.26±1.04^{C}	34.53±2.09^{C}
Ethanolic propolis extract	50 gm	21.7±0.83^{A}	43.4±1.67^{A}
Ethyl acetate propolis extract	50 gm	19.34±0.33^{B}	38.68±0.66^{B}
LSD($p<0.05$)		1.76	4.52

As shown in the table 1 and figure 1,2,3 Gas chromatography-mass spectrometry analysis results showed that watery propolis extract contains 16 different components. The analysis was found that this extract was opulent in the flavonoids content as of 5-hydroxy-7-methoxyflavanone (pinostrobin) 29.89%, 2-isopropenyl dihydrofuran 20.79%, other less percentage flavonoids compounds include 2H,8H-Benzo[1,2-b:3,4-b'] dipyran 2.1% and 2',4',6'-Trihydroxy chalcone 0.12%. Fatty acids and their esters constitute 8.68% from total watery extract content and include two compounds Tetra-decanoic acid 4.38%, Oleic acid acid 4.3%. two Aromatic acids and their esters include P-Coumaric acid 3.72%, Osthole 2.55%. In addition, 4 Aliphatic hydrocarbons and their esters n-heptacosane 7.79%, n-Hexadecanoic acid 5.92, 11-Octadecenoic acid methyl ester 2.55%, 9-Octadecenoic acid 6.47%. Terpenes constitute 0.13 and include one compound 8(5H),2'- [1,3] dioxane], 6,7,7b,10a-tetrahydro-1. Organic compounds E)-1-Indan-1-ylethanone oxime 5.42%. alcohols include 2-Methoxy-4-vinylphenol 0.37%, other compounds were 1,4-Naphthalenedione, 2-(1-buten) 3.49%.

The major phytochemical constituent in the ethanol extract was flavonoids compounds include 5-hydroxy-7-methoxyflavanone (pinostrobin), 2-isopropenyl dihydrofuran, 2H,8H-Benzo[1,2-b:3,4-b']dipyran, 2,2- dimethylene-8-oxo-3,4-dihydro-2HI,8HI- pyrano, Pinostrobin, pinocembrin, tectochrysin, But-2-enoic acid, 2-methyl, 1-methylethyl)-8-oxo-1,2-dihydrofurano (1.01, 3.99, 1.78, 4.16, 2.5, 7.14, 7.15, 4.69 and 11.53 %) respectively. 8.68% fatty acid and their esters include 4 compounds Tetradecanoic acid, Dodecanoic acid, Palmitoleic acid, Oleic acid (0.57, 1.15, 0.28, 3.08%) respectively. 17.58% Carboxylic acid and their esters include 2 chemicals 5methoxymethyl-2furoic acid, Malonic acid, 6-heptynyl (4.09, 13.49%) respectively. 14.68% Aromatic acids and their esters include 3 chemicals Ferulic acid, P-Coumaric acid, Osthole (6.36, 3.11, 5.21%). 1.82% Aliphatic hydrocarbons and their esters include 2 compounds 9-Octadecenoic acid, Hexadecanoic acid methyl ester (1.52, 0.3%) respectively. 0.91% organic compounds include 2 chemicals 2-Nonadecanone, Benzo[h]quinoline-3-carbonitrile (0.76, 0.15%) respectively. In addition, 0.33% 2-Methoxy-4-vinylphenol. 3.53% 7-Demethylsuberosin, 11.82% 1,4-Naphthalenedione, 2-(1-buten, 0.29% Benzene ethanol.

Regarding the ethyl acetate extract of propolis shows 24.21% flavonoid compounds, this percentage distributed into 4 different chemicals 1, 1.58, 21.23, 0.4 % for 2-isopropenyl dihydrofuran, 2H,8H-Benzo[1,2-b:3,4-b'] dipyran, 2H-1-Benzopyran-2-one, 7-methoxy, 1-methylethyl)-8-oxo-1,2-dihydrofurano respectively. Fatty acids and their esters constitute highly percentage 42.02% including 2 compounds Dodecanoic acid, 9-Tetradecynoicacid, methyl ester 3.76, 38.28% respectively. Carboxylic acid and their esters constitute 6.1% include acetic acid propyl ester, acetic acid butyl ester, and 5methoxymethyl-2furoic acid (2.87, 1.09, 2.14%) respectively. Limited quantity of aromatic acids and their esters 0.66% include Benzoic acid, phenylmethyl ester 0.28% and 2,5-dimethoxycinnamic acid 0.38%. highly content of Aliphatic hydrocarbons and their esters18% distributed n-Hexadecanoic acid 7.53%, and 9-Octadecenoic acid 10.47%. Terpenes 3.17% include two compounds Tetradecanoyl acid, 4-Methyl-m-dioxane 2.15 and 1.02 respectively. Organic compounds 1.73% include Heneicosane, 2-Nonadecanone, 1-Butanol, 2-Pentacosanone 0.65, 0.73, 0.14, and 0.21% respectively. Alcohols 1.28% include 2-Methoxy-4-vinylphenol, 2-buten-1-ol,2-methyl 0.32 and 0.96%. other compounds 7-Demethylsuberosin 0.74%, 1,4-Naphthalenedione 1.59% , 2-1-buten, Benzene ethanol 0.39%.

Table 2 Chemical characterization of three extracts of propolis by GC-MS

Compounds	Groups	Type of Extract (Area %)		
		Watery	ethanolic	Ethyl acetate
Flavonoids compounds		52.9	43.95	24.21
5-hydroxy-7-methoxyflavanone (pinostrobin)	Flavonoids	29.89	1.01	----
2-isopropenyl dihydrofuran	Isoflavonoids	20.79	3.99	1
2HI,8H-Benzo[1,2-b:3,4-b']dipyran	Flavonoids	2.1	1.78	1.58
2,2- dimethylene-8-oxo-3,4-dihydro-2H,8H- pyrano	Flavonoids	----	4.16	----
Pinostrobin	Flavonoids	----	2.5	----
pinocembrin	Flavonoids	----	7.14	----
tectochrysin	Flavonoids	----	7.15	----
But-2-enoic acid, 2-methyl	Flavonoids	----	4.69	----
2',4',6'-Trihydroxy chalcone	Flavonoids	0.12	----	----
2H-1-Benzopyran-2-one, 7-methoxy	Flavonoids	----	----	21.23
1-methylethyl)-8-oxo-1,2-dihydrofurano	Flavonoids	----	11.53	0.4
[illegible]		[illegible]	[illegible]	[illegible]

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Fatty acids and their esters		8.68	5.08	42.02
Tetradecanoic acid	Fatty acid	4.38	0.57	----
Dodecanoic acid	Saturated Fatty acid	----	1.15	3.76
Palmitoleic acid	Saturated fatty acid	----	0.28	----
Oleic acid	Fatty acids and their esters	4.3	3.08	----
9-Tetradecynoicacid, methyl ester	Fatty acid	----	----	38.28
Carboxylic acid and their esters		----	17.58	6.1
Acetic acid, propyl ester	----	----	----	2.87
Acetic acid, butyl ester	----	----	----	1.09
5methoxymethyl-2furoic acid	----	----	4.09	2.14
Malonic acid, 6-heptynyl	----	----	13.49	----
Aromatic acids and their esters		6.27	14.68	0.66
Benzoic acid, phenylmethyl ester	Aromatic ester	----	---	0.28
Ferulic acid	Aromatic acids	----	6.36	----
P-Coumaric acid	Aromatic acid and their esters	3.72	3.11	----
Osthole	Aromatic acid	2.55	5.21	----
2,5-dimethoxycinnamic acid	Aromatic acid	----	----	0.38
Aliphatic hydrocarbons and their esters		22.73	1.82	18
n-heptacosane	Aliphatic hydrocarbons	7.79	----	----
n-Hexadecanoic acid	Aliphatic acids and esters	5.92	----	7.53
11-Octadecenoic acid, methyl ester	Aliphatic acids and esters	2.55	----	----
9-Octadecenoic acid	Aliphatic acids and esters	6.47	1.52	10.47
Hexadecanoic acid, methyl ester	Aliphatic acids and esters	----	0.3	----
Terpenes		0.13	----	3.17
Tetradecanoyl acid	Triterpenoids	----	----	2.15
8(5H),2'- [1,3] dioxane], 6,7,7b,10a-tetrahydro-1	terpens	0.13	----	----
4-Methyl-m-dioxane	Terpenes	----	----	1.02
Organic compounds		5.42	0.91	1.73
Heneicosane	Organic compound	----	----	0.65
2-Nonadecanone	Organic compound	----	0.76	0.73
E)-1-Indan-1-ylethanone oxime	Organic compound	5.42	----	----
Benzo[h]quinoline-3-carbonitrile	Organic compound	----	0.15	----
1-Butanol	Organic compound	----	----	0.14
2-Pentacosanone	Organic compound	----	----	0.21
Alcohols		0.37	0.33	1.28
2-Methoxy-4-vinylphenol	Alcohols	0.37	0.33	0.32
2-buten-1-ol,2-methyl	alcohols	----	----	0.96
7-Demethylsuberosin	polyphenols	----	3.53	0.74
1,4-Naphthalenedione, 2-1-buten	naphthoquinone derivatives	3.49	11.82	1.59
Benzene ethanol	others	----	0.29	0.39

Figure 1 GC-mass analysis of watery propolis extract sample

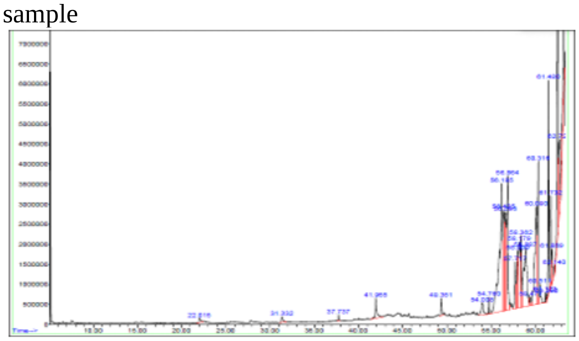

Figure 2 GC-mass analysis of ethanolic propolis extract sample

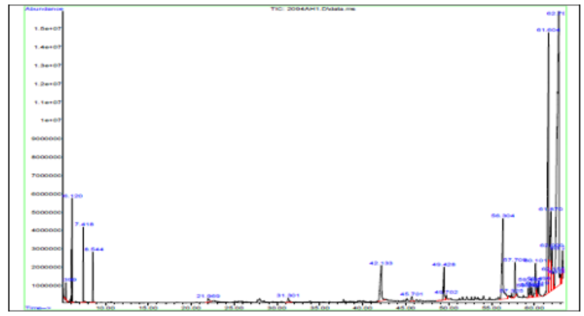

Figure 3 GC-mass analysis of ethyl acetate propolis extract sample

Discussion

The study was undertaken to reveal the chemical composition of different solvent extracted samples from local propolis. This resin has been employed in traditional medicine around the globe, and contemporary research is looking at natural materials as sources of novel compounds to treat a wide range of illnesses (4). Water, ethanol, and ethyl acetate solvents were used for extraction process. Propolis bee product is a vegetable putty that refers to a collection of gummy, balsamic resinous substances that bees primarily gather from bark and buds of a variety of plants, including birch, poplars, oaks, willows, conifers, and many more (17) Its rich in large number of biological active ingredients, these active substances can be obtained from raw materials by using different solvents and techniques. One of the most important biological attributes of propolis has been pointed out by various studies to be the antioxidants effects. There are a huge number of factors that affect the content of propolis of active ingredients, which are necessarily reflected in its biological and medical activities. High concentrations of flavonoids and phenolic substances have been demonstrated to have antioxidant and antibacterial properties (18). It has been shown that the kind and quantity of secondary metabolites recovered from medicinal plants or original materials are influenced by the solvents used during the extraction process (19). The efficiency of the solvent in extracting the chemical components of the plant or original materials is shown by the extraction yield, which is the mass of extract recovered in relation to the initial amount of original material (20). The selection of a suitable solvent, extraction techniques, phytochemical screening procedures, fractionation techniques, and identification techniques are the main steps involved in obtaining high-quality bioactive molecules (21).

In the present work, the maceration method was used for propolis extraction process which is traditional and most often used method to extract active ingredients of propolis (10). Numerous studies have been written on this technique, examining the effects of various factors such as temperature, time of extraction, solid-liquid ratio, and solvent type on the propolis extraction process' efficacy. To evaluate the result of the extraction process via using 3 different solvents, the amount of extracted matter, extraction content was observed. Results showed the mean values of the yield extracts presented as (%) were significantly ($P<0.05$) variations in crude propolis extract yield among the different solvents used for propolis extraction process. Many studies have reported the impact of different solvents on the content of secondary metabolites and/or their antioxidant activity (18). In the overall, highest yields were obtained in the ethanolic extract (43.4%), followed by ethyl acetate extract (38.68%). Lowest yields were recorded in the watery extract with (34.53%). The extraction yield, quantity of phenolic compounds, and evaluation of the material's antioxidant activity are the basis for propolis quality control (4). According to studies, the extract yield and antioxidant activity of phenolic components in plant material are greatly impacted by the solvent polarity (22). Water, methanol, ethanol, dichloromethane, acetone, chloroform, and ether are the normal solvents used in propolis extraction. For low-wax propolis extracts that are high in biologically active substances, ethanol is the most often utilized solvent (23). Getting the most extract that is enriched with the desired groupings of chemicals is one of the primary goals of the extraction process (24). Because propolis is derived from plant resins, regardless of their chemical composition, all types of propolis are typically soluble in organic solvents and have comparatively low solubility in water because resins are typically polar (10) The findings show that the ethanol extract outperformed the solvents ethyl acetate and water in terms of yield. According to Azwanida (25), the extraction of propolis differs from that of medicinal plants since propolis does not contain the insoluble cellular matrix found in plants. Significant amounts of polar, intermediate, and non-polar molecules can be extracted from various plant extracts using ethanol as a solvent (26).

Propolis's chemical components that give it its biological activity vary and differ among samples from various sources and extraction methods (4). GC-MS has a rather wide coverage of compound classes, such as organic and amino acids, sugars, sugar alcohols, phosphorylation intermediates, and lipophilic substances, despite the fact that no one analytical method can fully capture the metabolome in original materials (27). Using gas chromatography mass spectrometry to analyze propolis sample extracts could assist determine their composition and enhance their biological qualities (23). Due to a large number of compounds and isomers and their variety in natural products, it is very difficult to identify all the compounds by chromatography (28). It was feasible to recognize a number of chemicals from various classes in the chromatograms of the propolis extracts. Different intensities and area percentages of these chemicals were detected in each extracted sample. According the results of the present work and following GC-MS analysis, the separated chemical compositions of watery, ethanolic, and ethyl acetate propolis extracts from local propolis samples revealed the presence of several categories of chemicals, including phenolic compounds, fatty acids and their esters, terpenes, aromatic, aliphatic acids and their associated esters, and flavonoids and their derivatives with clear variations and different in the types and percentages of these chemicals according to the type of the solvent use. It is known that raw propolis contains more than 350 different chemical compounds (1). These compounds were separated from different samples and with different detection methods, and most of them were based on GC technology. Therefore, the type and quantity of materials separated depend mainly on the place of collection of the sample and the technique used for separation (29). Many data have been shown the solvent used and the extraction method play a vital role in the nature of the isolated compounds and, as a result, are reflected on their biological effectiveness (18). In general, the polarity of the solvent and propolis composition are the two most important factors able to influence the quality of extracts (5)

The present study revealed a difference in total phenolic content and total flavonoid content among different solvent extracts of local propolis. With regard of watery extract, the total flavonoid content was recorded 52.9% However, ethanolic 43.95%, ethyl acetate 24.21%. These results are in agreement with that found by other authors. Kujumgiev et al., (30) found flavonoids and phenolic acid esters as main constituents in Bulgarian propolis samples. The type and content of flavonoids in propolis may vary depending on the specific propolis raw material as well as the extraction and preparation methods (1). According to these findings, the main constituents were phenolics, which are consistent with published research, and propolis from China and Europe included many varieties of phenolic acid esters and flavonoids (30). Also, it was discovered that flavonoid levels affected the antioxidant more strongly action of these extracts as opposed to their antibacterial ((31). Many authors stated that the biological activities of propolis are mostly connected to the high levels of phenolic acids in propolis (18,32,33). The order of solvents with highest flavonoids content was watery > ethanol > ethyl acetate. Our results indicated that ethyl acetate propolis sample has low total polyphenol and flavonoids content compared with other samples. Ethyl acetate extract was more efficient solvent for the isolation of fatty acids and their esters compared to other solvents. In fact, every propolis extract has a unique combination of ingredients. The potential variation in biological activity of propolis extracts can be attributed to the diversity of their constituents and, as a result, their principal chemicals.

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Conflict of interest

No conflict of interest is found for the present study.

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