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RESEARCH ARTICLE

ANALYSIS OF THE CHEMICAL COMPOSITION OF LEBANESE PROPOLIS

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ABSTRACT

The aim of this work is to study the chemical composition of a Lebanese Propolis sample. Extraction of the sample with Ethanol 70% was first applied to a greenish powered propolis. The Total Phenolic Content and the Total Flavonoid Content of the Crude Ethanol Extract obtained showed 252.34 mg GAE/g propolis and 140 mg QE/g propolis respectively. This extract was then used for a Liquid-Liquid Fractionation process, using three successive organic solvents. Three different fractions were obtained: Hexane Extract, Methylene Chloride Extract and Ethyl Acetate Extract. By comparison with eight analytical standards, two Phenolic acids and six Flavonoids were identified and quantified in those organic extracts using a Reversed Phase High Performance Liquid Chromatography system (RP-HPLC). The chromatograms showed the presence of Ferulic acid (9.10%), Chrysin (5.94%), Pinocembrin (2.08%), Quercetin (1.91%), Kaempferol (0.48%), Galangin (0.26%), Caffeic acid (0.05%), and Apigenin (0.02%).

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INTRODUCTION

Propolis is a honeybee product with broad spectrum of biological properties (Osés *et al.,* 2016; Huang *et al.,* 2014). As a resinous substance, propolis is prepared by honeybees to seal the cracks, smooth walls, and to keep moisture and temperature stable in the hive all year round (Burdock *et al.,* 1998). Raw propolis is typically composed of 50% plant resins, 30% waxes, 10% essential and aromatic oils, 5% pollens and 5% other organic substances (Huang *et al.,* 2014). It has been reported that propolis is collected from resins of poplar, conifers, birch, pine, alder, willow, palm (Kosalec *et al.,* 2004). Propolis has been used as a popular remedy for several centuries for a wide array of ailments (Sawaya *et al.,* 2011). It is widely used to prevent and treat colds, wounds and ulcers, rheumatism, sprains, heart disease, diabetes (Li *et al.,* 2012; Hu *et al.,* 2005) and dental caries (De Castro Ishida *et al.,* 2011) due to its diverse biological properties such as anti-inflammatory (Huang *et al.,* 2014), antimicrobial (Kologeropoulos *et al.,* 2009; Mohammadzadeh *et al.,* 2007b; Tosi *et al.,* 2007; Mendes da silva *et al.,* 2006), antioxidant (Laskar *et al.,* 2010; Kologeropoulos *et al.,* 2009; Mohammadzadeh *et al.,* 2007a; Ahn *et al.,* 2007; Mendes da Silva *et al.,* 2006), antitumor, antiulcer, anti-HIV activities (Huang *et al.,* 2014), anti-parasitic and anti-viral/immune stimulating (Sawaya *et al.,* 2011). The wide application of propolis in modern medicine has drawn growing attention to its chemical composition (Luo *et al.,* 2011). Many studies revealed that the observed effects might be the result of synergistic action of its complex constituents (Osés *et al.,* 2016; Huang *et al.,* 2014; Amoros *et al.,* 1992). Previous reviews (Huang *et al.,* 2014; Bankova *et al.,* 2000;Marcucci, 1995; Ghisalberti, 1979) have covered the knowledge about the chemical composition and botanic origin of propolis throughout 20th century. Until 2000, over 300 chemical components belonging to the flavonoids, terpenes, and phenolics have been identified in propolis (Huang *et al.,* 2014). Propolis is usually consumed as an extract, so the type of solvent and extractive procedures employed further affect its composition (Sawaya *et al.,* 2011).Therefore, the methods used for the extraction, analysis of the percentage of resins, wax and insoluble material in crude propolis, determination of amino acid, heavy metal contents, phenolic compounds and flavonoid have been extensively studied (López *et al.,* 2014; Luo *et al.,* 2011; Sawaya *et al.,* 2011; Popova *et al.,* 2011). Moreover, with the development of separation and purification techniques such as High Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), Gas

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Chromatograhy (GC), as well as identification techniques, such as Mass Spectroscopy (MS), Gas Chromatography and Mass Spectroscopy (GC-MS), more compounds have been identified in propolis; including flavonoids, terpenes, phenolics and their esters, sugarsand hydrocarbons. In contrast, relatively common phytochemicals such as alkaloids, and iridoids are not been reported (Huang *et al.,* 2014). It has also been demonstrated that the propolis chemical composition is susceptible to the geographic location (Zhou *et al.,* 2008), botanical origin (Salatino *et al.,* 2014; Silici *et al.,* 2005), bee species (Silici *et al.,* 2005) and seasonal variations (Valencia *et al.,* 2012). No studies have ever been reported on Lebanese propolis, thus; in this study, we are interested in the determination of the chemical composition of a Lebanese propolis sample. A Reversed Phase High Performance Liquid Chromatography (RP-HPLC-DAD) system was used to analyze several propolis solvent extracts. The extraction was first performed by Ethanol 70% (Crude Ethanol Extract), followed by a Liquid/Liquid fractionation process using Hexane, Methylene Chloride and Ethyl Acetate successively. Eight compounds (two Phenolic Acids and six Flavonoids) were identified by comparison of Propolis Extracts chromatograms to analytical standards solutions of Phenolic acids and Flavonoids.

MATERIALS AND METHODS

Propolis and Source plant: The analyzed sample of greenish propolis was produced by honeybees (*Apismellifera* L.) in an apiary located in the citrus groves of the Lebanese Coast. The sample was ground prior to extraction.

Reagents and Chemicals: All analytical standards (Apigenin, Chrysin, Galangin, Kaempferol, Pinocembrin, Quercetin, Caffeicacid and Ferulic acid-trans) were purchased from Sigma-Aldrich (USA); their purities were above 98%. The extraction and fractionation solvents (Ethanol, Hexane, Methylene Chloride and Ethyl Acetate), also purchased from Sigma-Aldrich (USA), were of analytical grade. The HPLC mobile phase solvents (Acetonitrile/Formic acid), purchased from Sigma-Aldrich (USA), were of HPLC grade. Before use, all mobile phase solvents were filtered through a 0.45 µm membrane filter. The Folin-Ciocalteau reagent was purchased from Sigma-Aldrich (USA). Deionized water was prepared by a Milli-Q water purification system. Gallic acid, HCl, Na_2CO_3, Magnesium, and $AlCl_3$ used for the determination of the Total Phenolic and the Total Flavonoid Contents were provided by the Laboratories of the Lebanese University, Faculty of Sciences I.

Preparation of Propolis Extracts

Crude Ethanol Extract: 10 g of crude greenish powered propolis were extracted by maceration for 7 days in a shaker, regulated at a speed of 100 rpm and temperature of 30°C, with 50 mL of Ethanol 70% (Sawaya *et al.,* 2011; Cunha *et al.,* 2004). The insoluble portion was then separated by filtration and the filtrate was again extracted by maceration for 7 more days in the same conditions. After the second filtration, the filtrate was kept in a freezer at -18°C overnight and filtered again at this temperature to reduce the wax content of the extract. Solvent was then evaporated at reduced pressure with a rotary evaporator at a temperature of 50°C.The extract obtained was freeze-dried, weighed and labelled Crude Ethanol Extract.

Liquid-Liquid Fractionation process of the Crude Ethanol Extract: 43 g of crude greenish powered propolis were extracted by maceration exactly as described for the preparation of the Crude Ethanol Extract. The extract obtained was then used for a serial Liquid-Liquid Fractionation process using 3 successive organic solvents from the less polar one to the more polar one: Hexane, Methylene Chloride and Ethyl Acetate. Each fraction obtained was filtered, and concentrated at reduced pressure with a rotary evaporator at 40°C. Dry extracts of propolis obtained were freeze-dried, weighed and labelled as following: Hexane Extract; Methylene Chloride Extract, and Ethyl Acetate Extract.

Total Phenolic Content (TPC): The analysis of the Total Phenolic Compounds (TPC) was carried out according to the spectrophotometric method described by Folin-Ciocalteau (Walterman and Mole, 1994). Gallic acid was used as a standard. 0.5 mL of each Propolis Extract solution (1mg/mL) was mixed with 5 mL ultrapure water, 0.5 mL of Folin-Ciocalteau reagent and 0.5 mL Na_2CO_3 10%. The absorbance was measured at 760 nm after 2 hours of incubation in the dark at room temperature. A control study was conducted under the same conditions. The content of the Total Phenolic Compounds in the samples was determined based on a standard curve of Gallic acid. The results of the content of total phenols are expressed as Gallic acid Equivalents (mg GAE/g of propolis; w/w).Three replicates were done for each extracts and the results were expressed by the mean values.

Total Flavonoid Content (TFC): Generally, the presence of flavonoids in plant extracts can be determined by a first preliminary quick test. This test was used on the propolis different extracts studied. It consisted of adding some fragments of Magnesium and some drops of HCL 1N on 2 mL of the analyzed extract. If red coloration is observed, then the presence of flavonoid is detected, and thus the quantification could be carried on. The Total Flavonoid Content (TFC) of the Ethanol, Ethyl Acetate, Methylene Chloride and Hexane Propolis Extracts was determined according to the $AlCl_3$ method described in the literature (Vennat *et al.,* 1992). 1 mL of each Propolis Extract solutions (1mg/mL) was mixed with 1 mL $AlCl_3$ 2%. A control series were prepared in parallel, and after 1 hour at room temperature, the absorbance was measured at 430 nm. Quercetin was used as a standard. The Total Flavonoid Content was calculated as Quercetin Equivalents (mg QAE/g of propolis), after drawing a calibration curve. The results obtained for each of the extracts represent the mean value of three replicates.

Preparation of the analytical standards for the HPLC analysis: Two phenolic acids (Caffeic acid, Ferulic acid-trans) and six flavonoids (Apigenin, Chrysin, Galangin, Kaempferol, Pinocembrin, Quercetin) were investigated. Four levels of working analytical standards solutions were prepared and diluted as necessary with Acetonitrile to get appropriate concentrations of 5, 10, 20 and 50 ppm. Calibration curves were build upon the results of four replicates injections for each standard. A linear regression equation was drawn and the determination of the coefficient (R^2) was calculated by the

means of the least-squares analysis. The linear regression coefficients for the mostly compounds were above 99%.

RP-HPLC-DAD Analysis: The analysis of the Ethanol, Ethyl Acetate, Methylene Chloride and Hexane extracts of propolis by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) was performed on a HITACHI system equipped with Diode Array Detector (DAD) and Autosampler L-2200. 5 mg of each dried Propolis Extract were dissolved in 1 ml Acetonitrile. A 20 µL of each extract wasinjected into a C18 Column of Genesis type (150 mm ×4.6 mm) with 4 µm particle diameter. The column was maintained at 30°C. The mobile phase consisted of 2% Formic acid in water (Solvent A) and Acetonitrile (Solvent B). The applied elution conditions were with the following gradient of 30% Solvent B (0-20min), 60% Solvent B (20-25 min) and 75% Solvent B (25-50 min) at a flow rate of 0.8 mL/min. The detection was monitored at 280 and 340 nm according to the respective maximal absorption of the compounds investigated. Acquisition data and interpretation of peaks were performed by the software EZ Chrome Elite. Each compound was identified according its retention time by external standards method and sometimes by spiking with the appropriate analytical standards under the same conditions. The quantification was done according to the peak area measurements which were reported in calibration curves of the corresponding standards.

RESULTS AND DISCUSSION

Global yields of the Ethanol Propolis Extraction and the Organic Solvents Fractionation Process: As we mentioned before, powered crude propolis contains plant alcohol extractible resins, beeswax and insoluble material. Several procedures may be used to extract its bioactive components. One of the known methods is the one used by Cunha *et al.,* (2004) and by Sawaya *et al.,* (2011) that uses maceration with Ethanol 70%. This method was applied here, since we aim to mostly extract Phenolic Acids and Flavonoids that are responsible for most biological activities. Yield of this extraction procedure was 60.80% (w/w). Most of it was wax contents. This wax contents were eliminated by freezing the Ethanol Extract overnight (-16°C) and separating the precipitated wax by filtration at this temperature to obtain a finally global yield of 19.33% for the non-waxed extract (Table 1). Comparing the waxed extraction yield (60.80%) with literature results, we found that Nieva Moreno *et al.,* (2001) reported yields between 31 and 65% (w/w) for samples of Argentine propolis extracted with 80% Ethanol. Miyataka *et al.* (1997), while studying the anti-inflammatory effect of propolis, extracted several samples of Brazilian propolis with 99.5% Ethanol and with distilled water, reporting yields of 41-60% (w/w) for the ethanolic extracts and 4-14% (w/w) for the water extracts. In a similar procedure, Hayashi *et al.* (1999), studying the antioxidant effect of propolis, extracted a sample of Brazilian propolis with 70% Ethanol and with distilled water, with a yield of 44.5% (w/w) for the ethanolic extract and 11.1% (w/w) for the water extract. Cunha *et al.,* (2004) reported extraction yields with absolute alcohol by maceration between 48.41 and 59.48%, while studying the factors that influence the yield and composition of Brazilian propolis extracts. All the above results are in line with our own. Nevertheless, the variability of yield between the different countries in these studies while using approximately the same extractive procedure reflects the great variability of the composition of propolis that is related to the geographical location. The afterward fractionation with the three successive organic solvents used allowed the partition of the extracted compounds in these solvents. The global yields obtained for the different propolis extracts are presented in Table 1. If we consider that the 19.33% of Ethanol Extract are equal to 100% of dry matter, we found that 18.62%, 6.64% and 5.42% of the extracted molecules are found in the Hexane, Dichloromethane and Ethyl Acetate Extracts respectively. It is important to mention that the Fractionation process applied here have never been reported before in the literature in similar studies aiming to qualify and quantify the chemical content of propolis. Although, in this study, we decided to obtain different fractions with different solvents attempting to study the greatest number of bioactive compounds, including Phenolic Acids, Flavonoids and Terpenes in subsequent and further studies; since part of our research work showed that the organic solvent extracts including/especially Hexane Extract had an anti-bacterial and anti-cancer activity (results not shown here).

Total Phenolic Content (TPC)& Total Flavonoid Content (TFC): Logically, Phenolic Acids and Flavonoids will not be found in the Hexane Extract since Hexane is a non polar solvent. It is more expected to find this class of molecules in the two other solvents used (Methylene Chloride and Ethyl Acetate), as well as in the Crude Ethanol extract. The analysis of these specific classes of compounds can be firstly evaluated, for the Flavonoids, by the spectrometric method using $AlCl_3$ as reagent (Vennat *et al.,* 1992), with a calibration curve constructed using Quercetin, even if this flavonoid is not found in the sample analyzed (Sawaya *et al.,* 2011). For the Phenolic compounds, the total phenol content is a standard test, usually determined by the Folin-Ciocalteau method, with calibration curves using phenol or Gallic acid as standards (Walterman and Mole, 1994). Common legislation usually accepts minimum flavonoid contents of 0.5% (w/w) and minimum phenolic contents of 5% (w/w) in crude propolis (Sawaya *et al.,* 2011). The Total Phenolic Content quantified here in the different propolis extracts is expressed as Gallic Acid equivalents and presented in Table 2. The Crude Ethanol Propolis Extract showed relatively high content of phenolic compounds 252.34 mg GAE/g propolis (25.23%) (Table 2). This value is well in line with the minimum requirements according to the Regulation of Identity and Quality of Propolis that is, as mentioned before, 5% (w/w) for phenolic compounds (Cabral *et al.,* 2012). By comparison with literature, Bonvehi *et al.* (1994), found 10.10% of phenols (by spectrophotometry) in a sample of Brazilien propolis extracted with methanol. Woisky and Salatino (1998) reported a concentration of total phenolic substances between 8.8 and 13.7%. Cunha *et al.* (2004) obtained phenolic content values between 8.00 and 13.34%. And finally, Brazilian propolis analysis by Cabral *et al.* (2012) showed values of 1.48% and 16.96% for different samples analysis. The Total Phenolic Content (TPC) varied between the extracts after the fractionation process; the Ethyl Acetate Extract contained 212.21 mg GAE/g (21.22%) and the Methylene Chloride Extract contained 20.96 mg GAE/g (2.09%). Almost all the phenolic compounds extracted initially with ethanol are present in the most polar solvent used in the fractionation

process which is the Ethyl Acetate. The Hexane Extract presented the lowest value (1.77%). This is due to the polarity of the solvent: Hexane is a non-polar solvent that cannot solubilize polar phenolic compounds. Moreover, the sum of the phenolic compounds found in the fractionation extracts (250.94 mg GAE/g propolis) (Table 2) showed high recovery yield (99.44%) of this process. The Total Flavonoid Content quantified in the different propolis extracts, is expressed as Quercetin Equivalents and presented in Table 2. The Total Flavonoid Content (TFC) also varied between the extracts (Table 2). The Crude Ethanol Propolis Extract showed relatively high content of flavonoids (140.00 mg QE/g propolis or 14%), which fitted normally to the minimum requirements according to the Regulation of Identity and Quality of Propolis that is 0.5% (w/w) for flavonoids. Literature comparison pointed differences with a Brazilian propolis analyzed by Cabral *et al.* (2012) where values of 0.32% and 6.15% were found for different samples analysis of TFC. The sum of the flavonoids found in the fractionation extracts (135.09 mg GAE/g propolis) (Table 2) showed high recovery yield (96.49%) of this process. The flavonoids extracted with Ethanol were distributed between the Ethyl Acetate Extract which contained 91.71 mg QE/g (9.17%) and the Methylene Chloride Extract which contained 42.86 mg QE/g (4.29%). The Hexane Extract presented the lowest value (0.05%); this was the confirmation of the preliminary test for flavonoid detection, since no red coloration was observed in this extract. In the other extracts, the intensity of red color reflected the abundance of flavonoids. The Ethanol Extractpresented high coloration, followed by Ethyl Acetate and Methylene Chloride Extracts, which had almost same coloration.

High-Performance Liquid Chromatography (RP-HPLC-DAD): Figure 1 showed the retention times of the eight pure analytical standards solution mixture of Flavonoids and Phenolic compounds analyzed by the RP-HPLC system. Figure 2 showed the HPLC chromatograms for all the propolis extracts studied. Figure 3 represented the partition of the identified Phenolic Acids and Flavonoids in the organic solvents after the fractionation process. Table 3 represented the compounds identified in the extracts and their respective quantities. Peaks and the order of elution of compounds in the analytical standards solution mixture (Figure 1) were assigned based on the retention times obtained from separately chromatograms analysis of each standard. Thus, the order of elution was found to be as the following: Caffeic acid(2.267 min), Ferulic acid(3.193 min), Quercetin (6.4937 min), Apigenin(10.627 min), Kaempferol(11.967 min), Chrysin(28.373 min), Pinocembrin(29.773 min) and finally Galangin(35.187 min). The two Phenolic acids analyzed eluted first followed by the Flavonoids. The order of elution is well in line with similar studies (Yang *et al.,* 2013; Valencia *et al.,* 2012; Luo *et al.,* 2011; Laskar *et al.,* 2010; Kologeropulos *et al.,* 2009). Peaks and compounds in the different propolis extracts (Crude Ethanol, Ethyl Acetate, Methylene Chloride and Hexane) (Figure 2) were assigned by comparison with the retention time of the eight analytical standards in Figure 1.

Crude Ethanol Extract: The chromatogram of the Crude Ethanol Extract (Figure 2(a)) revealed the presence of Caffeic acid, Ferulic acid, Quercetin, Apigenin, Kaempferol, Chrysin, Pinocembrin, and Galangin. Therefore, it can be deduced that the Lebanese propolis sample analyzed contained all these molecules. Their amounts were quantified using the area values of their corresponding peaks in the chromatogram on standards calibration curves. Propolis content of each molecule was calculated in mg/g of dry matter in the extract. The quantification (Table 3) revealed high amounts of Ferulic acid (91 mg/g or 9.1%), as the principally Phenolic Acid found, followed by, from the highest to the lowest amounts, Chrysin (5.94%), Pinocembrin (2.08%), Quercetin (1.91%), Kaempferol (0.48%), Galangin (0.26%), Caffeic acid (0.05%), and Apigenin (0.02%). After the fractionation process, these molecules were distributed in the different organic solvent extracts (Figure 3).

Ethyl Acetate Extract: The chromatogram of the Ethyl Acetate Extract (Figure 2(b)) revealed the presence of Caffeic Acid (0.475 mg/g), Ferulic Acid (66.08 mg/g), Chrysin (50.139 mg/g) and Pinocembrin (17.543 mg/g) (Table 3). Furthermore, the total extracted Phenolic Acids were mostly present in the Ethyl Acetate Extract (66.555 mg/g) *(Table 3)*. This result confirmed the previous result obtained with the analysis of the TPC by spectrophotometry where the highest content (212.21 mg/g) (Table 2) of these molecules was present in this extract. The same deduce was observed concerning the Flavonoids content, where most of these (67.682 mg/g) (Table 3) were found in this extract, confirming the highest value of the TFC (91.71 mg/g)*(Table 2)* for the extract.

Methylene Chloride Extract: The chromatogram of the Methylene Chloride Extract (Figure 2(c)) revealed the presence of Ferulic Acid (22.79 mg/g), Quercetin (18.421 mg/g), Apigenin (0.134 mg/g), Kaempferol (3.583 mg/g), Chrysin (8.719 mg/g) and Pinocembrin (1.626 mg/g)*(Table 3)*. The total Phenolic Acids present in this extract (22.79 mg/g) *(Table 3)* confirmed the results obtained for the TPC (20.96 mg/g), which comes in second place (Table 2). The total Flavonoids content (32.483 mg/g) (Table 3) also confirmed the results obtained for the TFC (42.86 mg/g), which comes in second place (Table 2).

Hexane Extract: The chromatogram of the Hexane Extract (*Figure 2(c))* revealed relatively small quantities ofFerulic Acid (1.038 mg/g), Kaempferol(0.125 mg/g) and Galangin (1.909 mg/g) (Table 3). Above and beyond, the results in *Table 3*also revealed that the fractionation process conserved the extracted fraction of each molecule in the Crude Ethanol Extract. The sum of the Phenolic Acids (Caffeic and Ferulic acids) found in the fractionation extracts (90.384 mg/g) showed high recovery yield (98.74%) of this process, 91.53 mg/g were initially present in the Crude Ethanol Extract. The sum of the Flavonoids found in the fractionation extracts (102.199 mg/g) showed as well high recovery yield (95.48%) comparing with the initial Flavonoids extracted in the Crude Ethanol Extract (107.034 mg/g). Moreover, studying the partition of each compound (Phenolic Acids and Flavonoids) in the organic solvents extracts, unveiled the following (*Figure 3)*:

- ***Caffeic Acid*:** Almost all of the extracted Caffeic acid was found in the Ethyl Acetate Extract.
- ***Ferulic Acid*:** Ferulic Acid was distributed in the three solvents. The large quantity of it was found in the Ethyl Acetate Extract, followed by the Methylene Chloride and the Hexane Extracts.

Table 1. Global Yields of the Ethanol Propolis Extraction for the Organic solvents Fractionation Process

Propolis Extract	Ethanol (Crude)	Hexane	Methylene Chloride	Ethyl Acetate
Yields% (w/w)	19.33	18.62	6.64	5.42

Table 2. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of the Propolis organic solvent Extracts

Propolis Extract	Ethanol (Crude)	Hexane	Methylene Chloride	Ethyl Acetate
TPC (mg GAE/g propolis) (w/w)	252.34±0.03	17.77±0.05	20.96±0.02	212.21±0.02
TOTAL			**250.94 mg GAE/g propolis**	
TFC (mg QE/g propolis) (w/w)	140.00±0.09	0.52±0.03	42.86±0.01	91.71±0.05
TOTAL			**135.09 mg QE/g propolis**	

Table 3. Compounds identified in the Ethanol, Hexane, Methylene Chloride and Ethyl Acetate Propolis Extracts and their respective quantities

		Quantity of the compounds identified in the Propolis Extracts (mg/g)*				TOTAL (mg/g) After the fractionation process
	Compounds	Ethanol (Crude)	Hexane	Methylene Chloride	Ethyl Acetate	
[1]	Caffeic acid	0.533	n.d	n.d	0.475	0.475
[2]	Ferulic acid	90.999	1.038	22.790	66.080	89.908
TOTAL Phenolic Acids (mg/g)		**91.532**	**1.038**	**22.790**	**66.555**	**90.384**
[3]	Quercetin	19.099	n.d.	18.421	n.d.	18.421
[4]	Apigenin	0.170	n.d.	0.134	n.d.	0.134
[5]	Kaempferol	4.855	0.125	3.583	n.d.	3.708
[6]	Chrysin	59.463	n.d	8.719	50.139	58.858
[7]	Pinocembrin	20.819	n.d.	1.626	17.543	19.169
[8]	Galangin	2.628	1.909	n.d.	n.d.	1.909
TOTAL Flavonoids (mg/g)		**107.034**	**2.034**	**32.483**	**67.682**	**102.199**

* Quantities are expressed as percentage of dry matter in the propolis extract (w/w), mean of triplicate analyses for each sample. n.d.: not determined.

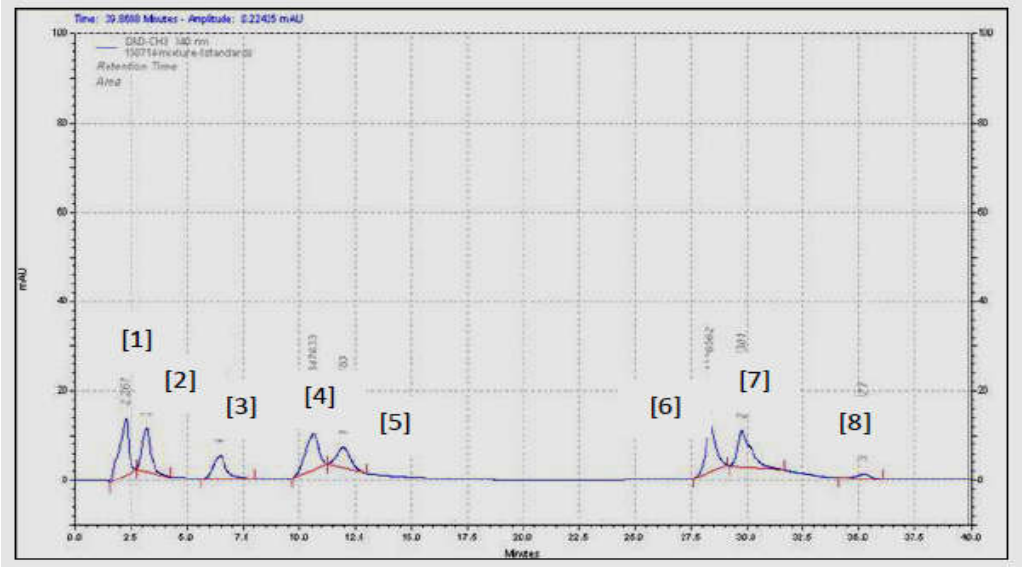

Figure 1. RP-HPLC-DAD Chromatogram of the standards solution mixture of Phenolic acids and Flavonoids. Numbers were inserted across the chromatograms to facilitate comparison

- ***Quercetin*:** All the Quercetin found in the Crude Ethanol Extract reappeared in the Methylene Chloride Extract.
- ***Apigenin*:** All the Apigenin found in the Crude Ethanol Extract reappeared in the Methylene Chloride Extract.
- ***Kaempferol*:** Kaempferol was distributed between the Methylene Chloride and the Hexane Extracts.
- ***Chrysin*:** Chrysin appeared mostly in the Ethyl Acetate Extract followed by the Methylene Chloride Extract.
- ***Pinocembrin*:** Pinocembrin appeared mostly in the Ethyl Acetate Extract and humbly inthe Methylene Chloride Extract.
- ***Galangin*:** All the Galangin found in the Crude Ethanol Extract reappeared in the Hexane Extract.

To recapitulate, Ferulic Acid, Chrysin, Pinocembrin and Quercetin had the highest amounts in the propolis sample analysed (Figure 3). The results obtained were compared with results on Propolis obtained in other countries, e.g. in Romania (Coneac *et al.,* 2008), the following results were found: Caffeic acid (6.34 mg/g), Quercetin (9.97 mg/g), Apigenin (8.24 mg/g), Kaempferol (2.93 mg/g) and Chrysin (9.81 mg/g). By comparing these results with the analysis of the Lebanese Propolis, it can be concluded, that the Lebanese Propolis is richer in Quercetin, Kaempferol and Chrysin (Table 3). On the contrary, the Lebanese Propolis is scarcer with Caffeic acid and Apigenin. In Nigeria (Yang *et al.,* 2013), similarity with the Lebanese Propolis were noticed; the Nigerian Propolis contained 2.78 mg/g of Galangin; the Lebanese Propolis contained 2.63 mg/g of Galangin.

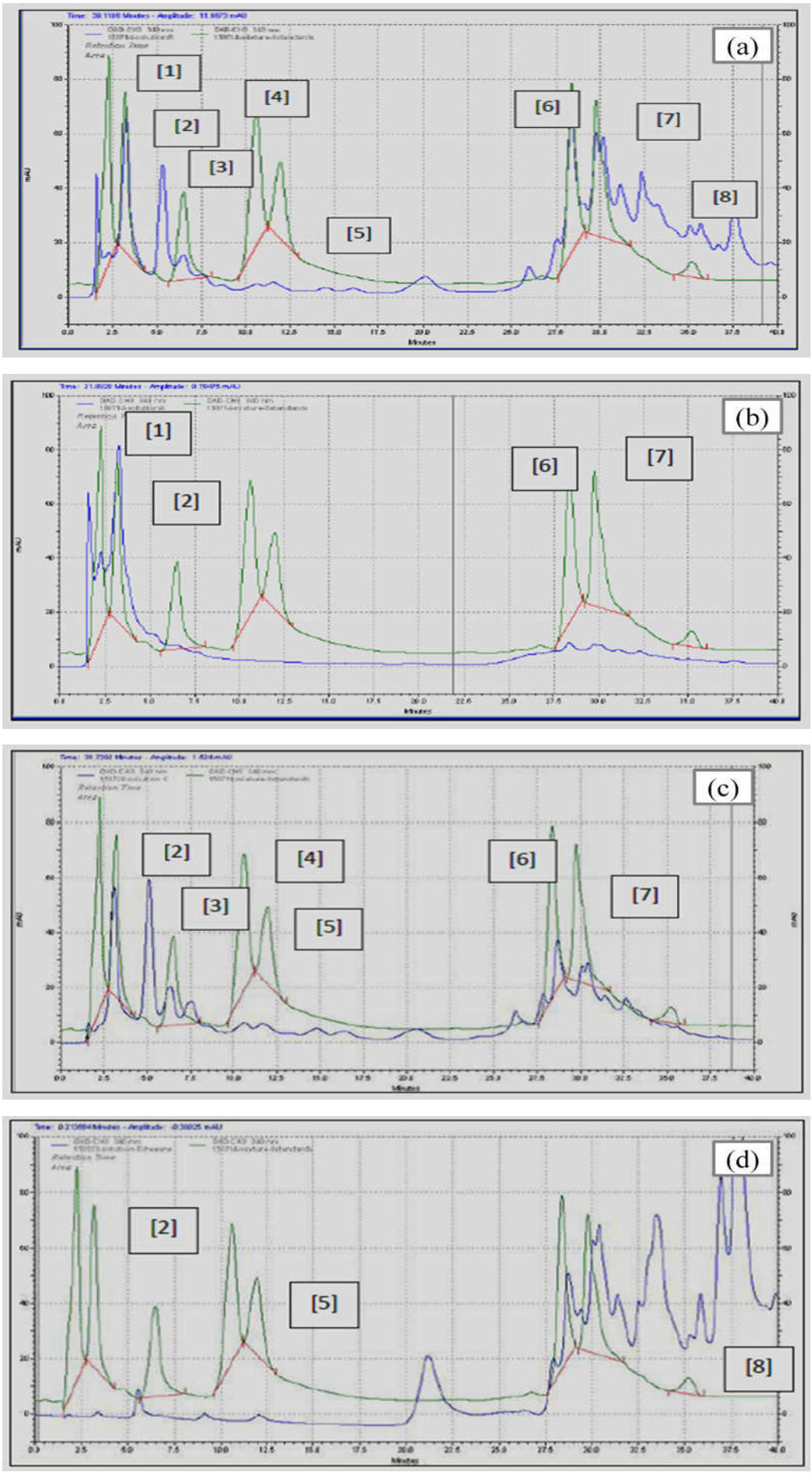

Figure 2. RP-HPLC-DAD Chromatograms of the Crude Ethanol Propolis Extract (a), Ethyl Acetate Propolis Extract (b), Methylene Chloride Propolis Extract (c) and Hexane Propolis Extract (d) at 340 nm. Numbers were inserted across the chromatograms to facilitate comparison

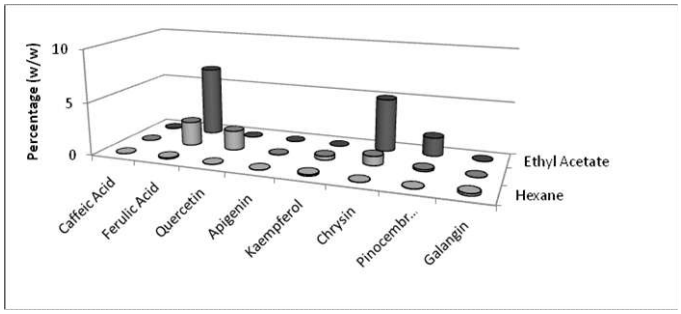

Figure 3. Partition of the identified Phenolic Acids and Flavonoids in the Hexane, Methylene Chloride and Ethyl Acetate Propolis Extracts after the fractionation process.=

The amounts of Quercetin were significantly different; 0.64 mg/g for the Nigerian Propolis *vs.* 19.1 mg/g for the Lebanese Propolis (Table 3). Since it has been demonstrated that the propolis chemical composition is susceptible to the geographic location, botanical origin (Salatino *et al.,* 2014; Silici *et al.,* 2005), and bee species (Silici *et al.,* 2005), the differences with other countries are legitimately tolerable.

Conclusion

The aim of this work was to study the chemical composition of a Lebanese Propolis sample. Extraction of the sample with Ethanol 70% revealed that the Total Phenolic Content (TPC) and the Total Flavonoid Content (TFC) were 25.23% (252.34 mg GAE/g propolis) and14% (140 mg QE/g propolis) respectively. The Liquid-Liquid Fractionation process applied on the Crude Ethanol Extract using three successive organic solvents unveiled the distribution of the extracted molecules among three different extracts: Hexane, Methylene Chloride and Ethyl Acetate. Two Phenolic acids and six Flavonoids were identified and quantified in these extracts using Reversed Phase High Performance Liquid Chromatography analysis. The results obtained showed that the compounds were mostly present in the Ethyl Acetate Extract, followed by the Methylene Chloride and the Hexane Extracts. Ferulic acid (9.1%), Chrysin (5.94%), Pinocembrin (2.08%), Quercetin (1.91%) were the prevailing quantified compounds followed by Kaempferol (0.48%), Galangin (0.26%), Caffeic acid (0.05%), and Apigenin (0.02%). It is important to mention that qualifying and quantifying flavonoids and phenolic components of Lebanese propolis is here done for the first time. Further chemical analysis investigation must be extended on propolis samples from different geographical locations in the country and different harvest seasons to depict more authentic biomolecules.

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