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Lebanese Propolis from Different Regions: Phytochemical Screening, Antioxidant Activity and Effect against Cancer Cells

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ABSTRACT

Aim: Apitherapy, preparations, and food and beverage additives all employ propolis. Chemical content, biological activities as well, highly depend on the origins of propolis. This study aimed to compare the Total phenolic contents, antioxidant activity, and cytotoxicity to cancer cell lines of Lebanese propolis extracts from four regions.

Materials and methods: The methanolic extracts of propolis collected were prepared in the first step. The samples were subjected to phytochemical screening for the first time. In a second time, samples were tested for total phenol content by the Folin–Ciocalteau method, and radical scavenging activity using a spectrophotometric method. Anticancer activity was assayed on human tumor cell lines MDA-MB-231 and A549, using MTT assay.

Results: The results show that the estimation of the total phenolics varies between 3.60 mg and 73.52 mg of GAE / g of propolis extract and / g of propolis extract. The inhibition concentration values of 1, 1-Diphenyl-2-picrylhydrazyl radical scavenging and ascorbic acid in propolis in Fakeha, Debaal, Wadi Faara, and Qlaileh were 25.9 ± 5, 26.3 ± 4, 57.1 ± 10 and 729.7 ± 42 (µg.mL^{-1}), respectively. Additionally, propolis extracts 1 mg.mL^{-1} from two regions, namely Wadi Faara (31.71%) and Debaal (23.3%) demonstrated the inhibitory effect on proliferation of A549 cancer cell lines at 72 hours in a dose-dependent manner. However, these extracts show the opposite effect on breast cancer cells.

Conclusion: These results are of interest since Lebanese propolis from some regions has antioxidant properties and decreases the percentage of cell viability of human tumor cells A549; thus, it has the potential to contain some chemical compounds acting as an anticancer drug.

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Introduction

Propolis is a natural resinous product assembled by honey bees (*Apis mellifera*) from different plant sources. It's used to make the protective shield at the entrance of beehives and it was already known for its medicinal powers for generations when the product was employed in embalming bodies in Egypt [1]. Nowadays, propolis piqued scientists' interest due to its broad range of activities that can be used in complementary therapies, based on the biological and pharmacological properties that have been demonstrated including antitumor, antibacterial, antioxidant, antifungal, and other activities [2-5].

Propolis is generally composed of 50% resin and balm (including phenolic compounds), 30% wax and fatty acids, 10% essential oils, 5% pollen, and 5% various organic and inorganic compounds. The specific composition of propolis depends on the vegetation at the site of collection [6].

Among all the bee products, propolis possesses the highest antioxidant activity [7]. The antioxidant potential of propolis was originated from their polyphenolic substances [8,9]. Thus, propolis can be used for the prevention and treatment of diseases related to the increase of oxidative stress such as cancer, aging, and cardiovascular diseases [10].

On the other hand, cancer has become one of the major diseases and problems that have caused predominant death, and it is considered the second cause of death after cardiovascular diseases [11]. The typical cancer treatment is generally based on using chemotherapy, radiotherapy, cytotoxic drugs, and surgery [12,13]. These conventional therapies are effective and can even cure many types of cancers including breast cancer, colon, pancreatic, testicular, ovarian, and certain lung cancers,

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but their effectiveness is often limited by toxic effects [14]. Thus, continued searching for a safer and more effective treatment is needed [15]. For many years' natural medicines have been used and are still used in developing countries as the primary source of medical treatment [16]. Among many other potential anti-cancer natural sources propolis was demonstrated to have different anti-cancer effects going from selectively cytotoxic, anti-proliferative, and pro-apoptotic against tumor cells, to anti-metastatic, anti-mutagenic, anti-invasive, and anti-angiogenic [17–20].

Propolis's physical, chemical, and biological properties vary according to its botanical and geographical origins, which lead to variation in the chemical composition [21–23]. Therefore, several types of propolis are known based on the geographical area of the hive, the plants present in this geographical area, the availability of the plants during the season, and the species of bees [24].

This work comes in the continuation of an investigation aiming to study the Lebanese propolis, and the effect of biological environments on the quality of the propolis produced. In the present study, methanolic propolis extracts from four different Lebanese locations were prepared. Thus, the effect of geographical origin on the phytochemical contents was assessed and results were compared for the antioxidant capacity in terms of free radical scavenging assay and reducing power assay.

Materials and Methods

Chemicals and instrumentation

All chemicals used were of analytical grade. Methanol (MeOH), Ethanol (EtOH), chloroform, Folin Ciocalteu, and ascorbic acid were purchased from BDH (England). 2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, sodium carbonates anhydrous (Na_2CO_3), potassium ferricyanide, trichloroacetic acid, and iron (III) chloride ($FeCl_3$), were purchased from Sigma Aldrich (USA). Samples were weighed using a RADWAG XA 82/220/2X laboratory balance. The absorbance values were measured using a VWR UV-6300PC double beam spectrophotometer and extracts were concentrated using HEIDOLPH (Germany) rotavapor apparatus.

Samples collection

In this study, four Lebanese *Apis mellifera* propolis samples were collected (April 2019) from four apiaries located in different Lebanese regions (Figure 1), more specifically from Debaal (33°15′02″ N, 35°20′56″ E, Fakeha (34°14′44″ N, 36°24′21″ E, Qlaileh (33°19′87″ N, 35°22′91″ E and Wadi-Faara (34°17′22.0″ N 36°18′15.8″ E). The main process to collect the raw propolis samples was started by the initial preparation to separate it from extraneous macro impurities if present. The obtained samples were frozen at -20°C until analysis.

Figure 1. Effects of PCM layer location variations on glove thermal protection performance (times to second-degree skin burn temperature (60°C)) for (a) explosive/flashover situation (heat flux at 83 kW/m^2) and (b) hazardous condition (heat flux at 8.3 kW/m^2) (PCM thickness of 1 mm).

Preparation of methanolic extract of propolis (MEP)

The methanolic extract of propolis (MEP) was obtained according to the procedure described by Li, et al. [25] with slight modifications. Briefly, one gram of each frozen brown to yellow propolis sample was chopped into small pieces and immediately homogenously pulverized. Then, each sample was sonicated in 20 mL of MeOH for 2 hours using an ultrasonic bath at 25°C. The mixture was then filtered through Whatman filter No.1, and the filtrate was evaporated under reduced pressure to produce the methanolic extract of propolis (MEP). Finally, the MEP was weighed and stored at 4°C for further use.

Determination of percentage yield (%)

The extraction yield was calculated according to the following equation (1):

$$\text{Yield } \% = \frac{w_2}{w_1} \cdot 100 \text{ (equation 1)}$$

Where W1 is the dry weight of the used matter and W2 is the weight of collected extract after evaporation of the solvent.

Phytochemical screening

The qualitative phytochemistry tests of the MEP samples were carried out through phytochemical characterization tests *via* coloring or precipitation reactions on the ex-

tracts [26,27].

Test for alkaloids

About 2 mL of extracts were treated with Dragendorff's reagent (solution of Potassium Bismuth Iodide). The formation of a red precipitate indicates the presence of alkaloids.

Anthraquinones (Borntrager's reaction for free anthraquinone)

In a dry test tube, 3 mL of MEPs were mixed with 10 mL of chloroform. This was steamed for 5 minutes in a steam bath and directly filtered before cooling. An equivalent volume of a 10% ammonia solution was added to the filtrate. This was shaken, and brilliant pink coloration in the upper aqueous layer was noticed, indicating the presence of Anthraquinones.

Test for terpenoids (Salkowski test)

1 mL of each extract was mixed in 2 mL of chloroform, and concentrated H_2SO_4 (3 mL) was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presence of Terpenoids [28].

Test for hydrolysable tannins

A few drops of 0.1% ferric chloride were added and observed for brownish green or a blue-black coloration.

Test for quinones

1 mL of concentrated hydrochloric acid (HCl) was added to one mL of MEP. The presence of quinones is confirmed by the appearance of yellow color.

Test for flavonoids

1 mL of KOH is added to 1 mL of each extract. The yellow shift shows the existence of flavonoids.

Test for Saponins (Frothing Test)

1 mL of extract was shaken with 2 mL of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Determination of total phenolic content (TPC)

The total polyphenol content in propolis extract was determined by using the Folin-Ciocalteu method [29] with some modifications. Briefly, 20 µL of the MEP extract was taken and mixed with 1500 µL distilled water, then 100 µL of aqueous Folin-Ciocalteu solution. After 5 min incubation at room temperature, 300 µL of sodium carbonate (7.5%) were added. The obtained mixture was allowed to stand for 40 min in the dark. After which the absorbance was read at 760 nm in a spectrophotometer. The TPC in the extract was extrapolated from the calibration curve derived by repeating the same procedure for different concentrations of methanolic solutions of gallic acid (30-270 $\mu g.mL^{-1}$), and results were expressed in mg of gallic acid equivalents per g of propolis extract (mg GAE/g).

Biological investigations

Antioxidant capacity: The antioxidant activity of MEP samples was determined using the traditional method of Blois[25], Diphenyl^{-1}-picrylhydrazyl (DPPH) radical scavenging assay, with a slight adjustment. First, the extracts were prepared by dissolving 10 mg of each sample in 5 mL of methanol in order to obtain a concentration of 2 $g.L^{-1}$. A stock solution of DPPH (32 $mg.L^{-1}$) was prepared in methanol. The reaction mixture consisted of 1 mL of diluted methanolic extract and 1 mL of DPPH solution. The mixture was incubated in the dark at room temperature for 30 min, and then the absorbance was taken at 520 nm, using a corresponding blank prepared by adding 1 mL of methanol instead of the extract solution in the reaction mixture. Ascorbic acid (0.54 – 10.82 $\mu g.mL^{-1}$) was used as a reference standard. All the reaction mixtures were carried out in triplicates. The absorbance reading was measured using a UV-Vis spectrophotometer. The percentage inhibition and IC50 were calculated using equation (2). All data were recorded as mean ± SD for three replicates.

DPPH scavenged (%) = $(A_{DPPH} - A_{sample})/A_{DPPH} \times 100$ (equation 2)

A_{DPPH} is the absorbance of the blank control; A_{sample} is the absorbance of the samples (extracts or ascorbic acid).

Determination of antitumor activity: Cell lines used in this study including the human breast cell line (MDA-MB231), and the human lung cancer (Lung carcinoma, A549), were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Both cell lines were cultured in DMEM growth medium (Dulbecco's modified eagle's medium) supplemented with 10% fetal bovine serum, and penicillin/streptomycin (10,000 U/mL penicillin, 10 mg/ml streptomycin) (Sigma-Aldrich, USA). The cells were incubated at 37°C, 5% CO_2 in a humidified atmosphere. Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazoliumbromide) assay. MTT is reduced intracellularly in a mitochondrion-dependent reaction to yield insoluble formazan crystals. The ability of cells to reduce MTT indicates mitochondrial activity and serves as a measure of cell viability. Briefly, MDA-MB-231 and A549 cells were seeded in 96-well plates (5×10^3 cells/well). After 24 h, cells were treated with the different extracts at concentrations 1, and 1.5 $mg.mL^{-1}$ in duplicate, and reincubated for 72 h. Following incubation, the cells are washed and brought into contact with a freshly prepared 0.5 $mg.mL^{-1}$ MTT solution, and the plate was incubated for a further 4 h at 37°C. The absorbance was measured spectrophotometrically with an ELISA microplate reader (ELISA reader/Biotech) at 570 nm wavelengths. The number of viable cells was directly correlated to the number of purple formazan crystals formed.

Statistical analysis: All the experiments for the determination of total phenolics, antioxidant and antitumor

assays were conducted in triplicates. The values were expressed as the mean ± standard deviation (SD). The statistical analysis of the results was done using GraphPad Prism software. The values of $p<0.05$ are considered statistically significant. Correlation coefficients (r) and coefficients of determination (r 2) were calculated using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

Results

The yield of the ethanol extract was 14%, 18%, 22%, and 26% w/w for Fakeha, Qlaileh, Debaal, Wadi Faara propolis in Lebanon, respectively. The phytochemical tests carried out in MEP samples display the appearance of coloration, a precipitate, or flocculation through certain specific reagents. The results of the tests, illustrated in (Table 1), reveal the presence of several biologically active constituents such as alkaloids, tannins (condensed and hydrolysable), quinones, anthraquinones, and the absence of others such as saponins. The concentration of the total phenolic compounds in MEP is depicted in Figure 2. The MEP derived from Wadi Faara (73.52 mg GAE/g) showed the highest values of total phenolic compounds followed by that from Debaal (69.6 mg GAE/g) and Fakeha (35.38 mg GAE/g) and that from Qlaileh (3.69 mg GAE/g) was the lowest content. The antioxidant activities of the different MEP samples were assessed by the DPPH method and are shown in Figure 3. The antioxidant activity of MEP from Fakeha, Debaal, Wadi Faara and Qlaileh presented IC50 in concentrations 25.9, 26.3, 57.1, 729.7 $\mu g.mL^{-1}$, respectively, higher compared to the positive control (Ascorbic acid, 5.8 $\mu g.mL^{-1}$) indicating an inferior antioxidant activity to the ascorbic acid drug.

The anti-tumor activity of the 4 samples was tested on the A549 (lung cancer) and MDA-MB231 (breast cancer) cell lines. The cells were treated with two concentrations (1 mg and 1.5 mg) of each of these samples for 72 hours (Figure 4). Then, cell viability was measured quantitatively by MTT colorimetric assay.

The obtained results showed that the extracts used have a significant effect on the cell viability of the lung cancer line (A549. Findings detect a significant reduction in viability of cells treated with 1 mg of Debaal (23.3%) and Wadi faara (31.71%) extracts. The extracts of Fakeha and Qlaileh have no significant effect with this concentration. However, the treatment of cells with 1.5 mg of the extracts of the four samples shows a significant reduction in cell viability (Figure 5. The most significant reduction is obtained with Wadi Faara extract (36.58%) followed by Debaal extract (33.26%).

Unexpectedly, these extracts show a positive effect on the cell viability of the breast cancer line (MDA MB231). A sig-nificant increase in viability of cells treated with 0.5 mg of Fakeha, and Qlaileh extracts was detected. Similarly, this increase is detected with 1 mg of Debaal, Wadi Faara, and Fakeha extracts.

Table 1. Phytochemical analysis for the MEPs

Phytochemical family	Debaal	Wadi Faara	Fakeha	Qlaileh
Alkaloids	+++	+++	+	++
Flavonoids	+++	++	+	+
Anthraquinones	+++	++	+	-
Terpenoids	+++	++	+	+
Hydrolysable tannins	+++	++	+	+
Condensed tannins	+++	+++	++	+
Quinones	+++	++	+	-
Saponins	-	-	-	-
Note: +++ denotes high; ++ denotes moderate; + denotes low; - denotes absence				

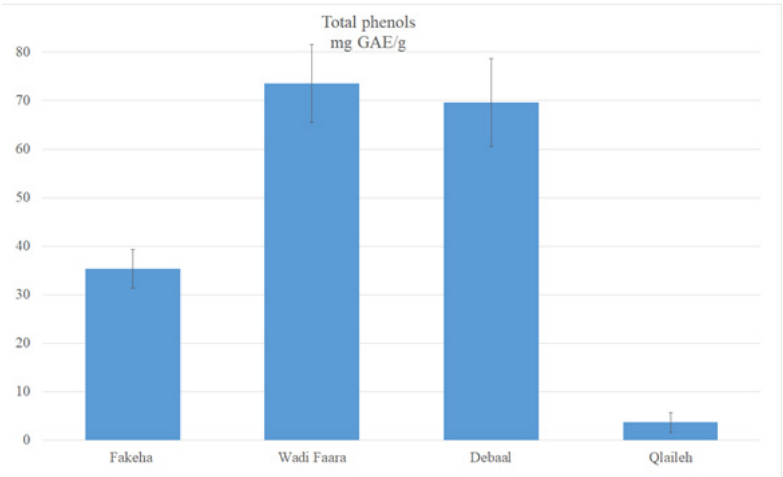

Figure 2. Comparison of total phenols (Gallic acid equivalents (GAE).g^{-1} of propolis extract) for the different regions. Mean values of three extractions ± SD

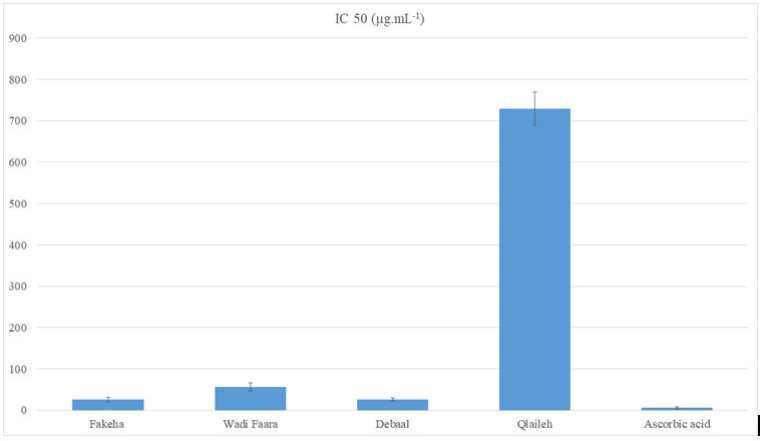

Figure 3. Average IC50 (µg mL^{-1}). Mean values of three extractions ± SD

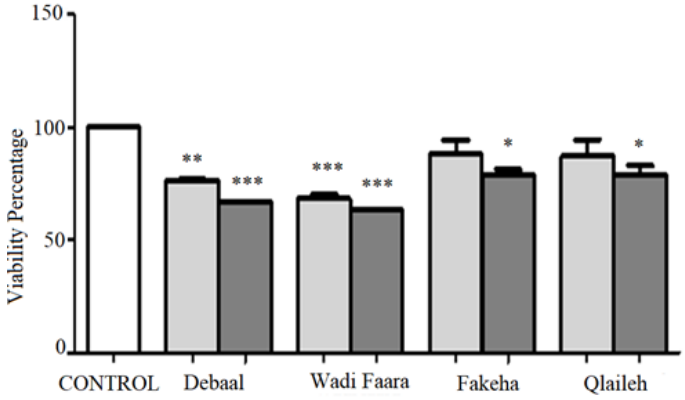

Figure 4. Effect of MEP from different regions on cell proliferation of A549 cell line presented as percentage of cell viability at two concentrations (72h) (mean ± SEM). Pvalue<0.05 (*), Pvalue<0.01 (**) and Pvalue< 0.001(***).

Note: (▭) - 1 MG; (▭) - 1.5 MG

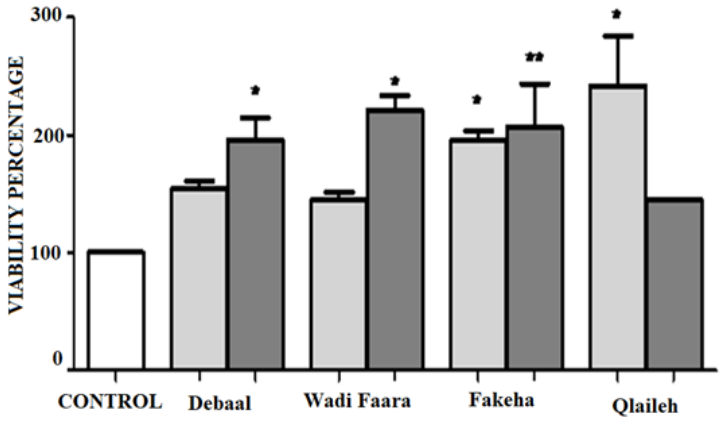

Figure 5. Effect of MEP from different regions on cell proliferation of MDA-MB-231 cell line presented as percentage of cell viability at two concentrations (72h) (mean ± SEM). Pvalue<0.05 (*), Pvalue<0.01 (**) and Pvalue< 0.001(***).

Note: (▭) - 1 MG; (▭) - 1.5 MG

Discussion

The obtained extraction yield ranged between 14%-26%. The extraction efficiency was in accordance with many The obtained extraction yield ranged between 14%-26%. The extraction efficiency was in accordance with many literature works. For instance, Woo et al., [30]. Showed that But Jeju (Korea) originated propolis yield was less than 10% yield until 60% ethanol, and the yield increases

at 10%~20% over 70% ethanol. In the work of Pobiega et al., the yields of extractions varied between 5.76 and 15.92% depending on the method [31]. In contrast, the work numbers were lower than many others, for example, those obtained by Trusheva et al., (41%–75%) [32]. and by Mircea et al., (37.1%–96.7%) [33]. The phytochemical screening of propolis extracts shows the presence of flavonoids, tannins, quinones, alkaloids, terpenes and anthraquinones, with absence of saponins. These findings are in good agreement with the work of Chamadi et al. on the Lebanese propolis [34].

The total polyphenol content of the studied propolis ranged from 3.69 to 73.52 mg GAE/g propolis. Depending on the standard and solvent utilized, literature references describe a variety of ranges for total phenolics of propolis from various geographical sources. The obtained range was consistent with the result of Ethiopian propolis [35]. propolis from some Turkish regions [36]. Malaysian Propolis [37]. Venezuelan propolis [38]. and Portuguese propolis [39]. On the contrary, the total phenolic content of Lebanese propolis was lower than others such as Brazilian propolis (277.81 mg-398.11 mg GAE/g) [40]. propolis from the Basque Country (Northeastern Spain) (200–340 mg/g propolis extracts) [41]. propolis from Western Romania (214 mg ± 48 mg GAE/g) [42]. and Argentinean propolis had great amounts of phenolics (ranging from 257 mg to 353 mg GAE/g) [43].

The IC50 value is a commonly used criterion for determining the antioxidant activity of test samples. It is determined as the antioxidant concentration required to reduce the initial DPPH concentration by 50% [44]. The highest antioxidant effect of MEP was that from Fakeha, while the lower from Qlaileh, presented IC50 25.9 and 729.7 $\mu g.mL^{-1}$ respectively. All the extracts present IC50 higher than the reference standard of the ascorbic acid. Similarly, findings were reported by many works such as for Ethiopian propolis (12.17 $\mu g.mL^{-1}$–22.07 $\mu g.mL^{-1}$) [35]. Portuguese propolis (7 $\mu g.mL^{-1}$-69 $\mu g.mL^{-1}$) [45]. and Indonesian propolis (25.54 $\mu g.mL^{-1}$-69.96 $\mu g.mL^{-1}$) The IC50 from Lebanese propolis was higher than Brazilian (102.94 µg.mL 1-47.42 µg.mL 1) [40], Western Romania (10 $mg.mL^{-1}$-0.3 $mg.mL^{-1}$) [46]. Mexican Brown Propolis 67.9 $\mu g.mL^{-1}$, and Malaysian propolis (4.27 $mg.mL^{-1}$) [47].

Indeed, several studies have demonstrated a positive relationship between the polyphenol content of natural material and its antioxidant capacity [48-50]. The correlation between phenolic composition and DPPH further suggests that the high antioxidant capacity of the extracts results mainly from the contribution of polyphenol compounds to the extracts. However, the exceptional result of Fakeha extract leads us to conclude that, the content of phenolic compounds is an important factor but it is not the only one, there are other criteria related to phenolic compounds to be considered in the interpretation of the antioxidant activity, such as the criterion of quality. Polyphenols have conjugated ring structures and hydroxyl groups that can scavenge free radicals and reactive oxygen species that are produced during oxidative reactions. The phenolic compounds respond differently in the analysis, depending on the number of phenolic groups and the total phenolic compounds do not necessarily incorporate all the antioxidants that may be present in an extract.

The anti-tumor effect of four propolis extracts was studied on the human breast (MDA-MB231) and lung (A549) cancer cells by MTT assay, hence our results reveal a probably anti-tumor effect of propolis extracts only on lung cancer cells. On the other hand, these extracts show the opposite effect on breast cancer cells. Findings from Brazilian green propolis, Thai propolis and Saudi Arabian propolis exhibit a similar effect of their extract against many cancer cell lines among them A549 in a dose-dependent manner. Yahima et al. revealed that propolis decreased mitochondrial membrane potential by overexpression of pro-apoptotic genes (*Bax and Noxa*) and reduction of the antiapoptotic gene Bcl-X_L. The expression level of other genes remained unchanged (p53, *Caspse-3* and *Bax*), whereas p21 expression was increased. In contrast to our results, Abutaha N., tested the cytotoxic potential of Jordanian propolis against different cell lines, and results showed that MEP exhibited cytotoxic potential against all cell lines tested (the IC50 value was 91.2 $\mu g.mL^{-1}$ for MDAMB231). The data obtained by the researcher lead him to suggest that the inhibition of the growth of MDA-MB-231 cancer cells was through the induction of apoptosis.

Conclusion

First, the phytochemical screening of MEP, as qualitative analysis, highlighted the presence and abundance in propolis of flavonoids, flavanones, tannins, alkaloids, resins, quinones, and anthraquinones that have a confirmed therapeutic activity. These characterizations also show the absence of saponines. The total phenols content in MEP from Wadi Faara and Debaal have the highest amount, 73.52 and 69.60 mg $GAE.g^{-1}$ of propolis respectively, while the best antioxidant activities were obtained with extracts from Fakeha and Debaal (IC50 0.0259 and 0.0263 $mg.mL^{-1}$ respectively). The anti-tumor effect of the four samples was studied and results reveal an anti-tumor effect of extracts only on lung cancer cells. Therefore, it would be interesting to study the effect of our extracts on other lung cancer lines and other types of cancer. In addition, a study on the effect of these extracts on the pro-apoptotic proteins *Bax* and *Bcl2* will allow us to detect the possibility of the effect of these extracts on apoptosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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