Chemical composition, antibiofilm and anticancer effect of different propolis extracts collected from Mila (Algeria)

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Abstract

The chemical contents, antibiofilm and anticancer effects of different propolis extracts (petroleum ether, chloroform, ethyl acetate and methanol) collected from Mila (Algeria) were investigated in this study. Chemical analysis showed the presence flavonoids as a major compound. The highest antibiofilm activity was observed at the 200 µg/ml methanol fraction (92%). Florescent microscope images also confirmed antibiofilm effect. Our results showed that the structure of biofilm treated with propolis was degraded and there were no water channels seen in the biofilm structure. In addition, the density of dead cells in the biofilm was also noted with fluorescent staining. According to FTIR analysis, it was found that propolis caused some changes in macromolecules on the cell surface. Cytotoxic activity of propolis (EC50: 97–117 µg/ml) was determined by MTT test. Propolis extracts caused a significant increase in mRNA levels of proteins involved in tumor suppression and apoptosis in the MDA-MB-231 cell line and induced apoptosis. Western blot analysis confirmed these results. As a result, it can be said that Algerian propolis has anticancer and antibiofilm effective compounds.

Introduction

Recently, antibiotic resistance has been the biggest challenge facing public health. If antibiotic resistance is not prevented, the danger awaiting us in the future is enormous. Soon, antibiotics may become completely ineffective in the treatment of infectious diseases and minor wound infections may result into death. Nowadays, the use of effective natural antibacterial products has been suggested to overcome antibiotic resistance. Therefore, new antibacterial and antibiofilm agents are screened. In our case, one of the recommended natural products is propolis. Propolis (bee-glue) has attracted the attention of human beings since ancient times. Propolis is collected by honeybees from botanical resinous sources. It is used for the protection of hives from several threats. The main chemical constituents of propolis are essential oils, phenolic, and flavonoids. It has been the subject of many scientific research because of its rich chemical content and wide biological effect. Especially, the antibiofilm effect of propolis has been extensively investigated recently (Veloz et al. 2019; Daikh et al. 2020). Biofilm is a complex of microbial structure that connect to natural or synthetic materials and are firmly embedded in the extracellular matrix (Roy et al. 2018). Biofilm is produced by pathogenic bacteria such as *Staphylococcus*, *Streptococcus*, *Pseudomonas* and human pathogenic yeasts such as *Candida albicans* and some non-pathogenic bacteria (Kumar et al. 2011; Villa et al. 2011). Microorganisms in biofilm exchange genetic material with each other. Therefore, the genetic modifications in pathogens cause resistance to antibiotics. Additionally, the effect of antimicrobials on bacteria in biofilm is limited. As a result, the antibiotic resistance has led to an increase in infectious diseases and epidemics. For example, *S. aureus* is a biofilm-forming human pathogen that causes serious hospital-related infections (Coates et al. 2014; Tong et al. 2015) and it often develops high resistance to various antimicrobial agents. Hence, there is a need for natural medicines that can penetrate the bacterial biofilm and disrupt the mechanism of action of the pathogen. For this reason, both herbal products and chemical substances produced by many living organisms provide an advantage in health research.

It is known that various products of plants, animals and microorganisms are rich in secondary metabolites that can be applied as chemotherapeutic agent. The antitumor and anticancer effects of propolis were reported in many studies. However, these activities can be changed due to propolis chemical composition that depend on its region of collection (Mercan et al. 2006; Shehata et al. 2020). Recently, the anticancer activity of Algerian propolis has been studied in different cell lines such as: Colorectal Cancer Cell Line (Caco-2) (Daikh et al. 2020), Human Pancreatic PANC-1 cancer (Rouibah et al. 2018), Human Lung Adenocarcinoma cell lines (A549) (Brihoum et al. 2018) and Chemo-resistant Human Lung Adenocarcinoma (A549/DOX) (Kebsa et al. 2018) and Melanoma (Benguedouar et al. 2015). However, the potential anticancer effect and molecular mechanism of Algerian propolis on Human breast adenocarcinoma (MDA-MB-231) cells have not been investigated.

Building on this, we focused on detecting the antibiofilm and anticancer activities of different extracts of Algerian propolis. In this present study, *Staphylococcus aureus* ATCC 29213 and Human breast adenocarcinoma (MDA-MB-231) cells have been used. Also, the bacterial cells have been analysed to discriminate between propolis-injured and intact cells by FT-IR. Biofilm structure has been imaged by fluorescence microscope. Over and above, the anticancer and apoptosis effect of extracts have been confirmed at the level of gene expression.

Materials And Methods

Extraction procedure

Propolis was collected from the region of Ares localized in the wilaya of Mila (Algeria) during summer of year 2014. It was ground on small pieces and extracts were prepared by using four solvents of varying polarity. The used solvents are petroleum ether (PE), chloroform (Cl), ethyl acetate (EA), and methanol (ME). The extraction for each solvent was repeated three times. After extractions, samples were filtered and solvents were evaporated by using Rotary evaporator.

Total phenolic and flavonoids contents

Folin-Ciocalteu colorimetric assay was performed to determine the total phenolic content (TP) of extracts as described by Singleton et al. (1999) with slight modifications. Briefly, a volume of 125 µl (1mg/1ml) of propolis extracts, and 500µl of distilled water were mixed with 125 µl of Folin-Ciocalteu reagent. 1250 µl of saturated sodium carbonate Na_2CO_3 (20g/l) were added after 3 min. The obtained solution was incubated for 90 min, then the developed colour was measured at 760 nm. Gallic acid was used as a reference and results were given as µg GAE/mg of extract.

The total flavonoid content (TF) were also determined in this study by using the aluminium nitrate colorimetric assay (Devequi-Nunes et al. 2018). In short, 1 mg of propolis extracts was dissolved in 1ml of methanol to obtain the solution S1. 500 µl of S1 were mixed with 1900 µl (MeOH). Then 50 µl (S2) (CH3COOK) and 50 µl ($Al(NO_3)_2 9H_2O$) were added. The absorbance was measured at 415 nm after 40 min. Results were given as a Quercetin equivalent (µg QE / mg of extract).

Biofilm inhibition analysis

Algerian propolis was appraised for its inhibition potential of biofilm ability for *Staphylococcus aureus* ATCC 29213. The cells were grown overnight in TSB (Merck) and 0.5 McFarland standard turbidity was used for cell growth. The cell culture was inoculated with propolis extracts in a 96-well polystyrene plate. Wells were washed with PBS (0.01 M potassium phosphate buffer, pH 7.2) after 24 h incubation at 37˚C and dyed by crystal violet (Merck) (0.1% w/v) for 20 min. In order to remove the dye, plates were rinsed with tap water. The amount of biofilm material was measured in 200 µL of 20% glacial acetic acid (Merck) by Microplate Reader at 570 nm. Biofilm formation in the tubes containing only medium and bacteria was accepted 100%. Each experiment was implemented in triplicates and biofilm reduction was computed by the following biofilm formula: Biofilm reduction (%) = [(Control OD – Sample OD) / Control OD] × 100.

Fluorescence microscope analysis

Biofilm was formed on the surface of microscope coverslips that were added to the bacterial suspension containing propolis with maximum dose of antibiofilm effect and incubated for 24 hours at 37˚C. Then, each coverslip was then cleaned with sterile dH_2O and a mixture of SYBR Green and Propidium iodide (SYBR Green/PI; z ratio of 1: 3 in 100µL dH_2O) was added to surface of coverslip. After 30 min incubation at dark, the immersion oil was dropped on the coverslip and the samples were analysed by Fluorescence Microscope at 100x magnification in green and red light.

FTIR analysis

Fourier transform infrared spectroscopy (FTIR) analysis was performed to investigate the modifications on the cell surface after propolis treatment. *S. aureus* was grown for 24 hours in TSB medium containing the desired concentrations of propolis. The cell culture was pelleted by centrifugation (6000 g, 5 min). The pellet was washed twice with dH_2O and was then submitted to FTIR analysis. Infrared spectra were performed between 4000 and 400 cm-1 using a Perkin Elmer UATR Two (FT-IR) Spectrophotometer device.

Cell culture and viability assay

The effect of propolis on cell viability for Human breast adenocarcinoma (MDA-MB-231) cell line was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Cancer cells were cultured by using RPMI 1640 (Sigma) containing 10%heat-inactivated fetal bovine serum (FBS, Capricorn), 2mM glutamine and 1% penicillin/streptomycin solution (Gibco) at 37˚C with 5% CO_2. After tyrpsinized, cells were counted and 2500 cell were seeded into 96 well plate. Then, cells were treated with propolis extracts at various concentrations (250, 100, 50, 25, 10, 5, and 2.5 µg/mL). After incubation for 24 h, MTT (5 mg/ml in phosphate buffer saline) were added to fresh medium containing wells and incubated for 4 h. The well content was thoroughly mixed with 50 µl of dimethyl sulfoxide (DMSO,

Sigma) after incubation period. The amount of formazan dye was measured with a microplate reader (Epoch, BioTek, USA) at 590 nm. Cell viability (percent) was calculated by using formula:

Cell viability% = ($OD_{treatment}$/ $OD_{control}$) x 100.

Apoptosis analysis

Apoptosis analyses were performed using the Annexin V-EGFP Apoptosis Kit (BioVision, USA). Briefly, propolis treated cells were collected after 24 hours of incubation and washed with Phosphate buffered saline (PBS). 100 µL of binding buffer was used for resuspension of the cells. In addition, 5µl Annexin-V and 1µL (Propidium iodide) PI dye were added to cell suspension, mixed gently and kept on ice for 15 minutes. Then, treated cells were analysed in Arthur Cell Counter (NanoEnTek, USA). Apoptotic, necrotic and alive cell percent were determined over the total cell population. Apoptotic cell percentage was given as the mean ± SEM.

Gel electrophoresis and western blotting

For SDS-PAGE and Western Blot analysis, MDA-MB-231 cells (treated with propolis extracts and control cells) were collected, and proteins were prepared. BCA method was used to determine the total cellular protein concentration by using BSA standards. 75–150 µg protein were separated by 8.5–12% polyacrylamide gels by using the method of Laemmli (1970). Protein level changes due to propolis were determined by Western blot analysis. Briefly, separated proteins were transferred to nitrocellulose membrane by using Tris–glycine buffer containing methanol, at 90 V for 90 min. After transfer, 5% non-fat dry milk in TBST [20 mM Tris-HCl, pH 7.4, 400 mM NaCl and 0.1% (v/v) Tween 20] was used to block the membrane. After 60 min, Membrane was incubated with anti-human Caspase 3, Bcl-2, Bax, CDK-4 and P53 antibodies (diluted 1:200-1/1000 in blocking solution). After being washed with TBST for three times, the blot was further incubated with alkaline phosphatase conjugated secondary antibodies for 2 hours. After that, membrane washed with TBST to remove unbound antibodies. The method described by Ey and Ashman (1986) was used to determine Alkaline phosphatase activity. The final images were obtained by using imaging instrument (GelLITE gel documentation system, United Kingdom). GAPDH was used as a housekeeping protein. Band densities were quantified using image analysing software (Scion Image Version Beta 4.0.2).

RNA Isolation, cDNA synthesis and real time PCR

MDA-MB-231 cells ($1x10^7$ cells per 75-cm^2 flask) have been exposed to EC50 values of different propolis extracts. RNA was extracted from cells by using the RNA isolation kit (innuPREP RNA Mini Kit 2.0, Analytic Jena, Germany) after 24h. The RNA amounts were determined at 260/280 nm, and agarose gel electrophoresis was used to check the integrity of RNA. OneScript® Plus cDNA Synthesis Kit (ABM, USA) was used for converting the RNAs to cDNA. Briefly, 2.5 µg of RNA, w0.5 µM oligo(dT)18 reverse transcription primer, dNTP, RT buffer, RNaseOFF Ribonuclease Inhibitor (20 Units), OneScript® Plus RTase (200 Units) and Nuclease-free water was incubated at 50˚C for 50 min. Synthesis reaction was stopped

by incubation at 85˚C for 5 min. Quantitative real-time PCR assays for Caspase-3, Bax, Bcl-2, CDK-4 and P53 were performed using Applied Biosystems™ StepOnePlus™ Real-Time PCR System (Thermo, USA). Reactions were performed in 10 µl volumes using ABM KiloGreen 2X qPCR MasterMix (ABM, USA). As a negative control, PCR-grade containing sample and a sample in which Reverse transcriptase was omitted were used. 2 − ΔΔCt method was used for determination of fold changes in mRNA levels and GAPDH was used as a housekeeping gene.

Statistical analysis

Experiments used in this study were run in triplicates. Statistical analyses were performed by using The VassarStats online statistical software package. Data were provided as means, including standard error of means (SEM). To compare the data, student's t-test was used and $p < 0.05$ was chosen for statistical significance.

Results And Discussions

Total phenolic and flavonoid contents

Total phenolic (TP) and flavonoids (TF) contents of Algerian propolis extracts were determined using Folin-Ciocalteu and aluminium nitrate methods. TP and TF of various propolis extracts ranged from 5.36 ± 1.14 to 187.16 ± 4.83 GAE µg/mg and 0.38 ± 0.41 to 63.53 ± 1.28 QE µg/mg, respectively. Methanol extract was found to be the richest extract on both phenolic and flavonoid contents (Table 1).

Table 1
Total phenolic and flavonoid contents of Algerian propolis extracts

Extracts	Total phenolic EGA (µg/mg)	Total flavonoid EQ (µg/mg)
PE	5.36 ± 1.14	0.38 ± 0.41
Cl	79.16 ± 2.66	16.25 ± 0.21
EA	119.94 ± 4.83	60.34 ± 1.39
ME	187.16 ± 4.83	63.53 ± 1.28

The methanol extract exhibited a higher TP and TF values compared to propolis collected from Khanchla and Ghardaia (TPC: 14.23–4.93 GAE µg/mg and TFC: 3.45–1.94 QE µg/mg, respectively) (Rebiai et al. 2014). TP and TF amounts obtained in this study were similar to those reported for propolis collected from Fundão Region in Portugal (151.00 GAE µg/mg) (Moreira et al. 2008) and Korean propolis collected from Yeosu (212.7 GAE µg/mg) (Choi et al. 2006). But the reported values were lower compared to Chinese samples collected from Hebei (302 GAE µg/mg) (Ahn et al. 2007) and Hubei (299 GAE µg/mg) (Kumazawa et al. 2004). The chemical composition of different propolis has been widely investigated. It was found to be very complex and varies according to the geographical region, climate or plant type (Mercan et al. 2006; Yasar et al. 2016). This chemical diversity is very important for biological and

medicinal properties of propolis such as antibiofilm, antimicrobial, antioxidant, anticancer, antiallergic and antiviral (Daikh et al. 2020; Yasar et al. 2016; Yildirim et al. 2016; Mercan et al. 2006). The most abundant biologically active components of propolis are flavonoids and phenolic acids. Moreover, therapeutic activity of propolis depends largely on polyphenol contents with particular flavonoids (Campos et al. 2015; Boulechfar et al. 2019). Among the biological activities linked to phenolic and flavonoids composition, antibacterial, antibiofilm and anticancer activities are largely documented. In addition, some researchers stated that propolis with rich content in aromatic acids (etc. caffeic acid and ferulic acid) and flavonoids (etc. chrysin and galangin) exhibited a strong antibacterial, antibiofilm and/or anticancer effect (Patel, 2016; Ahangari et al. 2018).

Biofilm inhibition activity

The biofilm inhibitory effects of Algerian propolis was determined against *S. aureus* ATCC 29213 by using different concentration (10 to 800 µg/mL). Results of the antibiofilm screening assay are given in Fig. 1. All tested extracts showed antibiofilm effect on staphylococcal biofilm. In general, the biofilm inhibition efficiency of extracts increased with the increasing concentration of propolis indicating that the biofilm inhibition activity of propolis acts in a dose-dependent manner. Similar results were found in study where Polish propolis showed dose-depended antibiofilm activity (Wojtyczka et al. 2013).

Methanol and chloroform extracts exhibited the highest potency compared to petroleum ether and ethyl acetate extracts against bacterial biofilm production. The minimum and maximum inhibition percentages of methanol extract were found as 55% (50 µg/mL) and 92% (200 µg/mL), respectively. The highest activity was achieved at concentrations of 400 µg/mL (82%) and 500 µg/mL (83%) in chloroform extract. Compared to methanol, chloroform extract inhibited only 55% of the bacterial biofilm at a concentration of 200 µg/mL. Antibiofilm assay revealed that about 800 µg/mL of extract was required for the high inhibition of petroleum ether (67%). However, the ethyl acetate extract decreased the biofilm inhibition by ~ 64% and 59% at 400 and 600 µg/mL concentrations, respectively. Our results showed that methanol extract was the most effective extract, undouptly due to its flavonoid content known to have antibacterial properties. As indicated by Kothari (2014), methanol is the most suitable solvent for the extraction of flavonoids. Furthermore, the high antibiofilm effect of propolis collected from the mountains of Kafkas and Ural is due to their rich flavonoid content (Bryan et al. 2015a). It is worth noting that methanol extract also acts at very low concentrations compared to other tested extracts.

Our previous results about antibiofilm activity of five different Algerian propolis on eight bacterial strains were quite similar to the results of this study. Several factors were investigated for their potential influence on biofilm formation (Daikh et al. 2020). All of these results revealed that the used solvent during extraction in association with propolis origin and the tested bacterial strains significantly affect biofilm formation. Moreover, caffeic and ferulic acids were pointed to be responsible of the observed antibiofilm. We also reported that the high antibiofilm effect of the methanol extract of Algerian propolis was due to the higher content of ferulic and caffeic acid compared to other extracts. Caffeic acid was also reported to exhibit a biofilm activity by Luis et al. (2014). Bioactive natural compounds not only

increase the permeability of the bacterial membrane, but they also reduce metabolic activity, mobility and membrane transport (Veloz et al. 2019). Stan et al. (2016) found that propolis extracts had a strong inhibition on clinical *S. aureus* strains adherence to the cellular substrate. Several possible mechanisms were suggested to understand the antibacterial and biofilm inhibition efficacy of propolis. Propolis can affect cell membrane permeability leading to a reduction in the production of adenosine triphosphate and can compromise bacterial mobility and other activities (Almuhayawi, 2020). Both activities could be due to the synergic effects of phenolics, in particularly flavonoids such as Galangin, Pinobanksin and Pinocembrin, and other propolis constituents (Castaldo and Capasso, 2002; El-Guendouz et al. 2016).

Fluorescent microscopy analysis

The bacterial biofilm structure, untreated (control group) and treated with propolis, was examined by Fluorescence Microscope (Fig. 2). It is clearly seen that the biofilm structure of *S. aureus* is compact and dense ensemble, and each community is interconnected by water channels (Fig. 2A and 2B). As seen in Fig. 2C-2J, the decrease in the amount of bacterial biofilm treated with propolis was significant compared to control group. Contrary of control biofilm, biofilm community and their water channels degraded after treatment with propolis. Moreover, the decrease in number of live cells (bright) and the increase in number of dead cells (opaque) were also showed. Particularly, the effect of methanol extract on biofilm structure was more pronounced (Fig. 2I and 2J).

The biofilm is viscous and has high water content. The structure of the biofilm is quite complex and connected to each other by water channels which provide nutrient and material accessibility to the deepest regions of the biofilm (Archer et al. 2011). Vikram et al. (2010) reported that flavonoids not only inhibited biofilm production of *E. coli* and reduced the virulence of *Vibrio harveyi*, but also modulated cell-cell communication. They also notified that among the tested flavonoids, naringenin showed nonspecific inhibition in auto inducer-mediated cell-cell signalling (Vikram et al. 2010). As indicated by Bryan et al. (2015b), complete inactivation of *S. aureus* and *E. coli* biofilms after 18 h of Russian propolis treatment was observed that was confirmed by the confocal microscopy. Moreover, the treatment with propolis caused lysis of cells and the decomposition of the cell walls was predominately caused by organic compounds in Russian propolis according to microscopic images (Ambi et al. 2017). In similar another study, the microscopic studies confirmed the antibiofilm action of propolis (Grecka et al. 2020). According to our microscopy images, the deterioration of biofilm structure showed that the cell-cell communication was disrupt. Particularly during our microscope examination, we observed that the water channels between the cell assemblages were interrupted, and the density of these channels was also less compared to the control. Thus, we assumed that the absence of water channels after treatment with propolis was evidence of lethal effect of propolis on bacterial cells. Furthermore, it was more and more pleasing for us that the results of fluorescence microscopy and antibiofilm activity confirmed each other.

FTIR analysis

The FTIR spectrum of *S. aureus* represented in Fig. 3 reveals the characteristic features of protein with the presence of strong band at 3288.76 cm^{-1} related to NH of amide A (Sionkowska et al. 2004; Kochan et al. 2020). Band at 2918.68 cm^{-1} could be attributed to amide B. In addition, the presence of bands at 1638.26 cm^{-1}, 1550.05 cm^{-1} and 1240.81 cm^{-1} is characteristic of functional group of amide I, II and III respectively. According to Davis and Mauer (2010), picks located between 1500 – 1200 cm^{-1} are both characteristic bands of fatty acid bending vibrations and proteins. While picks located between 1200 – 900 cm^{-1} are attributed to carbohydrates in cell walls.

The analysis of FTIR spectrum of cells reveals the presence of a band at 1400 cm^{-1} characteristic of fatty acid and amino acids and in general attributed to C = O symmetric stretching of COO- of the cited components. In addition, the presence of a band at 720 cm^{-1} assigned to C-H of > CH_2 of fatty acids and proteins is also observed. Band observed at 1080.31 cm^{-1} is assigned to DNA, RNA and phospholipids.

After treatment with chloroform (Cl), ethyl acetate (EA) and methanol (ME) extracts of Algerian propolis, all the mentioned bands remained and had the same intensity indicating no change on *S. aureus* proteins constitution (Fig. 3C, 3D, 3E). On the contrary, the treatment with petroleum ether extract caused a decrease of the intensity of protein bands (Fig. 3B). Additionally, bands at (2918.68 cm^{-1}, 1550.05 cm^{-1} and 1240.81 cm^{-1}) disappeared, concomitantly with the reduction of the intensity of the band appearing at 1638.26 cm^{-1} and increasing the band at 3288.76 cm^{-1} suggesting a change of secondary structure of *S. aureus* proteins and probably their denaturation.

FTIR spectra of Cl (Fig. 3C), EA (Fig. 3D) and ME (Fig. 3E) extracts seem practically superimposable, which means that the three tested extracts present the same chemical profile. The three tested extracts present characteristic bands of aliphatic compounds which appeared as strong band at 3283.76-3284.69 cm^{-1} and 2900 cm^{-1}. Band at 2850 is cm^{-1} is attributed to hydroxyl groups. Moreover, aromatic compounds and flavonoids are also present. The presence of such aromatic components could be detected by the presence of bands between 1650 – 1600 cm^{-1} and 1550 – 1400 cm^{-1} (Oliveria et al. 2016; Anjos et al. 2015; Afonso et al. 2020). Peaks at 1170.5-1171.41 cm^{-1} and 1234.64-1242.40 cm^{-1} are assigned to stretching vibrations of C-O and C-O-C in alcohols, esters and sugars present in propolis (El-Guendouz et al. 2016).

On the contrary, PE extract spectra are qualified by the presence of fatty acids with the presence of characteristic bands of C-H asymmetric stretching bands appearing near 2930 and 2870 cm^{-1}. Two other bands appearing around 1640 cm^{-1} and 1480 cm^{-1} could be attributed to the presence of aliphatic acids (El-Guendouz et al. 2019).

Cytotoxic activity

The cytotoxic efficacy of different propolis extracts was determined in breast cancer cell line (MDA-MB-231) using MTT assay. To assess the cytotoxic effect, cells were treated with various concentrations of

propolis extracts for 24 hours. The extracts showed cytotoxic activity in a dose-dependent manner. EC50 values were found in a range from 97 to 117 µg/mL (Fig. 4).

Most active extract was found to be ethyl acetate extract, showing an EC50 value of 97 µg/mL. The cytotoxic effect of propolis extracts was in the following order: Ethyl acetate Chloroform Petroleum ether Methanol. Our results are in accordance with those reported in the literature. Xuan et al. (2014) showed that ethanolic extract of Chinese propolis had cytotoxic effects on MCF-7 and MDA-MB-231 with a dose and time-dependent manner. Same results were reported by Milošević-Đorđević et al. (2015) for two Serbians propolis samples. In addition, Turkish propolis was also showed cytotoxic effect on MDA-MB-231 cells and the highest antiproliferative activity was obtained at 10 mg/ml concentration for 24 and 72 h (Uçar and Değer, 2019).

Natural products such as propolis contain many bioactive substances with cytotoxic and anticancer effects on different cancer cells. Caffeic acid phenyl esters (CAPE) are the most common compounds in propolis and it was inhibited MDA-MB-231 cell proliferation (Chang et al. 2017). Moreover, its cytotoxic effect was proven against human myeloid cancer cells (Jin et al. 2008). Algerian propolis is rich in biologically active phytochemicals and has a good immunomodulatory effect (Soltani et al. 2017). Another study showed that Algerian propolis inhibited lung cancer cells proliferation and induced apoptosis (Kebsa et al. 2018). Furthermore, the inhibitory effect of Algerian propolis on lung cancer cell proliferation was confirmed with *in vitro* tests (Brihoum et al. 2018). Algerian propolis was also reported to reduce melanoma tumor progression/dissemination and to extend mice life span. Galangin was pointed to significantly reduce melanoma cell proliferation and to induce autography/apoptosis dependently (Benguedouar et al. 2015).

Western blot analysis

Western blot analysis was performed to determine the effect of propolis on the level of proteins involved in apoptosis and cell cycle. As represented in Fig. 5, the densitometry analysis of the Caspase 3 protein level indicated that the expression was increased significantly 1.42-, 1.35-, 1.35- and 1.53-folds in PE, Cl, EA, ME extracts with respect to control, respectively ($p < 0.05$). Although PE and Cl extracts decreased the level of Bax protein (2- and 1.69-folds, respectively), EA and ME extracts increased Bax level (2.19- and 2.15-folds, respectively). The results of densitometry analysis of the Bcl-2 shown in Fig. 5 indicated that Bcl-2 expression was decreased 1.56-, 1.44-, 2.38- and 2.43-folds in PE, Cl, EA, ME extracts treated MDA-MB-231 cells ($p < 0.05$). Similarly, to Bcl-2, CDK-4 protein level was decreased significantly with propolis extracts treatment (2.86-, 1.13-, 2.85- and 3.44-folds, respectively, $p < 0.05$). While, P53 protein level was increased significantly in PE, Cl, EA, ME extracts treated cells (1.58-, 1.57-, 3.17- and 2.1-folds, respectively).

Results demonstrated that Algerian propolis caused a cell growth inhibition on the tested cells due to induction of apoptosis and cell cycle arrest. Intrinsic apoptotic pathway was induced by propolis treatment due to induction of caspase-3 and 9 activities (Kebsa et al. 2018). Algerian propolis ethanol

extract was also reduced the proliferation of LNCaP cells by arresting cell cycle at G0/G1 and inducing apoptosis (Zabaiou et al. 2019).

Real time PCR

The effects of propolis treatment on gene expression analysis of Caspase3, Bax, Bcl-2, CDK-4 and P53 were revealed in MDA-MB-231 cells. Real Time-PCR results of mRNA levels were represented in Fig. 6. Caspase3 mRNA level was increased 1.49-, 1.62-, 10.41-, 28.44-folds in PE, Cl, EA and ME extracts treated cells with respect to control, respectively ($p < 0.05$). Although, PE and Cl extracts decreased the level of Bax mRNA (8.6- and 6.98-folds, respectively). While PE and ME extracts increased Bax mRNA level (1.34- and 3.91-folds, respectively). On the other hand, Bcl-2 mRNA levels were decreased 2.08-, 1.36-, 1.81- and 3.63-folds due to extract treatment. P53 mRNA level was increased 2.27-, 3.16-, 11.79- and 24.16-folds because of PE, Cl, EA, ME extracts treatment (Fig. 6, $p < 0.05$). Similarly, to protein level, CDK-4 mRNA level was decreased significantly (5.02-, 1.38-, 2.79- and 1.34-folds, respectively).

Our results are in contradiction with those reported in the literature. Cuban propolis caused to decrease the expression of apoptosis-related genes such as Caspase3, P53, p21 and Bax in MDA MB-231 cells. Whereas, Bcl-2, Bcl-XL, Noxa and Puma expression was not changed (Frión-Herrera et al. 2019). Brazilian propolis caused reduction of mitochondrial membrane potential in A549 cells by overexpression of Bax and Noxa and suppression of the Bcl-XL. While, the expression level p53, Caspase3, bcl-2 genes remained unchanged, p21 expression was increased (Frión-Herrera et al. 2015). Brazilian propolis and caffeic acid, one of the main phenolic acids present in propolis, were also tested on Hep-2 cells. Both products induce apoptosis. Only propolis downregulates P53 expression. Caspase-3 expression was increased due to propolis treatment and that was correlated with induction of both early and late apoptosis due to propolis and caffeic acid treatment (da Silva et al. 2017).

Apoptosis analysis of Mila extracts

In this study, Annexin-V was used to analyse apoptosis. For quantification Arthur image-based cytometer was used. H_2O_2, was used as a positive control. As shown in Fig. 7, after 24 hours of application with extracts, apoptotic cell proportions were increased significantly. The ratio of apoptotic cells in propolis treatment was 6 to 10-folds higher than the control ones. These results confirmed that propolis extracts caused apoptosis and had a cytotoxic effect. Also, in all treated cells, there was a statistically significant decrease in the percentage of live cells compared to the control ones. Our results are in accordance with previous research on Chinese propolis. Xuan and co-workers studied the effects of Chinese propolis on Annexin A7 (ANXA7), P53, Reactive oxygen species levels, NF-κB p65 and mitochondrial membrane potential in MCF-7 and MDA-MB-231 (Xuan et al. 2014). They found that propolis caused significant induction of ANXA7 expression, NF-κB p65 and ROS level. Chinese propolis increased the tumor suppressor proteins (p21CIP1 and p53) in HCT116 cell line and induced apoptosis (Ishihara et al. 2009). Similarly, Turkish propolis increased in protein level associated with P53, Bax and P21 (CDKN1A) genes in MCF-7 cells and its activity was also confirmed by western blot (Misir et al. 2020).

Conclusion

We aimed in the present study to investigate chemical contents, antibiofilm and anticancer effects of Algerian propolis on MDA-MB-231 cell line. Based on our data, Algerian propolis exhibited an antibiofilm activity in a dose-dependent manner. In addition to that, a significant correlation between fluorescence microscopy and antibiofilm effect was detected. FTIR analysis also revealed that Algerian propolis affected functional groups on cell membrane of *S. aureus*. Furthermore, Algerian propolis was found to possess an interesting cytotoxic activity on MDA-MB-231 cell line. The tested propolis seems to induce cell death via apoptosis and caused a significant increase in mRNA levels of proteins involved in tumor suppression and apoptosis. Phenolic acids and flavonoids were identified as major types of components of the tested propolis extracts. Last of all, the results obtained from present study further verify our understanding of the antibiofilm and anticancer effects of propolis. Thus, from the human health point of view both antibiofilm and anticancer properties of propolis are worthy of further investigation.

Declarations

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Author contributions

NMD, SA and NS designed the experiment and wrote manuscript. FN determined the antibiofilm properties, anticancer activity, microscopy analysis and FTIR analysis. AD analysed chemical contents of propolis. NS and AD evaluated FTIR data. DM checked anticancer activity.

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Conflict of interest

The authors have no conflicts of interest to declare and all co-authors have seen and agreed with the contents of the manuscript. This manuscript has not been submitted to, nor is under review at another journal or other publishing venue. The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript. All authors have no conflict of interest with the editors and reviewers of 3Biotech journal. The subject of our research is microorganisms, which does not involve any human or animal subjects, and does not violate any ethical standards.

Data availability

The authors declare that data supporting the findings of this study are available within the article.

Ethical statement

The subject of our research are microorganisms and cancer cell line, which do not involve any human or animal subjects, and does not violate any ethical standards.

Informed consent

Not applicable.

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Figures

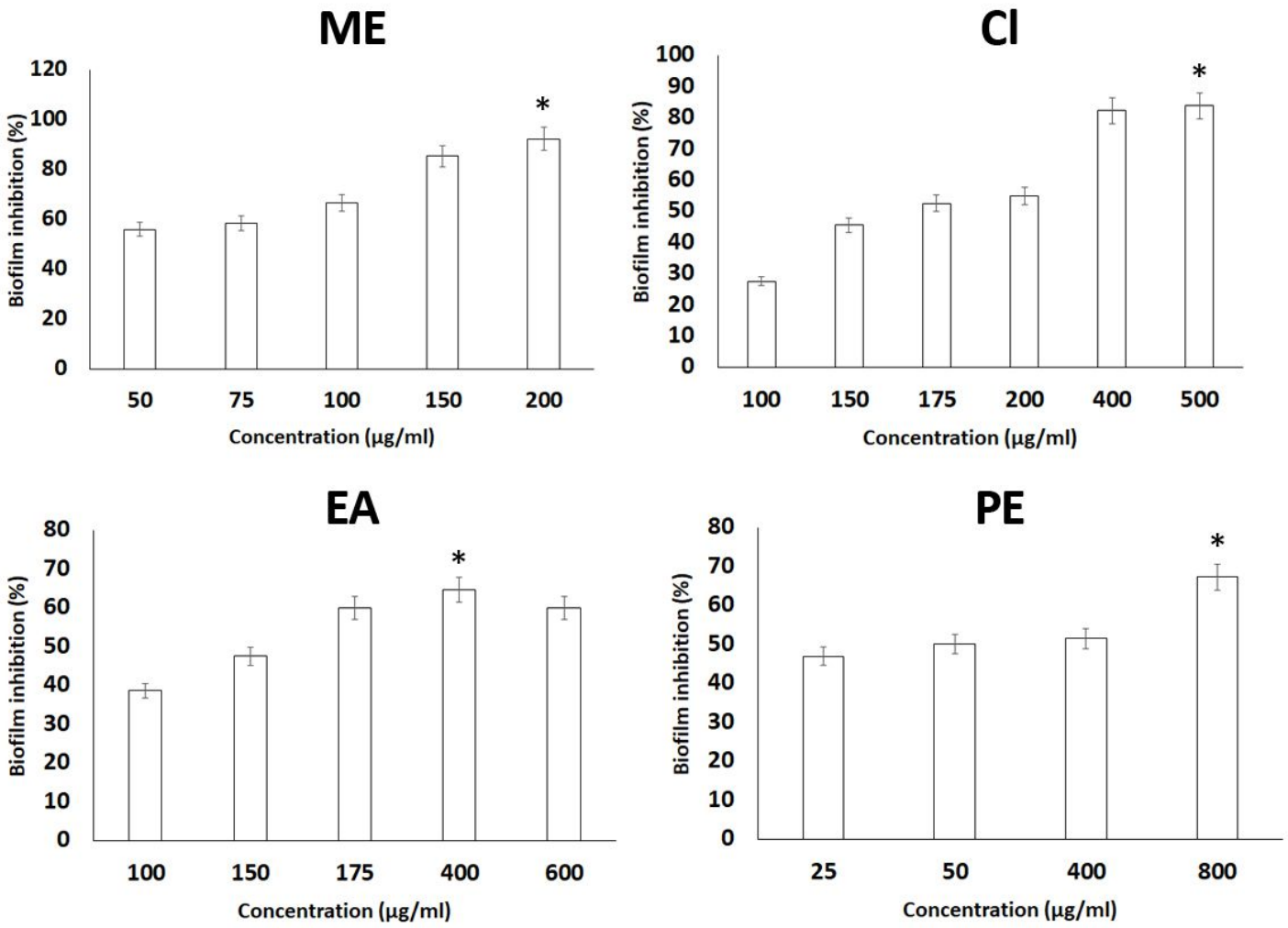

Figure 1

The biofilm inhibitory results of methanol (ME), chloroform (Cl), ethyl acetate (EA) and petroleum ether (PE) extracts (*: $p<0.05$).

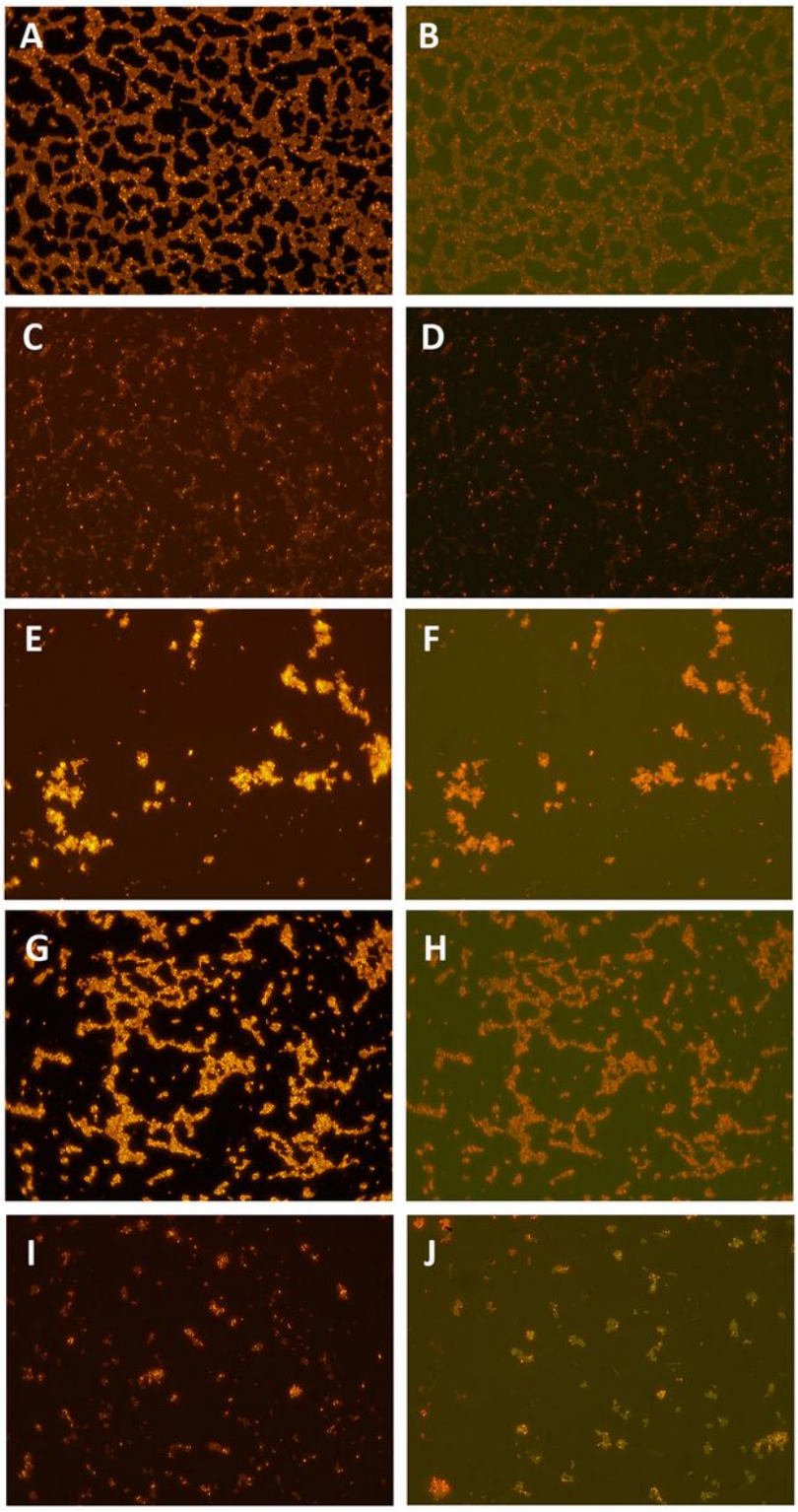

Figure 2

Biofilm images of *Staphylococcus aureus* ATCC 29213 (A, B, control). *S. aureus* was grown at the concentration of 800 µg/ml petroleum ether (C, D), 500 µg/ml chloroform (E, F), 400 µg/ml ethyl acetate (G, H) and 200 µg/ml methanol (I, J). Microscope photos were imaged at 100X objective.

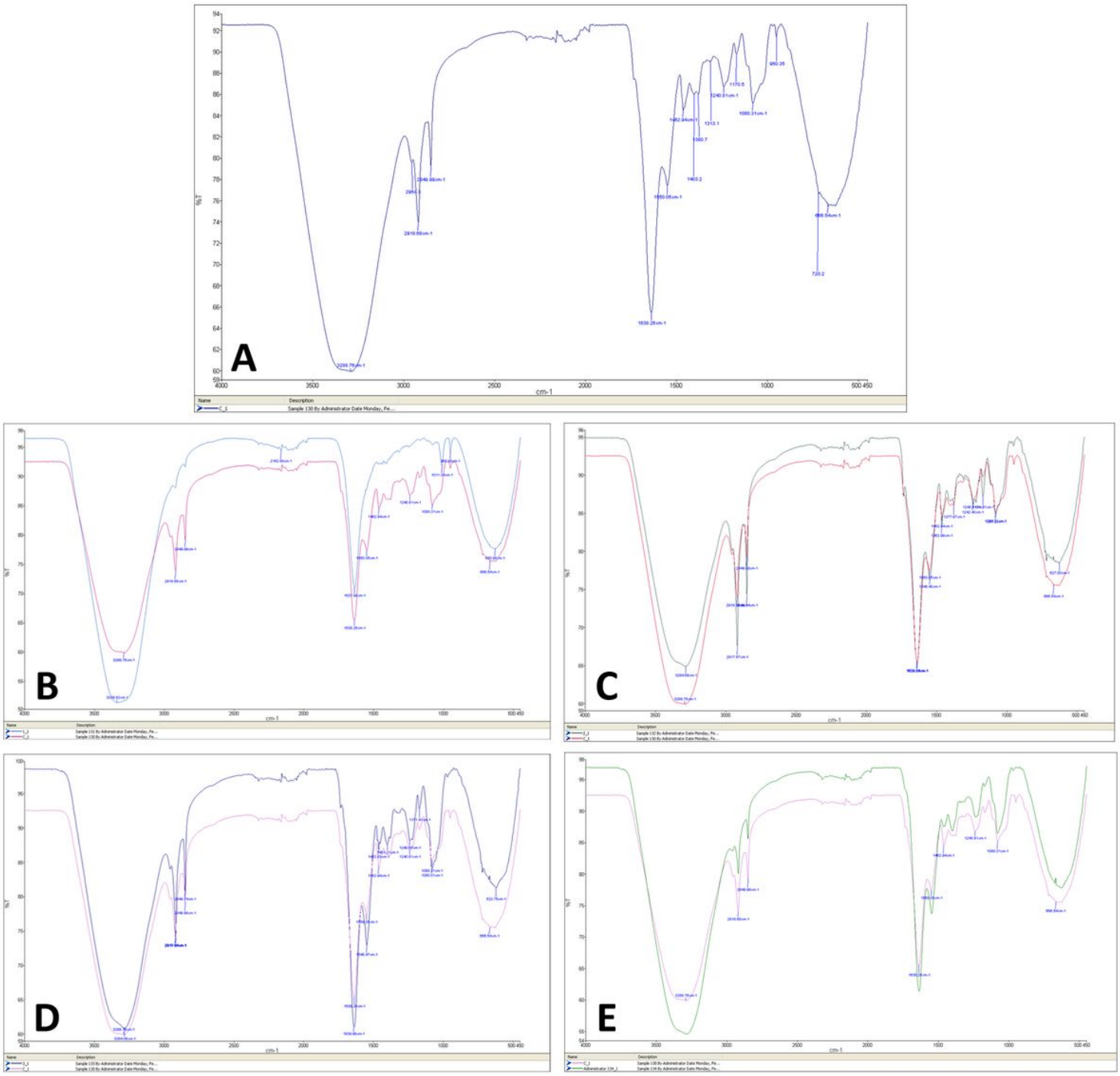

Figure 3

FTIR spectrum of *Staphylococcus aureus*. Picture A showed control group, untreated with propolis. Pictures B (Petroleum ether; PE), C (Cl, chloroform), D (EA, ethyl acetate) and E (methanol, ME) have represented for FTIR spectra after treatment propolis.

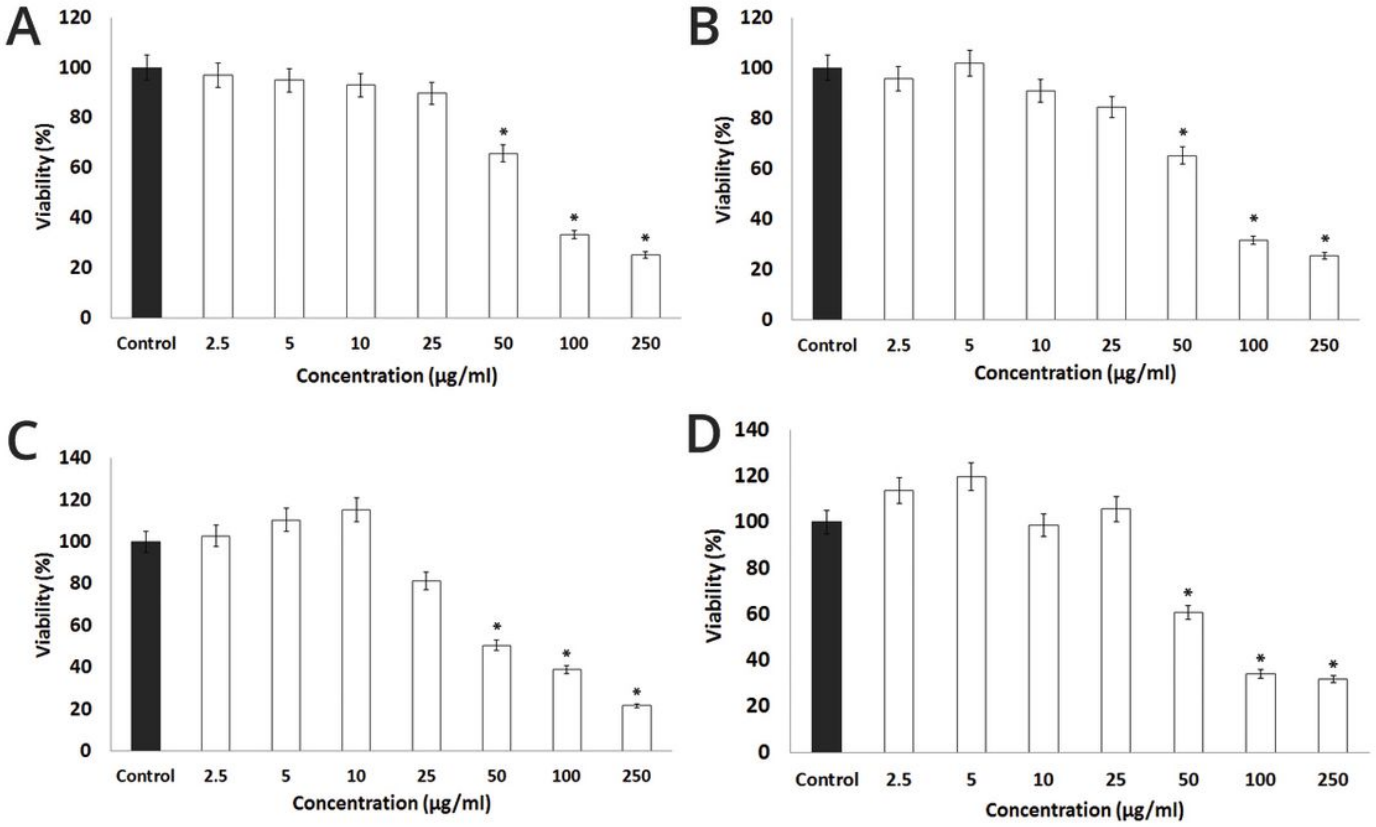

Figure 4

Assessments of cytotoxicity of propolis extracts on MDA-MB-231 cells by MTT assay. EC50 values was determined after 24 h treatment. (A) Petroleum ether (B) Chloroform (C) Ethyl acetate and (D) Methanol.

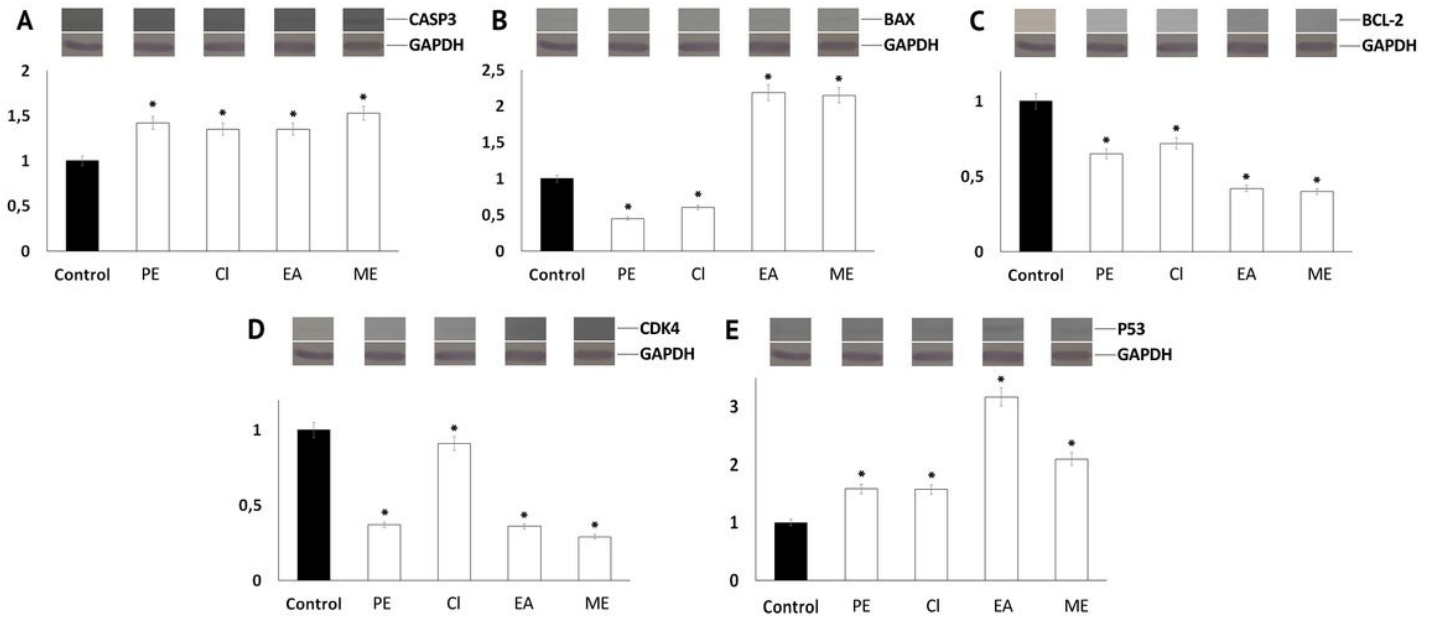

Figure 5

Effects of propolis extracts on Caspase3, Bax, Bcl-2, CDK-4 and P53 expression confirmed by Western blot analysis. Protein bands obtained from propolis treatment were expressed a fold-change of control ones. * p<0.05.

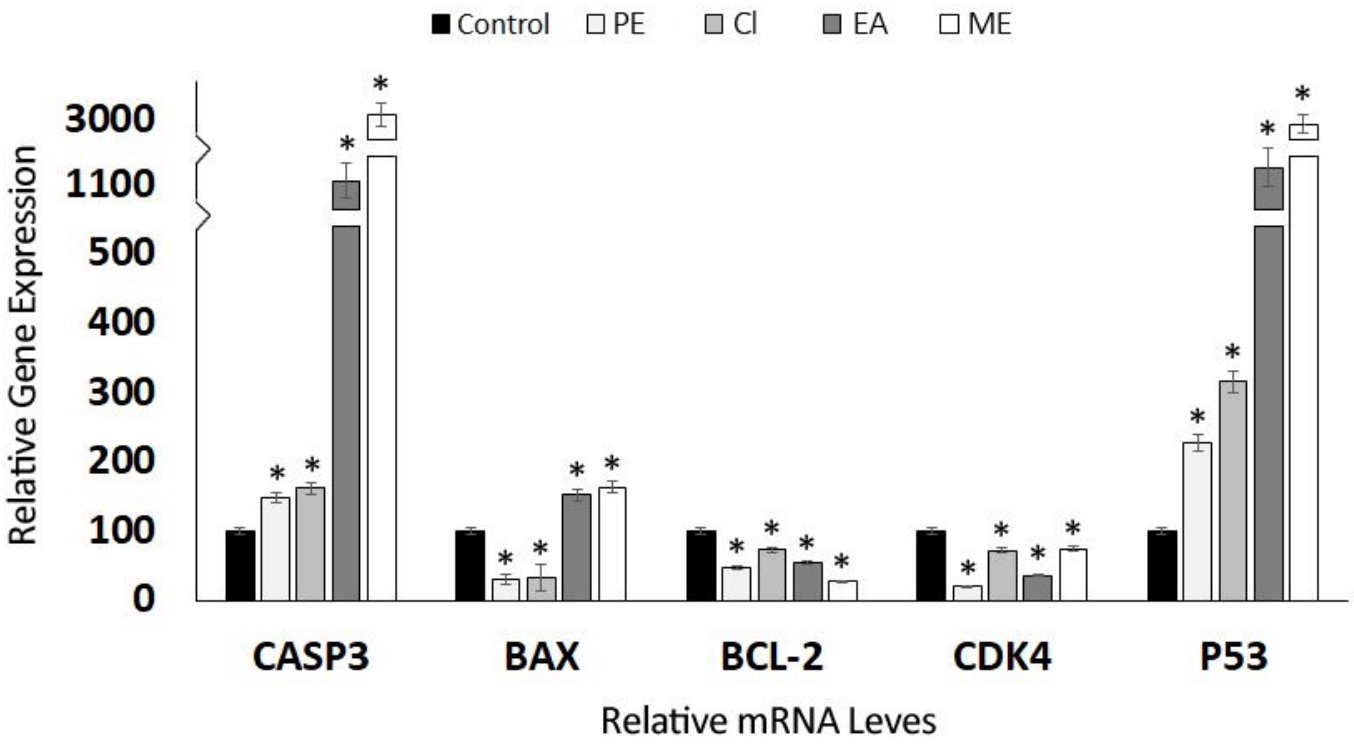

Figure 6

Caspase3, Bax, Bcl-2, CDK-4 and P53 genes expression of treated and control cells. GAPDH was used for normalization of mRNA levels. Values obtained from the treated cells were expressed as a percentage of control. *p<0.05.

Figure 7

Apoptosis analysis of propolis extracts by Arthur Cell Counter. After 24 h incubation of propolis extracts, cancer cells were analysed for apoptosis. H2O2 was used as a positive control. *p < 0.05.