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Anticancer Activity of Propolis Extract-ZnO Nanocomposite Against Human Lung Carcinoma Cell Line

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Abstract

This study aimed to evaluate the synergistic effect of propolis extract-zinc oxide (EEP-ZnO) nanocomposite against human lung carcinoma cell line. EEP-ZnO nanocomposite was formulated by the combination of Zinc Oxide nanoparticles (ZnO NPs) with ethanolic propolis extract (EEP) that was characterized by X-ray diffraction (XRD), Transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FT-IR). The MTT assay and Annexin V-PI apoptosis assay were used for cytotoxicity evaluation of prepared EEP-ZnO nanocomposite against human lung carcinoma cell line (A549) and human lung normal cell line (WI-38). Results showed that the propolis extract- zinc oxide nanocomposite significantly decreased cell viability and increased cell apoptosis, as compared to EEP and ZnO NPs alone. It was also observed that EEP, ZnO NPs, and EEP-ZnO nanocomposite are exhibited a less toxic effect on WI-38 comparable to A549.

Keywords: Propolis extract; Zinc oxide nanoparticles; MTT assay; Apoptosis

1. Introduction

According to relevant estimates, there are more than 100 different and unique forms of cancer, each of which occur due to the uncontrolled growth of abnormal cells, inactivation of apoptotic mechanisms and the acquisition of metastatic properties [1]. The leading cause of mortality globally during the past ten years has been lung cancer [2]. Both conventional treatment modalities (surgery, chemotherapy, and radiation therapy) and complementary and alternative therapeutic approaches are used to treat cancer. However, current forms of cancer treatments cause side effects and mainly affect the healthy and normal tissues or organs [3]. Various studies have demonstrated the effectiveness of several natural remedies for treating cancer, including the usage of propolis, as their therapeutic potential to treat several diseases is well shown [2]. Honeybees produce propolis, often called bee glue, from leaf buds and fissures in the bark of many tree species. Propolis is a sticky substance with a black colour. Once collected, this material is enriched with bee salivary and enzymatic secretions [4]. Propolis has a complicated chemical structure that is influenced by the flora that bees visit as well as the type of bees [5], and could change with the seasons. Numerous factors, including the floristic composition of the area and the site of the collection, have an influence on the chemical composition of propolis [6]. Propolis is a complex substance containing over 300 different chemicals, as it is usually composed of 50% resin, 30% wax, 10% essential oils, 5% pollen, and 5% other substances which include minerals and organic compounds [7].

Propolis has been used frequently in a variety of fields, most notably in traditional medicine to heal wounds, burns, and gastrointestinal diseases [8]. Propolis has a wide range of pharmacological properties, including anti-inflammatory, anti-cancer, anti-fungal, and wound healing. It also possesses a wide range of antimicrobial properties [9].

Nanotechnology is concerned with the production of materials with nanoscale dimensions in the range of 1 to 100 nanometers and their use in various disciplines in our environment. As a result of their large surface area to volume ratio, nanomaterials exhibit several superior chemical, thermal, optical, electronic, magnetic, and biological characteristics as compared to their bulk materials. Nanoparticles (NPs) are now attracting the attention of biological

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researchers due to their numerous therapeutic uses and their potential to be employed as a targeted gene and drug delivery systems in biomedicine [10]. Inorganic metal oxides are among the many types of nanoparticles that are utilized often because of their tiny size and advantageous surface chemistry. It has been reported that zinc oxide nanoparticles (ZnO NPs) exhibit higher selectivity, retention, and controlled drug release properties [11],[12].

ZnO NPs, has excellent ultraviolet (UV)-absorbing properties and transparency for visible light, rendering it an excellent sunscreen agent [13],[14]. Other properties, such as their antibacterial and anticancer activities, have also been explored, due to their ability to induce the production of ROS [15]. ZnO is comparatively inexpensive, biocompatible, and relatively less toxic compared with other metal oxide NPs, all of which increase the potential for its application [16]. All these properties make ZnO NP a suitable Nano carrier in cancer treatment [17].

Based on the previous studies, zinc oxide nanoparticles and propolis extract have positively effect on cancer treatment. Thus far, main aim of present study was evaluating the potential therapeutic effect of a novel nanocomposite of propolis extract with zinc oxide nanoparticles on human lung adenocarcinoma cell line (A549) and the human lung fibroblast normal cell line (WI-38).

2. Materials and methods

2.1. Chemicals

All materials were of analytical grade chemicals and were used without any further purification. Ethanolic Propolis Extract (EEP) was purchased from Plant Protection Research Institute, Agriculture Research Centre at Dokki, Giza, Egypt.

Zinc oxide nanoparticles (ZnO NPs) were purchased from Nano Gate Company, Egypt.

WI – 38 cells (Human lung fibroblast normal cells) and A549 cells (Human lung carcinoma cells) were imported from ATCC (the American Type Culture Collection (ATCC, Rockville, MD) via The Regional Center for Mycology & Biotechnology, Al-Azhar University. RPMI-1640 medium, glutamine and penicillin-streptomycin, 0.25% Trypsin-EDTA, Phosphate buffer saline (PBS) and fetal bovine serum (FBS) were purchased from Lonza (Belgium).

3-4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT), dimethyle sulphoxide (DMSO), trypan blue dye, absolute ethanol (99.9%) and FITC Annexin V, Propidium Iodide (PI) were purchased from Sigma (St. Louis, Mo., USA).

2.2. Preparation of ethanolic propolis extract (EEP)

Propolis samples were extracted by maceration at room temperature with occasional shaking in a proportion of 10 g of propolis to 100 ml of solvent (ethanol 80 % v/v), extracts were obtained after 7 days of maceration, and the ethanolic extracts were then filtered through Whatman (No.1) filter paper and incubated at room temperature until the ethanol evaporated and the product obtained a honey-like consistency are referred to as ethanolic extract propolis. This method was reported by Cunha *et al* [18].

2.3. Preparation of Propolis extract - Zinc oxide nanocomposite (EEP-ZnO nanocomposite)

Preparation of Propolis extract – Zinc oxide nanocomposite was prepared using the method of Soumya *et al*.[19], with some modifications. 2.5 gm of propolis extract was dissolved in 50ml of ethanol (90%) and mixed with 2.5 gm of ZnO NPs that were added slowly under constant sonication for 30 min. After the completion of sonication, the resulting suspension was centrifuged for 10 minutes at 10000 rpm and washed three times with ethanol/water solution.

The pellet was dried overnight at 60°C to obtain propolis extract –zinc oxide nanocomposite (EEP-ZnO nanocomposite).

2.4. Characterization techniques

2.4.1. X-ray diffraction (XRD)

The sample's crystal structure was examined using the X-ray diffraction technique (XRD; Shimadzu XRD-6000). At room temperature, XRD patterns were observed in the range of 2θ from 4◦ to 90◦. Cu Kα was used as a radiation source of wavelength λ = 0.15408 nm, scan rate 8◦/min, operation voltage 40 kV, and current 30 mA.

2.4.2. High Resolution Transmission Electron Microscopy (HR- TEM)

The morphology and particle size of ZnO NPs and EEP- ZnO nanocomposite were characterized by High Resolution- Transmission Electron Microscopy (JEOL, JEM-2100, Tokyo, Japan) with an operating voltage of 200 kV. The sample was dispersed in

ethanol before examination and deposited on carbon coated copper grid.

2.4.3. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was done using Vertex70 RAM II, Germany with a resolution of 4 cm^{-1}. Sample was compressed in potassium bromide (KBr) disks; and measurements were taken in a transmittance mode at a wavenumber range of 4000–400cm^{-1}.

2.5. Evaluation of the cytotoxicity of propolis extract (EEP), Zinc Oxide Nanoparticles (ZnO NPs) and EEP-ZnO nanocomposite against A549 and WI-38 cell lines using the MTT assay

The MTT assay is a colorimetric technique for measuring cell viability. To determine cell viability, 5 x 10^4 of WI-38 and A549 cell lines were plated separately in 96-well tissue culture plates and incubated at 37°C for 24 hours. Cells were treated with different concentrations (3.9-500 µg/ml) of EEP, ZnO NPs, and EEP-ZnO nanocomposite for 48 h at 37 °C and 5% CO_2 in a humidified atmosphere. Cells incubated alone in medium served as a negative control for cell viability (untreated cells). After treatment, the number of viable cells was determined by MTT assay. Briefly, remove the medium from the 96-well plate and replace with 100 µl of fresh RPMI 1640 medium.

Then add 10 µl of 12 mM MTT stock solution (5 mg MTT in 1 ml PBS) to each well including the untreated cells. The 96-well plate was then incubated for 4 h at 37°C and 5% CO2. Remove 85 µl aliquots of medium from the wells and add 50 µl DMSO to each well, mix well with pipette, and incubate at 37°C for 10 min[20]. The number of viable cells was determined by measuring optical density at 590 nm using a microplate reader (SunRise, TECAN, Inc., USA), and the percentage of viability was calculated as

[($OD\ of\ treated\ cells/OD\ of\ untreated\ cells$)] x 100 %

The 50% inhibitory concentration (IC_{50}), which is the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each concentration using Graphpad Prism 5 software. Three independent experiments were conducted.

2.6. Cell apoptosis assay

To determine the cell death mechanism of A549 cell line treated with IC_{50} of EEP, ZnO NPs and EEP-ZnO nanocomposite for 48h by flow cytometry. Annexin V-FITC-PI Detection Kit was used to measure apoptosis. Briefly, cells were harvested and washed twice with PBS, suspended in 100 µl 10x binding buffer, stained with 5 µl Annexin V-FITC and 5 µl PI, then incubated in the dark for 15 min before adding 400 µl 10x binding buffer, then incubate for 20 minutes in the dark then analyzed by (BD Accuri C6 Plus) Flow Cytometer, counting 50,000 events per sample. The results were divided into four quadrants: normal cells [An(-)/PI(-), at the lower left quadrant], early apoptotic cells [An(+)/PI(-), at the lower right], late apoptosis and [An(+)/PI(-), at the upper right] and necrotic cells [An(-)/PI(+), at the upper left] [21].

2.7. Statistical analysis

Experiments were performed in triplicate (n=3) and expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance ANOVA followed by Tukey's test processed by (GraphPad Software, version 5.0, GraphPad Software Inc., Sandiego, CA). P values below 0.05 were considered significant.

3. Results and Discussion

3.1. X-ray diffraction (XRD)

The XRD patterns of Propolis extract (EEP), ZnO NPs and propolis extract-zinc oxide (EEP-ZnO) nanocomposite shown in **Figure 1**. The propolis extract (EEP) has a wide peak around 23.16°, which implies the amorphous nature of EEP and the diffraction peaks of ZnO NPs were more intensive and narrower, implying that the nanostructures possess the good crystalline nature. these peaks at scattering angles (2θ) of 31.98°, 34.66°, 36.48°, 47.78°, 56.78°, 63.08°, 66.6°, 68.14°, 69.3°, 72.96°, 77.08° corresponding to (100), (002), (101), (102), (110), (103), (200), (112), (201) and (202) crystal planes, respectively . The 2θ values coincide with those of the standard hexagonal wurtzite structure ZnO reported in the literature [22]. In addition, characteristic peak of propolis extract disappeared from the nanocomposite due to the amorphous phase of propolis extract and most peaks in the ZnO NPs make their appearance in the nanocomposite, but with higher intensity (fig.1), confirming the successful coating of ZnO NPs with propolis extract without any changes in crystal structure of the ZnO NPs [23].

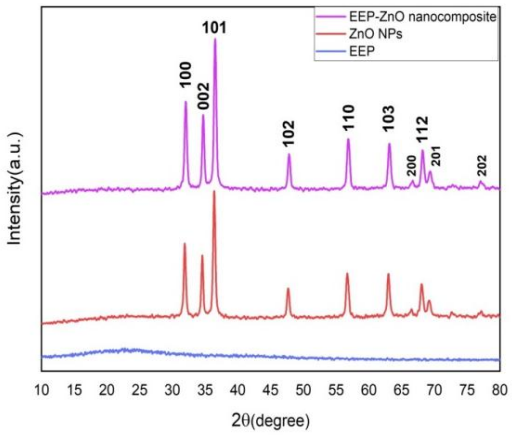
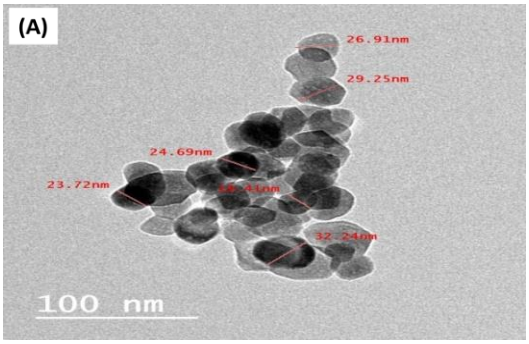


Fig. 1: XRD patterns of EEP, ZnO NPs and EEP-ZnO nanocomposite.

3.2. High Resolution Transmission Electron Microscopy (HR- TEM)

The complementary morphological description is achieved through the (HR- TEM) analysis. **Fig. 2(A)** shows (HR- TEM) image of ZnO NPs with an average size of 23.55±6.06 nm. It is evident from the figure that the particles are spherical in shape and uniformly distributed.

Fig. 2(B) shows (HR- TEM) image of EEP-ZnO nanocomposite, it is observed that there were some shadows around the ZnO-NPs, implying the existence of propolis extract, and accordingly propolis encapsulated ZnO NPs. The average diameter of ZnO-NPs in the nanocomposite is 22.92±4.67 nm. The average nanoparticle size for propolis extract encapsulated ZnO NPs does not show a significant differences in size compared to the uncoated ZnO NPs, due to the capping action of the active organic compounds such as (flavonoids and phenolic compounds) in the propolis extract that attached to the surface of zinc oxide nanoparticles which prevented agglomeration of the particles[24].



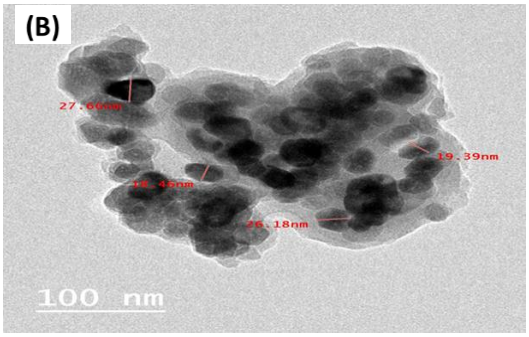


Fig. 2: HR-TEM of (A) ZnO NPs and (B) EEP-ZnO nanocomposite.

3.3. Fourier Transform Infrared Spectroscopy (FTIR)

The FT-IR analysis was performed to study the chemical structure of ZnO NPs, ethanolic propolis extract (EEP), and EEP-ZnO nanocomposite. As seen in **Fig.3**, the FT-IR spectrum of ZnO NPs showed band at 2977.61 cm^{-1} was attributed to the stretching vibration of (C–H) group [25].The band was observed at 1389.55 cm^{-1} due to symmetric stretching of carboxylate which became attached to the ZnO nanoparticles during synthesis [26].

The absorption peaks at 549.25 cm^{-1} and 494.02 cm^{-1} corresponds to the stretching vibration of (Zn-O) bond [27].

The spectrum of the propolis extract (EEP) **(Figure 3)** revealed band at 3284.32 cm^{-1} was due to the stretching vibrations of (O–H) group of phenol compounds [28].The bands at 2928.35 cm^{-1} and 2861.19 cm^{-1} were due to symmetrical stretches of CH_3 and asymmetrical stretches of CH_2, respectively, suggesting the presence of alkyl compounds in the propolis extract [29]. Whereas bands at region 1700–1400 cm^{-1} are indicating stretching vibration of carbonyl groups, ketone groups (C=O) and (C=C) related with lipids, flavonoids, phenolic compounds and aromatic ring deformations found in ethanolic propolis extract [30],[31].The bands at 1376.86, 1254.47, and 1168.65 cm^{-1} were attributed to C-O stretching and C-OH group bending of esters, alcohols, and carboxylic acids, mainly related to phenolic acids and flavonoids in propolis extracts. Band at 1027.61 cm^{-1} due to aromatic ether C-O-C bond stretching and band at 812.68 due to aromatic ring vibrations [32].

The EEP-ZnO nanocomposite spectrum(**Figure 3)** showed the Zn–O stretching vibrations at 549.25cm^{-1} and 494.02cm^{-1} shifted to 617.16 cm^{-1} and 543.28 cm^{-}

[1]. This change in the peak position of ZnO absorption bands attributed to Zn–O–Zn network is perturbed after the addition of propolis. Furthermore, the stretching vibration of (O–H) group at 3284.32 cm^{-1} is relatively weaker in EEP-ZnO nanocomposite than in propolis extract. Also, EEP-ZnO nanocomposite has strong intensity bands at 2977.61 and 1389.55cm^{-1} correspond to ν(C–H) and symmetric stretching of carboxylate, respectively in ZnO NPs due to the effect of the bands ν(CH_3) and ν(C-O) appearing at 2928.35 and 1376.86 cm^{-1} in the propolis extract and shifting of the propolis extract bands at 2861.19 ν(CH_2) to 2891.79 cm^{-1}, 1603.73 cm^{-1} ν(C=C) to 1585.82 cm^{-1}, 1444.77 ν(C-H) to 1487.31cm^{-1}, 1168.65 ν(C-O) to 1150 cm^{-1} and 1027.61 ν(C-O-C) to 1076.86 cm^{-1} in the nanocomposite. The bands at 1254.47cm^{-1} and 812.68 cm^{-1} of propolis extract was observed in EEP-ZnO nanocomposite. Therefore, FTIR results suggest that the existence of propolis extract and ZnO NPs in the nanocomposite.

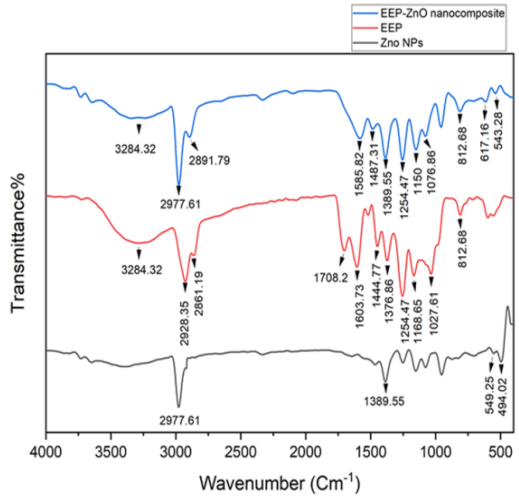


Fig. 3: FT-IR spectrum of ZnO NPs, propolis extract (EEP) and EEP-ZnO nanocomposite.

3.4. The cytotoxicity effect of EEP, ZnO NPs and EEP-ZnO nanocomposite on human lung carcinoma cells (A549) and human diploid lung fibroblast normal cell lines (WI-38)

The viability percentages (viability%) of the human epithelial lung carcinoma cell line (A549) and the human diploid lung fibroblast normal cell lines (WI-38) treated with different concentrations of EEP, ZnO NPs, and EEP-ZnO nanocomposite for 48hrs were decreased, but their toxicity percentages (toxicity%) were increased in a dose-dependent manner with increasing the compound concentrations, as demonstrated in **Figure 4**. The median growth inhibitory concentration (IC_{50}) values of EEP, ZnO NPs, and EEP-ZnO nanocomposite were 107.95±0.8 , 26.78±0.44, and 15.32±0.44 µg/mL for A549 cells, while IC_{50} values of EEP, ZnO NPs, and EEP-ZnO nanocomposite were 164.7±0.79, 81.91±0.96 and 44.2±0.45µg/ mL for WI-38 cells, respectively. EEP-ZnO nanocomposite improved the toxicity% on A549 cancer cells as compared to that obtained by EEP and ZnO NPs alone.

EEP, ZnO NPs, and EEP-ZnO nanocomposite are less toxic on normal lung cells (WI-38) comparable to lung cancer cells (A549).

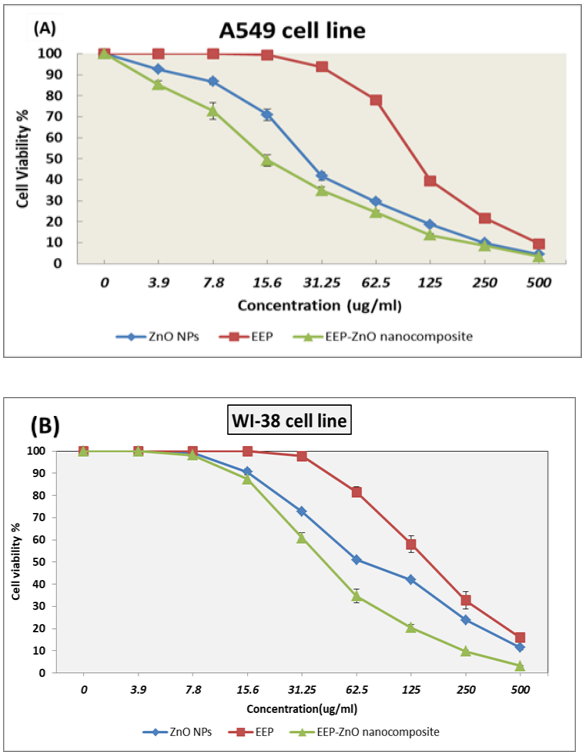


Fig. 4: The cytotoxicity effect of different concentrations of Propolis extract (EEP), Zinc Oxide Nanoparticles (ZnO NPs) and EEP-ZnO nanocomposite on A549 cell line (A) and WI-38 cell line (B) after 48 hr. Each value mean of triplicate ± standard deviation.

The results as in table **1** shows that there is a significant difference between IC_{50} values (µg/ml) of EEP-ZnO nanocomposite and IC_{50} values (µg/ml) of propolis extract and zinc oxide NPs after 48 hr on human lung cancer cell line (A549) and normal cell line (WI-38). The EEP-Zno nanocomposite is more targeted to cancer cell line than normal cell line.

Table 1. Comparison study of IC_{50} values (µg/ml) of EEP-ZnO nanocomposite, EEP and ZnO NPs on human lung carcinoma cells (A549) and human diploid lung fibroblast normal cell lines (WI-38) after 48 hr

Compound	IC50 (ug/ml)	
	A549 cell line	WI-38 cell line
EEP	107.95±0.80	164.7±0.79
ZnO NPs	26.78±0.44	81.91±0.96
EEP-ZnO nanocomposite	15.32±0.44[a,b]	44.2±0.45[a,b]

All data was represented by Mean ± SD.

[a] refers to that there is a statistically significant difference between IC50 values(µg/ml)of nanocomposite and EEP on A549 and WI-38 cell lines, *P value<0.05.

[b] refers to there is a statistically significant difference between IC50 values(µg/ml)of nanocomposite and ZnO NPs on A549 and WI-38 cell lines,*P value<0.05.

3.5. Cell apoptosis assay

Apoptosis and necrotic cell death were assessed using flow cytometric analysis with Annexin-V-FITC and PI dual-staining to evaluate the pro-apoptotic effects of EEP, ZnO NPs and EEP-ZnO nanocomposite on A549 cells. The cells were treated with the IC50 concentration (µg/ml) of ZnO NPs, EEP and EEP-ZnO nanocomposite for 48 h, partial apoptosis occurred in the EEP-ZnO nanocomposite group which was higher than that in the ZnO NPs group/the propolis extract group (**Figure 5**).

The results in **figure 6** show that the percentage of apoptotic cells, including early, late, and necrotic cells, in the treated cells (A549) with EEP, ZnO NPs, and EEP-ZnO nanocomposite groups was significantly higher than that of untreated cells (control) after 48 h.

From the results could conclude that for the anticancer effect of propolis extract due to that EEP possessed important phytochemical compounds that work excellently as antioxidants and anticancer agents [33]. Propolis has the ability to stop DNA synthesis in tumor cells and to cause aging of tumor cells (Apoptosis) [34],[35]. Propolis extract individually showed cytotoxic activity against A549 cancer cell line in the present study, which corroborates previous investigations using MCF-7 (human breast cancer), HT-29 (human colon adenocarcinoma), Caco-2 (human epithelial colorectal adenocarcinoma), and B16F1 (murine melanoma) cell lines, the result showed that EEP at a concentration of 250 μg/mL exhibited ≥50% mortality in all cell lines tested (i.e., IC50 value). EEP revealed a concentration and time dependent cytotoxic effect [36]. These findings were supported by Kouidhi *et al.* [37] study, who investigated the cytotoxic effects of The Tunisian EEP on normal (MRC-5) and cancer cell lines (HT-29, A549, Hep-2, Vero), A strong antiproliferative potencies of Tunisian EEP against all studied cancer cell lines with an IC50 ranged from 15.7± 3.4 to 200 ± 22.2 µg/ml.

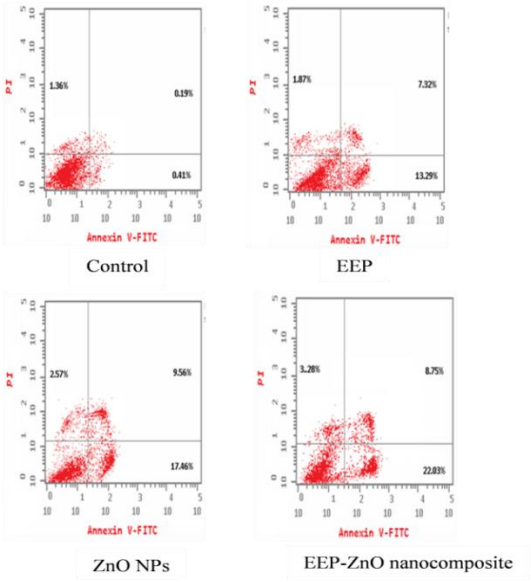


Fig. 5:Apoptosis induction by compounds treatment after 48 h on A549 cell line.

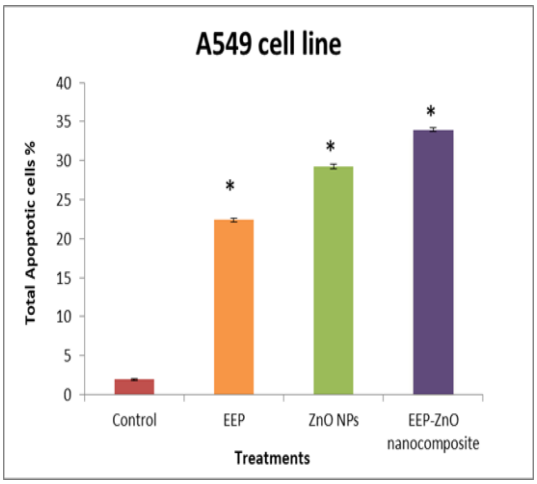


Fig. 6: Comparison of total apoptotic cells% in different treatments in related to control. Data are the mean ± SD. *(P value<0.05) a statistically significant difference as compared to control.

Salim *et al.*[38], concluded that EEP alone or in combination with DOX at both doses possesses greater antioxidant, antiproliferative and apoptotic effects against the PC3 cell lines as compared to treatment with DOX alone. Accordingly, EEP could be considered as a promising candidate for prostate cancer chemotherapy.

Gokduman [39], investigate the anti-cancer activity of propolis on liver hepatocellular carcinoma cells (HepG2 and Hep3B cells). The results showed greater antioxidant, antiproliferative and apoptotic effects against the (HepG2 and Hep3B cells). These results suggest the great potential of propolis as a potent anti-tumor compound in liver cancer treatment for further researches.

Upon treatment of A549 cancer cells with various concentrations of ZnO NPs, a gradual decline in the viability percent and gradual increase in the toxicity percent was observed. The interpretation for this inhibitory effect of ZnO NPs has been related to the rapid dissolution of ZnO NPs to Zn^{2+} ions, which takes place in acidic pH and this nature makes it suitable for use as nanocarriers in acidic tumor microenvironment.

By increasing the generation of ROS, damaging the DNA, and ultimately causing cell death through apoptosis, ZnO NPs can cause cytotoxicity in tumor cells [40]**.** The findings of the current study are consistent with those of Pandurangan *et al.* [41], who found that ZnONPs reduced HeLa cell viability from 5 to 50% in a dose-dependent manner, but had no effect on normal Madin-Darby Canine Kidney (MDCK) cells, which displayed 95% viability at a concentration of 0.06 mg/ml.

Majeed *et al.*[42], who reported that ZnONPs caused higher dose-dependent reduction in cell viability in HCT-116 cells via the induction of apoptotic cell death with IC50 value of 20 µg/mL as compared to normal Vero cell line with IC50 value of 30 µg/mL.

Fakhroueian *et al.*[43], studied the cytotoxic effect of ZnO NPs in MCF-7 cells. The result showed higher dose and time dependent cytotoxic effect in MCF-7 cells (IC50 value 41 µg/mL after 48 h) when compared to normal human fibroblast HFF-2 cells (IC50 value 105 µg/mL after 48 h).

More interestingly, a synergistic cytotoxic effect for EEP-ZnO nanocomposite against A549 cells was recorded, which could reveal the capacity of propolis extract and ZnO NPs to potentiate the cytotoxicity against lung cancer cells.

Conclusion

Based on the present results, it is possible to conclude that combination of propolis extract with zinc oxide nanoparticles exerted a better effect than that Propolis extract or ZnO NPs alone on human lung cancer cell line A549 after 48 hr.

Conflict of interest

The authors have no conflicts of interest to declare**.**

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