The Most cytotoxic Effect of Propolis Against Tumor Cells is Due To Apoptosis via Mitochondrial Pathway.

Khalid Mahdi Salih¹, Bedir Mohammed Al-Azawi², Nahi Yousif Yaseen³

¹ Department of Biology, College of Science, Al-Mustansiryah University
²,³ Iraqi Center of Cancer Research & Medical Genetics, Al-Mustansiryah University.

INTRODUCTION

Cancer is one of the major threats to public health in the developed world and increasingly in the developing countries (1). According to the World Health Organization, cancer accounted for 7.1 millions deaths in 2003, and it is estimated that the overall number of new cases will rise by 50% in the next 20 years (2). It has been estimated that 30-40 percent of all cancers can be prevented by life style and dietary measures alone (3). In recent years there is a growing interest in nutraceuticals which provide health benefits, disease prevention and
substitution of modern medicine (4). As many as 89% of patients with cancer or other chronic conditions use nutraceuticals, some of them show potential as adjuvants with conventional oncology treatment, others may be used to eliminate the side effects of conventional treatment (5).

In Iraq, several natural products have been investigated to evaluate their anti-tumor activity against tumor cells in vitro and in vivo such as green and black tea (6), crude extract of Miramia Salvia triloba (7), vegetable and callus parts of Melia azera (8), Rhubarb and Thyme crude extracts (9).

Propolis is a resinous substance with varying colors and consistencies, collected by Apes mellifera bees from several sources. The word propolis comes from the Greek pro meaning in defence of and polis meaning city, i.e. defence of the hives (10). It has been revealed that propolis possesses various biological activities such as anti-bacterial, antifungal, antiviral (11, 12), anti-inflammatory (13) and anticancer (14) properties. More than 300 different compounds have been identified so far in propolis (15), flavonoids are the ones which draw greater research interest (16). Recently, it has been reported that ethanolic extract of Brazilian propolis suppresses tumor-induced angiogenesis in vivo and tube formation of endothelial cells in vitro through induction of apoptosis in endothelial cells (17, 18). Furthermore, it was found that ethanolic extract of propolis-induced apoptosis is related to the selective activation of caspase-3 and induction of BCL-2/Bax regulation (19), or via augmentation of TRAIL-mediated apoptosis in cancer cells (20).

In this study, we investigated the cytotoxic and apoptotic effects of watery extract of Iraqi propolis on two tumor cell lines (Hep-2) and (AMN-3) and one normal cell line (Ref). We also evaluated the protein content in the secretions of these cell lines in their culture medium.

**MATERIALS & METHODS**

**Preparation of Propolis**

Natural propolis, dark brown in color, multifloral origin was used for experimentation. Propolis was collected from bee hives in Khan Dhari farm, Baghdad, Iraq. Coarsely powdered propolis was extracted, five volumes of water were added to 50g of propolis and stirred for 4h at 45°C. Following removal of insoluble materials by centrifugation at 470g for 15 min, the supernatant was dried and the resultant powder was stored at -20°C until used (21), Six concentrations (4, 2, 1, 0.5, 0.25, and 0.125mg/ml) of watery extract of propolis (WER) were prepared in serum-free RPMI-1640 medium (sigma) supplemented with benzylpenicillin (100,000 IU/ml) and streptomycin (100,000µg/ml), then sterilized by filtration through 0.22µm filter.

**Preparation of Cell lines**

Murine mammary gland adenocarcinoma (AMN-3), epithelial cell carcinoma of human larynx (Hep-2) and normal rat embryonic fibroblasts (Ref) cell lines were obtained from Iraqi Center of Cancer Research and Medical Genetics. All cell
lines were routinely kept in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO2 – 95% air incubator under standard conditions. Cell viability was measured by using trypan blue exclusion method to prepare a suitable concentration of cell suspension for tissue culture experiments (22).

**Cytotoxicity assay**

The cells were seeded in 96-well flat bottom plates at a concentration of 2×10⁶ cell/well and incubated at 37°C for 24h, then the old medium was discarded and the attached cells were treated with the various concentrations of propolis and reincubated for 48h (22). After treatment, the old medium was aspirated from each well and transferred into eppendorf tubes to estimate its protein content later. However, the wells were stained with crystal violet solution and reincubated for 20 min, then the stain solution was discarded and the plate washed with tap water and let to dry. The absorbance (optical density OD) of each well was determined at 492nm by Elisa plate reader (23).

**Determination of Protein**

The eppendorf tubes obtained from previous experiment were centrifuged to eliminate any associated cells. The supernatant was referred as cell line secretion and its protein content was determined by using Bradford method (24).

**Apoptosis Assay**

This assay was carried out according to Mitochondria Bioassay Kit (US, Biological Company) described by Chen et al (25). The cells of Hep-2 and AMN-3 lines were plated in 8-chamber tissue culture slide (LAB-TEK, Nunc, Inc.) at concentration of 5×10⁵ cells/chamber and incubated at 37°C for 24h, then the old medium was discarded from each chamber and the adherent cells were treated with the highest concentration of WEP (4mg/ml) and reincubated for 36h. After treatment, the medium was discarded and the cells were treated with the mitocapture reagent for 20 min and examined under the fluorescent microscope to count the number of apoptotic cells (green in color) and healthy cells (red in color).

**Statistical Analysis**

All data were expressed as Mean ± SE and the validity was tested by linear correlation (r) between the treatment and each parameter. However, the statistical significance of differences between treated and control groups was analyzed by Mann-Whitney at p<0.05 (26).
RESULTS AND DISCUSSION
The percent charge in OD of 3 cell lines after 48h exposure to various concentrations of WEP was referred as percent change in cell proliferation and illustrated in figure-1. Statistical analysis demonstrated that for concentrations of WEP (0.5, 1, 2, and 4mg/ml) caused significant reduction in cell proliferation of Hep-2 line (26.1%, 45.8%, 49% and 66.7% respectively), while three concentrations (1, 2 and 4mg/ml) caused significant reduction in AMN-3 line (27.2%, 53.7%and 63% respectively). However, all concentrations revealed no significant change in cell proliferation of Ref line.

The percent change of protein content in the secretions of treated groups were compared to those of their controls and represented in figure 2. Both Ref and Hep-2 cell lines showed significant direct correlation between WEP concentrations and protein content in their secretions (r = 0.86 and 0.93 respectively) with maximum increment about 84.7% in Ref line and 94.6% in Hep-2 line. However, those of AMN-3 line showed significant reverse correlation (r = -0.95) with maximum reduction about 79.1%.

Although several concentrations of WEP caused cytotoxic effects in tumor cell lines (Hep-2 and AMN-3), the highest concentration (4mg/ml) was chosen in apoptosis assay. Figure 3 showed complete apoptosis induction in treated group of Hep-2 cells (100%) and severe induction in AMN-3 cells (88.3%±3.53%) in comparison to their corresponding controls (10%±2.89% and 18.3%±4.41% respectively).

![Figure -1: Cytotoxic effect of 48hr exposure to different concentrations of WEP on the proliferation of 3 cell-lines.](image-url)
Figure- 2: modulating effect of 48hr exposure to different concentrations of WEP on the protein content in the secretions of 3 cell-lines.

Figure -3: Apoptotic effect of WEP on Hep-2 and AMN-3 cell-lines.

In this study, we found a potent cytotoxic and apoptotic activities of WEP against both tumor cell lines (figure 1,3). Understanding the mechanisms underlying the cytotoxic activity by propolis provide beneficial clue for the development of new drug and functional food candidates (21). Inducing apoptosis is one of the mechanisms for several therapeutic agents as shown in propolis by a number of studies (14, 27). It was reported that propolis can induce apoptosis in tumor cells by several pathways for instances; through activating caspases, Bid and
Cytochrome-c release (28), interfering with tumor suppressor protein such as P53 and P38 MAPK (29), inhibiting telomerase expression (30), and augmentation of tumor necrosis factor related apoptosis-inducing ligand (TRAIL) which is a natural occurring anticancer agent that preferentially induces apoptosis in cancer cells and is not toxic toward normal cells (20). Because the apoptosis detection kit that was used in this study distinguishes between healthy and apoptotic cells by detecting changes in the mitochondrial transmembrane potential (25), therefore, it can be suggested that propolis induced apoptosis in tumor cells via mitochondrial-dependent pathway because disruption of the mitochondrial transmembrane potential is one of the earliest intracellular event that occur following apoptosis induction (31).

On the other hand, this study demonstrated a contrast activity of WEP on protein content in the secretions of cell lines (figure 2). The elevation of protein content in the secretions of Hep-2 culture may be due to cellular disintegration that lead to release cytosol and increase the level of protein in the culture medium (22). Similarly, protein content was significantly increase in Ref culture, although its growth was not affected. Therefore, these findings need more investigations to interpret the behavior of Ref cells. However, the result of AMN-3 is quietly questionable because at the same conditions, propolis caused significant induction of apoptosis and growth inhibition (figure 1,3). Therefore, it can be suggested that propolis might partly inhibit either the synthesis or release of certain protein molecules such as growth factors, adhesion molecules, cytokines (32, 33). Furthermore, because the apoptosis that occurred in these cells was incomplete, thus the healthy cells may engulf the apoptotic bodies of the affected cells and prevent the accumulations of their protein in the surrounding environment, which is one of the characteristic features of the final stage in apoptosis process in vitro (31).

In conclusion, this study provides evidence that the WEP possesses cytotoxic activity toward tumor cells through the induction of mitochondrial pathway leading to apoptosis, also its ability to modulate the protein content in the secretion of tumor cell lines that need further qualitative and quantitative studies.

REFERENCES


The Most cytotoxic Effect of Propolis Against Tumor Cells is Due to Apoptosis via Mitochondrial Pathway.

Khalid, Bedir and Nahi