Journal of US-China Medical Science 14 (2017) 157-163
doi: 10.17265/1548-6648/2017.04.002

Elementery Analysis and anti-Tumor Activity on Glioblastoma Cell Lines of Propolis Samples from Giresun Province of Turkey

Meltem Maras Atabay[1], Orhan Sezgin[2], Mehmet Taspinar[3] and Veysel Yuksek[4]

1. Department of Science Education, University of Bülent Ecevit, Zonguldak, Kdz Ereğli 67300, Turkey

2. Department of Medical Biology, University, of Karadeniz Technical, Trabzon 61000, Turkey

3. Department of Medical Biology, University of Yüzüncü Yil, Van 65800, Turkey

4. Department of Medical Laboratory Technicianary, University of Yüzüncü Yil, Van 65800, Turkey

Abstract: One of the natural products which have been used as a folk medicine is propolis. It is reported to exhibit several biological activities. Trace and macro-element profiles in propolis provided information enough to develop the classification of the geological origin of propolis. The geological origin of propolis determines its biological properties. It has become a promising anticancer natural agent in the recent days. The first purpose of this study was to determine macro-element and heavy metal contents of raw propolis samples originating from Giresun, a rainy and temperate region of Turkey. The second purpose of this study was to assess cell viability on glioblastoma cell lines of propolis samples. Macro-element and heavy metal contents (Mg, Na, Al, Si, K, Fe, Cu, Zn, Mn) of raw propolis samples from Giresun, were evaluated using inductively coupled plasma spectrometer with mass detection. Glioblastoma cell lines were exposed to different concentrations of propolis, and the apoptotic levels were determined using apoptosis assay. Additional cell viability and proliferation were analyzed by XXT assay. We used the XTT assay to assess cellular proliferation and viability. Turkish propolis is rich in the minerals. Potasium content was at a relatively higher level than other elements in samples from various regions of Turkey. The results obtained indicate no pollution of the collection areas. Results showed that apoptotic cell population increased significantly in glioblastoma cell lines exposed to increasing concentrations of propolis extracts. We reported the antiproliferative activity of these propolis on cancer cells. We suggested the induction of apoptosis after treatment with Turkish propolis on glioblastoma cell lines. Further studies are needed to advance in the understanding of the molecular basis of apoptosis induction on by Turkish propolis.

Key words: Propolis, element analysis, apoptosis, cell viability, XXT assay.

1. Introduction

Propolis is a naturopathic formulation collected by honeybees from buds and exudates of conifer trees and plants. Propolis that has anti-mutagenic and anti-carcinogenic properties and collected from plants by honeybees has biological and therapeutic effects [1-3]. Bees use the propolis to protect and reinforce their hives, repair their structure, and to cover honeycombs [1-3].

Propolis, a natural product derived from plant resins collected by honeybees, has been used in traditional medicine all over the world for thousands of years. The composition of the propolis depends upon the vegetation of the area from which it was collected and on the bee species. Therefore, it has gained popularity also as a health drink and is extensively used in food to improve health and prevent diseases [1]. Propolis has gained popularity also as a health drink and is extensively used in food to improve health and prevent diseases [1-5].

The composition of propolis is dependent of the flora, season, and time of the collection [6]. Macro-element profiles in propolis provided information enough to develop the classification of the geological origin of

Corresponding author: Meltem Maras Atabay, Ph.D., assoc. prof., research field: molecular biology.

propolis [7]. Typically, propolis is broadly characterised into (1) samples from temperate regions mainly originating from poplar tree exudates and rich in phenolics such as flavonoids, aromatic acids and esters and (2) samples from tropical areas, devoid or containing traces of poplar constituents but rich in other substances including prenylated derivatives of p-coumaric acids, diterpenes and, prenylated benzophenones and prenylated flavonoids [8].

Recently, propolis has become the area of interest and plays an effective role in natural product studies because of its several biological and pharmacological properties. The identity and the mechanism of action of the chemical compounds present in the propolis are investigated [9]. To identify the cellular targets of any bioactive compounds is a very difficult task [10-12]. Apoptosis is a completely regulated process, with an important and central role in the development and homeostasis of multicellular organisms. The apoptosis deregulation participates directly in the carcinogenesis process, being associated with the overexpression of anti-apoptotic genes, which leads to a selective survival advantage that promotes the proliferation of tumoral cells [13, 14]. One of the mechanisms of the anti-tumor activity of propolis has been shown to be through the induction of apoptosis [7, 15]. Inducing apoptosis is one of the mechanisms proposed for the therapeutic effects of propolis [16, 17]. This natural compound was tested for its antiproliferative capacity on KB (human mouth epidermoid carcinoma cells), Caco-2 (colon adenocarcinoma cells) and DU-145 (androgen-insensitive prostate cancer cells) human tumor cell lines.

Because taking into consideration the fact that the cell death effects of propolis could be due to a combination of chemical compounds and concentrations, we decided to investigate the cell death effects of propolis by concentrating our experiments on alcoholic extracts of propolis. In this study, we investigated the anti-tumor activity on glioblastoma cell lines of propolis samples, provided by Giresun province, collected in a rainy and temperate region of Turkey. Our study investigates systematically by using functional genomics how propolis influences the metabolism of an organism.

2. Materials and Methods

2.1 Propolis Samples

A total of 15 different propolis samples collected from fifteen towns of Giresun, Turkey; the most abondent plants in this region are *Pinus sylvestris, Abies nordmanniana, Picea orientalis, Juniperus Communis, Ouercus pontica, Fagus orientalis, Carpinus betulus, Alnus cardifolia, Acer cappadocicum, Castanea sativa, Platanus orientalis, Betula pendula* (M. Ö zmenli). Material was obtained in the period of June to August 2015. It was chosen according to the sample of the hives from fifteen towns of Giresun.

2.2 Elementary Analysis of Propolis with ICP-MS

1000 mg of material were weighted and diluted with 20 ml of concentrated, pure nitric acid solution produced by Merck Campany. Quantative analysis of elements were determined using Perkin Elmer NexION 300D ICP – plasma spectometer with mass detection.

2.3 Preparation of Ethanol Extracts of Propolis (EEP)

10 gr of propolis samples was dissolved in 100 ml of 96% ethanol and incubated for 24 h at 60 ℃. After incubation, the sample was centrifuged for 10 min at 4000 rpm. The supernatant was filtered and evaporated to dryness in a rotavapor.

2.4 Glioblastoma Cell Culture

Glioblastoma cell line were cultured in six-well tissue culture plates at (3 × 105) ml/well in DMEM medium containing 10% Fetal Bovin Serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomicin. Cells were incubated in humidified 5% CO_2 at 37℃. The cells were incubated at different concentrations (25

µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml) of propolis for another 24 hours. Untreated glioblastoma cells were used as a control. At the end of incubation, apoptotic cell death was determined by the XTT method. Trypan blue and XTT methods were used to evaluate the cytotoxicity.

2.5 Cell Viability and XTT Assay

XTT is reduced to a soluble, brightly colored orange derivative to a mix of cellular effectors. This color change is accomplished by breaking apart the positively charged queternary tetra zole ring. Glioblastoma cells were added 30 µl XTT per well and incubated in 5% CO_2 at 37℃ for 4 h of the culture period tested. The medium was then decanted and 1 ml dimethylsulphoxide (DMSO, Sigma-Aldrich) was added to each well to ensure dissolving of the formazan salts. The concentration was measured at 450-690 nm in a UV spectrometer.

2.6 Trypan Blue Viability Test

Trypan Blue Test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue. In this test, cell suspension was mixed with trypan blue dye and examined immediately under invert microscope. Cell viability was calculated as the number of viable cells by the total number of cells within the thoma lam. If cells take up trypan blue, they are considered non-viable. To calculate the number of viable cells per ml a culture, was used the formula below.

2.7 Statistical Analysis

Differences for element means in raw propolis from Giresun province of Turkey were assessed by non-parametric statistics of one-way analysis of variance (ANOVA). $P < 0.05$ was considered significant. SPSS 10 software was used for analyses.

3. Results

3.1 Elementary Analysis of Propolis

The first series of experiments was related to the levels of the macro-element and heavy metals content (Mg, Na, Al, Si, K, Fe, Cu, Zn, Mn) of raw propolis samples originating from a rainy and temperate region of Turkey (Table 1).

Non-Parametric statistics were used to calculate mean and standard deviation for elements in samples. Potasium was the most abundant element in the samples. The potasium content mean was found at 0.385 ± 0.03 mg/kg. Potasium content was the highest in samples from Giresun province, whereas zinc element was the lowest in those of this region. The copper content mean was at 0.082 ± 0.03 mg/kg. Cu and Zn contents in propolises were within safe limits, and compared well with levels in foods from Turkish Food Codex [18], though Zn contents were high. Sodium content mean was 0.0834 ± 0.1 mg/kg. Interestingly, no sulphur (S) was detected in propolis samples. These propolis are rich in the minerals of K, Na, Mg, and could be more beneficial in human nutrition. The results obtained indicate no pollution of the collection areas.

The second series of experiments was related to verifying a working propolis concentration and determining how cell viability was affected.

Results showed that apoptotic cell population increased significantly in Glioblastoma cell lines exposed to increasing concentrations of propolis extracts.

Since 10 gr raw propolis was dissolved in 100 ml ethanol (96%), stok propolis solution was 100mg/ml PE extract (Fig. 1). Upon 5 min of exposure to 25 µg/ml propolis concentration, cells grown for 16 h have an increased number of cells suggesting early apoptosis (Fig. 1B). The results showed that the PE2 extracts at 25 µg/ml concentration induced apoptosis in association with increased number of XTT positive cells.

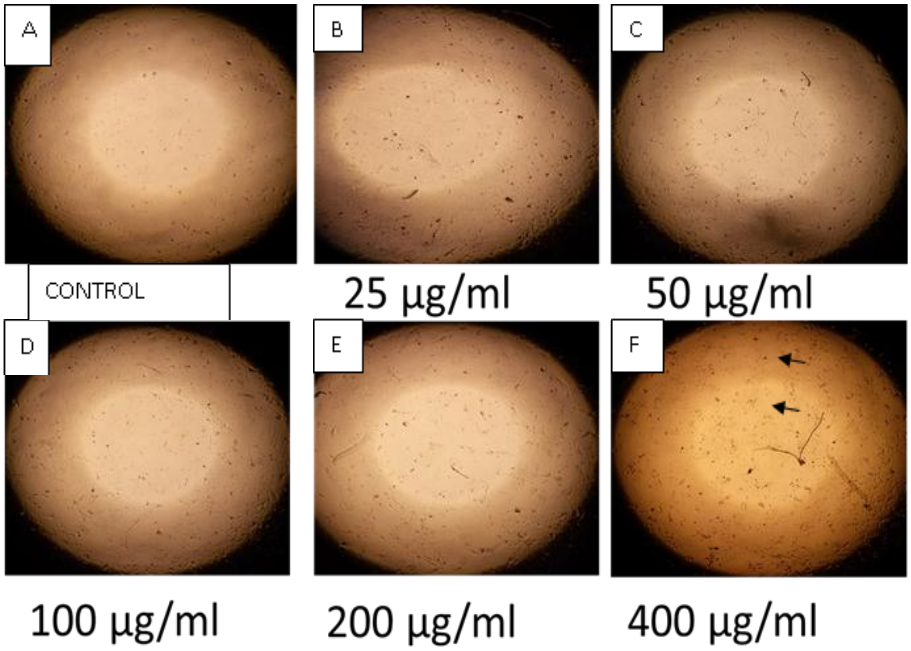

Fig. 1 Evaluation of XTT assay and Trypan Blue staining. (A) Control group glioblastoma cells after 24h of culture. (B, C, D, E, F) Propolis extracts (PE2, PE3, PE4, PE5, PE6) at different concentration induced apoptosis in association with increased number of XTT positive cells.

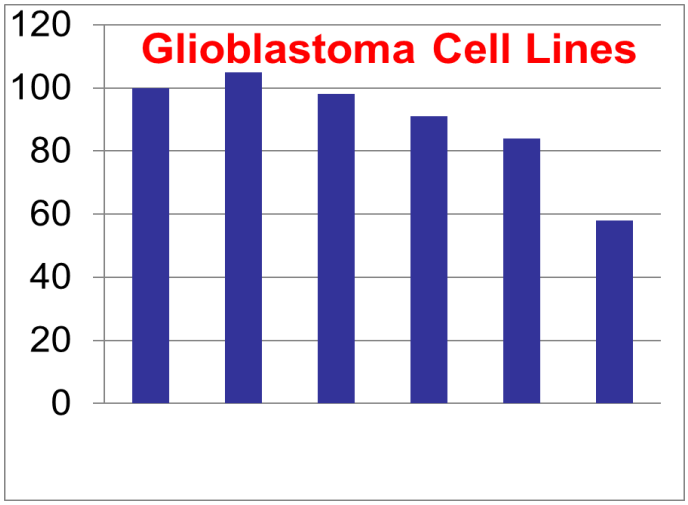

Fig. 2 XTT positive glioblastoma cell viability treated with different types of PE in 25, 50, 100, 200 and 400 µg/ml dilutions after 24h of culture.

PE 5 (Fig. 1E) extract at 200 µg/ml concentration was the most increased apoptotic dose. 42% of the cells observed to died through apoptotic cell death at PE6 (Fig. 1F) extract, suggesting late apoptosis, leading to secondary necrosis (Fig. 1C and D).

3.2. Cell Viability and Cytotoxicty

Glioblastoma cells were treated with propolis extracts at various concentrations for 24 h, and the cell viability was determined as described above by XTT assay. All propolis extracts at all concentrations inhibited the growth of Glioblastoma cells in a dose-dependent manner (Fig. 2).

Our results showed that PE 5 and PE 6 at 200 µg/ml and 400 µg/ml were more effective in inhibiting Glioblastoma cell growth when compared with other

Table 1 Recommended wave lengths and concentration mean of the different elements determined ICP-MS.

Element	Line (nm)	Concentration mean (mg/kg)	Standard deviation
Mg	279	0.357	0.02
Na	568	0.0834	0.1
Al	308	0.0921	0.05
Si	251	0.264	0.01
K	766	0.385	0.03
Fe	259	0.0207	0.01
Cu	324	0.082	0.03
Zn	213	0.0081	0.01
Mn	257	0.228	0.04

extracts and dilutions (Fig. 2).

3.3 Effect of Propolis Extracts on Apoptosis Induction of Glioblastoma Cells

To determine the occurrence of apoptosis in Gliobastoma cells treated with the seven types of propolis extracts, the cells were stained with trion blue dye. After treatment with different types and various concentrations of propolis extracts for 24 h, the numbers of XTT positive cells in all treated groups (Fig. 3B) were significantly greater when compared with the control group (Fig. 3A).

However, in Glioblastoma cells treated with propolis extracts PE 5 and PE 6, the number of XTT positive cells was greater than in the other propolis extracts (PE 1, 2 and PE 3) (Table 1). The propolis induced apoptosis in a dose-dependent manner and the most effective dilutions of propolis extracts PE 5 and PE 6 were 200 µg/ml and 400 µg/ml.

4. Discussion

Propolis is an important apicultural product with various chemical compositions and several pharmacological and nutritional applications. Moreover, it is a mixture of components collected by bees and found to have diverse biological properties. Its content has been found to be linked to the normal surrounding habitat, weather, and season collection. Diversity in chemical composition of propolis reflects the floral richness and the climate changes in the country [19]. Macro-element and heavy metal contents of raw propolis samples originating from Giresun, a rainy and temperate region of Turkey were evaluated using ICP-plasma spectometer with mass detection.

Geographical molecular marker is also important for analyzing the location of propolis taken [10]. The composition of propolis is dependent on the flora, season, and time of the collection [20]. In our study, sodium was low content in samples from Giresun province while higher level of Na in propolis was observed in samples from Artvin, İzmir and Bursa region in Doğan et al. study [21]. Mg was similar content with the ranges of their study. S level was not high enough for detection with the ICP-MS.

Propolis can contain heavy metals, which can significantly affect the biological properties of derived product [22]. We determined Zn, Al and Fe contents were at the lower level than in previous report [21]. But Mn was higher level in it. Since the low level or absence of heavy metals, propolis may be used as an indicator of environmental contamination, as has been suggested suggested [7].

The results obtained which indicate no pollution of collected areas. The propolis located there can be used for the elaboration of high-quality be products, free of toxic contaminants. We used Giresun propolis for activation of apoptosis in the Glioblastoma cell line for this reason.

The biological activities of propolis samples of different geographic origin have been investigated [1, 7]. Many reports have indicated that different types of propolis extracts significantly inhibit cell growth and reduce the differentiation or proliferation of tumor cells [22, 23]. However, the effect of propolis on cell death or growth is still questionable [15].

The biological effects exhibited by this *Chilean* propolis sample, under these experimental conditions, could be related to an overall effect of the phenolic compounds present in the extract (flavonol galangin; hydroxycinnamic acids, caffeic acid, p-cumaric acid,

ferulic acid and CAPE), which were detected by HPLC in the gradient mode the method of choice for the assay of propolis components. Recently, extracts of propolis were shown to trigger various apoptotic pathway in different types of cancer cells [24-28].

In the present study, the effects of propolis extract from Giresun, a rainy and temperate region of Turkey on the growth, cytotoxicty and apoptosis of Glioblastoma cells were investigated. The data demonstrated that propolis treatment was associated with a strong inhibition of growth and cell death via apoptosis in a dose-dependent manner. When different dilutions of extracts were used during treatment of cells, 200 µg/ml and 400 µg/ml dilutions significantly increased the cell death. The most effective propolis extracts and dilutions appeared to be PE 6 in 400 µg/ml concentrations.

We conclude that propolis may have anti-tumour effects by increasing apoptosis. Such propolis extracts may be important economically and allow development of a relatively inexpensive cancer treatment [15, 29]. Results showed that Turkish propolis sample exhibits interesting biological properties, correlated with its chemical composition and expressed by its capacity to scavenge free radicals and to inhibit tumor cell growth. More apoptotic cells were detected with lower concentration of propolis extracts because when the cells were treated with the higher concentration of propolis extracts, they die via the necrotic pathway before apoptotic pathway activation. Propolis is able to induce an apoptosis cell death response but that increased exposure to propolis provides a corresponding increase in the necrosis response [10].

In conclusion, The dosage of propolis extracts may therefore be important when planning optimum treatment. Propolis extracts exhibited a dose-dependent inhibition of cellular growth and activation of apoptosis in the Glioblastoma cell line.

This opens new possibilities for understanding and validating propolis as an alternative therapeutic agent. Overall, our results indicate that propolis extracts had a strong and selective apoptotic effect on tumor cells that may be useful in future treatment of hematological neoplasias [30].

References

[1] Russo, A., Cardile, V., Sanchez, F., Troncoso, N., and Vanella, A. 2004. "Chilean Propolis: Antioxidant Activity and Antiproliferative Action in Human Tumor Cell Lines." *Life Sci*. 76 (5): 545-58.

[2] Pagliarone, A. C., Missima, F., Orsatti, C. L., Bachiega, T. F., and Sforcin, J. M. 2009. "Propolis Effect on Th1/Th2 Cytokines Production by Acutely Stressed Mice." *Journal of Ethnopharmacology* 125 (7): 230-3.

[3] Valente, M. J., Baltazar, A. F., Henriqu, R., Estevinho, L., and Carvalho, M. 2011. "Biological Activities of Portuguese Propolis: Protection against Free Radical-Induced Erythrocyte Damage and Inhibition of Human Renal Cancer Cell Growth *in vitro*." *Food and Chemical Toxicology* 49 (1): 86-92.

[4] Cvek, J., Medic-Saric, M., Vitali, D., Vedrina-Dragojevic, I., and Smit, Z. 2008. "The Content of Essential and Toxic Elements in Croatian Propolis Samples and Their Tinctures." *Journal of Apicultural Research* 47 (1): 35-45.

[5] Anjanette, W., Gjertsen, D. D. S., Stothz, K. A., Kathleen, G., and Neiva, D. D. S. 2011. "Effect of Propolis on Proliferation and Apoptosis of Periodontal Ligament Fibroblasts." *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod*. 112 (4): 843-8.

[6] Lima, G. G., Souza, R. O., Bozzi, A. D., Poplawska, M. A., Devine, D. M., and Nugent, M. J. D. 2016. "Extraction Method Plays Critical Role in Antibacterial Activity of Propolis-Loaded Hydrogels." *Journal of Pharmaceutical Sciences* 105 (8): 1248-57.

[7] Alday, E., Valencia, D., Carreno, A. L., Picerno, P., and Piccinelli, A. L. 2015. "Apoptotic Induction by Pinobanksin and Some of Its Ester Derivatives from Sonoran Propolis in a B-cell Lymphoma Cell Line." *Chemico-Biological Interactions* 242: 35-44.

[8] Kardar, M. N., Zhang, T., Coxon, G. D., Watson, D. G., Fearnley J., and Seidel, V. 2014. "Characterisation of Triterpenes and New Phenolic Lipids in Cameroonian Propolis." *Phytochemistry* 106: 156-63.

[9] Wua, J., Omeneb, C., Karkoszkaa, J., Boslanda, M., and Eckarda, J. 2011. "Caffeic Acid Phenethyl Ester (CAPE), Derived from a Honeybee Product Propolis, Exhibits a Diversity of anti-Tumor Effects in pre-Clinical Models of Human Breast Cancer." *Cancer Letters* 308 (1): 43-53.

[10] de Castro, P. A., Savoldi, M., Bonatto, D., Barros, M. H., and Goldman, M. H. 2011. "Molecular Characterization of Propolis-Induced Cell Death in *Saccharomyces*

Cerevisiae." *Eukaryotic Cell* 10 (3): 398-411.

[11] Omene, C. O., Wu, J., and Frenkel, K. 2011. "Caffeic Acid Phenethyl Ester (CAPE) Derived from Propolis, a Honeybee Product, Inhibits Growth of Breast Cancer Stem Cells." *Invest New Drugs* [Epub ahead of print].

[12] Avci, C. B., Gündüz, C., Baran, Y., Sahin, F., and Yilmaz, S. 2011. "Caffeic Acid Phenethyl Ester Triggers Apoptosis through Induction of Loss of Mitochondrial Membrane Potential in CCRF-CEM Cells." *J. Cancer Res. Clin. Oncol*. 137 (1): 41-7.

[13] Braicu, C., Pileczki, V., Irimie A., and Berindan-Neagoe, I. 2013. "p53 si RNA Therapy Reduces Cell Proliferation, Migration and Induces Apoptosis in Triple Negative Breast Cancer Cells." *Mol. Cell Biochem*. 381 (1-2): 61-8.

[14] Wyllie, A. H. 1997. "Apoptosis: An Overview." *Brit. Med. Bull*. 53: 451-65.

[15] Vatansever, H. S., Sorkun, K., Deliloğlu, İ., Gurhan, S., and Ozdal-Kurt, F. 2010. "Propolis from Turkey Induces Apoptosis through Activating Caspases in Human Breast Carcinoma Cell Lines." *Acta Histochem*. 112 (6): 546-56.

[16] Aso, K., Kanno, S., Tadano, T., Satoh, S., and Ishikawa, M. 2004. "Inhibitory Effect of Propolis on the Growth of Human Leukemia U937." *Biol. Pharm. Bull*. 27 (5): 727-30.

[17] Muneo, M., Kyung, M. K., Seok-Jong, S., Young-Choon, L., and Yeon-Kye, K. 2008. "Propolis Induces Cell Cycle Arrest and Apoptosis in Human Leukemic U937 Cells, through Bcl-2/Bax Regulation." *Environmental Toxicology and Pharmacology* 26 (1): 61-7.

[18] Bonhevi, J., and Orantes Bermejo, F. J. 2013. "Element Content of Propolis Collected from Different areas of South Spain." *Environ. Monit. Assese.* 185 (7): 6035-47.

[19] Savaya, A. 2016. "Analytical Strategies for the Characterisation of Samples of Brazilian Propolis from Different Regions and Species of Bees." Propolis Human and Bee Health Conference, Glasgow, Scodland.

[20] Almutairi, S., Edrada-Ebel, R., Fearnley, J., Igoli, J. O., and Alotaibi, W. 2014. "Isolation of Diterpenes and Flavonoids from a New Type of Propolis from Suui Arabia." *Phytochemistry Letters* 10: 160-3.

[21] Dogan, M., Silici, S., Saraymen, R., and Ilhan, I. O. 2006. "Element Content of Propolis from Different Regions of Turkey." *Acta Alimentaria* 35 (1): 27-130.

[22] Marcucci, M. C. 1995. "Propolis: Chemical Composition, Biological Properties and Therapeutic Activity." *Apidologie* 26 (83): 89.

[23] Padmavathi, R., Senthilnathan, P., and Sakthisekaran, D. 2006. "Therapeutic Effect of Propolis and Paclitaxelonhepatic Phase I and II Enzymes and Marker Enzymes in Imethylbenz(a)anthracene-induced Breast Cancer in Female Rats." *Comp. Biochem. Physiol. C Toxicol. Pharmal*. 143 (3): 349-54.

[24] Chen, C. N., Wu, C. L., and Lin, J. K. 2007. "Apoptosis of Human Melanoma Cells Induced by the Novel Ompounds Propolin A and Propolin B from Taiwenese Propolis." *Cancer Lett*. 245 (1): 218-31.

[25] Izuta, H., Shimazawa, M., Tazawa, S., Araki, Y., and Mishima, S. 2008. "Protectiveeffects of Chinese Propolis and Its Component, Chrysin, against Neuronal Cell Death via Inhibition of Mitochondrial, Apoptosis Pathway in SH-SY5Y Cells." *J. Agric. Food Chem*. 56 (19): 8944-53.

[26] Jin, C. Y., Moon, D. O., Choi, Y. H., Lee, J. D., and Kim, G. Y. 2007. "Bcl-2 and Caspase-3 Are Major Regulators in Agaricus blazei-Induced Human Leukemic U937 Cell Apoptosis through Dephoshorylation of Akt." *Biol. Pharm. Bull.* 30 (8): 1432-7.

[27] Chen, M. F., Wu, C. T., Chen, Y. J., Keng, P. C., and Chen, W. C. 2004. "Cell Killing and Radiosensitization by Caffeic Acid Phenethyl Ester (CAPE) in Lung Cancer Cells." *J. Radiat. Res.* 45 (2): 253-60.

[28] Paulino, N., Teixeira, C., Martins, R., Scremin, A., and Dirsch, V. M. 2006. "Evaluation of the Analgesic and anti-Inflammatory Effects of a Brazilian Green Propolis." *Planta Med.* 72 (10): 899-906.

[29] Banskota, A. H., Nagaoka, T., Sumioka, L. Y., Tezuka, Y., Awale, S., Midorikawa, K., Matsushige, K., and Kadota, S. 2002. "Antiproliferative Activity of the Netherlands Propolis and Its Active Principles in Cancer Cell Lines." *Journal of Ethnopharmacology* 80 (1): 67-73.

[30] Cavaliere, V., Papademetrio, D. L., Lorenzetti, M., Valva, P., and Preciado, M. V. 2009. "Caffeic Acid Phenylethyl Ester and MG-132 Have Apoptotic and Antiproliferative Effects on Leukemic Cells But Not on Normal Mononuclear Cells." *Transl Oncol*. 2 (1): 46-58.