RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Investigation of *In-Vitro* Cytotoxic and Potential Anticancer Activities of Flavonoidal Aglycones from Egyptian Propolis

Reham S. Ibrahim[a,*], Ahmed A. Seif El-Din[a], Marwa Abu-Serie[b], Nevine M. Abd El Rahman[c], Maha El-Demellawy[b,c], Aly M. Metwally[a]

[a] *Department of Pharmacognosy, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt*

[b] *Medical Biotechnolog Department, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City, Egypt*

[c] *Pharmaceutical & Fermentation Industries Development Center, City of Scientific Research and Technological Applications, Alexandria, Egypt*

Recieved on: 17.06.2017
Revised on: 30.07.2017
Accepted on: 02.08.2017

Keywords

Propolis
Flavonoidal aglycones
In-vitro anticancer activity

Abstract

The *in-vitro* anticancer activity of Egyptian propolis ethanol extract as well as two isolated flavonoidal aglycones, tectochrysin and quercetin-3,3'-di-O-methylether,was assayed using neutral red uptake method on human breast cancer (MCF-7) and colorectal adenocarcinoma (Caco-2) cell lines, in addition to their *in-vitro* cytotoxic effect to normal human peripheral blood mononuclear cells (PBMCs). Egyptian propolis ethanol extract and quercetin-3,3'-di-O-methylether showed both high activity and selectivity towards the two tested cell lines suggesting them as effective, safe and selective anticancer candidates against both breast and colorectal cancer.

1. Introduction

Propolis or bee glue (CAS No. 9009-62-5) is the generic name for the resinous substance produced by honey bees by mixing resins from various plant sources with wax from their glands (Burdock, 1998). Hive bees utilize propolis for sealing crevices and holes in their honeycombs, hence, protect against invaders and smoothing out the internal walls, hence, reduce microbial growth on hive wall. In this manner, propolis can be considered as a mean of maintaining homeostasis of the nest environment (Simone-Finstrome and Spivak, 2010). Propolis is composed mainly of resin (50%), wax (30%), volatile compounds (10%), pollen (5%) and other organic substances (5%) including amino acids and trace elements such as iron, copper, manganese and zinc (Barbaric et al., 2011). The local flora at the site of collection utilized by bees for manufacturing of propolis determines its chemical composition. This fact results in the striking diversity of propolis chemical composition (Bankova, 2005a). It became clear that comparing propolis samples from different regions of the world might be the same as comparing extracts of two plants that belong to different plant families (Bankova, 2005b). The biological activity of propolis is mainly attributed to the components of its resin. Propolis possesses antihepatotoxic, antitumour, antioxidative, antimicrobial and antiinflammatory properties (Banskota et al., 2001)

The cancer inhibitory effects of phenolic compounds in propolis have been confirmed in a variety of culture cell lines (Russo et al., 2004). Propolis resin contains mainly polyphenols the major of which are flavonoids such as pinocembrin, galangin and pinobanksin in addition to phenolic acids and their esters such as

***Corresponding author**
Business Tel: +0201223821098
Fax: +2-03-4871668
E-mail: rehamsaid84@yahoo.com

esters of coumaric and caffeic acid (Castaldo and Capasso, 2002). In spite of the huge number of diverse chemical constituents that were isolated from propolis worldwide, there were no studies concerning the isolation of chemical constituents from Egyptian propolis except two studies that reported the isolation of four prenylated flavones (El-Bassuony, 2009; El-Bassuony and AbouZid, 2010). On the other hand, we had reported the isolation and identification of eleven phenolic compounds from Egyptian propolis for the first time; these compounds were pinostrobin, izalpinin, tectochrysin, pinocembrin, galangin, chrysin, quercetin-3,3'-di-O-methyl ether, kaempferol-3-O-methyl ether, quercetin-3,7-di-O-methyl ether, isoferulic acid and galangin-5-O-methyl ether (Ibrahim et al., 2014)

In the present study, tectochrysin and quercetin-3,3'-di-O-methylether, were tested for their anticancer activity on human breast cancer (MCF-7) and colorectal adenocarcinoma (Caco-2) cell lines. The choice of these two compounds was based on that there were no previous studies performed to assay their anticancer effect on MCF-7 or Caco-2 cell lines using neutral red uptake (NRU) method, except one study performed on tectochrysin isolated from *Friesodielsia discolor* which was tested for its cytotoxic activity on MCF-7 cell line and it exhibited cytotoxicity with IC_{50}= 4.49 µg/ml, using the sulforhodamine B (SRB) assay (Prawat et al., 2012)

Moreover, as several studies had revealed that ethanol extract of propolis samples collected abroad exhibited anticancer activity on MCF-7 (Kamiya et al., 2012; Xuan et al., 2014; Omene, 2013) and Caco-2 ((Russo et al., 2004; Choudhari et al., 2013; Ishihara et al., 2009) cell lines, the present study is considered to be the first to screen the anticancer activity of Egyptian propolis against MCF-7 and Caco-2 cell lines.

2. Materials and methods

2.1. Chemicals

Ficoll-Paque™ Plus (density 1.077 g/L), RPMI 1640 medium, DMEM medium, HEPES buffer, L-glutamine and Fetal Bovine Serum (FBS) were obtained from Lonza (USA), Trypan Blue, mitomycin C and fixation buffer (0.5% formalin with 1% calcium chloride) were from Sigma (USA), Neutral Red was from Bio Basic Inc. (Canada), extraction buffer (destain buffer) [50% ethanol from Fisher Scientific (USA) with 1% glacial acetic acid from Sigma (USA) in distilled water] and DMSO from Fisher Scientific (USA).

2.2. Cell lines

Cell lines obtained from American Tissue Culture Collection (ATCC®, USA), MCF (ATCC® Number: HTB-22™) and Caco-2 (ATCC® Number: HTB-37™).

2.3. Samples preparation

Propolis sample was collected in October 2011 at noon time from University of Alexandria Agricultural Research Farm in Abees, Alexandria, Egypt, by the aid of professional entomologists. A voucher specimen of this propolis sample was kept in refrigerator at 2° C in department of Pharmacognosy, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt.

Crushed propolis sample (2.0 g) was extracted by maceration in 20 mL of 70% ethanol at room temperature for 2 days, with occasional shaking. The extract was filtered through a filter paper. The filtrate was evaporated under reduced pressure using rotary evaporator at 50 ˚C till dryness.

A 2 mg/mL stock solution of dry propolis ethanol extract or isolated compound (tectochrysin or quercetin-3,3'-di-O-methylether) was prepared by dissolution in least amount of DMSO/ethanol mixture and completing the volume with supplement medium (RPMI-1640) followed by sterilization using a 0.2 µm syringe filter. Five different concentrations of propolis extract/compound (2.00, 1.00, 0.50, 0.25 and 0.125 µg/mL) were prepared by serial dilution from each stock solution in a 96-well plate using complete culture medium.

2.4. In-vitro cytotoxic activity of propolis ethanol extract, tectochrysin and quercetin-3,3'-di-O-methylether on human peripheral blood mononuclear cells (PBMCs) using neutral red uptake method

In-vitro cytotoxicity assay was performed to assess the viability of normal cells (Human peripheral blood mononuclear cells, PBMCs) after incubation for 72 hours with propolis ethanol extract, tectochrysin and quercetin-3,3'-di-O-methylether. Viability of cells was measured using neutral red uptake (NRU) assay as described by (Borenfreund and Puerner, 1984) to determine the non-cytotoxic concentration (safe dose) of each extract or isolated compound. This assay depends on the fact that neutral red dye can be incorporated into the lysosomes of living cells (Fotakis and Timbrell, 2006) providing a quantitative assay to the cytotoxic effects.

Cytotoxicity assay involved three main steps. First, the isolation of PBMCs from freshly collected blood sample, then incubating PBMCs with different concentrations of propolis samples ethanol extracts, tectochrysin and quercetin-3,3'-di-O-methylether and finally, measuring cells' viability using neutral red uptake assay.

2.4.1. Isolation of PBMCs from freshly collected blood samples

PBMCs were isolated from heparinized healthy volunteer peripheral blood by density gradient centrifugation technique as described by (Boyum 1968). Blood samples were freshly collected into heparinized sterile tubes. Blood was diluted using equal volume of RPMI-1640 medium supplemented with 25 mM HEPES buffer and 4mM L-glutamine. Diluted blood was layered over equal volume of Ficoll-Paque™ Plus (density 1.077 g/L) (lymphocyte Separation Medium LSM) and centrifuged at 2000 rpm for 30 minutes with no acceleration and break at room temperature.

The buffy mononuclear cell layer was collected using sterile Pasteur pipette into 50 mL sterile Falcon tube and washed twice in phosphate buffered saline (PBS) using centrifugation at 1650 rpm for five minutes. The isolated PBMCs viability was assessed by using Trypan blue (TB) exclusion assay. The PBMCs were re-suspended at 1 x 106 cells/mL in RPMI-1640 medium containing 25 mM HEPES, 4mM L-glutamine supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS).

2.4.2. *Incubation of PBMCs with different concentrations of propolis ethanol extract tectochrysin and quercetin-3,3'-di-*O*-methylether*

Tested extract's/compounds' wells (treated wells) were prepared by adding in each well 100 µL of their previously prepared concentrations to 100 µL of suspended PBMCs at 1 x 10^6 cells/mL (final concentrations of each extract/compound were (1.00, 0.50, 0.25, 0.125 and 0.0625 µg/mL) and final cell number/well was equal to 1×10^5 PBMCs).

Solvent (blank) wells were prepared by adding 100 µL of the solvents in the same proportions used for dissolution of each extract/compound to 100 µL of suspended PBMCs at 1×10^6 cells/mL.

Control wells were prepared by adding 100 µL of culture medium to 100 µL of suspended PBMCs at 1×10^6 cells/mL. Each set of samples was pipetted in duplicate. The dosed plate was then gently shaken then incubated at 37 ºC, 5% CO_2 for 72 hours.

2.4.3. Measuring PBMCs viability using NRU assay:

After incubation, the plate was centrifuged at 2000 rpm for ten minutes. The media were discarded by inversion over absorbent filter paper. Neutral red stain working solution (80 µg/mL) was prepared, and 100 µL of this solution was added to each well, then the plate was gently shaken. Followed by incubation at 37 ºC in humidified 5% CO_2 for three hours to allow neutral red uptake by cells and then centrifuged at 2000 rpm for ten minutes. Excessive dyes were discarded and cells were washed three times using PBS and centrifugation at 2000 rpm for 5 minutes. The stained cells were fixed with 100 µL fixing solution (0.5% formalin with 1% calcium chloride) for one minute. Color was extracted from cells using 100 µL/well destain solution (50% ethanol with 1% glacial acetic acid) for five minutes with shaking to solubilize neutral red. The stain intensity was assayed using automated microplate reader spectrophotometer (SPECTROstar Nano, BMG LABTECH, Germany) adjusted at 540 nm. The absorbance values were then used to determine the viability of each well by comparing the absorbance of propolis extract/compound treated well (E) relative to control untreated wells (C). The % cell inhibition of each blank control well was added to the % cell viability of its corresponding treated well in order to cancel the cytotoxic effect due to the solvent (ethanol and DMSO in different proportions) used for dissolving the extract/compound. Hence, viable cell fraction was calculated according to the following expression of cell viability:

$$\text{Cell viability (\%)} = (E / C) * 100$$

$$\text{Cell inhibition (\%) by blank} = 100 - [(B / C) * 100]$$

$$\text{Actual cell viability (\%)} = \text{Cell viability (\%)} + \text{cell inhibition (\%) by blank}$$

Where:

E: The mean absorbance of extract/compound treated wells.

B: The mean absorbance of blank control wells.

C: The mean absorbance of control wells.

2.4.4. Statistical analysis

Results were interpreted to calculate the effective concentration that kills 50% of cells (EC_{50}) for propolis extract/compound using GraphPad InStat 3.0

software.

The maximum safe concentration (safe dose) that keep 100% cell viability (EC_{100}) for each extract/ compound was also calculated using GraphPad InStat 3.0 software in order to be used as the maximum concentration in the dose-response curve for the following anticancer assays.

2.5. In-vitro anticancer activity of propolis ethanol extract, tectochrysin and quercetin-3,3'-di-O-methylether on human breast cancer (MCF-7)and colorectal adenocarcinoma (Caco-2) cell lines using neutral red uptake method

2.5.1. Seeding of 96-well plates with MCF-7 and Caco-2 cells:

MCF-7 cells were routinely maintained as adherent cells in RPMI-1640 medium while Caco-2 cells were maintained in DMEM medium both supplemented with 10% FBS at 37 ºC in a humidified air incubator containing 5% CO_2. Cells were subcultured for two weeks before assay. Cell viability was assessed using Trypan blue (TB) exclusion assay.

MCF-7 and Caco-2 cells were washed twice in their respective media supplemented with 4 mM L-glutamine and 25 mM HEPES buffer. The MCF-7 cells were suspended at 3 x 10^4 cells/mL in RPMI culture medium (RPMI supplemented medium and 10% FBS) while Caco-2 cells were suspended at 4 x 10^4 cells/mL in DMEM culture medium (DMEM supplemented medium and 10% FBS). The appropriate number of cells (seeding cell density) was chosen to be 3 x 10^3 cells/well (100 µL of the prepared suspension) for MCF-7 cells and 4 x 10^3 cells/well (100 µL of the prepared suspension) for Caco-2 .The cells were left to adhere on the polystyrene 96-well plates in the incubator at 37 ºC, 5% CO_2 and 95% humidity for 24 hours.

2.5.2. Incubation of MCF-7 and Caco-2 cells with different concentrations of propolis ethanol extracts tectochrysin and quercetin-3,3'-di-O-methylether:

Different concentrations of propolis ethanol extract or isolated compound (tectochrysin or quercetin-3,3'-di-O-methylether) were prepared based on the determined safe doses using serial dilution in a 96-well plate. Tested extracts/ compound wells were prepared by adding 100 µL of the previously prepared concentrations to a 100 µL of cancer cells' suspension. Parallel concentrations of the solvent were prepared to be used as blank controls. Mitomycin C (0.09 µg/ml) was used as a positive anticancer drug control.

Control wells were prepared by adding 100 µL culture media to a 100 µL of cancer cells suspension. Each set of samples was pipetted in duplicate. The plate was gently shaken, then incubated at 37 ºC, 5% CO_2 for 72 hours. Cancer cells viability was measured using neutral red uptake assay as described previously. Percent cell inhibition was calculated from the following expression:

Cell inhibition (%) by sample =100- [(E /C) X 100]

Cell inhibition (%) by blank =100- [(B /C) X 100]

Actual cell inhibition (%) = Cell inhibition (%) by sample - Cell inhibition (%) by blank

Where:

E: The mean absorbance of extract/compound treated wells.

B: The mean absorbance of blank control wells.

C: The mean absorbance of control wells.

2.5.3. Statistical analysis

The half maximal inhibitory concentration (IC_{50}) values were determined from the GraphPad InStat 3.0 software.

3. Results

The results of the *in-vitro* cytotoxic activity on normal PBMCs and *in-vitro* anticancer activity on MCF-7 and Caco-2 cell lines of propolis ethanol extract, tectochrysin and quercetin-3,3'-di-O-methylether expressed as EC_{50} or IC_{50} (µg/mL) are listed in (**Table 1**)

4. Discussion

Several mechanisms contribute to the overall cancer preventive and antitumor effects of propolis and its phenolic components. Further studies demonstrated that flavonoids, phenolic acids, as well as ethanol extract of propolis inhibit the cancer cell cycle progression, cell proliferation and tumor growth, prevent tumor metastasis, induce cell-cycle arrest and apoptosis (Aso et al., 2004; Scheller et al. 1989; Orsolic et al., 2005a; Orsolic et al., 2005b)

The role of host immune functions has become increasingly important in our understanding of the mechanisms involved in prevention of malignant diseases. Ethanol extract of propolis stimulated nonspecific immunity, activated humoral immunity

Table (1): *In-vitro* cytotoxic activity on normal PBMCs and anticancer activity on MCF-7 and Caco-2 cell lines of propolis ethanol extract, tectochrysin and quercetin-3,3'-di-O-methylether expressed as EC_{50} or IC_{50} (µg/mL)

Sample	PBMCs EC_{50}(µg/mL)	MCF-7 IC_{50}(µg/mL)	Caco-2 IC_{50}(µg/mL)
Propolis ethanol extract	9.112±0.639 [b,c]	2.630±0.112[b,c]	1.165±0.123
Tectochrysin	1.894±0.363 [a]	1.247±0.090 [a,c]	0.739±0.0515
Quercetin-3,3'-di-O-methylether	1.232±0.072 [a]	0.355±0.021	0.221±0.002 [a,b]
Mitomycin C	N.D.	0.036 ±0.026	0.023±0.012

All values are expressed as the mean ± SEM. Significance ($p < 0.05$) compared with: [a] Propolis ethanol extract, [b] tectochrysin and [c] quercetin-3,3'-di-O-methylether.

and enhanced cell-mediated immunity [Orsolic et al., 2005a; Sforcin, 2007; Orsolic, 2003; Krol et al., 2007; Blonska et al., 2004). The enhancement of host immune function by propolis may be beneficial to cancer chemoprevention.

For the development of a new anticancer drug, it should be evaluated for its efficacy, safety and cost (Garattini and Bertele', 2002) Based on these criteria, propolis ethanol extracts, tectochrysin and quercetin-3,3'-di-O-methyl ether are evaluated in terms of their efficacy, safety and cost as candidate breast or colorectal anticancer drugs.

4.1 Efficacy of propolis ethanol extract, tectochrysin and quercetin-3,3'-di-O-methyl ether as candidate anticancer drugs

According to the US NCI (National Cancer Institute) plant screening program guidelines, it was recommended that a crude extract is generally considered to have *in-vitro* cytotoxic activity if the IC_{50} value (concentration that causes a 50% cell kill) in carcinoma cells, following incubation between 48 and 72 hours, is less than 20 µg/mL, and less than 4 µg/mL for pure compounds (Boik, 2001)

Hence, according to NCI guidelines the tested propolis sample ethanol extract exerts potential *in-vitro* anti-breast cancer activity with IC_{50} less than 20 µg/mL (2.630 µg/mL) on MCF-7 cell line and extremely potential *in-vitro* anti-colorectal cancer activity with IC_{50} 1.165 µg/mL on Caco-2 cell line (**Table 1**).

Upon comparing the obtained results performed on Egyptian propolis with other propolis types, it was astonishing to observe that ethanol extracts of both Brazilian and Chinese propolis didn't exhibit any inhibitory activitiy on Caco-2 (with IC50 >50 µg/mL) (Ishihara et al., 2009). However, Brazilian red propolis (Kamiya et al., 2012) and ethanol extract of Chinese propolis (Xuan et al., 2014) significantly reduced MCF-7 cell viability. In addition, Caco-2 cells didn't show high sensitivity to Chilian propolis extract with 45% of cell viability after 72 hrs (Russo et al., 2004). Moreover, cell viability in case of Indian propolis at 12 hrs on MCF-7 and Caco-2 cells was found to be, 12% and 40%, respectively (Choudhari et al., 2013).

Furthermore, the two tested pure compounds tectochrysin and quercetin-3,3'-di-O-methylether are also considered to have potential *in-vitro* breast and colorectal anticancer activities with IC_{50} less than 4 µg/mL (1.247 µg/mL and 0.355 µg/mL, respectively) on MCF-7 cell line and (0.739 µg/mL and 0.221µg/mL, respectively) on Caco-2 cell line (**Table 1**).

4.2. Safety and selectivity of propolis ethanol extract, tectochrysin and quercetin-3,3'-di-O-methyl ether as candidate anticancer drugs

It is important to establish that the candidate anticancer drug has anticancer activity at concentrations that can be achieved *in-vivo* without inducing toxic effects to normal cells. The relative effectiveness of the candidate anticancer drug in inhibiting cancerous cells compared to inducing normal cell death is defined as the therapeutic or selectivity index (S.I) and can be calculated using the following expression (FDA, 2006)

$$S.I. = EC_{50} \text{ value}/IC_{50} \text{ value}$$

Where:

Table (2): Selectivity of propolis ethanol extract, tectochrysin (T) and quercetin-3,3'-di-O-methylether (Q) towards breast or colorectal cancer *in vitro*:

Sample	Selectivity index (S.I.) towards MCF-7	Selectivity index (S.I.) towards Caco-2
Propolis ethanol extract	3.46	7.82
Tectochrysin	1.52	2.56
Quercetin-3,3'-di-O-methylether	5.57	3.47

EC_{50}: Concentration that kills 50% of the normal cells (PBMCs in the present study).

IC_{50}: Concentration that kills 50% of the cancerous cells (MCF-7 or Caco-2 in the present study).

It is desirable to have a high therapeutic index giving maximum anticancer activity with minimal normal cell toxicity. Studies determining cytotoxicity and therapeutic indexes should be conducted before the initiation of phase 1 clinical studies (FDA, 2006)

S.I. value indicates selectivity of the candidate drug to the cell lines tested. Drugs with SI value greater than 3 are considered to have high selectivity (Mahavorasirikul et al., 2010)

Calculated S.I. of propolis ethanol extract, tectochrysin and quercetin-3,3'-di-O-methyl ether and the corresponding selectivity towards breast or colorectal cancer *in-vitro* are presented in (**Table 2**).

Upon scrutinizing the obtained data (**Table 2**), we can observe that propolis ethanol extract and quercetin-3,3'-di-O- methylether have selectivity towards both breast and colorectal cancer *in-vitro* (selectivity index greater than 3), while tectochrysin has lower selectivity towards both cancer types.

However, in order to confirm the efficacy, safety and selectivity of Egyptian propolis ethanol extract as well as Quercetin-3,3'-di-O- methylether as candidate breast and colorectal anticancer drugs, further *in-vivo* testing on animal models is of great importance and highly recommended.

5. Conclusion

Quercetin-3,3'-di-O- methylether appears to have potent anticancer activity against the two tested breast (MCF-7) and colorectal (Caco-2) cancerous cell lines; with IC_{50} = 0.35 and 0.22 µg/mL, respectively. Moreover, it shows selectivity towards both cell lines; with S.I.= 5.57 and 3.47, respectively. Hence, quercetin-3,3'-di-O- methylether is considered to be an effective, safe and selective anticancer candidate against both breast and colorectal cancer.

Furthermore, the propolis ethanol extract has high anticancer activity against the two tested breast (MCF-7) and colorectal (Caco-2) cancerous cell lines; with IC_{50}= 2.63 and 1.17 µg/mL, respectively. Moreover, It shows selectivity towards both cell lines; with S.I.= 3.46 and 7.82, respectively. Hence, propolis ethanol extract is considered to be an inexpensive (2 L.E./g), effective, safe and selective anticancer candidate against both breast and colorectal cancer.

6. Conflict of interest

The authors report no declaration of conflict of interest.

7. References

Aso, K., Kanno, S., Tadano, T., Satoh, S., Ishikawa, M. 2004. Inhibitory effect of propolis on the growth of human leukemia U937. Biol Pharm Bull. 27, 727-730.

Bankova, V., 2005. Recent trends and important developments in propolis research. Evid Based Complement Alternat Med. 2, 29-32.

Bankova, V., 2005. Chemical diversity of propolis and the problem of standardization. J Ethnopharmacol. 100,114- 117.

Banskota, A. H., Tezuka, Y., Kadota, S., 2001. Recent progress in pharmacological research of propolis. Phytother Res. 15, 561-571.

Barbaric, M., Miskovic, K., Bojic, M., Loncar, M. B., Smolcic-Bubalo, A., Debeljak, Z., Medic-Saric, M.,

2011. Chemical composition of the ethanolic propolis extracts and its effect on Hela cells. J Ethnopharmacol. 135, 772-778.

Blonska, M., Bronikowska, J., Pietsz, G., Czuba, Z. P., Scheller, S., Krol, W., 2004. Effects of ethanol extract of propolis (EEP) and its flavones on inducible gene expression in J774A.1 macrophages. J Ethnopharmacol. 91, 25-30.

Boik, J., 2001. Natural compounds in cancer therapy: Promising non toxic antitumor agents from plants and other natural sources. Oregon Medical Press, Minnesota, USA..

Borenfreund, E., Puerner, J., 1984. A simple quantitative procedure using monolayer cultures for cytotoxicity assay. J Tissue Cult Methods. 9, 7-9.

Boyum, A., 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1g. Sc and J Clin Lab Invest Suppl. 97, 77-89.

Burdock, G. A., 1998. Review of the biological properties and toxicity of bee propolis (propolis). Food Chem Toxicol. 36, 347-63.

Castaldo, S., Capasso, F., 2002. Propolis, an old remedy used in modern medicine. Fitoterapia. 73, S1-S6.

Choudhari, M. K., Haghniaz, R., Rajwade, J. M., Paknikar, K. M. 2013. Anticancer Activity of Indian Stingless Bee Propolis: An *In-Vitro* Study. Evid Based Complement Alternat Med, Article ID 928280.

El-Bassuony, A. A., 2009. New prenilated compound from Egyptian propolis with antimicrobial activity. Rev Latinoam Quím. 37, 85-90.

El-Bassuony, A., AbouZid, S., 2010. A new prenylated flavanoid with antibacterial activity from propolis collected in Egypt. Nat Prod Commun. 5, 43-45.

Fotakis, G., Timbrell, J. A., 2006. *In-vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett.60, 171-177.

Garattini, S., Bertele', V., 2002. Efficacy, safety, and cost of new anticancer drugs. BMJ, 325, 269-271.

Guidance for industry. Antiviral product development. U.S. Department of Health and Human Services. Food and Drug Administration (FDA). Center for Drug Evaluation and Research (CDER).2006.

Ibrahim, R. S, Wanas A. S., Seif El-Din, A. A., Radwan, M. M., Elsohly, M. A., Metwally, A. M., 2014. Planta Med. 80, 810. Proceedings of American Society of Pharmacognosy 2014 Annual Meeting – Held in conjunction with the 14th Annual Oxford International Conference on the Science of Botanicals, Oxford, Mississippi, USA. 2014 August 2–6

Ishihara, M., Naoi, K., Hashita, M., Itoh, Y., Suzui, M., 2009. Growth inhibitory activity of ethanol extracts of Chinese and Brazilian propolis in four human colon carcinoma cell lines. Oncol Rep. 22, 349-354.

Kamiya, T., Nishihara, H., Hara, H., Adachi, T., 2012. Ethanol extract of Brazilian red propolis induces apoptosis in human breast cancer MCF-7 cells through endoplasmic reticulum stress. J Agric Food Chem. 60, 11065-11070.

Krol, W., Scheller, S., Czuba, Z., Matsuno, T., Zydowicz, G., Shani, J., Mos, M., 1996. Inhibition of neutrophils' chemiluminescence by ethanol extract of propolis (EEP) and its phenolic components. J Ethnopharmacol. 55, 19-25.

Mahavorasirikul, W., Viyanant, V., Chaijaroenkul, W., Itharat, A., Na-Bangchang, K., 2010 Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells *in-vitro*. BMC Complement Altern Med. 10, 55.

Omene, C., Kalac, M., Wu, J.; Marchi, E., Frenkel, K., O'Connor, A., 2013. Propolis and its active component, caffeic acid phenethyl ester (CAPE), modulate breast cancer therapeutic targets via an epigenetically mediated mechanism of action. J Cancer Sci Ther. 5, 334–342.

Orsolic, N., Basic, J., 2003. Immunomodulation of water-soluble derivate of propolis: a factor of antitumor reactivity. Biol Pharm Bull. 84, 265-273.

Orsolic, N., Sver, L., Terzic, S., Basic, J., 2005. Peroral application of water-soluble derivate of propolis (WSPD) and its related polyphenolic compounds and their influence on immunological and antitumour activity. Vet Res Commun. 29, 575-593.

Orsolic, N., Terzic, S., Mihaljevic, Z., Sver, L., Basic, J., 2005. Effects of local administration of propolis and its polyphenolic compounds on tumor formation

and growth. Biol Pharm Bull. 28, 1928-1933.

Prawat, U., Phupornprasert, D., Butsuri, A., Salae, A-W., Boonsri, S., Tuntiwachwuttikul, P., 2012. Flavonoids from Friesodielsia discolor. Phytochem Lett. 5, 809-813.

Russo, A., Cardile, V., Sanchez, F, Troncoso, N., Vanella, A., Garbarino, J. A., 2004. Chilean propolis: antioxidant activity and antiproliferative action in human tumor cell lines. Life Sci. 76, 545-558.

Scheller, S., Krol, W., Swiacik, J., Owczarek, S., Gabrys, J., Shani, J., 1989. Antitumoral property of ethanolic extract of propolis (EEP) in mice-bearing Ehrlich carcinoma. Z. Naturforsch. 44C, 1063-1065.

Sforcin, J. M.., 2007. Propolis and the immune system: a review. J Ethnopharmacol. 13, 1-14.

Simone-Finstrome, M., Spivak, M., 2010. Propolis and bee health: the natural history and significance of resin use by honey bees. Apidology. 41, 295-311.

Xuan, H., Li, Z., Yan, H., Sang, Q., Wang, K., He, Q., Wang, Y., Hu, F., 2014. Antitumor activity of Chinese propolis in human breast cancer MCF-7 and MDA-MB-231 Cells. Evid Based Complement Alternat Med., Article ID 280120.