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ORIGINAL ARTICLE

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Chemical and biological diversity of propolis samples from Bulgaria, Libya, and Egypt

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ABSTRACT

Introduction: Honey bee propolis is an important product because it contains bioactive substances such as total phenolics, flavonoids, and organic acids for treatment of several diseases including cancer.

Aim: In this study, the identity of chemical composition and biological activity of propolis samples from Bulgaria, Libya, and Egypt were examined.

Methods: Chemical composition and biological activity of propolis samples from Bulgaria, Libya, and Egypt were examined using analytical methods and 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide cell-based assay against liver (HepG2), breast (MCF-7), and colorectal (Caco-2) cancer cell lines.

Results: The results showed that Libyan propolis proved to has the most cytotoxic activity among all tested propolis samples HepG2 (84.99%), MCF-7 (83.57%), and Caco-2 (79.63%).

Conclusion: Propolis could be used as cancer therapeutic agent or to complement conventional cancer treatments. It is important for propolis users, such as companies producing propolis preparations, to know the characteristic concentrations of the above-mentioned constituents of this propolis type to guarantee a good quality product with better biological activity.

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Introduction

Cancer is a large group of disorders characterized by uncontrolled cellular proliferation. Cancer cells are also capable of metastasizing to other regions causing a number of devastating outcomes [1]. Nearly all body organs are vulnerable to cancer with liver, colon, and breast being the most common ones. Hepatic cancers are the third leading cause of cancer associated deaths worldwide, and currently, the frequent cause of deaths in cirrhotic patients [2]. Colon cancer has an estimated incidence of over 1 million new cases annually worldwide [3]. Almost one of three patients with colon cancer dies from the disease. Colon cancer also more often affects people of well-developed countries in comparison to less developed countries [4]. Breast cancer is the leading cause of female mortality, more than half a million deaths were reported in 2012. It continues to represent the most frequently diagnosed cancer in females with more than 1.7 million new cases diagnosed in 2012; it represents 25% of all new cancer cases diagnosed in women [5].

Does this mean that we are helpless against cancer? The answer to this question was presented in 1994 in Cancer Letters by Badria [6]. On the other hand, 62 Egyptian food and medicinal preparations were extensively examined for antimutagenic/anticarcinogenic activity using short-term and host-mediated assays. The antimutagenic activity of the substances examined was ranked as follows: 13 (strong), 7 (mild), and 5 (weak) after metabolic activation. Metabolic activation seems to be necessary for most antimutagenic substances in this

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study, e.g., radish inhibits 29% of mutagenicity produced in direct antimutagenic assay and inhibits 89% of mutagenicity induced in host-mediated assay. So, there is an urgent need for the discovery of new regimen for hepatocellular carcinoma (HCC) treatment. Recently, many anti-tumor compounds with new structural features and mechanism of action have been isolated from natural products. Natural products serve as a good and affordable source for new drug entities. Different vaccines and biologics have been inspired from natural products structures, such as betulinic acid and its analogous had an inhibitory activity against topoisomerase [7]. Oleogum resin of *Boswellia carterii* showed anti-proliferative activity on T-lymphocyte culture [8]. Moreover, the combination of *Boswellia serrata*, licorice root (*Glycyrrhiza glabra*), and Tumeric root (*Curcuma longa*) was used in the control of bronchial asthma because of their leukotriene inhibition, anti-inflammatory, and antioxidant activity, respectively [9]. These combinations of terpenoids were also used in the treatment of knee osteoarthritis and hepatitis C [10]. Cucurbitacin proved to have potent *in vitro* and *in vivo* activities toward HCC [11]. Recently, cucurbitacin B used as anti-tumor activity against ovarian cancer cell line (A2780) and as a chemosensitizer for cisplatin cytotoxicity in cisplatin-resistant ovarian cancer cell line (A2780CP) in 2-D and 3-D culture models [12]. Recently, many antitumor compounds with new structural features and mechanism of action have been isolated from natural products. Natural products serve as a good and affordable source for new drug entities [13–19].

Apitherapy (*Apis* is a Latin word means bee) is the practice of using bee products such as propolis for disease prevention or treatment. It can be also described as the science (and art) of using honey bee products, to maintain health and assist the individual in regaining health [20]. Propolis is the resinous mixture that honey bees collect from different sources to use it as a sealant for unwanted open spaces in hives. At least 200 propolis constituents have been identified so far. The most important constituent of propolis seems to be phenolics which constitutes more than 50% of its total weight and are related to a substantial part of its biological activity [21,22]. Thus, the relationship between the flavonoids and propolis biological effects reveal the interest of quantifying these constituents in propolis preparations [23,24]. Propolis as anti-inflammatory, anti-oxidant, anti-infective, and anti-cancer agents have been studied [25]. The chemical constituents responsible for its beneficial biological activities, and especially for its antimicrobial and antioxidant properties, are well documented: flavonoids (including flavones, flavonols, flavanones, and dihydroflavonols) and other phenolics (mainly substituted cinnamic acids and their esters) [26]. It is important for propolis users, such as companies producing propolis preparations, to know the characteristic concentrations of the above-mentioned constituents of this propolis type to guarantee a good quality product and a reasonable degree of antibacterial activity.

Apitherapy could be used as cancer therapeutic agent or to complement conventional cancer treatments.

Materials and Methods

Ten honey bee colonies with almost the same equal powers and the number of combs were selected, and divided into two groups, each of five colonies:. Group A has Queen of Carniolan hybrid and Group B was headed by the Queen of Italian hybrid**.**

Materials

Propolis

Propolis samples were obtained from three different sources (Figure 1). Two honeybee hybrid strains colonies propolis were collected (Carniolan–Italian). Scraping propolis from the hive is done monthly at the end of each month throughout the season study. The weight of the amount of propolis obtained monthly for a year of study was compared between the amount of propolis obtained from the two hybrid strains (Carniolan–Italian) and the amounts of propolis obtained in different seasons (Autumn–Winter–Spring–Summer).

Materials used in the biological assays

Cell lines, Hep-G2, Caco-2 and MCF-7 (Holding Company for Biological Products and Vaccines, VACSERA, Agouza, Giza, Egypt), Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), an antibiotic/anti-mycotic solution containing 1,000 U/ml penicillin, 1,000 µg/ml streptomycin, and 25 µg/ml fungisone, Phosphate Buffer saline, Dimethylsulfoxide (DMSO), and 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO), 96-well plates, Tissue Culture Treated Polystyrene (#3512, Corning Inc., NY), enzyme linked immunosorbent assay (ELISA) BioTek Lx800 microplate reader

Figure 1. Propolis (Egyptian, Libyan, and Bulgarian).

(BioTek, Bedfordshire, UK) were used for cell-based cytotoxicity assay.

Methods

Propolis

The crude propolis was collected and prepared for chemical and biological studies during 2013–2015. Three honeybee colonies sources, Egyptian, Libyan, and Bulgarian, were used to collect and prepare the propolis samples. They were obtained by scraping propolis from the hive at the end of each month throughout the season.

The scraped resinous material was shade dried, ground in a tooth miller. Each propolis powder was then extracted by 70% methanol under sonication for 1 hour at 60°C until exhaustion. After filtration, the solvent was removed by rotary evaporation under reduced pressure at temperatures below 45°C. Crude extracts were refrigerated at 0°C until used in the assay. DMEM medium was used to prepare serial dilutions from DMSO stock solutions, DMSO limit 0.02% v/v.

Cytotoxicity assay using MTT

Activity as cytotoxic agents against hepatic, colorectal, and breast cancers were tested in a cell-based assay using Hep-G2, Caco-2, and MCF-7 cell lines, respectively, by MTT assay. Cell lines were cultured in complete growth DMEM media containing 10% FBS, 1% penicillin/streptomycin, and incubated at 37°C with 5% CO_2 and 90% relative humidity. All cell passages used were between passages 30–40. Five-Flouro uracil (FU) or cisplatin was used as standard cytotoxic agent. The compounds were dissolved in DMSO free media (water soluble components) or DMSO/Media vehicle so that the DMSO limit doesn't exceed 0.05%.

The viability of cells was measured colorimetrically using MTT assay which indicates mitochondrial metabolic activity. This assay depends on measuring the activity of mitochondrial reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductase that converts the yellow tetrazolium salt, MTT, to a purple formazan product that is water insoluble. After solublization of formazan crystals, the purple solution is easily measured quantitatively using ELISA plate reader at wavelength of 540 nm [27,28].

Cell lines were cultured in 96-well plates (1×10^5 cells/ml). After incubation, the medium was removed and the wells were treated with 100 µl of 5 mg/ml MTT and incubated for 4 hours at 37°C. Then, 100 µl of solubilizing solution, DMSO, were added to each well, and the produced purple solution was quantified colorimetrically at 540 nm.

Statistical analysis

The cytotoxic activity of the test compounds was indicated by the ratio of tested well to negative control and half maximal inhibitory concentration (IC_{50}) was calculated. Cytotoxic activity was calculated from the following formula:

$$\text{Percentage of activity} = \frac{(\text{OD\_(negative control)} - \text{OD\_test})}{\text{OD\_(negative control)} \times 100}$$

where OD means "optical density."

Analysis of data was done using GraphPad Prism V6.01 (GraphPad Software Inc, San Diego, CA). Mean comparisons were made with significant differences reported at $P < 0.05$, replication $n = 3$.

Results

Data summarized in Table 1 and Figure 2 show that in Hep-G2, the Libyan propolis recorded the highest activity with 84.99% inhibition followed by Bulgarian powdered propolis, 58.97%, while the lowest value was with Egyptian propolis, 7.87%. Cinnamic acid showed 12.52% inhibition. In Caco-2, the Libyan propolis recorded highest inhibition, 79.63%, followed by Bulgarian powder propolis, 51.87%. The lowest value was recorded with Cinnamic acid, 22.82%, followed by Egyptian Propolis, 27.73%. While in MCF-7, the Libyan propolis recorded highest activity, it showed 83.57% inhibition, followed by Bulgarian powder propolis, 75.65%. The lowest value recorded was Ferulic acid, 14.89%, followed by Cinnamic acid, 27.06% inhibition.

Table 1. Percentage of cytotoxicity of different propolis with respect to their sources as well as their collection methods, in HEP-G2, Caco-2, and MCF-7 cell lines.

Honey bee products*	% inhibition activity		
	HEP-G2	Caco-2	MCF-7
Control			
5-Flouro uracil*	12.92	69.93	0.00
Cis-platin*	85.30	90.24	86.23
Propolis			
Bulgarian powder propolis	58.97	51.87	75.65
Egyptian propolis	7.87	27.73	73.92
Libyan propolis	84.99	79.63	83.57
Propolis's main components			
Caffeic acid	0.00	0.00	0.00
Cinnamic acid	12.52	22.82	27.06
Ferulic acid	15.11	30.32	14.89

*All compounds were used in concentration of 100 µg/ml.

Discussion

Propolis consider as a traditional medicine and dietary natural products and have recently become the focus of attention in the treatment of certain diseases as well as promoting overall health and well being. There is strong evidence supporting the positive role of natural food and food product on the induction of apoptosis in different tumor cells. In this regard, we investigated the effect of propolis against liver (HEP-G2), breast (MCF-7), and colorectal cancer (Caco-2).

We come to the conclusion that propolis treatments have effect against liver, breast, and colorectal cancer as showed in Figure 2. The affect was ranged between the lowest inhibition activity 7.87% in Egyptian propolis on Hep-G2 and the highest inhibition activity 84.99% in Libyan propolis on Hep-G2. The effects were very clear The IC_{50} for venom at Hep-G2 was 84.99, 58.97, and 7.87 in Libyan propolis, Bulgarian powder propolis, Egyptian propolis, respectively. As the IC_{50} at Caco-2 was 79.63, 51.87, and 27.73, in Libyan propolis, Bulgarian powder propolis, Egyptian propolis, respectively, while the IC_{50} at MCF-7 was 83.57, 75.65, and 73.92 in Libyan propolis, Bulgarian powder propolis, Egyptian propolis, respectively.

Propolis showed remarkable effects on MCF-7, Hep-G2, and Caco-2. It inhibited the growth of the cells. The cytotoxic activity of the different Propolis types can be correlated to their composition. The most active as anticancer were those with higher flavonoids and total phenolics composition content. These results are in agreement with Li et al. [29] who discussed the cytotoxicity of 13 cylcoartane-type triterpenes and four prenylated flavanones, isolated from propolis collected in Myanmar against a panel of six different cancer cell lines [30,31]. The hypothesis is that breast cancer cells' viability gradually decreases depending on the increasing dose of caffeic acid phenethyl ester (CAPE). The estimated IC_{50} value by Wu et al. [32] amounted to 15 µM for MDA-MB-231 and MCF-7 cell lines was only slightly higher than the results obtained in our experiment, with 14.08 and 8.01 µM for MDA-MB-231 and Hs578T, respectively [33]. Propolis and the CAPE substantially inhibit the growth of the cells of triple-negative breast cancer of the lines MDA-MB-231 and Hs578T. The cytotoxic activity of compounds depends on the time of exposure and the concentration of the CAPE and ethanol extract of propolis [34]. Propolis using both anti-inflammatory (tumor necrosis factor- α (TNF- α), cyclooxygenase-1 (COX-1), and cyclooxygenase-2 (COX-2)) and anti-colon cancer (DLD-1 colon cancer cell viability) assays; and determined the phenolic compounds responsible for the activity. Propolis tincture solids had very high levels of the dihydroflavonoids pinocembrin and pinobanksin-3-O-acetate, and high levels of the dimethylallyl, benzyl and 3- methyl-3-butenyl caffeates relative to CAPE. It showed good broad-spectrum activity in anti-proliferative assays against three other gastro-intestinal cancer cell lines, HCT-116 colon

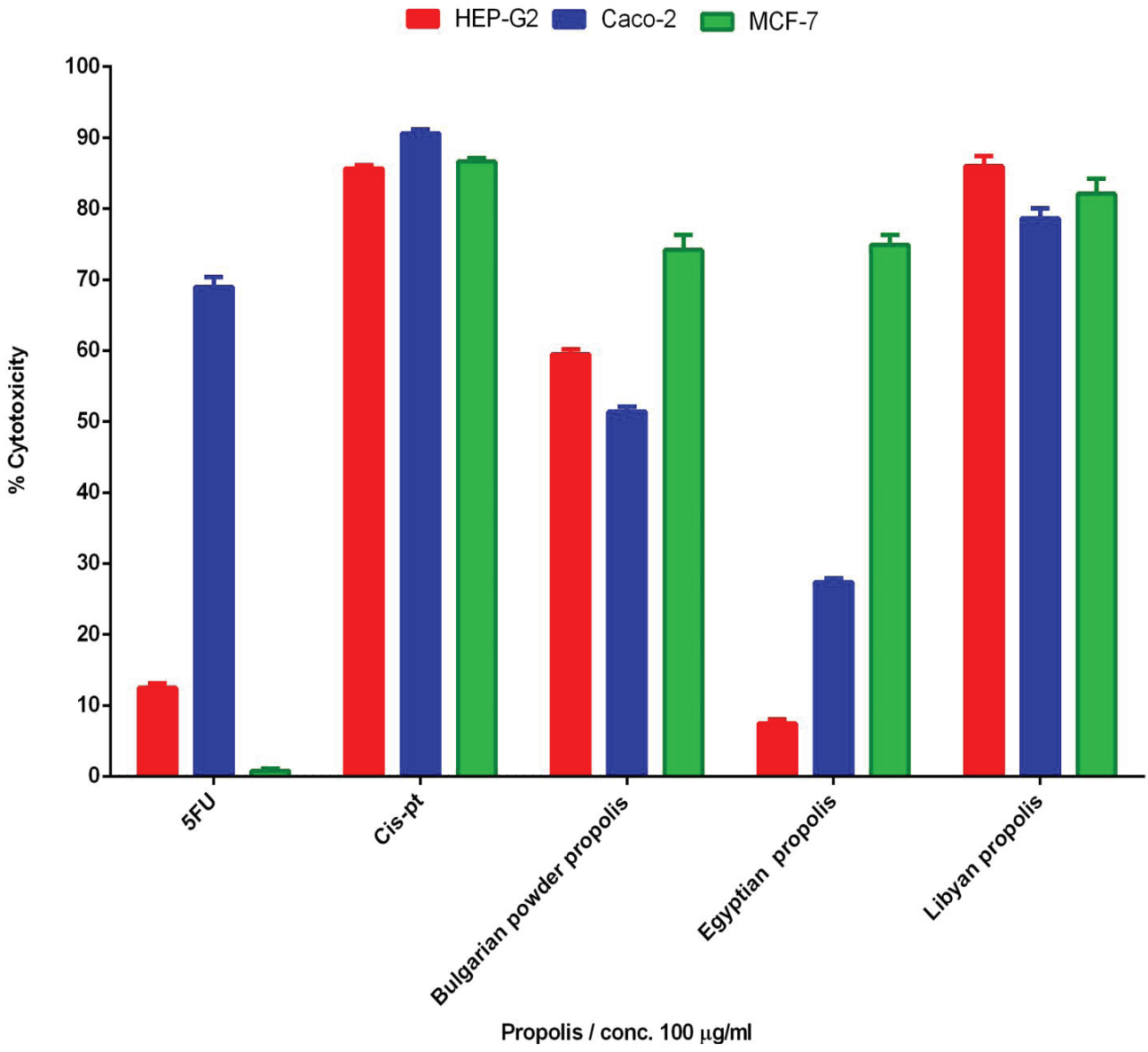

Figure 2. Percentage of cytotoxicity of different propolis at 100 µg/ml against Hep-G2, Caco-2, and MCF-7 cell lines, using 5-FU and cis-platin (cis-pt) as positive control.

carcinoma, KYSE-30 oesophageal squamous cancer, and NCI-N87 gastric carcinoma [35].

We suggested that the effects of propolis on breast cancer (MCF-7) may alter the activity of carcinogen biotransformation enzymes by modulating Phases I and II enzymes [36], and inhibit angiogenesis diminished VEGF expression. It suppresses metastatic growth by decreasing hypoxic survival and STAT3 activation [37], inhibits HDAC8 enzymatic activity [38]. As liver cancer (HEP-G2), it reduces inflammation by decreasing the expression of COX-2 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) p65 levels, induces apoptosis by decreasing the levels of p53, Bax, caspase 3, β-arrestin and Bcl xL [39], attenuates the canonical Wnt protein and NF-κβ signaling pathways, up regulates apoptotic gene expression [40], and down regulates the β-catenin expression [41] and colorectal cancer (Caco-2). It inhibits cell proliferation, recovers antioxidant mineral levels, and reduces nitrosative stress [42].

From the previous results, it could by summarized that propolis under treatments exhibits potential cytotoxic effect on Hep-G2, Caco-2, and MCF-7.

Conclusions

The results showed that propolis is an interesting agent that has valuable activities against Hep-G2,

Caco-2, and MCF-7 with propolis being an effective agent that might be incorporated in cancer remedy regimens after further studies.

Conflict of Interest

There is no conflict of interest to disclose.

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