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Antioxidant and Anti-Mycotoxin Activities, and Cytotoxicity Properties in Vitro of Propolis Extracts

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Abstract

The present study aims to determine the antioxidant activity and cytotoxic activity of propolis and evaluate the anti-mycotoxin activity of different propolis extracts. The three extracts of propolis (ethyl acetate, ethanol (75%), and water) were subjected to testing of the antioxidant potential, and total phenolic and flavonoid contents. determination of the cytotoxicity activity by standard cell culture method against colon and liver and the ability of propolis extracts to control aflatoxin (AF) production. The results revealed that the highest value of antioxidant activities was found for propolis ethyl acetate extract with an average % inhibition of 63.42%. the highest amount of propolis's total phenolic and flavonoid contents using ethyl acetate extract were 149.5 µg gallic acid equivalent/mg extract and 218.1 µg quercetin equivalent/mg extract, respectively. The three propolis extracts affect liver and colon human cell cancer. Ethyl acetate extract of propolis showed the lowest IC_{50} with the highest anti-cancer activity on the liver cancer cell line. In comparison, water extract showed the lowest IC_{50} with the greatest anticancer activity on the colon cancer cell line (50 and 54 µg/ml, respectively). Using different concentrations of propolis to reduce AF levels led to the complete disappearance of AF production at 100, 200, and 400 mg/ml concentrations of Ethyl acetate extract against AF production. In conclusion, these obtained findings indicated that propolis extracts exhibited substantial antioxidant and anti-cancer activities.

Keywords: *Propolis extracts, Antioxidant, Aflatoxin production, Flavonoids, Phenols, Cytotoxicity.*

1. Introduction

Propolis is a naturally occurring and sticky compound, similar to bee glue, that honey bees (*Apis mellifera*) make from resins, saps, and mucilage taken from different parts of the plant and mixed with beeswax and many bee enzymes [1]. Honey bees use propolis to repair hive damage, improve interior walls, keep the hive at a steady humidity and temperature, and provide protection for the colony from predators, parasites, and pathogenic microorganisms [2]. Propolis was historically preferred by the ancient Egyptians as an embalming substance because it was the ideal plastic substance to shield the mummy from bacteria, fungi, and viruses [3]. The medicinal action of propolis has been extensively used in traditional medicine across various cultures [4]. It has various biological activities, including antioxidant, antibacterial, antifungal, immunomodulatory, anti-inflammatory, anticancer, and antibiotic properties [5,6].

Propolis compounds have anticancer activity, affecting key cancer development processes like cell proliferation, angiogenesis, invasion, evading apoptosis, and metastasis. Propolis and its constituents also chemo-sensitize cancer cells with multidrug resistance and can be beneficial for patients receiving chemotherapy and radiotherapy to reduce their negative effects [7]. Propolis has been reported as a potent anti-inflammatory, immunomodulatory, and anticarcinogenic agent in Asian medicine, while it has been used in South America as an antioxidant and antimicrobial agent [8, 9]. Recent studies have shown the anticancer activity of propolis both *in vitro* and *in vivo*, and its mechanism of action, including its active compounds (flavonoids) against various cancer cell lines [10, 11].

Aflatoxins (AF) are poisonous secondary metabolites produced by Aspergillus species, some of which have been linked to human cancer (AF of the B and G series) [12]. The high chemical stability of these substances makes them resistant to heat, extreme pH values, high pressure, and mild chemical treatments, resulting in contamination in processed products, including animal-derived ones, meat, milk, and eggs, mainly from *in vivo* hydroxylation reactions. AF contamination is a significant issue in tropical and subtropical areas because the conditions are favorable for fungal growth. Mediterranean regions have been severely contaminated with AF due to temperature rise, climate change, and droughts [13]. Consumption of food contaminated with AF-B1 caused deleterious effects on different body systems, making it a risk to human and animal health and it was responsible for economic losses [14]. Propolis showed an effective role against many pathogenic microorganisms and counteract the effects of toxic material [15]. Therefore, the current study aims to determine the antioxidant activity, and anticancer properties of propolis and evaluate the anti-mycotoxin activity of propolis extracts.

2. Materials And Methods

Materials

Chemicals, reagents, and cell lines

Solvents (Ethyl acetate, Ethanol (75%), and dimethyl sulfoxide (DMSO) were purchased from El-Gomhouria Co, Egypt. Cell lines and culture conditions were obtained from the National Cancer Institute, Cairo, Egypt. Propolis powder was purchased from Imtenan health shop, in Egypt.

Methods

Preparation of propolis extracts

According to Wagner- Huber, et al., [16], the extraction was performed. Three extracts were prepared (ethanol 70%, ethyl acetate, warm distilled water up to 40°C). The propolis powder was dissolved in each of the 1:10 Stock solution organic solvents until exhaustion (1-3 times), with frequent shaking occasionally. The extracts were filtered and concentrated at a reduced temperature (40°C) in an oven for *72* hours. Then, the obtained extracts were stored in a refrigerator at 4ºC in dark glass bottles.

Determination of the total phenolic compounds

The propolis' total phenolic content was assessed by the spectrophotometric microplate reader FluoStar Omega using gallic acid as a standard according to the Folin–Ciocalteu method [17]. In a 96-well microplate, 10 µl of sample or standard were mixed with 100 µl of Folin-Ciocalteu reagent (Diluted 1: 10). About 80 µl of 1M Na2CO3 was added to the mixture, incubated at room temperature for 20 min in the dark, and the resulting blue complex color was measured at 630 nm. A stock solution of gallic acid was prepared as 1 mg/ml in methanol, and the various dilutions were prepared as follows: 100, 200, 400, 600, 800, and 1000 µg/ml. The sample was prepared at a concentration of 4mg/ml DMSO.

Determination of flavonoid content

The propolis' flavonoid contents were assessed using a spectrophotometer (microplate reader FluoStar Omega) at 420 nm [18]. A stock solution of standard rutin was prepared at 1000 µg/mL in methanol. Then several dilutions were prepared: 10, 50, 100, 400, 600, and 1000 µg/ml. The sample was prepared at a concentration of 4mg/mL DMSO using 15 µl of sample or standard, followed by the addition of 175 µl of methanol and 30 µl of 1.25 % AlCl3. Then, 30 µl of 0.125 M C2H3NaO2 was added to the mixture and incubated for 5 min. Finally, the obtained yellow color was measured at 420 nm.

Determination of propolis as an anti-free radical (DPPH)

According to Devequi-Nunes et al. [19] used 1, 1-diphenyl-2-picrilidrazil (DPPH) to determine the antioxidant activity of propolis. The IC50 value was calculated by the line equation according to the levels of extracts and their corresponding proportions of radical DPPH absorption. After 20 min incubation at room temperature, the absorbance inside the plate was measured at 540 nm. Using the formula RSA = 100 (A control – A sample)/A control to determine the extract's radical scavenging activity (RSA).

Cytotoxic activity

The human hepatocellular carcinoma cell line (HepG2) and human colorectal cancer cell line (HCT116) were grown at 37 °C in a humidified environment supplied with 5% CO2 in Dulbecco's Modified Eagles Medium (D-MEM) containing 5% fetal calf serum, 100 UI/mL penicillin, 100 g/mL streptomycin, and 0.2% sodium bicarbonate. The cells' viability was determined by Orellana and Kasinski in vitro Sulforhodamine B test [20]. Cell lines were diluted and tested for cytotoxicity using SRB assay. The

cells were planted in 96-well plates with trichloroacetic acid (TCA), then 100 mL of a new medium comprising different concentrations of propolis was added and incubated for 48 hrs, then treated with 50 µL of the cold TCA to terminate the assay. The media was removed, washed, and dried. Control was drug-free culture media. Each well received 50 µL of 0.4% w/v SRB in 1% acetic acid and was incubated at room temperature for 20 min. After staining, the washing of plates was done with 1% acetic acid and air-dried. Trizma base eluted bound stain and the absorbance of the cells was measured at 540 nm and 640 nm. Plate-by-plate percent growth was determined compared to control wells.

Detection of AF

High-performance liquid chromatography (HPLC) assessed AF generated by A. parasiticus in sub-MIC extract quantities. Five milliliters of spore suspension containing 0.4 104 CFU/ml in PDB medium then incubated for seven days at 35 °C. Each well was centrifuged for 5 min at 3500 rpm. HPLC was used to identify AF in culture media (Scanning Fluorescence Detector Water 474 at 365 nm); AF in samples was quantified by comparing the under-curved region to genuine standards [21]. The experiment was carried out at the Animal Health Research Institute, Agricultural Research Center, Ministry of Agriculture, Cairo, Egypt.

Statistical analysis

All tests were done in triplicate. Statistical analysis was done using GraphPad Prism 5.01 software. Comparison of data groups was done by one-way ANOVA followed by Newman-Keuls post hoc test. All results were expressed as means ± SD.

3. Results and Discussion

Phenols, flavonoid content, and antioxidant activity of propolis extracts.

The active compounds in propolis extracts were estimated quantitatively and the results of the phytochemical analysis of propolis extracts were presented in Figure (1). The highest contents of both total phenolic and flavonoid in propolis were obtained using ethyl acetate extract, 149.5 µg gallic acid equivalent/mg extract, and 218.1 µg quercetin equivalent/mg extract, respectively. However, the ethanol extract contained the lowest amounts of total flavonoid content (17.8µg quercetin equivalent/mg extract), and the lowest amount of total phenolic content was observed in the water extract. The results obtained agree with the finding of Miłek, et al., [22] which indicated that a high content of phenolic compounds was found in all propolis extracts. One of the most significant classes of bioactive components in propolis is known to be phenolic compounds, particularly the portion of flavonoids and phenolic acid derivatives [23]. Similar results were obtained in the previous study [24] declaring that the ethanolic extract of Polish propolis contained 137.19 mg GAE/g of extract. While the flavonoid content ranged from 18.76 - 93.13 mg QE/g for 70% ethanol extract of Polish propolis as reported by Wezgowiec, et al., [25]. Poplar propolis is primarily composed of phenolic acids, flavonoids, and esters [26].

Natural products, including alkaloids, tannins, flavonoids, and phenolic compounds, have therapeutic benefits as traditional medicines for treating diseases, providing valuable knowledge for drug discovery. Plants contain natural antioxidant compounds that can prevent free radical-mediated oxidative reactions, potentially benefiting the human body from diseases [27]. Flavonoids' pharmacological activity is primarily attributed to their tricyclic compound structure and the radicals that are attached to their rings [28]. Flavonoids and phenolic acid have been attributed to the successful use of propolis as an anti-inflammatory and healing agent [29]. The flavonoids and phenolic chemicals that are present in propolis can eliminate free radicals and protect lipids and vitamin C from destruction in the oxidative process [30]. Therefore, propolis is increasingly popular among consumers due to its inclusion in various products such as drinks, foods, cosmetics, chewing gum, and toothpaste [31]. Phenols and flavonoids in propolis act as scavengers for free radicals in the human body [32]. The high content of phenolic compounds in all propolis extracts constitutes one of the most crucial bioactive substances in propolis [23].

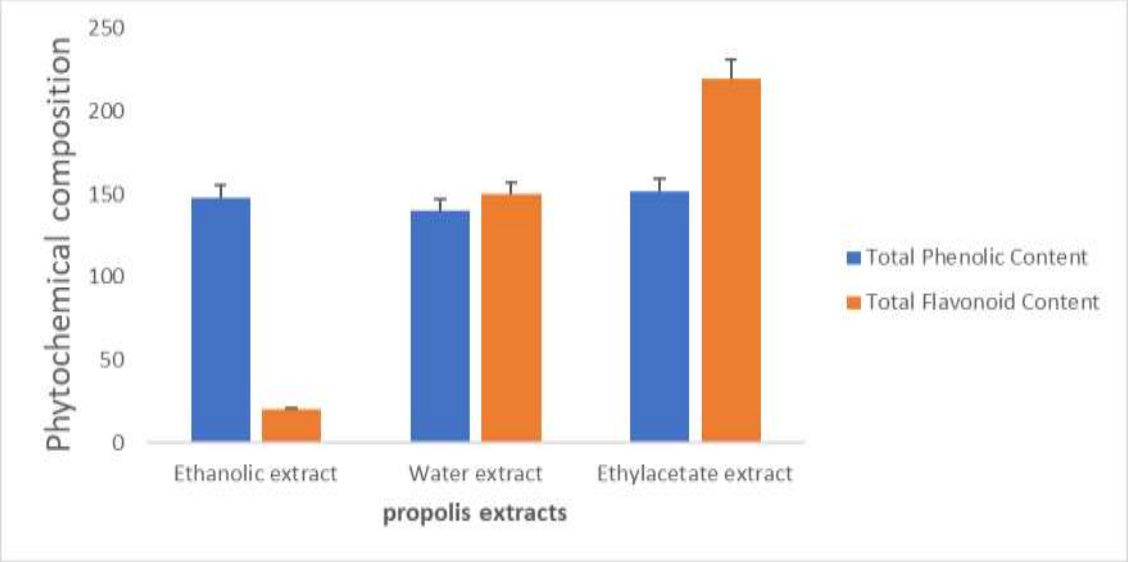

Fig 1. Phytochemicals analysis of phenolic and flavonoid contents of extracts

Evaluation of propolis antioxidant activity by DPPH assay using Trolox as standard

The antioxidant activity of propolis extracts was assessed using the DPPH method. The results showed the antioxidant capabilities of propolis (Table 1). Data revealed that the highest value of DPPH scavenging activity was for propolis ethyl acetate extract revealing that the trolox equivalent value was equal to 552.5 µM T eq/mg with average inhibition percent 63.42%, followed by water extract, and then ethanol extracts, which contained 613.3 and 1009.8 µM T eq/mg, respectively. The results agree with that obtained by Sun, et al., [33] who stated that the propolis extracts with complex phenolic composition have higher antioxidant activities compared to those with lower phenolic content.

These activities are attributed to phenolic compounds, therefore, it serves as a convenient source of natural antioxidants and a dietary supplement, enhancing human health and preventing oxidation-related illnesses [34]. Studies using the DPPH method evaluated propolis' antioxidant activities and reported a correlation between flavonoids and phenol content and their antioxidant effect [35]. Propolis samples with the most flavonoids and phenol content showed the highest antioxidant effect [36]. Propolis is rich in organic compounds, including polyphenolics (58%), and flavonoids (28%), which are essential for its antioxidant properties [37]. It was reported that propolis has higher antioxidants than honey [38].

Plant metabolism produces bioactive substances with diverse chemical properties and various biochemical and physiological activities. These adaptable molecules are difficult to determine their exact function, but they share common antifungal and antioxidant properties, making them adaptable molecules [39, 40].

Table 1. Antioxidant activity (DPPH assay) of propolis extracts

Propolis Extraction	The extracts		
	Ethanol extract	Ethyl acetate	Water extract
DPPH scavenging activity (µM TE/mg)	1009.8±44.70	552.5±18.40	613.3±21.20
Average % inhibition	30.95	63.42	68.58

-Data is expressed as mean ± SD.

Cytotoxicity and growth inhibitory potential on HepG2 and HCT 116 cancer cell lines *in vitro* (MTT assay)

The three propolis extracts were prepared with different concentrations of 500, 250, 125, 62.5, and 0 µg/mL, against liver HepG2 and colon HCT 116 cancer cell lines.

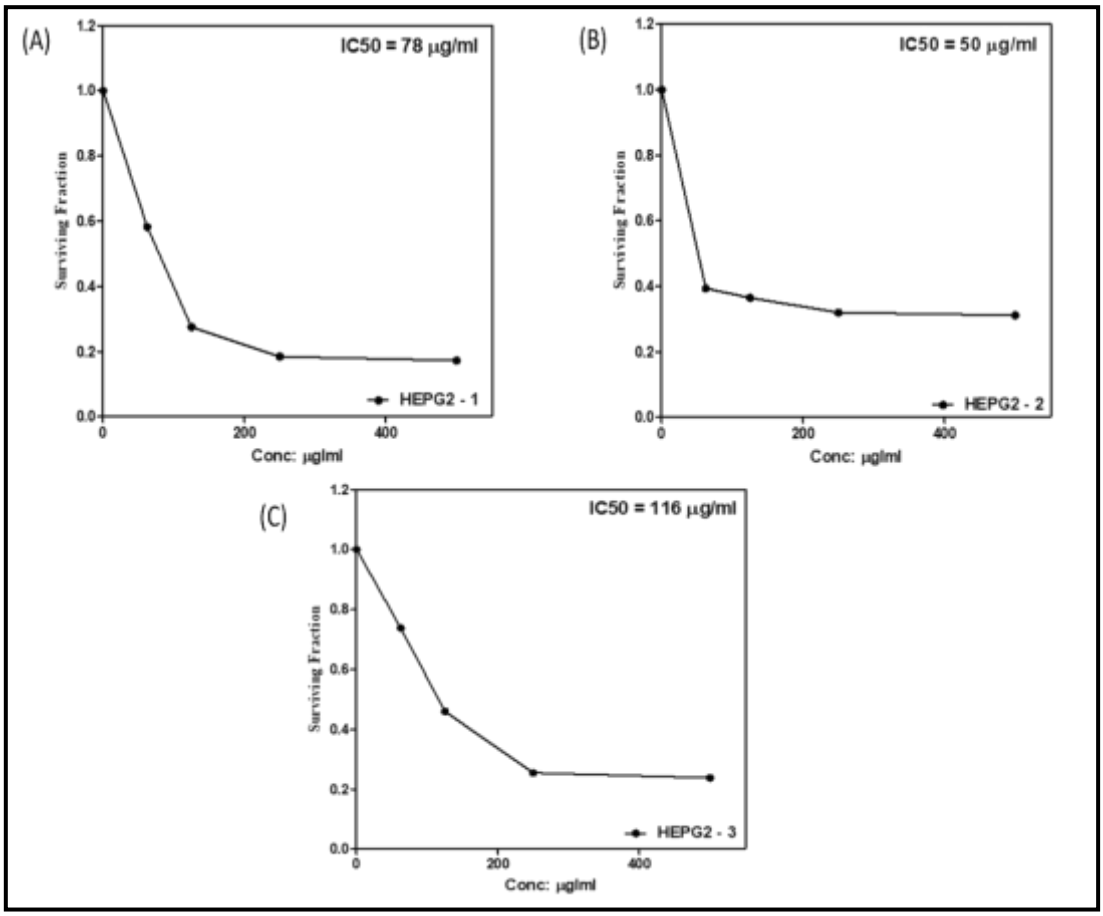

Fig 2. Cytotoxic effects of different propolis extracts on liver HepG2 cancer cell line (A); Ethanol extract (B); Ethyl acetate extract (C); Water extract.

The results are presented in Figures 2 & 3. The data illustrated in Table (2) showed decreased percentages of survival rates against different concentrations of propolis extracts against human liver HepG2 cancer cell lines. All propolis extracts (fig. 2) of liver HepG2 cancer cell lines against all the concentrations 62.5, 125, 250, and 500 µg/ml with a positive correlation between propolis concentrations and cell destruction. All concentrations of propolis extracts appreciably reduced the percentage of live and apoptotic tumor cells in a dose-dependent manner, again leading to an increase in the number of dead cells with increasing doses. In these conditions, the Ethyl acetate extract of propolis showed the lowest IC50 with the highest anti-cancer activity (50 µg/ml), followed by the ethanolic extract (78 µg/ml), and finally, the aqueous extract of propolis showed the highest IC50 with the lowest anti-cancer potential when compared to aqueous extract (116 µg/ml). The data shown in Fig. (3) showed a decrease in survival rates against different concentrations of propolis extracts against HCT 116 human colon cancer cell lines.

Using propolis extracts of different concentrations in Table 3 showed that the cytotoxicity of HCT 116 human colon cells exposed to concentrations 62.5, 125, 250, and 500 µg/ml were decreased in a dose-dependent manner up to concentration 500 µg/ml, with a positive correlation between propolis extracts concentration and cell destruction. The aqueous propolis extract showed the lowest IC50 with the highest anticancer activity, followed by ethyl acetate extract, and finally, the ethanolic extract of propolis showed the highest IC50 with the lowest anti-cancer potential (54, µg/ml) compared to the ethanol and ethyl acetate extracts (88, and 77 µg/ml, respectively). Briefly, based on MTT assay results the anti-cancer activity of propolis ethyl acetate and water extract showed the lowest IC50 value in examined both liver and colon cancer cell lines with values equal to 50 and 54 µg/ml respectively. Propolis can inhibit the growth of HepG2 and HCT 116 cancer cell lines. In this regard, propolis extract is more effective in treating liver and colon cancer with fewer side effects. At the same time, warm water extract of propolis can be used orally for the treatment of colon cancer.

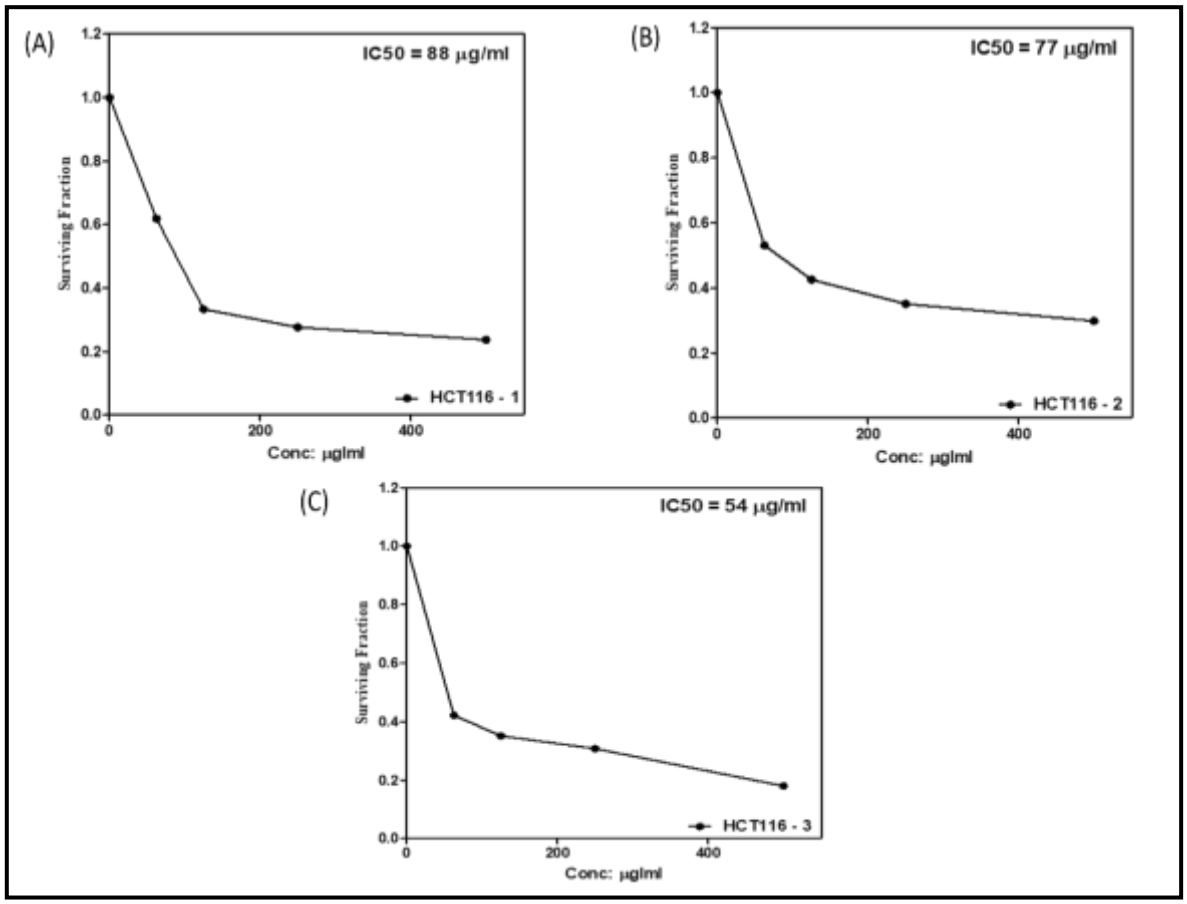

Fig 3. Cytotoxic effects of different propolis extracts on colon HCT 116 cancer cell line (A); Ethanol extract (B); Ethyl acetate extract (C); Water extract.

In line with the current results, Elkhenany et al., [41] accounted for the free radical scavenging and cytotoxicity of propolis to its various natural compounds. Propolis a long-standing known for a long time for its anesthetic, antioxidant, anti-tumoral, anti-cancer, anti-hepatotoxic, anti-septic, anti-mutagenic, and cytotoxic activity [42]. Turkish propolis extract induces apoptosis of cancer cell lines and stimulates the production of cell cycle p21 proteins, both of which result in cell cycle arrest [43]. These same propolis samples exhibited moderate antiproliferative effects on cancer cell lines when tested with the MTS technique. Propolis could inhibit colon, breast, liver, and lung cancer cell line proliferation [44]. The cytotoxic activity of dietary flavonoids on various human cancer types showed proapoptotic activity in the HepG2 cell line [45, 46].

Flavonoids from plant foods and bee products may offer cancer chemoprevention and anti-cancer phytotherapy, causing cytotoxicity, apoptosis, and cell cycle arrest in cancer cells. These properties are related to the flavonoid content of propolis [47]. Different honey samples collected from different places in Palestine and Morocco exhibit a significant cytostatic effect upon the treatment of HCT cells [48]. In addition, the cytostatic activity of MCF cells is strongly correlated with the antioxidant content (phenols, flavonoids, and flavonols). Research has demonstrated the anticancer properties of various propolis varieties from diverse geographical regions, using human cancer-derived cell lines [49]. Additionally, the ethanoic extracts of propolis are rich in phenolic acid and flavonoids, which may have chemo-protective properties in cancer cells by scavenging free radicals [50].

Several cancer cell lines showed antiproliferative activity when exposed to propolis. Reports suggest that propolis can inhibit oncogene signaling pathways, reduce cell proliferation, and increase apoptosis. In addition to antiangiogenic effects, and modification of the tumor microenvironment [26, 51]. Brazilian green propolis has been found to significantly contribute to the management of chemokine-mediated inflammation [52]. Moreover, the natural extracts can be combined with conventional chemotherapeutic regimens to offer a safer and more effective cancer treatment [53]. It also could be used as functional polymer microparticles for encapsulating biologically active compounds [54]. It is worth mentioning that Egyptian propolis' ethanolic extract has cytotoxic effects in various cancer cell lines, including colon cancer, MDA-MB-231, MCF-7, and HeLa, making it a potential addition to conventional chemotherapeutic regimens for safer and more effective cancer treatment [55].

Effect of propolis extracts on AF production

Table 2 presents the levels of different concentrations (40 - 400 mg/ml) of propolis and its effect on AF Production (B1, B2, and G1 AF). By using different concentrations of propolis in Table (2) data showed a reduction of the AF's levels with complete disappearance of AF reported at 100, 200, and 400 mg/ml concentrations of ethyl acetate extract, followed by ethanol extract (200 and 400 mg/ml concentration respectively. Whereas water extract had the lowest response against AF production (B1, B2, and G1 AF). Conclusively, propolis extract demonstrated the ability to inhibit Aspergillus brasiliensis growth, which reduces AF production. In this respect, Shehata, et al., [56] investigated the antifungal activities of propolis ethanol extract. The Egyptian and Chinese propolis ethanol extracts demonstrated strong antifungal potency against high AF-producing Aspergillus flavus ITEM 698 and Aspergillus parasiticus ITEM 11, indicating their potential as effective antifungal agents against toxigenic fungi.

Table 2. Effect of various concentrations of propolis extracts (**µg/ml**) on AF Production

Propolis Extracts	Concentration of propolis mg/ml	AFB1	AFB2	AFG1
Ethanol extract	40	22.3	4.50	0.50
	60	8.70	ND	0.90
	80	0.85	ND	0.20
	100	0.30	ND	ND
	200	ND	ND	ND
	400	ND	ND	ND
Ethyl acetate extract	40	19.50	2.40	0.25
	60	7.30	ND	1.30
	80	0.57	ND	ND
	100	ND	ND	ND
	200	ND	ND	ND
	400	ND	ND	ND
Water extract	40	25.4	7.50	0.75
	60	9.6	2.40	1.50
	80	1.2	0.57	ND
	100	0.6	ND	ND
	200	0.2	ND	ND
	400	ND	ND	ND

AF: AF, ND: Not detectable

Loi et al., [57] suggest that bioactive phenolic compounds and propolis ethanol extract may regulate or suppress mycotoxin production during fungal growth or life cycle. AF, a deadly mycotoxins class, has been linked to various adverse effects in mammals, including mutagenic, carcinogenic, hepatotoxic, teratogenic, immunosuppressive, estrogenic, and histopathologic effects [58]. AF poisoning, a food-borne disease, has been reported in numerous countries to cause severe illness and death in humans and animals after consuming contaminated foods [59]. Bioactive compounds have been found to reduce the ability of toxigenic fungi to contaminate food due to their anti-mycotoxigenic potential and decreased AF levels. These compounds inhibit Aspergillus growth, secondary metabolism, and AF production, degrade AF, and, in some cases, detoxify them [57].

4. Conclusion

Propolis extracts revealed significant antioxidant and anticancer activity and the ability to control AF production. The antioxidant properties of propolis extracts are crucial in combating oxidative stress and reducing cellular damage caused by free radicals, suggesting their possible application in cancer prevention and treatment strategies. These extracts have shown promising potential as natural sources of antioxidants for various health benefits. The ability of propolis extracts to inhibit AF production highlights their potential in food safety and toxin control measures. Further research and in vivo studies are required to confirm these findings and explore their potential therapeutic applications.

Compliance with the ethical statement

All authors of this paper have no conflict of interest.

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Contribution

The authors were equally involved in writing the manuscript.

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