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Anti-fungal activity of five studied plant extracts compared with propolis and their effect on aflatoxigenic production and expression of aflatoxin biosynthesis genes of studied *Aspergillus flavus* isolates

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Abstract: This study was designed to evaluate fungal activity of five plant extracts namely; *Ammi majus*, *Camellia sinensis*, *Trifolium alexandrinum*, *Humulus lupulus,* and *Linum usitatissimum* (seeds), and propolis against an aflatoxin-producing *Aspergillis flavus* isolate. Their ability to inhibit production of four aflatoxins (B_1, B_2, G_1 and G_2) was studied, too. Four isolates of *Aspergillus flavus* were isolated from infected wheat grain collected from local markets at Alexandria governorate, Egypt. Fungal isolates were molecularly identified as; *A. flavus* 1 (acc#: OR477304), *A. flavus* 2 (acc#: OR478624), *A. flavus* 3 (acc#: OR478625) and *A. flavus* 4 (acc#: OR477305). Six concentrations of the five plant extracts and propolis were used (0.0, 0.01, 0.05, 0.1, 0.5 and 1 g/50 ml) to study their anti-fungal activity against *A. flavus 3* (OR478625). All treatments produced significant reduction in the percentage of fungal biomass's growth when compared to control. The highest reduction in fungal biomass by *A. majus* (50.8%) and *T. alexandrinum* (54.6%) appear at 0.01 and 0.5 g/50ml. For *C. sinensis* the highest reduction in growth biomass (60.6%) appear at 0.01 g/50ml. In contrast to *H. lupulus* and *L. usitatissimum,* the highest reduction in growth biomass appears at 1 g/50ml are 38.4% and 61.6% respectively. Propolis exhibited the highest reduction in fungal biomass (48.2%) at 0.5 g/50 ml. Production of AFs by *A. flavus* OR478625 in broth medium were significantly affected by plant extracts, in contrast to propolis. The plant extract of *A. majus* cause complete inhibition of the four types of AFs production (B_1, B_2, G_1 and G_2) at all studied concentrations. As for *C. sinensis* extract complete inhibition appeared for only three types of AFs (B_2, G_1 and G_2) under all concentrations. In addition, *H. lupulus* extract caused complete inhibition for the four AFs at 0.5 and 1% concentrations. As for *T. alexandrinum* extract complete inhibition of the four AFs was achieved at 0.01 and 1%. Regarding *L. usitatissimum* extract AFG_1 was inhibited at all concentrations. Propolis completely inhibited the four studied aflatoxins at 0.5 and 1 %. Quantity of studied secondary metabolites varies from one extract to others. Phenolics ranged from 121 to 322 mg/g, flavonoids from 50 to 85 mg/g, ascorbic acid from 10 to 36 mg/g, saponins from 17 to 36 mg/g and tannins from 11 to 35 mg/g, for studied plant extracts. Three methods were used for studding antioxidant activity. For FRP and DPPH methods the highest antioxidant activity appeared with *A. majus* (35.8±3 mg/g and 81±3%) respectively, but for PMA methods the highest antioxidant activity appeared with *H. lupulus* (30±2

mg/g). Meanwhile, propolis exhibited 30.8, 76.4 and 11.5 antioxidant capacity using FRP, DPPH and PMA methods, respectively. Additionally, treatment of *A. flavus* 3 with *A. majus,* showed remarkable variations in genes expression when compared to control. This study presented the antioxidant properties, antifungal capacity and inhibitory effect on aflatoxin production of the studied plant extracts and propolis, as well.

Keywords: *Aspergillus flavus*; aflatoxins regulation; plant extracts; *A. majus*; *C. sinensis*; *T. alexandrinum*; *H. lupulus*; *L. usitatissimum*; propolis.

1. Introduction

Aflatoxins (AFs) are mostly predominant in main food crops such as sorghum, maize, wheat, and in groundnuts, tree nuts, spices as well as milk and meat products [1,2]. Aflatoxin contamination relies mainly on growing and species of *Aspergillus* and storage conditions, combined with management practices employed and many further factors [3,4]. Accumulation of aflatoxins can make through the food chain which considered a severe health impact to humans [5]. Unfortunately, exposure of human to low doses of aflatoxins may resulted in stunted young children growth, cancer induction and immune suppression [6], so a worldwide priority for human health is controlling the spread of aflatoxigenic *A. flavus* in cereals and food products during storage [7]. Climate changes one of the important factors which effect on pro and post-harvest contamination with aflatoxins [1,3]. These climate changes have great effect on the relations among various mycotoxigenic species and the relative mycotoxin contamination of staple commodities [8]. Number of climate factors as temperature and water availability have important effect of growth of aflatoxins producing organisms [9].

Production of these highly toxic aflatoxins which are considered one of the highly toxic secondary metabolites of *A. flavus, A. parasiticus*, and *A. nomius* [10]. *Aspergillus* species are filamentous fungi that are spreaded widely in variety of environments and substrates, decaying vegetation, seeds and grains, where they live as saprophytes, but some species as parasites in a wide range of hosts including humans and animals [11]. Most *Aspergillus* species have versatile features enabling them to persist in various ecological environments [12,13].

Several methods have been developed to control aflatoxins production through controlling aflatoxins producing fungi. These methods are including natural methods as using plant extracts which considered as ecofriendly, safe, cheap, biodegredable and alternative treatments to chemicals [14]. Plant leaves and fruits are famous by containing numerous compounds with biological and medicinal properties. Plant extracts are considered a high source of valued products that are valuable as useful drugs, or drug adjuvants, pharmaceutical uses, in food industry as food constituents, food flavors, and antioxidants as well. For example, bioactive compounds which could have anticarcinogenic, antioxidant, and antimicrobial abilities may be tannins, alkaloids, flavonoids, phlobatannins, terpenoids, and glycosides [15]. Add to that, linolenic acid and allylphenol that are characterized by their reduction of fungal growth as *Rhizoctonia sp* and *Pythium sp* and also decreased biomass production and they have been stated to be active against a number of other plant pathogens [16].

Ammi majus (*A. majus*) a wild plant with medicinal uses, belongs to the *Apiaceae* family [17]. It is traditionally used as a safe and defensible treat in various chronic ulcers. It exhibited noteworthy biological activity as antioxidant, antiviral, antimicrobial, relaxant and cardiovascular effect as well as hypotensive etc. [18].

Regarding *Camellia sinensis* L., (*C. sinensis*) it is used as a food additive and is characterized by its therapeutic properties on metabolic dysfunctions. Furthermore, these plants and their active

constituents blocked the release of inflammatory intermediaries and oxidant factors. Moreover, it showed antidiabetic, anti-inflammatory, antioxidant, lipolysis, hepato-protective, cardio-protective effects [19], anticancer [20,21], anti-atherosclerotic and antihypertensive [22,23], and role in decreasing lipid content [24]. In this regard, many findings proposed that its tea could improve fatty liver diseases by regulating gut microbiota [25] due to containing minerals, proteins, and different amino acids combined with the rich constituent of gallic acid, caffeine, and catechin [26].

Furthermore, *Trifolium alexandrinum* commonly known as berseem, belongs to a family Fabaceae and is considered an important winter fodder crop in Egypt since ancient times [27-29]. It possesses antibacterial [30], hepatoprotective [31], phytoremediation activities [32]. Moreover, they attained medicinal importance as analgesics and antiseptics [30]. These medicinal herbs are effective for the treatment of pneumonia, sinus infections, fevers, encephalitis, and a febrile sensation in the body [33] add to that, dried flowers are used to cure asthma, congestion, and ulcer.

Humulus lupulus L. (hops) is an effective medicinal aromatic plant with importance in industry. It has hundreds of phytochemicals many active secondary metabolites such as phenolic, flavonoids, tannins, etc. which have positive impact pharmacological and medicinal value [34]. Hop extracts are important substitutes in antimicrobial, cancer, metabolic syndrome, and hormone replacement therapy treatments, as well as insecticides, preservatives, and fragrances [35].

Linum usitatissimum Linn, popularly known as Flaxseed or Linseed is one of the essential herbs which has a strong historical platform of having therapeutical properties to treat various diseases [36]. It belongs to family Linaceae, and is traditionally used by local healers to treat different disease conditions and has been documented by various authors for its ethnomedicinal uses. The plant is world distributed in many countries [37]. It has been broadly utilized in numerous ethnic worldwide as a primary medicinal plant because of its health benefits in various types of ailments. *L. usitatissimum* has antimicrobial activity and their extract considered as antibacterial and antifungal agent [37-39].

In addition to mentioned plant extracts, Propolis known as (bee glue) is a complex resinous mix formed by honeybees (*Apis mellifera*). From ancient times its used for its medicinal properties. It is substances collected from several plants are mixed with many enzymes as β-glycosidase of the honeybee saliva, digested, and combined to beeswax to formula the final product. Propolis is a highly complex mixture which have documented valuable health benefits. The chief constituents of propolis comprise resins, waxes, oils, and pollen, combined with various organic compounds, amongst which are phenolics, flavonoids, terpenes, esters, and alcohols, combined with antioxidants including beta-carotene, caffeic acid, and kaempferol [40]. This biochemical structure diversity of propolis is exceedingly variable, and this is expressively affected by the geographical origin, climate, water availability, and many other environmental factors. But all types of propolis have antimicrobial, anti-neoplastic, anti-inflammatory, antioxidant, hepatoprotective, cariostatic, and immunostimulatory activity [41].

The role of molecular detection, identification and fingerprinting of microorganisms has moved progressively from the academic world to the diagnostic laboratory. Differential Display Reverse Transcriptase polymerase chain reaction (DDRT-PCR), a proficient, reflective and reproducible technology, is more valuable in plentiful ways than other attitudes of gene expression analysis [42]. Besides, the technological simplicity and wider applicability made this technique very novel. It has successfully utilized in a number of organisms starting from yeast to mammals. This technique was introduced and developed to accelerate the identification of differentially expressed genes to overcome the shortcomings of earlier known methods which were sensitive to error, unresponsive and strenuous.

The aim of the current work is to evaluate the antimicrobial activity of five plant extracts (*Ammi majus*, *Camellia sinensis*, *Trifolium alexandrinum*, *Humulus lupulus* and the seeds of *Linum usitatissimum*) and propolis against aflatoxin producing *A. flavus* isolates, and against the ability of fungi to produce aflatoxins; B_1, B_2, G_1 and G_2. Antioxidant activity of the above mentioned extracts and propolis was evaluated, too.

2. Materials and Methods

2.1. Fungal Isolation and Identification

Twenty *Aspergillus* strains were isolated from different wheat grains collected from local markets at Alexandria governorate, Egypt. These fungal isolates were then purified, morphologically characterized through defining the color of the colony, color and size of spore, conidiophore structure, and vesicle form according to the available keys [43,44]. Finally, the isolated fungi were checked for their aflatoxin production through Thin Layer Chromatography (TLC).

2.2. Extraction and Quantification of Aflatoxins Produced by A. flavus Studied Isolates

2.2.1. Chemicals Used

Standards of AFs (B_1, B_2, G_1 and G_2) crystalline materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Each stock standard solution of AF with concentration 1 ng/µl was dissolved in benzene: acetonitrile (98: 2, v: v), were kept at 4°C in the dark. Stock solutions were evaporated to dryness under reduced pressure before being used, then they were derivatized with trifluoroacetic acid (TFA) as the working standards.

Solvents like chloroform, benzene, ethyl acetate, formic acid, toluene and methanol were purchased from El-Nasr Company for pharmaceutical chemicals, Cairo, Egypt. Acetonitrile was purchased from from Merck-Schuchardt Hohenbrunn bei Munchen, West Germany.

2.3. Extraction of AFs from the Fermented Media

The extraction protocol was carried out according to Eppley [45] with slight modification. At the end of the incubation period of *A. flavus* isolates, 25 ml of filtrate was added to 25 ml chloroform and was shaked for 1/2 h on a rotary shaker at 120 rpm. Then transferred to a separating funnel which was allowed to stand for some time until two layers formed. The lower chloroform layer was filtered over anhydrous sodium sulfate in a 250 ml beaker, evaporated approximately to dryness in a water bath (70-80°C), and the residue was taken by washing twice using 1-2 ml of chloroform into a small glass vial which evaporated till dryness (dry film).

2.4. Molecular Identification

2.4.1. DNA Purification

The fungal strain with the maximum production of AFs was molecularly identified via PCR amplification and sequencing of the ITS region for molecular identification, and the fungi were grown on potato dextrose broth media (PDB; 20 g dextrose and 200 g of potatoes /Litter of distilled water) for 6 days and after collecting the fungal disk from margin of fungal colony. DNA was extracted by CTAB protocol designated by Umesha et al [46]. Measuring of the DNA concentration and purity were made in a Nanodrop 2000 spectrophotometry (Thermo Fisher Scientific, Wilmington, USA), kept at -20 °C in a concentration of 200 ng/µL until further use.

2.4.2. PCR Amplification

The amplification of rDNA-ITS (ITS 1 and 4) region was made using specific PCR primers: (ITS1;TCCGTAGGTGAACCTGCGG and ITS4; TCCTCCGCTTATTGATATG) according to White et al. [47]. In 20 µl total volume PCR reaction consists of; 10 µl of 2x Multiplex PCR master mix (Qiagen), 1 µl (0.3 µM) of each primer and 1 µl of purified DNA template (100 ng) and 7µl of sterile ddH_2O. The amplification conditions were as follow: initial denaturing at 95°C for 5 minutes, followed by 35 cycles of; denaturing 95°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 1 minute, with a final cycle of extension at 72 °C for 10 minutes. The PCR amplicons were electrophoresed using 1.5 % agarose gel, visualized, purified, and sent for sequencing (Macrogen Company, Korea).

2.4.3. DNA Sequencing and Phylogenetic Construction

The achieved DNA sequences were aligned using DNA Blast, and the clean sequences were submitted to the NCBI GenBank database (https://www.ncbi.nlm.nih.gov), for the accession number. The DNA sequences were aligned with other fungal isolates of the same species available in the GenBank database, and the phylogenetic trees were created using maximum- likelihood (ML) method in MEGA 11 software [48].

2.5. Effect of Plant and Propolis Extracts on Biomass of a Selected isolate of A. flavus

To prepare the extracts, the dried aerial parts of 4 plants (*Ammi majus*, *Camellia sinensis*, *Trifolium alexandrinum* and *Humulus lupulus*), and the seeds of *Linum usitatissimum* were collected from the local market in Alexandria, washed with water, left to air-dry, and then powdered by a blender. Then, 10 g of each plant wee extracted with 100 ml of 80 % methanol, and put on shaker for 48 h. After extraction, filtration with Whatman No.1 filter paper was done, and the solvent was removed under reduced pressure at 50 °C. The remaining dried powder of each plant was further stored at 4 °C for being used in further biological studies [49,50]. Meanwhile, propolis was scraped off the top of the frames and inner wall boxes of Egyptian bee colonies at El-Manzala, Dakahlia, Egypt. After that, propolis was extracted with 80 % methanol (10 g of propolis/ 100 ml of 80% methanol), then evaporated to dryness as previously described [51,52].

2.6. Effect of the Extracts on Fungal Biomass

According to Yousef et al. [50], the five previously prepared plant extracts (4 plant extracts and propolis) were added to each flask of 50 ml of sterilized Potatoes Dextrose Broth medium (PDB; 200 g Potatoes, 20g dextrose /litter of distilled water), separately, at 6 concentrations (0.0, 0.01, 0.05, 0.1, 0.5 and 1 g/ 50 ml). Then each concentration was inoculated with five small disks (0.5 cm in diameter) of ten days old *A. flavus* 3 (478625) mycelium grown on Czapeck-Dox agar plate and incubated at 28 ±2°C for 10 days on shaker at 150 rpm. A Whatman No.1 filter paper was used for filtration of broth inoculated flasks after days of incubation. Filter paper take few seconds to get rid of any excess media. Filter papers with mycelium were weighed three time. Mean weight of the mycelium was measured after deducting the weight of filter paper. The weight of biomass in the presence of treatments compared with control considered as antimicrobial effect of the six studied concentrations against four isolates of aflatoxin producing *A. flavus*.

2.7. Phytochemical Screening and Antioxidant Activity of Studied Plants and Propolis Extracts

Methanolic extracts previously prepared from the dried powder of the studied plant extracts and propolis were used to assess some antioxidant compounds as described by Sobhy et al. [53]. Firstly, saponins were assessed quantitatively according to Hiai et al. [54]. Tannin contents were evaluated by the method of Broadhurst and Jones [55]. Afterwards, flavonoids, phenolics, ascorbic acid and total amino acids were assayed by the methods of Chang et al. [56], Jindal and Singh [57], Oser [58] and Lee and Takahashi [59] , orderly.

Finally, the antioxidant activity of the product was assayed by three different methods; ferric reducing power (FRP), phosphomolybdate assay (PMA) and Diphenyl -1-picrylhydrazyl (DPPH), according to Oyaizu [60], Jayaprakasha et al. [61], Brand-Williams et al. [62] and Bondet et al. [63], respectively.

2.8. Differential Display for A. flavus under Different Concentration of the Dyes Using Chitinase Primer

2.8.1. Extraction of Total RNA from Fungal Mycelium

The mRNA was extracted from fungal mycelia using QIAGEN mRNA extraction kit according to the manufacture instructions (Qiagen Ltd., Germantown, Maryland, USA).

2.8.2. Reverse Transcription of RNA

Reverse transcription reactions were achieved using oligo dT primer (5`-TTTTTTTTTTTTTTT-3`). Each 25 µl reaction mixture containing 2.5 µl 5 x buffer with $MgCl_2$, 2.5 µl 2.5 mM dNTPs, 1 µl 10 pmol primer, 2.5 µl RNA and 0.2 µl reverse transcriptase and completed with dH_2O to 25 µl. PCR amplification was completed in a thermal cycler programmed at 95 °C for 5 min, 42 °C for 1 hr, 72 °C for 10 min and stored at 4 °C until used [64].

2.8.3. Differential Display Polymerase Chain Reaction (DD-PCR)

The reaction mixture for DD-PCR was carried out in a final volume 25 µl comprising 2.5 µl 10 x buffer with $MgCl_2$, 2 µl 2.5 mM dNTPs, 1 µl 10 pmol primer (5`CAGGCCCTTCCAGCACCCAC 3`), 2 µl of resulting cDNA and 0.2 µl Taq DNA polymerase (5 units/µl). PCR amplification was performed in a thermal cycler automated for single cycle at 95 °C for 5 min. Afterwards, 40 cycles were made as pointed: 30 sec at 95 °C for denaturation, 1 min at 45 °C for annealing and 2 min at 72 °C for elongation. Then reaction was incubated for 10 min at 72 °C for ending extension [64]. 2 µl of loading dye was added prior to loading of 10 µl per gel pocket. Electrophoresis was achieved at 80 Volt in 1.5% agarose gel with addition of ethidium bromide solution. Lastly, gel documentation system was used for gel visualization and photographing.

2.9. Statistical Analysis

The performed experiments were entirely randomized design. The means of each treatment were compared by least statistically difference test (LSD) and considered statistically significant at $P \leq 0.05$. Statistical analysis was completed with the aid of analysis of variance technique (ANOVA) using CoStat program version 6.303 (CoHort software, Monterey, CA, USA).

3. Results

3.1. Fungal Identification

In this study, the aflatoxigenic *A. flavus* fungal isolates were used as a high aflatoxin producer and its morphological characteristics were performed as mentioned by Klich [43] as the fungal colonies were olive-green, velvety flat with a raised texture, forming dark brown sclerotia. The conidiophores were colorless, rough, and thick-walled, bearing vesicles globose to sub-globose with radiated sterigmata and globose, thin-walled, slightly rough conidia ranging from 250 to 450 µm.

The ITS1 and ITS4 sequence product marked the isolates as *A. flavus*, and the sequences were put in the GenBank database under accession number as follow; *A. flavus* 1: OR477304, *A. flavus* 2: OR478624, *A. flavus* 3: OR478625 and *A. flavus* 4: OR4773.

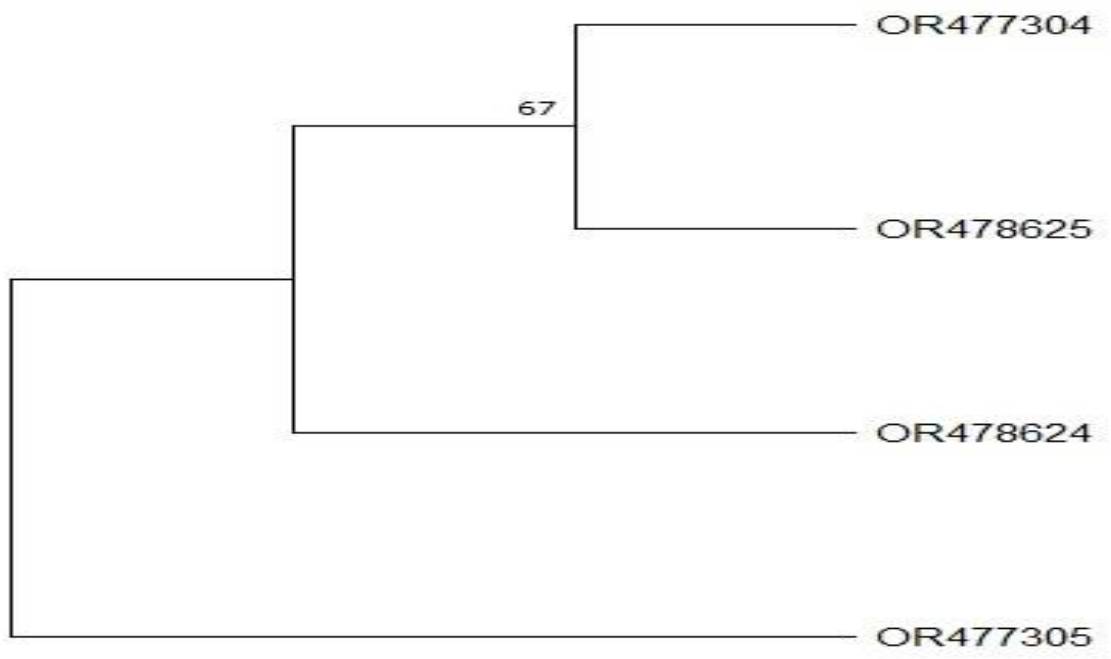

Figure 1. Illustrate, the phylogenetic tree of the four studied aflatoxigenic *A. flavus* isolates (*A. flavus* 1: OR477304, *A. flavus* 2: OR478624, *A. flavus* 3: OR478625 and *A. flavus* 4: OR477305) collected from wheat grains from different markets of Alexandria, Egypt.

Figure 2. The morphological appearance of the six treatments: A: *Ammi majus*, B: *Camellia sinensis*, C: *Trifolium alexandrinum*, D: *Humulus lupulus*, E: seeds of *Linum usitatissimum,* and F: propolis.

3.2. Effect the Extracts on the Growth of A. flavus No.3 (OR 478625)

The effect of different concentrations of the five treatments (plant extract of *A. majus*, *C. sinensis*, *T. alexandrinum*, *H. lupulus*, and seed of *L. usitatissimum*) as well as propolis on the growth mass of *A. flavus No.3* (OR478625) was illustrated in Fig. (3). All treatments appear significant reduction of fungal growth biomass at different concentration compared with control. The data revealed that; the highest percentage of inhibition of C. *sinensis* and *A. majus* extracts which led to significant reduction in fungal growth are 0.1, 0.5 and 1 g/50 ml but the highest reduction in mycelium growth biomass appears at 0.1 and 1 g/50 ml with *T. alexandrinum* and propolis extracts. For *H. lupulus* extract the highest degree of inhibition appear at 0.01 g/50 ml, this reduction is decreasing by increasing the concentration. For *L. usitatissimum* the value of reduction in fungal biomass slightly different by increasing the concentration of extract and give the highest reduction at 0.1 and 1 g/50 ml

respectively. Where, the percentage of reduction= (weight of biomass for control-weight of biomass of treatment/weight of control) %.

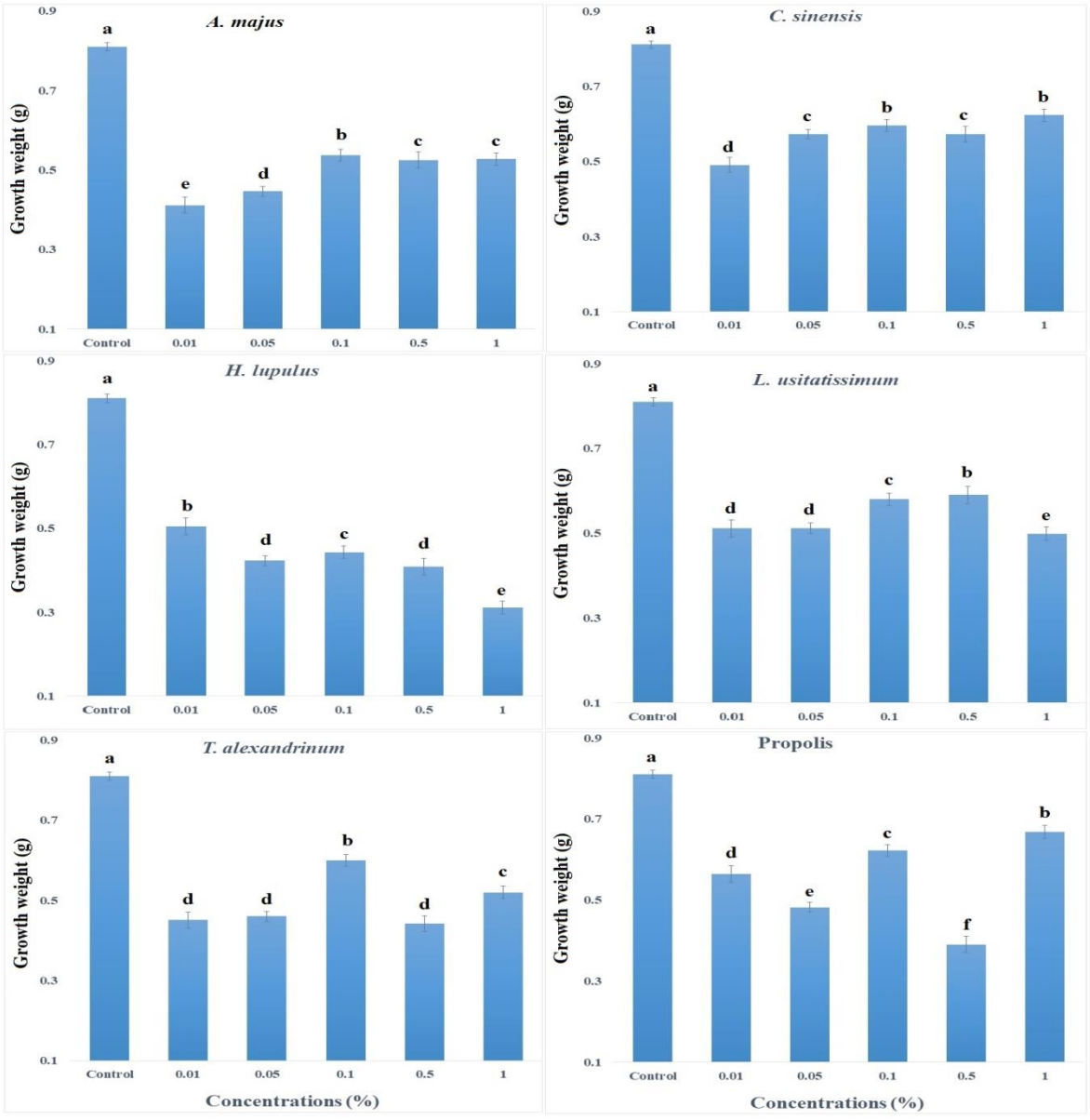

Figure 3. Effect of different concentrations of plant extracts; *A. majus*, *C. sinensis*, *T. alexandrinum*, *H. lupulus*, and *L. usitatissimum* and propolis (0.0, 0.01, 0.05, 0.1, 0.5 and 1 % (g/50 ml)) on *A. flavus No.3* (OR 478625) growth weight. Data are means of three replicates ± SD. Different letters refer to significant difference at *P< 0.05*.

3.3. Effect of the Plant Extracts and Propolis on Production of Aflatoxins by A. flavus No.3 (OR478625)

The results displayed in Table (1) revealed that, addition of *A. majus* plant extract to fungal growth medium exhibited complete inhibition for production of the four studied AFs (B_1, B_2, G_1 and G_2) under all concentrations when compared with control. The treatment with *C. sinensis* extract cause complete inhibition for production of three types of AFs (B_2, G_1 and G_2) under all concentrations, but at 0.05, 0.5 and 1 % for AF B1. The *H. lupulus* extract completely inhibited all types of AFs at 0.5 and 1%. The *T. alexandrinum* extract inhibited the production of all AFs at 0.01 and 1 % concentration, completely. Meanwhile, at concentration 0.5% three AFs production B_2, G_1 and G_2

were inhibited, completely, but the aflatoxin B_1 was inhibited when compared with control (441 μg/L). At 0.05 and 0.1% concentrations only AF G_1 production were inhibited completely. Extracts of *H. lupulus, L. usitatissimum* and *T. alexandrinum* at all concentration caused complete inhibition for AF G1 production. The production of AF B1, AF B2 and AF G2 were the same quantity at 0.1 and 0.5 % extract of *L. usitatissimum* with noticeable reduction compared with the control as well as at 1% μg/L there is higher reduction compared with control (Table 1).

Table 1. Effect of different concentration of plant extracts and propolis on AFs production by *A. flavus* OR478625.

Plant extracts	**Conc. (%)**	**Aflatoxin (μg/L) Mean ± SD**				
		B_1	**B_2**	**G_1**	**G_2**	**Total**
Control	0.0	2498	150	23	0.16	2671.16
Ammi majus	0.01	0.0	0.0	0.0	0.0	0.0
	0.05	0.0	0.0	0.0	0.0	0.0
	0.1	0.0	0.0	0.0	0.0	0.0
	0.5	0.0	0.0	0.0	0.0	0.0
	1	0.0	0.0	0.0	0.0	0.0
***Camellia sinensis* (green tea)**	0.01	220	0.0	0.0	0.0	220
	0.05	0.0	0.0	0.0	0.0	0.0
	0.1	220	0.0	0.0	0.0	220
	0.5	0.0	0.0	0.0	0.0	0.0
	1	0.0	0.0	0.0	0.0	0.0
Humulus lupulus	0.01	2207	19	0.0	0.032	2226
	0.05	882	0.0	0.0	0.0	882
	0.1	1765	19	0.0	0.032	1784
	0.5	0.0	0.0	0.0	0.0	0.0
	1	0.0	0.0	0.0	0.0	0.0
Trifolium alexandrinum	0.01	0.0	0.0	0.0	0.0	0.0
	0.05	3089	157	0.0	0.076	3246.08
	0.1	3089	193	0.0	0.23	3282.23
	0.5	441	0.0	0.0	0.0	441.00
	1	0.0	0.0	0.0	0.0	0.0
Linum usitatissimum	0.01	1324	19	0.0	0.032	1343.03
	0.05	441	0.0	0.0	0.0	441.00
	0.1	3089	193	0.0	0.23	3282.23
	0.5	3089	193	0.0	0.23	3282.23
	1	1324	19	0.0	0.032	1343.03

In contrast to the control, the effect of propolis on production of four aflatoxin types (B_1, B_2, G_1 and G_2) were displayed in Table (2). At the same concentrations (0.0, 0.01, 0.05, 0.1, 0.5 and 1 %), the obtained data revealed that 0.5 and 1 % of propolis extract completely inhibited the four types of aflatoxin (0.0 μg/L), while at 0.05 % significantly inhibited production of AF B_2, AF G_1 and AF G_2, furthermore, at 0.01 inhibited AF G_1 and significantly decreased B_1, B_2, and G_2 types compared to control.

Table 2. Effect of different concentration of propolis on AFs production by *A. flavus* OR478625.

Propolis Conc. (%)	Aflatoxin (μg/L) Mean ± SD				
	B_1	**B_2**	**G_1**	**G_2**	**Total**
0.0	2498±6[a]	150±2[a]	23±1[a]	0.16±0[a]	2671±13[a]
0.01	2207±3[a]	19±1[b]	0.0±0[b]	0.032±0[b]	2226±15[a]
0.05	220±1[b]	0.0±0[c]	0.0±0[b]	0.0±0[c]	220±3[b]
0.1	2648±12[a]	157±2[a]	24±1[a]	0.15±0[a]	2829±11[a]
0.5	0.0±0[c]	0.0±0[c]	0.0±0[b]	0.0±0[c]	0.0±0[c]
1	0.0±0[c]	0.0±0[c]	0.0±0[b]	0.0±0[c]	0.0±0[c]

*Data are means of three replicates ± SD.
*Different letters refer to significant difference at *P< 0.05*.

3.4. Phytochemical Constituents of the Studied Plant and Propolis Extracts

Secondary metabolites; saponin, tannin, flavonoids, Phenolic, ascorbic and total amino acids from six studied treatments (*A. majus*, *C. sinensis*, *T. alexandrinum*, *H. lupulus*, seeds of *L. usitatissimum* and propolis) were quantitatively studied as described in Table (3). Quantity of secondary metabolites varies from one extract to another. For phenolics its quantity ranges from 121 to 322 mg/g, flavonoids from 50 to 85 mg/g, ascorbic acid from 10 to 36 mg/g, saponins from 17 to 36 mg/g but for tannin range from 11 to 35 mg/g. For phenolics and flavonoids its highest quantity appears in *C. sinensis* extract, for ascorbic acid its highest quantity appear in propolis followed *L. usitatissimum* extract, but saponin concentration the higher quantity appear with propolis followed by *A. majus* extract. The highest quantity of tannin appears in propolis extract.

Table 3. Phytochemical screening of five studied plant extracts and propolis.

Parameter	Quantity of the phytochemical in mg/g (Mean± SD)					
	A. majus	***C. sinensis***	***T. alexandrinum***	***H. lupulus***	***L. usitatissimum***	**Propolis**
Phenolic	211±2[b]	322±12[a]	121±7[e]	152±2[d]	180±8[c]	181.4±7[c]
Flavonoids	51±2[c]	85±6[a]	50±2[c]	65±2[b]	51±2[c]	47.7±0.7[c]
Ascorbic	14±1[bc]	12±2[cd]	10±1[d]	15±1[b]	36±2[a]	35.1±0.8[a]
Saponin	36±3[b]	32±2[b]	17±1[c]	22±1[c]	18±2[c]	41.1±5[a]
Tannins	14±2[de]	25±3[c]	15±1[d]	35±2[b]	11±1[e]	100.8±3[a]

*Data are means of three replicates ± SD.
*Different letters refer to significant difference at *P< 0.05*.

3.5. Antioxidant Activity of the Studied Plant Extracts and Propolis

Three methods FRP, PMA and DPPH were used for studying antioxidant activity of five plant extracts and propolis as illustrated in Table (4). For FRP and DPPH methods the highest antioxidant activity (35.8±3 mg/g and 81±3%) appear with *A. majus,* respectively. Meanwhile, PMA method, the highest antioxidant activity appear with *H. lupulus* (30±2). However, the antioxidant activity of propolis was recorded as 30.8±1, 76.4±1 and 11.5±2 for FRP, DPPH and PMA methods, respectively (Table 4).

3.6. Differential Display for A. flavus OR478625 Under Different Concentrations (0.5 and 1 %) of the Dyes Using Chitinase Primers

RNA arbitrarily primed PCR technique is based upon the use of reverse transcribed RNA as a template to identify differentially expressed genes in a manner analogous to that of arbitrarily primed PCR (AP-PCR) Welsh and McClelland [65], which uses genomic DNA as a template. AP-PCR has proven to be a powerful method for the detection and isolation of differentially expressed genes in several systems including tumor cells [66,67].

Table 4. Antioxidant activity of five studied plant extracts and propolis by FRP, PMA and DPPH methods.

Parameter	*A. majus*	*C. sinensis*	*T. alexandrinum*	*H. lupulus*	*L. usitatissimum*	Propolis	Unit
FRP	35 ± 3^{a}	33 ± 3^{ab}	31 ± 1^{bc}	26 ± 2^{d}	28 ± 1^{cd}	30.8 ± 1^{bc}	mg/g
PMA	27 ± 3^{bc}	25 ± 1^{c}	29 ± 1^{ab}	30 ± 2^{a}	22 ± 1^{d}	11.5 ± 1^{e}	
DPPH	81 ± 3^{a}	79 ± 1^{ab}	65 ± 5^{c}	62 ± 5^{c}	73 ± 3^{b}	76.4 ± 2^{ab}	%

*Data are means of three replicates ± SD.
*Different letters refer to significant difference at $P< 0.05$**.**

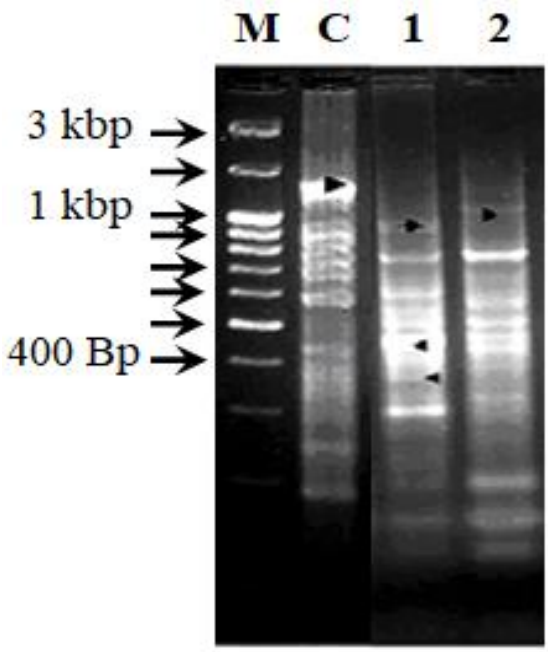

Figure 4. Differential display PCR using arbitrary primers for *A. flavus* treated with *Ammi majus*. Lane 1: 3 kbp DNA marker, Lane 2: Control, Lanes 3, 4: Treated samples with 0.5 and 1% concentrations.

A. flavus OR478625 was treated using different concentrations from *A. majus* (0.5 and 1%). The total RNA was extracted, and the differential display analysis was achieved for the treated and control samples using arbitrary primers. Data presented in (Figure ,4) showed that the differential display for the samples; control, 3 and 4 revealed that, two up-regulated bands with different molecular weights are appeared. These bands appeared only with sample 3 (arrow indicated to right) but they are not shown with control. One of the important things is the disappearance of the down-regulated band from treated samples but it was highly expressed in the control ones (arrow indicated to right).

4. Discussion

Aflatoxins are severe, toxic and carcinogenic mycotoxins that are produced by fungi belonging to genus *Aspergillus spp*, primarily *A. flavus* and *A. parasiticus* [68]. Within section *Flavi*, there are about 18 species that produce aflatoxins but most of them have little economic or agricultural importance [69]. Aflatoxin poses serious health threats to both human and livestock [4,16]. Different studies indicate that some plants have numerous constituents such as peptides, aldehydes, many essential oils, phenols, etc. with potentially important therapeutic application against human pathogens, comprising bacteria, fungi, and virus [16, 70-72].

In this study, four aflatoxigenic *A. flavus* isolates were morphologically and genetically identified and deposited their sequences in the GenBank database and identified as; *A. flavus* 1 (OR477304), *A. flavus* 2 (OR478624), A. *flavus* 3 (OR478625) and *A. flavus* 4 (OR477305). The effect of different concentrations of five plant extracts (*A. majus*, *C. sinensis*, *T. alexandrinum*, *H. lupulus*, and *L. usitatissimum*) as well as propolis on *A. flavus 3* (OR478625) growth biomass. All treatments appear significant reduction of fungal growth biomass at different concentrations. The data revealed the concentration which cause highest percentage of inhibition varies according to type of plant extract, [49, 73,74]. The highest reduction in mycelium growth appears at 0.1 to 1 g/50ml for *A. majus*. For *C. sinensis* the highest reduction in growth appears at 0.01 g/50ml. Contrast to *H. lupulus,* the highest reduction in growth appears at 1 g/50ml and for *L. usitatissimum*. The highest of decreasing in fungal biomass was achieved by propolis as well as for *T. alexandrinum* is 0.5 g/50ml.

The AFs regulation effect by extract of *Ammi majus*, *Camellia sinensis*, *Humulus lupulus*, *Trifolium alexandrinum*, *Linum usitatissimum* and propolis. Many articles using plant extracts as effective factor inhibiting aflatoxins production, [75]. Methanolic plant extracts as *Zingiber officinale* and *Hyoscyamus muticus* were effective and completely inhibited the production of AFs with *A. flavus* G14 at all studied concentrations. A like, *Cinnamomum zeylanicum*, *Artemisia absinthium*, *Piper nigrum*, *Camellia sinensis*, *Cichorium endivia*, *Sonchus oleraceus* and *Chenopodium murale* inhibit AFs production at most concentrations were used. [73] presented that, aqueous extracts of *Lupinus albus*, *Ammi visnaga* and *Xanthium pungens* suppressed the growth and biosynthesis of AFs by *A. flavus* and the inhibitory consequence of these extracts was comparative to their concentrations. In this respect, Qin [76] found that three kinds of extracts of green tea *(Camellia sinensis)* were effective for their effects on AFB_1-induced hepato-carcinogenesis in rats.

Many workers have reported antimicrobial activity of studied plant extracts and showed the importance of natural chemicals as possible source of non-phytotoxic, systemic and easily biodegradable alternative pesticides. Corresponding to [49,73,77] different extracts of plant species exhibited antifungal activity of aflatoxin producing *Aspergillus* species (*Thymus vulgaris, Eucalyptus globulus*, *Allium sativum*, *Mentha viridis*, *Nigella sativa* and *Allium cepa*) and reducing the fungal dry weight. The aqueous plant extract of *Allium sativum*, *Thymus vulgaris*. rhizome of *Zingiber officinalis*, *Olea europaea* and *Eucalyptus globulus* were strongly inhibited aflatoxin B1production. Moreover, T. *vulgaris* and extract of *Ocimum basilicum* leaf showed the strongest inhibition of Aflatoxin B2 production. Generally, several plant extracts which illustrate antifungal activities, they can be divided into groups according to the chemical structure of their volatile oil. High fungicidal activities can be found in the phenol-containing lamiacaea species, 1,8-cineole showed antifungal activity against aflatoxin producing *Aspergillus* species ([78-81]. According to Dubey and Dwivedi [82], no plant extract acts against all properties of fungus by the same rate, but in most cases, plant extract affects by different rates on the different biological characters of the fungus.

Addition of natural extracts to the fungal growing media resulted in decrease fungal growth and inhibition of AFs production [50], these findings are in accord with Abdel-Wahab [75] which reported that; AF production by studied isolates of *A. flavus* G14 was completely inhibited by methanolic extracts of *Zingiber officinale* and *Hyoscyamus muticus* at all concentrations (0.01, 0.05, 0.1, 0.5 and 1 % w/v.

Also, propolis (bee-glue)-a natural product made by bees from the resins of *Populus nigra* and chestnut trees [83] displayed antimicrobial activity against *Aspergillus flavus* [84], *A. sulphureus* [85], *Sclerotium cepivorum* [86-88], soybean and sunflower wilt disease [89] and soil-borne fungi [90]. Ghaly *et al*. [91] established that propolis ethanolic extract concentrations reduced the percentage of conidial germination of *A. flavus* of studied isolates, reduced its mycelial dry mass and reduce AFB_1 production with *A. flavus* isolates compared with control, where Pepeljnjak *et al*. [85] found that propolis extract inhibit growth of *A. flavus*, *A. ochraceous*, *Penicillium viridicatum* and *P. notatum* and had antifungal activity towards *A. sulphureus*.

The antifungal efficacy of the studied extract specially *A. majus* may be attributed to its antioxidant compounds and activity. Saponins are steroidal or tri-terpenoid complexes that are commonly glycosylated at one end of the compound, possess soap-like properties in H_2O [92]. Several saponins have antimicrobial and anti-insects properties and are considered protective agents towards potential pathogens [93]. Besides, Tannins; a secondary metabolite and has a role as potential biological antioxidants used for defense of oxidative stress imposed by fungi, which has been implicated widely [94].

In our study, the capacity of studied extracts as antioxidant was shown to be influenced by the total phenolic and total flavonoid contents. Further, Flavonoids are important natural group of phenolics and possess wide spectrum chemical and biological characteristics including radical scavenging properties and described to have antioxidant, anticarcinogenic, and antimicrobial properties [95]. Furthermore, the reducing capability of the mentioned extracts may have considered as a substantial indicator of its potential antioxidant capacity which have a role in changing free radicals to more steady products and hence ending free radical originated chain reactions [96]. Moreover, the DPPH method is known to provide consistent information regarding the antioxidant capacity of definite extracts, products or compounds. The A. majus extract studied showed high antioxidant activity attaining (81 %) of DPPH scavenging.

Molecular techniques have been used increasingly over the past decade to improve the sensitivity, specificity and turn-around time in the clinical laboratory. In this study, *A. flavus* strains was subjected to different treatments to examine the productivity of AFs. Molecular studies based on the differential display were carried out to examine the down regulated and the up regulated bands (genes) [66]. We show that strains can be distinguished by comparing polymorphisms in genomic fingerprints and also the changes inside the genome under different stress. the genetic variation which examined, might happen for the treated fungus compared with the control (non-treated). The genetic variations after two steps of PCR observed in the level of messenger RNA and the response of the organism toward the treatment will be followed with a high amount of mRNA for the influenced genes. The appearance of a new band in the treated fungus it means a gene (s) expression (a gene opening) (up regulated band) as a response for the new conditions. But the disappearance of band it means that a gene shutdown (down regulated band). The obtained results are showed there are many up regulated bands in different molecular weights, these actually belong many different genes in the aflatoxins pathway. But there is only one down regulated band appeared with the control sample. These results come in agreement with Williams *et al*. [97] and Caceres, et al. [98]. Generally, this band appeared in 75% with the control samples, which pushed us to examine this band to discover in which gene in the aflatoxins pathway it is responsible. Now it is clear that the down regulated band means the aflatoxins biosynthesis gene and the absence of this band in the treated samples means that, all the treatment makes a blocking for the biosynthesis gene or at least make inhibition for that gene. In addition, the disappearance of this band in the treated fungus may not be completely inhibition of the biosynthesis gene and may be expressed but with very low level, which prevent the polymerase to see it and the result no amplicon. Actually, mRNA differential display (DDRT-PCR), is gel based transcript profiling system based on electrophoretic fingerprinting of amplified cDNA fragments [99]. Therefore, we utilized differential display to identify potential factors produced by some plant extracts on the fungus aflatoxin production [100]. In conclusion, some of examined treatments affect the aflatoxin biosynthesis gene. But the others may inhibit another gene in the same pathway.

5. Conclusions

This study recognized the anti-fungal effect of five plant extracts and propolis against the isolate *A. flavus 3* (OR478625). It is worthy mentioned that all treatments produced significant reduction of the fungal biomass's growth when compared to control. The highest reduction was exhibited by *C. sinensis* (60.6%), and *A. majus* (50.8%) at 0.01 g/50 ml. Meanwhile, *T. alexandrinum*

exhibited 54.6% reduction at 0.5 g/50 ml, followed by *L. usitatissimum* and *H. lupulus* which produced 61.6 and 38.4% reduction at 1 g/50 ml, respectively. Propolis exhibited 48.2% reduction at 0.5 g/50 ml. AF production (B_1, B_2, G_1 and G_2) was completely inhibited by *A. majus* extract at all concentrations, and by propolis and *H. lupulus* extract at 0.5 and 1%. Three types of AFs (B_2, G_1 and G_2) were completely inhibited by *C. sinensis* extract at all concentrations, and by *T. alexandrinum* extract at 0.01 and 1%. Whilst, only AFG_1 was inhibited by *L. usitatissimum* extract at all concentrations. The plant extracts showed an average of 221.5, 67.5, 23, 26.5 and 23 mg/g of phenolics, flavonoids, ascorbic acid, saponins and tannins, respectively. *A. majus* exhibited the highest antioxidant activity (35.8±3 mg/g and 81±3%) using FRP and DPPH methods, respectively. Meanwhile, *H. lupulus* exhibited the highest antioxidant activity (30±2 mg/g) using PMA method. Additionally, treatment of *A. flavus* 3 with *A. majus* resulted in genetic variations when compared to control. This study presented the high antioxidant activity, the high antifungal capacity, and the remarkable aflatoxin inhibition of the studied plant extracts and propolis, as well.

Patents: N/A

Supplementary Materials: N/A

Author Contributions: GFHA-W, SS, FHG, AMS and MMM designed the experiments; GFHA-W, and SS conducted the experiment; analyzed the results; GFHA-W prepared a manuscript draft; FHG, AMS and MMM edited the manuscript; GFHA-W, SS, FHG, AMS and MMM revised the manuscript for technical and scientific accuracy. All authors have read and agreed to the published version of the manuscript.

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References

1. Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahiye, A.M., Misore, A., DeCock, K., Rubin, C. (2005). Kenya Aflatoxicosis Investigation Group. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. Environ Health Perspect., 113(12):1763-1767. doi: 10.1289/ehp.7998.
2. Iqbal, S.Z., Jinap, S., Pirouz, A.A., Ahmad, F.A.R. (2015). Aflatoxin M1 in milk and dairy products, occurrence and recent challenges: A review. Trends in Food Science & Technology, 46(1): 110-119. https://doi.org/10.1016/j.tifs.2015.08.005.
3. Paterson, R.R., Lima, N. (2010). Toxicology of Mycotoxins. Molecular, Clinical and Environmental Toxicology, 100: 31-63. http://dx.doi.org/10.1007/978-3-7643-8338-1_2.
4. Khalifa, E., Marwa, T.M., Monga, I.M., Piekutowska, M., Alsuhaibani, A.M., Basel, A.A., Sotohy, A.S., Soumya, G., Yosra, A.H., Mohamed, H., Ahmed, M.A. (2022). Diversity of Toxigenic Fungi in Livestock and Poultry Feedstuffs. Int. J. Environ. Res. Public Health, 19(7250): 1-16. DOI: 10.3390/ijerph19127250.
5. Sherif, S.H., Salama, E., Mosaad, A.A. (2009). Mycotoxins and child health: The need for health risk assessment. Int. J. Hygiene Environ. Health, 212: 347-368. 10.1016/j.ijheh.2008.08.002.
6. Ogodo, A., Ugbogu, O. (2016). Public Health Significance of Aflatoxin in Food Industry– A Review. European J. Clin. Biomed. Sci., 2: 51-58. 10.11648/j.ejcbs.20160205.14.
7. Olana, A. (2022). Aflatoxin producing fungi and its management: Review Article. J. Plant Pathol. Microbiol., 13(10): 1-6.
8. Magan, N., Aldred, D., Medina, A. (2012). Abstracts. Quality Assurance and Safety of Crops & Foods, 4: 145-145. https://doi.org/10.1111/j.1757-837X.2012.00152.x
9. Perrone, G., Ferrara, M., Medina, A., Pascale, M., Magan, N. (2020). Toxigenic Fungi and Mycotoxins in a Climate Change Scenario: Ecology, Genomics, Distribution, Prediction and Prevention of the Risk. Microorganisms, 29;8(10):1496. doi: 10.3390/microorganisms8101496.
10. Payne, G.A., Brown, M.P. (1998). Genetics and physiology of aflatoxin biosynthesis. Annu. Rev. Phytopathol., 36: 329-362. 10.1146/annurev.phyto.36.1.329.

11. Khaneghah, A.M., Ismail, E.S., Susan, R., Yadolah, F. (2010). The Food processor's guide to your facility's pest control. J. Food Safety, 37(6): 1-7. https://doi.org/10.1111/jfs.12532.
12. Kwon-Chung, K.J., Sugui, J.A. (2013). *Aspergillus fumigatus*-what makes the species a ubiquitous human fungal pathogen? PLoS Pathol., 9(12):e1003743. doi: 10.1371/journal.ppat.1003743.
13. Paulussen, C., Hallsworth, J.E., Alvarez-Perez, S., Nierman, W.C., Hamil, P.G., Blain, D., Lievens, B. (2019). Ecology of aspergillosis: insights into the pathogenic potency of *Aspergillus fumigatus* and some other *Aspergillus* species. 10(2): 296-322. doi: 10.1111/1751-7915.12367.
14. Shabeer, S., Asad, S., Jamal, A., Ali, A. (2022). Aflatoxin Contamination, Its Impact and Management Strategies: An Updated Review. Toxins, 14(5): 307. https://doi.org/10.3390/toxins14050307.
15. Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D.G., Lightfoot, D.A. (2017). Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. Plants (Basel, Switzerland), 6(4): 42. https://doi.org/10.3390/plants6040042.
16. Youssef, N.H., Qari, S.H., Matar, S., Hamad, N.A., Dessoky, E.S., Elshaer, M.M., Sobhy, S., Abdelkhalek, A., Zakaria, H.M., Heflish, A.A. (2021). Licorice, Doum, and Banana Peel Extracts Inhibit *Aspergillus flavus* Growth and Suppress Metabolic Pathway of Aflatoxin B1 Production. Agronomy, 11: 1587. https://doi.org/10.3390/agronomy11081587.
17. Al-Hadhrami, R.M.S., Hossain, M.A. (2016). Evaluation of antioxidant, antimicrobial and cytotoxic activities of seed crude extracts of *Ammi majus* grown in Oman. Egy. J. Basic App. Sci., 3(4): 329-334. DOI: 10.1016/j.ejbas.2016.08.001.
18. Hossain, M.A., Al Touby, S. (2020). *Ammi majus* an Endemic Medicinal Plant: A Review of the Medicinal Uses, Pharmacological and Phytochemicals. Ann. Toxicol., 2(1). DOI: 10.36959/736/634.
19. Anaeigoudari, A., Safari, H., Khazdair, M.R. (2021). Effects of *Nigella sativa, Camellia sinensis,* and *Allium sativum* as Food Additives on Metabolic Disorders, a Literature Review. Front. Pharmacol., 12: 762182. https://doi.org/10.3389/fphar.2021.762182.
20. Dias, T.A., Duarte, C.L., Lima, C.F., Proença, M.F., Pereira-Wilson, C. (2013). Superior anticancer activity of halogenated chalcones and flavonols over the natural flavonol quercetin. Eur. J. Medic. Chem., 65: 500-510. https://doi.org/10.1016/j.ejmech.2013.04.064.
21. Thitimuta, S., Pithayanukul, P., Nithitanakool, S., Bavovada, R., Leanpolchareanchai, J., Saparpakorn, P. (2017). *Camellia sinensis* L. Extract and Its Potential Beneficial Effects in Antioxidant, Anti-Inflammatory, Anti-Hepatotoxic, and Anti-Tyrosinase Activities. Molecules (Basel, Switzerland), 22(3): 401. https://doi.org/10.3390/molecules22030401.
22. Hodgson, J.M., Devine, A., Puddey, I.B., Chan, S.Y., Beilin, L.J., Prince, R.L. (2003). Tea Intake Is Inversely Related to Blood Pressure in Older Women. The J. Nut., 133(9): 2883-2886. https://doi.org/10.1093/jn/133.9.2883.
23. Al Disi, S.S., Anwer, M.A., Eid, A.H. (2016). Anti-hypertensive herbs and their mechanisms of action: Part . Fron. Pharmacol., 6:2015. https://doi.org/10.3389/fphar.2015.00323.
24. Huang, H.C., Lin, J.K. (2012). Pu-erh tea, green tea, and black tea suppresses hyperlipidemia, hyperleptinemia and fatty acid synthase through activating AMPK in rats fed a high-fructose diet. Food and Function, 3(2): 170-177. https://doi.org/10.1039/c1fo10157a.
25. Liu, B., Mao, Q., Zhou, D., Luo, M., Gan, R., Li, H., Huang, S., Saimaiti, A., Shang, A., Li, H. (2021). Effects of Tea against Alcoholic Fatty Liver Disease by Modulating Gut Microbiota in Chronic Alcohol-Exposed Mice. Foods. 10(6): 1232. https://doi.org/10.3390/foods10061232.
26. Ning, J.-M., Ding, D., Song, Y.-S., Zhang, Z.-Z., Luo, X., Wan, X.-C. (2016). Chemical Constituents Analysis of white tea of Different Qualities and Different Storage Times. Eur. Food Res. Technol., 242(12): 2093-2104. 10.1007/s00217-016-2706-0.
27. Sabudak, T., Guler, N. (2009). *Trifolium* L.- Arevie on its phytochemical and pharmacological profile. Phytotherapy Research, 439-446. www.interscience.wiley.com. Doi: 10.1002/ptr.2709.
28. 28. Bakheit, B.R. (2013). Egyptian Clover (*Trifolium alexandrinum* L.) Breeding in Egypt: A Review. Asian J. Crop Sci., 5: 325-337. doi: 10.3923/ajcs.2013.325.337.
29. Behiry, S.I., Hamad, N.A., Alotibi, F.O., Al-Askar, A.A., Arishi, A.A., Kenawy, A.M., Elsamra, I.A., Youssef, N.H., Elsharkawy, M.M., Abdelkhalek, A. (2022). Antifungal and Antiaflatoxigenic Activities of Different Plant Extracts against *Aspergillus flavus*. Sustainability, 14: 12908. https://doi.org/10.3390/su141912908.

30. Khan, A.V., Ahmed, Q.U., Shukla, I., Khan, A.A. (2012). Antibacterial activity of leaves extracts of *Trifolium alexandrinum* Linn. against pathogenic bacteria causing tropical diseases. Asian Pacific J. Trop. Biomed., 2(3): 189-194. https://doi.org/10.1016/S2221-1691(12)60040-9.
31. Sakeran, M.I., Zidan, N., Rehman, H., Aziz, A.T., Saggu, S. (2014). Abrogation by *Trifolium alexandrinum* root extract on hepatotoxicity induced by acetaminophen in rats. Redox Report: Communications In Free Radical Research, 19(1): 26-33. https://doi.org/10.1179/1351000213Y.0000000068.
32. Ali, H., Naseer, M., Sajad, M.A. (2012). Phytoremediation of heavy metals by *Trifolium alexandrinum*. Agris On-line Papers in Economics and Informatics, 2: 1459-1469. 10.6088/ijes.002020300031.
33. Khan, S., Khatoon, S. (2007). Ethnobotanical studies on some useful herbs of Haramosh and Bugrote Valleys in Gilgit, Northern Areas of Pakistan. Pak. J. Botany, 40(1): 43-58.
34. Keskin, M. (2022). *Humulus lupulus* L. (hops) based silver nanoparticles: synthesis, characterization and enzyme inhibition effects. Acta Sci. Pol. Hortorum Cultus, 21(4): 11-20. https://doi.org/10.24326/asphc.2022.4.2.
35. Korpelainen, H., Pietiläinen, M. (2021). Hop (*Humulus lupulus* L.): Traditional and Present Use, and Future Potential. Econ. Bot., 75: 302-322. https://doi.org/10.1007/s12231-021-09528-1.
36. Radadiya, K.V., Panara, K., Acharya, R. (2023). A comprehensive ethnopharmacological review on *Linum usitatissimum* Linn. (Atasi). Int. J. Botany Stu., 8(2): 47-54.
37. Hussien, Z.G., Aziz, R.A. (2021). Chemical Composition And Antibacterial Activity Of *Linum usitatissimum* L. (Flaxseed). Syst. Rev. Pharm., 12(2): 145-147. doi:10.31838/srp.2021.2.15.
38. Ouf, J.M., Ali, G.H., Hafez, S.M., Youssef, W., Mosa, A.M.M.A. (2023). Shelf life of chilled broiler which fed on mycotoxins contaminated ration. Egyp. J. Animal Health, 3(3): 204-214. https://ejah.journals.ekb.eg/.
39. Baqer, G.K., Madhi, K.S., Baqer, F.K., Baqer, L.K., Abbas, B.A. (2023). Antibacterial Activity and Phytochemical Screening of *Linum usitatissimum* L. on Bacteria Isolated from Wound Infections. Iran. J. War Public Health, 15(4): 347-352. 10.58209/ijwph.15.4.347.
40. Pascoal, P.M., Narciso, I.S., Pereira, N.M. (2014). What is sexual satisfaction? Thematic analysis of lay people's definitions. Journal of Sex Res., 51(1): 22-30. https://doi.org/10.1080/00224499.2013.815149.
41. Kurek-Górecka, A., Górecki, M., Rzepecka-Stojko, A., Balwierz, R., Stojko, J. (2020). Bee Products in Dermatology and Skin Care. Molecules, 25(3): 556. doi: 10.3390/molecules25030556.
42. Singh, V., Ali, M.N. (2016). Analysis of Differential Gene Expression under Salinity through Differential Display Reverse Transcription Polymerase Chain Reaction (DDRTPCR) Technique: A Review. Electronic J Biol., 12:4.
43. Klich, M.A. (2002). Identification of Common *Aspergillus* Species. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, P: 124.
44. Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C., Varga, J. (2007). Diagnostic tools to identify black aspergilli. Studies in Mycology, 59, 129-145. https://doi.org/10.3114/sim.2007.59.13.
45. Eppley, R.W. (1968). An Incubation Method For Estimating The Carbon Content Of Phytoplankton In Natural Samples. Limnology and Oceanography, 13. doi: 10.4319/lo.1968.13.4.0574.
46. Umesha, S., Manukumar, H.M., Raghava, S. (2016). A rapid method for isolation of genomic DNA from food-borne fungal pathogens. 3 Biotech., 6(2): 123. doi: 10.1007/s13205-016-0436-4.
47. White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W. (1990). Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. Book: PCR - Protocols and Applications - A Laboratory Manual, Publisher: Academic Press.
48. Tamura, K., Stecher, G., Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution, 38(7): 3022-3027. https://doi.org/10.1093/molbev/msab120.
49. Mossa, M.I. (2021). Antifungal Effect of *Thymus vulgaris* methanolic extract on growth of aflatoxins producing *Aspergillus parasiticus*. Egypt. J. Bot., 61(2): 349-359. DOI: 10.21608/ejbo.2019.12540.1311.
50. Yousef, H., Metwaly, H.A., Hassanin, M.M.H. (2022). effect of plant extracts on suppression of *Aspergillus flavus* growth and Aflatoxin productionin Peanuts. Egypt. J. Phytopathol., 50(2): 25-32. DOI: 10.21608/ejp.2022.150154.1064.
51. El-Sakhawy, M. (2023). Propolis harvesting and extraction. Egypt. J. Chem., 66(1): 313-321.
52. Gniewose, M., Pobiega, K., Olbrys, N., Krasniewska, K., Synoiec, A. (2023). The effect of ethanol propolis extracts on inhibition of growth of *Fusarium solani* on hen eggs. J. App. Sci., 13(1): 315. https://doi.org/10.3390/app13010315.

53. Sobhy, S., Allah, K., Kassem, E., Hafez, E., Sewelam, N. (2019). Seed priming in natural weed extracts represents a promising practice for alleviating lead stress toxicity. Egypt. J. Exp. Biol., 15: 453. *(PDF) Antioxidant activity, antibacterial behavior, and anticancer impact of Egyptian propolis*. Available from: https://www.researchgate.net/publication/388840454_Antioxidant_activity_antibacterial_behavior_and_anticancer_impact_of_Egyptian_propolis.
54. Hiai, S., Oura, H., Hamanaka, H., Odaka, Y. (1975). A color reaction of panaxadiol with vanillin and sulfuric acid. Planta Med., 28(6): 131-138.
55. Broadhurst, R., Jones, W.T. (1978). Analysis of condensed tannins using acidified vanillin. J. Sci. Food Agric., 29(9): 788-794. DOI: 10.1002/jsfa.2740290908.
56. Chang, C.-C., Yang, M.-H., Wen, H.-M., Chern, J.-C. (2002). Estimation of total flavonoid content in propolis by two complementary colometric methods. J. Food Drug Analy., 10(3), Article 3. Available at: https://doi.org/10.38212/2224-6614.2748.
57. Jindal, K.K., Singh, R.N. (1975). Phenolic Content in Male and Female *Carica papaya:* A Possible Physiological Marker for Sex Identification of Vegetative Seedlings. Physiologia Plantarum, 33: 104-107. https://doi.org/10.1111/j.1399-3054.1975.tb03774.x.
58. Oser, B. (1979). Hawk's Physiological Chemistry, 15th ed., McGrow Hill Publish. Company, New York.
59. Lee, Y.P., Takahashi, T. (1966). An improved colorimetric determination of amino acids with the use of ninhydrin. Analytical Biochem., 14(1): 71-77. https://doi.org/10.1016/0003-2697(66)90057-1.
60. Oyaizu, M. (1986). Studies on product of browning reaction prepared from glucose amine. Japanese J. Nut, 44(1986): 307.
61. Jayaprakasha, G.K., Singh, R.P., Sakariah, K.K. (2001). Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models in vitro. Food Chem., 73(3): 285-290.
62. Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity, LWT - Food Sci. Technol., 28(1): 25-30. https://doi.org/10.1016/S0023-6438(95)80008-5.
63. Bondet, V., Brand-Williams, W., Berset, C. (1997). Kinetics and Mechanisms of Antioxidant Activity using the DPPH. Free Radical Method, LWT- Food Sci. Technol., 30(6):609-615. https://doi.org/10.1006/fstl.1997.0240.
64. Chen, X.Q., Bonnefoi, H., Pelte, M.-F., Lyautey, J., Lederrey, C., Movarekhi, S., Schaeffer, P., Mulcahy, H.E., Meyer, P., Stroun, M., Anker, P. (2000). Telomerase RNA as a detection marker in the serum of breast cancer patients. Clin. Cancer Res., 6: 3823-3826.
65. Welsh, J., McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research, 18(24): 7213-7218. https://doi.org/10.1093/nar/18.24.7213.
66. Wong, K., Mok, C., Welsh, J., McClelland, M., Tsao, S., Berkowitz, R. (1993). Identification of differentially expressed RNA in human ovarian-carcinoma cells by arbitrarily primed PCR fingerprinting of total RNAs. Int. J. Oncol., 3(1): 13-17. doi: 10.3892/ijo.3.1.13.
67. Nelson, J.B., Chan-Tack, K., Hedican, S.P., Magnuson, S.R., Opgenorth, T.J., Bova, G.S., Simons, J.W. (1996). Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. Cancer Res., 56(4): 663-668. PMID: 8630991.
68. Sserumaga, J.P., Ortega-Beltran, A., Wagacha, J.M., Mutegi, C.K., Bandyopadhyay, R. (2020). Aflatoxin-producing fungi associated with pre-harvest maize contamination in Uganda. Int. J. Food Microbiol., 16(313): 108376. doi: 10.1016/j.ijfoodmicro.2019.108376.
69. Frisvad, J.C., Hubka, V., Ezekiel, C.N., Hong, S.B., Nováková, A., Chen, A.J., Arzanlou, M., Larsen, T.O., Sklenář, F., Mahakarnchanakul, W., Samson, R.A., Houbraken, J. (2019). Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. Studies in Mycol., 93: 1-63. doi: 10.1016/j.simyco.2018.06.001.
70. Saad, S.M., Ramadan, M.S., Reham, A.A.A., Khalifa, E.A.A. (2015). The using of essential oils in improving mycological status of some meat products. Benha Vet. Med. J., 29(1): 85-96.
71. El-Nagerabi, S., Al-Maqbali, M., Alabri, S., Elshafie, A. (2021). An *in vitro* Antifungal and Antiaflatoxigenic Properties of *Commiphora myrrha* and *Prunus mahaleb*. J. Food Res., 10(6): 10-21. DOI:10.5539/jfr.v10n6p10.
72. Sobhy, M., Ali, S.S., Cui, H., Lin, L., El-Sapagh, S. (2023). Exploring the potential of 1,8-cineole from cardamom oil against food-borne pathogens: Antibacterial mechanisms and its application in meat preservation. Microbial Pathogenesis, 184(2023): 106375. https://doi.org/10.1016/j.micpath.2023.106375.
73. Mahmoud, A.L. (1999). Inhibition of growth and aflatoxin biosynthesis of *Aspergillus flavus* by extracts of some Egyptian plants. Let. App. Microbiol., 29(5): 334-336. https://doi.org/10.1046/j.1472-765x.1999.00636.x.

74. Mossa, M.I., Abdul Wahid, O.A. (2016). Effect of *Thymus vulgaris, Salvia* and *Artemisia* extracts against filamentous fungi on North Sinai. Egypt J. Bot., Special Issue For 6th International Conference on Biotechnology and its Applications in Botany and Microbiology, 11-12 May, 2016, Menoufia Univ., P: 301-316.
75. Abdel-Wahab, M., Mostafa, M., Sabry, M., el-Farrash, M., Yousef, T. (2008). Aflatoxins as a risk factor for hepatocellular carcinoma in Egypt, Mansoura Gastroenterology Center study. Hepatogastroenterology, 55(86-87): 1754-1759. PMID: 19102385.
76. Qin, G.Z. (1991). Effects of green tea extract on the development of aflatoxin B1-induced precancerous enzyme-altered hepatocellular foci in rats. Chinese J. Preventive Med., 25(6): 332-334.
77. Abeer, E-A., Monira, A.-O., Saleh, A.-S., Mahmoud, M., Kasi, M. (2012). Prevention of aflatoxin contamination of maize by *Aspergillus flavus* through aqueous plant extracts in Saudi Arabia. Afr. J. Microbiol. Res., 6: 6931-6935. 10.5897/AJMR12.1455.
78. Adjovi, Y.C.S., Koulony, R., Atindehou, M.M., Ahehehinnou, H.U., Dadavodou, J., Sanni, A. (1919). *Laurus nobilis* L.a ntural alternative against *Aspergillus flavus* and aflatoxins. Int. J. Dev. Res., 9(05): 27692-27697.
79. Alizadeh-Salteh, S. (2015). Growth Inhibition of *Aspergillus flavus* Isolated from Pistachio by Secondary Metabolites. J. Nuts, 6(2): 85-94.
80. Kim, H.-M., Kwon, H., Kim, K., Lee, S.-E. (2018). Antifungal and Antiaflatoxigenic Activities of 1,8-Cineole and t-Cinnamaldehyde on *Aspergillus flavus*. App. Sci., 8(9): 1655. https://doi.org/10.3390/app8091655.
81. Abdelaziz, A.M., El-Wakil, D.A., Attia, M.S., Ali, O.M., AbdElgawad, H., Hashem, A.H. (2022). Inhibition of *Aspergillus flavus* Growth and Aflatoxin Production in *Zea mays* L. Using Endophytic *Aspergillus fumigatus*. J. Fungi (Basel, Switzerland), 8(5): 482. https://doi.org/10.3390/jof8050482.
82. Dubey, R.C., Dwivedi, R.S. (1991). Fungitoxic properties of some plant extracts against vegetative growth and sclerotial viability of *Macrophomina phaseolina*. Ind. Phytopathol., 44: 411-413.
83. Anjum, S.I., Ullah, A., Khan, K.A., Attaullah, M., Khan, H., Ali, H., Bashir, M.A., Tahir, M., Ansari, M.J., Ghramh, H.A., Adgaba, N., Dash, C.K. (2019). Composition and functional properties of propolis (bee glue): A review. Saudi J. Biologic. Sci., 26(7): 1695-1703. https://doi.org/10.1016/j.sjbs.2018.08.013.
84. Lotfy, M. (2006). Biological Activity of Bee Propolis in Health and Disease. Asian Pacific J. Cancer Prevention, 7: 22-31.
85. Pepeljnjak, S., Maysinger, D., Jalsenjak, I. (1982). Effect of propolis extract on some fungi. Scientia Pharmacentica, 50: 165-167.
86. Fahmy, F.G., Omar, M.O.M. (1988). Effect of Propolis extracts on certain potato viruses., Fourth International Congress on Agriculture in Tropical Climates, Cairo, Egypt, 6-10, November.
87. Fahmy, F.G., Omar, M.O.M. (1989). Potential use of Propolis to control white rot disease of onion. Assiut J. Agric. Sci., 20(1): 265-275.
88. Bellik, Y., Boukraa, L. (2012). Beneficial effects of propolis on human health and chronice diseases, The antimicrobial properties of propolis, Chap x, book, Nova Science Publishers, Inc., P: 1-17.
89. Saeed, F.A., Mohamed, S.H. (1992). The influence of propolis extract on soybean and sunflower wilt disease. Assiut J. Agric. Sci., 23(2): 179-190.
90. Abdul Salam, K.S., Mohamed, M.I. (1995). Effect of propolis on some *Fusarium* spp. Egypt J. Phytopathol., 21(2): 149-155.
91. Ghaly, M.F., Ezzat, S.M., Sarhan, M.M. (1998). Use of propolis and ultragriseofulvin to inhibit aflatoxigenic fungi. Folia Microbiologica, 43(2): 156-160. https://doi.org/10.1007/BF02816502.
92. Price, K.R., Johnson, I.T., Fenwick, G.R. (1987). The chemistry and biological significance of saponins in foods and feedstuffs. Crit. Rev. Food Sci. Nutr., 26: 27-135. doi: 10.1080/10408398709527461.
93. Gallardo, F., Boethel, D.J. (1990). Effects of the Allelochemical, α-tomatine, on the Soybean Looper (Lepidoptera: Noctuidae). J. Entomol. Sci., 25(3): 376-382. https://www.doi.org/10.18474/0749-8004-25.3.376
94. Kehrer, J.P. (1993). Free radicals as mediators of tissue injury and disease. Crit. Rev. Toxicol., 23(1): 21-48. https://doi.org/10.3109/10408449309104073.
95. Ghafar, M.F., Prasad, K.N., Weng, K.K., Ismail, A. (2010). Flavonoid, hesperidine, total phenolic contents and antioxidant activities from *Citrus* species. Afr. J. Biotechnol., 9(3).
96. Ardestani, A., Yazdanparast, R. (2007). Antioxidant and free radical scavenging potential of *Achillea santolina* extracts. Food Chem., 104(1): 21-29.

97. Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18(22): 6531-6535. https://doi.org/10.1093/nar/18.22.6531.
98. Caceres, I., Khoury, A.A., Khoury, R.E., Lorber, S., Oswald, I.P., Khoury, A.E., Atoui, A., Puel, O., Bailly, J.D. (2020). Aflatoxin Biosynthesis and Genetic Regulation: A Review. Toxins, 12(3): 150. https://doi.org/10.3390/toxins12030150.
99. Brown, S.A., Venkatesh, V. (2005). Model of adoption of technology in households: A baseline model test and extension incorporating household life cycle. MIS quarterly: 399-426. https://doi.org/10.2307/25148690.
100. Yu, J. (2012). Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. Toxins, 4(11): 1024-1057. https://doi.org/10.3390/toxins4111024.