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ORIGINAL PAPER

Effect of Plant Extracts on Suppression of *Aspergillus flavus* Growth and Aflatoxins Production in Peanuts

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ABSTRACT

Peanuts (*Arachis hypogaea* L.) represent one of the most vulnerable crops to infection by *Aspergillus flavus* under storage conditions. Accordingly, peanuts represent a major source of aflatoxin. The mycelial growth diameter of *A. flavus* was dramatically reduced by all tested plant extracts and their various concentrations as compared to the control. Thyme extract was the most effective in reducing the linear growth and the total aflatoxin production of *A. flavus in vitro*. Application of plant extracts on peanuts before the artificial infection with *A. flavus* under storage conditions showed high efficiency of reducing total aflatoxin production compared to the control. Thyme extract at concentration 5% was the most effective treatment in reducing total aflatoxin production. According to the protein electrophoresis results of *A. flavus* treated with rosemary, thyme, and basil plant extracts, thyme treatment caused the development of newly produced proteins with about 25 kDa molecular weights. Whereas in *A. flavus* isolate treated with rosemary, two bands with molecular weights of 63 and 75 kDa, respectively, entirely were vanished. Similarly, *A. flavus* isolate treated with basil resulted in disappearance of one band with molecular weight of 63 kDa.

Keywords: Peanuts, *Arachis hypogaea*, *Aspergillus flavus*, Aflatoxins, Protein, VICAM, Plant extracts.

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INTRODUCTION

One of the most significant oilseed crops in the world is groundnut (*Arachis hypogaea* L.), which is grown in more than 100 nations (Dwivedi *et al*., 2003). With a total yearly yield of 43.98 million tons, groundnuts are grown on 27.66 million hectares around the world (FAOSTAT, 2018). It serves as a significant source of both income and food (Izge *et al*., 2007). The producing area in Egypt is 142,642 feddan and the production is of 198,763 tons (Mahmoud *et al.*, 2021). Peanuts provide the body with protein, fat, and vitamins (Settaluri *et al*., 2012) as well as minerals that are important for health and widely consumed by all ages (Lavkor and Var, 2017). However, peanuts can cause allergic reactions for some people (Shreya, 2016), and the greatest contamination risk is the presence of aflatoxins (Nyirahakizimana *et al*., 2013). Horn *et al.* (1995) mentioned that peanuts are considered to be one of the most vulnerable crops because *Aspergillus flavus* or *A. parasiticus* can infect peanut kernels. As a result, peanuts represent a major source of aflatoxin in people's diets (Mutegi *et al*., 2013). Aflatoxins are potent liver carcinogens and are now listed by the International Agency for Research on Cancer as Group 1 carcinogens. (Anonymous., 2002) Additionally, aflatoxins possess acute, immunosuppressant, and chronic genotoxic properties (Williams *et al*., 2004). The main producers of aflatoxins, as secondary metabolites, are *Aspergillus flavus, A. parasiticus* and *A. nomius* (Pitt and Hocking, 2009). Different strategies have been investigated to prevent aflatoxins from contaminating peanuts. One of the best strategies to reduce mycotoxin contamination in food and feed is to prevent the growth of toxic fungus on sensitive substrates. (Kabak *et al*, 2006). Despite rising public concern over chemical residues and the spread of resistance in pathogen populations, the chemical fungicides are nevertheless extensively used to control plant diseases (Tripathi and Dubey, 2004). The increasing risk of health caused by fungicides residues in food, such as cancer, liver diseases, and endocrine disruption, has prompted the quest for sustainable agricultural management alternatives (da Silva, 2020). Humans have relied on medicinal plants and their products from prehistoric times all over the world. Basil (*Ocimum basilicum L*.) has already been demonstrated that it has antimycotoxigenic, antiradical, antioxidant, antifungal, and antibacterial properties (Mezeyová, 2020).

Alkadi (2021) reported that *Ocimum basilicum* extract has antifungal properties against *A. parasitiucus* and *A. flavus*. Rosemary (*Rosmarinus officinalis L.*) is widely used as an anti-inflammatory, carminative agent, circulatory stimulus, and alleviates rheumatic muscle (Borges *et al.,* 2019). Moreover, it has antiaflatoxigenic and antifungal properties against *A. flavus* (Prakash *et al.,* 2015). Thyme (*Thymus vulgaris L.*) is a medicinal aromatic herb; it has antibiotic, antiseptic and antifungal activities (Ekoh *et al.,* 2014). Střelková *et al.* (2021) reported the potential antifungal effects of thyme, against certain fungi including *Aspergillus* spp. The major goals of the current study are to ascertain the antifungal properties of thyme, rosemary, and basil as plant extracts as well as how they affect the growth of *A. flavus*' mycelium. Using the VICAM technique, it is also possible to assess how these extracts affected the synthesis of aflatoxins.

MATERIALS AND METHODS

Preparation of plant extracts:

Fresh leaves from thyme, rosemary, and basil plants were gathered, properly rinsed with tap water, and then allowed to air dry. Plant tissue weighing 100 g was mashed in a mortar and pestle. Each plant sample was extracted separately using an equal volume (100 mL) of sterilized distilled water for 24 hours at room temperature. The resulting extracts were separated, filtered, and heated to 40°C. The dried crude extracts were then dissolved in sterilized distilled water to create stock solutions of each extract, which were then stored in the fridge until needed.

Impact of plant extracts on mycelial growth of *A. flavus* on PDA *in vitro*:

To obtain the concentrations of 1, 3, and 5% (v/v), extracts of sweet basil, rosemary, and thyme were introduced separately to PDA flasks that had been sterilized. *A. flavus* discs (5 mm in diameter) obtained from peanuts were inoculated onto three PDA medium plates, and morphological characteristics were examined under a microscope to identify. For Petri plates, a 25 °C incubator was utilized. After three days, the radial growth of the tested fungus was measured in each treatment and compared to the control. According to the methodology proposed by Topps and Wain (1957), the percentage of fungal growth inhibition was computed when the fungal growth of the control plates (without treatments) entirely filled the plates.

$$\% \text{ Inhibition} = A - B / A \times 100$$

A= the linear growth in control treatment.

B= the linear growth of treated fungus.

Impact of plant extracts on suppression of aflatoxins production by *A. flavus* on potato dextrose broth (PDB) *in vitro*:

Plant extracts were used to culture *A. flavus* in 250 mL-Erlenmeyer flasks containing 50 mL PDB. *A. flavus* spore suspension comprising 2.5 mL and 1 10^8 spores/mL was added to the flasks, along with various plant extracts at final concentrations of 1, 3, and 5% (v/v). As a control, PDB was inoculated with 2.5 mL of an *A. flavus* spore suspension (1×10^8 spores/mL). The mycelial biomass of *A. flavus* was then measured after being incubated for 7 days at 28 °C on a 150-rpm rotary shaker. The total aflatoxin concentrations in culture broth filtrates were measured using the immunoaffinity fluorometric technique with the AflaTest® Fluorometer Series-4 (VICAM 1. P, USA). A blender jar was filled with 5 g of sodium chloride and 50 mL of culture broth filtrate after they had been combined and weighed. The jar was then mixed at a high speed for 1 minute to extract the aflatoxins using 100 mL of an 80:20 by volume mixture of methanol and water. The mixture was filtered through Whatman filter paper No. 1 before being diluted four times with distilled water and going through a glass-microfiber filter once more. The filtrate was injected into a 2 mL Aflatest® immunoaffinity column and allowed to elute at a rate of 1-2 drops/sec. Aflatoxin residue was removed from the column using 1 mL of high-performance liquid chromatography (HPLC) grade methanol after the column had been washed twice with 1 mL of distilled water. The total aflatoxin concentration was measured using a pre-calibrated VICAM Series-4 Fluorometer set at 360 nm absorption and 450 nm emissions after 1 mL of bromine developer was added to the methanol elute. The averaged results for each sample were provided in part per billion (Kaaya and Eboku, 2010).

Impact of plant extracts on suppression of aflatoxins Production by *A. flavus* in peanuts under storage conditions:

The surface of the peanut kernels was sterilized for one minute with 5% NaOCl, followed by three rinses in sterilized water. 25 g of peanut kernels were sprayed with 2 mL of each treatment separately in a Petri dish. 25 g of peanuts were sprayed with 2 mL of sterile, distilled water as the control. For each treatment, there were three dishes in replicate. Afterward, 1 mL of *A. flavus* spore suspension (1×10^8

spores/mL) was added to the peanut kernels as an inoculant. The growth of *A. flavus* on kernels was visually assessed after incubation at 28°C for 7 days. AflaTest Immunoaffinity column (VICAM) Chromatography test, the technique previously indicated, was used to measure the concentration of aflatoxins production linked to peanut kernels in each treatment. There were three times the trials were carried out.

Extraction of proteins from *A. flavus* isolates:

Proteins were made using the Guseva and Gromova-described techniques (1982). The mycelium that had been cultured for 8 days at 20 to 30°C in the liquid Czapeck's medium was extracted by filtration through cheesecloth, numerous washes with distilled water, and freeze-drying. This frozen mycelium was extensively mixed with glass beads before being ground to a fine powder in liquid nitrogen. It was then floated in phosphate buffer pH 8.3 (1-3 mL/g mycelium). Centrifugation of the ground mycelium took place at 0°C for 30 minutes at 19,000 rpm. Bradford (1976) established the protein concentration of the supernatant by utilizing bovine serum albumin as a reference protein.

Electrophoresis of dissociated protein (SDS-PAGE):

Each supernatant was combined with an equivalent amount of a solution containing 64% buffer (0.15 M Tris-HCl, pH 6.8), 20% glycerol, 6% SDS, 10% 2-mercaptoethanol, and 0.1% bromophenol blue before boiling in a water bath for 3 minutes. Electrophoresis was performed on samples of 20 microliters (40 g of protein) using a 7.5 polyacrylamide gel produced in 0.1% SDS and 3.5% stacking gel (Laemmli, 1970). In a 7.5% polyacrylamide gel with a 3.5% stacking gel, electrophoresis was carried out at 10°C for 4 hours at 15 and 30 mA, respectively, until the dye band reached the bottom of the separating gel (Laemmli, 1970). A vertical slab mould (Hoefer Scientific Instruments, San Francisco, CA, USA, model LKB 2001, measures 16 × 18 × 0.15 cm) was used for electrophoresis. Three hours of 30 milli-Amper electrophoresis at 10°C were used. Silver nitrate was used to stain gels in order to identify protein bands (Sammons *et al*., 1981).

Gel analysis:

According to Sneath and Sokal (1973), gel documentation system (Uvitec, Cambridge, UK) used the unweighted pair group technique of arithmetic means (UPGMA) to cluster the protein patterns acquired by PAGE and SDS-PAGE.

Statistical analysis:

To validate the findings, these tests were carried out three more times. Statistical analysis software "COStat 6.4" was used to compare means using Duncan's test at p 0.05 and determine the standard error (CoStat, 2005).

RESULTS AND DISCUSSION

Impact of plant extracts on mycelial growth diameter and mycelial growth biomass of *A. flavus* on PDA *in vitro*:

Data presented in Table (1) show that all tested plant extracts and their different concentrations significantly decreased the mycelial growth diameter of *A. flavus* grown on PDA compared to the control. This reduction was directly proportional to the increase in the concentration of the tested plant extracts from 1 to 5 %. In this respect, the linear growth of *A. flavus* was most effectively inhibited by thyme extract, followed by basil and rosemary extracts as they recorded averages of growth reduction, being 81.33, 84.73, and 86.55%, respectively, compared to control. Concerning mycelial dry weight, data (Table, 1) show the same pattern. Generally, the reduction in mycelial dry weight of *A. flavus* grown in PDB was directly proportional to the increase in concentration of the tested plant extracts and showed significant reduction with averages of 69.85, 72.21 and 63.26%; respectively, compared to control. Application of the extracts, even if crude, would probably show better antifungal activities because the bioactive compounds are usually concentrated by the extraction process. The reduction of mycelial growth percentages may be due to the presence of phenolic compounds in thyme such as thymol. Positive effectiveness of the present extracts on mycelial growth inhibition is somewhat similar to those reported by Ismail *et al.* (1989), Khan *et al.* (1993), Zedan *et al.* (1994), Shafie (2004), Halawa (2004), and Hassanin (2013). Also, the obtained results showed an increase in the inhibitory effect for each extract by increasing its concentration are coincide with Baiuomy (1997), El-Habaa *et al.* (2002), and Shafie (2004). On the other hand, these extracts might contain fungicidal substance(s), causing inhibition to fungal spores' germination and preventing the formation of reproductive organs of the tested fungus. These findings concur with those made public by Centeno *et al.* (2010) and Mohammedi and Atik (2013).

Impact of plant extracts on suppression of aflatoxins production by *A. flavus* on PDB *in vitro*:

Tabulated data (Table, 2) represent a gradual suppression of total aflatoxin production by *A.*

flavus due to using all tested plant extracts and their different concentrations. The percent inhibition of total aflatoxin production ranged from 74.15 to 92.64 %, compared to controls. In this respect, thyme extract was the most effective in reducing the total aflatoxin production, followed by basil and rosemary extracts, as they recorded averages of aflatoxins concentrations, being 3.9, 6.5, and 9.5 ppb, respectively, compared to control. This inhibition of activity in total aflatoxin production may be attributed to the presence of phenolic compounds with different amounts and types of the tested medicinal and aromatic plant extracts such as thymol in thyme. Due to the inclusion of flavonoids, betalain, phenolics, phytoalexins, and thiosulfonates, plant extracts are utilized to detoxify microorganisms. But mostly antimicrobial and antioxidant activities of plant extracts are due to their phenolic alignments (Jayaprakasha *et al.*, 2002). However, thymol, salicylic acid, cinnamic acid, vanillyl acetone, and vanillin are phenolic compounds that ceased *A. flavus* growth by targeting oxidative mitochondrial stress as defense system (Kim *et al.*, 2006). These results are in harmony with those reported by Mohammedi and Atik (2013) and Nikan and Jafari (2015).

Table (1): Impact of plant extracts and their concentrations on mycelial growth diameter and mycelial growth biomass of *A. flavus in vitro*.

Treatments	Conc. (%)	Mycelial growth diameter (cm)	Inhibition of mycelial growth (%)	Mycelial dry weight (mg)	Inhibition of growth weight (%)
Rosemary	1	2.61 b	65.93	7.31 bc	38.26
	3	1.58 b	79.37	6.43 bc	45.69
	5	1.43 b	81.33	3.57 f	69.85
Thyme	1	1.56 b	79.63	7.82 b	33.95
	3	1.22 b	84.07	6.19 bcd	47.72
	5	1.03 b	86.55	3.29 f	72.21
Basil	1	1.60 b	79.11	5.72 cde	51.69
	3	1.53 b	80.03	4.67 def	60.56
	5	1.17 b	84.73	4.35 ef	63.26
Control	-	7.66 a	-	11.84 a	-
L.S.D. at 5%		1.70	-	1.70	-

According to Duncan's multiple range analysis, the means in each column that are denoted by similar letters do not differ significantly (P≤0.05).

Table (2): Impact of plant extracts and their concentrations on suppression of aflatoxins production by *A. flavus* in PDB *in vitro*.

Treatments	Con. (%)	Aflatoxins conc. (ppb)	Inhibition of Aflatoxins production (%)
Rosemary	1	13.7 b	74.15
	3	12.0 b	77.36
	5	9.5 c	82.08
Thyme	1	6.3 de	88.11
	3	5.7 e	89.25
	5	3.9 f	92.64
Basil	1	7.7 d	85.47
	3	6.2 de	88.30
	5	6.5 de	87.74
Control		53.0 a	-
L.S.D. at 5%		1.70	

According to Duncan's multiple range analysis, the means in each column that are denoted by similar letters do not differ significantly (P≤0.05).

Impact of plant extracts on suppression of aflatoxins production by *A. flavus* in peanut kernels under storage conditions:

Results in Figures (1 and 2) show the effect of using plant extracts in peanuts kernels infected with *A. flavus* with their high efficiency for reducing total aflatoxin production comparing to the untreated control. Among all treatments, thyme extract at concentration 5% was the most effective treatment, recorded 1 ppb; whereas the lowest efficiency was recorded when peanuts were treated with rosemary extract at concentration 1% (2.8 ppb). Furthermore, basil extract at concentration 5% recorded 1.5 ppb compared to the untreated control. The percent inhibition of total aflatoxin production ranged from 82.50 to 91.88 %, compared to controls. The action of their phenolic components, including eugenol, linalool, thymol, and carvacrol, may be responsible for the reduction of aflatoxins' harmful effects in inoculated stored peanut kernels. The acidic character of the hydroxyl group in these phenolic compounds, which forms

a hydrogen bond with enzyme activity sites, is assumed to be responsible for the mycotoxin-suppressing potential of these compounds (Mohammedi and Atik, 2013 and Nikan and Jafari, 2015).

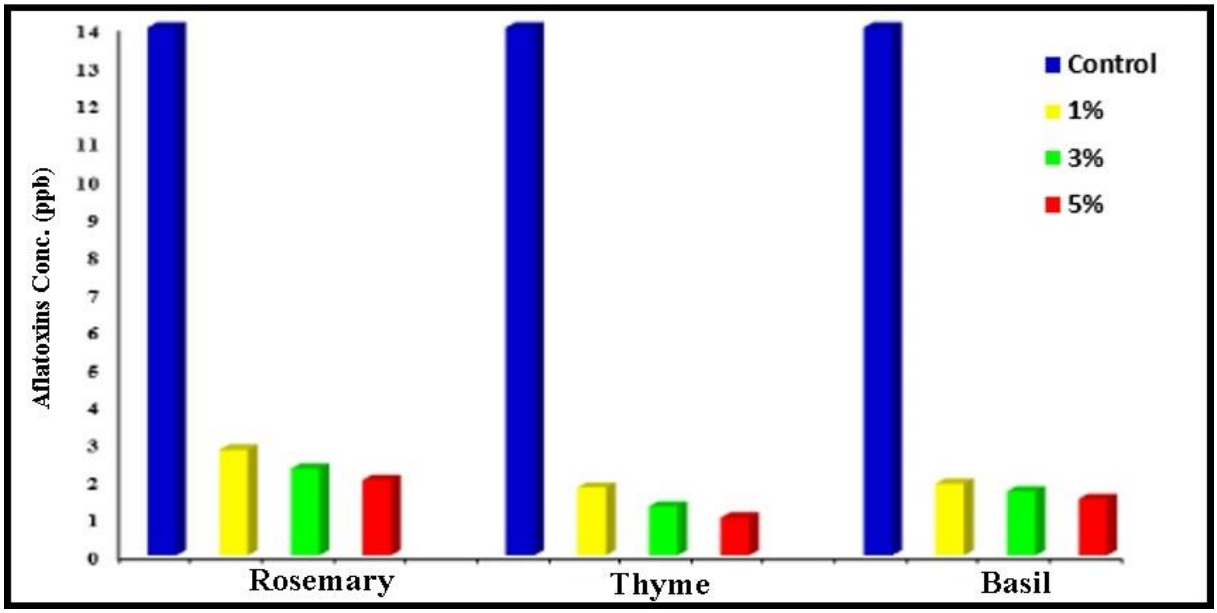

Figure (1): Aflatoxin concentrations (ppb) in peanut kernels treated with each of rosemary, thyme, and basil plant extracts under storage conditions.

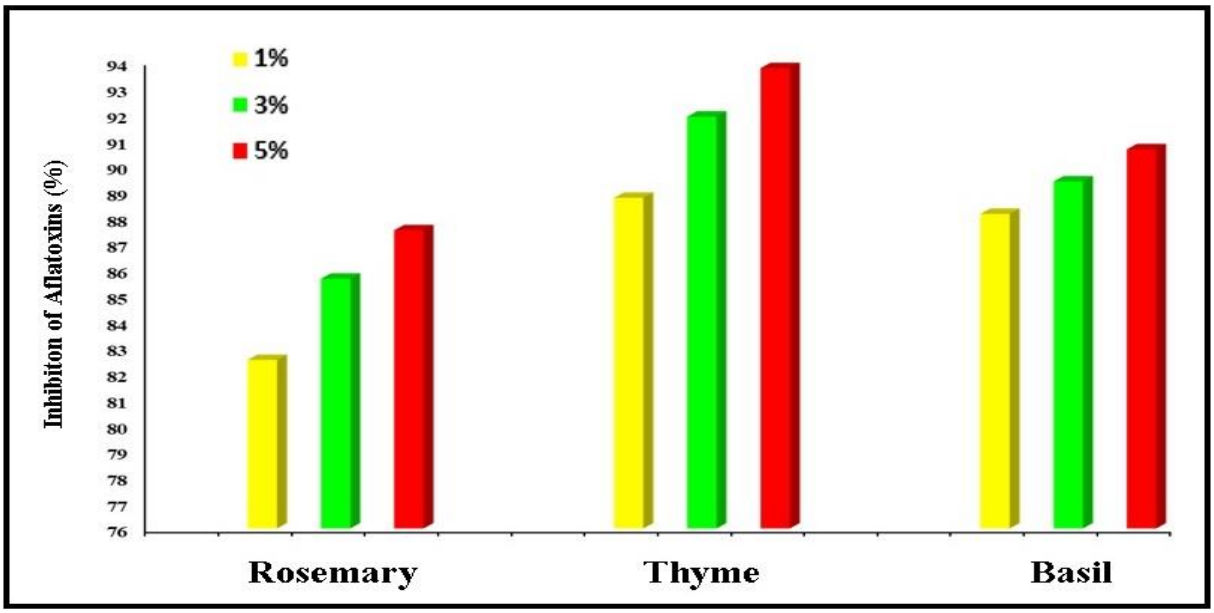

Figure (2): Percent inhibition of aflatoxin in peanuts treated with rosemary, thyme, and basil plant extracts under storage conditions.

Electrophoresis of dissociated protein (SDS-PAGE):

SDS-PAGE analysis was done to assess how the gene expression of *A. flavus* changed after being exposed to extracts from the herbs rosemary, thyme, and basil. Other treatments did not reveal all of the protein bands found in the *A. flavus* isolates. Protein patterns in the control group produced bands of altered proteins. The protein marker contained seven major bands, including ones that were 66, 45, and 22 kDa, respectively. Two bands with molecular weights of 63 and 75 kDa completely vanished in an *A. flavus* isolate that had been exposed to rosemary. Similarly, after basil treatment of an *A. flavus* isolate, one band with a molecular weight of 63 kDa vanished. While in the thyme-treated *A. flavus* isolate, this led to the creation of newly expressed proteins with molecular weights of about 25 kDa (Figure 3). The outcomes are relatively comparable to those mentioned by Liang *et al* (2015). The processing of plant extracts causes several biological reactions, such as oxidative stress-stimulated metabolic

alterations, which successively alter the level of protein synthesis (Kulatunga *et al*., 2016). The toxic effects of plant extracts on fungal cells originate from significant metabolic changes in some protein synthesis, as demonstrated by the lack or presence of some crucial protein synthesis (Alghuthaymi *et al*., 2020).

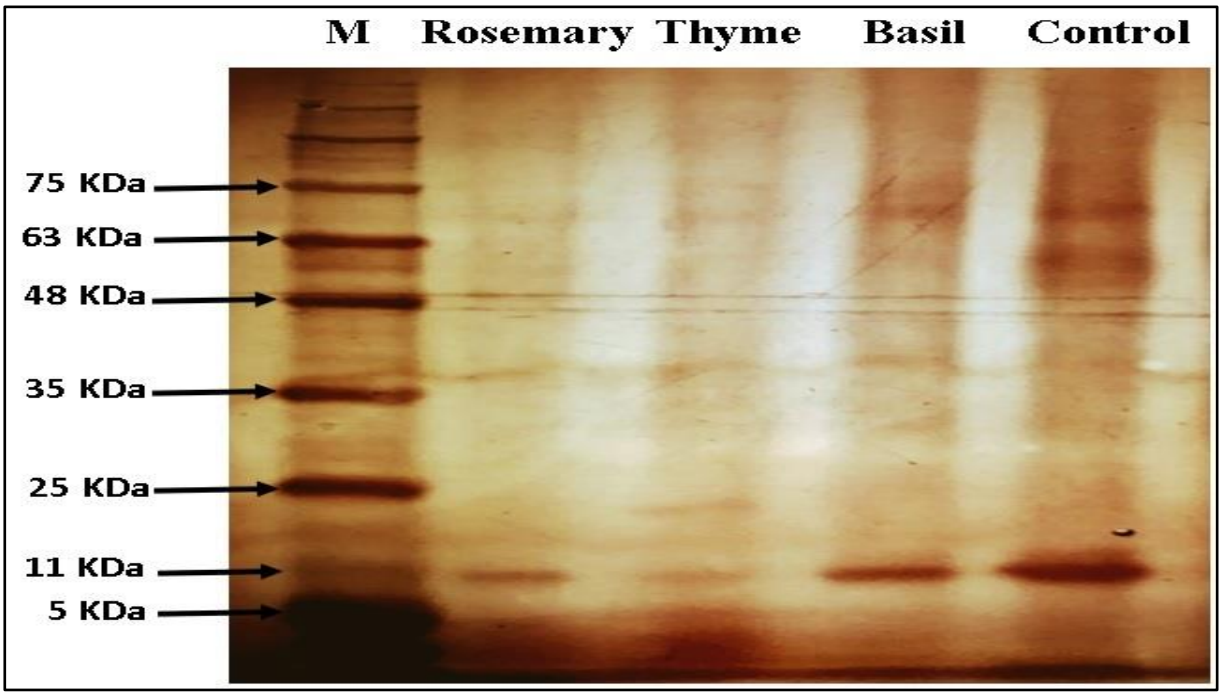

Figure (3): SDS-PAGE protein expression profile from *A. flavus* mycelium treated with basil, thyme, and rosemary plant extracts. A standard protein molecular weight marker can be seen in Lane M.

CONCLUSION

In conclusion, this study revealed that rosemary, thyme and basil plant extracts have the capability for managing peanut postharvest disease caused by *A. flavus* and reducing aflatoxin production under storage conditions. Moreover, they are effective defense treatments due to their low cost and safe to inhibit aflatoxin contamination in peanuts.

CONFLICTS OF INTEREST

The author(s) declare no conflict of interest

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