Antifungal activities of ethanol extract from walnut husk of Juglans sp. against Alternaria alternate.

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Abstract:

This study was conducted in the laboratories of the Khabat Technical Institute / Erbil Technical University in the winter of 2022-2023. In order to compare the effects of the pesticide Topsin (Thiophanate methyl 70% WP) at a concentration of 1 g/100 ml culture medium against the growth of Alternaria alternate, the study will test an alcoholic extract of the green outer husk of walnut fruits, Juglans regia, at four concentrations (0, 1, 1.25, and 1.5 g/100 ml culture medium). The findings demonstrated the existence of inhibitory activity against the fungus at all concentrations tested, with the greatest outcomes occurring at 1.5 g/100 ml of culture medium, when the inhibition rate reached 94%. This extract's laboratory result was examined using a number of treatments on some growth characteristics and yield of the Vicia faba (number of pods, number of seeds, length of the plant, percentage of protein in the seeds, percentage of seed germination) in greenhouse conditions, and the results were good when the plant was treated (directly with the extract), The results reached (4.96, 18.78, 81.16 cm, 22.74%, 84%), respectively, compared to the comparison treatment. For the treatment (fungi with addition of the extract directly), it also gave good results, and the number of pods, number of seeds, plant length, percentage of protein in seeds, and germination percentage were as follows (5.04, 19.88, 79.52 cm, 21.64%, 76%), respectively.

The following phenolic compounds were found in the plant extract when using (HPLC) analysis: galactic acid, caffeic acid, chlorogenic acid, rutin, coumaric, rosmaric acid, quercetin, cinnamic acid, and apigenin .

Keywords: Husk. Phenols. Vicia sp. Alternaria sp .

Introduction:

The temperate regions of the Northern Hemisphere and South America are home to Juglans regia walnut trees. In the United States, walnut trees are grown for their fruit and timber. A walnut normally consists of three layers: the kernel, the hard husk, and the green outer husk. You can roast, eat uncooked, or extract oil from walnut kernels [11]. Walnuts include significant levels of water, protein, dietary fiber, sugar, starch, and unsaturated and saturated fats, they are also a good source of omega-3 fatty acids, which are thought to have many health benefits. Along with a variety of vitamins, such as A, C, E, and K, they are a significant source of a wide range of minerals and salts, including calcium, iron, magnesium, manganese, phosphorus, potassium, selenium, and zinc [22]. Antioxidant compounds are abundant in walnuts. Among its many uses, hard veneers are used as thickeners, abrasives, fillers, and pigments [11]. Phenolic chemicals are abundant in walnut fruits [24]. The genetic composition of the different cultivars, geographic location, climate, and other

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environmental factors all affect the amount of phenolic chemicals present. The nut fruit's developmental stage affects the phenolic concentrations [23 .[

Walnuts' outer green husk is rich in phenoxal chemicals, which are antioxidants [5] According to [15], the walnut tree's stem, leaves, husk, green husk, and kernel all have the potential to be antioxidants. But they infrequently, as opposed to the kernel and husk. The walnut's outer green husk is frequently discarded after being used in commerce. Green husks have the potential to be antioxidants because of their high phenolic content [31 .[

Walnuts' outer green husk have demonstrated an antibacterial action against Gram-positive bacteria [27]; they can also be utilized as a broad-spectrum biocide [6]. Herbicide applications for walnut shells are also possible [2, 25.[

Phenolic compounds are the most abundant secondary metabolites in plants, showing a wide range of distinct biological activities, have received more and more attention in recent years [34]. Phenols influence fungi by interfering with their metabolism and preventing them from growing by preventing them from constructing cell walls, among other fundamental biological functions [17]. One of the fungi that infects the native Vicia faba bean crop is Alternaria alternative. This causes spots to form on the leaves of the plant, which lowers the efficiency of photosynthesis and impacts plant growth. Both the ultimate crop's quality and quantity may decline as a result of the disease.

Thus, the aim of this study was to determine how an extract from the green outer husk of Juglans regia walnut fruits affected the growth of Alternaria alternate in the laboratory. Also, The effect of some treatments on some growth characteristics, yield, protein percentage, and germination of the seeds of the bean plant Vicia faba, as well as knowing the phenolic substances, their type, and their concentration rate in the extract of the green outer shell of walnut fruits using different types of solvents by using the HPLC method.

Materials and methods:

The experiment was carried out in the agricultural season of 2022–2023 at the Khabat Technical Institute. For almost a month, pea plants were cultivated inside the greenhouse in plastic pots with five seeds each pot and daily monitoring. The green outer husk of Juglans regia walnut trees was harvested in late spring from the Huraman region in northern Iraq during the plant's growth, since it was discovered that April has the largest percentage of active components in the green outer husk of walnut trees [23]. then ground and dried in the lab. Subsequently, the extraction was carried out utilizing a soxolite apparatus in accordance with the procedure [18] with an ethanol solvent, and the material was kept refrigerated until needed.

In order to identify the chemical components in the plant extract, particularly phenols and flavonoids, samples were transported to Warmi University's central laboratory in Iran for chemical analysis using high-performance liquid chromatography (HPLC). It was extracted using the following solvents: ethanol - ethanol, ethanol - acetone, acetone - ethanol, and acetone - acetone .

Each 0.5 g sample from the ground plant was combined with 10 mL of ethanol, placed in a bath at 50°C for 40 minutes, then centrifuged for 10 minutes at 8000 rpm. At 40°C, the supernatant was dried out using a rotating vacuum evaporator. To determine which solvent yields the best results, the dried materials were dissolved in 5 mL of ethanol

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and kept at 4°C for analysis. This sample, called ethanol-ethanol [23], was deemed the first sample, and so on for the remaining samples.

Using the PCR method, the fungus was discovered after it was isolated from the remnants of the previous season's bean plant in the institute's field. The pesticide Topsin was used as a control at a concentration of 1 g/1000 ml PDA (per the instructions on the package), and the extract was tested on the fungus in the lab at four concentrations (0, 0.5, 1, and 1.5 g/100 ml PDA) (Parveen et al.,2013) The Growth Inhibition percentage (GI %) was calculated as follows [29:[

$$\frac{\text{Hyphal growth in Control Petri dish} - \text{Hyphal growth in treating Petri dish}}{\text{Hyphal growth in Control Petri dish}} \times 100 =$$

Next, the plant within the greenhouse was used to test the best concentration. Five replicates of each of the following treatments were tested: Spray the plant with the fungus and then directly with the extract (before symptoms appear), Spray the plant with the extract only, Spray the plant with the fungus and then directly with the pesticide (before symptoms appear), Spray the plant with fungi only, Control without any treatment, Spray the plant with the fungus and then with the extract (after the spots appear), Spray the plant with the fungus and then with the pesticide (after the spots appear.(

The number of pods, number of seeds, length of the plant, percentage of protein in the seeds, and percentage of seed germination were all studied.

The data was analyzed statistically for all of the studied traits [9], according to analyses of variance using the Statistical Analysis System [20]. Duncan's multiple range test (DMRT) at the 5% of significance was used to the compare among means [28.[

Results and discussion:

The results of the PCR analysis showed that the fungus isolated from the remains of the bean plant is Alternaria alternate.

Table No. (1) below illustrates how different extract concentrations affect the fungus's ability to grow. The concentration of 1.5 g/100 ml of PDA was found to be the most effective in inhibiting the growth of the fungus, with a growth rate of 0.44 cm and an effect that was very similar to that of the pesticide. In contrast, the fungal growth rate was 3.48 cm and 1.84 cm, respectively, when two concentrations of PDA (0.5, 1) g/100 ml were used. This could be because the plant's antimicrobial properties are attributed to its phenolic compounds and flavonoids. Research by [7] and [33] also revealed that the plant contains other compounds in addition to flavonoids and phenolic compounds, which are responsible for the plant's antimicrobial properties. And [1] demonstrated the antifungal properties of flavonoids and phenolic compounds .

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Table (1) The effect of different concentrations of walnut shell extract and the pesticide on the fungus Alternaria alternate in the laboratory.

	Extract concentrations				**Topsin pesticide**
	0g/100 ml	0.5g/100ml	1g/100ml	1.5g/100ml	1g/ 1000 ml
Fungi growth/cm	8.6 a	3.48 b	1.84 c	0.44 d	0
Growth Inhibition %	0	59%	78%	94%	100%

It is clear from Table No. (2) that when using the (extract) treatment before spots appeared on the plant, it gave the best results compared to the comparison treatment in terms of the number of pods, the number of seeds, the length of the plant, the percentage of protein in the seeds, and the germination percentage, which reached (4.96, 18.78, 81.16 cm, 22.74%, and 84%, respectively.

The number of pods, number of seeds, length of the plant, percentage of protein in the seeds, and percentage of the plant were 5.04, 19.88, 79.52 cm, 21.64%, and 76%, respectively, indicating that the treatment (fungi with the extract directly) had positive results.

As for the treatment (fungi only), as well as the treatment (fungi with the addition of the extract after the appearance of the spots) and the treatment (the fungi with the addition of the pesticide after the appearance of the spots), we did not obtain good results because the spots appeared and affected the studied characteristics [10.[

Table (2) Results of the effect of different treatments on the studied characteristics

	Pods no.	Seed no.	Length / cm	Protein %	Germination%
fungi + direct extract	5.04 a	19.88 ab	79.52 b	21.64 bc	76 a
Extract only	4.96 a	18.78 abc	81.16 ab	22.74 ab	84 a
fungi + direct pesticide	5.16 a	20.78 a	83.16 a	23.984 a	100 a
Fungi only	3.28 b	12.20 cd	61.68 d	18.954 d	8 b
control	4.28 ab	18.56 abc	87.86 b	21.918 bc	80 a
fungi + extract after the appearance of spots	3.08 b	10.68 d	68.64 c	19.342 c	28 b
Fungi + pesticide after the appearance of stains	3.56 b	13.44 bcd	66.56 c	20.94 c	20 b

The findings of the HPLC analysis used to identify the different types of phenols and flavonoids and their percentages in various solvents are displayed in table no 3. Even though the extraction technique was the same, the results varied because of the different solvents used. The two solvent combinations that produced the best outcomes were ethanol - acetone and acetone - acetone. The following chemicals were present in varying amounts in them:

-1Gallic acid: known for its wide range of medicinal properties, including antioxidant, antimicrobial, anti-inflammatory, and anticancer properties [13], its percentage was high when employing solvents (ethanol - acetone) and reached 56 ppm, which is regarded as a high percentage compared to other samples.

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-2Caffeic acid: using the second sample (ethanol – acetone), the percentage of this compound was significant, reaching 16.5 ppm. In the same way, the proportion increased to 14 ppm in the fourth sample (acetone - acetone). It is well-known for having anti-inflammatory and antioxidant properties; research indicates that these attributes are linked to the presence of numerous bioactive compounds that are members of the flavonoid, kaempferol, and quercetin families of flavonoids, as well as the phenolic acid, caffeoylquinic acid, caffeic acid, and quinic acid families [3.[

-3Chlorogenic acid exhibited a high percentage of 238.1 ppm when the fourth sample (acetone – acetone) was used, and 155 ppm when the second sample (ethanol – acetone) was used. According to reports, it has bactericidal properties [12]. Chlogenic acid has been shown to have antifungal properties [26.[

-4Rutin: Using the fourth sample (acetone - acetone), its proportion was high, reaching 41.2 ppm. However, it reached 38 ppm in the second sample (ethanol – acetone). Due to its strong antioxidant and anti-inflammatory characteristics, rutin, a dietary flavonoid found in fruits and vegetables, offers a variety of medicinal uses [4.[

-5Coumaric: Using the first sample (ethanol - ethanol), its percentage was high, reaching 61.8 ppm. Similarly, the percentage of the second sample (ethanol - acetone) was 27.7 ppm. According to [16], their biological actions include those of an antioxidant, anti-inflammatory, anti-cancer, antibacterial, and antiviral .

-6Rosmarinic acid: using the second sample (ethanol – acetone), the percentage of this compound was high, reaching 11.0 ppm. In the same way, the proportion of the fourth sample acetone - reached 9.4 ppm. It has been established that rosmarinic acid possesses significant biological qualities, including antibacterial, anti-inflammatory, and antioxidant capabilities [8.[

-7Quercetin: When the second sample (ethanol and acetone) was used, quercetin's percentage increased to 40.8 ppm. Similarly, the proportion of the first sample (ethanol - ethanol) was 12.6 ppm. It has been found that quercetin strongly inhibits the growth of several Gram-positive and Gram-negative bacteria [14] and inhibits the activity of certain fungi [19]. One of the strongest antioxidants derived from plants is quercetin [15.[

-8Cinnamic acid: Its percentage was high, reaching 7.1 ppm when the fourth sample (acetone - acetone) was employed. Similarly, the percentage of the second sample (ethanol– acetone) was 4.0 ppm. Because of its antibacterial and antiseptic properties, cinnamic acid is frequently used to preserve agricultural products [32.[

-9Apigenin: When using the fourth sample (acetone - acetone), the proportion of apigenin was significant, reaching 62.4 ppm. Similarly, the percentage of the second sample (ethanol + acetone) was 26.3 ppm. apigenin have antimicrobial activity [21.[

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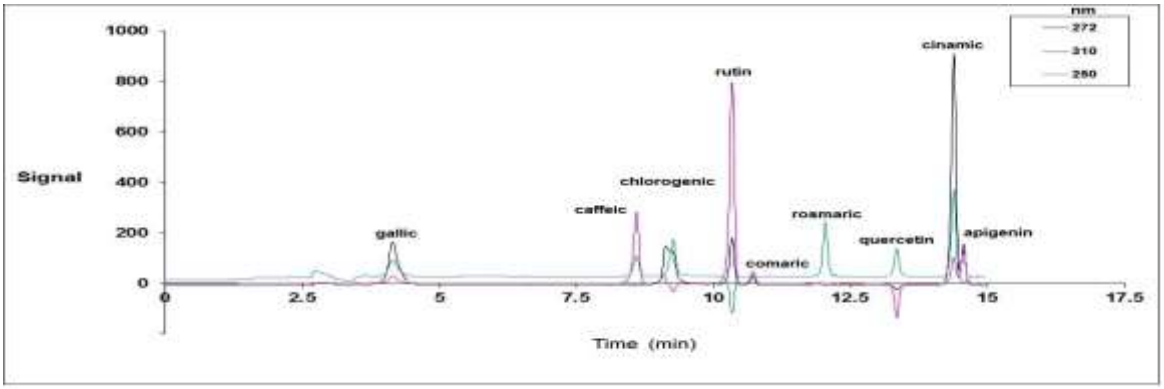
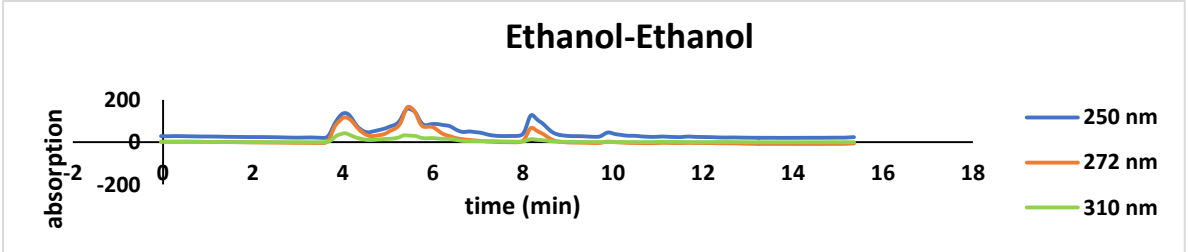
Table (3) shows the results of analyzing extracts when using different solvents by the HPLC method

Compounds	gallic acid	caffeic acid	chlorogenic acid	rutin	coumaric	Romaric acid	quercetin	cinnamic acid	apigenin
sample	(ppm) or mg/kg	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
ethanol - ethanol	14.0	1.9	14.3	5.7	61.8	1.0	12.6	1.0	7.3
ethanol-acetone	56.0	16.5	155.0	38.0	27.7	11.0	40.8	4.0	26.3
acetone - ethanol	8.7	0.8	5.6	4.1	1.5	1.0	0.5	0.0	0.1
acetone - acetone	17.4	14.0	238.1	41.2	12.5	9.4	0.1	7.1	62.4

The curves showing the four plant extract samples as they appeared in the HPLC analysis are shown below, together with the standard curve for the methanolic extract of the green outer husk of the walnut fruit. The HPLC system measures polyphenolic chemicals at three different wavelengths: 250, 272, and 310 nm. These compounds include nine different compounds of gallic acid, caffeic acid, chlorogenic acid, rutin, coumaric acid, rosmarinic acid, quercetin, cinnamic acid, and apigenin. The x-axis represents the amount of time that has passed since the sample was injected into the HPLC, and the y-axis represents the peak's height.



Standard curve showing the absorption time of chemical compounds



The curve of the first sample shows the absorbance over time

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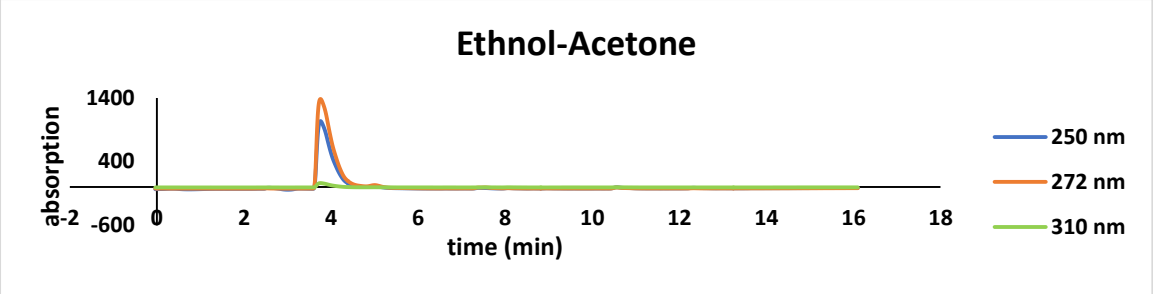
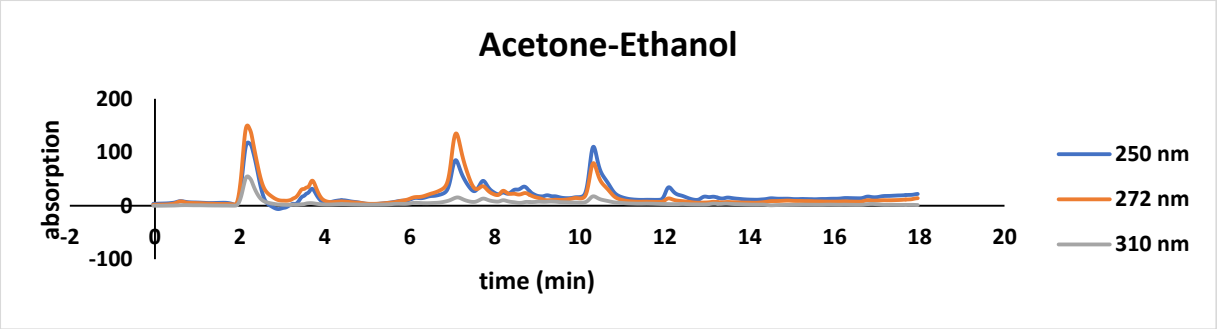
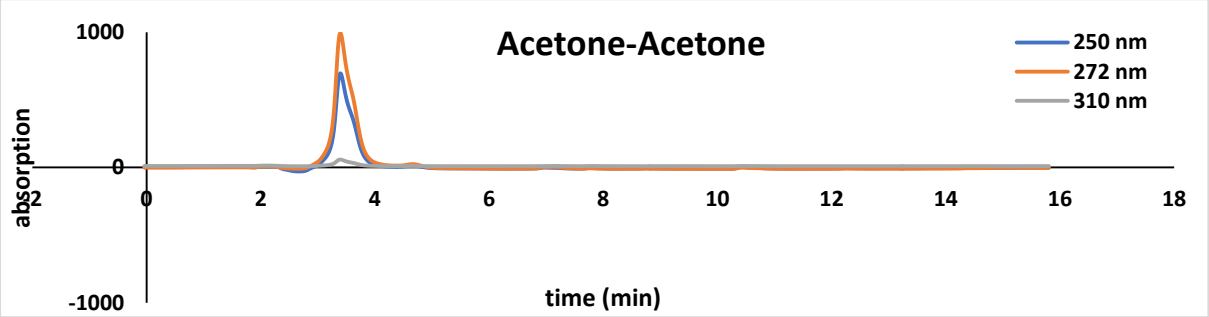


The curve of the second sample shows the absorbance over time



The curve of the thread sample shows the absorbance over time



The curve of the fourth sample shows the absorbance over time

Table (3)shows the concentrations of phenolic compounds and the time elapsed after injecting the sample into the HPLC

Sample 1 Ethanol-Ethanol	compound	Percentage of the compound%	Retention time
	Gallic acid	0.0014	4.02
	Caffeic acid	0.0002	8.30
	Chlorongenic acid	0.0014	8.95
	Rution	0.0006	11.13
	Comaric	0.0062	11.35
	Rosmaric acid	0.0001	11.67
	Quercetion	0.0013	13.55
	Cinnamic acid	0.0001	14.23
	apigienin	0.0007	14.99
Sample 2 Ethanl-Acetone			

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	Gallic acid	0.0056	3.75
	Caffeic acid	0.0017	8.075
	Chlorongenic acid	0.0155	8.97
	Rution	0.0038	10.56
	Comaric	0.0028	10.69
	Rosmaric acid	0.0011	11.50
	Quercetion	0.0041	13.76
	Cinnamic acid	0.0004	14.7
	apigienin	0.0026	14.81
Sample 3 Acetone-Ethanol			
	Gallic acid	0.00087	4.43
	Caffeic acid	0.00008	8.76
	Chlorongenic acid	0.00056	9.23
	Rution	0.00041	10.32
	Comaric	0.00015	10.54
	Rosmaric acid	0.00010	11.59
	Quercetion	0.00005	13.48
	Cinnamic acid	0.00000	14.87
	apigienin	0.00001	14.89
Sample4 Actone-Acetone			
	Gallic acid	0.00174	3.39
	Caffeic acid	0.001402	8.16
	Chlorongenic acid	0.023809	9.52
	Rution	0.004116	10.44
	Comaric	0.001251	10.59
	Rosmaric acid	0.000935	11.02
	Quercetion	0.00001	13.22
	Cinnamic acid	0.000714	14.41
	apigienin	0.006236	14.56

Conclusions:

An effective antifungal can be made by extracting the green outer husk of walnut trees. Neither the protein ratio nor the rate of germination was impacted by the extract of the green outer husk of walnut trees, nor the growth features of the plant. This allows us to apply the extract straight to the plant. The findings verified the existence of beneficial and physiologically active compounds such flavonoids and phenols. Compared to synthetic chemicals that harm human health and the environment, these materials are safer.

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