In-vitro antifungal activity of water and acetone extracts of Lawsonia inermis L. and Punica granatum L. and calcium carbonate against Aspergillus fumigatus Fres

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Summary:

Water and acetone extracts of Lawsoma mermis L. leaves and Punica granatum L. peels, and calcium carbonate, alone or in combination, were used against the germination, sporulation, and spore growth of Aspergillus fumigatus Fres. The combination of acetone extracts of Linermis and P. granatum and that of calcium carbonate alone were found to be the most effective against Aspergillus fumigatus. The acetone extract of L. inermis was very Effective inhibitor for the fungus radial growth when it was used alone or in combination with other treatments. The water extract of L. inermis and P. granatum clearly increased the fungus sporulation and spore ermination. It was concluded that the acetone extracts of L. inermis and P. granatum alone or in ombination with calcium carbonate were considered to have a good in-itro antifungal activity against 1. fumigates.

Introduction:

The fungus A. fumigatus is one of the common human pathogens that causes aspergillosis and produces a toxin named hemolytic toxin (15). It produces abundant small conidia that are easily aerosolized. In immunocompromised patients, the conidia may germinate and produce hyphae that invade the lungs and other tissues (5).

L. inermis is a shrub belonging to the family Lythraceae. It was locally named "Henna." The extract of this plant was found to have in-vitro antimicrobial activity against Brucella species and Neisseria catarrhalis (12). Also, its ether extract has an in-vitro inhibitory effect against dermatophytes such as Trichphyton and Microsporium (9). (14) reported that the extract of this plant has antimycotic activity against Pestalotiopsis mangiferae

P. granatum (Pomegranate) is a tree belonging to the family Punicaceae. It was locally

named "Rumman." Many studies have revealed that the water extract of this plant material has antifungal activity against A. niger and some bacteria (3) and many other fungi, especially dermatophytes (6).

Calcium carbonate (CaCO3) was also shown to have antifungal activity against A. flavus and was used to control it in grain stores (13).

This study was intended to elucidate the effects of the acetone and water extracts of the above plants, in addition to the effect of CaCO3 and their combinations on the colony radial growth, sporulation, and spore germination of A. fumigatus.

Materials and Methods:

Plant materials

The leaves of L. inermis were collected from the Agricultural Research Station, College of Agriculture, while the peels of P. granatum were freshly brought from the local market at Basrah

city. The leaves and peels were airily dried and well milled.

Preparation of plant extracts:

Ten grams of dried materials of each sample were placed in thumbs of a soxhlet extractor and extracted separately with 200 ml of either distilled water or acetone for 24 hours. The extracts were evaporated by a rotary evaporator (Switzerland RM Scientific LTD. This method was replicated three times to obtain a sufficient quantity of dried material extracted. The dried residue was kept in tightly closed vials in a deep freeze away from light until the time of use (modified method of Harborne, 1984).

Isolation of the fungus

A. fumigatus was isolated from orange fruits on PDA, purified, and identified according to (16).

Radial growth and sporulation:

Potato dextrose agar (PDA) was prepared in a 150 ml tlask containing 99 ml of medium

Autoclaved at 121°C and 15 pounds per inch for 20 minutes. One gram of plant extracts or calcium carbonate (CaCO3) was added to each flask to obtain a concentration of 1% of each treatment as follows:

1-1 g of L. inermis acetone extract (L.A.).

2-1 g of L. inermis water extract (L.W.).

3-1 g of P. granatum acetone extract (P.A.).

4-1 g of P. granatum water extract (P.W.).

5-1 g of calcium carbonate (CaCO3).

6-0.5 g of L.A. + 0.5 g of P.A.

7-0.5 g of L.W. 0.5 g of P.W.

8-0.5 g of L.W. 0.5 g of P.A

9-0.5 g of L. A + 0.5 g of P. W.

10-0.5 g of L. A + 0.5 g of L. A + 0.5 g of CaCO3

11-0.5 g of L. W - 0.5 g of CaCO3

12-0.5 g of P. A + 0.5 g of CaCO3

13-0.5 g of P. W + 0.5 g of CaCO3

14- Control (only PDA).

The flasks were shaken well to be homogenized. Twenty milliliters of PDA were

poured into each sterile Petri dish 9 cm in diameter | with three replicates for each treatment. Each sterile Petri dish was inoculated with an agar disk (5 mm diameter) of a 7-day-old culture of A. fumigatus.

All Petri dishes were incubated at 25 2" C. The radial growth was measured daily until the mycelium growth had reached the edge of the Petri dish in the control treatment.

The percentage of inhibition of radial growth was estimated according to the modified method of Abbott (1925) as follows:

$$\% \text{ Inhibition of radial growth} = \frac{Radial\ growth\ average - Radial\ growth\ average\ of\ control\ of\ treatment}{Radial\ growth\ average\ of\ control} \times 100$$

To determine the sporulation an agar disk (3 mm diameter)

To determine sporulation, an agar disk (3mm diameter) of 7-day-old culture of the fungus was taken from the edge of the colony for each treatment by cork borer and placed in each vial containing 5 ml of FAA (Formaline Acetic Acid: Alcohol at the ratio of 1:1:8). All vials were shaken well for five minutes, then the spores were counted using a haemocytometer (Bedan, 1996). The sporulation was calculated as follows:

$$Sporulation = \frac{Number\ of\ spores\ in\ all\ squares \times 10x\ dilution}{Number\ of\ counted\ squares}$$

Spore germination:

One gram of each dried residue of plant extracts or calcium carbonate was added to each flask containing 99 ml sterile distilled water to obtain a concentration of 1% for each treatment as shown in section 2-4. Ten milliliters of each treatment were taken from each flask and placed in

A vial. An agar disk (5 mm diameter) of a 7-day-old culture of the fungus was placed in each vial and shaken well to remove the spores from the conidiophores. One drop from each vial was taken by a dropper and placed on a slide placed in a Petri dish containing filter paper soaked with distilled water. All these steps were carried out under sterile conditions (modified method of Bedan, 1996). The percentage of germinated spores was estimated after 6, 12, and 18 hours as follows:

$$\% \text{ Germinated spores} = \frac{Number\ of\ germinated\ spores}{Number\ of\ total\ spores} \times 100$$

Statistical analysis:

Statistical analysis was performed computerically by ANOVA analysis using Minitab 11 statistical program

Results:

Inhibition of radial growth:

The inhibition percentage of radial growth of A. fumigatus was estimated. Statistically, a high significant inhibition ($P<0.001$) occurred in the treatments L.A., L.AP.A, L.WP.A, L.AP.W, L.A. $CaCO_3$, and P.A+ $CaCO_3$, with inhibition percentages of (81.6, 92.33, 78.76, 84.48, 65.56, and 54.02)%, respectively, while the effects of the other four treatments P.A., L.WP.W, L.W. $CaCO_3$, and P.W- $CaCO_3$ were significant at the statistical level of $P<0.01$ with inhibition percentages of (31.41, 44.82, 33.33, and 27.01)%, respectively (Table 1). It was also found that L.W. and P.W. treatments had no effects.

Sporulation:

Spores were counted. The L.A.P.A. treatment revealed a high significant inhibition ($P<0.001$) against fungus sporulation. It reduced the number of spores up to 2437 spores in comparison with control treatment (12087 spores) followed by ($CaCO_3$ and P.W + $CaCO_3$) treatments at a level of $P<0.01$, which decreased the number of spores up to 5758 and 8483 spores in

respectively (Table 2).

comparison with control treatment (12087 spores) followed by (CaCO3 and P.W + CaCO3) treatments at level of P<0.01 which decreased the number of spores up to 5758 and 8483 spores respectively (Table 2).

Table 1. Inhibition percentage of radial growth of A. fumigatus on PDA by plant extracts and CaCO

Treatments	Percentage of inhibition (Mean = SEM)	Significance with respect to control
L. A	81.6 ± 0.66	***
L. W	12.64 = 0.66	NS
L. P	31.41 ± 12.03	**
L. P	1.49 ± 0.98	NS
CaCO3	28.35 ± 2.99	*
L.A + P.A	92.33 ± 0.38	***
L.W +P.W	44.82 ± 1.33	**
L.W - P.A	78.76 1.54	***
L.A- P.W	84.48 ± 0.99	***
L.A- CaCOs	65.56 = 0.99	***
L.W+	33.33 1.52	**

$CaCO_3$		
P.A-$CaCO_3$	54.02 - 0.58	***
P.W-O_3	27.01 ± 1.52	**

***P<0.001,P<0.01,P<0.05

It was also found that the treatments L.W.P., W.L.W.-CaCO3, and 1 W P.A. significantly ($P<0.01$) increased sporulation up to 20,985, 23,570, and 25,671 spores, respectively, compared with the control treatment (12,087 spores), while L.W. treatment had no effect (Table 2).

Table 2. Number of spores of A. fumigatus counted by agar disk hemocytometer (3 mm diameter) for all treatments

Treatments	Spores 100 (Mean = SEM)	Significance with respect to control	Effect
L.A.	157.08 ± 1.82	*	Δ
L.W.	101.08 ± 1.24	NS	↔
P.A.	74.75 ± 0.72	*	▼
P.W	81.67 ± 1.1	*	▼
$CaCO_3$	57.58 ± 2.17	**	▼
L.A-P.A	24.37 ± 0.36	***	↙
L.W+P.W	209.58 ± 1.5	***	▼
L.W-P.A	256.71 ± 4.49	***	Δ
L.A-P.W	161.00 ± 1.52	*	Δ
L.A + $CaCO_3$	182.08 ± 2.06	**	Δ
L.W-$CaCO_3$	235.70 ± 4.05	***	Δ
P.A - $CaCO_3$	84.83 ± 2.31	*	▼
P.W CaCO3	45.58 ± 0.44	**	▼
Control	120.87		

***P < 0.001 **P<0.01 *P < 0.05 NS P > 0.05

Increase ↑ Decrease ↓ No effect ⟷

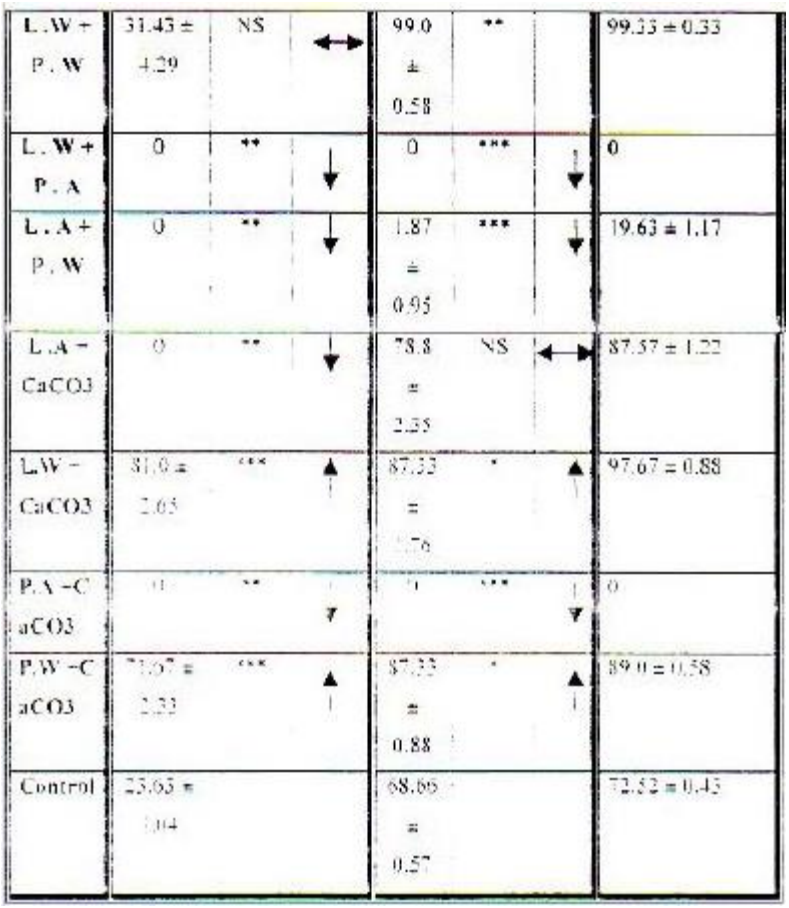
3-3- Spores germination:

The percentage of germinated spores was estimated as shown in Table (3), which explained that the treatments P. A, CaCO3, L. A-P. A, L. W+P. A and P. A CaCO3 were very effective and significantly inhibited spores germination after 6, 12 and 18 hours by 100% for all treatments The treatments L.A. and LA CaCO3, inhibited the germination percentage up to 100% after 6 hours only while they increased the percentage of germinated spores after 12 and 18 hours up to (85.67, 78.8, 97.67 and 87.57)% respectively compared with control treatments which were (68.66 and 72.52)% respectively, also it was noticed that the treatments L.W.P.W.L.W CaCO3 and P.W-CaCO3 increased spores germination after 6, 12 and 18 hours in comparison with control treatments in contrast L.W.P.W. and LA +

$CaCO_3$ had no effects after 6 and 12 hours, respectively (Table 3).

Table 3. Percentage of spore germination of A. fumigatus after three incubation periods of 6, 12, and 18 hours

Treatments	6 hours			12 hours			18 hours
	% spores germination Mean ± SEM	Significance with respect to control	Effect	% spores germination Mean ± SEM	Significance with respect to control	Effect	% spores germination Mean ± SEM
L. A	0	**	↓	85.67 ± 0.88	*	↑	97.67 ± 0.88
L. W	92.67 ± 2.03	***	↑	99.0 ± 0.58	**	↑	99.33 ± 0.33
P. A	0	**	↓	0	***	↓	0
P. W	96.0 ± 1.15	***	↑	97.0 ± 0.58	**	↑	98.0 ± 0.58
CaCO3	0	**	↓	0	***	↓	0
L. A + P. A	0	**	↓	0	***	↓	0



L.W + P.W	31.43 ± 4.29	NS	↔	99.0 ± 0.58	**	99.33 ± 0.33
L.W + P.A	0	**	↓	0	***	↓ 0
L.A + P.W	0	**	↓	1.87 ± 0.95	***	↓ 19.63 ± 1.17
L.A + CaCO3	0	**	↓	78.8 ± 2.35	NS	↔ 87.57 ± 1.22
L.W + CaCO3	81.0 ± 2.65	***	▲	87.33 ± 1.76	*	▲ 97.67 ± 0.88
P.A + CaCO3	0	**	↓	0	***	↓ 0
P.W + CaCO3	71.67 ± 2.33	***	▲	87.33 ± 0.88	*	▲ 89.0 ± 0.58
Control	23.63 ± 1.04			68.66 ± 0.57		72.53 ± 0.45

P0.001 P0.01 * P<0.05 NS P>0.05

Increase Δ Decrease ↓ No effect ↔

Discussion:

Acetone extracts of L. inermis and P. granatum were the most effective inhibitors against the radial growth, sporulation, and spore germination of A. fumigatus. This inhibitory effect could be due to the acetone-soluble constituents present in L. inermis and P. granatum plants, such as tannins (17) and resins (8), which are found in different quantities in these plants (2).

Calcium carbonate also inhibited sporulation.

spore germination and to a lesser extent radial growth of A. fumigatus, especially when mixed with acetone extract of P. granatum. This inhibitory effect may be a result of its effect on the important enzymes needed by the fungus for growth (13).

Acetone extract of L. inermis with its combinations such as the acetone extract with either water extract of P. granatum and/or CaCO3 respectively were effective to prevent hyphae formation in vitro: this may be caused by the ability of L. inermis to prevent utilization of

Carbon and nitrogen sources, which were essential for fungal growth (18:11).

Acetone, rather than water extract of L. inermis and P. granatum, seems to be a promising antimycotic agent against A. fumigatus.

The main pathogenicity of A. fumigatus was caused by hyphae, which tend to invade preexisting cavities or blood vessels (5).

Further studies are needed to prove these preliminary results and compare them with currently reported antimycotic drugs (7).

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والأسيتوني النباتي الحنةLawsonia inermis L

والرمان Punica granatum L.وكاربونات

الكالسيوم ضد الفطر Aspergillus fumigatus Fres

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الخلاصة:

أستخدم المستخلص الماني ومستخلص الأسيتون الأوراق الجنة Punica granatum وقشر الرمان Lawsonia inermis L.اوكاربونات الكالسيوم . (CaCO3) 1%كل على حدة أو مع بعضها ضد الفطر Aspergillus funigatus Fres المعرفة تأثيرها في النمو الشعاعي والتجرثم وأنبات أبواغ الفطر المذكور.

وقد وجد أن خليط مستخلصات الأسيئون لكل من الجنة والرمان أو كاربونات الكالسيوم لوحدها كان لها التأثير الأكبر ضد القطر . fumigatus كما تبين أن مستخلص الأسيتون الأوراق الحنة قد ثبط النمو القطري للقصر بشكل فعال سواء أستخدم لوحده أو مع المعاملات الأخرى ، في حين أدى المستخلص المالي الأوراق الحسنة وتشور الرمان إلى زيادة تجرثم الفطر وإثبات أبواغه بشكل ملحوظ.

يستنتج من ذلك أن مستخلص الأسيتون لكل من أوراق الحنة وقشور الرمان سواء كان لوحده أو بالأتحاد مع كاربونات الكالسيوم يمكن إعتباره مضادا فطريا" جيدا ضد الفطر fumigatus …