Bas.J.Vet.Res., Vol.4,No.2,2005.

IN-VITRO ANTIFUNGAL ACTIVITY OF WATER AND ACETONE EXTRACTS OF *LAWSONIA INERMIS, PUNICA GRANATUM* AND CALCIUM CARBONATE AGAINST *ASPERGILLUS FUMIGATUS.*

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(Received 11 December 2004 ,Accepted 4 July 2005)

Keywords: Antifungal activity, *Aspergillus fumigatus*, , *Lawsonia inermis* L..

ABSTRACT

Water and acetone extracts of the leave of *Lawsonia inermis* L., the peels of *Punica granatum* L. and calcium carbonate (1%), alone or in combination were used against the fungus *Aspergillus fumigatus* radial growth, sporulation and spore germination. The combination of acetone extracts of *L. inermis* and *P. granatum*, and that of CaCO3 alone, were found to be the most effective against *A. fumigatus*. The acetone extract of *L. inermis* was very effective inhibitor of radial growth of the fungus when used alone or in combination with other treatments. The water extract of *L. inermis* and *P. granatum* increased sporulation and spore germination of the fungus. It was concluded that the acetone extracts of *L. inermis* and *P. granatum* alone or in combination with CaCO3 were considered to have a good in vitro antifungal activity against *A. fumigatus*.

INTRODUCTION

The fungus *A. fumigatus* is one of the common human pathogen which causes aspergillosis and produces a toxin named haemolytic toxin (Rao,1993). It produces abundant small conidia which are easily aerosolized. In immunocompromised patients, the conidia may germinate and produce hyphae which invade the lungs and other tissues (Brooks *et al*, 1998).

L. inermis is a small tree from the family Lythraceae, it was locally named as 'Henna'. The extracts of this plant was found to have an in vitro antimicrobial activity against *Brucella* species and *Neisseria catarrhalis* (Oswalds *et al*, 1971). Its ether extract has an in vitro inhibitory effect against dermatophytes such as *Trichophyton* and *Microsporium* (Ghani and Yahya, 1987). Rai(1996) reported that the extract of this plant has an antimycotic activity against *pestalotiopsis mangiferae*.

P. granatum is a tree plant of the family Punicaceae; it was locally named 'Rumman'. Many studies revealed that the water extracts of the plant materials have an antifungal activity against the fungus *Aspergillus niger* and some bacteria (Anesini and Perez, 1993) and many other fungi especially dermatophytes (Dutta *et al*, 1998).

Calcium carbonate (CaCO3) was also shown to have an antifungal activity and used to control the fungus *Aspergillus flavus* in the stores (Qassim,1998).

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

This study is elucidate the effect of the acetone and water extracts of the above cited plants, in addition to the effect of CaCO3 and their combination, on the 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 – Basrah University, while , the peels of *P. granatum* were brought fresh from the market in Basrah, Iraq. The leaves and peels were air dried and well milled.

Preparation of plant extracts.

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

Isolation of the fungus.

A. fumigatus was isolated from the hydatid cysts of sheep, purified and identified according to Raper and Fennel (1965).

Radial growth and Sporulation

Potato dextrose agar (PDA) was prepared in a 150 ml flasks containing 99 ml of media and autoclaved at 121°C and 15 Pounds/ inch² for 20 minutes. One gram of plant extracts or calcium carbonate (CaCO3) were added to each flask to obtain a concentration of 1% of each treatment as follows:

1- 1g of *L. inermis* acetone extract (L/A).
2- 1g of *L. inermis* water extract (L/W).
3- 1g of *P. granatum* acetone extract (P/A).
4- 1g of *P. granatum* water extract (P/W).
5- 1g of calcium carbonate (CaCO3).
6- 0.5g of L/A +0.5g of P/A.
7- 0.5g of L/W +0.5g of P/W.
8- 0.5g of L/W +0.5g of P/A.
9- 0.5g of L/A +0.5g of P/W.
10- 0.5g of L/A +0.5g of CaCO3.
11- 0.5g of L/W +0.5g of CaCO3.
12- 0.5g of P/A +0.5g of CaCO3.
13- 0.5g of P/W +0.5g of CaCO3.
14- Control (PDA) only.

The flasks were shacked well to be homogenous. 20 ml of PDA were poured in each sterile Petri dish (9 cm diameter) with three replicates for each treatment. Each sterile Petri dish was inoculated with an agar disk (5mm diameter) of 7- day- old culture of A. *fumigatus*. All Petri dishes were incubated at 25 ± 1°C. The radial growth was daily measured until the mycelium growth had reached the edge of Petri dish in control treatment.The percentage inhibition of radial growth was estimated according to the modified method of Abbott (1925) as follows:

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

To determine the sporulation, an agar disk (3 mm diameter) of 7-day-old culture of the fungus was taken from the edge of colony for each treatment by cork borrer and placed in each vial contained 5 ml of FAA (Formaline: Acetic acid: Alcohol at the ratio of 1:1:8). All vials were shacked well for five minutes, then the spores were counted by using haemocytometer.

Spore germination

One gram of each dried residue of plant extracts or calcium carbonate were 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 (5mm diameter) of 7-day-old culture of the fungus was placed in each vial and shacked well to remove the spores from the conidiophores. One drop from each vial was taken by a dropper and placed on a slide putting it in a Petri dish containing filter paper soaked with distilled water; all under sterile conditions. The percentage of germinated spores was estimated after 6, 12 and 18 hours.

statistical analysis

Statistical analysis was performed computerly by ANOVA analysis using GPIS statistical program.

RESULTS

Inhibition of radial growth

The percentage inhibition of radial growth was estimated. Statistically, highly significant inhibition occurred with (L/A, L/A+P/A, L/W+P/A, L/A+P/W, L/A+CaCO3 and P/A+CaCO3) treatments ($P<0.001$). While the effect of another four treatments (P/A, L/W+P/W, L/W+CaCO3 and P/W+CaCO3)were significant at statistical level of $P<0.01$(table 1).

Table 1: Percentage inhibition of radial growth of *Aspergillus fumigatus* on PDA media containing plant extracts and CaCO3 according to listed treatments compared with control.

Treatments	Percent inhibition (Mean ± SEM)	Significance with Respect to control
L/A	81.6 ± 0.66	***
L/W	12.64 ± 0.66	NS
P/A	31.41 ± 2.03	**
P/W	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+ CaCO3	65.56 ± 0.99	***
L/W+ CaCO3	33.33 ± 1.52	**
P/A+ CaCO3	54.02 ± 0.58	***
P/W+ CaCO3	27.01 ± 1.52	**

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

sporulation:

Fungal spores were counted to estimate the sporulation activity. The treatment (L/A+P/A) revealed highly significant inhibition of fungus sporulation compared to control (P<0.001), followed by P/w+ CaCO3 and CaCO3 at (P<0.01).

The treatments L/w+P/W, L/W+P/A and L/W+ CaCO3, on the other hand, significantly increased sporulation compared tocontrol (P<0.01), table 2 .

Table 2: Number of spores x100/agar disk (3mm diameter) of *Aspergillus fumigatus* counted by haemocytometer for each treatment compared with control.

Treatments	Spore x100 (Mean ± SEM)	Significance With Respect to control	effect
L/A	157.08 ± 1.82	*	↑
L/W	101.08 ± 1.24	NS	↔
P/A	74.75 ± 0.72	*	↓
P/W	81.67 ± 1.1	*	↓
CaCO3	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	***	↑
L/A+P/W	161.00 ± 1.52	*	↑
L/A+ CaCO3	182.08 ± 2.06	**	↑
L/W+ CaCO3	235.70 ± 4.05	***	↑
P/A+ CaCO3	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

Spore germination:

The percentage of germinated spores was estimated. Table 3 showed that treatment with P/A,CaCO3,L/A+P/A,L/W+P/A or P/A+ CaCO3 was very effective and inhibited spore germination significantly after 6,12,18 hours.The treatments L/A and L/A+ CaCO3 inhibited spore germination after 6h; the inhibition was increased after 18h compared with control. The treatments L/W, P/W, L/W+ CaCO3 and P/W+ CaCO3, on the other hand, increased spore germination after 6,12,and 18 hours.

Table 3: Percentage of spore germination of A. fumigatus estimated after 6, 12, 18 hours according to listed treatments compared with control.

Treatments	6 hours			12 hours			18 hours		
	Percent spore germination (Mean ± SEM)	Significance with Respect to control	Effect	Percent spore germination (Mean ± SEM)	Significance with Respect to control	Effect	Percent spore germination (Mean ± SEM)	Significance with Respect to control	Effect
L/A	0	**	↓	85.67±0.88	*	↑	97.67±0.88	***	↑
L/W	92.07 ±2.03	***	↑	99 ± 0.58	**	↑	99.33±0.33	***	↑
P/A	0	**	↓	0	***	↓	0	***	↓
P/W	96 ± 1.15	***	↑	97±0.58	**	↑	98±0.58	***	↑
CaCO3	0	**	↓	0	***	↓	0	***	↓
L/A+P/A	0	**	↓	0	***	↓	0	***	↓
L/W+PW	31.43 ±4.29	NS	↔	99±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 ± 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 ± 0.58	**	↑
Control	23.63±1.04			68.66±0.57			72.52±0.43		

*** P< 0.001 ** P<0.01 * P<0.05 NS P>0.05
↑ Increase ↓ Decrease ↔ no effect

DISCUSSION

The acetone extracts of L. inermis and P. granatum, were the most effective inhibitors of radial growth, sporulation and spore germination of A. fumigatus. This inhibitory effect could be due to the acetone soluble constituents presents in L. inermis and P. granatum plants such as tannins (Tyler et al,1988) and resins (Gennaro, 1995) which are found in different quantities in these plants (Al-Rawi and Chakravarty,1988).

Calicium Carbonate also inhibited sporulation,spore germination and to a lesser extent radial growth, especially when mixed with acetone extract of *P. granatum*. This inhibitory effect may result from its effect on the important enzymes needed by the fungus for growth (Qassim, 1998).

The main pathogenic effect of A. *fumigatus* was formed by hyphae which have a tendency to invade preexisting cavities or blood vessels (Brooks *et al*, 1998).

The acetone extract of *L. inermis* with its combinations such as the (acetone

extract with either water extract of *P. granatum* or CaCO3 respectively) were effective in preventing 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
(Westergaard and Mitchell, 1947 ; Hirsch, 1954).

The acetone rather than water extract of *L. inermis* and *P. granatum* seems to be a promising antimycotic agents against A. *fumigatus* .

Further studies are required to prove these preliminary results, comparing them with currently reported antimycotic drugs (Faergemann and Fredriksson, 1980).

الفعالية المضادة للمستخلص المائي والاسيتوني لنبات الحناء والرمان وكاربونات الكالسيوم ضد الفطر Aspergillus fumigatus خارج الجسم الحي

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الخلاصة

استخدم المستخلص الاسيتوني لاوراق الحناء وقشور الرمان وكاربونات الكالسيوم تركيز 1% ضد النمو الشعاعي وسبورات وابواغ فطر الاسبرجلس .

وادى استخدام مزيج من المستخلص الاسيتوني لنبات الحناء والرمان وكاربونات الكالسيوم لوحدها الى زيادة الفعالية المضادة للفطر

كانت الفعالية التثبيطية للمستخلص الاسيتوني لنبات الحناء عالية جدا ضد النمو الشعاعي للفطر عند استخدامة مفردا او مع المستخلصات الاخرى , بينما ادى استخدام المستخلص المائي لنبات الحناء والرمان الى زيادة عملية التبويغ

يمكن الاستنتاج بان استخدام المستخلص الاسيتوني لنبات الحناء والرمان او مزجهم مع كاربونات الكالسيوم ادى الى زيادة الفعالية التثبيطية ضد الفطر Aspergillus fumigatus خارج الجسم الحي

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