Sensitivity of *Aspergillus flavus* to methanolic and aqueous extracts of some medicinal plants

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Abstract

The study was conducted to investigate the antimicrobial activity of methanolic and aqueous extracts of: *Melia azedarach* L. fruit; Merlia stem, *Lantana camara* flower, *Lantan camara* fruit, *Cordia dichotoma* fruit, Ricinus seeds, Pomegranate peel, Senna leaves, using well, and discs agar diffusion methods in addition to find Minimum Inhibitory Concentration, against *Aspergillus flavus* which was isolated from different feed sources. Out of the two crude extracts, the methanol extracts showed the highest activity. Pomegranate peel extracts of both methanol and aqueous extract showed the best anti aspergillus activity. Well agar diffusion method showed better results in comparison with disc diffusion method. The results justify their use in traditional herbal medicine for the management of fungal based diseases.

Key words: Sensitivity; plant extract, Aspergillus.
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حساسية فطر الاسبريكلاس لمستخلص الميثانول ومستخلص الماء لبعض النباتات الطبية

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الخلاصة

أنجزت الدراسة لبحث الفعالية المضادة لفطر الاسبريكلاس لمستخلص الميثانولي والمستخلص المائي لبعض النباتات الطبية: ثمار السبحبح، سيقان السبحبح، أزهار النتانا، ثمار النتانا، ثمار البمبر، بذور الخروع، قشرة لب الرمان، اوراق السنا، باعتماد طريقتي الحفر والأقراص، فضلا عن ايجاد التركيز الادنى المثبط للفطر المعزول من مصادر الغذاء المختلفة. اظهرت النتائج ان مستخلصات الميثانول هي الأفضل، وان مستخلصات لب الرمان كانت الأفضل كمضادات للاسبريكلاس. طريقة الحفر اعطت نتائج افضل بالمقارنة مع طريقة الاقراص. وهذا يشير إلى إمكانية اعتمادهما كأساس لعلاج الأمراض ذات الأساس الفطري.

الكلمات المفتاحية: الحساسية، مستخلصات النباتات، الاسبريكلاس

Introduction

Many plants species reported to have pharmacological properties as they are known to possess various secondary metabolites like glycosides, saponins, flavonoids, steroids, tannins, alkaloids, tripenes (1, 2, 3). Research on medicinal plants attracted a lot of attentions globally. Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value, therefore microbiologists used phytochemicals for their exploration as antimicrobial s such plant products would be biodegradable and safe to human health (4). Plant produced compounds are of interest as sources of safer or more effective substitutes for synthetically produced antimicrobial agents. The use of herbal medicine predates the introduction of antibiotics and predates social, economic, and religious barriers. The aims of this study were to explore the anti- *Aspergillus flavus* effects of aqueous and methanol extracts of some medicinal plants, in addition to compare the disc and well diffusion methods.

Materials and Methods

- **Test medicinal plants:**

- **Crude plant extracts preparation:** The fruits, leaves, flowers of plants were either purchased from local markets (Senna leaves) or collected (Pomegranate fruit, Cordia fruit, Lantana flowers, Lantana fruits, Ricinus seeds, Melia fruits) from farms in Baquba city, Diyala province, Iraq from December 2015- May 2016.

- **Preparation of extracts**

- **Alcoholic extract:** The collected fruits, leaves, flowers of plant used in the study, were air- dried under the shade at room temperature, ground into powder and extracted using Methanol, and water according to standard extraction methods (5). Total of thirty-forty grams of shade dried, powder of plant materials (Cordia fruit; Melia stem; Melia Fruit; Lantana flower; Lantana fruit; Senna leaves and pomegranate peel) were filled separately in the thimble and extracted successively with 250- 300 ml methanol (100%) using a Soxhlet extractor for 48 hours. While Ricinus seeds extract prepared according to (6) in which the husks of the seeds were manually removed, the wet flesh then pressed by compressor to remove the oil present. The powder left either macerated in distilled water or methanol to prepare the aqueous or methanolic extract. The extracts were concentrated using rotary evaporator. After complete solvent evaporation, each extract was weighed and preserved at 4^{o}C in airtight bottles until further use (5).

- **Aqueous extract:** Total of thirty-forty grams of shade dried, powder of plant materials (Cordia fruit; Melia stem; Melia Fruit; Lantana flower; Lantana fruit; Senna leaves; Pomegranate peel, Ricinus seed powder) were put separately in 300 ml distilled water on magnetic steror and left for 24 hours. Then filtered through double layer guaze, then through filter paper. The extracts were concentrated using rotary evaporator. After complete solvent evaporation each extract was weighed and preserved at 4^{o}C in airtight bottles until further use (5).

- **Sample reconstitution and preparation:** Standard concentrations were prepared by dissolving 1.5 g of dried extract in 5 ml of methanol or water to obtain 300mg/ml. and used as the test extracts for antimicrobial activity assay(7, 8).

- **Antimicrobial activity:** Disc and well agar diffusion techniques were used as the standard method for antimicrobial activity and minimum inhibitory concentrations (MICs) for active extracts against *Aspergillus flavus*. Stock solution at concentration 300 mg/ ml) was prepared for each plant tested. Three dilutions were prepared (100, 200, and 300 mg/ml) from each stock solution. Filter paper discs of 5 mm diameter were prepared and used for each concentration by pipetting 100 µl onto individual paper discs (5 mm) drop by drop using micropipette. Paper discs with crude extracts were transferred onto plates and inoculated with 1 ml of standard inoculum. The plates were incubated at 25 ^{o} C for *Aspergillus flavus*. Nystatin was used as standard antifungal, while discs with extraction solvents only were used as controls. These were done in triplicates under sterile conditions and results recorded after 24, 48, 72, 96 and 120 hours. The antimicrobial activity was determined by measuring clear inhibition zones diameters (including diameter of paper discs) using the caliper (9). Minimum inhibitory concentrations were determined by recording the lowest concentration of the active extracts that inhibited growth of the microorganisms, (9). Methanolic and aqueous extracts of each plant added separately by limited volume from standard concentration of each extract to limited volume of potato dextrose agar before solidification, after

completer mixing obtain (100, 200, 400, 800 and 1600 mg/ ml), then agar poured to Petri dishes glass of 9 cm diameter after solidification discs from periphery of culture fungal colony (1 week old) through cork porer of 0.5 cm and placed in center of petri dishes under sterile conditions. The plates incubated in versal position at 27 ^{o} C for 1 week.

- **Minimum inhibitory concentrations (MIC):** MIC of each extracts and standards were able to kill or inhibit the growth of fungus in this study was exhibited by extracts that were active at concentration of 100 to 1600 mg/ml.
- **Statistics:** In order to analyze data, multiple way ANOVA was used to determine significant factors in production of inhibition zones (10).

Results

The results of the study revealed that in aqueous extracts, disc diffusion method, the lowest inhibitory zone was shown by Melia stem extract at 100 mg/ ml (6.75 ± 0.25mm) while the highest inhibitory zone was exhibited by pomegranate peel extract at 300 mg /ml (27.17 ± 0.17 mm). The inhibitory zones were concentration dependent. At 100, 200 and 300 mg /ml the highest zone exhibited by pomegranate peel extract. (Table1).

Table (1) The diameter of inhibitory zone of Aqueous extract: Disc diffusion method.

Extract	Concentration of extract mg /ml		
	100	200	300
Melia Stem	6.75± 0.25	9.75 ± 1.75	15.83 ±1.17
Pomegranate peel	14.25± 2.25	22.50 ± 3.33	27.17 ± 0.17
Senna leaves	9.83± 0.33	12.0 ± 2.36	15.75 ± 0.25
Cordia fruit	12.17 ± 1.09	13.0 ± 0.50	14.5 ± 1.42
Lantana flower	8.67 ± 1.01	9.0 ±1.0	16.5 ± 2.75
Melia fruit.	10.50± 0.50	13.17 ± 1.33	17.5 ± 1.49

Values are Mean± S.E.

The result revealed that the highest zone of inhibition (32.5 ±1.00 mm) was in 300 mg/ ml exhibited by pomegranate peel extract. While the lowest zone of inhibition (6.26 ± 0.25 mm) exhibited by Melia stem in 100 mg/ ml. the highest inhibitory zone was exhibited by pomegranate peel extract followed by Senna leaves, Melia stem, Cordia fruit, lantana flower, Melia fruit respectively. In comparison with control all above control (Table -2-).

Table (2) Diameter of inhibitory zone Aqueous extract: well diffusion method

Extract	Concentration of extract mg /ml		
	100	200	300
Melia Stem	6.26 ± 0.25	11.75 ±2.75	13.75 ± 0.00
Pomegranate peel	9.75 ± 2.25	25.0 ± 0.00	32.5 ± 1.00
Senna leaves	10.0 ± 0.00	13.0± 3.50	15.50 ± 1.00
Cordia fruit	8.25 ± 0.72	9.25 ± 4.33	11.75 ± 1.25
Lantana flower	6.50 ± 0.50	9.75± 0.75	11.25± 0.00
Melia fruit	7.0± 0.50	8.25 ± 0.75	10.5± 0.50
Control	6.00 ±1.00	6.25 ±0.25	9.0 ± 0.0

Values are Mean± S.E.

The results showed that the highest inhibitory zone exhibited by pomegranate peel extract (21.5 ± 1.66 mm) in 300 mg/ ml, and lowest (7.33±0.44 mm) was exhibited by lantana fruit extract in 100 mg/ ml. the inhibition was concentration dependent, the highest inhibitory zone was exhibited by pomegranate peel, in 100, 200 and 300 mg/ml (Table 3).

Table (3) Diameter of inhibitory zones; methanol extract, disc diffusion method

Extract	Concentration of extract mg /ml		
	100	200	300
Cordia fruit	8.25 ±0.25	8.5 ±0.29	9.0 ± 0.50
Pomegranate peel	13.17 ± 1.42	16.17± 1.45	21.5 ± 1.66
Ricinus seed	9.0 ±0.50	11.25 ±1.75	12.5± 2.57
Lantana fruit	7.33 ±0.44	11.33± 0.17	16.33± 2.19
Senna leaves	9.0 ± 1.04	12.25 ± 0.75	13.33 ±1.20
Control	-	6.75 ± 0.75	7.0 ± 1.0

Values are Mean± S.E.

The result indicated that the highest inhibitory zone (21.25 ±1.75 mm) exhibited by pomegranate peel extract in 300 mg/ ml, while lowest inhibitory zone (8.5 ± 0.5 mm) was shown by pomegranate peel. in 100 mg/ ml the highest inhibitory zones were exhibited by Senna leaves. While in 200 and 300 mg/ ml, the highest zone was exhibited by Pomegranate, peel (Table 4).

Table (4) Diameter of inhibitory zones; Methanol extract; dip diffusion method

Extract	Concentration of extract mg /ml		
	100	200	300
Cordia fruit	9.5 ±0 .5	10.5±0.3	12.5 ± 0.5
Pomegranate peel	8.5± 0.5	16.25 ±0.25	21.25 ± 1.75
Ricinus seed	9.5± 0.5	13.25± 0.75	16.5 ±2.35
Lantana fruit	9.25 ± 0.75	10 ± 0.0	17± 3.0
Senna leaves	11.75 ± 0.25	13.25 ± 0.75	16.25 ± 0.75
control		9±0.65	9±0.25

Values are Mean± S.E.

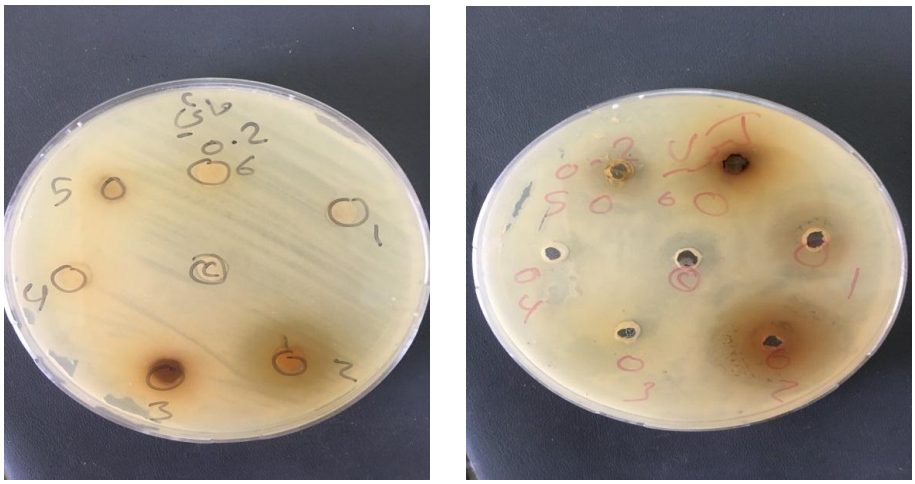
- **Minimum inhibitory concentration aqueous extract:** The minimum inhibitory concentration in aqueous extract was exhibited by pomegranate peel and Senna leaves at 1600 mg /ml. While in alcoholic extract the pomegranate peel, Ricinus seed and Senna leaves exhibited minimum inhibitory concentration at 400, 800, 1600 mg/ ml, while lantana flower exhibited at 1600 mg/ ml the minimum inhibitory concentration (Table 5 and 6).

Table (5) Minimum inhibitory concentration; aqueous extract

Extract	Minimum Inhibitory Concentration 0f aqueous extract				
	100	200	400	800	1600
Melia Stem	19	14	12	9.5	12.25
Melia Fruit	3	4	5	8	10
Pomegranate	26.5	30	31.5	28.5	0
Senna	28.75	14	8	31.25	0
Cordia Fruit	26.5	27	3	8	10
Lanata flower	26	19	19	23	26.5
Ricinus seed	34.75	41	38.75	42.75	35
Control	52.17				
Nyst.	37.5				

Table (6) Showing the diameter of inhibitory zones; Minimum Inhibitory Concentration, methanol extract

Extract	Minimum Inhibitory Concentration of methanol extract				
	100	200	400	800	1600
Cordia fruit	22.83	27.83	37.33	28.33	32.6
Pomegranate peel	8.5	13.5	0	0	0
Ricinus seed	29.25	20	0	0	0
Lantana fruit	37	33	20.5	22	0
Senna leaves	14	21	0	0	0
Melia fruit	22	26.75	27.5	20	25.5
Nystatin					
Control	10.17				



Pictures showing the diameter of inhibitory zones of plant extracts against the isolated *Aspergillus flavus*

Discussion

The preliminary phytochemical study of *Ricinus communis* revealed the presence of steroids, saponins, alkaloids, flavonoids and glycosides (11). The different solvent extracts of roots of *Ricinus communis* possess anti- fungal activities against *Candida albicans* and *Aspergillus niger* (12). Preliminary tests for the phytochemicals of *Cordia myxa*, revealed the presence of Flavonoids, Tannins and Saponins (13). Cordia myxa showed negative results against fungal strains (*Aspergillus niger*; Penicillium spp., Scytalidium spp.) Pomegranate peel is an important sources of bioactive compounds such as phenolics, flavonoids, ellagitannins and proanthocyanidin compounds (14), Pomegranate fruit extracts constituents possess immense biological activities such as antifungal (15). (16) reported that among selected fungal cultures the highest antifungal activity was against *Aspergillus parasiticus*. The peel extracts were shown no activities against *Aspergillus flavus*. The Pomegranate's peel is an important sources of bioactive compounds such as phenols, flavonoids, ellagitannins and proanthocyanidin compounds (14) and complex polysaccharides (17). Fungistatic activity of pomegranate peel varied with test organisms as it inhibited the growth of *Penicillium citrinum* for 8 days, *Penicillium patulum* for 4 days and *Penicillium roquefortii* and *Aspergillus ochraceons* for 3 days (18). (16) reported that among the selected fungal cultures, the highest antimicrobial activity was recorded against *Aspergillus parasiticus* was recorded. The peel extract were shown no activities against *Aspergillus flavus*. Majority of the *Lantana camara* activity is due to bioactive compounds viz, flavones, isoflavones, flavonoids, isocatechins. alkaloids, tannin, saoponins and triterpenoids. The polymethoxylated flavone, isolated from the methanol extract of dried leaves of *Lantana camara* exhibited the antibacterial and antifungal properties (19). The essential oil containing beta – caryophyllene, geranyl acetate, terpinyl acetate, bornyacetate and limonene and showed wide spectrum of antifungal activities (20-23). *Lantana camara* Linn Extracts from the leaves exhibit fungicidal (24)The activity of ethanolic leaf, seed and fruit extracts from *Melia azedarach* in controlling plant and human pathogenic fungi as *Aspergillus flavus, Fusarium monitiform, Microsporum canis* and *Candida albicans* has been reported (25). (26) Recorded all *Melia azedarach* Methanol, Ethanol, Petroleum ether and water showed significant activity against *Aspergillus niger, Aspergillus flavus, Fusarium oxisoporum, Rhizopus stolonifera*. Carpinella, *et al*., (27) reported that ethanolic extract of the Senna plant showed high activity against dermatophytic fungi and

Microsporum. It has been observed that antimicrobial activity of the plants is associated with the presence of some chemical components such as phenols, tannis, saponins, alkaloids, steroids, flavonoids, and carbohydrates. Methanolic extract of Senna showed the highest activity than the water extracts. (28) documented that Minimum inhibitory concentration of the plant extract was low on all fungal agents except *Aspergillus niger*. Extracts of the leaves of the *Cassia alata* showed significant in vitro antifungal activity against various fungi viz *Aspergillus niger, R. japonicum*, *Candida albicans, C. tropiathius* and *R. glutinis* (29, 30). In a study by (31). *Senna alata* Linn was found to have antifungal activities. The methanolic extract was the most active of the two crude extracts tested against fungal organisms (31). From the biological activity results obtained in this study (31) the most active component exhibited low MIC against the mould at 70 µg/ ml while higher activity was recorded against all the test organisms at 860 µg/ ml. Study indicated that *Senna alata* Extract showed antifungal activity to the dermatophytes, this is in agreement with (32). From literature search, there is no evidence that flavonoid glycoside, a main constituent of the leaf extract of *S. alata* is responsible for antifungal activity. In a recent review, the methanolic fraction of the leave has been shown to be active against *Trichophyton mentagrophytes* at a concentration of 50 mg/ ml but has no activity against moulds and *candida albicans*. Much earlier, the antifungal activity of *Cassia alata* leaf extracts has been reported (33). The organic crude extracts showed more inhibition than the aqueous extracts on the average for all plants extracts tested (34). It is possible that the aqueous crude extracts may contain antimicrobial constituents insufficient for efficacy and which may explain why large amounts of the decoctions must be drunk by the patients (34). Those observation is supported by (35). Who also found out that, aqueous extracts showed little or no antimicrobial activity in contrast to those made using organic solvents. All the *S. didymobotrya* extracts were in active against *A.Niger*) Filamentous fungus) and *C. albicans* (yeast fungus). The inactive of the extracts against the two fungi, may be due to differences in cell wall composition (35). The antimicrobial effects can be attributed to the phytochemicals of the plants in this study: in phenols and polyphenols include phenolic toxicity to microorganisms which include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (36). Quinones they provide a source of stable free radicals and are also known top complex irreversibly with nucleophilic amino acids in protein (36), often leading to inactivation of the protein and loss of function. They may also render substrates unavailable to the microorganism. Flavones, flavonoids and flavonols, they are known to be synthesized by plants in response to microbial infection (37) and found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (38). Tannins one of their molecular actions is to complex with proteins through so- called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (36). The studies documented the inhibitory activities of tannins and reported them to be toxic to filamentous fungi, yeast and bacteria (39). Terpenoids and essential oils, the activities of terpenenes or terpenoids have been demonstrated against Fungi (40). Lectins and polypeptides, Peptides are inhibitory to microorganisms are often positively charged and contain disulfide bonds. The formation of ion channels in the microbial membrane (41) or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors (42). could is their mechanism, of

action. **Conclusion**: In study, the extracts of Pomegranate peel have high potential as antimicrobial agent.it showed varying degrees of activities against tested aspergillus. **Recommendations:** As only in vitro method was used in assessing the antimicrobial activity of the plant crude extracts, further investigations using bioassay guided fractionations are recommended to isolate and identify the pure compounds responsible for antimicrobial activity

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