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ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS. *Part II*

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Antifungal activity of the essential oils was studied against the strains of *Aspergillus niger, A. parasiticus, A. flavus, A. oryzae, A. fumigatus, Penicillium digitatum, Trichoderma* Spp. and *Helminthosporium oryzae* by zone inhibition method. These oils were also tested for their fungicidal/fungistatic activity. Varaible toxicity was observed against one or more of the test fungi. The essential oil of seed and gum portion of *Ferulassafoetida* exhibited antifungal activity against all of the 8 test fungi.

Key words: Antifungal activity, Oils, Inhibition zone, Fungicidal/fungistatic activity.

Introduction

Antifungal activity of essential oils has been studied since dawn of early medical practices till the present times of scientific investigations [1]. Antifungal activity of essential oils has been reported by Maruzzella Jasper *et al.* [2], N. Martinez *et al.* [3] Karapinar Mehmet [4] and A.K. Singh *et al.* [5]. Although greater emphasis seems to have been placed in the pursuit of antibacterial activity of essential oils [6], some studies have actively been devoted to the antifungal activity recently. Dikshi [7] and Brud [8] have found inhibitory effects of essential oils against a number of fungi. Some of the investigators [9] have tested essential oils in vitro against human fungal pathogens but only qualitative methods were employed. In the present study, essential oils from *Ferrula assafoetida, Citrus limon, Schinus terebinthifolius and Cypress sempervirens* were tested for their antifungal activity against strains of *Aspergillus niger, A. parasiticus, A. flavus, A. oryzae, A. fumigatus, Penicillium digitatum, Trichoderma* spp. and *Helminthosporium oryzae* by zone inhibition method. Screening was carried out with pure essential oil, as extracted. Zone diameters were measured after every 24 hrs for 4 days to determine the percentage of change in the zones.

Results of antibacterial activity of these essential oils against *Escherichia coli, Shigella dysenteriae, Bacillus subtilis* and *Staphylococcus albus* have been reported in Part-I under publication [10].

Fungal species are so widely distributed in the environment [11,12] that practically every ecological site - not only has its fungal inhabitants but has staggeringly large number of them. Many of these fungi are pathogens of plants grown for food, shelter and clothing while a smaller number are agents of disease in animals, including man. Many saprophytic fungi attack and degrade raw or manufactured materials of every kind such as foodstuffs, timber, leather, paint, glue, plastics, chemicals, kerosene and diesel oil [13]. Although, only a few species commonly infect humans, yet taken together they rank closely behind viruses and bacteria as causes of disease in humans. One of the test fungi, *Aspergillus* species may cause allergic, colonizing and invasive diseases [14]. For example, *Aspergillus fumigatus* may colonize and then invade tissues in a traumatized cornea or in burns, wounds or the external ear. It and other *Aspergillus species* become opportunistic invaders in immunodeficient persons or individuals with anatomic abnormalities of the respiratory tract. Another form is allergic pulmonary aspergillosis with asthma, eosinophilia with minimal tissue invasion but abnormal bronchograms. Various species of *Aspergillus* produce aflatoxins in foods [14]. *P. digitatum*, another fungal species included as a test organism in the present studies, causes penecillosis [15]. *Helminthosporium* and *Trichoderma* spp., also included in the tests, are phytopathogenic [16, 17].

Any agent, essential oil or some thing else, with a basis for being effective against one or more fungi is worth exploring and development as a medicine against the specific fungal pathogen.

The purpose of the present study is accordingly to screen out important indigenous medicinal plants of Pakistan for their efficacies of antimicrobial activity against the test fungi.

Experimental

Test organisms. Standard cultures of the following were obtained from the Fermentation Section, Biotech. & Food Research, PCSIR Laboratories Complex, Lahore:

(1) *Aspergillus niger* (2) *A. parasiticus* (3) *A. oryzae* (4) *A. flavus* (5) *A. fumigatus* (6) *Penicillium digitatum* (7) *Trichoderma spp.* (8) *Helminthosporium oryzae.*

Stock cultures were maintained on agar slants.

Test media. Czapek Dox media was used for the stock

TABLE 1. ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL OF GUM OF *FERULA-ASSAFOETIDA* AGAINST TEST FUNGI (pH 7.3, TEMP. 30°C).

S. No.	Test Organisms	Inhibition zone (mm) after 24 hrs	48 hrs	72 hrs	96 hrs	Fungicidal/ Fungistatic
1.	*A. flavus*	None	28	25	21 25*	Fungistatic
2.	*A. fumigatus*	None	22	25	17 22.72	Fungistatic
3.	*A. niger*	None	23	20	15 34.8	Fungistatic
4.	*A. oryzae*	None	25	22	21 16	Fungistatic
5.	*A. parasiticus*	None	25	25	22 12 12	Fungistatic
6.	*H. oryzae*	None	18	17	15 16.66	Fungistatic
7.	*P. digitatum*	None	21	19	19 9.52	Fungistatic
8.	*Trichoderma Spp*	None	29	28	26 10.34	Fungistatic

* % age decrease in inhibition zone after 96 hrs.

culture of the fungal strains and for the petriplate screening work. This test medium serves as an assay medium because it does not inactivate the antibiotics and supports the growth of the test organisms.

The Czapek Dox medium and the Czapek Dox broth were made according to the standard ingredients and procedures.

Czapek Dox broth was used for the preparation of inoculum and the pH was adjusted to 7.3.

TABLE 2. ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL OF SEEDS OF *FERULA-ASSAFOETIDA* AGAINST TEST FUNGI (pH 7.3, TEMP. 30°C).

S. No.	Test Organisms	Inhibition zone (mm) after 24 hrs	48 hrs	72 hrs	96 hrs	Fungicidal/ Fungistatic
1.	*A. flavus*	None	28	28	27 3.57*	Fungistatic
2.	*A. fumigatus*	None	28	26	23 17.87	Fungistatic
3.	*A. niger*	None	40	38	35 12.5	Fungistatic
4.	*A. oryzae*	None	43	40	40 6.97	Fungistatic
5.	*A. parasiticus*	None	25	23	22 8	Fungistatic
6.	*H. oryzae*	None	23	23	23 0	Fungicidal
7.	*P. digitatum*	None	25	25	23 8	Fungistatic
8.	*Trichoderma Spp*	None	27	27	27 0	Fungicidal

* % age decrease in inhibition zone after 96 hr.

TABLE 3. ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL OF LEAF OF *CYPRESS SEMPERVIRENS* AGAINST TEST FUNGI (pH 7.3, TEMP. 30C°).

S. No.	Test Organisms	Inhibition zone (mm) after 24 hrs	48 hrs	72 hrs	96 hrs	Fungicidal/ Fungistatic
1.	*A. flavus*	None	None	None	None	-
2.	*A. fumigatus*	None	None	None	None	-
3.	*A. niger*	None	None	None	None	-
4.	*A. oryzae*	None	None	None	None	-
5.	*A. parasiticus*	None	38	35	35 13.5*	Fungistatic
6.	*H. oryzae*	None	29	29	29 0	Fungistatic
7.	*P. digitatum*	None	29	27	27 6.89	Fungistatic
8.	*Trichoderma Spp*	None	27	26	26 3.70	Fungistatic

* % age decrease in inhibition zone after 96 hr.

Essential oils. Essential oils of the plants (1) *Ferula assafoetida*, family Umbelliferae (gum oil), (2) *Ferula assafoetida* (seed oil), (3) *Citrus limon* var. Ureka lemon family Rutaceae (peel oil), (4) *Citrus limon* var. Lemon, family Rutaceae (peel oil), (5) *Schinus terebinthifolius*, family Anacardaceae (leaf oil) and (6) *Cypress sempervirens*, family Cuperessaceae (leaf oil) were recovered by steam distillation using an all-glass distillation assembly according to Ernest [18] and Bhatty [19].

Method of assay. Antifungal activity of the essential oils was carried out by the cavity method of Fleming [20] modified by Johnson *et al.* [21] against the said eight pathogenic

TABLE 4. ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL OF LEAF OF *SCHINUS TEREBINTHIFOLIUS* AGAINST TEST FUNGI (pH 7.3, TEMP. 30C°).

S. No.	Test Organisms	Inhibition zone (mm) after 24 hrs	48 hrs	72 hrs	96 hrs	Fungicidal/ Fungistatic
1.	*A. flavus*	None	28	25	25 10.71*	Fungistatic
2.	*A. fumigatus*	None	27	27	27 3.70	Fungistatic
3.	*A. niger*	None	None	None	None	-
4.	*A. oryzae*	None	None	None	None	-
5.	*A. parasiticus*	None	25	22	19 24	Fungistatic
6.	*H. oryzae*	None	30	28	27 10	Fungistatic
7.	*P. digitatum*	None	32	30	28 12.5	Fungistatic
8.	*Trichoderma Spp*	None	25	20	18 28	Fungistatic

* % age decrease in inhibition zone after 96 hr.

TABLE 5. ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL OF PEEL OF *CITRUS LEMON* (BAR LEMON) AGAINST TEST FUNGI (PH 7.3, TEMP. 30C°).

S. No.	Test Organisms	Inhibition zone (mm) after				Fungicidal/ Fungistatic
		24 hrs	48 hrs	72 hrs	96 hrs	
1.	*A. flavus*	None	25	25	23 8*	Fungistatic
2.	*A. fumigatus*	None	None	None	None	-
3.	*A. niger*	None	None	None	None	-
4.	*A. oryzae*	None	28	23	19 32.14	Fungistatic
5.	*A. parasiticus*	None	35	30	28 20	Fungicidal
6.	*H. oryzae*	None	None	None	None	-
7.	*P. digitatum*	None	30	28	25 16.66	Fungistatic
8.	*Trichoderma Spp*	None	20	19	17 15	Fungicidal

* % age decrease in inhibition zone after 96 hr.

TABLE 6. ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL OF PEEL OF *CITRUS LIMON* (VAR. UREKA LEMON) AGAINST TEST FUNGI (PH 7.3, TEMP. 30C°).

S. No.	Test Organisms	Inhibition zone (mm) after				Fungicidal/ Fungistatic
		24 hrs	48 hrs	72 hrs	96 hrs	
1.	*A. flavus*	None	27	23	23 14.8*	Fungistatic
2.	*A. fumigatus*	None	30	29	25 16.66	Fungistatic
3.	*A. niger*	None	None	None	None	-
4.	*A. oryzae*	None	23	23	19 17.39	Fungistatic
5.	*A. parasiticus*	None	18	18	17 5.55	Fungistatic
6.	*H. oryzae*	None	20	19	16 20	Fungicidal
7.	*P. digitatum*	None	28	25	24 14.28	Fungistatic
8.	*Trichoderma Spp*	None	25	25	23 8	Fungistatic

* % age decrease in inhibition zone after 96 hr.

fungal strains in batches. In each batch two dishes were taken as control, one for the medium and the other for the test organism. Six petriplates were used for the experiments.

To find antifungal activity, cultures of 96 hrs were seeded by swabbing on 7 of the 8 petridishes. Well was cut with a sterile cork borer in the inoculated dishes. Wells (cavities) of 6 inoculated dishes were filled with 0.1 ml of oil. Plates were incubated for 96 hrs at 30°C. The procedure for the preparation of inoculum was adopted from Meena *et al.* [22].

Measurement of inhibition zones. After every 24 hrs upto a total of 96 hrs, the inhibition zones were measured with the help of a scale to the nearest mm. Color photographs of the plates were taken for elucidation of the mechanisms of antifungal action of the essential oils.

Fungicidal/fungistatic activity. The above essential oils were also tested for their fungicidal/fungistatic activity, by the modified technique of Gerber and Houston [23]. This was done by re-inoculating the portion of inhibited zone of each pathogen on the Czpek Dox medium separately. Mycelial growth on seventh day indicated fugistatic activity, while its complete absence denoted fungicidal nature of the oil.

Results and Discussion

The antifungal activity of *F. assafoetida* is given in Table 1. *Ferula* gum oil showed fungistatic activity against all of the 8 test fungi. Maximum inhibition was obtained after 48 hrs, which exhibited a decrease in all cases after 96 hrs.

Maximum zone of inhibition was shown by *Trichoderma* spp. i.e. 26 mm and minimum by *A. niger* and *H. oryzae* i.e. 15 mm. *Aspergillus oryzae, A. flavus, A. fumigatus, A. parasiticus and Penicillium digitatum* gave 21, 21, 17, 22 and 19 mm zones of inhibition respectively. The percentage decrease in zones of inhibition after 48 hrs is also shown in the Table.1

Penicillium digitatum showed 9.52% decrease which is the minimum, while *A. niger* exhibited maximum decrease in its efficacy - 34.8%. The percentage decrease in inhibition zone of *A. flavus, A. fumigatus, A. parasiticus, A. oryzae, H. oryzae* and *Trichoderma* spp. was 25, 22.72, 12, 16, 16.66 and 10.34% respectively.

Antifungal activity of essential oil of seeds of *F. assafoetida* against test fungi is given in Table 2. The oil showed fungicidal effect against *H. oryzae* and *Trichoderma spp.* and fungistatic activity against the rest of the test fungi. The oil retained maximum efficacy against *H. oryzae* and *Trichoderma* spp. even after 96 hrs.

Antifungal activity of leaf oil of *Cypress sempervirens* against test fungi is shown in Table 3. The oil showed fungistatic activity against 5 test fungi only. The essential oil showed maximum activity against *A. parasiticus* indicated by 35 mm zone and minimum activity against *Trichoderma* spp. by 26 mm zone. The oil was totally inactive against 4 species of Aspergillus i.e. *A. niger, A. oryzae, A. flavus,* and *A. fumigatus.*

Results of leaf oil of *Schinus terebinthifolius* against the test fungi are given in Table 4. The oil had fungistatic activity against all except *A. niger* and *A. oryzae* which were not affected.

Results of peel oil of *C. limon* (*var. Lemon*) peel oil, and of *C. limon (var. Ureka lemon)* are given in Table 5 and 6 respectively.

TABLE 7. SUMMARY OF ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS AGAINST TEST ORGANISMS.

Fungus / Essential Oil	*A. flavus*		*A. fumigatus*		*A. niger*		*A. oryzae*		*A. parasiticus*		*H. oryzae*		*P. digitatum*		*Trichoderma spp.*		Efficacy
	Z. Dia. mm	% decr.	Z. Dia. mm	% decr.	Z. Dia. mm	% decr.	Z. Dia. mm	% decr.	Z. Dia. mm	% decr.	Z. Dia. mm	% decr.	Z. Dia. mm	% decr.	Z. Dia. mm	% decr.	
F. assafoetida (Gum)	28'	25	22'	23	23'	35	25'	16	25'	12	18'	17	21'	10	29'	10	V. Good
F. assafoetida (Seeds)	28'	4	28'	18	40'	12	43'	7	25'	8	23*	0	25'	8	27*	0	Excellent
S. terebinthiofolius (Leaves)	28'	11	27'	4	-	-	-	-	25'	24	30'	10	32'	13	25'	28	Good
C. limon (lemon) Peel	25'	8	-	-	-	-	28'	32	35*	20	-	-	30'	17	20*	15	Good
C. limon (Ureka) Peel	27'	15	30'	17	-	-	23'	17	18'	6	20*	20	28'	14	25'	8	Good
C. sempervirens (Leaves)	-	-	-	-	-	-	-	-	38'	13	29'	0	29'	7	27'	4	Poor

(i) Z. Dia = Inhibition Zone dia. in mm after 48 hours; (ii) % decr. = Percentage decrease in zone dia. after 48 hours; (iii) ' = Fungistatic activity (iv) * = Fungicidal activity.

Summary of the results is given in Table 7. Remarks in the column of efficacy are based on the following observations:

(1) Number of test organism inhibited, (2) Zone diameters, (3) Nature of activity whether cidal or static and (4) Percentage decrease in zone diameters.

The analysis of these results will be reported in Part-IV of this series.

The fungi toxicity of essential oils appear to be neither a genus nor a family character. Different members belonging to same genus or a single family yield oils that behave differently.

Very few antifungal substances are known or available in the market as compared to the antibacterials[24] Stock has emphasized the need for discovery of powerful and specific antimycotic agents on an increasing scale to combat fungal infections.

The discovery of the essential oils exhibiting antifungal toxicity against specific fungal species in the present investigations may prove useful in the development of effective antidermatomycotic treatments.

The active components in the chemical compositions of these oils may be responsible for the antifungal as well as antibacterial activities observed in the present studies. It is also possible that the essential oil supresses mycelial growth of the fungi or inhibits sporulation, thus results in reduced growth of the fungi within the region of the inhibition zone.

It will be of considerable theoretical and practical future importance if some common factors are studied for the discovery of both antibacterial as well as antifungal activity exhibited by some specific essential oil components.

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