Research Paper :

Evaluation of rhizospheric fungi and extract of *Melissa officinalis* for antimicrobial and proteolytic activities

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SUMMARY

The antimicrobial activity of root and shoot extract of *Melissa officinalis* extracted in different solvents and potential effect of rhizospheric fungal strains against most serious plant pathogens of agricultural crops as well as human diseases were investigated during the year 2008-09. The petroleum ether root extract of *Melissa officinalis* was highly active against *Bacillus* sp. (+ve) bacterium whereas root and shoot extracts from chloroform and methanol extraction were proved more antagonistic to *Klebsiella* sp. at higher concentrations within 13.67 –15.39 mm diameter range of zone inhibition. However, hydrophobic plant extracts were more effective towards *Fusarium, Alternaria* and *Penicillium* sp. Maximum antagonistic activity was pronounced at higher concentration in petroleum ether, methanol and chloroform solvents. The cell free supernatant of three rhizospheric fungi *Aspergillus fumigatus, Mycelia sterilia* (white) and *Penicillium* MP-2 showed good antibacterial effect against *Pseudomonas* sp., *Salmonella typhi*, *S. paratyphi* and *Shigella* sp. while *Aspergillus fumigatus* and *Penicillium* sp. MP-3 showed inhibitory effect against pathogenic fungi. Only one isolate, *Mycelia sterilia* (black) amongst 8 rhizospheric fungi was able to produce good amount of proteolytic activity that can be exploited for enzyme production in different types of industries.

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Microorganisms and medicinal plants are rich source of secondary metabolites which are potential sources of useful products (Vining, 1990). The advantage of microbial systems in the search of novel and commercially important components is defined by rapid growth, low cost and extreme sensitivity. Fungi play an essential role in the soil environment as major decomposers of plant residues, releasing nutrients that sustain and stimulate plant growth in the process. Some fungi possess antagonistic properties toward plant pathogens (Nascimento *et al*., 2002, Smith *et al*., 1999) which make them beneficial to agriculture and industry.

Melissa officinalis commonly known as lemon balm member of Lamiaceae family is considered one of the important medicinal plant species (Lauk *et al*., 2003). Today it is used in different branches of industry such as medicine, perfume, cosmetic and food etc. in many countries of the world. The main components of lemon balm essential oil ranged from 0.01 to 0.25%, 39% citronellal, 33% citral (*Citransellol linalal*), thymol (0.4-11.94%), β-caryophyllene (5.91-7.27%), Spathulenol (2.06%) and geraniol (2.2%). It is traditionally used as mild sedative, spasmolytic and antibacterial agent (Adinee *et al*., 2008, Cosge *et al*., 2009). This particular composition comprising of *Melissa* leaf extract can be used for cosmetic and pharmaceutical dietric Schnitzler *et al*., 2008) exerting direct antiviral effects. Though today also plant derived source components remain a significant fraction of pharmaceuticals employed clinically. Soil under medicinal plants can also be considered a novel enrichment environment because of presence of plant litters, residues and secretion going continuously into the soil, biodegraded and remained there. Microorganisms adapted to this type of environment may have the potential to yield idiolites and unexpected biological activities.

The present investigation determines the evaluation of fungal isolates and plant extract of *Melissa officinalis* for production of bioactivities such as antibacterial, antifungal and proteolytic to combat economically important diseases of agriculture crops and incorporation in medicines or cosmetic industries for human use.

MATERIALS AND METHODS

Screening of fungal strains for antimicrobial and proteolytic activities:

Soil samples from *M. officinalis* were used to enumerate and isolate the fungi. Isolations were made by using dilution plate method (Johnson, 1957) in Potato dextrose agar medium. The isolated fungi were identified according to their microscopic, morphological, cultural and biochemical characteristic as per their genera (Gilman, 1959). All these strains were maintained on PDA at 4^0C and subcultured on the same medium periodically at 28^0C.

The cell free supernatant of all the selected 10 fungal isolates and 9 bacterial isolates of rhizosphere of *M. officinalis* were tested for their antimicrobial activities. The activities were assayed by spot and well plate methods using 100µl cell free culture supernatants. In spot plate method, each isolated fungal strain was spotted on the surface of nutrient agar plates, already lawned with 24 hours old culture of indicator bacteria. While in well plate method 100µl of 4 days old cell culture supernatant of each fungal strain added to each well on Nutrient agar plates lawned with indicator test bacteria with the help of sterile cotton swabs. Plates were incubated at 37°C for 24 to 28 hours. The antibacterial activity measured in terms of mm diameter of clear zone produced around the bit or well. In a same way, the antifungal activity was measured.

Preparation of plant extracts and its assay for biological activity:

Collected plant materials from different areas of Himachal Pradesh were dried under shade and converted into moderately coarse powder. The crude powder (100g) of root and shoot was extracted in solvent apparatus separately (Peach and Tracey, 1979). The extracts obtained were evaporated to dryness under vacuum. The residues collected were determined for antimicrobial and proteolytic activities after making different concentrations in dimethyl sulfoxide (DMSO) (w/v). About 100 ul of each extracted sample at different concentrations *viz.,* 50, 100, 150 and 200 ug/ul was used to assay the biological activities by well plate method. Activity was expressed in terms of mm diameter of zone produced around the well at 37^0C for bacteria and 28^0C for fungi after 24 hours.

Proteolytic activity:

Spot and well plate methods (Iida *et al*., 1982) were assayed for determination of proteolytic activity . The test was carried out on Skim milk agar plates (1%) that contains sterile skim milk + 100 ml Potato nutrient agar.

Statistical analysis:

The results obtained in all the above experiments were subjected to analysis of variance using Completely Randomized Design by keeping three replications in each (Gomez and Gomez, 1976) .

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been presented under following heads:

Enumeration and antimicrobial response of rhizospheric fungi:

The viable counts (cfu) of isolated fungi from *M. officinalis* ranged upto 5.53 cfu and the rhizosphere soil constituted of 8 predominant fungi including one unidentified strain such as *Aspergillus fumigatus, A. tamarii, Mycelia sterilia* (black), *Mycelia sterilia* (white), *Penicillium* sp. MP-1, *Penicillium* sp. MP-2 and *Penicillium* MP-3. Amongst the fungal strains, *M. sterilia* (black), *A. fumigatus*, *Penicillium* MP-3 and unidentified strain possessed maximum antifungal action to most of the tested fungi such as *Aspergillus* sp. (75 mm), *Trichoderma* sp. (45mm) , *Fusarium* (44mm), *Alternaria* (40mm), *Rhizoctonia* (14mm) and *Sclerotium* (12mm) by using cell free supernatant (Table 1).

Out of all the fungal strains isolated from soils of *M. officinalis*, *Aspergillus fumigatus* and *Penicillium* sp. showed antibacterial effect against *Pseudomonas* sp. and *S. paratyphi , S. typhi,* respectively. However, *Mycelia sterilia* (white) reported weak inhibition against *Salmonella paratyphi* and *S. typhi* while *Penicillium* showed strong effect against *Klebseilla, Shigella* and *Salmonella* spp. A good amount of antifungal activity was noticed in *Mycelia sterilia* (black), *Penicillium* sp. MP-3 and unidentified strain to maximum number of test fungi when tested with spot plate method (Table 2). However, *A. fumigatus* and *Mycelia sterilia* (white) have shown strong activity to *Pseudomonas* sp., *S. typhi, S. paratyphi* while *Mycelia sterilia* (Black) did not react to

Table 1 : Antifungal activity and antibacterial activity of cell free supernatant of fungal strains from *Melissa officinalis* by well plate method

Fungal strains *Melissa officinalis*	Antifungal activity* (mm dia) *Alternaria* sp.	*Aspergillus* sp.	*Fusarium* sp.	*Penicillium* sp.	*Phytophthor* sp.	*Trichoderma* sp.	*Rhizoctonia* sp.	*Pythium* sp.	*Trichothecium* sp.	*Sclerotium* sp.
Aspergillus fumigatus	40		44				17		8	12
Aspergillus tamarii		25		30		25				
Mycelia sterilia (black)	33	55	12		11	40				
Mycelia sterilia (white)	28	75	w	10			11			
Penicillium sp. MP 1					12	w			11	
Penicillium sp. MP 2	50	48	12	w						
Penicillium sp. MP 3	44	62	11			45	12		w	
Unidentified strain	50	60			w	55				11
	Antibacterial activity** (mm dia)									
Aspergillus fumigatus				w				27		
Aspergillus tamarii							8	15		
Mycelia sterilia (black)			w							
Mycelia sterilia (white)			w	20						
Penicillium sp. MP 1						w	10			
Penicillium sp. MP 2			35	11			12			
Penicillium sp. MP 3				38			12			
Unidentified strain			w				11			

Indicates no activity ; Indicates activity; w Indicates weak activity

* Antibacterial activity expressed in terms of clear zone (mm dia.) produced around the well (8mm) by 100µl of supernatant at 28±2°C for 72 hrs of incubation

**Antibacterial activity expressed in terms of clear zone (mm dia.) produced around the well (8mm) by 100µl of supernatant at 37°C for 24 hrs of incubation

Table 2 : Screening of fungal strains from the rhizosphere soil of *Melissa officinalis* for their antifungal and antibacterial activity by spot plate method

Fungal strains *Melissa officinalis*	*Alternaria* sp.	*Aspergillus* sp.	*Fusarium* sp.	*Penicillium* sp.	*Phytophthor* sp.	*Trichoderma* sp.	*Rhizoctoni a* sp.	*Pythium* sp.	*Trichothecium* sp.	*Sclerotiu m* sp.
	Antifungal activity* (mm dia)									
Aspergillus fumigatus	17		12				w		w	w
Aspergillus tamarii		7		10		12				
Mycelia sterilia (black)	11	18	w		w	15				
Mycelia sterilia (white)	8	22	w	w			w			
Penicillium sp. MP 1					w	w			w	
Penicillium sp. MP 2	16	15	w	w						
Penicillium sp. MP 3	15	18	w			15	w		w	
Unidentified strain	16	18			w	20				w
	Antibacterial activity** (mm dia)									
Aspergillus fumigatus				w				8		
Aspergillus tamarii							w	w		
Mycelia sterilia (black)			w							
Mycelia sterilia (white)			w	8						
Penicillium sp. MP 1						w	10			
Penicillium sp. MP 2			15	w			w			
Penicillium sp. MP 3				w			w			
Unidentified strain			w				w			

Indicates no activity ; Indicates activity; w Indicates weak activity

* Antifungal activity expressed in terms of clear zone (mm dia.) produced around the spot at 28 2°C for 72 hrs of incubation

** Antibacterial activity expressed in terms of clear zone (mm dia.) produced around the spot at 37°C for 24 hrs of incubation

any bacterium. Similar effect of strain of penicillin producing fungus, *P. chrysogenum* produced a quinine sorrentanone that was active against Gram (+)ve and Gram (-) ve bacteria and this supported the present finding of the research (Miller and Huang, 1995). The isolated strains belonging to genera *Aspergillus*, *Penicillium* MP-3 and unidentified strain in our study were found to be a potent producer of biological activity. Similarly lipopeptides inhibitors of fungal glucan biosynthesis called echinocandins are very promising groups of antifungal agents produced by *Aspergillus nidulans* (Sakuda *et al*., 1995). Whereas a mutant of patulin producing strain of *Penicillium urticase* investigated produced new azaphilone epoixe and patulodin that showed weak antagonism against fungi (Azad *et al*., 1985).

Evaluation of plant extracts of *M. officinalis* for biological activity:

Methanol extract of both root and shoot comparatively gave more antagonistic effect to *Klebsiella* sp. in range of 13.20-13.44 mm and 13.33-13.67 mm diameter at 150 and 200 µg concentrations followed by chloroform. Petroleum ether however reported active only against Gram (+) ve bacterium *i.e. Bacillus* sp. by giving 10.79 mm inhibition at 200 µg. High concentration in general induced more antimicrobial activity in comparison to low concentration. Similar effect was recorded with regards to antifungal activity. Maximum suppression was obtained in plant extract, which was extracted in methanol solvent that allowed high inhibition of *Fusarium* sp., *Alternaria* sp. and *Penicillium* sp. than petroleum ether and chloroform extraction (Table 3). The extracts of above *Melissa officinalis* showed antifungal behaviour to *Alternaria* sp., *Fusarium* sp. and *Penicillium* sp. Among the solvent extracts tested, methanol gave more effect than ethanol chloroform, benzene and petroleum ether except for *Polyalthia longifolia* where petroleum ether extract recorded highly significant antifungal activity than other solvent extracts (Grange

Table 3 : Antibacterial and antifungal activity of root and shoot extracts of *Melissa officinalis* by well plate method

Antibacterial*/ antifungal activity** (mm dia)

Plant extracts	Indicator test bacteria/Fungi	Plant extract (µg /100µl) 50	100	150	200	Marginal mean
Root						
Petroleum ether (60-80^{0}C)	*Bacillus* sp.	8.00	8.10	9.48	10.79	9.09
Chloroform	*Klebseilla* sp.	10.82	11.15	11.37	12.00	11.33
Methanol	*Klebseilla* sp.	11.27	12.39	13.20	13.44	12.57
Shoot						
Chloroform	*Klebseilla* sp.	8.39	10.73	12.67	15.39	11.79
Methanol	*Klebseilla* sp.	9.67	11.57	13.33	13.67	12.06
Marginal mean		9.63	10.79	12.01	13.06	-
Root						
Petroleum ether (60-80^{0}C)	*Fusarium* sp.	8.12	10.68	10.81	11.00	10.15
Chloroform	*Fusarium* sp.	8.00	8.43	9.20	12.25	9.47
Methanol	*Alternaria* sp.	11.48	11.57	12.00	12.10	11.79
	Penicillium sp.	10.54	11.00	12.33	12.64	11.63
Shoot						
Chloroform	*Penicillium* sp.	10.48	10.87	11.00	11.00	10.84
Methanol	*Fusarium* sp.	10.72	11.53	11.57	12.00	11.45
Marginal mean		9.89	10.68	11.15	11.83	-

*Antibacterial activity expressed in terms of clear zone (mm dia) produced around the well (8mm) by 100 µl plant extract at 37^{0}C for 24 hours of incubation.

Effect	$CD_{0.05}$
Concentrations	0.6182
Indicator test bacteria	0.06912
Concentration × Indicator test bacteria	1.37

** Antifungal activity expressed in terms of clear zone (mm dia) produced around the well (8 mm) by 100 µl plant extract at 28± 2^{0}C for 72hrs of incubation.

Effect	$CD_{0.05}$
Concentrations	0.5479
Indicator test bacteria	0.6710
Concentration × Indicator test bacteria	1.32

and Davey, 1990). The essential oil of lemon balm extracted from this plant is known to give inhibitory action (Pasqua *et al*., 2005). The antagonistic behaviour of plant extracts thus attributed in this investigation may be due to some of the metabolites present in plant. *M. officinalis* showed antimicrobial activity against *Lactobacillus*, *Enterococcus, Pseudomonas* and *Staphylococcus aureus* strains. Its essential oils observed to be bacteriostatic and bacteriocidal against *E. coli* 0157-H7 ATCC35150 and *Salmonella typhimurium* ATCC6994 (De Sousa *et al*., 2004). The most powerful scavenging compounds were identified as monoterpene aldehydes and ketones and mono and sesquitapone hydrocarbons (Darouche *et al*., 2006). Extracts from plants of the Lamiaceae family have been described for antifungal (Bozin *et al*., 2007; Fang *et al*., 2005 and Kaur *et al*., 1989).

Proteolytic activity:

Four fungal strains *viz., Mycelia sterilia* (black and white), *F. oxysporum* and *T. harzianum* produced good amount of proteolytic activity in range of 8-16 mm diameter of clear halo (Table 4) with maximum in well plate method. The rest of the strains possessed weak or no proteolytic activity. Some proteases especially elastase, substilisin and pronase also possessed lytic properties against different gram positive and negative bacteria. Four fungal strains, *M. sterilia* (black and white), *F. oxysporum* and *Trichoderma harzianum* produced good amount of proteolytic activity while rest of the strains possessed weak activity. The intra and extra cellular amino peptidase activity was observed in *M. officinalis* within range of 81.8- 88.9 % intercellular and 11.1-18.2% extracellular, respectively (Korenova *et al*., 2007). Two strains Act-M-1 and Act-M-8 actinomycetes in the present study exhibited more potential in imparting antagonistic effect against *Pythium* and *Phytophthora* sp. while strain M-2 recorded highest proteolytic activity of 40 mm clear zone formation on Skim milk agar (Kaur *et al*., 2008).

Table 4 : Proteolytic activity of rhizospheric fungi from *Melissa officinalis* assessed by spot and well plate method using cell free supernatant

Rhizospheric fungi	Proteolytic activity (mm dia.)	
	Spot plate*	Well plate**
1. *Aspergillus fumigatus*	-	-
2. *Aspergillus tamarii*	+w	+w
3. *Mycelia sterilia*(black)	+8	+ 16
4. *Mycelia sterilia* (white)	-	-
5. *Penicillium* sp. MP-1	+w	+w
6. *Penicillium* sp. MP-2	-	-
7. *Penicillium* sp. MP-3	-	-
8. Unidentified strain	+w	+w

- Indicates no activity; + Indicates activity;
w Indicates weak activity

* Proteolytic activity expressed in terms of clear zone (mm dia) produced around the fungal spot at 37^0C for 24 hrs of incubation

** Proteolytic activity expressed in terms of clear zone (mm dia) produced around the well (8 mm) by 100 µl of supernatant at 37^0C for 24hrs of incubation.

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