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Phytochemical and Antifungal Screening of *Medicago sativa* and *Zinnia elegans*

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Abstract: Roots of *Medicago sativa* (Lucern) and whole plant of *Zinnia elegans* (Zinnia) were subjected to phytochemical and antifungal screening. *M. sativa* was found to contain 17% saponins, while *Z. elegans* had 7.5%, on dry weight basis. Crude saponins isolated from *M. sativa* showed antifungal activity against *Colletotricum falcatum* with minimum inhibitory concentration (MIC) of 289μg/ml, while *Z. elegans* inhibited the growth of *Fusarium moniliforme* up to the concentration of 207μg/ml. Crude saponins of *M. sativa* and *Z. elegans* were fractionated qualitatively into four and five fractions by thin layer chromatography and quantitatively by column chromatography, respectively. Three major fractions of crude saponins isolated from these plants were also checked for their antifungal activity. Fraction A of *M. sativa* was effective against *C. falcatum* with MIC of 31μg/ml, while fraction B of *Z. elegans* showed pronounced antifungal activity against *F. moniliforme* having MIC value of 34.5μg/ml.

Key words: *M. sativa, Z. elegans,* saponins, phytochemistry, antifungal screening

Introduction

Antibiotic or antimicrobial substances like saponins, glycosides, flavonoids and alkaloids etc. are found to be distributed in plants, yet these compounds were not well exploited due to the lack of knowledge and techniques. The phytochemical screening has been under taken by various research workers in different countries of the world. (Badar *et al.*, 2000; Nishimuta *et al.*, 1999; Rastrelli *et al.*, 1999 and Li *et al.*, 1999). The phytoconstituents i.e., phenols, anthraquinones, alkaloids, glycosides, flavonoids and saponins are antibiotic principles of plants. From these phytoconstituents, saponins have been reported to exhibit antifungal (Nagata *et al.*, 1985), anti-inflammatory (Takagi *et al.*, 1980) fungistatic (Zehavi and Segal, 1986), molluscidal (Sati *et al.*, 1987), pesticidal (Fatima, 1988) hemolytic and foaming activity (Feroz *et al.*, 1993).

Pakistan's main cash crops include cotton, sugarcane, rice, chickpea and sunflower. All of these crops are attacked by a variety of fungal infestations which resulted in the tremendous loss of yield. *Fusarium oxysporum* (Halila *et al.*, 1984) attacked chick pea and *Colletotricum falcatum* Went is responsible for red root of sugarcane (Gill and Saleem, 1982). Sunflower is attacked by *Macrophomina phaseolina* causing charcoal rot (Hafeez and Ahmad, 1997). *Fusarium moniliforme* is the causative of bakanae disease of rice (Ilyas *et al.* 1996). Cotton is attacked by *Rhizoctonia solani* causing cotton root rot. To combat with these fungal infestations a variety of synthetic chemicals and formulations are available in the market. But extensive use of these chemicals for long time have resulted in a number of serious health problems due to their residues in food chain and the resistance acquired by micro-organism and their ecological effects. More than half of the cases of poisoning and three fourth of deaths were reported from third world countries. So, there is a great need to go back towards natural plant protection methods. Efforts are being made all over the world to isolate natural antifungal and antimicrobial agents which could be employed as plant protection measures (Zhang and Lewis, 1997).

So, *M. sativa* (Lucern) and *Zinnia elegans* (Zinnia) was planned to screen for their constituents and to study the antifungal activity of saponins.

Materials and Methods

The project was laid out in Department of Chemistry, University of Agriculture, Faisalabad during June to October 2000. Whole plant of *Z. elegans* and roots of *M. sativa* were dried in hot air oven at 70°C for 5-6 hours and pulverized in an electric grinder. Methanol extract of these plants were prepared and tested against different phytoconstituents like alkaloids, steroids, saponins, flavonoids, anthraquinones and glycosides by using the standard methods of Brain and Turner (1975).

Extraction of crude saponins: Pulverized plant material was defatted with petroleum ether in a Soxhelt extractor. The defatted, solvent free plant material was again extracted with methanol to get crude saponins. The solvent was removed under reduced pressure yielding the crude saponins which were identified by their frothing as well as hemolytic activities (Feroz *et al.*, 1993). Antifungal activities of the crude saponins and the fractionated samples were determined following the procedure of Mobeen *et al.* (1984).

Qualitative separation of crude saponins by thin layer Chromatography: The pre coated TLC plates (Silica gel) were activated at 105°C for one hour. Then these plates were spotted with crude saponins solution in methanol with the help of capillary tube, 3 cm apart and 3 cm above the base. Hot air dryer was used to prevent spreading of spots during application of samples on plates. Loaded plates were placed in developing tank, pre-saturated with solvent system methanol, chloroform and water (30:65:10). After 3-4 hours the plates were removed from the tank, air dried and sprayed with vapors of 50% sulphuric acid as locating agent, followed by heating at 90-95°C. The R_f values of different components on TLC plates were noted (Wolf, 1970).

Quantitative fractionation of isolated crude saponins by column chromatography: Aluminum oxide was used as adsorbent and mixture of methanol and dimethyl ether were employed as eluent. The 50g of alumina and 100ml mixture of dimethyl ether in methanol was transferred to the column. Elution was carried out with 25, 50, 75 and 100% methanol in dimethyl ether in case of *Z. elegans*, while in *M. sativa* 60, 70, 80, 90 and 100% methanol in dimethyl ether was used. Each fraction was collected separately in china dish. The solvent was evaporated under reduced pressure (Gestetner *et al.*, 1965).

Estimation of antifungal activity: Antifungal activity of crude saponins and their fractions were determined against phytopathogenic fungi, *M. phaseolina, F. oxysporum, R. solani, C. falcatum* and *F. moniliforme* by using the procedure of Mobeen *et al.* (1984). The identified fungi were obtained from the Plant Pathology Section, Ayub Agricultural Research Institute, Faisalabad.

A set of six test tubes was serially numbered. The 5ml CH_3OH soln. was added in each tube and 5ml stock solution of saponins (1%) was transferred to test tube No. 1. Then 5ml soln. from Ist tube was transferred to 2nd test tube, this two fold serial dilution was continued up to the sixth test tube and then discarding the 5ml from the last tube. Now the contents of all the test tubes were separately added in already sterilized petri plates. The CH_3OH was allowed to evaporate under laminar flow. Then Potato Dextrose Agar (PDA) medium was prepared (Cruickshank, 1975), approximately 15 ml of which the was added in each plate and allowed to set as a firm gel. The pour plates were incubated over night at 37°C and those plates showing no contamination were finally selected for use.

The positive control was maintained simultaneously by a standard antifungal agent "Benlate" while a plate simply with PDA served as a negative control. All petri plates were inoculated under laminar flow by embedding in the center, equal quantities of the actively growing culture of desired fungus and placed in incubator at 29°C. The antifungal activity of saponins and standard antifungal agent was

determined by noting the colony diameter (Robert, 1978).
The data were statistically analyzed by applying factorial arrangements of treatment under Complete Randomized Design (Steel and Torrie, 1992).

Results and Discussion

Phytochemical analysis of *M. sativa* and *Z. elegans* showed 17 and 7.5% saponins respectively (Table 1). Levy *et al.* (1989), Feroz *et al.* (1993) and Small (1996) reported the presences of saponins in *M. sativa* and Athar *et al.* (1990) found saponins in *Z. elegans* with minute variation in the percentage of saponins, which depends upon the maturity of the plants used.
Crude saponins extracted from roots of *M. sativa*, on qualitative separation by TLC showed 5 spots with R_f values of 0.85, 0.75, 0.5, 0.34 and 0.15. While *Z. elegans* showed 4 spots with R_f values, 0.7, 0.92, 0.15 and 0.36. Quantitative separation of crude saponins by column chromatography showed that *M. sativa* and *Z. elegans* were fractionated into five (A, B, C, D and E) and four fractions (A, B, C and D), respectively.

Table 1: Detection of important phytoconstituents in plant extracts

Phytochemical constituents	*M. sativa*		*Z. elegans*	
	+Ve/-Ve	%	+Ve/-Ve	%
Alkaloids	+Ve	-	-Ve	-
Saponins	+Ve	17	+Ve	7.5
Steroids	+Ve	-	+Ve	-
Flavonoids	-Ve	-	+Ve	-
Glycosides	+Ve	-	+Ve	-
Anthraquinone	-Ve	-	-Ve	-

Antifungal activity of crude saponins: Crude saponins isolated from *M. sativa* inhibited the growth of *C. falcatum* (Table 2), out of five test fungi. The statistical analysis showed highly significant difference among dilution (D), fungus (F) and FXD. At different levels of dilution, the colony diameter of fungus was changed. The colony diameter of *C. falcatum* at control (0 mg) was 6.5cm, while was 25 mg conc. had diameter of 4.5 cm and the value of diameter was changed with the change in concentration of saponins. DMR test (Data not shown) at 99% confidence interval showed that 289μg/ml was the minimum inhibitory concentration (MIC) from crude saponins of *M. sativa*.
Crude saponins extracted from *Z. elegans* inhibited the growth of *F. moniliforme* (Table 3). The statistical analysis shows highly significant difference among dilutions, Fungus and FXD. At control (0 mg), the diameter of fungus growth was 5.8 cm (Table 3). Similarly, when the concentration of saponins decreased gradually, the growth of fungus was also changed. The DMR test at 99% confidence interval showed that 207mg/ml was the MIC of saponins extracted from *Z. elegans*. Standard antifungal agent "Benlate" inhibited the growth of all fungi (Table 4). There was highly significant difference among F, D and FXD for Benlate. While DMR test revealed that 13.5 μg/ml was the MIC. On comparing the antifungal activities of crude saponins with Benlate, *M. sativa* showed 4.6% and *Z. elegans* showed 6.5% of the activity of Benlate.
These observations of antifungal activity of saponins are supported by the results of various scientists work. Nagata *et al.* (1985) isolated two triterpenoid saponins from the aqueous methanolic extract of *Camellia* (*C. japonica*) leaf, which showed antifungal activity as characterized by abnormal germination of conidia. Shimoyamada *et al.* (1990) detected antifungal activity in the crude saponins obtained from bottom cut of *Asparagus officinalis*. This activity was specific to certain fungi, e.g., *Candida*, *Erytococcus*, *Trichophyton*, *Microsporum* and *Epidermophyton*.

Table 2: Colony diameter of five test fungi and PDA at various dilutions of saponins isolated from *M. sativa*

Test fungi	Colony diameters (cm) at different dilutions							
	0	25mg ml^{-1}	12.5mg ml^{-1}	6.25mg ml^{-1}	3.125mg ml^{-1}	1.563mg ml^{-1}	0.781mg ml^{-1}	Means
F. oxysporum	6.5b	6.1bcd	6.4dc	6.3bcd	6.2bcd	6.4bc	6.5b	6.3b
F. moniliforme	5.9bcd	5.7bcd	5.4d	5.5cd	5.7bcd	5.8bcd	5.8bcd	5.7c
R. solani	5.7bcd	5.6cd	5.8bcd	5.9bcd	5.5cd	5.6cd	5.7bcd	5.7c
M. phaseolina	7.9a	7.7a	7.4a	7.5a	7.7a	7.8a	7.8a	7.7a
C. falcatum	6.4bc	4.5e	4.6e	4.7e	5.7bcd	5.9bcd	6.1bcd	5.4d
Means	6.5a	5.9c	5.9c	6.0bc	6.2abc	6.3ab	6.4a	-

Means having different letters differ from each other at 99% confidence interval.

Table 3: Colony diameter of five test fungi on PDA at various dilutions of saponins isolated from *Z. elegans*

Test fungi	Colony diameters (cm) at different dilutions							
	0	25mg ml^{-1}	12.5mg ml^{-1}	6.25mg ml^{-1}	3.125mg ml^{-1}	1.562mg ml^{-1}	0.781mg ml^{-1}	Means
F. oxysporum	6.7b	6.4b-e	6.2b-g	6.5b-d	6.4b-e	6.6bc	6.7	6.5b
F. moniliforme	5.8c-h	3.2j	3.4j	3.6j	3.7j	4.5i	5.3	4.2e
R. solani	5.7e-h	5.6e-h	5.4gh	5.3hc	5.5f-h	5.4gh	5.5	5.5d
M. phaseolina	7.7a	7.4a	7.6a	7.5a	7.4a	7.5a	7.6	7.5a
C. falcatum	6.3b-f	6.0b-h	5.8d-h	6.2b-g	5.9c-h	6.1b-g	6.2	6.0c
Means	6.4a	5.7d	5.7d	5.8cd	5.8cd	6.0bc	6.2ab	-

Means having different letters differ from each other at 99% confidence interval.

Table 4: Colony diameter of five test fungi on PDA at various dilutions of standard fungistatic agent "Benlate"

Test fungi	Colony diameters (cm) at different dilutions							
	0	25mg ml^{-1}	12.5mg ml^{-1}	6.25mg ml^{-1}	3.125mg ml^{-1}	1.562mg ml^{-1}	0.781mg ml^{-1}	Means
F. oxysporum	6.5c	1.7op	1.9no	2.2mn	2.3m	3.9fg	4.1	2.7d
F. moniliforme	5.8d	2.5lm	2.8kl	3.0jk	3.7gh	3.9fg	4.2	3.2c
R. solani	8.4a	2.8kl	3.2lj	3.5nl	3.9fg	4.1ef	4.3	2.8d
M. phaseolina	7.6b	0.2r	0.8q	1.5p	2.7l	3.2lj	3.4	3.7b
C. falcatum	6.4c	0.3r	1.0q	1.8op	2.5lm	3.7fg	4.2	4.3a
Means	6.9a	1.5j	1.9f	2.4e	3.0d	3.8c	4.0b	-

Means having different letters differ from each other at 99% confidence interval.

Table 5: Colony diameter of *C. falcatum* at various dilutions of fraction A from *M. sativa*

Test fungi	Colony diameters (cm) at different dilutions						
	0	2.5mg ml^{-1}	1.25 mg ml^{-1}	0.625mg ml^{-1}	0.312mg ml^{-1}	0.156mg ml^{-1}	0.0781mg ml^{-1}
C. falcatum	6.4a	3.7c	4.0bc	4.1bc	4.2bc	4.5b	4.7b

Means sharing different letters differ from each other at 99% confidence interval.

Table 6: Colony diameter of *F. moniliforme* at various dilutions of fraction B of *Z. elegans*

Test fungi	Colony diameters (cm) at different dilutions						
	0	2.5mg ml^{-1}	1.25 mg ml^{-1}	0.625mg ml^{-1}	0.312mg ml^{-1}	0.156mg ml^{-1}	0.0781mg ml^{-1}
F. moniliforme	5.9a	4.0c	4.2c	4.3bc	4.5bc	4.6bc	4.9b

Means sharing different letters differ from each other at 99% confidence interval.

. Parveen *et al.* (2001) isolated saponins from *C. album* which inhibited the growth of *R. solani* and *C. falcatum*, while *T. foenum-graecum* inhibited the growth of *C. falcatum and F. monileforme*.

Antifungal Activity of Individual Fractions: Three major fractions (A, C and D) of *M. sativa* were tested for their antifungal activity against *C. falcatum*. Only fraction A, showed highly significant effect (Table 5) on the growth of *C. falcatum* (with MIC, 31μg/ml). The 3 major fractions (A, B and C) of *Z. elegans* were also tested for its antifungal activity by applying the same methods against *F. moniliforme*. Fraction B showed highly significant effect (Table 6) on the growth of *F. moniliforme* with 34.5 μg/ml MIC) as compared to the fractions A and D.

On comparing the antifungal activities of individual fractions of crude saponins with "Benlate" it was found that most effective fraction of *M. sativa* i.e. fraction A showed 43.5% while most effective fraction of *Z. elegans* i.e., fraction B showed 39.1% of the activity of Benlate. These results are invigorated by the observations of Christian and Hadwiger (1989), isolated saponins like compounds from peas and tested for antifungal activity. Growth of *Fusarium solani* f. sp. and f. sp. *pisi macroconidia* was inhibited by saponins at concentration of 150 and 300 mg/ml, respectively. Li *et al.* (1999) showed that antifungal assay guided isolation of the 95% ethanol extract of stem of *Colubrian retusa* yielded saponins, were marginally active against only *C. neoformans*, with MIC of 50 μg/ml. Zehavi and Segal (1986), Athar (1990) and Feroz *et al.* (1993) reported similar results.

Keeping in view the importance of antifungal activity of saponins extracted form *M. sativa* and *Z. elegans*, it can be suggested that farmers should also use the saponins besides synthetic antifungal agents. By this method we can decrease the expenditures of such an expensive antifungal agents present in market.

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Sustainable Agriculture Management of Plant Diseases

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Abstract: This review aims to achieve sustainable management of the fungal pathogens diseases by regulation and exploitation of the microbial diversity without causing degradation of environment and health problems. Development of sustainable, integrated pest management (IPM) approaches for plant diseases control; ecology and characterization of plant pathogens and biocontrol agents. Restoring beneficial organisms that attack, repel, or otherwise antagonize disease-causing pathogens will render a soil disease-suppressive. Plants growing in disease-suppressive soil resist diseases much better than in soils low in biological diversity. Beneficial organisms can be added directly, or the soil environment made more favorable for them through use of compost and other organic amendments. Compost quality determines its effectiveness at suppressing soil-borne plant diseases. More recently, a larger portion of the strategies utilized in agriculture have been biological control practices. In the broad sense, host genetics, soil amendments, fertilizer effects on pathogens, etc., are all part of the IPM picture.

Key words: IPM, plant diseases, sustainable agriculture

Introduction

Sustainable Agriculture defined as an integrated system of plant production practices having a site-specific application that will, over the long term: satisfy human food and fiber needs; enhance environmental quality and the natural resource base upon which the agriculture economy depends; make the most use of nonrenewable resources and on-farm resources and integrate, where appropriate, natural biological cycles and controls; sustain the economic viability of farm operations and enhance the quality of life for farmers and ranchers and society as a whole (Gliessman, 1990; Mahaffee and Kloepper, 1994; Neate, 1994). Sustainable agriculture is a way of farming that can be carried out for generations to come (Folgarait, 1998). This long-term approach to agriculture combines efficient production with the wise stewardship of the earth's resources. Sustainable agriculture include the following:

1) Meet human needs with a safe, high-quality and affordable supply of food and fiber.
2) Protect the natural resource base and prevent the degradation of air, soil and water quality.
3) Use nonrenewable resources efficiently.
4) Use natural biological cycles and controls.
5) Assure the economic survival of farming and the well-being of farmers, their families and communities.
6) Creation of institutional incentives and funding that focus public and private research, education, and technology development on integrating agricultural productivity and profitability with environmental stewardship.

New technology in all areas has improved agricultural production, thus its sustainability. Today's agriculture is using best management practices (BMP's), by targeting many of its applications, not broadcasting as was done in the past. New disease resistant hybrids, biological pest control, reduced fungcide use, cultural practices that reduce the incidence of diseases and better placement and reduced amounts of fertilizers are all being employed (Cook, 1994; Nisbet and Fox, 1991).

General suppression

Disease suppressive soils: There are two types of disease suppression, specific and general. Specific suppression results from one organism directly suppressing a known pathogen. These are cases where a biological control agent is introduced into the soil for the specific purpose of reducing disease incidence. General suppression is the result of a high biodiversity of microbial populations that create conditions unfavorable for plant disease development (Nisbets and Fox, 1991; Neate, 1994; Harrison and Frank, 1999).

Introducing a single organism to soils seldom achieves disease suppression for very long. If not already present, the new organisms may not be competitive with existing microorganisms. If soil conditions are inadequate, the introduced beneficial organism will not survive. This practice is not sufficient to render the soil "disease suppressive;" it is like planting flowers in the desert and expecting them to survive without water. With adequate soil conditions, inoculation with certain beneficials should only be needed once. A soil is considered suppressive when, in spite of favorable conditions for disease to occur, a pathogen either cannot become established, establishes but produces no disease, or establishes and produces disease for a short time and then declines (Schneider, 1982; Hermosa *et al.*, 2000). Suppressiveness is linked to the types and numbers of soil organisms, fertility level, and nature of the soil itself (drainage and texture). The mechanisms by which disease organisms are suppressed in these soils include induced resistance, direct parasitism (one organism consuming another), nutrient competition and direct inhibition through antibiotics secreted by beneficial organisms. Additionally, the response of plants growing in the soils contributes to suppressiveness. This is known as induced resistance and occurs when the rhizosphere (soil area around plant roots) is inoculated with a weakly virulent pathogen. After being challenged by the weak pathogen, the plant develops the capacity for future effective response to a more virulent pathogen. In most cases, adding mature compost to a soil induces disease resistance in many plants. The level of disease suppressiveness is typically related to the level of total microbiological activity in a soil. Larger the active microbial biomass, greater the capacity to utilize carbon, nutrients and energy in the soil, thus lowering their availability to pathogens. In other words, competition for mineral nutrients is high, as most soil nutrients are tied up in microbial bodies. High competition—coupled with secretion of antibiotics by some beneficial organisms and direct parasitism by others -makes for a tough environment for the pathogen (Chen *et al.*,1993). Our goal is to create soil conditions with all three of these factors present. Therefore we want high numbers and diversity of competitors, inhibitors and predators of disease organisms, as well as food sources on which these organisms depend. The food for beneficial organisms comes either directly or indirectly from organic matter and waste products from the growth of other organisms. It should be noted that general suppression will not control all soil-borne diseases. *Rhizoctonia solani* and *Sclerotium rolfsii*, for example, are not controlled by suppressive soils—their large propagules make them less reliant on external energy or nutrient sources and therefore they are not susceptible to microbial competition. With these two pathogens, "specific" beneficial organisms such as *Trichoderma* and *Gliocladium* will colonize the harmful propagules and reduce the disease potential (Granatstein, 1998).

Crop rotation and disease suppression: Avoiding disease buildup is probably the most widely emphasized benefit of crop rotation in vegetable production. Many diseases build up in the soil when the same crop is grown in the same field year after year. Rotation to a non-susceptible crop can help break this cycle by reducing pathogen levels. To be effective, rotations must be carefully planned. Since diseases usually attack plants related to each other, it is helpful to group vegetable rotations by family e.g., nightshades, alliums, cole crops, cucurbits. The susceptible crop, related plants and alternate host plants for the disease must be kept out of the field during the rotation period (Karlen *et al.*, 1994). Since plant pathogens persist in the soil for different lengths of time, the length of the rotation will vary with the disease being managed. To effectively plan a crop rotation it is essential to know what crops are affected by what disease organisms. In most cases, crop rotation effectively controls those pathogens that survive in soil or on crop residue. Nor will it help control pathogens that can survive long periods in the soil without a host—*Fusarium*, for example. Rotation, by itself, is only effective on pathogens that can overwinter in the field or be introduced on infected seeds or transplants. Of course, disease-free transplants or seed should be used in combination with crop rotation. The period of time between susceptible crops is highly variable, depending on the disease . For example, it takes seven years without any cruciferous crops for clubfoot to dissipate. Three years between parsley is needed to avoid damping off. Three years without tomatoes to avoid *Verticillium* wilt on potatoes. A three-year crop rotation is the standard recommendation for control of black rot (*Ceratocystis fimbriata*), stem rot (*Fusarium* oxysporum), and scurf (*Monilochaetes infuscans*) in sweet potatoes.

Plant nutrients and disease control: Soil pH, calcium level, nitrogen form, and availability of nutrients can play a major role in disease management. Adequate crop nutrition makes plants more tolerant of or resistant to disease. Also, the nutrient status of the soil and the use of particular fertilizers and amendments can have significant impacts on the pathogen's environment. One of the most widely recognized associations between fertility management and a crop disease is the effect of soil pH on potato scab. Potato scab is more severe in soils with pH levels above 5.2. Below 5.2 the disease is generally suppressed. Sulfur and ammonium sources of nitrogen acidify the soil, also reducing the incidence and severity of potato scab. Liming, on the other hand, increases disease severity. While lowering the pH is an effective strategy for potato scab, increasing soil pH or calcium levels may be beneficial for disease management in many other crops. Adequate levels of calcium can reduce clubroot in crucifer crops (broccoli, cabbage, turnips, etc.). The disease is inhibited in neutral to slightly alkaline soils (pH 6.7 to 7.2) (Campbell and Arthur, 1990). A direct correlation between adequate calcium levels, and/or higher pH and decreasing levels of *Fusarium* occurrence has been established for a number of crops, including tomato, cotton, melons and several ornamentals (Jones *et al.*, 1989 ; Yamazaki and Hosina, 1995). Calcium has also been used to control soil-borne diseases caused by *Pythium*, such as damping off. Crops where this has proved effective include wheat, peanut, peas, soybeans, peppers, sugarbeet, beans, tomato, onions, and snapdragon (Ko and Ching-Wen, 1989). Researchers in Hawaii reported reduction of damping off in cucumber after amending the soil with calcium and adding alfalfa meal to increase the microbial populations (Ko and Ching-Wen, 1989). Potassium fertility is also associated with disease management. Inadequate potash levels can lead to susceptibility to *Verticillium* wilt in cotton (Obrien-Wray, 1995). Phosphate can also be critical. Increasing phosphorus rates above the level needed to grow the crop can increase the severity of *Fusarium* wilt in cotton and muskmelon (Jones *et al.*, 1989). In general, the combination of lime, nitrate nitrogen and low phosphorus is effective in reducing the severity of *Fusarium*.

Biological control: Biological control of plant disease is defined as the involvement of the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens and the diseases they cause (Lewis and Papavizas, 1991). These "specialized" fungi and bacteria are microorganisms that normally inhabit most soils. In their native habitat they compete with other microorganisms for space and food and in some cases they produce toxic substances that parasitize and/or kill other soil-inhabiting microorganisms such as *Pyhtium* sp., *Phytophthora* sp., *Rhizoctonia* sp., and other plant pathogens (Lorito *et al.*, 1996). There are four different mechanisms by which biocontrol agents control other microorganisms. Most biocontrol agents apply only one of these four mechanisms, however, some may employ more than one.

- **Direct competition with the target organism:** In this case the biocontrol agent out competes the target organisms for nutrients and space.

- **Antibiosis:** With antibiosis, the biocontrol agent produces an chemical compound such as an antibiotic or some type of toxin that kills or has some sort of detrimental effect on the target organism.

- **Predation or parasitism of the target organism:** In this case the biocontrol agent can attack and feed directly on the target organism or the biocontrol agent can produce of enzymes and some sort of toxin that kills the target organism and then the biocontrol agent feeds on the dead target.

- **Induced resistance of the host plant.** It has been know for decades that once a plant is infected with a pathogen, that infection triggers some sort of reaction in the infected host plant that helps keep it from being infected with other pathogens. The infected plant becomes more "resistant" to other infections.

In the area of greenhouse floriculture and perennial production there are about a half dozen products that are currently popular. of these root shield appears to be the most widely used. Root shield is the T-22 strain of the soil inhabiting fungus *Trichoderma harzianum* (TH). It uses both antibiosis and predation against many common soil inhabiting fungi that cause root and crown rots such as *Pythium, Rhizoctonia, Fusarium* and *Sclerotinia*. It appears to be one of the most popular biofungicides in the greenhouse industry and can be an asset to a disease management program if used properly. In order for any of these biological control agents to work for you, two simple rules must be followed. First off, all of these products must be used in conjunction with standard disease cultural controls. Cultural controls include, growing plants in a well drained media, not over watering, keeping the greenhouse relative humidity below 85%, practicing strict sanitation and making sure that the nutrient and pH conditions of the host plant a within the ideal range for proper growth and development.

Biocontrol of soil borne disease: Chemical control of soil borne plant diseases is frequently ineffective because of the physical and chemical heterogeneity of the soil , which may prevent effective concentrations of the chemical from reaching the pathogen. Biological control agents colonize the rhizosphere, the site requiring protection and leave no toxic residues, as opposed to chemicals. Micro organisms have been used extensively for the biological control of soil borne plant diseases as well as for promoting plant growth. Fluroscent pseudomonas are the most frequently used bacteria for biological control and plant growth promotion, but *Bacillus and Streptomyces* species have also been commonly used. *Trichoderma, Gliocadium* and *Coniothyrium* are the most commonly used fungal biocontrol agents (Hoitink, 1986). Competition as a mechanism of biological control has been

exploited with soil borne Plant pathogens as with the pathogens on the phylloplane. Naturally occuring, nonpathogenic strains of *Fusarium* oxysporium have been used to control wilt diseases caused by pathogenic *Fusarium* spp.
Molecular techniques have also facilitated the introduction of beneficial traits into rhizosphere competent organisms to produce potential biocontrol agents. Chitin and b -(1,3) - glucan are the two major structural components of many plant pathogenic fungi, except by oomycetes, which contain cellulose in their cell wall and no appreciable levels of chitin. Biological control of some soil borne fungal diseases has been correlated with chitinase production, bacterial producing chitinases or glucanases exhibit antagonism in vitro against fungi (Haran *et al.*, 1996; Baek *et al.*, 1999).

Biocontrol of airborne diseases: Many naturally occurring microorganisms have been used to control diseases on the aerial surfaces of plants (Elad, 2000). The most common bacterial species that have been used for the control of diseases in the phylloshpere include *Pseudomonas syringae, P. fluorescens, P. cepacia, Erwinia herbicola* and *Bacillus subtilis*. Fungal genera that have been used for the control of air borne diseases include *Trichoderma, Ampelomyces* and the yeasts *Tilletiopsis* and *sporobolomyces* (Haggag and El-Gamal, 2001). Phytopathogenic bacteria possess several genes that encode phenotypes that allow them to parasitize plants and overcome defense responses elicited by the plant. In addition, phytopathogenic bacterial possess pathogenicity genes. Isogenic avirulent mutants can be produced by insertional inactivation of genes involved in pathogenicity. Nonpathogenic mutants of *Erwinia amylovora*, produced by transposon mutagenesis, have also been used in the biological control of fire blight. Antibiosis has been proposed as the mechanism of control of serveral bacterial and fungal diseases in the phyllosphere. Molecular biology techniques could be used to enhance the efficacy of biocontrol agents that use antibiosis as a more of action (Garcia *et al.*, 1994). Biocontrol agents must normally achieve a high population in the phyloshpere to control other strains, but colonization by the agent may be reduced by competition with the indigenous microflora. Integration of chemical pesticides and biocontrol agents have been reported with *Trichoderma* spp. and *P. syringae*. Biocontrol agents tolerant to specific pesticides could be constructed using molecular techniques. Resistance to the fungicide benomyl is conferred by a single amino acid substitution in one of the B-tubulins of *Trichoderma viridae*. The corresponding gene thereby producing a biological control agent that could be applied simultaneously or in alternation with the fungicide.

Inoculum products: The following is a partial list of soil inoculum and biocontrol products available for control of soil-borne diseases on a variety of plants:

- Liquid drench containing *Bacillus subtilis* GB03 for horticultural crops at seeding or transplanting or as a spray for turf target pathogen/disease is *Rhizoctonia, Pythium, Fusarium* and *Phytophthora*.
- Peat-based dried biomass from solid fermentation; aqueous suspension of *Burkholderia cepacia* for control of *Rhizoctonia, Pythium, Fusarium* and disease caused by lesion, spiral, lance and sting nematodes. Used in alfalfa, barley, beans, clover, cotton, peas, grain sorghum, vegetable crops and wheat as a seed treatment, in drip irrigation or as a seedling drench.
- Dry powder formulation of *Bacillus subtilis* for control of *Rhizoctonia solani, Fusarium* spp., *Alternaria* spp. and *Aspergillus* spp. attacking roots of cotton and legumes. Can be added to a slurry, or mixed with a chemical fungicide for commercial seed treatment.
- A seed inoculant of *Pseudomonas cepacia* for control of *Rhizoctonia solani, Fusarium* spp., *Pythium* spp. in corn, vegetables and cotton.
- *Agrobacterium radiobacter* strain K-84 for control of crow gall disease caused by *Agrobacterium tumefaciens* in fruit, nut and ornamental nursery stock. Used as a dip or spray for root, stems or cuttings.
- *Streptomycetes* soil drench for suppression of *Fusarium, Alternaria* and *Phomopsis*. *Trichoderma* fungus for suppression of *Pythium, Rhizoctonia solani* and *Fusarium* spp. Applied as granules or wettable powder mixed with soil or potting medium or as a soil drench. Crops include trees, shrubs, transplants, all ornamentals, cabbage, tomato and cucumber.
- *Gliocladium virens* GL-21 for damping-off and root rot pathogens especially *Rhizoctonia solani* and *Pythium* spp. of ornamental and food crop plants grown in greenhouses, nurseries, homes, and interior-scapes. Sold as granules.
- *Bacillus subtilis* GB03 plus chemical pesticides. Used as a dust seed treatment in the planter box for seedling pathogens of barley, beans, cotton, peanut, pea, rice and soybeans.
- *Trichoerma huzianum* Rifai strain KRL-AG2 for control of *Pythium* spp. *Rhizoctonia solani, Fusarium* spp. and *Sclerotinia homeocarpa* in bean, cabbage, corn, cotton, cucumber, peanut, potato, sorghum, soybean, sugarbeet, tomato, turf and greenhouse ornamentals. Applied as in-furrow granules, broadcast to turf, mixed with greenhouse soil, or mixing powder with seeds in the planter box or in commercial seed treatment.

The *Trichoderma* system: *Trichoderma* are one of a small group of beneficial fungi, which has proven commercially viable as a biological control agent. This micro-organism is now registered as a bio-fungicide in France, the UK, Switzerland, Sweden, Belgium, Chile, New Zealand and the USA and regulations are pending in several other countries. *Trichoderma* thrives in the leaf litter or mulch in orchard situations and it requires a minimum organic carbon level of 1% to ensure proliferation in cropping locations. This species is a mycoparasite or saprophyte, which feeds on pathogenic fungi. There is now a body of photographic evidence highlighting this phenomenon where *Trichoderma* are seen actively parasitic basideomycetes including *Armillaria, mellea, Rhizoctonia solani* and *Chondrostereum purpureum*. In fact, *Trichoderma* can control the growth of many opportunistic, wood-infecting, decay fungi, as well as many soil-borne fungi responsible for seedling wilt and damping off (e.g. *Fusarium* and *Pythium*) (Baek *et al.*, 1999; Elad, 2000). *Trichoderma* is completely safe for humans and livestock. In 55 years of research there has never been a recorded adverse reaction. The predatory qualities of *Trichoderma* are a big part of the appeal of this species of fungus for commercial applications, but there are other associated benefits that warrant consideration (Lorito *et al.*, 1996).

Compost and disease suppression: Compost has been used effectively in the nursery industry, in high-value crops and in potting soil mixtures for control of root rot diseases (Haggag, Wafaa and Saber, 2000, 2001). Adding compost to soil may be viewed as one of a spectrum of techniques—including cover cropping, crop rotations, mulching, and manuring—which add organic matter to the soil (Hoitink *et al.*, 1991; Logsdon, 1995). The major difference between compost-amended soil and the other techniques is that organic matter in compost is already "digested". Other techniques require the digestion to take place in the soil, which allows for both anaerobic and aerobic decomposition of organic matter. Properly composted organic matter is digested chiefly through aerobic processes. These differences have important implications for soil and nutrient management, as well as plant health and pest management (Trankner, 1992; Hudson ,1994). Compost is effective because it fosters a more diverse soil environment in which a myriad of soil organisms exist. Compost acts as a food source and shelter for the antagonists that compete with plant pathogens, for those organisms that prey on and parasitize pathogens and for those beneficial that produce antibiotics. Root rots caused by *Pythium*

and Phytophthora are generally suppressed by the high numbers and diversity of beneficial microbes found in the compost. Such beneficial prevent the germination of spores and infection of plants growing on the amended soil (Goldstein, 1998; Harrison and Frank, 1999). Systemic resistance is also induced in plants in response to compost treatments. Hoitink *et al.* (1997) has now established that composts and compost teas indeed activate

disease resistance genes in plants. These disease resistance genes are typically "turned on" by the plant in response to the presence of a pathogen. These genes mobilize chemical defenses against the pathogen invasion, although often too late to avoid the disease. Plants growing in compost, however, have these disease-prevention systems already running (Goldstein, 1998). Induced resistance is somewhat pathogen-specific, but it does allow an additional way to manage certain diseases through common farming practices. It has become evident that in disease management, using a "one size fits all" approach to composting will not work. Depending on feed stock, inoculum and composting process, composts have different characteristics affecting disease management potential. For example, high carbon to nitrogen ratio (C:N) tree bark compost generally works well to suppress *Fusarium* wilts. With lower C:N ratio composts, *Fusarium* wilts may become more severe as a result of the excess nitrogen, which favors *Fusarium*. (Hoitink *et al.*, 1997). Compost from sewage sludge typically has a low C:N ratio. Some of the beneficial microorganisms that re-inhabit compost from the outside edges after heating has subsided include several bacteria (Bacillus species, *Flavobacterium balustinum* and various Pseudomanas species) and several fungi (*Streptomyces, Penicillin, Trichoderma* and *Gliocladium* virens). The moisture content following peak heating of a compost is critical to the range of organisms inhabiting the finished product. Dry composts with less than 34% moisture are likely to be colonized by fungi and therefore are conducive to *Pythium* diseases (Hoitink *et al.*,1997). Compost with at least 40 to 50% moisture will be colonized by both bacteria and fungi and will be disease suppressive (Hoitink *et al.*,1997). Water is typically added during the composting process to avoid a dry condition. Compost pH below 5.0 inhibits bacterial biocontrol agents. Three approaches can be utilized to increase suppressiveness of compost. First, curing the compost for four months or more; second, incorporating the compost in the field soil several months before planting and third, inoculating the compost with specific biocontrol agents (Hoitink *et al.*,1997). Two of the more common beneficial used to inoculate compost are strains of *Trichoderma* and *Flavobacterium*, added to suppress *Rhizoctonia solani*. *Trichoderma harzianum* acts against a broad range of soil-borne fungal crop pathogens, including *R. solani*, by production of anti-fungal exudates. The key to disease suppression in compost is the level of decomposition—as the compost matures, it becomes more suppressive. Readily available carbon compounds found in low-quality, immature compost can support *Pythium* and *Rhizoctonia*. As these compounds are reduced during the complete composting process, saprophytic growth of these pathogens is dramatically slowed (Nelson *et al.*, 1994). Beneficial such as *Trichoderma hamatum* and *T. harzianum*, unable to suppress *Rhizoctonia* in immature composts, are extremely effective when introduced into mature composts. For *Pythium suppression*, a direct correlation has been shown between general microbial activity and amount of microbial biomass and the degree of suppression. *Pythium* is a nutrient-dependent pathogen with the ability to colonize fresh plant residue, especially in soil that has been fumigated to kill all soil life. The severity of diseases caused by *Pythium* and *R. solani* relates less to the inoculum density than to the amount of saprophytic growth the pathogen achieves before infection (Cook, 1994). Consequently, soils that are antagonistic to saprophytic growth of *Pythium*—such as soils amended with fully decomposed compost—will support lower disease levels. As for *Rhizoctonia*, this fungus is highly competitive in colontizing fresh organic matter (Chung *et al.*, 1988). Its ability to colonize decomposed organic matter is decreased or non-existent. There is a direct relationship between a compost's level of decomposition and its suppression of Rhizoctonia—again pointing to the need for high-quality, mature compost. Like compost, raw manure is conducive to diseases at first, then becomes suppressive after decomposition. In other words: organic amendments supporting high biological activity (i.e., decomposition) are suppressive of plant-root diseases, while raw organic matter will often favor colonization by the pathogen.

Determining and monitoring compost quality: It is clear that compost maturity is a key factor in its ability to suppress disease. The challenge involved in achieving and measuring that maturity is the primary reason why compost is not more widely used. Certainly, immature compost can be used in field situations, as long as it is applied well ahead of planting, allowing for eventual stabilization. However, good disease suppression may not develop because of other factors. For example, highly saline compost actually enhances *Pythium* and *Phytophthora* diseases unless applied months ahead of planting to allow for leaching. High-quality compost should contain disease-suppressive organisms and mycorrhizal inoculum (Hoitink *et al.*, 1997).

Direct inoculation with beneficial organisms: There are a number of commercial products containing beneficial, disease-suppressive organisms. These products are applied in various ways—including seed treatments, compost inoculants, soil inoculants and soil drenches. Among the beneficial organisms available are *Trichoderma, Flavobacterium, Streptomycetes, Gliocladium* spp., *Bacillus* spp., *Pseudomonas* spp. and others. A partial list of these products can be found in the resources section. These companies will send you their product and technical information upon request. Consider your cost and overall soil health before trying these products. *Trichoderma* and *Gliocladium* are effective at parasitizing other fungi, but they stay alive only as long as they have other fungi to parasitize. In soils with low fungal biomass (soils with low organic matter and plenty of tillage) these two beneficial have nothing to feed on. Compost is a great source of both the organisms and the food they need to do their jobs. A great diversity of bacteria and fungi occur in good compost.

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Phytochemical and Antifungal Screening of *Medicago sativa* and *Zinnia elegans*

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Abstract: Roots of *Medicago sativa* (Lucern) and whole plant of *Zinnia elegans* (Zinnia) were subjected to phytochemical and antifungal screening. *M. sativa* was found to contain 17% saponins, while *Z. elegans* had 7.5%, on dry weight basis. Crude saponins isolated from *M. sativa* showed antifungal activity against *Colletotricum falcatum* with minimum inhibitory concentration (MIC) of 289μg/ml, while *Z. elegans* inhibited the growth of *Fusarium moniliforme* up to the concentration of 207μg/ml. Crude saponins of *M. sativa* and *Z. elegans* were fractionated qualitatively into four and five fractions by thin layer chromatography and quantitatively by column chromatography, respectively. Three major fractions of crude saponins isolated from these plants were also checked for their antifungal activity. Fraction A of *M. sativa* was effective against *C. falcatum* with MIC of 31μg/ml, while fraction B of *Z. elegans* showed pronounced antifungal activity against *F. moniliforme* having MIC value of 34.5μg/ml.

Key words: *M. sativa, Z. elegans,* saponins, phytochemistry, antifungal screening

Introduction

Antibiotic or antimicrobial substances like saponins, glycosides, flavonoids and alkaloids etc. are found to be distributed in plants, yet these compounds were not well exploited due to the lack of knowledge and techniques. The phytochemical screening has been under taken by various research workers in different countries of the world. (Badar *et al.*, 2000; Nishimuta *et al.*, 1999; Rastrelli *et al.*, 1999 and Li *et al.*, 1999). The phytoconstituents i.e., phenols, anthraquinones, alkaloids, glycosides, flavonoids and saponins are antibiotic principles of plants. From these phytoconstituents, saponins have been reported to exhibit antifungal (Nagata *et al.*, 1985), anti-inflammatory (Takagi *et al.*, 1980) fungistatic (Zehavi and Segal, 1986), molluscidal (Sati *et al.*, 1987), pesticidal (Fatima, 1988) hemolytic and foaming activity (Feroz *et al.*, 1993).

Pakistan's main cash crops include cotton, sugarcane, rice, chickpea and sunflower. All of these crops are attacked by a variety of fungal infestations which resulted in the tremendous loss of yield. *Fusarium oxysporum* (Halila *et al.*, 1984) attacked chick pea and *Colletotricum falcatum* Went is responsible for red root of sugarcane (Gill and Saleem, 1982). Sunflower is attacked by *Macrophomina phaseolina* causing charcoal rot (Hafeez and Ahmad, 1997). *Fusarium moniliforme* is the causative of bakanae disease of rice (Ilyas *et al.* 1996). Cotton is attacked by *Rhizoctonia solani* causing cotton root rot. To combat with these fungal infestations a variety of synthetic chemicals and formulations are available in the market. But extensive use of these chemicals for long time have resulted in a number of serious health problems due to their residues in food chain and the resistance acquired by micro-organism and their ecological effects. More than half of the cases of poisoning and three fourth of deaths were reported from third world countries. So, there is a great need to go back towards natural plant protection methods. Efforts are being made all over the world to isolate natural antifungal and antimicrobial agents which could be employed as plant protection measures (Zhang and Lewis, 1997).

So, *M. sativa* (Lucern) and *Zinnia elegans* (Zinnia) was planned to screen for their constituents and to study the antifungal activity of saponins.

Materials and Methods

The project was laid out in Department of Chemistry, University of Agriculture, Faisalabad during June to October 2000. Whole plant of *Z. elegans* and roots of *M. sativa* were dried in hot air oven at 70°C for 5-6 hours and pulverized in an electric grinder. Methanol extract of these plants were prepared and tested against different phytoconstituents like alkaloids, steroids, saponins, flavonoids, anthraquinones and glycosides by using the standard methods of Brain and Turner (1975).

Extraction of crude saponins: Pulverized plant material was defatted with petroleum ether in a Soxhelt extractor. The defatted, solvent free plant material was again extracted with methanol to get crude saponins. The solvent was removed under reduced pressure yielding the crude saponins which were identified by their frothing as well as hemolytic activities (Feroz *et al.*, 1993). Antifungal activities of the crude saponins and the fractionated samples were determined following the procedure of Mobeen *et al.* (1984).

Qualitative separation of crude saponins by thin layer Chromatography: The pre coated TLC plates (Silica gel) were activated at 105°C for one hour. Then these plates were spotted with crude saponins solution in methanol with the help of capillary tube, 3 cm apart and 3 cm above the base. Hot air dryer was used to prevent spreading of spots during application of samples on plates. Loaded plates were placed in developing tank, pre-saturated with solvent system methanol, chloroform and water (30:65:10). After 3-4 hours the plates were removed from the tank, air dried and sprayed with vapors of 50% sulphuric acid as locating agent, followed by heating at 90-95°C. The R_f values of different components on TLC plates were noted (Wolf, 1970).

Quantitative fractionation of isolated crude saponins by column chromatography: Aluminum oxide was used as adsorbent and mixture of methanol and dimethyl ether were employed as eluent. The 50g of alumina and 100ml mixture of dimethyl ether in methanol was transferred to the column. Elution was carried out with 25, 50, 75 and 100% methanol in dimethyl ether in case of *Z. elegans*, while in *M. sativa* 60, 70, 80, 90 and 100% methanol in dimethyl ether was used. Each fraction was collected separately in china dish. The solvent was evaporated under reduced pressure (Gestetner *et al.*, 1965).

Estimation of antifungal activity: Antifungal activity of crude saponins and their fractions were determined against phytopathogenic fungi, *M. phaseolina, F. oxysporum, R. solani, C. falcatum* and *F. moniliforme* by using the procedure of Mobeen *et al.* (1984). The identified fungi were obtained from the Plant Pathology Section, Ayub Agricultural Research Institute, Faisalabad.

A set of six test tubes was serially numbered. The 5ml CH_3OH soln. was added in each tube and 5ml stock solution of saponins (1%) was transferred to test tube No. 1. Then 5ml soln. from Ist tube was transferred to 2nd test tube, this two fold serial dilution was continued up to the sixth test tube and then discarding the 5ml from the last tube. Now the contents of all the test tubes were separately added in already sterilized petri plates. The CH_3OH was allowed to evaporate under laminar flow. Then Potato Dextrose Agar (PDA) medium was prepared (Cruickshank, 1975), approximately 15 ml of which the was added in each plate and allowed to set as a firm gel. The pour plates were incubated over night at 37°C and those plates showing no contamination were finally selected for use.

The positive control was maintained simultaneously by a standard antifungal agent "Benlate" while a plate simply with PDA served as a negative control. All petri plates were inoculated under laminar flow by embedding in the center, equal quantities of the actively growing culture of desired fungus and placed in incubator at 29°C. The antifungal activity of saponins and standard antifungal agent was

determined by noting the colony diameter (Robert, 1978).
The data were statistically analyzed by applying factorial arrangements of treatment under Complete Randomized Design (Steel and Torrie, 1992).

Results and Discussion

Phytochemical analysis of *M. sativa* and *Z. elegans* showed 17 and 7.5% saponins respectively (Table 1). Levy *et al.* (1989), Feroz *et al.* (1993) and Small (1996) reported the presences of saponins in *M. sativa* and Athar *et al.* (1990) found saponins in *Z. elegans* with minute variation in the percentage of saponins, which depends upon the maturity of the plants used.
Crude saponins extracted from roots of *M. sativa*, on qualitative separation by TLC showed 5 spots with R_f values of 0.85, 0.75, 0.5, 0.34 and 0.15. While *Z. elegans* showed 4 spots with R_f values, 0.7, 0.92, 0.15 and 0.36. Quantitative separation of crude saponins by column chromatography showed that *M. sativa* and *Z. elegans* were fractionated into five (A, B, C, D and E) and four fractions (A, B, C and D), respectively.

Table 1: Detection of important phytoconstituents in plant extracts

Phytochemical constituents	*M. sativa*		*Z. elegans*	
	+Ve/-Ve	%	+Ve/-Ve	%
Alkaloids	+Ve	-	-Ve	-
Saponins	+Ve	17	+Ve	7.5
Steroids	+Ve	-	+Ve	-
Flavonoids	-Ve	-	+Ve	-
Glycosides	+Ve	-	+Ve	-
Anthraquinone	-Ve	-	-Ve	-

Antifungal activity of crude saponins: Crude saponins isolated from *M. sativa* inhibited the growth of *C. falcatum* (Table 2), out of five test fungi. The statistical analysis showed highly significant difference among dilution (D), fungus (F) and FXD. At different levels of dilution, the colony diameter of fungus was changed. The colony diameter of *C. falcatum* at control (0 mg) was 6.5cm, while was 25 mg conc. had diameter of 4.5 cm and the value of diameter was changed with the change in concentration of saponins. DMR test (Data not shown) at 99% confidence interval showed that 289μg/ml was the minimum inhibitory concentration (MIC) from crude saponins of *M. sativa*.
Crude saponins extracted from *Z. elegans* inhibited the growth of *F. moniliforme* (Table 3). The statistical analysis shows highly significant difference among dilutions, Fungus and FXD. At control (0 mg), the diameter of fungus growth was 5.8 cm (Table 3). Similarly, when the concentration of saponins decreased gradually, the growth of fungus was also changed. The DMR test at 99% confidence interval showed that 207mg/ml was the MIC of saponins extracted from *Z. elegans*. Standard antifungal agent "Benlate" inhibited the growth of all fungi (Table 4). There was highly significant difference among F, D and FXD for Benlate. While DMR test revealed that 13.5 μg/ml was the MIC. On comparing the antifungal activities of crude saponins with Benlate, *M. sativa* showed 4.6% and *Z. elegans* showed 6.5% of the activity of Benlate.
These observations of antifungal activity of saponins are supported by the results of various scientists work. Nagata *et al.* (1985) isolated two triterpenoid saponins from the aqueous methanolic extract of *Camellia* (*C. japonica*) leaf, which showed antifungal activity as characterized by abnormal germination of conidia. Shimoyamada *et al.* (1990) detected antifungal activity in the crude saponins obtained from bottom cut of *Asparagus officinalis*. This activity was specific to certain fungi, e.g., *Candida*, *Erytococcus*, *Trichophyton*, *Microsporum* and *Epidermophyton*.

Table 2: Colony diameter of five test fungi and PDA at various dilutions of saponins isolated from *M. sativa*

Test fungi	Colony diameters (cm) at different dilutions							
	0	25mg ml^{-1}	12.5mg ml^{-1}	6.25mg ml^{-1}	3.125mg ml^{-1}	1.563mg ml^{-1}	0.781mg ml^{-1}	Means
F. oxysporum	6.5b	6.1bcd	6.4dc	6.3bcd	6.2bcd	6.4bc	6.5b	6.3b
F. moniliforme	5.9bcd	5.7bcd	5.4d	5.5cd	5.7bcd	5.8bcd	5.8bcd	5.7c
R. solani	5.7bcd	5.6cd	5.8bcd	5.9bcd	5.5cd	5.6cd	5.7bcd	5.7c
M. phaseolina	7.9a	7.7a	7.4a	7.5a	7.7a	7.8a	7.8a	7.7a
C. falcatum	6.4bc	4.5e	4.6e	4.7e	5.7bcd	5.9bcd	6.1bcd	5.4d
Means	6.5a	5.9c	5.9c	6.0bc	6.2abc	6.3ab	6.4a	-

Means having different letters differ from each other at 99% confidence interval.

Table 3: Colony diameter of five test fungi on PDA at various dilutions of saponins isolated from *Z. elegans*

Test fungi	Colony diameters (cm) at different dilutions							
	0	25mg ml^{-1}	12.5mg ml^{-1}	6.25mg ml^{-1}	3.125mg ml^{-1}	1.562mg ml^{-1}	0.781mg ml^{-1}	Means
F. oxysporum	6.7b	6.4b-e	6.2b-g	6.5b-d	6.4b-e	6.6bc	6.7	6.5b
F. moniliforme	5.8c-h	3.2j	3.4j	3.6j	3.7j	4.5i	5.3	4.2e
R. solani	5.7e-h	5.6e-h	5.4gh	5.3hc	5.5f-h	5.4gh	5.5	5.5d
M. phaseolina	7.7a	7.4a	7.6a	7.5a	7.4a	7.5a	7.6	7.5a
C. falcatum	6.3b-f	6.0b-h	5.8d-h	6.2b-g	5.9c-h	6.1b-g	6.2	6.0c
Means	6.4a	5.7d	5.7d	5.8cd	5.8cd	6.0bc	6.2ab	-

Means having different letters differ from each other at 99% confidence interval.

Table 4: Colony diameter of five test fungi on PDA at various dilutions of standard fungistatic agent "Benlate"

Test fungi	Colony diameters (cm) at different dilutions							
	0	25mg ml^{-1}	12.5mg ml^{-1}	6.25mg ml^{-1}	3.125mg ml^{-1}	1.562mg ml^{-1}	0.781mg ml^{-1}	Means
F. oxysporum	6.5c	1.7op	1.9no	2.2mn	2.3m	3.9fg	4.1	2.7d
F. moniliforme	5.8d	2.5lm	2.8kl	3.0jk	3.7gh	3.9fg	4.2	3.2c
R. solani	8.4a	2.8kl	3.2lj	3.5nl	3.9fg	4.1ef	4.3	2.8d
M. phaseolina	7.6b	0.2r	0.8q	1.5p	2.7l	3.2lj	3.4	3.7b
C. falcatum	6.4c	0.3r	1.0q	1.8op	2.5lm	3.7fg	4.2	4.3a
Means	6.9a	1.5j	1.9f	2.4e	3.0d	3.8c	4.0b	-

Means having different letters differ from each other at 99% confidence interval.

Table 5: Colony diameter of *C. falcatum* at various dilutions of fraction A from *M. sativa*

Test fungi	Colony diameters (cm) at different dilutions						
	0	2.5mg ml^{-1}	1.25 mg ml^{-1}	0.625mg ml^{-1}	0.312mg ml^{-1}	0.156mg ml^{-1}	0.0781mg ml^{-1}
C. falcatum	6.4a	3.7c	4.0bc	4.1bc	4.2bc	4.5b	4.7b

Means sharing different letters differ from each other at 99% confidence interval.

Table 6: Colony diameter of *F. moniliforme* at various dilutions of fraction B of *Z. elegans*

Test fungi	Colony diameters (cm) at different dilutions						
	0	2.5mg ml^{-1}	1.25 mg ml^{-1}	0.625mg ml^{-1}	0.312mg ml^{-1}	0.156mg ml^{-1}	0.0781mg ml^{-1}
F. moniliforme	5.9a	4.0c	4.2c	4.3bc	4.5bc	4.6bc	4.9b

Means sharing different letters differ from each other at 99% confidence interval.

. Parveen *et al.* (2001) isolated saponins from *C. album* which inhibited the growth of *R. solani* and *C. falcatum*, while *T. foenum-graecum* inhibited the growth of *C. falcatum and F. monileforme*.

Antifungal Activity of Individual Fractions: Three major fractions (A, C and D) of *M. sativa* were tested for their antifungal activity against *C. falcatum*. Only fraction A, showed highly significant effect (Table 5) on the growth of *C. falcatum* (with MIC, 31μg/ml). The 3 major fractions (A, B and C) of *Z. elegans* were also tested for its antifungal activity by applying the same methods against *F. moniliforme*. Fraction B showed highly significant effect (Table 6) on the growth of *F. moniliforme* with 34.5 μg/ml MIC) as compared to the fractions A and D.

On comparing the antifungal activities of individual fractions of crude saponins with "Benlate" it was found that most effective fraction of *M. sativa* i.e. fraction A showed 43.5% while most effective fraction of *Z. elegans* i.e., fraction B showed 39.1% of the activity of Benlate. These results are invigorated by the observations of Christian and Hadwiger (1989), isolated saponins like compounds from peas and tested for antifungal activity. Growth of *Fusarium solani* f. sp. and f. sp. *pisi macroconidia* was inhibited by saponins at concentration of 150 and 300 mg/ml, respectively. Li *et al.* (1999) showed that antifungal assay guided isolation of the 95% ethanol extract of stem of *Colubrian retusa* yielded saponins, were marginally active against only *C. neoformans*, with MIC of 50 μg/ml. Zehavi and Segal (1986), Athar (1990) and Feroz *et al.* (1993) reported similar results.

Keeping in view the importance of antifungal activity of saponins extracted form *M. sativa* and *Z. elegans*, it can be suggested that farmers should also use the saponins besides synthetic antifungal agents. By this method we can decrease the expenditures of such an expensive antifungal agents present in market.

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