Antifungal Potential of *Ganoderma Lucidum* Extract Against Plant Pathogenic Fungi of Calendula Officinalis L.

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***Abstract*—** *Ganoderma lucidum* has antifungal bioactive compounds. The present research was focused to check the best extract of *G. lucidum* against plant pathogenic fungi (*Fusarium oxysporum, Alternaria alternata*) isolated from *Calendula officinalis* (marigold). Infected marigold plant samples were collected from Lahore in order to isolate *F. oxysporum,* and *A. alternata*. Organic and aqueous extract of *G. lucidum* were evaluated as a biological control agent. Concentrations (5%, 10%, 15% and 20%) of the extract were applied by Agar absorption, Agar well diffusion and Vapor assay techniques. It was concluded that methanolic extract of *G. lucidum* has more antagonistic potential as compared to acetone extract in all concentrations and maximum inhibitory value was 64% by adopting agar absorption method while the least inhibitory value 38% was observed in aqueous extract of *G. lucidum* by using Agar well diffusion method. These information will be highly useful to control plant pathogenic diseases of marigold.

***Keywords*—** Leaf Spots, Fusarium wilt, Marigold, *Fusarium oxysporum*, *Alternaria alternate* and *Ganoderma lucidum*..

I. Introduction

MARIGOLD (*Calendula officinalis* L.) has its vernacular name as "pot marigold" and it is an herbaceous plant belongs to the family *Asteraceae* (*Compositae*). This family is reported to have its origin from Mediterranean and West Asia while marigold has been also originated fsrom Southern Europe and East Mediterranean [1]. Marigold (*Calendula officinalis* L.) was used to grow as major ornamental flowering crop until its bioactive as well as medicinally important compounds were recognised. Oil extracted from this plant also has its pharmaceutical significance [2], [3]. Anti-viral and antimicrobial potential of marigold is also reported [4]. This horticultural crop is threatened by different soil borne and air borne plant pathogens.

Leaf spot and flower blight caused by *Alternaria* sp Pape, have been observed in causing heavy losses among common cultivars of both African and French marigold in the areas. *Alternaria* spp. are mould fungi and present in soils, plants, food and indoor air. The genus *Alternaria* consist of both saprobes and plant pathogens which have been described worldwide infecting crops in the field and causing post-harvest decline of many plant products [5]. For controlling and curing of injuries to diseased plants many medicinal plants and their extracts have been used other than fungicides. Curative mushrooms are also significant with respect to new drug and pharmacological research growth. These mushrooms are still unidentified biological assets to use as agricultural chemicals. *Ganoderma* species, specifically *Ganoderma lucidum* is vastly classified medicinal mushroom in oriental traditional medicine that has been extensively used for the treatment of chronic infections of numerous etiologies [6]. Due to its capability of medication among various diverse infections it acknowledged terms like “Elixir of life", “Food of Gods", “Mushroom of the Universe". Its intracellular and extracellular polysaccharides showed inhibition of the growth of several types of cancer cells [7], [8]. *G. lucidum* extract has been described dynamic against *Bacillus subtilis* and *Pseudomonas syringes* which are plant pathogens [9]. It also has been tested against *Aspergillus niger, Curvularia lunata* and *Drashlera sp.* [10].

The present work is focused on the isolation of the pathogens involved in wilting in *C. officinales* and to check the antifungal potential of methanol, acetone and aqueous extract of *G. lucidum* against fungi isolated from *C. officinales*.

II. Materials & Methods

A. Collection of Infected Calendula officinalis

Infected Calendula officinalis plants were collected from the Jinnah Garden, Lahore with the courtesy of Parks and Horticulture Authority (PHA), Lahore, Government of the Punjab. Plant samples with infected roots as well as floral parts were collected on the basis of their symptoms. Along with the plants, adhered soil was also collected. Rhizospheric soil was collected by digging 6cm deep in the soil and about 1kg of soil was collected in sterilized polythene bags. These specimens were brought to PARB project no. 437 Laboratory at Institute of Agricultural Sciences, University of the Punjab, Lahore and processed accordingly.

B. Isolation of Pathogenic Fungi from Infected Roots and Leaves

Infected roots and leaves were cut into 1 cm small pieces and surface sterilized with 5 % NaOCl solution for two minutes. Pieces were then washed with distilled water for one minute and dried over a blotting paper. Plates were prepared containing Potato Dextrose Agar (PDA) and pieces of roots and leaves were then inoculated on media plates under a-septic conditions. Plates were then incubated at 28oC for four to five days.

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C. Microscopy

As there were two kinds of fungi observed on the plates which were inoculated with infected leaves and roots, that's why they were studies under microscope separately. Slides were prepared by using Trypan blue as stain and the slides were carefully observed under Binocular microscope at different lens powers such as 10X, 40X and 100X.

D. Collection of Ganoderma lucidum

Ganoderma lucidum samples were collected from the field near STC (Student Teacher Center), Botanical Garden and PU Graveyard in Quaid-e-Azam Campus, University of the Punjab, Lahore. The main hosts were Eucalyptus citriodora, Dalbergia sisso, Acacia Arabica. The mushroom basidiocarps were identified by their physical characteristics. Specimens collected from above mentioned places were brought to laboratory and rinsed with tap water. Furthermore, dried under heater fan at 40oc for 2 hours and then preserved in freezer at -200C to use for further processing.

E. Organic and aqueous extraction of Ganoderma lucidum

The anti-fungal compounds were extracted from G. lucidum with aqueous and organic solvents, in order to separate the chemical constituents. The solvents used in making extract were acetone, methanol and water. To make 20% stock solution, twenty grams of chopped G. lucidum was soaked in 100 ml of methanol, acetone and water for 24hrs by keeping in shaker at 100 rpm. Extract was filtered through a double layered muslin cloth followed by Whatman No. 1 filter paper. The crude extracts were dissolved in dimethyl sulfoxide (DMSO) to equal 100 mL as stock solution. The lower concentrations of 5, 10, and 15% were prepared by adding appropriate quantity of DMSO into the 20% stock solution [11]

F. Antifungal Assays of *G. lucidum* extracts

G. lucidum extracts gotten from different solvents (i.e. acetone, methanol, aqueous) were applied in various concentrations on fungal cultures isolated from marigold to test the antifungal activity. The experiments were done in triplicate. The methods used to test the antifungal activity of Ganoderma lucidum extract were in three types.

G. Vapors diffusion assay

Petri dishes containing PDA were point inoculated with the investigated fungi. In the lid of the petri dish a sterile filter paper was placed and 200ul of various percentage solutions of methanol, acetone and aqueous extracts were dropped to the filter paper. Pathogenic fungi were inoculated into the wells prepared on medium. Petri dishes were sealed with parafilm and incubated in reversed position for 3 days at 28oc. Colony diameters were measured [12].

H. Well diffusion method

Wells were made on the agar surface with 8mm cork borer. 200µl extracts from each prepared percentage solution was poured into the well using sterile micro pipette. The pathogenic fungal spores were spread to the whole plate. The plates were pragmatic for the zone development around the wells. The percentage of inhibition zone was calculated [13].

I. Agar absorption assay

This method was opposite to the well diffusion method. 200ul of prepared solutions were spread with glass made spreader to the whole plate. The fungal culture plates were inoculated in 8mm prepared well. The percentage of inhibition was calculated [14]

J. Data calculation

Treated (*T*) and control (*C*) Petri dishes were measured diametrically in three different directions till the fungal growth in the control dishes was nearly far-reaching. The percentage of growth inhibition (*I*) was calculated using the formula [15]

$$I\ (\%) = [(C - T)/C] \times 100$$

K. Statistics

All the experiments were done in triplicates and the results are expressed in mean values and standard deviation (SD).

III. Results

A. Morphological study of the infected root and leaves samples

Samples were observed carefully on the basis of their symptoms and it was observed that stems were wilted from the root to the apex. All the infected plant samples were having the similar symptoms which are usually caused by the soil borne pathogens. Roots were also having a stunted growth due to fungal infection and root system was not much developed which are the indications of having the attack of *Fusarium oxysporum* which is a soil borne pathogens and responsible for causing wilt disease in a great number of hosts. Leaves were deformed and of brown to dark brown colour at the apex Fig. 1.

Fig. 1: Infected stem and leaves of *C. officinales* showing the symptoms of Blossom Blight

B. Isolation of Pathogenic Fungi from Infected Roots and Leaves

After four days whitish cottony mycelium was observed on the infected roots which were inoculated on PDA medium. Mycelium was observed to grow within the root tissues. That's why this mycelium was supposed to be the pathogenic fungi and processed further for microscopy and identification. After three to four days dark brown to black mycelium growth was observed on the surface of infected leaves which were previously inoculated on PDA plates. Mycelium growth was fast with a fluffy appearance. At the same time another type of mycelium was also observed which were growing from the tissues of infected leaves. This mycelium was of dark grey to brown in colour with a mild growth Fig. 2.

Fig. 2: Fungal growth on infected leaves *C. officinales* inoculated on PDA plates

C. Microscopic Study

Under the microscope, septate, hyaline and slightly branched hyphae were observed. Conidia are sickle shaped and occasionally found in chain at a monophyloid conidiophore. The fungus was identified as *Fusarium oxysporum* which is responsible for permanent wilting disease in Marigold and other ornamental as well as agricultural crops. Under the microscope hyphae from the black culture were observed to be septate with light brown to brown conidia. Conidia were horizontally septate. The fungus was identified as *Alternaria alternata* which is responsible for the Leaf spot disease in Marigold Fig.3.

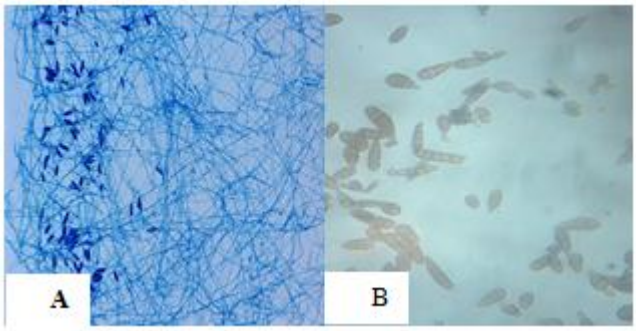

Fig. 3: A. Myeclia and conidia of *Fusarium oxysporum*
B. Conidia of *Alterneria alternata*

D. Antifungal Assays of Ganoderma lucidum extracts

Result for antifungal potential of various concentrations (5%, 10%, 15%, and 20%) of *G. lucidum* was determined by vapors diffusion assay, agar well diffusion and agar absorption assay. Methanol, acetone and aqueous extracts of *G. lucidum* were found as the effective and remarkable antifungal agent when tested against a panel of plant pathogenic fungi. In experiments antifungal potential of various extracts were increasing with the rise of percentage.

In vapours diffusion assay it was observed that all the extracts (methanolic, acetone and aqueous) demonstrated various degrees of antifungal activities. When isolated fungi were treated with the 20% of methanolic extract, the highest antifungal activity was demonstrated against *F. oxysporum* 57% and the inhibitory growth of *A. alternata* was reduced up to 52% as compared to control Fig. 4(a). Acetone extract demonstrated 52% inhibition against *A. alternata* and 49% against *F. oxysporum* at 20% concentration Fig. 4 (b). With the usage of aqueous extract, *F. oxysporum* showed 40% inhibitory effect and *A. alternata* demonstrated 33% in hibition Fig. 4 (c).

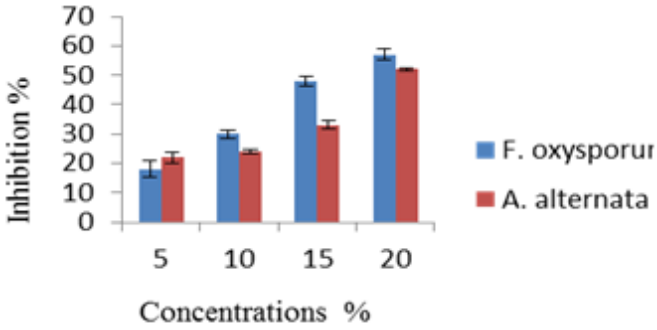

Fig. 4 (a): Evaluation of antifungal activity of Methanolic extract of *G. lucidum* by vapors diffusion assay

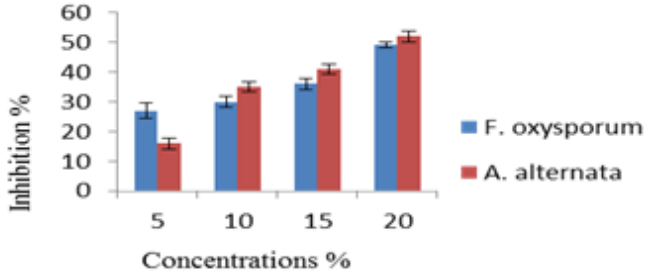

Fig. 4 (b): Evaluation of antifungal activity of Acetone extract of *G. lucidum* by vapors diffusion assay

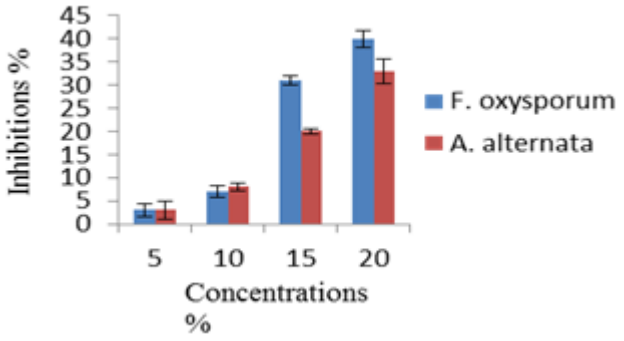

Fig. 4 (c): Evaluation of antifungal activity of aqueous extract of *G. lucidum* by vapors diffusion assay

In agar absorption assay methanolic extract showed maximum inhibitory growth against *F. oxysporum* 64% inhibitory effect and *A. alternata* had 47% inhibitory growth at 20% concentration as compared to control. Acetone extract showed 57% inhibition against *F. oxysporum* and *A. alternata* showed 50% inhibitory growth. Aqueous extract were also showing its inhibition 47% against *F. oxysporum* and 43% against *A. alternata* at 20% concentration Fig. 5 (a,b,c).

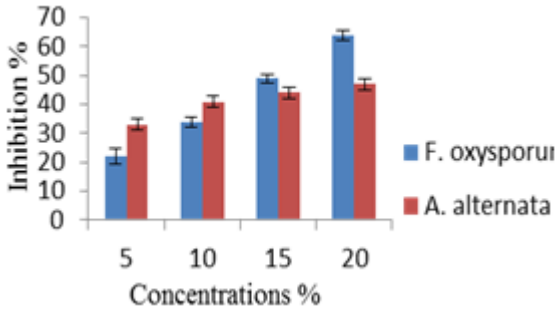

Fig. 5 (a): Evaluation of antifungal activity of Methanol extract of *G. lucidum* by Agar Absorption assay

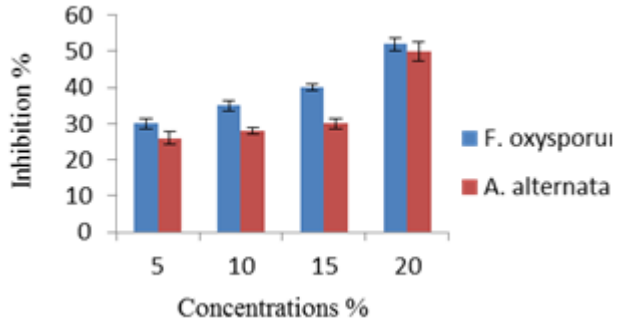

Fig. 5 (b): Evaluation of antifungal activity of Acetone extract of *G. lucidum* by Agar Absorption assay

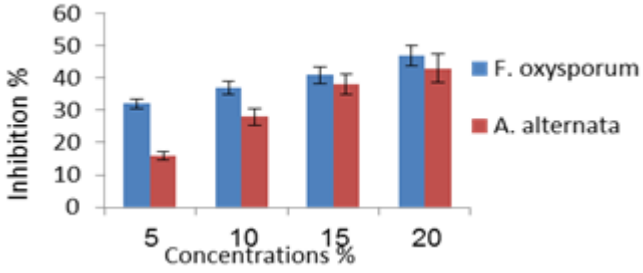

Fig. 5 (c): Evaluation of antifungal activity of aqueous extract of *G. lucidum* by Agar Absorption assay

With the agar well diffusion method *A. alternata* demonstrated 49% and *F. oxysporum* reduced its zone at 38% with 20% methanol extract. With acetone extract of *G. lucidum* the highest zone of inhibition was demonstrated by *A. alternata* 64% with 20% concentration. While in aqueous extract of agar well diffusion method *A. alternata* demonstrated best inhibition zone of 64% with the concentration of 20% Fig. 6(a,b,c).

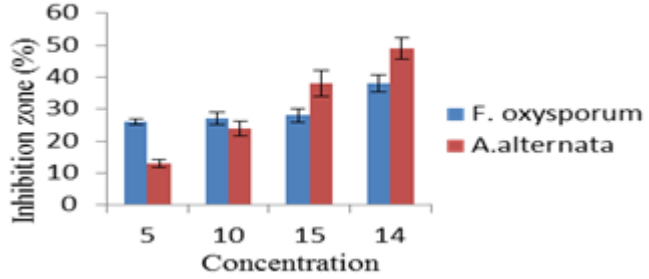

Fig. 6 (a): Evaluation of antifungal activity of Methanolic extract of *G. lucidum* by Agar Well Diffusion Method

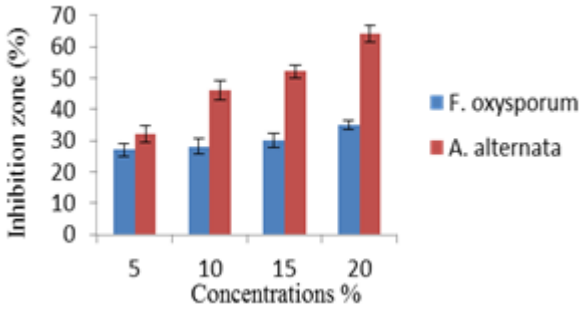

Fig. 6 (b): Evaluation of antifungal activity of Acetone extract of *G. lucidum* by Agar Well Diffusion Method

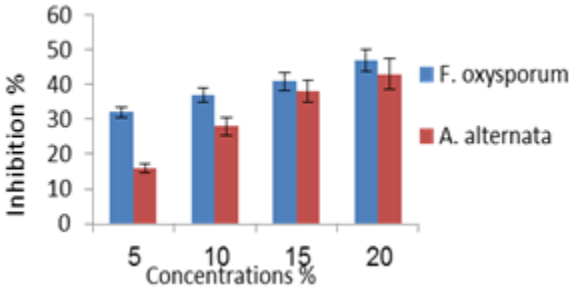

Fig. 6 (c). Evaluation of antifungal activity of Aqueous extract of *G. lucidum* by Agar Well Diffusion Method

IV. Discussion

During the detailed survey which was conducted to collect the samples of infected *Calendula officinalis* (Marigold) the plants with stunted growth, floral blackening, and discoloration of leaves and deformation of the roots were observed and collected Fig. 1. Similar symptoms were observed in *Fusarium* wilt of banana [16], whereas deformation, withering and blackening of floral apex and leaves in leaf spot and blight diseases of marigold were examined [17]. Infected samples were inoculated on PDA plates after disinfecting the surface contaminants and also to let the tissue invading mycelium to sprout on the plate. After incubation whitish mycelium growth was observed which was later identified as *Fusarium oxysporum*. As this fungus is soil borne and have ability to invade vascular bundle of the host plant, therefore, the symptoms observed were confirmed. *F. oxysporum* was confined the vascular tissues [18]. The pathogen sporulates further to parenchymatous tissues after the plant dies and become visible by disrupting the root epidermis Fig. 2. From the inoculated leaves heavy infestation of dark brown to black mycelium was observed, it was observed to have comparatively fast growth than *F.oxysporum* Fig. 2. Dark brown to black, fast and fluffy mycelial growth was observed on leaves [19]. Mycelial morphology was discussed while studying *F. oxysporum* and *A. alternata* [20]. In antifungal activity, acetone extract had the more potential, but potential of methanol extract was also remarkable. The aqueous extract displayed least antifungal activity than the methanol and acetone extracts. *Ganoderma lucidum* has been used as biocontrol agent. Acetone extract (1000mg/ml) possess strong

antifungal activity against F. *oxysporum* and *C. lunata*. All the values of inhibition increased with the increasing concentrations of every extract [21]. The most active compounds are commonly insoluble in water, so it is projected that low polarity organic solvents would produce best energetic extracts [22]. The potential of aqueous extracts from G. lucidum were observed and found the inhibitory action of the extracts [23]. Methanol extract of *G. lucidum* displayed significant activity against fungi [24]. Some Phytochemicals are more soluble in alcohol than in water [25]. Because of strong molecular configuration acetone was better than methanol extract. The high level of effectiveness of acetone as solvent of extraction could be linked with higher concentration of metabolites extracted.

V. Conclusion

It was concluded from the whole research work that spreading technique had shown the best inhibitory potential against different plant pathogenic fungi as compared to vapor assays and agar well diffusion methods. Similarly, among organic and aqueous extracts the acetone extract provided the highest growth inhibition. In all techniques, all extracts were showing their maximum inhibition at 20% concentration while the inhibition was increasing by increasing the concentration from 5-20%. So, in a nut shell there is a dire need to explore the science of organic pesticides in some more dimensions in order to find some more viable, cost effective and environmental friendly suggestions as well as strategies.

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