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Exploring the Phytochemicals of *Delphinium ajacis* and their Applications in Biocontrol Activity against Some Plant Pathogens

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ABSTRACT

The diversity of phytochemicals could serve as a source of useful drugs against many plant diseases. The present study deals with phytochemical analysis of larkspur (Delphinium ajacis) and their antimicrobial activity against Colletotrichum gloeosporioides, Alternaria solani, Pyricularia oryzae and Rhizoctonia solani. In this paper, the dried stems, leaves and flowers of Delphinium ajacis were extracted using standard assays to evaluate the phytochemical constitution. Qualitative analysis of phytochemical constituents was performed by the well-known tests protocol available. The phytochemical screening revealed the extract richness in alkaloids, tannins, terpenoids, phenols and saponins. The study also includes preparation of different extracts by successive solvent extraction for detail analysis. Fluorescence analysis of these extracts were also noted under ordinary and UV light to signify their characteristics. The in vitro antimicrobial activities were also investigated by using disc diffusion methods. The leaf extract exhibit marked antimicrobial activity against plant pathogens. It may lead to development of potentially effective and environmental friendly alternative chemicals to control plant diseases.

Keywords: *Delphinium ajacis;* Phytochemicals; Antimicrobial activity; Alternaria solani; Pyricularia oryzae; Rhizoctonia solani; Colletotrichum gloeosporioides.

INTRODUCTION

From ancient times, plants from the genus *Delphinium* have been used not only for medicinal purposes but also used as insecticide and poison. These activities are linked to the phytochemicals present in these plants [1] and these substances are of great interest because of their versatile applications. *Delphinium ajacis* is an annual flowering plant of the family *Ranunculaceae*. It is frequently grown in gardens for its spikes of blue, pink or white flowers [2]. Almost all parts of the plants contain these bioactive compounds, although the amount present varies [3]. These chemical compounds after undergoing limited safety studies are proved to be used as a drug for therapeutic purpose. Plants contain many active compounds such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids [4-10]. They are present in leaves, flowers, bark, seeds, fruits, root, etc [10-13]. The analysis of the compounds revealed the presence of alkaloids, flavonoids and phenols in large amount whereas phytosterols and saponins are present in smaller quantities. These phytochemicals in various combinations are proved to have beneficial medicinal effects but with a focus on activity against human diseases [14-17].

Recently these phytochemicals have received attention for controlling plant diseases and reported to show inhibitory effects against many phytopathogens due to the presence of effective chemotherapeutants, which can prove to be a valuable sources of natural pesticides [18-24]. It was reported that the inhibitory effect of the plant extracts may be due to the hydrophobic nature of the plant extracts and their constituents, and was suggested that the antimicrobial components of the plant extracts cross the cell membrane by interacting with the enzymes

and proteins of the membrane, so producing a flux of protons towards the cell exterior which induces change in the cell and ultimately their death [25,26].
Synthetic chemical pesticides are known to be the most effective way to control plant pathogens, to improve the quality and quantity of the plant and their products, but they are not considered as long-term solution as they are not eco-friendly and have toxic effects on the environment, soil, plant, beneficial microbes present in soil [27] and adversely effects the human and animal health [28,29]. Extracts of many higher plants have been reported to exhibit antifungal properties under laboratory trails [30-33].
Hence the plant metabolites and the plant based pesticides can prove to be better alternatives as compared to the synthetic pesticides as they show no residual toxicity in soil and plant products. This prompted us to explore and study some plants for their phytochemicals for controlling phytopathogens from the surrounding areas of our locality. In this paper, larkspur (*Delphinium ajacis)* was selected for its application as pesticides, thus evolving environmentally safe and alternative methods to control plant diseases.

MATERIALS AND METHODS

Plant material

The plants *Delphinium ajacis* were collected from pots planted in the campus of Guru Nanak Girls College, Yamuna Nagar situated in the northern India and nearby hilly and plane areas in the month of January-March. The plant was identified taxonomically. The leaves, stems and flowers were thoroughly washed with tap water to remove soil particles and then shade dried at room temperature. Plant material was milled to make fine powder and stored in air tight containers.

Soxhlet Extraction

The plant extract were prepared by refluxing the dried and powdered plant parts of the *Delphinium ajacis* for 72 hrs, by hot continuous percolation method using Soxhlet apparatus. The plant extract were prepared in different solvent of varied polarity.
The extracts were concentrated to a dry mass under the reduced pressure using rota-evaporator at 40^{o}C and the residues were stored (Amarasingham et al., 1964).The qualitative phytochemical and secondary metabolite screening were carried out and studied the antimicrobial activity of the prepared plant part extract. The preliminary qualitative phytochemical analysis revealed that methanol extracts exhibited maximum diversity of chemical constituents being a bipolar solvent and had high % yield of the extract. The % yield of the plant part extract of *Delphinium ajacis* prepared in methanol is tabulated in Table 1.

Table 1: The ethnobotanical data of the plant parts employed and extract percentage yield

Plant species	Family	Plant Part	Yield extract (%)
Delphinium ajacis	*Ranunculaceae*	Leaves	11.55
		Stem	10.75
		Flowers	7.69

Phytochemical isolation

Different qualitative chemical tests can be performed for the chemical composition of the extract [34-36] . The following tests were performed on extracts prepared for qualitative analysis of phytochemicals present in them:

Detection of Alkaloids

50 mg extract stirred with dilute hydrochloric acid and filtered. The filtrate is tested carefully with various alkaloidal reagents are:

Mayer's test: To the filtrate, a drop or two of Mayer's reagent are added by the side of the test tube. A white or creamy precipitate indicates the presence of alkaloids.

Wagner's test: To the filtrate, 1 or 2 ml of Wagner's reagent are added by the side of the test tube. A reddish-brown precipitate confirms the presence of alkaloids.

Dragendorff's test: To the filtrate, 1 or 2 ml of Dragendorff's reagent are added. A prominent yellow precipitate indicates presence of alkaloids.

Detection of Carbohydrates [37]

The extract (100 mg) is dissolved in 5 ml of water and filtered. The filtrate is subjected to the following tests:

Molisch's test: To 2 ml of filtrate, two drops of alcoholic solution of α-naphthol added, the mixture is shaken well and 1 ml of concentrated sulphuric acid is added slowly along the sides of the test tube and allowed to stand. A violet ring indicates the presence of carbohydrates.

Fehling's test: One ml of filtrate is boiled on water bath with 1ml each of Fehling solutions A and B. A red precipitate indicates the presence of sugar.

Barfoed's test: To 1 ml of filtrate, 1ml of Barfoed's reagent is added and heated on a boiling water bath for 2 min. Red precipitate indicates presence of sugar.

Benedict's test: To 1 ml of filtrate, 1 ml of Benedict's reagent is added. The mixture is heated on a boiling water bath for 2 min. A characteristic red precipitate coloured precipitate indicates the presence of sugar.

Detection of Glycosides

Borntrager's test: 50 mg of extract is hydrolysed with concentrated H_2SO_4, heat for 5 min and filtered. To 2 ml of filtered hydro-lysate, 3 ml of chloroform is added and shaken, chloroform layer is separated and 10% ammonia solution is added to it. Rose pink colour in the ammonical layer indicates the presence of glycosides.

Detection of Saponins [34]

The extract (50 mg) is diluted with distilled water and made up to 20 ml. The suspension is shaken in a graduated cylinder for 15 min. A 2 cm layer of foam indicates the presence of saponins.

Detection of Proteins and Amino acids [38,39]

The extract (100 mg) is dissolved in 10 ml of distilled water and filtered through whatman no.1 filter paper and the filtrate is subjected to tests for proteins and amino acids.

Millon's test [40]: To 2 ml of filtrate, 1 ml of Millon's reagent is added. A white precipitate indicates the presence of proteins.

Biuret test: An aliquot of 2 ml filtrate is treated with one drop of 2 % copper sulphate solution. To this, 1 ml of ethanol (95%) is added, followed by excess of potassium hydroxide pellets. Pink colour in the ethanolic layer indicates the presence of proteins.

Ninhydrin test [41]: Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) are added to 2 ml of aqueous filtrate. A characteristic purple colour indicates the presence of amino acids.

Detection of fixed Oils and Fats [34]

Spot test: A small quantity of extract is pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oil.

Saponification test: A few drops of 0.5 N alcoholic potassium hydroxide solution is added to a small quantity of extract along with a drop of phenolphthalein. The mixture is heated on water bath for 2 h. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

Detection of Phenolic compounds

Ferric chloride test: The extract (50 mg) is dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution is added. A dark green colour indicates the presence of phenolic compounds.

Lead acetate test: The extract (50 mg) is dissolved in distilled water and to this, 3 ml of 10% lead acetate solution is added. A bulky white precipitate indicates the presence of phenolic compounds.

Detection of Tannins

1 ml of the extract was added in 2 ml of water in a test tube. 2 to 3 drops of diluted ferric chloride solution was added and observed for green to blue-green (cathechic tannins) or a blue-black (gallic tannins) coloration.

Test for flavonoids [42]

Dilute ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 ml) was added. A yellow coloration that disappears on standing indicates the presence of flavonoids.

Detection of Terpenoids [43]

Salkowski test: To 0.5 g each of the extract was added 2 ml of chloroform. Concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Detection of Steroids

2 ml of acetic anhydride was added to 0.5 g of the extract of each with 2 ml of H_2SO_4. The colour changed from violet to blue or green in some samples indicates the presence of steroids.

Preliminary phytochemical analysis of extract of leaf, stem and flower of *Delphinium ajacis* in different solvent of varied polarity tabulated in Table 2, 3 and 4. Fluorescence analysis of these extracts noted under ordinary and UV light to signify their characteristics tabulated in Table 5.

Antifungal activity

Test Microorganisms: Cultures of different microbes were obtained Indian Agricultural Research Institute (IARI), New Delhi. All the isolates were checked for purity and were maintained on their respective media as per guidelines of IARI. Test microbes employed were:

Fungal pathogens

(i) *Alternaria solani* (ATCC 4632)
(ii) *Rhizoctonia* solani (ATCC 6841).
(iii) *Colletotrichum gloeosporioides* (ATCC 6848)
(iv) Pyricularia oryzae (ATCC 7019)

Assay of antifungal activity

Antifungal activity of plant extracts were carried out against fungal pathogens *Alternaria solani, Rhizoctonia solani, Colletotrichum gloeosporioides and Pyricularia oryzae*. The stock cultures of fungal pathogens were revived by inoculating in potato dextrose agar medium and grown at 25^{o}C for 5-7days. The plant extract dissolved in methanol, sterilized in disposable millipore filter (0.22 µm pores) and mixed with sterile potato dextrose agar (PDA) medium to obtain the final concentration of 10 mg/ml of each plant extract and then poured in sterile petri dishes (60 mm diameter). The media loaded by methanol was considered as a control. Allowed it to solidify and then discs of 6mm diameter plug cut from 7 days old fungal culture of the test organisms were placed at the centre of the amended agar plates. Each treatment was replicated four times. Plates were incubated at 25 ± 2°C for 7 days and diameter of the fungal colony in petri plates with extract and control was measured and percentage of mycelial inhibition was calculated using the following formula:

$$\text{Percentage of mycelial inhibition} = [C - T / C] \times 100$$

where, C and T are the growth diameter (mm) in control and treatment, respectively and data of the mean diameter of the mycelia growth and % of mycelial growth inhibition is tabulated in Table 6.

Different concentrations of the each plant part extract (0, 2, 4, 8 and 16 mg/ml) were prepared and sterilized in disposable Millipore filters (0.22 µm pores). The PDA was sterilized and different concentrations of the plant extracts were amended into it when the temperature of the medium decreased to below 40°C. The amended medium was allowed to solidify. PDA media loaded by methanol was considered as a control. To compare efficacy of plant extracts with that of fungicide (carbendazim) in controlling phytopathogenic fungi, different concentrations (0, 2, 4, 8 and 16 ppm) of carbendazim were prepared by mixing weighted fungicide with a known volume of sterile (PDA) and then poured in sterile petri dishes (60 mm diameter) and allowed to solidify. A fungal disc of 6 mm placed at the center of Petri dish in potato dextrose agar medium with plant extracts of various concentrations and fungicide. The cultures were incubated at 25 ± 2°C and the diameter of colony was measured after 6 days. Fungicidal effect of the plant extracts was measured and MIC as well as MFC were determined and then compared with fungicidal effect of the reference fungicide with four replications.

RESULTS

The methanolic extract of plant part extract of *Delphinium ajacis* (leaves stem and flowers) were studied and evaluated the antifungal activities against phytopathogenic fungi *Alternaria solani, Rhizoctonia solani, Colletotrichum gloeosporioides and Pyricularia oryzae.* The plant part extracts were effective at 10 mg/ml in inhibiting the growth of the fungal colony as compared to control. Assays showed that plant extract provide a significant inhibition to mycelia growth of the phyto-pathogenic fungi and their sensitivity to a given plant part extract varied greatly. *The* extract of *Delphinium ajacis* leaves showed complete inhibition on colony growth of *Pyricularia oryzae*, followed by inhibition on colony growth of *Colletotrichum gloeosporioides*, which was

slightly less than complete inhibition whereas low inhibition was observed against *Rhizoctonia solani* and *Alternaria solani. Delphinium ajacis* stem extract showed complete inhibition on colony growth of *Colletotrichum gloeosporioides, Pyricularia oryzae* and Rhizoctonia *solani, followed by inhibition on colony growth of Alternaria solani. Delphinium ajacis* flowers extract completely reduced the growth of plant pathogenic fungi Colletotrichum *gloeosporioides, followed by Pyricularia oryzae* and least inhibition observed against *Alternaria solani*.

MIC and MFC of plant extracts compared with carbendazim as a reference fungicide to study the fungicidal nature of the extract prepared. Carbendazim, an effective fungicide showed complete inhibition to the mycelial growth of Alternaria *solani, Rhizoctonia solani, Colletotrichum gloeosporioides and Pyricularia oryzae*. *Pyricularia oryzae* was more sensitive to carbendazim whereas *Rhizoctonia* solani were least sensitive at the same concentration. The concentration effect of the reference fungicide carbendazim on the mycelial growth is presented in Figure 1 and result are shown in Table 7 where the inhibitory effect started at 2 ppm and increased in proportion to carbendazim concentration and reached to maximum of complete inhibition. Similarly with the increase in concentration of the plant part extract, increase in the inhibition in the mycelial growth observed. The concentration effect of extract of leaves, stem and flowers of *Delphinium ajacis* on the mycelial growth is shown in Figure 2, 3 and 4 and also reported in Table 8.

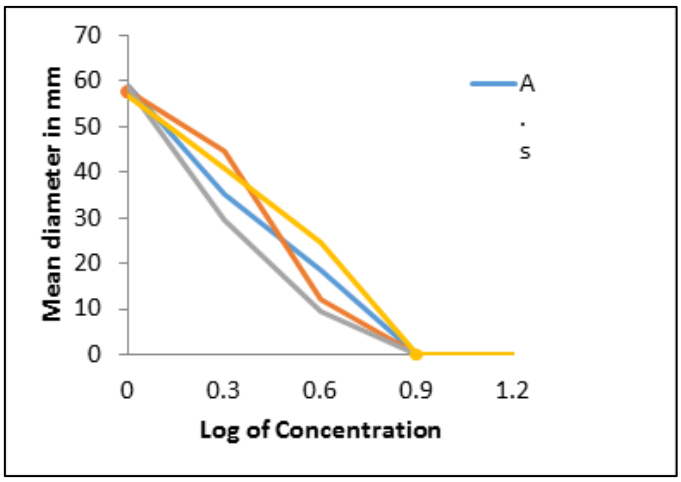

Figure 1: Effect of concentrations of reference fungicide (Carbendazim) on mycelia growth of fungi: *A.s (Alternaria solani); C.g (Colletotrichum gloeosporioides); P.o (Pyricularia oryzae); R.s (Rhizoctonia solani)*

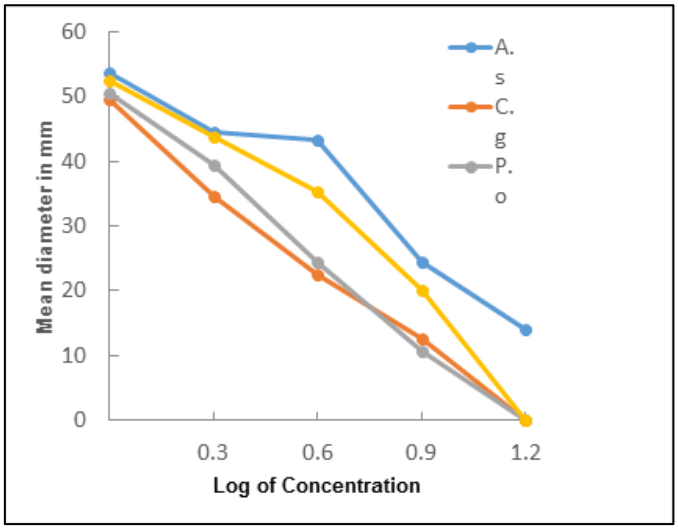

Figure 2: Effect of concentration of leaves methanolic extract on mycelia growth of fungi: *A.s (Alternaria solani); C.g (Colletotrichum gloeosporioides); P.o (Pyricularia oryzae); R.s (Rhizoctonia solani)*

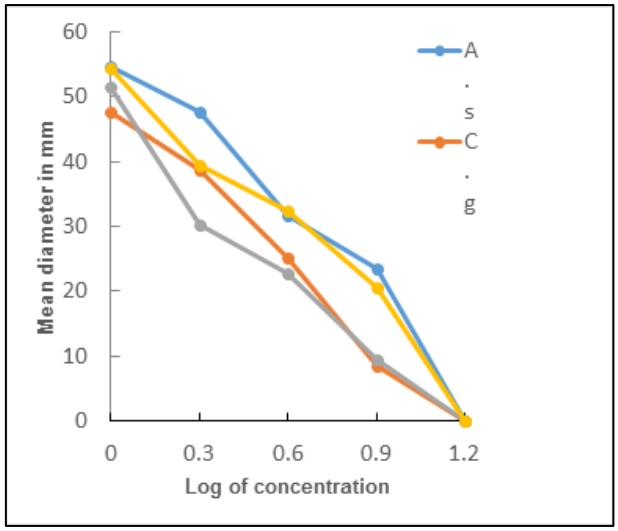

Figure 3: Effect of concentration of stem (methanolic) extract on mycelia growth of fungi: *A.s (Alternaria solani); C.g (Colletotrichum gloeosporioides); P.o (Pyricularia oryzae); R.s (Rhizoctonia solani)*

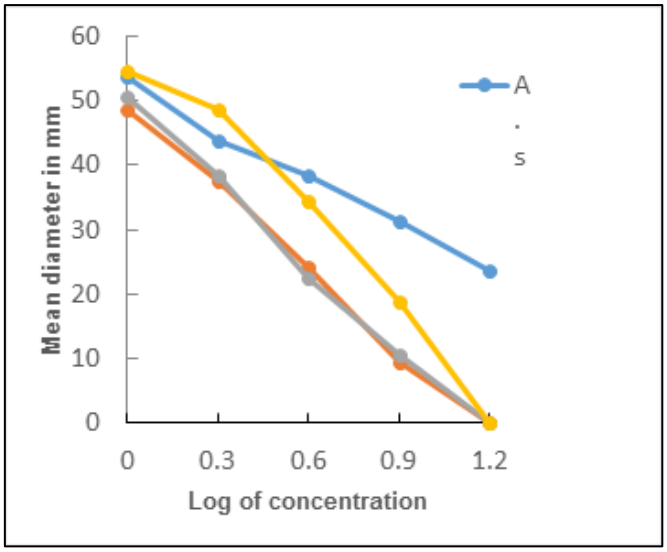

Figure 4: Effect of concentration of flowers (methanolic) extract on mycelia growth of fungi: A.s (*Alternaria solani*); C.g (*Colletotrichum gloeosporioides*); P.o (*Pyricularia oryzae*); R.s (*Rhizoctonia solani*)

DISCUSSION

Plant diseases have a great influence on humans, as humans depends for food, clothing pharmaceutical, household products and many more, on plants and their products. Pathogenic fungi ranked second to insects are the major cause of plant diseases, which result in heavy loss of plant products. To prevent the decrease in the yield of the crops and major plant products, from plant diseases, variety of control measures are presently in use. The chemical compounds are the highly effective and most commonly used means for controlling the plant diseases. The use of chemicals has been found to be very effective in controlling plant fungal diseases but its continued application results in increase of toxicity level in the soil, environment, and plant products which affects the human health and even destroys the beneficial micro-organisms present in the soil. To overcome the adverse effects of synthetic chemicals on the environment, plant products and human health, there is a need to search for new and safer pesticides which are eco-friendly and effective in nature. Results from the study indicates that plant parts (leaves, stem , and flower) of *Delphinium ajacis*, showed inhibition on the growth of phyto-pathogens such as *Colletotrichum gloeosporioides , Alternaria solani, Pyricularia oryzae and Rhizoctonia solani*. Some plant part showed complete inhibition, some showed lesser inhibition, but all showed inhibition. The study of MIC and MFC of the fungi toxicants as plant part extract in comparison with reference fungicide done to evaluate their efficacy of suppressing the mycelia growth of the phyto-pathogenic fungi. It

was observed, that when carbendazim, an effective fungicide used for suppressing the growth of phyto-pathogenic fungi was compared with the extracts of different plant parts of *Delphinium ajacis* on mycelia growth of the phytopathgenic fungi, carbendazim completely inhibited the mycelia growth at 8 ppm while the diverse plant extract showed complete or effective inhibition at a higher concentration of plant extracts in order to attain the same effect. Although, inhibition against fungus by plant extract showed at a higher concentration than the reference fungicide but being eco-friendly, plant extracts application can be seen as an effective bio controlling agents in controlling mycelia growth. Hence the present study indicates that the plant extracts of diverse plant parts can be an alternative method of utilizing the plant extract as a natural source of antimicrobial agent, which are safe, involving the use of natural product and phytochemicals to control plant diseases, without leaving adverse effect in the biosphere.

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