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MOLECULAR DOCKING AND INHIBITION STUDIES ON THE INTERACTION OF *PROSOPIS JULIFLORA* ALKALOIDS AGAINST *FUSARIUM SOLANI* (MART.) SACC.

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Abstract- The alkaloid fraction of leaves of *Prosopis juliflora* were isolated by acid-base fractionation. The antifusarial activity of the alkaloid fractions against *Fusarium solani* were evaluated by disc diffusion assay and minimal inhibitory concentration (MIC). The effect of the active alkaloid fraction on *F. solani* mycelium and conidia was studied using mycelium growth inhibition assay, biomass production, release of cellular material, spore germination assay, light microscopy and scanning electron microscopy. The total alkaloid fraction subjected to TLC eluted 4 bands with Rf values of 0.52, 0.6, 0.81, 0.84. All the bands were subjected to antifusarial activity. Band II (Rf value 0.6) showed significant antifusarial activity with zone of inhibition of 39.3 mm and MIC of 40 µg/ml against *F. solani*. The LC-MS analysis of Band II indicated the presence of Juliprosopine and Prosopine. At 50 µg/ml concentration, the active alkaloid fraction showed significant reduction in mycelial growth, biomass production and spore germination which was confirmed by microscopic studies. The active alkaloid fraction also effected the fungal cell wall leading to the leakage of cellular material. Molecular docking using ligand fit protocol with Autodock tool was carried out to understand the interaction of β-glucosidase of *F. solani* with active alkaloid fraction to propose the possible mechanism of action for the antifusarial activity. The ligands Juliprosopine and Prosopine showed hydrogen bond interaction with active sites of the protein at minimum binding energy. The present study indicates the strong inhibition potential of the active alkaloid fraction against *F. solani*.

Keywords- *Prosopis juliflora*, Juliprosopine, Prosopine Anti-fusarial activity, *Fusarium solani*, Molecular docking.

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Introduction

Fusarium solani (Mart.) Sacc. (Teleomorph = *Nectria haematococca* (Berk. & Br.)) is an important phytopathogenic fungus which is known to cause several plant diseases like root, crown and fruit rot of *Cucurbita* sp., root and stem rot of pea, sudden death syndrome of soybean, foot rot of bean, dry rot of potato, corn rot, damping-off, surface rot in many seeds and deterioration of several storage grains like Sorghum, Maize, Millets [1, 30]. *Fusarium solani* (*F. solani*) is seed-borne, both internal and external, survive more than 1-2 years in seed and causes significant reduction in seed germination. Post-harvest losses due to development of *F. solani* during storage and distribution of harvested fruits and vegetables is very high. Some strains of *F. solani* also cause infections in humans [2].

Disease management practices are in vogue to control diseases caused by *F. solani*. Carbendazim (Commercial name: Bavistin) is widely used for the management of *Fusarium* diseases [3]. But in recent years, there is a development of resistant pathogens to these chemical fungicides and there is a growing concern on the effect of these fungicide on non-target species [4]. Thus, biological control utilizing natural products is an important alternative management strategies. Hence large number of workers are currently working on the utilization of secondary metabolites of plant origin for plant disease management. Bioactive compounds from many of the medicinal plants have been found to be effective against plant pathogenic fungi in general and *F. solani* in particular. The plants showing significant inhibitory activity against *F. solani* are *Azardirachta indica*, *Artemissia annua*, *Rheum emodi, Eucalyptus globulus, Ocimum sanctum*, Chili, Lantana, Lemon grass and Onion seeds [3, 5].

Beta-glucosidase is a glycoside hydrolase 3 (GH3) enzymes present in *F. solani* and is related with the synthesis of cell wall [6]. Thus, it is an attractive target for the development of selective inhibitors/ antifungal agents against β-glucosidase to control the *Fusarium* diseases in plants. Computational techniques are the vital processes to understand interaction between the fungi protein and antifungal compound isolated from *P. juliflora*. *In silico* screening of antifungal compound help a lot for reducing the number of candidate molecules for synthesis and experiments [7].

In the present study, *Prosopis juliflora* an invasive weed plant with unique biology was selected for the management of *F. solani*. The plant is rich in large number of secondary metabolites, which are produced from different parts of the plant. The alkaloids of *P. juliflora* is reported to possess good antimicrobial activity against a number of plant and human pathogens [8, 9].

Antifungal and antibacterial potential of the alkaloid fraction of the leaves of *P. juliflora* against important pathogens has been reported and in some cases its potential for the management of some seed borne pathogens has been established [9, 10]. However, the efficacy of alkaloid fraction of *P. juliflora* against *F. solani* and determination of the possible mechanism of action by molecular docking studies has not been done. Thus, the aim of present study is to evaluate the inhibitory potential of alkaloids of *P. juliflora* against *F. solani* and understand *in silico* protein-ligand interactions between the alkaloids and β-glucosidase.

Materials and Methods

Isolation of alkaloids from *P. juliflora*

Dried leaves were powdered and mixed with Methanol for extraction. The methanol extract was subjected to acid/basic fractionation process for the isolation of alkaloid fraction using the protocol of Singh et al [11]. The fraction was subjected to Dragendroff's reagent test for the confirmation of alkaloids.

Separation of alkaloid fraction by thin layer chromatography

Total alkaloid fraction was spotted on the TLC plate and run on the elutant;

Methanol: Acetic acid (100:1). All the band that appeared on Silica gel separating different alkaloids were scraped from TLC plates, dissolved in methanol (HPLC grade) and filtered through Whatmann No. 1 Filter paper and were subjected to Antifusarial activity.

Fusarium solani strain

F. solani was isolated from infected maize seed samples and morphologically identified using standard manual [12]. The culture was maintained on Potato dextrose agar medium at 25±2^{o}C for further studies.

Preparation of inoculum

The inoculum of *F. solani* was prepared from 7 day old culture grown on Potato dextrose agar medium. The Petri dish was flooded with 8 to 10 ml of distilled water and the conidia was scraped using sterile spatula. The spore density of was adjusted with spectrophotometer (A_{590} nm) to obtain a final concentration of approximately 10^5 spores/ml [13].

Antifusarial activity by disc diffusion method and MIC

Antifusarial activity of alkaloids fraction was studied by disc diffusion method following Clinical and Laboratory Standard Institute (CLSI) methodology [14]. 100 µl of the inoculum of *F. solani* was seeded on the plates containing CDA medium. The plates were allowed to dry for 3-5 min. 50 µl of the active alkaloid fraction (5 mg/ disc) was loaded to the sterile discs of 6 mm diameter and positioned on the test plates. The plates were incubated at 25±2°C for 7 days. The diameter of the inhibition zones if any were measured in mm. Discs loaded with respective solvents exclusive of extract served as control. All the tests were performed in triplicates.

The minimal inhibitory concentration (MIC) value for the active alkaloid fraction against *F. solani* was determined by serial plate dilution assay in accordance with Clinical and Laboratory Standard Institute (CLSI) methodology [14]. In, 96-well plate, 100 µl of alkaloid fraction (2-fold serially diluted), 100 µl of Czapek dox broth and 100 µl of the inoculum of *F. solani* were added and incubated at 25± 2°C for 7 days. After incubation, the optical density was measured to check the inhibition at 590 nm. The clear solution indicated inhibition of *F. solani*.

LC-MS analysis of the active alkaloid fraction of _P. juliflora_

The band showing antifusarial activity were subjected to LC-MS analysis out using Waters Acquity System consisting of a degasser, binary pump, auto sampler, and column heater. The column outlet was coupled to a Thermo-fleet (LCQ-Fleet) Ion Trap mass spectrometer equipped with an ESI ion source [31].

Effect of the active alkaloid fraction on _F. solani_ mycelium growth

A 6-mm diameter agar plug of *F. solani* was placed on Capek Dox agar (CDA) medium containing different concentrations of the active alkaloid fraction (0, 25, 50, 100, 150, and 200 µg/mL). The plates were incubated at 25±2^{0}C for 3 to 10 days. The study was done in triplicates. Mycelial radial growth was measured, when the growth in control plate reached more than 2/3 diameter, the activity was expressed as EC50 (the active alkaloid fraction concentration inhibiting growth by 50%). EC50 value was calculated as logarithm value of X to the regression when Y = 5. The rate of inhibition (as probability value Y) and concentrations of alkaloid fraction (as logarithm X). The linear regression equation was fit as (Y=a+bx) and coefficient (r) was calculated [15].

Effect of the active alkaloid fraction on biomass production of _F. solani_

The biomass production of *F. solani* was determined as mycelial dry weight. The active alkaloid fraction was added to 50 ml potato dextrose broth so as to reach final concentrations of 0, 25, 50, 100, 150 and 200 µg/mL and three 6 mm-diameter fungal agar discs of *F. solani* were inoculated into it. The flasks were kept at 25°C under gentle shaking for 96 h, the mycelial mat was filtered (Whatmann No. 1 filter paper) and dried in oven for at 60^{0}C for 2 h. The weight of the mycelia was determined by subtracting the initial weight of the filter paper from the weight of mycelia and filter paper. The fungal biomass was calculated as the mean value of three independent samples [16].

Effect of the active alkaloid fraction on fungal cell wall

The effect of alkaloid fraction on fungal cell wall was studied by releases of cellular material. Three agar discs of 6 mm diameter of *F. solani* was taken and placed in 50 ml of Potato dextrose broth. The setup were incubated in shaker incubator at 25°C, 150 rpm for 1 h with Alkaloid fraction at the concentration of 0, 25, 50, 100, 150 and 200 µg/ml. After 1h, the mycelium was centrifuged at 10,000 ×*g* at 4°C for 10 min. The mycelia was washed three times with PBS buffer (0.05 mol/l, pH 7.0) and re-suspended in the same. The release of cellular material was determined in each supernatant by UV absorption at 260 nm [17].

Effect of active alkaloid fraction on spore germination

50 µl of conidia suspension of *F. solani* was transferred to a cavity slide, to it 40 µl of alkaloid fraction (50 µg/mL) was added and using sterile distilled water the concentration was made up to 100 µl per slide. The slide was incubated at 25±2^{0}C for 18h. The percentage of germinated conidia was determined from at least 100 conidia per cavity by microscopic examination [16]. Percentage spore germination is calculated according to the following formula:

$$\text{Spore germination } (\%) = \frac{Germinated\ spores (No.)}{Total\ spores\ (No.)} \times 100$$

Microscopic studies

A mycelial agar disc of *F. solani* was placed in the center of PDA plate containing 50 µg/mL the active alkaloid fraction and incubated at 25±2^{0}C for 3 days under dark. The agar disc incubated in the absence of alkaloid fraction was served as control [18]. Thin layers (1 mm) of agar blocks containing mycelia was cut off from the growing edges of the colonies for examination under light microscopy and Scanning electron microscopy to observe recognizable morphological and cytological changes [15].

***In-silico* studies by molecular modelling**

The interaction between active alkaloid fraction (Juliprosopine and Prosopine) from *P. juliflora* and *F. solani* cell was studied using molecular docking studies. Sequence of Beta-glucosidase of *F. solani* was selected from NCBI and using Swiss–Model workspace server the protein structure predicted was Beta-glucosidase with PDB ID: 3AHZ. The Structures of Juliprosopine and Prosopine were retrieved from NCBI PubChem and using Marvin sketch (Freeware version) the 2D and 3D structure were cleaned. The active site prediction of Proteins was done using PDBsum and CASTp. The grid generation wizard was used to define the docking space. The molecular interaction between Beta-glucosidase and active alkaloid fractions and accurate docking of ligands into the active sites of Proteins was done using Ligand Fit protocol available in Accelrys Discovery studio 2.5 (Accelrys, San Diego, CA, USA). AutoDock 4.2 workspace was used for all the steps involved in ligand preparation, protein preparation, and Induced Fit Docking (IFD) [19].

Results

Isolation of alkaloids from _P. juliflora_

Four fractions were obtained from acid/base fractionation of methanol extracts of leaves. The fourth fraction was total alkaloid fraction (TAF), Orange red color precipitate when subjected to Dragendorff's reagent, confirmed the presence of alkaloids.

Separation of alkaloid fraction by thin layer chromatography

Thin layer chromatography of total Alkaloid fraction showed the presence of 4 bands with Rf values of 0.52, 0.6, 0.81, 0.84 [Fig-1].

Antifusarial activity by disc diffusion and MIC

All the 4 bands obtained in TLC were subjected to antifusarial activity. Only band II with Rf value 0.6 showed antifusarial activity. The active alkaloid fraction (AF) present in Band II showed significant antifusarial activity against *Fusarium solani* with zone of inhibition 39.3±0.66 mm in comparison standard fungicide Carbendazim (Commercial name: Bavistin) with zone of inhibition 10.2±0.34 mm

[Fig-2]. The minimal inhibitory concentrations of the active alkaloid fraction of *P. juliflora* against *F. solani* was found to be 40 µg/ml.

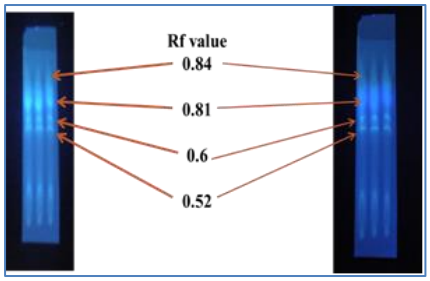

Fig-1 TLC of Total Alkaloid fraction showing 4 bands at different Rf values

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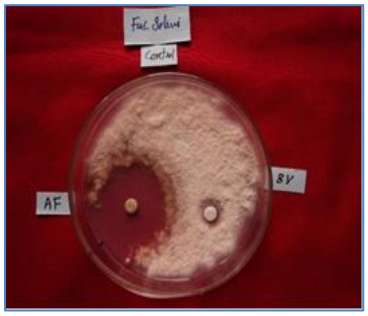


Fig-2 Antifusarial activity of the active alkaloid faction

LC-MS analysis of the active alkaloid fraction of *P. juliflora*

The Band II obtained in TLC were subjected to LC-MS analysis showed 6 peak the highest peak with 51.66% area at the Retention Time 1.16 [Fig-3] gave a protonated molecular ion [M+H]+ at m/z of 630.5713 and 316.2318. Based on Literature the 630.5713 mass is of alkaloid Juliprosopine and 316.2318 mass is of Prosopine [Fig-4].

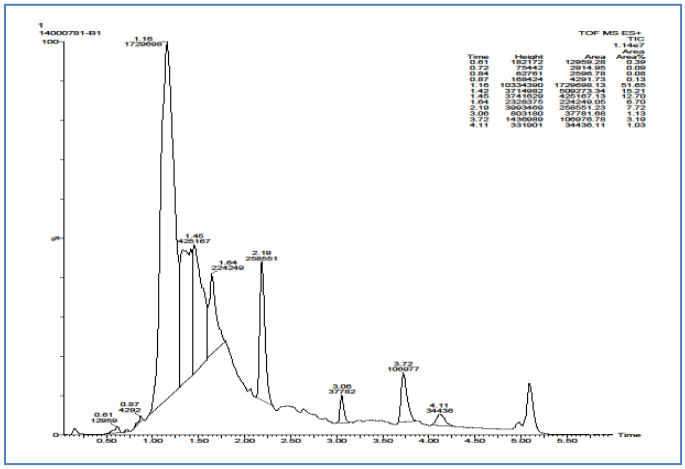


Fig-3 LC Chromatogram showing highest peak with 51% at the Retention Time 1.16

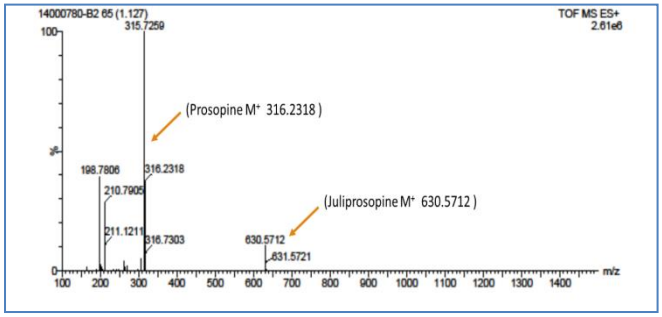


Fig-4 Mass spectrum of the active alkaloid fraction showing $[M^+ H]^+$ mass of Juliprosopine and Prosopine

Effect of the active alkaloid fraction on *F. solani* mycelium growth

The mycelia of *F. solani* was sensitive to the active alkaloid fraction and the EC50 values are 52.47 with R^2 value 0.987. The mycelial growth of *F. solani* was inhibited by alkaloid active fraction (AF) in dose dependent manner. Different concentrations of active fraction (in log) and Percentage of inhibition are present [Fig-5].

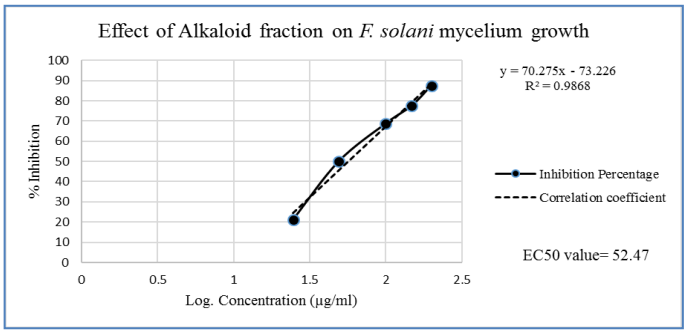


Fig-5 Linear regression analysis showing mycelium growth inhibition

Effect of the active alkaloid fraction on biomass production of *F. solani*

The biomass production of *F. solani* showed significant difference in the biomass production on Control and different treatments. At concentration of 200 µg/ml, the dry mycelial weight was 1.2 g whereas in control it was 11.92 g. The concentration of Active fraction (AF) at 50 and 100 µg/ml showed constant decrease in biomass production was observed [Fig-6].

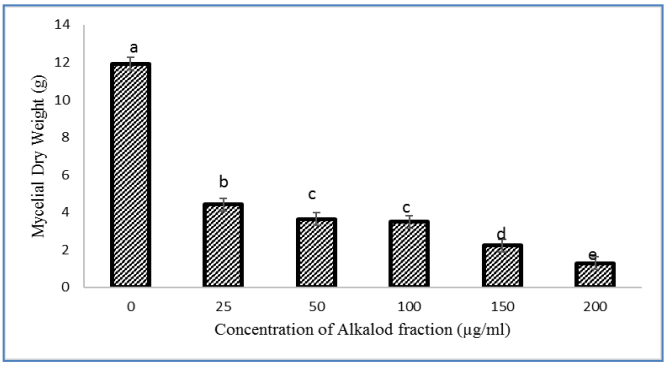


Fig-6 Effect of different concentrations of active alkaloid fraction (AF) on mycelial dry weight of *F. solani*. Vertical bars are denoted with Standard Error and different letters indicating significant difference in student's t-test (P=0.05).

Effect of the active alkaloid fraction on fungal cell wall

Different concentrations of alkaloid active fraction showed significant effect on the cell wall of *F. solani* cells. The leakage of UV-absorbing cellular components from fungal cell was observed at 50 µg/ml and complete release of cellular component was observed at 200 µg/ml [Fig-7].

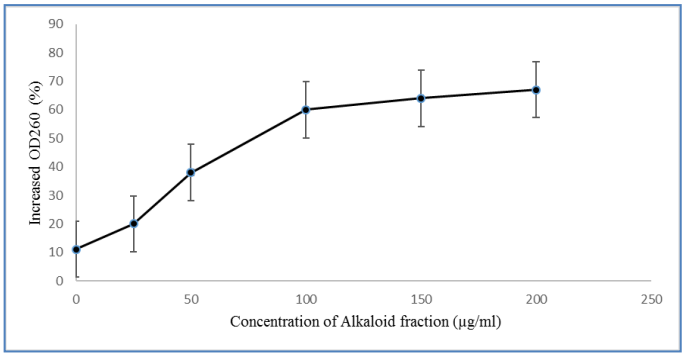
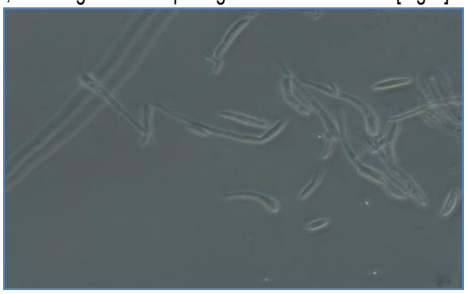


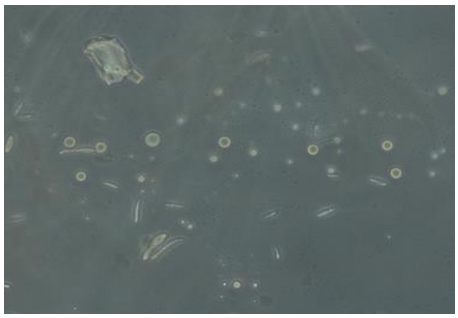
Fig-7 Effect of Alkaloid fraction on the release of cellular materials from *F. solani*.

Effect of active alkaloid fraction on spore germination

Average spore germination was 83% in control, while in active alkaloid fraction (AF) it was nil, resulting in 100% spore germination inhibition [Fig-8].



(A)



(B)

Fig-8 *F. solani* spore germination in (A) Control and (B) treatment with Alkaloid fraction showing no germination

Microscopic studies

The light microscopic studies revealed reduction in the size of micro-conidia and macro-conidia and degraded hyphae and mycelium in comparison with control [Fig-9a], [Fig-9d). The SEM analysis showed inhibition of hyphal growth and hyphal morphology defects such as cell wall disruption, Shriveled and withered hyphae and excessive septation were observed [Fig-9e],[Fig-9f]. In control cultures, the mycelia organization revealed by SEM also showed an extracellular material around the hyphae, resembling a bio-film and smooth cell wall [Fig-9b], [Fig-9c].

***In silico* studies by molecular modeling**

Considering the well obtained *in vitro* results, the molecular docking studies was performed for Juliprosopine [Fig-10a] and Prosopine [Fig-10d]. Molecular modelling is a technique to study the interaction between two molecules in a best orientation with minimum binding energy. The minimum binding energy found in the interaction of between beta-glucosidase and Juliprosopine was -2.91 kcal/mol with 2 Hydrogen Bond and Prosopine was - 2.79 kcal/mol with 4 Hydrogen bonds. The best orientations of hydrogen bonds and hydrophobic interaction of docked molecules are presented [Fig-10c], [Fig-10e]. The *in silico* studies revealed that all the synthesized molecules showed good binding toward the target proteins thus can act as an inhibitor for Beta-glucosidase. The minimum binding energy of Juliprosopine and Prosopine is due to dipole-dipole and hydrogen bond interaction with amino acids of targeted protein. Docked ligand molecules are represented as [Fig-10 a-f].

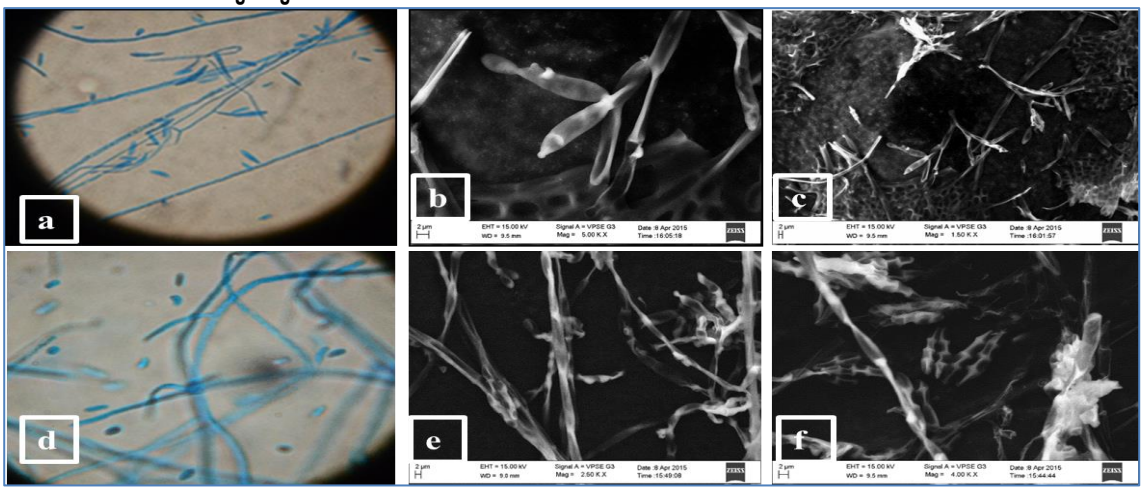


Fig-9 Effect of Alkaloid fraction on hyphae morphology of *F. solani* (40X magnification. Hyphae and Conidia (a) in control (d) in treatment with 50 µg/mL of Alkaloid fraction. (b) and (c) Scanning electronic microscopy of *Fusarium solani* in control showing Macro conidia and hyphae. (e) and (f) Scanning electronic microscopy of *F. solani* in Treatment with alkaloid fraction (50µg/mL) showing shriveled hyphae and reduced conidia.

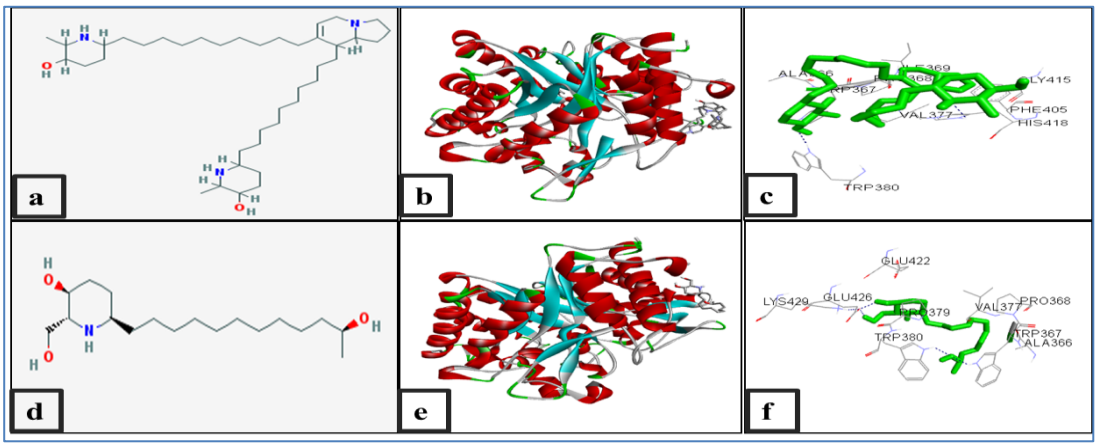


Fig-10 (a) Juliprosopine and (d) Prosopine retrieved from NCBI PubChem. (b) and (c) Ligand fit interaction between Beta-glucosidase and Juliprosopine. (e) and (f) Ligand fit interaction between Beta-glucosidase and Prosopine.

Discussion

P. juliflora is a fast growing species introduced in India that has spread rapidly and has occupied a vast area of non-arable land [8]. Even though it is considered as an important weed, the ability of this plant to produce a large biomass of leaves is an important character that could be exploited, as it is a source for important bioactive alkaloids. The antifungal potential of leaves of *P. juliflora* and preliminary studies on the utility of the same to manage seed borne fungi of sorghum [9]. Reports on the isolation and characterization of the various alkaloids from *P. juliflora* are available [20, 11].

Literature survey reveals the paucity of information on the antifusarial potential of the alkaloid fraction from *P. juliflora*. None of the earlier workers have evaluated the *invitro* and *in-silico* antifusarial efficacy of the active alkaloids fraction against an important phytopathogen *F. solani*. Juliprosopine and Prosopine are the important alkaloids isolated from leaves of *P. juliflora* [21]. The biological activity of Prosopine from *P. juliflora* has not been recorded. Considering these, in the present study the antifusarial potential of alkaloids from *P. juliflora* was evaluated. Alkaloid fraction isolated from leaves of *P. juliflora* showed significant antifusarial activity against this potent pathogen in disc diffusion assay. The zone of inhibition of alkaloid fraction was 39.3 mm, which is four times higher than that of Carbendazim at the same concentration. Results of the study suggest high potential of these alkaloids for managing *F. solani* in crop disease management.

The study has shown that the alkaloids fraction inhibits both the mycelial growth and spore germination whereas earlier reports have shown either inhibition of mycelium or inhibition of spore germination on other fungi [22, 15]. The present study revealed a positive correlation between inhibitions of mycelial growth with increase in the concentration of alkaloid fraction. Similar observations were also found with reference to biomass production suggesting the high inhibitory potential. Hasan, [23] also recorded decrease in biomass production of *F. graminearum* with increase in concentration of vinclozolin.

Conidia germination is the growth stage most sensitive to inhibition by many compounds [15, 24]. Present investigation shows that alkaloid fraction isolated from *P. juliflora* was very effective in preventing germination of conidia of *F. solani*. Some studies have shown a relationship between the onset of sporulation and mycotoxin production [22, 24]. A study showed that Chemical compounds that inhibit sporulation in *Aspergillus parasiticus* and *A. nidulans* also promoted the inhibition of aflatoxin and sterigmatocystin production, respectively [25, 26]. These studies indicate that on treatment with chemical compounds there is reduction in both sporulation and mycotoxin levels. Thus in our study the complete inhibition of spore germination may also be related to reduction in mycotoxin production in *F. solani*.

The experiment conducted to understand the effect of the active alkaloid fraction on the cell wall revealed the ability of the alkaloid to lyse the fungal cell wall at 50 µg/ml suggests the fungicidal potential of the alkaloids. These observations were further corroborated by light microscopy and SEM studies where in deleterious morphological manifestation of the fungal hyphae were clearly notices in the form of shriveling and deformation of the hyphae and hyphal tips. Shriveled hyphae was commonly observed compared with the normal mycelia. These alterations in the cellular wall may be related to the chemical characteristic of alkaloids present in leaves of *P. juliflora* as it contains an indolizidine ring in the center of the molecule and specific functional groups in positions 3 and 3' in the heterocyclic rings, gives polar and nonpolar ends that might facilitate their interactions with fungal cell membranes [27]. These interactions would increase the possibility of permeability of alkaloids to the cell wall and cytoplasmic membrane which leads to the disruption of enzymatic reaction involved in cell wall synthesis and integrity [32]. Similarly, Tyagi and Malik [28] have reported that yeast cells on treatment with essential oil of *Cymbopogon citratus* showed shrinkage of cells due to loss of cytoplasmic contents. This mechanism leads to leakage of the cytoplasmic contents and changing the structure of several layers of polysaccharides, fatty acids, and phospholipids [28].

A large number of studies on antimicrobial action of plant based bioactive compounds show the disruption of fungal and bacterial membranes [15]. According to Silva et al. [29] the alkaloids present in the extract of leaves of *P. juliflora*, possess chemical characteristics, which would lead to the breakdown of the fluid mosaic structure of the plasma membranes of cell [32]. The microscopic studies on Alkaloid fraction treated *F. solani*, mycelium appeared thin by reduction of cytoplasmic contents and the Micro-conidia and Macro-conidia are highly reduced in structure in comparison to control where the number and Size of Micro-conidia and Macro-conidia is larger.

Thus to understand the interaction of alkaloid of *P. juliflora* on the F. solani cell wall *in silico* study was conducted. The Beta glucosidase enzyme present in the cell wall of *F. solani* showed the positive interaction with both Juliprosopine and Prosopine alkaloids with minimum binding energy at its active site. The docking studies indicate that the alkaloids inhibit the cell wall synthesis enzymes leading to cell wall disruption and leakage of cytoplasmic content of the cell. The molecular docking studies ratify the observations of spore germination, inhibition of mycelial growth and fungicidal effect by lysis of cell wall. The results of the present investigation confirms the high potential of the two alkaloids as an important candidate for further studies in the utilization of leaves of *P. juliflora* for management of diseases caused by *F. solani*.

Conclusion

The present investigation has confirmed the antifusarial potential of the Band II (Rf value 0.6) of the alkaloid fraction isolated from leaves of *P. juliflora*. The inhibition studies have shown that the ability of the alkaloid faction to lyse the fungal cell wall and inhibit the spore germination. The molecular docking studies have also confirmed the inhibitory potential of Juliprosopine and Prosopine. Thus, these alkaloids can be further explored in agriculture for plant disease management.

Conflicts of interest: none declared

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