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ISOLATION AND CHARACTERIZATION OF CHEMICAL COMPOUNDS FROM FRUIT PULP OF CASSIA FISTULA AND THEIR ANTIMICROBIAL ACTIVITY

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ABSTRACT
In the present study effect of chloroform fraction of Cassia fistula fruit pulp on cyto-morphological parameters like mycelium width and conidial size of Alternaria solani has been studied. Column chromatography of chloroform extract and TLC fingerprinting of column fractions were also done. Column fractions were screened for antifungal activity and fraction showing best activity was further subjected to GC MS analysis for the purification and identification of the structure of active compound. Result suggested that mycelium width of Alternaria solani increased up to 77.89% and conidia size of the was reduced up to 97.61% at 1.25 mg/ml (Sub MIC) concentration of the chloroform extract. Eight fractions obtained from column chromatography and fraction no. 2 (FPF-2) showed maximum inhibition i.e. 98.25% against Alternaria solani. Rf values of TLC bands of column fractions were found between the range from 0.60 to 0.97cm. GC-MS analysis reveals the presence of butanoic acid, 2-methyl-, Penthiophane (2H-Thiopyran, tetrahydro) and Isopropyl acetate (Acetic acid, 1-methyl ethyl ester). These three compounds are responsible for the antimicrobial activity of Cassia fistula fruit pulp.

Keywords: Column chromatography, cytomorphology, Gas chromatography/mass, spectrometry Alternari solani, Cassia fistula

INTRODUCTION
Recent trends favour the use of alternative substances derived from natural plant extracts to control pests. The use of natural products for the control of fungal diseases in plants is considered as an interesting alternative to synthetic fungicides due to their less negative impacts on the environment. These natural products or plant extracts can be exploited either as leads for chemical synthesis of new agrochemicals, or as commercial products in their own right, or as a source of inspiration to biochemists for the development of new bioassays capable of detecting other, structurally simpler, compounds with the same mode of action¹ (Lange et al., 1993).

Plants have endless ability to synthesize various secondary metabolites which acts as main agents for plant defence mechanisms against microorganisms. These secondary metabolites are antimicrobial in nature and the use of various plant extracts for growth inhibition of plant pathogenic fungi. The biological and molecular action of plants secondary metabolites induces various morphological and cytological changes in the microorganisms including fungi and bacteria² (Wilson et al., 1997).

All morphological/cytomorphological alterations may be related to the effect of secondary metabolites on enzymatic reactions/specific enzymes regulating cell wall synthesis, changing/disturbing membrane permeability, thickening of cell wall by binding to the receptors as well as disruption of cell membrane³ (Polya 2003).
Cassia fistula (Linn.) belongs to family Fabaceae and Sub–family Caesalpinioideae. It is a very common plant known for its medicinal properties and is semi-wild in nature. It is distributed in various regions including Asia, South Africa, China, West Indies and Brazil. It is commonly known as Amaltas and in English it is popularly called “Indian Laburnum” and has been extensively used in Ayurvedic system of medicine for various ailments. It is widely used in traditional medicinal system of India. The plant parts are used in folk remedies for tumors of the abdomen, glands, liver, stomach, throat cancer carcinomata and impostumes of the uterus. Root is useful in fever, heart diseases, retained excretions and biliousness1 (Nadkarni, 2009).

Alternaria solani is the causal agent of early blight and important foliar pathogen of potato worldwide. It belongs to large long beaked and noncatenated spores group of the genus Alternaria2 (Simmons, 2000). It shows dark black to brown circular colony morphology on Potato dextrose agar (PDA) media. The mycelium consisted of septate, branched, light brown hyphae which turned darker with age. They reproduce asexually by means of conidia these Spores or conidia are the primary agent for infecting host plants for many plant pathogenic fungi3 (Heaney et al., 2000).

Due to the damage caused by plant diseases, continuous research is essential for developing new control methods to increase or even maintain current levels of crop production. Nowadays, the natural products and medicinal plants are a subject of great global interest for the discovery of new antimicrobial agents. One of the main procedures used in search of new biologically active substances is the systematic screening of plant extracts for their antimicrobial activity. This procedure has been a source of useful agents to control the microbial survival4 (Tuzun & Kloepper, 1995).

In the present study effect of different concentrations ranging up to MIC of chloroform fraction of Cassia fistula fruit pulp on cyto-morphological parameters like mycelium width and conidial size of Alternaria solani has been studied. Column fractions of chloroform extract were also screened for antifungal activity and fraction showing best activity was further subjected to GC MS analysis for the purification and identification of structure of active compound.

MATERIALS AND METHODS

Effect of chloroform extract on morphology of Alternaria solani

Chloroform extract was prepared by the hot extraction method suggested by Harborne, 1984. Minimum Inhibitory Concentration (MIC) of this extract was determined by broth dilution method5 (Collee et al., 1996). Effect of chloroform extract on various morphological and cytological parameters of Alternaria solani was studied. Test fungus was treated with increasing concentrations of the extract up till MIC. A small fungal biomass consisting of mycelium, and spores were removed from each tube and microscopic examination was done after staining with cotton blue and mounting in lacto-phenol. Changes in mycelium width, conidia size and no. of conidia were also observed with the help of Olympus trinocular research microscope BX- 51 and analyzed by ocular micrometer using microscope. Conidia/ spore counting were done by haemocytometer.

Column chromatography of chloroform extract

10 gm of dried and partially purified chloroform extract was dissolved in their mobile phase i.e. 50 ml Chloroform, 50 ml ethyl acetate, 30 ml ethanol and 100 μl acetic acid. Thus prepared solution was subjected to column chromatography. Glass column (Merck: 120–240 mm) filled with 650 gm of silica gel was used for column chromatography. Different fractions of extract containing different secondary metabolites were collected according to the color bands developed in column. These fractions were dried in rotatory vacuum evaporator under reduced pressure. Dried fractions were screened for their antifungal activity. The fraction showing best antifungal activity was subjected to further purification and characterization for active molecule via gas chromatography and mass spectrometry.

TLC Fingerprinting of column fractions

TLC fingerprinting of chloroform fraction of fruit pulp performed using precoated silica gel 60 F254 TLC plates (E-Merck) of uniform thickness (20mm x 20mm). A 10 cm length of TLC plate was cut and marked carefully. 10μl of plant extract was spotted onto the marked plate with the help of a capillary tube or pipette. TLC finger printing was derivatized with anisaldehyde sulphuric acid reagent followed by heating at 100°C till coloured bands of various secondary metabolites appeared. The observations were taken before and after derivatization, in visible as well as UV rays. Rf value of the extracted secondary metabolites were calculated as follows:

\[
\text{Rf} = \frac{\text{Distance traveled by substance}}{\text{Distance traveled by solvent}}
\]

Assay of Antifungal Activity of column fractions

Antifungal activity of various column fractions against Alternaria solani was done by Poison food technique6 (Grover and Moore, 1962). 100 mg of extract was dissolved in 10 ml solvent (acetone) to prepare stock solution of 10mg/ml concentration. 9 ml of molten PDA medium was poured into test tubes and then autoclaved. The molten sterilized medium along with 1 ml of stock solution was poured into Petri plates. In the control set no extract was used. After the solidification of the media, 6 mm inoculum disc of 7 days old culture of the fungus was aseptically inoculated upside down in the centre of the petriplate and incubated at 27±2°C. Culture control and acetone control were also maintained along with test samples. Antifungal activity was measured as a function of increase in growth of 6 mm disc of inoculum.

The average diameter of the fungal colonies was measured on the 7th day of incubation and percentage of mycelial growth inhibition was calculated by the following formula given below.

Percent Mycelial growth inhibition = gc-gt / gc× 100
Where,

gc = Growth of mycelial colony after incubation period in control set subtracting the diameter of inoculums disc.

gt = Growth of mycelial colony after incubation period in treatment set subtracting the diameter of inoculum disc.

Identification and Structure Determination by GC-MS (Gas chromatography/mass spectrometry)

For identification of active antifungal compound from selected fraction, the sample was sent to Sophisticated Instrumentation Centre for Applied Research and Testing (SICART) Anand (Gujarat, India). The GC MS analysis were performed on a GC (Perkin-Elmer) system coupled to Perkin Elmer Turbo Mass MS. HP1-MS capillary column (30m× 0.25µm ×0.25 µm) was used under the following conditions: oven temperature programmed from 70°C for 10 min, then gradually increased at 290°C at 3 min; injector temperature, 250°C, carrier gas Helium, flow rate 1 ml/min; the volume of injected sample was 1µl; split ratio 1:60; ionization energy 70eV: Run time 40 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The identification of the separated compounds was achieved through retention indices and mass spectrometry by the comparing mass spectra of the unknown peaks with those stored in the NIST/EPA/NIH Mass Spectral Library 2014.

RESULTS AND OBSERVATIONS

Effect of chloroform extract on mycelial width and conidia size of Alternaria solani are presented in Figure 1 and 2. A gradual decrease in conidia size, while swelling of hypha was observed due to treatment with extract. Mycelium width of Alternaria solani increased up to 77.89% at 1.25 mg/ml concentration of the extract. Conidia size of the Alternaria solani was reduced up to 97.61% at 1.25 mg/ml (Sub MIC) concentration of the chloroform extract. The inhibition of conidia and mycelia formation was observed at MIC of the extract i.e. 2.5 mg/ml.

Some abnormalities were also observed in reproductive structures of Alternaria solani after treatment with extracts. At 1.25 mg/ml concentration of extract dichotomous branching in the conidiophores was observed and conidia were found directly attached to the mycelia.

Eight fractions obtained from column chromatography were subjected to thin layer chromatography. TLC of each fraction showed presence of more than one band. Bands on TLC plates were observed before and after derivatization under UV light at 360 nm (Figure 3 A, B, C). Colour of bands changed after spraying anisaldehyde on the TLC plates.
At different Rf values, bands of various colours viz. dark yellow, light yellow, light green, dark green, light grey, yellow green, light brown, dark brown, and light pink were observed for TLC developed from column fractions of chloroform fraction of fruit pulp extract. It indicates that this fraction contains a group of compounds. Rf values of TLC bands of column fractions were found between the range from 0.60 to 0.97cm.

All column fractions showed significant antifungal activity but fraction no. 2 (FPF-2) showed maximum (98.25%) inhibition followed by fraction no. 1 (86.46%), fraction no. 4 (83.41%) and fraction no. 5 (62.45%). The percent mycelial growth inhibition observed with fraction no 3, 6, 7, 8 was 61.14%, 59.83%, 53.28% and 47.16% respectively (Figure 4).

The fraction no. 2 (FPF-2) showed most significant activity against test fungus was subjected to GC MS analysis for the separation and identification of active principle. The chromatogram obtained in GC MS analysis is given in Figure 5. Three compounds identified, the most prevailing compounds were Butanoic acid, 2-methyl- (90.36%) at retention time 3.68, Penthiophane (2H-Thiopyran, tetrahydro) (6.16%) at retention time 2.80 and Isopropylacetate (Acetic acid, 1-methylethyl ester) (3.48%) at retention time 2.04. The presence of compounds was confirmed after comparing with NIST/EPA/NIH Mass Spectral Library 2014. The mass spectrum of identified compounds is given in Figure 6 to 8.
DISCUSSION

Herbal remedies and alternative medicines are used throughout the world and in the past herbs have often represented the original sources of most drugs\textsuperscript{11} (Cooper, 2005). Results showed concentration dependent plant extract inhibition of fungal growth that may be due to increase in the concentration of secondary metabolites/active components on increasing the concentration. Tsai \textit{et al.} (1999) suggests that some fungal pigments are natural products and associated with development of reproductive structures\textsuperscript{12}. Similarly, dark brown pigment called melanin is formed by oxidative polymerization of phenolic compounds and synthesized during spore formation. Alkaloids such as solanine and chaconine are discussed as resistance factors of potato against \textit{Alternaria solani}\textsuperscript{13} (Sinden \textit{et al.}, 1972).

Versha \textit{et al.} (2003) reported that various fractions i.e. petroleum ether, chloroform, ethyl acetate and methanol of \textit{Alstonia scholaris} leaf powder exhibit significant antimicrobial activity against the test pathogens and strongest antifungal activity against \textit{A. niger} and \textit{A. flavus} was observed with chloroform fraction\textsuperscript{14}. Antifungal activity of petroleum ether, chloroform and acetone and ethanol extracts of \textit{Calendula officinalis} against \textit{A. fumigatus}, \textit{Rhizopus japonicum}, \textit{C. albicans}, \textit{C. tropicalis} etc. has been investigated by Kasiram \textit{et al.}, 2000. Rao \textit{et al.} (2006) reported that alcohol extracts of some medicinal plants showed most significant antifungal activity as compared to other extracts prepared in different solvents\textsuperscript{15, 16}. Presence of anthraquinone glycosides, sennosides A & B, rhein and its glucoside, barbaloin, aloin, formic acid, butyric acid and their ethyl esters and oxalic acid, pectin and tannin in \textit{Cassia fistula} fruit pulp has been reported by Agarwal and Paridhavi, 2005\textsuperscript{17}.

Further separation and characterization of active principle from column fractions of \textit{C. fistula} using GC-MS analysis reveals the presence of butanoic acid, 2-methyl-, Pentiophane (2H-Thiopyran, tetrahydro) and Isopropylacetate (Acetic acid, 1-methylethyl ester). The antifungal activity of butanoic acid, 2-methyl- and Pentiophane (2H-Thiopyran, tetrahydro) was already reported (Singh \textit{et al.}, 2003; Mickevičienė \textit{et al.}, 2015)\textsuperscript{18, 19}. However, Isopropylacetate (Acetic acid, 1-methylethyl ester) not yet reported for antimicrobial activity in literature. The presence of these compounds in \textit{C. fistula} fruit pulp extracts was also supported by Anitha and Miruthula, (2014)\textsuperscript{20}. Hence, present study deals with the extract preparation, fractionation and characterization of active antimicrobial compounds from \textit{Cassia fistula} fruit pulp.

CONCLUSION

In the present study it can be concluded by the observations that treatment with chloroform extract of \textit{Cassia fistula} leads the inhibition of conidiation, mycelial growth and morphological alterations in conidiophore hence responsible for conversion of pathogenic form of test fungus into non pathogenic form. Separation and identification of active compounds was done by column chromatography (CC) and GC-MS. Three compounds were obtained in GC MS analysis.
These compounds are responsible for antimicrobial activity of *Cassia fistula* fruit pulp. Further studies, will be included incorporation of active compounds to NMR and IR for molecular characterization and subsequent drug designing process.

REFERENCES

Effect of Different Physical Factors on *Cassia Fistula* Fruit Pulp Extract and their Herbal Formulation Efficacy

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**Abstract**

In the present study, effect of different physical factors like sunlight, heat, pH and long-term storage on extract and herbal formulation has been studied to determine its stability under varying physical conditions. Extract and herbal formulation were exposed to varying conditions of the parameters chosen for a specific time period, and then observed the effect as a function of change in MIC of extract against the *Alternaria solani*. Active principles present in *Cassia fistula* are highly susceptible to change in physical environment. However, it is found that extract and herbal formulation of *Cassia fistula* can be stored for 12 months, remains stable at alkaline pH, can stand with exposure to sunlight and high temperature. Hence a little favourable manipulation of physical conditions could improve its shelf life and can be used as fungicides for controlling microorganisms.

**Keywords:** Physical factor; Sunlight; Heat; Ph; Storage

**Introduction**

Amount of active constituents in the plant material affected by various physical factors, climatic conditions, geographical location of plants, seasonal variations, edaphic factors etc. Ashebir & Ashenafi [1]. Excess heating during extract preparation often affect biologically active substances such as flavonoids, essential oils and other heterogeneous phytoconstituents present in the plant extract which might influence their respective activity Scalber [2]. Herbal formulation will be commercially viable, if its stability can be maintained at varying physical conditions. The prerequisite conditions for use of plant extracts in such formulations are that their physical and chemical properties should not undergo any drastic change due to change in temperature, pH or exposure to sunlight and it should have a long shelf life at least 6 months and there should not be any reduction in its antimicrobial activity. Hence, before using the plant extract for making any herbal formulations, detailed studies to check stability of extract in varying conditions of physical factors such as pH, temperature etc. and its effect on MIC should be conducted.

Several workers have checked the stability of extract in the presence of different physical factors. Gupta & Viswanathan [3] reported decrease in antimicrobial activity of garlic extract against B. cereus when stored at room temperature but when the same extract was stored at 8 °C the antimicrobial activity was not changed. Tyneca et al. [4] reported that antimicrobial activity of Allium ursinum juice decreases on storage above 4 °C. Moore & Atkins [5] suggested that inhibitory property of garlic extract was unaffected by storage temperatures. Shahi et al. [6] observed effect of pH on antidermatophytic activity of stored essential oils and found that efficacy of oils was enhanced at altered pH. Heat stable activity of combination of ethanolic extract of *Cassia alata* and *Ocimum sanctum* was reported by Ranganathan & Balajee [7]. Rath et al. [8] studied effect of high, temperature and 0.5M sucrose on the activity of turmeric oil. Effect of different temperature, autoclaving, illumination and pH values on ninety six plant extracts was investigated by Wang and Ke-Qiang [9] who reported that at higher temperature activity decreased while after steam sterilization there was no change in activity. Rong et al. [10] studied insecticidal activity of *Ailanthus altissima* extract and found that the activity is greater in light than in dark.

and found that heat treatment above 75 °C reduced the inhibitory activity while inhibitory activity is stable between pH 2.0 to 8.0. Similarly Di Mambro et al. [13] studied the combined effect of temperature and relative humidity on the antioxidant effect of different plant extracts. Doughari [14] reported significant increase in bioactivity of compounds of root extracts of Carica papaya L. directly proportional with increase in temperature and inversely proportional to increase in pH. Arabshahi et al. [15] studied the effect of pH, temperature and storage on the antioxidant activity of drumstick leaves (Moringa oleifera), mint leaves (Mentha spicata) and carrot tuber (Daucus carota).

Srinivasan et al. [16] reported that Allium sativum extract stored at room temperature showed inhibitory activity against the tested pathogens up to seven days. When the extract was stored at 4 °C, it exhibited moderate activity till 60 days and if the same extract was stored at 20 °C the antimicrobial activity decreased. He also reported that activity of the same extract decreased at alkaline pH. Mehrrota et al. [17] reported that bioactive components of ethanol extract of Syzygium aromaticum were stable over a wide range of pH values and temperatures. Magdy et al. [18] reported no change in activity of plant extracts exposed to different temperatures ranging from 4, 30, 60, and 90 °C. This showed that phytoconstituents are thermostable. Ghogare et al. [19] reported slight decrease in the antimicrobial activity of Z. officinale and A. sativum extracts on increasing the pH of the extract. Barpete et al. [20] observed that combination of low light intensity, phytagel as gelling agent and thidiazuron (TDZ)-α-naphthalene acetic acid (NAA) was very effective for high frequency shoot regeneration of Lathyrus sativus. It is very important to determine the effect of physical factors on extract as well as herbal formulation, to improve their storage condition and maintenance of efficacy for prolonged period. Hence, in the present study, effect of pH, storage, temperature, sunlight etc. on MIC of extract and herbal formulation has been studied to determine its stability under varying physical conditions. MIC and MFC of chloroform fraction of Cassia fistula fruit pulp was determined at 2.5mg/ml and 5 mg/ml respectively.

Materials and Methods

Effect of physical factors such as heat, temperature, pH, sunlight etc. was studied by exposing the extract and herbal formulation to varying conditions of the parameters chosen for a specific time period, and then observing the effect as a function of change in MIC of extract against the test organism. Tubes containing MIC of extract, herbal formulation and extract free medium were maintained for comparison in each set of experiment against Alternaria solani. In the present study 100% alcoholic crude extract and partially purified chloroform extract of Cassia fistula fruit pulp and best ratios (8, 12, 18, 22) of herbal formulation which is made by combining plant extract, elicitor (neem oil cake) and binder (Cow dung) were used for the experiments. All ingredients of herbal formulation were used in following ratio:

- **Formulation ratio no. 8 (100% alcohol crude extract (4ml): 100% neem oil cake (3ml): 100% cow dung (3ml)).**
- **Formulation ratio no. 12 (100% alcohol crude extract (2ml): 100% neem oil cake (6ml): 100% cow dung (2ml)).**
- **Formulation ratio no. 18 (Partially purified chloroform extract (3ml): 100% neem oil cake (3ml): 100% cow dung (4ml)).**
- **Formulation ratio no. 22 (Partially purified chloroform extract (6ml): 100% neem oil cake (2ml): 100% cow dung (2ml)).**

These extracts and herbal formulation were found to be most potent. Experiments were repeated thrice and three replicates were maintained.

**Effect of sunlight**

Effect of sunlight on the viability of extracts and herbal formulation was studied according to the method suggested by Wang & Ke-Qiang [9]. Sterile vials containing 5ml of 100% alcoholic crude extract, partially purified chloroform extract and herbal formulation (ratio no. 8,12,18,22) were placed in sunlight for 15h and 30h. After which effect on efficacy of extract and herbal formulation was assayed by tube dilution method.

**Effect of heat**

Efficacy of extract and herbal formulation was assayed according to the method suggested by Rath et al. [8]. Effect of dry heat was studied by exposing sterile glass vials containing 100% alcoholic crude extract, partially purified chloroform extract and herbal formulation (ratio no. 8,12,18,22) to 40 °C and 90 °C for 4h in hot air oven while in case of wet heat; extract and herbal formulation was kept at 50 °C and 100 °C in water bath for 4h. Effect on activity of extract and herbal formulation was then assayed by tube dilution method. One tube containing untreated extract as well as herbal formulation (room temperature) was maintained as control for comparison.

**Effect of pH**

Effect of varying pH i.e. 4, 7 and 9 on efficacy of extract and herbal formulation was studied by method suggested by Dixit et al. [21]. Natural pH of extract and herbal formulation is 7. 0.1 N HCl and 0.1 NaOH were used to change the pH to 4 and 9 respectively. Culture medium was then added to tubes containing extract and herbal formulation and the tubes were inoculated with Alternaria solani. Inoculated tubes were incubated at 27±1 °C for 72 h and observed for change in herbal formulation and MIC of extract.

**Effect of storage**

Effect of storage on antifungal activity of extract and herbal formulation was assayed by method suggested by Rath et al. [8]. Extract and herbal formulation were stored at room temperature and change in their activity was assayed at regular intervals of 6 month up to 24 months by tube dilution method.
Results and Observations

The results of effect of different physical factors like sunlight, heat, pH and long-term storage on extract and herbal formulation of C. fistula fruit pulp are given in (Table 1-8).

Table 1: Effect of Sunlight Exposure on Crude and Partially Purified Extract of C. fistula Fruit pulp against A. Solani.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Extract</th>
<th>Unexposed Condition</th>
<th>15h</th>
<th>30h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>100% Alcoholic crude</td>
<td>No growth</td>
<td>No growth</td>
<td>Slight growth</td>
</tr>
<tr>
<td>2.</td>
<td>Partially purified chloroform</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>3.</td>
<td>Control (Without Extract)</td>
<td>Abundant growth</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Effect of Sunlight Exposure on Herbal Formulation against A. Solani.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Herbal formulation ratio number</th>
<th>Unexposed Condition</th>
<th>15h</th>
<th>30h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>8</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>2.</td>
<td>12</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>3.</td>
<td>18</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>4.</td>
<td>22</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>5.</td>
<td>Control (Without Extract)</td>
<td>Abundant growth</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Effect of Heat on Crude and Partially Purified Extract of C. fistula Fruit pulp against A. Solani.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Extract</th>
<th>Wet Heat</th>
<th>Dry Heat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R.T.</td>
<td>50 °C</td>
</tr>
<tr>
<td>1.</td>
<td>100% Alcoholic crude</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>2.</td>
<td>Partially purified chloroform</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>3.</td>
<td>Control (Without Extract)</td>
<td>Abundant growth</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Effect of Heat on Herbal Formulation against A. Solani.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Herbal formulation ratio number</th>
<th>Wet Heat</th>
<th>Dry Heat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R.T.</td>
<td>50 °C</td>
</tr>
<tr>
<td>1.</td>
<td>8</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>2.</td>
<td>12</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>3.</td>
<td>18</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>4.</td>
<td>22</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>5.</td>
<td>Control (Without Extract)</td>
<td>Abundant growth</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Effect of pH on Crude and Partially Purified Extract of C. fistula Fruit pulp against A. Solani.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Extract</th>
<th>Control (pH7)</th>
<th>pH4</th>
<th>pH9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>100 % Alcoholic crude</td>
<td>Slight growth</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Partially purified chloroform</td>
<td>Slight growth</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Control (Without Extract)</td>
<td>Abundant growth</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6: Effect of pH on Herbal Formulation against A. Solani.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Herbal formulation ratio number</th>
<th>Control (pH7)</th>
<th>pH4</th>
<th>pH9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>8</td>
<td>Slight growth</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>12</td>
<td>Slight growth</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>18</td>
<td>Slight growth</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>22</td>
<td>Slight growth</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Control (Without Extract)</td>
<td>Abundant growth</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Effect of Storage on Crude and Partially Purified Extract of C. fistula Fruit pulp against A. Solani.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Extract</th>
<th>Fresh Extract</th>
<th>6 Months</th>
<th>12 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>100 % Alcoholic crude</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>2.</td>
<td>Partially purified chloroform</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>3.</td>
<td>Control (Without Extract)</td>
<td>Abundant growth</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 & 2 shows that no changes in efficacy of chloroform extract and herbal formulation observed due to direct exposure to sunlight for 15h and 30h. In 100% alcoholic crude extract 15h exposure had no effect but after 30h exposure a slight decrease in activity was observed for A. solani. Table 3,4 depict the effect of wet as well as dry heat on extract and herbal formulation efficacy. Results indicate that 100% alcoholic crude extract and herbal formulation ratio number 8 and 12 up to 50 °C of wet heat and 40 °C of dry heat did not affect the activity of extract; however, heating at 100 °C of wet heat and 90 °C of dry heat for...
4h resulted in slight decrease in extract and herbal formulation efficacy as a slight growth of test fungus was observed. Wet as well as dry heat treatment had no effect on activity of chloroform extract and herbal formulation ratio number 18 and 22.

Table 5 & 6 shows the result of effect of pH on the efficacy of plant extract as well as herbal formulation. There was no inhibitory effect observed on efficacy of extract and herbal formulation at neutral and alkaline pH up to 9 but there was decrease in antifungal activity at acidic pH (pH-4) against Alternaria solani.

Table 7 & 8 shows the result of effect of long term storage of extract and herbal formulation at room temperature. Storage for 6 and 12 months had no effect on efficacy of extract and herbal formulation and the antifungal activity was same as the fresh extract.

Table 8: Effect of Storage on Herbal Formulaion against A. Solani.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Herbal Formulation Ratio Number</th>
<th>Fresh Extract</th>
<th>6 Months</th>
<th>12 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>8</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>2.</td>
<td>12</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>3.</td>
<td>18</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>4.</td>
<td>22</td>
<td>No growth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>5.</td>
<td>Control (Without Extract)</td>
<td>Abundant growth</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Herbal formulations using plant extracts are beneficial in the treatment of various diseases as they have no side effects as compared to synthetic antimicrobial drugs. Herbal formulations may be viable only when they have the ability to maintain stability and physical factors do not affect the activity of these formulations. Antimicrobial property of extract as well as herbal formulation may be affected by various physical factors such as pH, temperature, sunlight exposure etc. because all these factors are responsible for bringing the change in the chemical nature of compounds responsible for the antimicrobial activity.

Results showed that no change observed in antifungal activity of chloroform extract and herbal formulation after exposure to direct sunlight indicates that active principles of chloroform extract and herbal formulation are light stable and do not undergo photo oxidation. Whereas 100% alcoholic crude extract retained its antifungal potential up to 15h exposure of sunlight. Wang & Ke-Qiang [9] have reported similar results. Probably sunlight exposure do not destruct the active molecules of chloroform extract of Cassia fistula possess antifungal potential.

Effect of Heat on 100% alcoholic crude extract and herbal formulation ratio number 8 and 12 showed that the active principles can withstand the wet heat and dry heat up to 50°C and 40 °C respectively. While prolonged exposure of extract with 100 °C wet heat and 90 °C dry heat destroyed its antifungal potential whereas it has no effect on chloroform extract and herbal formulation ratio number 18 and 22. Singh et al. [22] had also concluded the same for antifungal and antioxidative potential of Foeniculum vulgare volatile oil and its acetone extract. Magdy et al. [18] also reported that the activity of Cinnamomum cassia, Allium sativum, Syzygium aromaticum, Punica granatum, Citrus lemonium and Hibiscus sabdariffa plant extracts were not affected when exposed to different temperatures ranging from 4 °C, 30 °C, 60 °C and 90 °C. The temperature resistance studies indicate that the phytoconstituents are thermostable, but heating at 120 °C or beyond leads decrease/loss in the antimicrobial activity, this may be due to volatilization of components and/or due to some physical and chemical changes in molecules of natural products during heating.

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The antifungal activity of extract and herbal formulation of Cassia fistula fruit pulp was found to be stable at the pH 7 and 9. Decrease in the activity of the same at pH 4 was observed. These results suggest that the active principles of the extract are better active at neutral pH. Nishihara et al. [23] suggested that the presence of a high concentration of salt interfere with the binding of cationic peptides to the cell surface of B. subtilis, which are required for its growth. Yen & Duh [24] reported that a methanol extract from peanut hulls had a higher antioxidant activity at neutral and acid pH. Increase in activity of phytoconstituents in the presence of acidic medium has been reported by Doughari [14]. Azizah et al. [25] reported the antioxidant activity of different extracts from cocoa by-products was higher at alkaline pH.

Jeffery [26] also investigated the effect of various other physical factors like heat and temperature etc. on antimicrobial activity of pepper leaf extracts. Arabshahi et al. [15] suggested that antioxidant activity of extract of mint leaves, carrot and drumstick varies with the change in pH. Yang et al. [27] investigated the effect of pH on antibacterial activity of Propolis ethanol extract against Streptococcus mutans and reported that the active molecules are highly stable at acidic pH followed by neutral and then alkaline pH. Srinivasan et al. [16] also reported decrease in the antimicrobial activity of Allium sativum extract on increasing pH value and it was least at pH 9. Bayliak et al. [28] reported that antioxidant activity of aqueous extracts of Rosa canina, Rhodiola rosea, Hypericum perforatum and Gentiana lutea is decreased at alkaline pH while prooxidant activity increase at same pH.
Storage studies results suggest that there was no effect of long term storage on the efficacy of extract and herbal formulation. During storage combinations of physical factor not as much affect the efficacy of extract as well as herbal formulation than individually affect. Arias et al. [29] investigated that aqueous and ethanolic extracts of Acacia aroma possess antibacterial activity against gram +ve and gram -ve bacteria and also evaluated that stored extracts have similar antibacterial activity as the fresh extracts.

Conclusion

The Results suggested that the active principles present in Cassia fistula are highly susceptible to change in physical environment. However it is found that it can be stored for 12 months, remains stable at alkaline pH, can stand with exposure to sunlight and high temperature. Hence a little favourable manipulation of physical conditions could improve its shelf life and can be used as fungicides for controlling microorganisms.

Acknowledgment

One of the authors (Deepa Hada) is thankful to University Grant Commission (UGC), New Delhi, India, for providing financial assistance.

References

IN VIVO MANAGEMENT OF EARLY BLIGHT DISEASE OF POTATO WITH HERBAL FORMULATION

Deepa Hada* and Kanika Sharma

Department of Botany, Mohanlal Sukhadia University, Udaipur-313001 (Rajasthan), India.

ABSTRACT

In the present study investigates the in vivo management of early blight disease of potato by seed dipping and foliar spray with herbal formulation from Cassia fistula L. fruit pulp extract in combination with neem oil cake and cow dung. The preventive action was studied as a function of decrease in disease severity, change in growth characteristics of host plant such as number of leaves/plant, plant height, number of tubers, tuber weight, and tuber size of healthy, infected and treated plants. Six treatments i.e. T1, T2, T3, T4, T5 and T6 were applied in different combinations. Four different controls i.e. C1, C2, C3 and C4 were also maintained. Results suggested that T4 treatment not only reduces the infection but also leads to increased growth, health and vigour of the host plant as compare to other treatments. This combination observed to show significant activity against Alternaria solani and can help to minimize the economical loss of potato crop. Data were subjected to analysis of CD and CV value and statistical analysis at 5% and 1% CD revealed that all the treatments are significant.

KEYWORDS: Herbal formulation, neem oil cake, cow dung, potato, Alternaria solani.

INTRODUCTION

Potato (Solanum tuberosum L.) is an annual, herbaceous plant of family Solanaceae. Although, it originated in South America in the region between Peru and Bolivia in the 16th century, it is now a globally important crop, exceeded only by maize, rice and wheat and is consumed by billions of people across the globe, half of which are in the developing countries. It is the fourth important crop worldwide by volume of production. It is high yielding, has a high nutritive value and is grown in about 140 countries.[1] The present area under potato cultivation in India is about 1.4 million hectares. India produces a total of about
Potatoes are produced on a large scale, with about 25-28 million tones of potatoes every year. Potato is an economical food and a source of low-cost energy to the human diet. It is also used for production of high quality starch, alcohol, etc. and its starch (farina) is used in laundries for sizing yarn in textile mills. It is also used for the production of dextrin and glucose. Potato tubers contain about 77.8% water, 22.6% carbohydrates, 2.1% protein, 0.3% fat, 1.1% crude fibre, 0.9% ash, 40 IU vitamin A, 12 mg ascorbic acid per 100 g of edible portion etc. This crop is highly susceptible to early blight caused by *Alternaria solani*.

Early blight is a very common disease of both potato and tomato. It is caused by the fungus, *Alternaria solani* (Ellis & G. Martin) L.R. Jones & Grout, which survives in infected leaf or stem tissues on or in the soil. Spores form on infested plant debris at the soil surface or on active lesions over a fairly wide temperature range, especially under alternating wet and dry conditions. The pathogen can also attack potato tubers and symptoms are circular to irregular lesions that are slightly sunken and often surrounded by a raised purple to dark brown border and produce a shallow, dry, corky rot.

Estimating total annual crop losses due to any particular disease is difficult to do accurately. Yield losses up to 79 per cent from early blight damage have been reported from India. Apart from the use of crop rotation, certified disease-free seeds and resistant varieties, and control measures are important to minimize infection. It is usually necessary to apply fungicide sprays to fully protect plants from early blight. Fungicide alternatives that have been fungicidal effect on disease incidence.

The overzealous and indiscriminate use of most of the synthetic fungicides has created different types of environmental and toxicological problems. Bavistin, mancozeb and thiram are the most commonly used plant fungicides. Such synthetic fungicides bring about the inhibition of pathogens by either destroying their cell membrane or its permeability or by inhibiting metabolic processes of the pathogens and hence are extremely effective. The flip side of this is that synthetic chemicals are harmful for human as well as soil health. They decrease soil fertility, enter the food chain, pollute the environment and cause several deleterious effects on human health and biosphere, contributing to significant declines in populations of beneficial soil organisms, soil acidification and compaction, thatch accumulation, and diminished resistance to diseases. Use of alternative methods which are sustainable and eco-friendly is therefore the answer to this problem.
Thus, current thinking about plant and environment protection suggests alternatives to pesticides and use of other strategies in addition to well known disease management methods such as crop rotation use of resistant cultivars, planting disease free seeds, biological control etc. for control of fungal diseases.\cite{9,10} One of these alternative methods is use of natural formulations prepared from plants. Plants possess natural antimicrobial compounds (which can be used to develop eco-friendly and effective fungicidal formulations). Although several plants extracts have been screened for in vitro antifungal activity against plant pathogenic fungi.\cite{11,12} But not much work has been done on in vivo study of preventive or therapeutic effect of plant extracts.

The present study investigates the in vivo management of early blight disease of potato by seed dipping and foliar spray with herbal formulation from *Cassia fistula* L. fruit pulp extract in combination with neem oil cake and cow dung. A comparison with synthetic fungicide has also been done. Innovative part of this study is use of combinations of plant extract with elicitors and binders to develop protective measure against this plant disease. The preventive action was studied as a function of decrease in disease severity, change in growth characteristics of host plant such as number of leaves/plant, plant height, number of tubers, tuber weight, and tuber size of healthy, infected and treated plants.

**MATERIALS AND METHODS**

**Test crop and fungus:** *Alternaria solani* was isolated from infected leaves of potato collected from the nearby fields of Udaipur (Saveenakheda, Manwakheda etc). Identification of fungus was done by Dr. T. Prameela Devi (Head, Division of Plant Pathology, Indian Type Culture Collection, IARI, New Delhi with I.D. No. 9944.15). Potato tubers were procured from the local market of Udaipur. Healthy seed tubers of approximately same size and having 3-5 eyes were used for experiment.

**Inoculum development:** Potato dextrose agar (PDA) medium which was sterilized in an autoclave at 15 psi for 20 minutes was used for culture of *Alternaria solani*. The inoculated petriplates were incubated at $25^\circ C$ for seven days. Pure culture thus obtained was used for mass culture of *Alternaria solani*.

Mass culture of *Alternaria solani* was done in 250 ml flasks containing 25 ml of autoclaved potato dextrose broth. Each flask was inoculated with 6 mm diameter of the fungus taken from the margins of a week old culture of *Alternaria solani*, grown on PDA medium. Then
flasks were incubated at 25 ± 20c for 72h. 25 ml of *Alternaria solani* suspension per pot of soil was used as inoculum for experimentation.

**Preparation of pots and soil:** Pots (30 cm Diameter) were sterilized with 20% CuSO4 solution whereas soil was autoclaved and then cooled. Sterile soil was mixed with inoculum and filled in the pre-sterilized pots. Since autoclaving of soil makes it nutrient deficient hence organic manure was added to the soil of each pot before sowing.[13]

**Preparation of herbal formulations and standard solution**

*In vivo* study of preventive /protective action of herbal formulation based on results of *in vitro* studies. Herbal formulations were prepared by using *Cassia fistula* fruit pulp extracts, powder, elicitor and binder in different ratio. 100% of neem oil cake and 100% of cow dung were mix with 100% alcoholic crude, partially purified chloroform extract and fruit pulp powder. All ingredients of herbal formulation were used in following combinations:

- Crude extract + elicitor + binder
- Partially purified extract + elicitor + binder
- Fruit pulp powder + elicitor + binder.

Mancozeb was used as standard antifungal and 10 mg/ml concentration was prepared in sterile water.

**Treatments and experimental design:** The study was conducted during the December, 2015 to March 2016 in the Botanical Garden, University College of Science, M.L.S. University, Udaipur, (Rajasthan). Seed dipping method and foliar spray were used for the study of preventive effect of herbal formulation.[14,15]

On the basis of results obtained from *in vitro* studies six treatments were applied in following combinations.

T1: Healthy tubers were treated with formulation no. 8 (100% alcoholic crude extract (4ml): 100% neem oil cake (3ml): 100% cow dung (3ml)).

T2: Healthy tubers were treated with formulation no. 12 (100% alcoholic crude extract (2ml): 100% neem oil cake (6ml): 100% cow dung (2ml)).

T3: Healthy tubers were treated with formulation no. 18 (Partially purified chloroform extract (3ml): 100% neem oil cake (3ml): 100% cow dung (4ml)).
T4: Healthy tubers were treated with formulation no. 22 (Partially purified chloroform extract (6ml): 100% neem oil cake (2ml): 100% cow dung (2ml).
T5: Healthy tubers were treated with fruit pulp powder (60gm): neem oil cake (20gm): cow dung (20gm).
T6: Healthy tubers were treated with fruit pulp powder (40gm): neem oil cake (30gm): cow dung (30gm).

Healthy seed tubers dipped in respective herbal formulations were planted in pre-sterilized soil pots containing 10 kg soil infested with Alternaria solani inoculum showed in Fig.7 & 8.

Four different controls were also maintained respectively showed in Fig. 7. These were as follows
C1: Healthy tubers were treated with 10mg/ml concentration of Mancozeb sown in soil inoculated with Alternaria solani.
C2: Untreated healthy tubers sown in unsterilized and uninoculated soil.
C3: Untreated healthy tubers sown in sterilized soil inoculated with Alternaria solani.
C4: Untreated healthy tubers in sterilized uninoculated soil

Three tubers were planted per pot at a depth of 5 cm. First foliar spray was given after 21days and second after 42 days from the date of sowing. 25 ml of Alternaria solani suspension was sprayed per plant and the treated plants were covered with poly bags for 48h. After 48hours 30 ml of herbal formulation was sprayed per plant and left for 90 days for assessing the disease severity.\[16\]

The tubers were harvested after 90 days from the date of sowing and observations were recorded as the number of leaves/plant, plant height of potato, total tuber weight /pot, tuber size of potato, total number of tubers/pot. Percent disease infestation on tubers was recorded with the comparative study of positive controls. Data were subjected to analysis of CD and CV value. Three replicates were maintained with each experiment.\[13,17\]

**Disease Severity**

Disease severity is the measure of sickness of diseased plant. It is a qualitative trait, which measures the effect of disease on a plant tissue, intensity of symptoms or damage.\[18,19\]

Disease severity was calculated by using following formula:

Disease Severity = Area of tissue infected/ Total area X 100
Growth Parameters
Following parameters as described in Technical bulletin of Central Potato Research Institute, ICAR, Shimla was measured by standard methods in healthy, infected and treated plants:
• Number of leaves/plant
• Plant height
• Total tuber weight/pot
• Tuber size
• Number of tubers /pot

RESULTS AND OBSERVATIONS
Disease Severity: Results of effect of herbal formulations on disease severity are listed in table no.1 & Fig. 1. Maximum disease severity was observed with C3 (88.5%) followed by C2 (60.14%) and C4 (57.14%) respectively. As compared to this all treatments as well as mancozeb were effective in reducing the disease severity. Maximum decrease in disease severity was observed with T4 (12.5%) followed by C1 (18.75%), T3 (22.42%), T1 (30.57%), T2 (38.44%), T6 (43.24%), and T5 (47%) treatment.

Reduction in disease severity was comparable in case of C1 i.e. mancozeb and T4 i.e formulation no. 22 prepared from partially purified chloroform extract (6ml): 100% neem oil cake (2ml): 100% cow dung (2ml). Although T4 provided slightly better protection. Mancozeb proved to be better as compared to T1, T2, T3, T5 and T6. Amongst the various treatments applied reduction in disease severity was best with T4 followed by T3, T1, T2, T6 and T5 (Fig. 1). Statistical analysis at 1% and 5% CD values reveals that all the treatments are significant to reduce disease severity in field trials.

Growth Parameters
Results of effect of herbal formulations prepared from *Cassia fistula* fruit pulp alcoholic crude extract and partially purified chloroform fraction, powder, neem oil cake and cow dung on following growth parameters of potato crop are summarized in table no. 1 & Fig. 2 to 6.

Data clearly indicate that, treatment with herbal formulations reduces disease severity which results into significant improvement of growth of host plant.

• **Number of Leaves/plant:** Results of effect of different treatments and synthetic fungicide on number of leaves/plant are given in table no. 1 & Fig. 2. The data indicate slight reduction
in number of leaves/plant due to infection. But significant increase in number of leaves/plant was observed due to treatment with herbal formulations as well as mancozeb. Maximum increase in number of leaves/plant was observed with T4 (54.66) followed by C1 (52.66), T5 (48.33), T6 (47.33), T3 (46) and C4 (44.66) respectively. Results obtained with T1 and T2 are comparable with C3. Statistical analysis at 5% and 1% CD revealed that all the treatments are significant.

- **Plant Height:** Results of effect of different treatments on height of plant are depicted in table no. 1 & Fig. 3. Similar course of growth improvement, as observed in case of no. of leaves was recorded for plant height after treatment with herbal formulations. Treatment with formulations and mancozeb resulted in increase of plant height as compared to control. Maximum plant height observed with T4 (25.33 cm) treatment followed by T6 (24.33 cm), and T5 (23.33 cm). Amongst the treatments applied, plant height observed for T3, T1, and T2 was found to be similar to C1. At 5% and 1% CD value all the treatments were found to be significant for field trials.

- **Total Tuber Weight /Pot:** Table no. & Fig. 4 list the results of effect of treatment on average tuber weight. Significant decrease in tuber weight was observed due to infection (C2, C3 and C4). All the treatments significantly improve tuber weight as compared to maintained controls. As observed in previous cases, T4 treatment was found to be the most effective and it resulted in maximum increase in tuber weight as compared to standard i.e. C1, and other controls i.e., C2, C3 and C4. Among controls, C1 (199.44 gm) treatment was found to be better than T5 (189.21 gm) and T6 (185.5 gm). Amongst various treatments T4 (245.45 gm) was best followed by T3 (223.47 gm), T2 (210.26 gm) and T1 (201.9 gm). Statistical analysis at 5% and 1% CD revealed that all the treatments are significant.

- **Tuber Size:** Results of effect of different treatments on tuber size are given in table no. 1 & Fig. 5. Minimum tuber size was observed with C3. Slight increase in tuber size was observed with C4 followed by C2. As compared to this all treatments as well as mancozeb were effective in increasing the tuber size. Maximum increase in tuber size was observed with T4 (6.86 cm) followed by T3 (6.3 cm), T2 (5.86 cm) and T1 (5.46 cm) respectively. C1 (5.36 cm) treatment was found to be effective over T5 (5.16 cm) and T6 (4.96 cm) treatment. Statistical analysis at 5% and 1% CD revealed that all the treatments are significant.
Number of Tubers/Pot: Results of effect of different treatments and synthetic fungicide on number of tubers/pot are given in table no. 1 & Fig. 6. Almost 50% reduction in tuber numbers was observed due to infection (C3 and C4). Significant improvement in number of tubers was observed with T4 treatment i.e. 18.33. T3 (16.66) was the second most effective treatment followed by T2 (14.66). Amongst the treatments applied, number of tubers/pot observed for T1 (13.66) was found to be similar to C1 and C2. C1 (13.66) and C2 (13.33) treatments were found to be effective over T5 (12.66) and T6 (10.66) treatment. Statistical analysis at 5% and 1% CD value all the treatments were found to be significant for field trials.

Table 1: Effect of Treatments on Disease Severity and Growth parameters of Potato

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Percent Disease Area</th>
<th>Number of Leaves/Plant</th>
<th>Plant Height (cm)</th>
<th>Total Tuber Weight (gm)/Pot</th>
<th>Tuber Size (cm)</th>
<th>Number of Tubers/Pot</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>30.57</td>
<td>41.66</td>
<td>23</td>
<td>201.9</td>
<td>5.46</td>
<td>13.66</td>
</tr>
<tr>
<td>T2</td>
<td>38.44</td>
<td>41.33</td>
<td>22.66</td>
<td>210.26</td>
<td>5.86</td>
<td>14.66</td>
</tr>
<tr>
<td>T3</td>
<td>22.42</td>
<td>46</td>
<td>23</td>
<td>223.47</td>
<td>6.3</td>
<td>16.66</td>
</tr>
<tr>
<td>T4</td>
<td>12.5</td>
<td>54.66</td>
<td>25.33</td>
<td>245.45</td>
<td>6.86</td>
<td>18.33</td>
</tr>
<tr>
<td>T5</td>
<td>47</td>
<td>48.33</td>
<td>23.33</td>
<td>189.21</td>
<td>5.16</td>
<td>12.66</td>
</tr>
<tr>
<td>T6</td>
<td>43.24</td>
<td>47.33</td>
<td>24.33</td>
<td>185.5</td>
<td>4.96</td>
<td>10.66</td>
</tr>
<tr>
<td>C1</td>
<td>18.75</td>
<td>52.66</td>
<td>21.66</td>
<td>199.44</td>
<td>5.36</td>
<td>13.66</td>
</tr>
<tr>
<td>C2</td>
<td>60.14</td>
<td>32.33</td>
<td>20.66</td>
<td>165.88</td>
<td>4.86</td>
<td>13.33</td>
</tr>
<tr>
<td>C3</td>
<td>88.5</td>
<td>43.66</td>
<td>16</td>
<td>125.16</td>
<td>3.16</td>
<td>12.66</td>
</tr>
<tr>
<td>C4</td>
<td>57.14</td>
<td>44.66</td>
<td>12.66</td>
<td>155.15</td>
<td>4.23</td>
<td>9.66</td>
</tr>
<tr>
<td>Mean</td>
<td>41.9</td>
<td>45.6</td>
<td>21.3</td>
<td>190.1</td>
<td>5.2</td>
<td>13.6</td>
</tr>
<tr>
<td>SD</td>
<td>22.8</td>
<td>6.2</td>
<td>4.0</td>
<td>34.7</td>
<td>1.0</td>
<td>2.6</td>
</tr>
<tr>
<td>SE m+</td>
<td>4.17</td>
<td>1.13</td>
<td>0.72</td>
<td>6.34</td>
<td>0.19</td>
<td>0.47</td>
</tr>
<tr>
<td>CD (P=0.05)</td>
<td>*7.47</td>
<td>*5.06</td>
<td>*4.59</td>
<td>*9.49</td>
<td>*3.26</td>
<td>*3.92</td>
</tr>
<tr>
<td>CD (P=0.01)</td>
<td>*9.33</td>
<td>*5.87</td>
<td>*5.04</td>
<td>*12.36</td>
<td>*3.96</td>
<td>*4.51</td>
</tr>
<tr>
<td>CV (%)</td>
<td>54.49</td>
<td>13.54</td>
<td>18.58</td>
<td>18.27</td>
<td>19.95</td>
<td>18.81</td>
</tr>
</tbody>
</table>

Each data represent the average of three replicates* Significance.

Figure.1: Effect of Treatments on Disease Severity of Potato
Figure 2: Effect of Treatments on Number of Leaves/Plant of Potato

Figure 3: Effect of Treatments on Plant Height (cm) of Potato

Figure 4: Effect of Treatments on Total Tuber Weight (gm)/Pot of Potato
Figure 5: Effect of Treatments on Tuber Size (cm) of Potato

Figure 6: Effect of Treatments on Number of Tubers/Pot of Potato

Figure 7: (a) Healthy tubers dipped in various treatments (b) Controls (C1, C2, C3 C4) against *Alternaria solani*
India rank 4th in area and it is the 3rd largest country in world in production of potato after China and Russian Federation. Potato is grown almost in all states of India. In India Rajasthan state has a very negligible area under potato cultivation and restricted to limited pockets like Kota, Dholpur, Bharatpur and Alwar districts.\(^{20}\) Early blight, caused by *Alternaria solani*, is a common potato (*Solanum tuberosum* L.) disease. The disease often occurs initially on older, less productive foliage, followed by a gradual upward progression within the canopy, resulting in premature leaf senescence.\(^{21,22}\) If the inoculum load is high during favourable environmental conditions, early blight may become severe enough to cause significant reductions in yield.\(^{23}\) Under such conditions, frequent applications of protectant fungicides are often required to reduce foliar disease severity and subsequent yield loss.

*Alternaria solani* is a very destructive fungus for potato crop, but with the utilization of advanced techniques it becomes easier to control this cosmopolitan fungus. One of the most commonly used method is the use of fungicides, but these fungicides causes serious health hazards to human beings and also they cause environmental pollution. Hence, now a days more emphasis is given on other methods of disease control like growing disease resistant varieties, alterations in agronomic practices, use of plant and natural products and herbal
formulations etc. because they are more economical, eco-friendly and safe. An effective control programme also combines cultural practices, fungicides, biological control, and solarization.\textsuperscript{[24]}

Avoiding or minimizing the pesticide residues is required in the marketable products of potatoes. Therefore, one important aspect is the development of alternative control treatments based on plant extracts and herbal formulations. The fungicidal activity of some plant extracts in controlling different plant pathogens have been reported by several workers.\textsuperscript{[25,26,27]} Management of plant diseases by the use of plant extracts has also been reported.\textsuperscript{[28,29]}

Plants possess various inducible defense mechanisms to protect themselves against pathogen attack. Yadav \textit{et al.} isolated compound which showed antimicrobial activity against Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Escherichia coli, Aspergillus niger and Fusarium oxysporum.\textsuperscript{[30]} Ali \textit{et al.} reported that the antibacterial and antifungal activities of \textit{C. fistula} and \textit{M. ferrea} extracts were tested on 14 bacteria and 6 fungi. \textit{C. fistula} extracts showed stronger antibacterial activity than \textit{M. ferrea}.\textsuperscript{[31]} Sundararaju \textit{et al.} reported that 100% mortality was recorded from the \textit{C. fistula} extract at 48 h at 50 and 100% concentrations. At 72 h, 100% mortality was observed in all extracts at all three concentrations. The mortality rate was minimum at 24 h in all three extracts. All plant extracts exhibited a high degree of nematicidal action against the adults and juveniles of \textit{P. coffeae}.\textsuperscript{[32]}

Matsuzaki \textit{et al.}, reported that soil with cow manure amendments is best treatment for reducing the severity of the disease and improving the final tubers yield of potato.\textsuperscript{33} Kimaru \textit{et al.}, 2004 investigate effect of Neem Kernel Cake Powder (NKCP) on development of tomato \textit{Fusarium} wilt.\textsuperscript{[34]} Hamid \textit{et al.}, used cow manure and soil solarisation treatment for effective suppression of potato disease caused by \textit{R. solani} and subsequent improvement of the final tuber yield.\textsuperscript{[35]}

Healthy seed tubers, treated with herbal formulation and mancozeb respectively for a comparative study, were sown in inoculated soil. Untreated tubers sown in inoculated soil served as positive control. During \textit{in vitro} screening of synthetic fungicides mancozeb was found to be the most effective synthetic fungicide against \textit{A. solani} hence it was used as a standard control.
Novelty of this study is the use of combination of plant extract of different purity with elicitor i.e. neem oil cake and binder i.e. cow dung, which is not yet reported in searched literature. These combinations observed to show significant activity against *A. solani* and can help to minimize the economical loss of potato crop.

A comparative study of effect of herbal formulation versus mancozeb reveals that T4 treatment i.e. formulation no. 22 was found to be significantly improved all the growth parameters as compared to other treatments. In case of number of leaves/plant and disease severity mancozeb was found to be good after T4 treatment. Results of growth parameters obtained for T1, T2, T3, T5 and T6 treatments were also significant as compared to healthy controls. Results of the comparative study between the preventive effects of herbal formulations and synthetic fungicide indicate that protection offered by treatment no. 4 was more effective over mancozeb treatment. As compared to other treatments T4 treatment significantly reduces *A. solani* infection. It may be due to the fact that the group of active metabolites responsible for antifungal activity. Preventive effect of herbal formulation on disease severity might be due to presence of secondary metabolites present in plant extract. *Cassia fistula* fruit pulp contains anthraquinone, glycosides, sennosides A & B, rhein and its glucoside, volatile oil, barbaloin, aloin, formic acid, butyric acid and their ethyl esters and oxalic acid, presence of pectin and tannin is also reported.\[36\]

Results of present study indicate that treatment with herbal formulation especially T4 treatment not only reduces the infection but also leads to increased growth, health and vigour of the host plant as compared to infected plant and synthetic fungicide treatment. Chakraborty and Patil, have suggested that in addition to the preventive effect natural formulations also maintain the soil health which results in healthy growth of crop.\[37\] Terpenes play an important role as signal compounds and growth regulators (phytohormones) of plants. Gershenzon and Dudareva, reported the role of terpenes in growth and development of plant.\[38\] Fungal pathogens can invade and occupy living plant cells, diverting nutrients to the growing fungus and suppressing plant defense mechanisms.\[39\]

**CONCLUSION**

Exploitation of preparations based on natural substances, which can limit plant pathogens development comes into higher and higher prominence, especially restricting traditional chemical preparation application. It results from comparable efficiency of bio-preparations to pesticides. In the present investigation, reduction in disease severity after treatment with
herbal formulations was found to be comparable with synthetic fungicides. Hence these formulations can be used to develop eco-friendly alternatives for management of early blight diseases of potato.

Among the various treatments, T4 (Formulation no.22) treatment was found to be effective against *Alternaria solani* both during *in vitro* as well as *in vivo* trials. This treatment was not only found to be reducing the disease incidence but also helpful in improvement of all the growth parameters. However characterization of this active treatment via advanced molecular techniques like IR, NMR, GC-MS etc. and further quality assurance and then field trials could be very helpful to overcome the problem of non targeted fungicides.

**ACKNOWLEDGMENT**

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COMPARATIVE ANALYSIS OF DIFFERENT ELICITORS AND BINDERS AND DEVELOPMENT OF HERBAL FORMULATION CONTAINING CASSIA FISTULA L. FRUIT PULP AGAINST ALTENARIA SOLANI

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ABSTRACT

In the present study various concentration (10% to 100%) of elicitors like neem, mustard and coconut oil cake and binders like cow dung, guar gum and gum acacia were used with the aim to study impact of concentration on antifungal activity. 20gm elicitors and binders were dissolved in 100 ml of autoclaved water for 24 h. The mixture was then filtered and used for antifungal activity. Results suggested that antifungal activity increased with increasing concentration of elicitors and binders. Maximum activity was observed with 100% Concentration. Hence this concentration was used for the preparation of herbal formulation. 30 ratios of herbal formulations were prepared and assayed for antifungal activity against Alternaria solani by poison food technique. Among the formulations prepared optimum activity was observed for formulation number 8, 12, 18 and 22 i.e. 62.92%, 62.05%, 92.62%, 95.24% respectively against Alternaria solani. On the basis of results obtained, best herbal formulation will be used for in vivo experiments. The purpose of this work was to evaluate antifungal activity of herbal formulation against Alternaria solani, which cause early blight disease in potato crop.

KEYWORDS: Herbal formulation, Elicitors, Binders, Oil Cakes, Cow Dung

INTRODUCTION

India is the largest manufacturer of medicinal plants and is called as botanical garden of the world (Seth & Sharma, 2004). In the last few years there has been an exponential development in the field of herbal drugs and these medicines are gaining popularity in all over the world because of their less side effects and natural origin. Medicinal plants are widely distributed in different regions of India and comprise important components of flora. Attention need to be given to assess the medicinal value of such plants to explore the potential drugs out of it. Many ancient medicines in use are obtained from medicinal plants, minerals and organic matter (Grover et al, 2002). In herbal preparations of Indian traditional health care systems the large number of medicinal plants are present which are used traditionally for over 1000 years named rasayana (Scartezzini & Sroni, 2000). Most practitioners formulate and dispense their own recipes in Indian systems of medicine (Seth & Sharma, 2004). Traditional natural products are effective sources of new agrochemicals for control of plant diseases (Kagale et al, 2004; Maya and Thippanna, 2013). Use of plant based products in agriculture is an environmentally safe and economically viable strategy for the control of diseases. Bio-agents and herbal product preparations do not leave any toxic residues and therefore can effectively replace synthetic fungicides.
Cassia fistula (Linn.) is a common plant known for its medicinal properties and is a semi-wild in nature and belongs to family Fabaceae and Sub-family Caesalpinioideae. It is distributed in various regions including Asia, South Africa, China, West Indies and Brazil (Pashanth et al., 2006; Blalerao et al., 2012). This plant has been extensively used in Ayurvedic system of medicine for various ailments, is commonly known as Amaltas and in English popularly called “Indian Laburnum”. It is deciduous and mixed-monsoon forests throughout greater parts of India, ascending to 1300 m in outer Himalaya, is widely used in ancient medicinal system of India (Gupta, 2010). The plants are used in folk remedies for tumors of the glands, abdomen, liver, stomach, and throat cancer carcinoma, and impostumes of the uterus. Root is useful in heart diseases, fever, retained excretions and biliousness (Nadkarni, 2009).

Potato (Solanum tuberosum L.) is grown in about 140 countries and fourth important crop worldwide by volume of production; it is high yielding and having a high nutritive value (Malik and Tufail, 1984). Potato is an cheap food and a source of low cost energy to our diet. This crop is excessively susceptible to early blight caused by Alternaria solani. Foliar symptoms of early blight disease first appear small, irregular to circular dark brown spots on the lower (older) leaves, too much defoliation may lead to death of the plant and high yield loss. The Alternaria solani can also attack potato tubers and symptoms are circular to irregular lesions that are slightly sunken and surrounded by a raised purple to dark brown border and produce a shallow, dry, corky rot (Folsom and Bonde, 1925, O’Brien and Rich, 1976, Wharton and Kirk, 2007).

In plant biology elicitors are extrinsic, or foreign, molecules often associated with diseases or synergistic organisms and plant pests. Molecules of elicitor can attach to specific receptor proteins located on plant cell membranes. The molecular pattern of elicitors are recognized by receptors and trigger intracellular defence signalling via the Octadecanoid pathway. The response results in the enhanced synthesis of metabolites which decrease damage and increase resistance to pest, disease or environmental stress (Bektas & Eulgem, 2015). Oil cake is one of the natural organic fertilizers with high nitrogen content, which is the residues of neem seeds, mustard, peanut seeds, sesame, coconut etc. after oil extraction process of the processing plant.

Neem (Azadirachta indica) is a monumental tree of Meliaceae family coming from the Indian subcontinent. Actually, distribution and importance of neem is increasing all over the World due to its important and beneficial properties, which reported by WHO/UNEP1989. Neem is considered to be one of the most valuable trees of the 21st century for its great potential in pest management, effective source of environmentally powerful natural pesticides, environmental protection and medicine. The waste by-product remaining after the oil extraction processes is neem cake. Neem is considered devoid of toxicity, as tested also by the old traditional use. Neemo cake has been successfully utilized as livestock feed for growing goats (Rao et al., 2003).

Binder is a material which holds or draws other materials together to form a cohesive whole mechanically, chemically, or as an adhesive. Generally materials labeled as binders in various proportions or uses can have their roles reversed with what they are binding. Guar gum, also known as guaran, is primarily the ground endosperm of guar beans. The guar seeds are delihked, milled and screened to get the guar gum. It is produced as a free-flowing, off-white powder. The color of guar gum powder is whitish and yellowish having slight odor. Acacia gum has long been used in everyday applications and in traditional medicine. The material is used by Egyptians as glue and as a pain-reliever base. Arabic physicians with the gum treated a wide variety of ailments, resulting in its current name (Dobelis, 1986). Presently, it is used widely in the cooking industry to give body and texture to processed food products and in the pharmaceutical industry.
as a demulcent. It is also used to stabilize emulsions. The bark fibers are used to make cordage (Duke, 1985).

Cattle rearing in India has been a tradition and intimately limited to agricultural economy. Different products used widely in number of Ayurvedic formulations are obtained from cow milk, ghee, curd, urine, and dung. In Indian sub-continental farming cow dung is traditionally used as organic fertilizer for centuries. The addition of cow dung increases the mineral status of soil, also increases resistance of plant against pests and diseases; increase plant growth and other beneficial activities such as sulphur oxidation and phosphorous solubilization. The Composition of cow dung is around 80% water and supports a matrix of undigested plant material that is rich in nutrients, micro-organisms, and their byproducts (Naskar & Ray, 2003).

There are several traditional agricultural practices followed by farmers to control plant diseases. Probably the oldest document was Kautilya’s Arthasastra, which reported the use of organic materials to control the crop disorders. Formulation is a cheap, environmentally safe fungicide made by combining plant extracts and organic materials to control plant diseases. Many of such techniques of traditional agriculture that require validation, such as use of organic materials (cow dung, oil cakes etc.) for control of plant diseases (Nene, 2003). The present study has been done to develop herbal formulation from Cassia fistula L. fruit pulp extract in combination with neem oil cake and cow dung for control of early blight of potato caused by Alternaria solani.

MATERIALS AND METHODS
Preparation of Extracts

- 100% alcoholic Crude extract of Cassia fistula L. was prepared by according to the modified cold extraction method suggested by Shadowy and Inggraff, (1974). 20 gm dried and powdered plant material was suspended in 100 ml of solvent (100% alcohol) for 24-48 hrs. Whatman filter paper no.1 was used for filtration of suspension then vacuum dried with the help of rotary vacuum evaporator.

- Partially purified chloroform extract was prepared by according to the hot extraction method suggested by Harborne, (1984). 40 gm dry plant powder was kept in Soxhlet extraction unit and extracted with 280 ml solvent.

- 20gm elicitors like neem, mustard and coconut oil cake and binders like cow dung, guar gum and gum acacia were dissolved in 100 ml of autoclaved water for 24 h. The mixture was then filtered and used for antifungal activity.

Antifungal Activity of Elicitors and Binders Individually

10% to 100% solution of elicitors like neem, mustard and coconut oil cake and binders like cow dung, guar gum and gum acacia were prepared and assayed for antifungal activity against Alternaria solani by poison food technique. Various concentrations (10% to 100%) of elicitors and binders were used with the aim to study impact of concentration on antifungal activity.

Preparation of Herbal Formulations

Herbal formulations were prepared by using plant extracts, elicitor and binder in different ratio. 100% of neem oil cake and 100% of cow dung were mix with 100% alcoholic and partially purified extract. All ingredients of herbal formulation were used in following ratio:

www.jprr.org  editor@jprr.org
• Crude extract : elicitor : binder
• Partially purified extract : elicitor : binder

Antifungal Activity of Herbal Formulations

Antifungal activity of 30 formulation ratios was done by poison food technique against Alternaria solani (Groover, & Moore, 1962). 9 ml of molten PDA medium was poured into test tubes and then autoclaved. The molten sterilized medium along with 1 ml of formulation (extract: neem oil cake: cow dung) was placed into Petri plates and in the control set no formulation was used. After the solidification of the media, 6mm inoculum disc of 7 days old culture of the fungus was aseptically inoculated upside down in the centre of the Petri plate and incubated at 25±2°C.

On the 7th day of incubation average diameter of the fungal colonies was measured and percentage of mycelial growth inhibition was calculated by the following formula given below.

\[
\text{% Mycelial growth inhibition} = \frac{\text{gc} - \text{gt}}{\text{gc}} \times 100
\]

Where,

\[
\text{gc} = \text{Growth of mycelial colony after incubation period in control set subtracting the diameter of inoculum disc;}
\]

\[
\text{gt} = \text{Growth of mycelial colony after incubation period in treatment set subtracting the diameter of inoculum disc.}
\]

RESULTS

Results suggested that antifungal activity increased with increasing concentration of elicitors and binders. Maximum activity was observed with 100% concentration. Hence this concentration was used for the preparation of herbal formulation. Among the elicitors and binders 100% neem oil cake and 100% cow dung gave 47.33 % and 31.01 % mycelial growth inhibition respectively showed in figure 1, 2 and 3.

30 ratios of herbal formulations were prepared and assayed for antifungal activity against Alternaria solani. According to results, Among the formulations prepared optimum activity was observed for formulation number 8, 12, 18 and 22 and percent mycelial growth inhibition is 62.92 %, 62.05 %, 92.62 %, 95.24 % respectively against solani showed in table 1 and figure 4. The second highest inhibition showed by formulation number 7, 10, 13, 20, 21, and 29 against Alternaria solani. The inhibitory activity of herbal formulation was compared with standard fungicides like mancozeb and bavistin are presented in table 2. Water used as control in which no herbal formulation and fungicides are present and all data were compared with water. On the basis of results obtained, best herbal formulation will be used for in vivo experiments.
Comparative Analysis of Different Elicitors and Binders and Development of Herbal Formulation Containing Cassia Fistula L. Fruit Pulp Against Alternaria Solani

Figure 1: Antifungal Activity of Oil Cakes (Elicitors) Against Alternaria Solani

Figure 2: Antifungal Activity of Binders Against Alternaria Solani

Figure 3: Antifungal Activity of Neem Oil Cake and Cow Dung

Table 1: In Vitro Antifungal Activity of Herbal Formulation Against Alternaria Solani

<table>
<thead>
<tr>
<th>Ratio No</th>
<th>Formulation Type</th>
<th>Ratio</th>
<th>Growth Diameter after 7 days (mm) ± SD</th>
<th>% Mycelial Growth Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.</td>
<td>100% alcoholic crude: neem oil cake: cow dung</td>
<td>6:2:2</td>
<td>30.03 ± 0.30</td>
<td>60.65</td>
</tr>
<tr>
<td>8.</td>
<td>100% alcoholic crude: neem oil cake: cow dung</td>
<td>4:3:3</td>
<td>28.3 ± 0.2</td>
<td>62.92</td>
</tr>
<tr>
<td>10.</td>
<td>100% alcoholic crude: neem oil cake: cow dung</td>
<td>2:5:3</td>
<td>29.86 ± 0.47</td>
<td>60.88</td>
</tr>
<tr>
<td>12.</td>
<td>100% alcoholic crude: neem oil cake: cow dung</td>
<td>2:6:2</td>
<td>28.96 ± 0.25</td>
<td>62.05</td>
</tr>
<tr>
<td>13.</td>
<td>100% alcoholic crude: neem oil cake: cow dung</td>
<td>3:4:3</td>
<td>30.16 ± 0.32</td>
<td>60.48</td>
</tr>
<tr>
<td>18.</td>
<td>Chloroform extract: neem oil cake: cow dung</td>
<td>3:3:4</td>
<td>5.63 ± 0.32</td>
<td>92.62</td>
</tr>
</tbody>
</table>
Table 1: Contd.,

<table>
<thead>
<tr>
<th>S. No</th>
<th>Chloroform extract : neem oil cake: cow dung</th>
<th>Growth Diameter After 7 Days (mm) ± 5d</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.</td>
<td>5:3:2</td>
<td>9.03 ± 0.47</td>
<td>88.16</td>
</tr>
<tr>
<td>21.</td>
<td>8:1:1</td>
<td>8.66 ± 0.30</td>
<td>88.65</td>
</tr>
<tr>
<td>22.</td>
<td>6:2:2</td>
<td>3.63 ± 0.41</td>
<td>95.24</td>
</tr>
<tr>
<td>29.</td>
<td>4:2:4</td>
<td>8.06 ± 0.51</td>
<td>89.44</td>
</tr>
</tbody>
</table>

Table 2: Antifungal Activity of Standard Fungicides with Water Control Against Alternaria Solani

<table>
<thead>
<tr>
<th>S. No</th>
<th>Standard Fungicides and Water Control</th>
<th>Growth Diameter After 7 Days (mm) ± 5d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mancozeb</td>
<td>14.67 ± 1.52</td>
</tr>
<tr>
<td>2</td>
<td>Bavistin</td>
<td>35.67 ± 1.52</td>
</tr>
<tr>
<td>3</td>
<td>Water</td>
<td>76.33 ± 0.57</td>
</tr>
</tbody>
</table>

DISCUSSIONS

Plants have thousands of constituents and are important sources of new agrochemicals and biologically active molecules show antimicrobial property. Many plant and plant products have been reported as having antimicrobial activities against plant pathogenic fungi (Sokovicet, 2009). Natural products with fungicidal activity have been and are being explored in order to make fungicides which are easily biodegradable, selective and can be locally produced, especially for farmers who cannot afford expensive synthetic fungicides. At present, serious attention is drawn to extracts from higher plants that contain antifungal substances in form of secondary metabolites, which help in resisting the pathogens. The extraction of any plant material with solvent will yield a mixture of compounds. The extract may contain a wide variety of compounds like alkaloids, phenols, tannins, flavonoids, Volatile oils, Saponins and Carbohydrates etc. (Cowan, 1999).

Use and exploitation of preparations based on natural products, which can limit plant pathogens growth comes into higher and higher prominence, especially limiting synthetic fungicide/ traditional chemical application. It results from
equivalent efficiency of bio-preparations to pesticides. Plant preparations have been used for centuries in medicine and pest control. Farmers in India use neem leaves to protect their stored grain from insects. Herbs and spices, such as basil and clove, have been used by many workers to protect food from spoilage, as both have antimicrobial properties (Manohar et al., 2001).

The antimicrobial activities of Cassia fistula plant parts have been studied earlier by many scientists (Hajra et al., 2011; Bhalodia et al., 2012). Significant reduction in growth of pathogen like Fusarium oxysporum, Rhizopus stolonifer by ethanolic leaf extract of cassia fistula has been reported (Hajra et al., 2011). Cassia fistula fruit pulp extract showed antifungal activity against Aspergillus niger, Aspergillus clavatus, Candida albicans (Bhalodia et al., 2012).

Matsuzaki et al., (1998) investigated that soil with cow manure amendments is ideal treatment for decreasing the severity of the disease and improving the final tubers yield of potato. Similar findings were expressed by Davis et al., 1996; Ivanyuk et al., 1996. Reduction in the stem infection has also been noticed when oats preceded potato as a green manure crop (Lootsma and Scholte, 1996). Solarizing soils plus use of appropriate organic materials have also been observed to actuate a chain reaction of chemical and microbial degradation, which increase toxicity against soil flora and fauna, especially soil borne plant pathogens. These possibly contributed to the high nutrient contents, which could be available with organic manure amendment (Gamlid et al., 2000).

Field trials against foliar and tuber disease of potato by using three types of organic material including thermal compost, static wood chips and vermin castings has been conducted by Al-Mughrabi et al. (2006). Infection of R. solani afflicted the normal growth and yield of potato tubers. However treatment with extract was been helpful in begin again normal yield (Khair and Haggag, 2007).

Alternaria solani is one of the most common and destructive pathogen of potato and so far no control practices have been found to manage the potato early blight disease efficiently. Though, a number of chemical compounds have been introduced in recent years to overcome this pathogen, but due to certain limitations including environmental pollution, mutagenic deterioration and ecotoxicological effects, no one could be known as ideal for effective and safe management of potato early blight. Feeling the gravity of scenario, a rapid upsurge for the development of herbal formulation has been observed in recent years to manage the harmless decline of potato crop. Several studies have pointed out the potential of neem (A. indica) tree to control plant pathogenic fungi that could be listed it as top fungicide and harmless bio-control agent (Abbasi et al., 2003; Dubey et al., 2009). In present study, we examined the herbal formulation which contains Cassia fistula fruit pulp crude and partially purified extract, neem oil cake and cow dung against Alternaria solani to manage potato crop.

CONCLUSIONS

Best herbal formulation will be used for in vivo experiments. The results of the present studies would suggest that use of herbal formulation from Cassia fistula L. fruit pulp extract holds promise control of early blight of potato compared to fungicides which are costly and hazardous.

ACKNOWLEDGMENTS

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REFERENCES


Traditional herbal medicines are moving from fringe to mainstream use with a large number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. The *Cassia fistula* is used to cure haematemesis, constipation, chlorosis, urinary disorders, biliousness, rheumatic condition, wounds, ulcers, skin diseases, diabetes. The present study was aimed to investigate the preliminary phytochemical screening of *Cassia fistula* fruit pulp. The fruit pulp extract of *Cassia fistula* were prepared using different solvents like Petroleum ether, Benzene, Chloroform, Acetone, Alcohol, Methanol, and Water. The phytochemical screening of the fruit pulp extracts was performed. The thin layer chromatography (TLC) of Chloroform extract was studied. The extracts were analyzed for the presence and absence of alkaloids, steroids, volatile oils, tannins, carbohydrates, flavonoids and saponins. After derivatization seven bands were found in TLC plate of chloroform fraction. Rf value of bands observed for this fraction lies between 0.12 to 0.75. These studies provided referential information in regard to its identification parameters assumed significantly in the way of acceptability of herbal drugs in present scenario of lack of regulatory laws to control quality of herbal drugs.

**Key-Words:** *Cassia fistula*, fruit pulp, phytochemical screening, TLC, Secondary metabolites

### Introduction
India is considered to be a country having rich emporia of medicinal plants and where ancient systems of medicine such as Ayurveda, Siddha and Unani medicines have been in practice for many years. Ayurveda (4000-600 B.C.), Rigveda (4500-1600 B.C.) and Atharvaveda (1200 B.C.) are traditional indigenous systems of medicines (Mahesh B, Satish S., 2008). Ayurveda literally means “Science of life”. According to Ayurveda, health is an indication of normal biological processes, which help to maintain mental and physical alertness and happiness of human beings (Sukh dev 1997). Charak Samhita is the first recorded treatise on Ayurveda which was followed by Sushruta Samhita around 900 B.C. charak samhita dealt primarily with medicine while Sushruta Samhita was concerned with the advanced state of knowledge on the general principles and details of treatment (Solecki, 1975; Kirtikar and Basu, 1984; Sharma et al., 2008). Each plant species in this universe has its own specific set of secondary metabolites and these secondary metabolites are widely present in medicinal herbs and plants (Harborne, 1984). Sandhu and Arora (2000) have reported that these secondary metabolites are responsible for antimicrobial activity of plant extracts.

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These secondary metabolites such as phenols, flavonoids, quinones, essential oils, alkaloids, sterols, thymol, coumarines and triterpenoids are untapped reservoirs of various valuable chemicals (Lefevre et al., 2008). Natural plant-based remedies are used for both acute and chronic health problems, from treating common colds to controlling blood pressure and cholesterol. Herbal plant formulations also have preventive effect against plant pathogenic microbes. *Cassia fistula* (Linn.) belongs to family Fabaceae and Sub–family Caesalpinioideae is a very common plant known for its medicinal properties are a semi-wild in nature. It is distributed in various regions including Asia, South Africa, China, West Indies and Brazil. It is commonly known as Amaltas and in English popularly called “Indian Laburnum” has been extensively used in Ayurvedic system of medicine for various ailments. It is deciduous and mixed-monsoon forests throughout greater parts of India, ascending to 1300 m in outer Himalaya, is widely used in traditional medicinal system of India (Prashanth et al., 2006; Gupta, 2010). It is a deciduous tree with greenish grey bark, compound leaves, leaflets are each 5-12 cm long pairs. It is a semi-wild tree known for its beautiful bunches of yellow flowers and also used in traditional medicine for several indications. A fruit is cylindrical pod and seeds many in black, sweet pulp separated by
transverse partitions. The long pods which are green, when unripe, turn black on ripening after flowers shed. Pulp is dark brown in colour, sticky, sweet and mucilaginous. (Danish et al., 2011; Bhalerao and Kelkar, 2012).

*C. fistula* is widely used in traditional medicines for various medicinal properties. The pulp of the ripe pods possesses a mild, pleasant purgative action (Bahorun et al., 2005). Various biological activities of the pod pulp such as antibacterial, antifungal, antioxidant, antileishmanial, and hypolipidemic activity were reported (Duraipandiyan and Ignacimuthu, 2007; Siddhuraju et al., 2002; Satorelli et al., 2007; Gupta and Jain, 2009).

In Ayurvedic medicinal system, *C. fistula* was used against various disorders such as haematemeses, pruritus, constipation, chlorosis, urinary disorders, biliousness, rheumatic condition, wounds, ulcers, skin diseases, diabetes, and other ailments (Alam et al., 1990; Asolkar et al., 1992).

Combined knowledge of biological activity and chemical constituents of the plant is desirable for discovery of new class of compounds. Thin layer chromatography (TLC) is routinely used as a valuable tool for qualitative determination of small amounts of impurities. Molecular markers generally refer to biochemical constituents, including primary and secondary metabolites and other macromolecules such as nucleic acids (Kalpana, et al., 2004). TLC has been used as a broad spectrum screen for detection of drug abuse. TLC results are only qualitative and cannot be quantified (Andrew et al., 1988; Jones and Gierasch, 1994).

In the present study partially purified extracts of *Cassia fistula* fruit pulp were subjected to rapid qualitative phytochemical tests for confirming the presence of primary and secondary metabolites. TLC was used to isolate the active compound from most effective fraction.

**Material and Methods**

**Collection of plant material**

The healthy, infection free, mature pods were collected from the campus of University College of Science, Mohanlal Sukhadia University, Udaipur, Rajasthan, India. The herbarium specimen was identified from Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India. Where a voucher number RUBL 211505 specimen was deposited. The pods were shade dried at room temperature and broken with the help of a pestle to extract out the pulp. The pulp was grounded in an electrical grinder after removal of the seeds from the pulp. The ground material was passed through sieve of mesh size 60 to obtain a fine powder which was used to prepare the extract.

**Preparation of extracts**

Reflux method of solvent extraction was used for successive separation of different partially purified organic constituents present in dried plant material (Harborne, 1984). Solvent series used for successive separation was as follows:

- Petroleum ether → Benzene → Chloroform → Acetone → Alcohol → Methanol → Water

40 gm dry fruit pulp powder was kept in Soxhlet extraction unit and extracted with 280 ml petroleum ether till all petroleum ether soluble fractions was extracted. Residue was dried in an oven below 50°C and used for extraction with next solvent in series. Fractions obtained at every step including aqueous fraction were vacuum dried in a rotary evaporator and the dried residue was used as extract.

**Phytochemical study of fruit pulp extracts**

Qualitative methods were used for the identification of different secondary metabolites or phytochemicals present in the plant extracts. Various fractions of fruit pulp obtained by successive extraction were then subjected to qualitative test suggested by Kokate et al., 1990. The extracts were analyzed for the presence and absence of alkaloids, steroids, volatile oils, tannins, carbohydrates, flavonoids and saponins.

**Tests for Detection of Secondary Metabolites**

**Alkaloids**

Alkaloids are compounds having one or more nitrogen containing heterocyclic ring. Presence of alkaloids in the partially purified fractions was tested by performing Mayer's test or Wagner's test or Hager's test. Reaction with Mayer's reagent produces a cream coloured precipitate; Hager's reagent gives yellow precipitate while Wagner's reagent results in formation of reddish brown precipitate. Small amount of extract was stirred with few drops of dilute HCl and filtered. The filtrate was tested with various alkaloid reagents and observed for development of coloured precipitate.

**Volatile Oils**

The odorous volatile chemical constituents of plants are known as volatile or essential oils. Sudan III test was used to detect presence of volatile oils. Development of red colour on mixing with Sudan III indicates the presence of volatile oils. Small amount of extract was mixed with Sudan III dye and observed for development of red colour.

**Tannins**

Chemically, tannins contain the mixture of complex organic substances in which polyphenols are present. Development of green colour indicates the presence of condensed tannins whereas blue colour indicates the presence of hydrolysable tannins.
Small amount of extract was taken and treated with alcohol FeCl₃ solution and observed for colour development.

**Saponin**
Saponins are complex glycoside compounds in which the a glycone is triterpenoid or steroidal in nature. Foam test was used to detect presence of saponin. Small amount of extract was diluted with 20 ml of distill water; then shaken in graduated cylinder for 15 minutes. Formation of a layer of foam at surface indicates the presence of saponin.

**Carbohydrates**
Carbohydrates are widely distributed in plants and can be detected by Molish’s test and Fehling’s test. Small amount of extract was dissolved in 5 ml distilled water and filtered. Development of purple colour on addition of few drops of α-napthol and conc. H₂SO₄ to the filtrate indicates presence of sugars. Similarly small quantity of filtrate was heated with equal amount of Fehling A and Fehling B solution. Development of brick red colour indicates the presence of carbohydrates.

**Flavonoids**
Flavonoids usually occur in plants as glycosides in which one or more of phenolic hydroxyl groups are combined with sugar residues. Alkaline reagent test was used to detect flavonoids. Small amount of extract was mixed with aqueous NaOH. Development of reddish brown colour shows presence of flavonoids.

**Sterols**
Sterols are triterpenes which are based on cyclopentane perhydroxy phenanthrene ring system. They are also called as phytosterols; Liebermann’s Burchard test was used for detection of phytosterols. Small amount of extract was mixed with 2 ml CHCl₃ and 1 ml of acetic anhydride. Subsequently concentrated H₂SO₄ was added gradually through the side of the test tube. Formation of brown coloured ring at junction of two layers indicates the presence of sterols.

**Development of solvent system for TLC (Thin layer chromatography)**
TLC is used for separation of mixtures and identification of constituents from extract using different solvents. A number of solvent and solvent mixtures were tried for separation of constituents from chloroform extract of *Cassia fistula* fruit pulp.

**TLC Fingerprinting of chloroform extract**
TLC fingerprinting of chloroform fraction of fruit pulp performed using precoated silica gel 60 F254 TLC plates (E-Merck) of uniform thickness (20mm x 20mm). A 10 cm length of TLC plate was cut and marked carefully. 10µl of plant extract was spotted onto the marked plate with the help of a capillary tube or pipette. Chloroform: ethyl acetate: ethanol: acetic acid (5 ml: 5 ml: 3 ml: 10 µl) was used as mobile phase. The TLC plate was kept in a chromatographic chamber containing the solvent system and the chamber was covered with glass plate to prevent evaporation of solvent. The plate was allowed to remain in the chamber till the solvent reached up to 8 cm distance. The plate was then observed at short and long wavelengths under UV-fluorescence analysis cabinet.

**Visualization of TLC Plate**
The TLC finger printing was derivatized with anisaldehyde sulphuric acid reagent followed by heating at 100°C till coloured bands of various secondary metabolites appeared. The observations were taken before and after derivatization, in visible as well as ultraviolet light. Rf values were calculated as follows:

\[
Rf = \frac{\text{Distance traveled by substance}}{\text{Distance traveled by solvent}}
\]

**Results and Discussion**
Observation of Phytochemical screening of partially purified fractions of *Cassia fistula* fruit pulp showed in table 1. Volatile oils were present in benzene, chloroform, acetone and alcohol fractions and flavonoids in benzene, chloroform, acetone and methanol fractions. Saponins were found only in petroleum ether and benzene fractions. Carbohydrates were present in benzene, chloroform and aqueous fractions. Alkaloids were absent in all fractions while steroids were present in chloroform and aqueous fractions. Only acetone fraction showed presence of tannins. Presence of anthraquinone glycosides, sennosides A & B, rhein and its glucoside, barbaloin, aloin, formic acid, butyric acid and their ethyl esters and oxalic acid, pectin and tannin in *Cassia fistula* fruit pulp has been reported by Agarwal and Paridhavi, (2005); Khare, (2007).

Ellof (1998) reported that tannins, saponins polypeptides and reducing sugars are soluble in water whereas terpenoids, flavonoids, alkaloids, and fatty acids are soluble in organic solvents. Similar findings have been reported by several workers (Scalbert, 1991; Zhang and Lewis, 1997). Tannins and reducing sugars are soluble in both water as well as organic solvents but their solubility is more in organic solvents as compared to water. Harborne (1984), Kokate et al., (1990) suggested that extraction of secondary metabolites from plant material by hot extraction with petroleum ether separates sterols, waxes and fatty acids.
leaving behind residue containing the defatted plant materials. Subsequently extraction of this residue with benzene separates out sterols and flavonoids. Terpenoids and flavonoids get extracted with chloroform. The last solvents i.e. alcohol removes alkaloids, flavonoids, polyphenols, tannins and reducing sugar from residue. Finally extraction with water yields remaining water-soluble metabolites such as anthocyanin, starch, tannins, saponins, reducing sugar and polypeptides (Scalbert, 1991; Zhang and Lewis, 1997). All the active principles present in plants are saturated organic compound so they get extracted in ethanol or methanol (Cowan, 1999).

The extraction of any plant material with solvent will yield a mixture of compounds. The extract may contain a wide variety of compounds like alkaloids, phenols, tannins, flavonoids, Volatile oils, Saponins and Carbohydrates etc. for the separation and identification of mixtures of constituents from partially purified extract, TLC is commonly performed using different combination of solvents. Higher the retention speed or the low retention time on TLC, better the solvent would be and vice versa. Among the different solvent system (table-2) for TLC of fruit pulp of Cassia fistula, Chloroform: ethyl acetate: ethanol: acetic acid in ratio of 5 ml: 5 ml: 3 ml: 10 µl appeared as ideal solvent for resolution of maximum number of constituents. After derivatization of TLC, seven bands were observed for chloroform fraction of Cassia fistula fruit pulp. Changes in the colour of bands suggest that there is presence of different secondary metabolites in extract. Rf value of bands observed for this fraction lies between 0.12 to 0.75. The Rf value and colour of bands of chloroform fraction has been summarized in table-3, figure-1 and 2.

TLC profiling of chloroform extracts gives an impressive result that directing towards the presence of number of phytochemical. The TLC method is best choice for the identification of secondary metabolite present in plants. Here the different Rf values indicate the presence of different nature of phytoconstituents in single extracts. Different Rf values of the compound also reflects an idea about their polarity. This information will help in selection of appropriate solvent system for further separation of compound from these plant extracts.

Conclusion

In the present study the qualitative tests of extracts showed significant indication about the presence of metabolites. Preliminary phytochemical investigations tests are useful to isolate the pharmacologically active principles present in the plant. Cassia fistula is known as a rich source of tannins, flavonoids and glycosides present in it, might be medicinally important and/or nutritionally valuable. It is an important medicinal plant with diverse pharmacological spectrum. The plant shows the presence of many chemical constituents which are responsible for varied pharmacological and medicinal property. The evaluation needs to be carried out on Cassia fistula in order to uses and formulation of the plant in their practical clinical applications, which can be used for the welfare of the mankind and formation of herbal drugs.

Acknowledgement

One of the authors (Deepa Hada) is thankful to University Grant Commission (UGC), New Delhi, India, for providing financial assistance.

References

10. Eloff J.N. (1998). Which extract should be used for the screening and isolation of


Table 1: Phytochemical screening of various fractions of *Cassia fistula* fruit pulp extract

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Alkaloids</th>
<th>Steroids</th>
<th>Volatile oils</th>
<th>Tannins</th>
<th>Carbohydrates</th>
<th>Flavonoids</th>
<th>Saponins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Benzene</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Chloroform</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Acetone</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Alcohol</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Methanol</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Aqueous</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

+ve = Presence, -ve = Absence

Table 2: Composition and Resolution of developing solvent system for TLC

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Solvent system</th>
<th>Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Petroleum ether</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>2.</td>
<td>Toluene</td>
<td>Not good</td>
</tr>
<tr>
<td>3.</td>
<td>Chloroform</td>
<td>Significant</td>
</tr>
<tr>
<td>4.</td>
<td>n- butanol</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>5.</td>
<td>Ethyl acetate</td>
<td>Significant</td>
</tr>
<tr>
<td>6.</td>
<td>Acetone</td>
<td>Not good</td>
</tr>
<tr>
<td>7.</td>
<td>2- propanol</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>8.</td>
<td>Ethanol</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>9.</td>
<td>Methanol</td>
<td>Good</td>
</tr>
<tr>
<td>10.</td>
<td>Water</td>
<td>Not good</td>
</tr>
<tr>
<td>11.</td>
<td>Acetic acid</td>
<td>Not good</td>
</tr>
<tr>
<td>12.</td>
<td>Chloroform: ethyl acetate</td>
<td>Not good</td>
</tr>
<tr>
<td>13.</td>
<td>Chloroform: ethyl acetate: ethanol</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>14.</td>
<td>Chloroform: ethyl acetate: ethanol: acetic acid</td>
<td>Significant</td>
</tr>
</tbody>
</table>

Table 3: Rf Values of TLC Fingerprinting of chloroform fraction of *Cassia fistula* fruit pulp

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Total number of Bands</th>
<th>Colour of Bands</th>
<th>Rf Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before Derivatization</td>
<td>After Derivatization</td>
</tr>
<tr>
<td>1.</td>
<td>7 Bands</td>
<td>Light brown</td>
<td>Dark brown</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>Light green</td>
<td>Dark green</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>Light yellow</td>
<td>Dark yellow</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>Light brown</td>
<td>Dark brown</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td>Light green</td>
<td>Dark green</td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td>Light pink</td>
<td>Dark pink</td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td>Dark green</td>
<td>Dark green</td>
</tr>
</tbody>
</table>
Fig. 1: TLC plate of chloroform extract (Before derivatization)

Fig. 2: TLC plate of chloroform extract (After derivatization)

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In the present study antifungal activity of crude and partially purified extracts of *Cassia fistula* L. fruit pulp has been assayed against *Alternaria solani* which is responsible for early blight of potato. Cold and hot extracts of fruit pulp was prepared in different organic solvents, which were subsequently recycled by rotary vacuum evaporator. Antifungal activity of different fractions was determined by poison food technique. Maximum percent extractive value was obtained with alcoholic extract. Maximum inhibitory activity was observed with 100% alcohol crude extract and partially purified chloroform extract against *Alternaria solani*. Mancozeb and bavistin were used as standards. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of chloroform fraction of *Cassia fistula* fruit pulp was investigated against *Alternaria solani*. Results suggest that *Cassia fistula* L. fruit pulp extract can be used to develop a biocontrol agent against *Alternaria solani*. The antifungal activity of the *Cassia fistula* was due to the presence of various secondary metabolites. Hence, this plant can be used to discover bioactive natural products that may serve as leads in the development of new pharmaceuticals research activities.

**Keywords:** Antifungal activity, Cold extraction, Hot extraction, Poison food technique, MIC, MFC.

**INTRODUCTION**

Drugs derived from natural sources play a significant role in the prevention and treatment of diseases. In many countries, traditional medicine is one of the primary health care systems [1]. Herbs are widely exploited in the traditional medicine and their curative potentials are well documented. Natural products of higher plants, may give a new source of antimicrobial agents with possibly novel mechanisms of action [2].

In the recent years, researches on medicinal plants have attracted a lot of attention globally. Evidences have been accumulated to demonstrate the promising potential of medicinal plants used in various traditional, complementary, and alternative systems of treatment of human diseases. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, and glycosides etc., which have been found in vitro to have antimicrobial properties [3]. Herbal medicines have been known to man since centuries. Therapeutic efficacy of many indigenous plants for several disorders has been described by practitioners of traditional medicine. Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. Traditional medicine continues to be a valuable source of remedies that have been used by millions of people around the world to secure their health [4].

*Alternaria solani* is the causal agent of early blight of potato that leads to major damages to potato crop. It is a major foliar disease of potato crop and losses due to early blight typically are around 20-25%; however, there have been cases of 70-80% losses [5]. It produces irregular to circular dark brown spots on the lower (older) leaves, excessive defoliation may lead to death of the plant and consequent yield loss [6]. *A. solani* overwinters as mycelium or conidia in plant debris, soil, infected tubers or on other host plants of the same family. The disease is controlled primarily through the use of cultural practices such as resistant cultivars and foliar fungicides, crop rotation, removal and burning of infected plant debris, and eradication of weed hosts helps reduce the inoculum level for subsequent plantings [7]. The most common and effective method for the control of early blight is through the application of foliar fungicides. Protectant fungicides recommended for early blight control are maneb, mancozeb, chlorothalonil, and triphenyl tin hydroxide [8]. But negative side of the use of synthetic fungicides is that they are harmful to human, soil as well as wildlife health, and enter the food chain and cause several deleterious effects on biosphere, contributing to significant declines in populations of beneficial soil organisms, soil acidification, and diminished resistance to diseases [9]. Natural plant products are important sources of new agrochemicals for the control of plant diseases. A search for an environmentally safe and economically viable strategy for the control of diseases has led to an increased use of plant based products in agriculture [10].

*Cassia fistula* (Linn.) belongs to family Fabaceae and Sub-family Caesalpinoideae is a very common plant known for its medicinal properties are a semi-wild in nature. It is distributed in various regions including Asia, South Africa, China, West Indies and Brazil. It is commonly known as Amaltas and in English popularly called “Indian Laburnum” has been extensively used in Ayurvedic system of medicine for various ailments. It is deciduous and mixed monsoon forests throughout greater parts of India, ascending to 1300 m in outer Himalaya, is widely used in traditional medicinal system of India [11]. There are several reports of antimicrobial activities of *Cassia fistula* [12, 13, 14]. The present paper reports the antifungal activity of crude and partially purified extracts of *Cassia fistula* L. against *Alternaria solani*.

**MATERIALS AND METHODS**

**Collection of plant material**

The healthy, infection free, mature pods were collected from the campus of University College of Science, Mohanlal Sukhadia University, Udaipur in May-June 2013, and were dried in shade. The pods were broken with the help of a pestle to extract out the pulp. The pulp was ground in an electrical grinder after removal of the seeds from the pulp. The ground material was passed through sieve of mesh size 60 to obtain a fine powder which was used to prepare the extract.

**Isolation of pathogenic fungus**

*Alternaria solani* was isolated from infected leaves of potato by single spore inoculation method. Fungus was purified; pure culture was maintained on PDA (Potato dextrose agar) and stored in refrigerator at 4 °C.

**Inoculum disc**

Seven day old culture of the test fungus was used for the preparation of inoculum disc of 6 mm in diameter.
Preparation of Plant extract

**Cold Extraction**

Crude extract was prepared by according to the modified cold extraction method suggested by Shadomy and Ingraff [15]. 100% alcohol, 50% alcohol as well as 100% aqueous extract of fruit pulp was prepared by dissolving 20 gm dried and powdered plant material in 100 ml of solvent (alcohol/ water) for 24 h. The suspension was filtered through Whatman filter paper no.1 then vacuum dried with the help of rotary vacuum evaporator. The dried residue was used as extract and solvent was recycled.

**Hot Extraction**

Reflex method of solvent extraction was used for successive separation of different partially purified organic constituents present in dried plant material [16]. Solvent series used for successive separation was as follows:

Pet. ether → Benzene → Chloroform → Acetone → Alcohol → Methanol → Water

This method involves continuous extraction of powdered dried plant material in soxhlet apparatus with a series of organic solvents. Each time before extracting with next solvent the plant material was dried in an oven below 50°C. 40 gm dry plant powder was kept in Soxhlet extraction unit and extracted with 280 ml solvent.

**Percent Extractive Value**

Crude extract and fractions obtained at every step including aqueous fraction were vacuum dried in a rotary evaporator. The dried extract and fractions were weighed and their percentage in terms of the dry weight of the plant material was estimated by the following formula given below:

\[
\text{Percent extractive} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant material}} \times 100
\]

**Antifungal activity of Plant extracts**

Antifungal activity of crude 100% alcohol, 50% alcohol as well as 100% aqueous extract and partially purified fractions of fruit pulp of *Cassia fistula* against *Alternaria solani* was done by Poison food technique [17]. 100 mg of extract was dissolved in 10 ml solvent to prepare stock solution of 10 mg/ml concentration. 9 ml of molten PDA medium was poured into test tubes and then autoclaved. The molten sterilized medium along with 1 ml of stock solution was placed into Petri plates and in the control set no extract was used. After the solidification of the media, 6 mm inoculum disc of 7 days old culture of the fungus was aseptically inoculated upside down in the centre of the Petriplate and incubated at 25±2°C. The average diameter of the fungal colonies was measured on the 7th day of incubation and percentage of mycelial growth inhibition was calculated by the following formula given below.

\[
\text{Mycelial growth inhibition} = \frac{gc-gt}{gc} \times 100
\]

Where, $ge = \text{Growth of mycelial colony after incubation period in control set}$ subtracting the diameter of inoculums disc; $gt = \text{Growth of mycelial colony after incubation period in treatment set}$ subtracting the diameter of inoculum disc.

**Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)**

Minimum inhibitory concentration (MIC) was determined by broth dilution method [18]. Potato dextrose broth (PDB) was used for determining inhibitory activity. 200 mg of the extract was dissolved in 10 ml of acetone to prepare stock solution of 20 mg/ml. Two fold serial dilution method was used for the preparation of 10 mg/ml to 0.0195 mg/ml concentration from the stock solution. Thus prepared concentration was serially diluted with sterile broth medium to attain final concentration of 1000 μg/ml to 1.95 μg/ml. All these tubes were than respectively inoculated with 100 μl of spore suspension (1×10⁶ spores/ml) and incubated at 25 ± 2°C for 72 h. One tube containing extract free autoclaved medium was used as control. Three replicates of each concentration were maintained and experiment was repeated thrice. A loopful of fungal biomass from each tube containing 9 ml broth medium and MIC as well as all concentrations were streaked onto the surface of sterile PDA slants and incubated at 25 ± 2°C for 72 h. Presence or absence of growth was observed after respective incubation time. Appearance of growth indicates that the extract concentration is just fungistatic and absence of growth indicates that extract concentration is fungicidal.

**RESULTS AND DISCUSSION**

Plants have a variety of secondary as tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosides and volatile oils. These metabolites play very important role in defense against insects, herbivores and microorganisms [19, 20]. The antifungal activity of different organic solvent extracts of *Cassia fistula* was reported by Hajra et al. [15].

In the present study *C. fistula* fruit pulp extracts prepared in water, alcohol and various organic solvents were screened for antifungal activity. Results of percent extractive value of various fractions of crude and hot extracts are depicted in table 1 and 2. Maximum percent extractive value was obtained with 50% alcoholic crude extract i.e. 56.05% and alcohol fraction of partially purified extract i.e. 28.5%. Results of antifungal activity of crude as well as partially purified extracts of fruit pulp against *Alternaria solani* are presented in table 3 and 4. The inhibitory activity of the extracts was compared with standard fungicides like mancozeb and bavistin are presented in table 5.

According to results, best antifungal activity was observed with 100% alcoholic crude extract and partially purified chloroform fraction against *A. solani* and % mycelial growth inhibition is 53.27% and 93.88% respectively given in table 3, 4. The second highest inhibition showed by 100% aqueous crude extract and benzene, petroleum ether, acetone fractions of partially purified extracts. 50% alcoholic crude extract and alcohol, methanol and water fractions of partially purified extracts showed less inhibition against *A. solani*. All data were compared with water as control in which no plant extract and fungi used.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Type of Extract</th>
<th>Weight of dried extract (gm)</th>
<th>% Extractive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100% alcohol</td>
<td>4.59</td>
<td>22.9</td>
</tr>
<tr>
<td>2</td>
<td>100% aqueous</td>
<td>9.81</td>
<td>49.05</td>
</tr>
<tr>
<td>3</td>
<td>50% alcohol</td>
<td>11.21</td>
<td>56.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Type of Extract</th>
<th>Weight of dried extract (gm)</th>
<th>% Extractive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Petroleum ether fraction</td>
<td>0.05</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>Benzene fraction</td>
<td>0.1</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>Chloroform fraction</td>
<td>0.06</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>Acetone fraction</td>
<td>0.43</td>
<td>1.07</td>
</tr>
<tr>
<td>5</td>
<td>Alcohol fraction</td>
<td>11.4</td>
<td>28.5</td>
</tr>
<tr>
<td>6</td>
<td>Methanol fraction</td>
<td>3.33</td>
<td>8.32</td>
</tr>
<tr>
<td>7</td>
<td>Water fraction</td>
<td>4.02</td>
<td>10.05</td>
</tr>
</tbody>
</table>
REFERENCES

CONFLICT OF INTERESTS

ACKNOWLEDGMENT

One of the authors (Deepa Hada) is thankful to University Grant Commission (UGC), New Delhi, India, for providing financial assistance.

CONFLICT OF INTERESTS

Declared None

REFERENCES

7. Raju RA. Transformation of Herbicidal Technology Chemical based to Ecological Concern. Published by Daye publishing house, Delhi; 2001.

The results of MIC and MFC of chloroform fraction of Cassia fistula fruit pulp was observed at 2.5 mg/ml and 5 mg/ml respectively.

Table 3: Antifungal Activity of Crude Extract of Cassia fistula Fruit pulp against A. solani

<table>
<thead>
<tr>
<th>S. No</th>
<th>Type of Extract</th>
<th>Growth Diameter after 7 days (mm) ± SD</th>
<th>% Mycelial growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100% alcohol</td>
<td>35.67 ± 1.52</td>
<td>53.27</td>
</tr>
<tr>
<td>2</td>
<td>100% aqueous</td>
<td>43.33 ± 1.15</td>
<td>43.23</td>
</tr>
<tr>
<td>3</td>
<td>50% alcohol</td>
<td>47 ± 1.73</td>
<td>38.42</td>
</tr>
</tbody>
</table>

Table 4: Antifungal Activity of Various Partially Purified Fractions of Cassia fistula Fruit pulp Extract against A. solani

<table>
<thead>
<tr>
<th>S. No</th>
<th>Type of Extract</th>
<th>Growth Diameter after 7 days (mm) ± SD</th>
<th>% Mycelial growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Petroleum ether</td>
<td>13.67 ± 1.15</td>
<td>82.09</td>
</tr>
<tr>
<td>2</td>
<td>Benzene</td>
<td>9 ± 1.00</td>
<td>88.20</td>
</tr>
<tr>
<td>3</td>
<td>Chloroform</td>
<td>4.67 ± 0.57</td>
<td>93.88</td>
</tr>
<tr>
<td>4</td>
<td>Acetone</td>
<td>17.67 ± 1.15</td>
<td>76.85</td>
</tr>
<tr>
<td>5</td>
<td>Alcohol</td>
<td>44.67 ± 1.15</td>
<td>41.40</td>
</tr>
<tr>
<td>6</td>
<td>Methanol</td>
<td>46.33 ± 2.00</td>
<td>39.30</td>
</tr>
<tr>
<td>7</td>
<td>Water</td>
<td>54.67 ± 1.15</td>
<td>28.37</td>
</tr>
</tbody>
</table>

Table 5: Antifungal Activity of standard fungicides with water control against A. solani

<table>
<thead>
<tr>
<th>S. No</th>
<th>Standard fungicides and water control</th>
<th>Growth Diameter after 7 days (mm) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mancozeb</td>
<td>14.67 ± 1.52</td>
</tr>
<tr>
<td>2</td>
<td>Bavistin</td>
<td>35.67 ± 1.52</td>
</tr>
<tr>
<td>3</td>
<td>Water</td>
<td>76.33 ± 1.15</td>
</tr>
</tbody>
</table>

Harborne, Cowan and Kolate et al., suggested that successive extraction of plant secondary metabolites should be done in petroleum ether followed by benzene, chloroform, acetone, alcohol, methanol and finally with water i.e. from non polar to polar solvents [16,19,21]. Extraction of secondary metabolites from plant material by hot extraction with petroleum ether separates sterols, waxes and fatty acids leaving behind residue containing the defatted plant materials. Subsequent extraction of this residue with benzene separates out sterols and flavonoids. Terpenoids and flavonoids get extracted with chloroform. The last solvent i.e. alcohol extracts alkaloids, flavonoids, polyphenols, tannins and reducing sugar from the residue. Finally extraction with water yields remaining water soluble metabolites such as anthocyanins, starch, tannins, saponins, reducing sugars and poly peptides [22,23].

CONCLUSION

The present study proved that antimicrobial properties of Cassia fistula fruit pulp on Alternaria solani. In the current investigations, 100% alcoholic crude extract and partially purified chloroform extract of Cassia fistula fruit pulp was found to be active on Alternaria solani as compared to standard fungicides. Further studies are necessary to isolate and reveal the active compounds of the extract. Further, purifications of these potent partially purified fractions will be used to develop natural fungicides which will do not have any environmental hazards.

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