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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 solania, Cassia fistula*

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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 action[1] (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 bacteria[2] (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 membrane[3] (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 biliousness[4] (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 *Alternaria*[5] (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 reproduces asexually by means of conidia these Spores or conidia are the primary agent for infecting host plants for many plant pathogenic fungi[6] (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 survival[7] (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[8]. Minimum Inhibitory Concentration (MIC) of this extract was determined by broth dilution method[9] (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 rotary 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 F_{254} 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. R_f value of the extracted secondary metabolites were calculated as follows:

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

Assay of Antifungal Activity of column fractions

Antifungal activity of various column fractions against *Alternaria solani* was done by Poison food technique[10] (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±2ºC. 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.

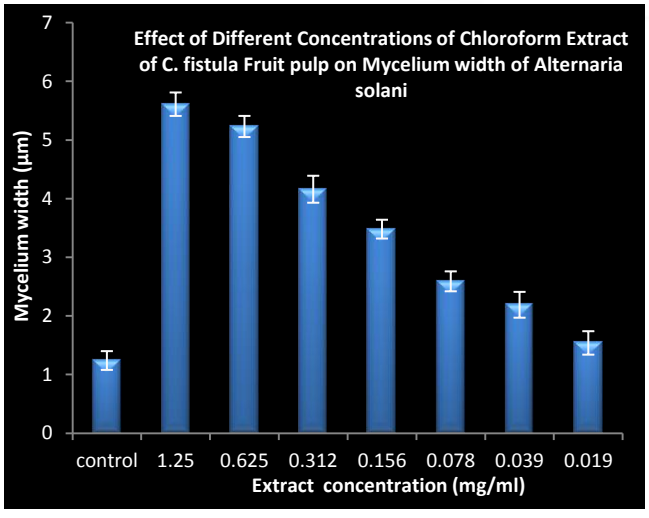

Figure 1: Effect of Different Concentrations of Chloroform Extract of *Cassia fistula* Fruit pulp on Mycelium width of *Alternaria solani*

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.

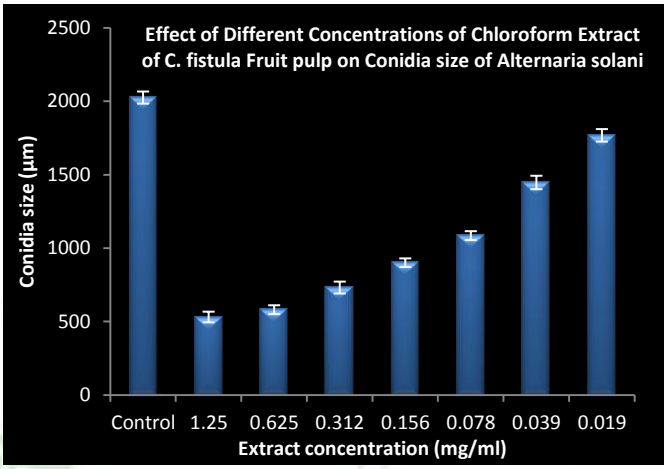

Figure 2: Effect of Different Concentrations of Chloroform Extract of *Cassia fistula* Fruit pulp on Conidia size of *Alternaria solani*

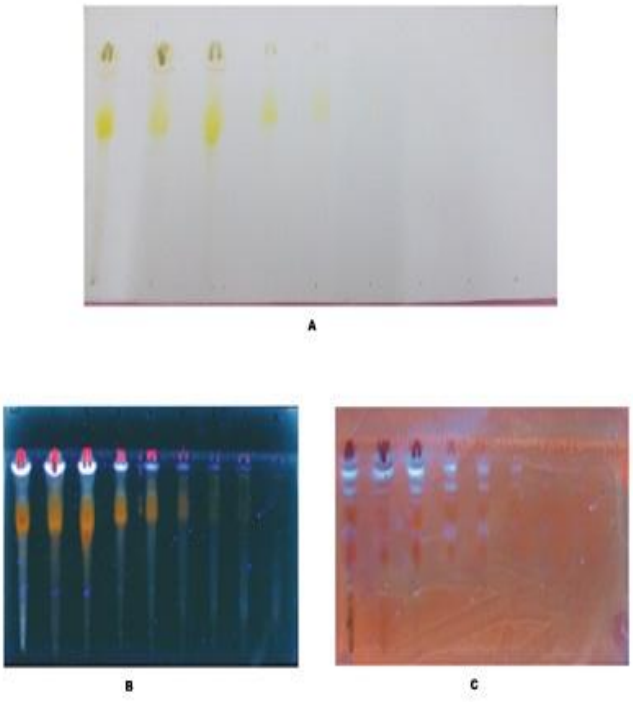

Figure 3: Thin Layer Chromatography (TLC) of Various Column Fractions of Chloroform Extract of *Cassia fistula* Fruit pulp

A: Under visible light
B: Under UV light (Before derivatization)
C: Under UV light (After derivatization)

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).

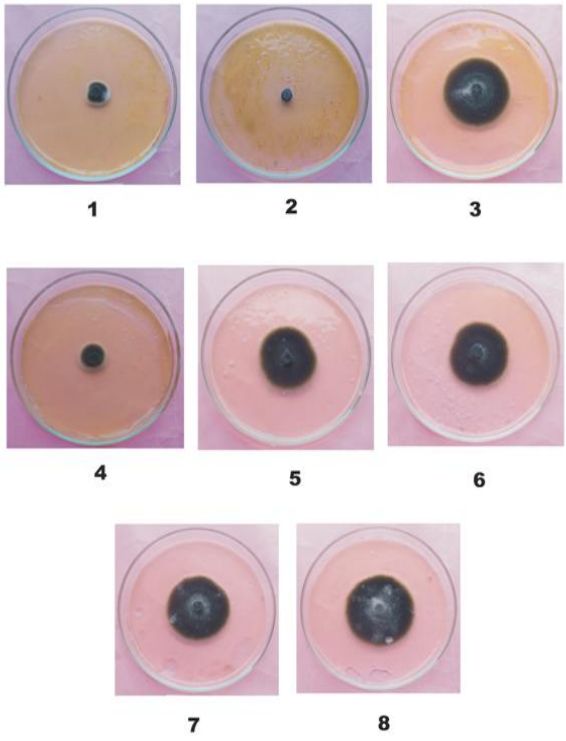

Figure 4: Antifungal Activity of Column Fractions (1-8 Numbers) of Chloroform Extract of *Cassia fistula* Fruit pulp against *Alternaria solani*

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.

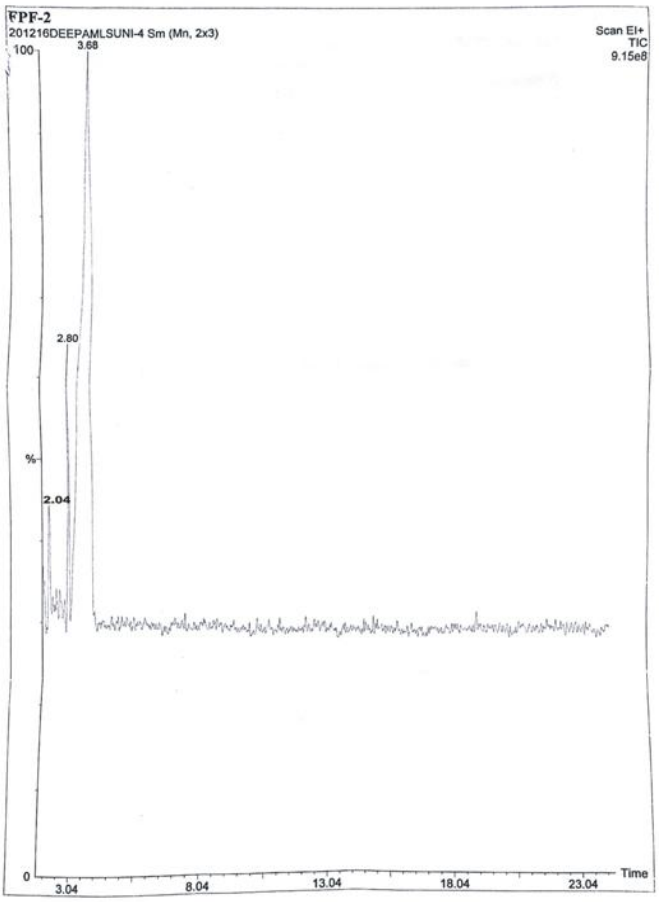

Figure 5: Gas Chromatography- Mass Spectrometry Analysis of Column Fraction no. 2 of Chloroform Extract of *Cassia fistula* Fruit pulp

Isopropylacetate (Acetic acid, 1-methylethyl ester)

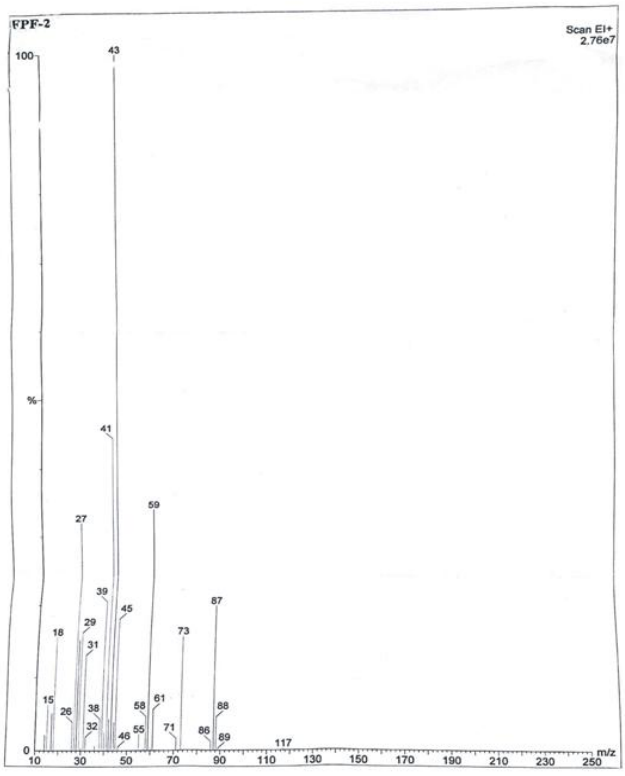

Figure 6: Mass Spectrum of Isopropylacetate (Acetic acid, 1-methylethyl ester)

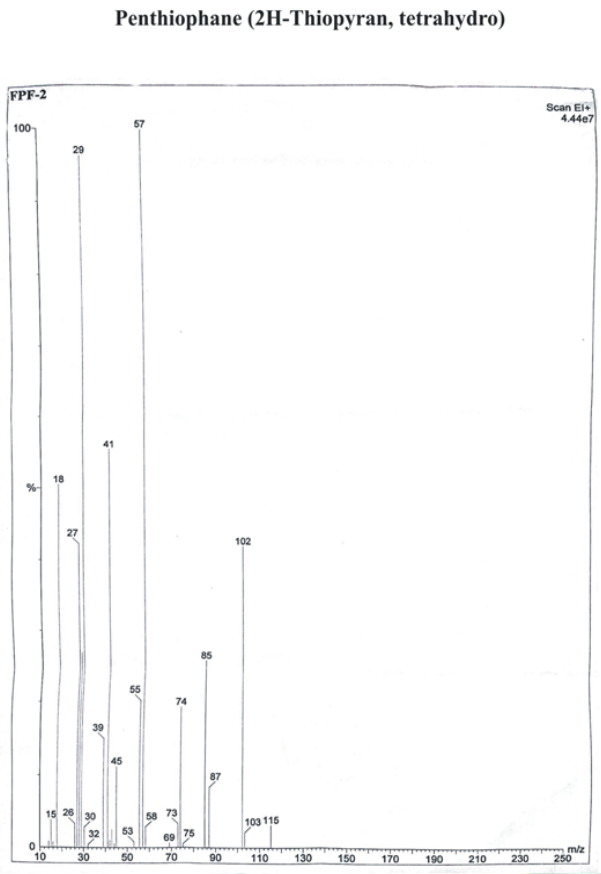

Figure 7: Mass Spectrum of Penthiophane (2H-Thiopyran, tetrahydro)

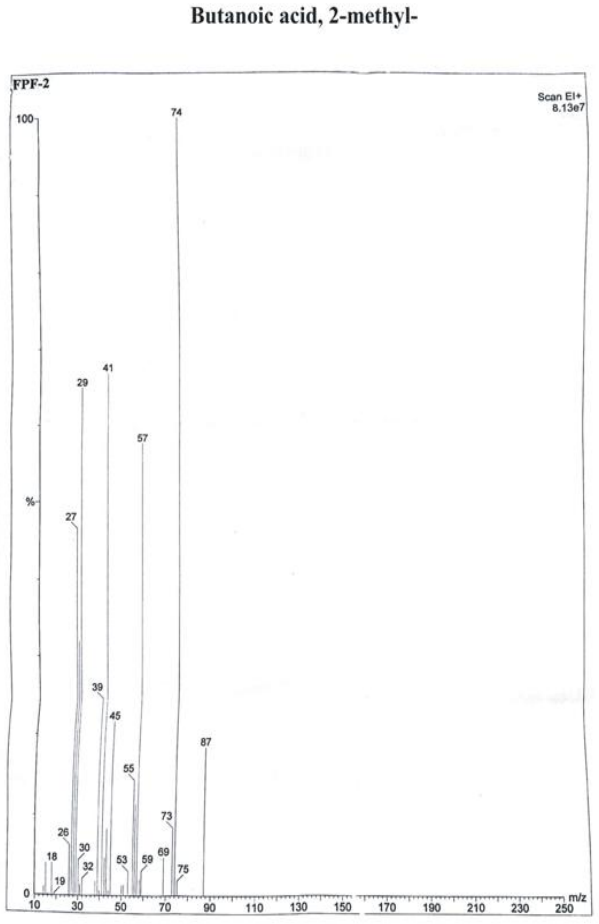

Figure 8: Mass Spectrum of Butanoic acid, 2-methyl-

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[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 *et al*. (1999) suggests that some fungal pigments are natural products and associated with development of reproductive structures[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 *Alternaria solani*[13] (Sinden *et al*., 1972).

Versha *et al*. (2003) reported that various fractions i.e. petroleum ether, chloroform, ethyl acetate and methanol of *Alstonia scholaris* leaf powder exhibit significant antimicrobial activity against the test pathogens and strongest antifungal activity against *A. niger* and *A. flavus* was observed with chloroform fraction[14]. Antifungal activity of petroleum ether, chloroform and acetone and ethanol extracts of *Calendula officinalis* against *A. fumigatus, Rhizopus japonicum, C. albicans, C. tropicalis* etc. has been investigated by Kasiram *et al*., 2000. Rao *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[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 *Cassia fistula* fruit pulp has been reported by Agarwal and Paridhavi, 2005[17].

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

CONCLUSION

In the present study it can be concluded by the observations that treatment with chloroform extract of *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.

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