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ORIGINAL RESEARCH ARTICLE

Identification of Antifungal Effect in the Leaf Extract of *Cassia fistula* L. BY Agar Cup Method and Isolation of 7-Hydroxyflavone by UV Spectral Analysis

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ABSTRACT

The present investigation was taken up with a view to raise an ecofriendly, safe, green control measure against fungi over hazardous synthetic antifungal agent. Aqueous ethanolic extract of leaves of *Cassia fistula* L. and it's different solvent fractions were tested for their antifungal potentialities. Highest antifungal activity was found in chloroform fraction which was subjected to thin layer chromatography and two bands were detected in solvent system benzene: methanol (95:5). Antifungal activity was located in Band I and Band I was found to be homogeneous in solvent systems TBA (butanol: glacial acetic acid: water, 3:1:1) and HOAc (glacial acetic acid: water, 3:17) with Rf 0.89 and 0.29 respectively. The bioactive sample was characterized as 7-hydroxyflavone by UV spectral analysis. The present study infers that the active compound isolated from *Cassia fistula* L. can be a non-hazardous ecofriendly measure against fungal infection of plants.

Key words: Antifungal potentiality, *Cassia fistula* L., 7-hydroxyflavone, ecofriendly.

INTRODUCTION

Cassia fistula L. (Fabaceae) is a tree, also known as golden shower or Indian laburnum, Amaltas, *Bactyrilobum fistula* wild (synonym) or *Cathartocarpus fistuloidis*(*Collad*) G Don(synonym), native to India [1, 2]. It is a very important medicinal plant with ethnomedicinal value and contains numerous active principles of therapeutic importance. In Ayurveda, *Cassia fistula* has been reported as "disease killer". From ancient period the use of *Cassia fistula* L. for treatment in various ailments was reported in Sushruta Samhita and Charaka Samhita [3]. *Cassia fistula* is used as natural laxative. It is applied against fevers, arthiritis, hematemesis, in cardiac and stomach problems and also effective in lowering bloodsugars, cholesterol. *Cassia fistula* can be employed as a remedy for tumors of abdomen, gland, liver, stomach and used against cancer, epilepsy and also have antioxidant and astringent property. It has antitumor activity against Elrich Ascites Carcinoma [4]. *Cassia fistula* leaves has antiperiodic, ulcer healing, anti-rheumatic properties and also effective against pain, inflammation, cough and ring worm [5 ,6, 7]. 4′-HydroxyBenzoic acid hydrate crystal from *Cassia fistula* exhibited antifungal activity against *Trichophyton mentagrophylis* and *Epidermophyton floccosum* [8]. In this paper we report isolation and identification of 7-hydroxyflavone from the leaves of *Cassia fistula* L. and its antifungal potentiality was established by an internationally accepted bioassay called agar cup method [9]. The present work may be cited as an approach towards a non-toxic, ecofriendly measure to control phytopathogenic fungi using green chemical from nature.

MATERIALS AND METHODS

Specimen collection:

Healthy, disease free, matured leaves of *Cassia fistula* were collected during the month of May-July, 2006, from Bolpur,Birbhum,West Bengal, India. A voucher specimen has been preserved in our laboratory.

Extraction, fractionation and separation of antifungal part:

500 gm sundried leaves of *Cassia fistula* were ground to a fine powder and then extracted in 1 Lit. of 50% aqueous ethanol at room temperature for five days. The extract was filtered and the

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filtrate was charcoalised. The charcoalised fraction was filtered repeatedly through Whatman No. 42 filter paper and a clear brown filtrate was obtained. The filtrate was concentrated under reduced pressure in a vacuum evaporator and a deep brown colored residue was obtained which was found to have fungicidal action. The residue was partitioned successively over petroleum ether (60–80°C), diethyl ether and chloroform. Each solvent fraction was collected separately, dried over anhydrous sodium sulphate and was concentrated under reduced pressure. The three fractions were subjected to fungicidal bioassay and the diethyl ether and chloroform fractions were found to have fungicidal property. The most active part was chloroform soluble fraction.

Fungicidal bioassay:

Preparation of sample solution:

The test solution was prepared by dissolving the dark brown residual solid in few drops of propylene glycol (a commonly used vehicle) and then diluting with sterile water [10] in the concentration of 10 mg ml^{-1}, 30 mg ml^{-1}, and 50 mg ml^{-1}. Few drops of propylene glycol diluted with sterile water were used as control. All the dilutions were sterilized by filtration using membrane filter (0.02µ pore size).

Fungal strains:

Pure culture of *Fusarium oxysporum*, *Phytophthora infestans*, *Curvularia lunata* were procured from the Department stock culture, Department of Botany, University of Kalyani, Kalyani, Nadia.

Antifungal assay by agar cup method:

Medium used- Potato Dextrose Agar (PDA) medium having following compositions-

Peeled and sliced potato = 250gm

Agar=20gm

Dextrose = 20 gm

Distilled water = 500 ml

The pH of the medium was maintained at 6.8–7. The fungal cultures used for this bioassay were 7 days (after full growth) old for *Fusarium oxysporum*, 5 days (after full growth) old for *Phytophthora infestans* and *Curvularia lunata*. Fungal suspension was prepared to contain approximately 101 1×106 cfu ml^{-1}. An overnight broth culture was used to seed sterile molten PDA medium maintained at 45°C. Small cylinders of agar were cut and scooped out using 7mm sterile cork borer. 0.1 ml of test solutions of different abovementioned concentrations were loaded separately to each cup with the help of micropipette. Propylene glycol with sterile water was loaded to maintain control. The test solutions were allowed to diffuse into the agar from the cup. The diameter of the fungal inhibition zone was measured every after the first visible colony appeared and the final measurement was taken when the control reached the size of the Petridish. If a culture grew in an irregular shape, two or more measurements were made and the average was recorded.

Thin layer chromatography of bioactive chloroform fraction:

A fungicidal bioassay of different solvent fractions revealed that the most active fraction was chloroform fraction. bioactive chloroform fraction was purified by repeated preparative thin layer chromatography.

Purification of the chloroform fraction and isolation of the bioactive compound

Glass plates (20cm×14 cm) were coated with silica gel G layers, 0.25mm thick, with slurry of silica gel G. Chloroform fraction was spotted on thin layer chromatography plates and the chromatograms were developed using solvent system benzene: methanol (95:5). The developed chromatoplates were air dried and placed in an iodine vapour chamber for visualization. The chloroform fraction was separated into two bands (Band I and Band II). Both the bands were scraped off and eluted in diethyl ether. The eluents obtained from Band I and Band II were subjected to antifungal screening by agar cup method against *Fusarium oxysporum*, *Phytophthora infestans*, *Curvularia lunata* and was administered in five different doses viz. 10 mg ml^{-1}, 30 mg $ml^{-1}$and 50 mg ml^{-1}. Antifungal activity was located in Band I as indicated by the results (vide Table 3). The homogeneity of the active Band I was tested in the solvent systems, TBA (butanol: glacial acetic acid:water, 3:1:1) and HOAc (glacial acetic acid: water, 3:17) and was found to be homogenous (Rf 0.89 and 0.29, respectively). The sample had a melting point 267 °C and appeared fluorescent pale yellow under UV light and appeared fluorescent bright yellow UV NH_3.

UV spectral analysis of the bioactive compound:

1. Stock solution was prepared by dissolving a small amount of the compound (about 0.1 mg) in about 10 ml of spectroscopic methanol. The concentration was then adjusted so that the optical density of the major absorption peak between 250 and 400 nm gave an optical density (O.D.) reading in the region 0.6 to 0.8.

2. The methanol spectrum was measured at normal scan speed (about 50 nm per minute) using 2-3 ml of the stock solution of the compound.
3. The methanol spectrum was measured at slow scan speed (about 10nm per minute) in the regions of the peak maxima in order to determine the wavelength (λ) of each maximum more accurately.
4. The NaOMe spectrum was measured immediately after the addition of three drops of the NaOMe stock solution to the solution used for steps 2 and 3. After 5 minutes the spectrum was rerun to check for flavonoid decomposition. The solution was then discarded.
5. The $AlCl_3$ spectrum was measured immediately after the addition of six drops of the $AlCl_3$ stock solution to 2-3 ml of fresh stock solution of the compound.
6. The $AlCl_3$/HCl spectrum was recorded immediately after the addition of three drops of the stock HCl solution to the cuvette containing the $AlCl_3$ (from step 5). The solution was then discarded.
7. The NaOAc spectrum of the flavonoid was determined as follows. Excess coarsely powdered anhydrous reagent grade NaOAc was added with shaking to a cuvette containing 2-3 ml of fresh stock solution of the compound. About a 2mm layer of NaOAc remained on the bottom of the cuvette. All the NaOAc spectra presented in this volume were recorded within 2 min after the addition of the NaOAc to the solution. A second spectrum was run after 5-10 min to check for decomposition.
8. The NaOAc/H_3BO_3 spectrum was determined as follows. As no decomposition was observed when NaOAc spectrum was rerun after 5 min, sufficient powdered anhydrous reagent grade H_3BO_3 to give a saturated solution was added with shaking to the cuvette (from step 7) which contained the NaOAc. The solution was discarded after the spectrum was recorded.
With the pure sample fungicidal bioassay was again performed against *Fusarium oxysporum, Curvularia lunata* and *Phytophthora infestans* and the antifungal action were confirmed.

RESULTS AND DISCUSSION

(Table 1 A) indicated petroleum ether fraction (60°-80°C) did not show any antifungal activity against the *Fusarium oxysporum*. But, diethyl ether and chloroform fractions showed antifungal activity towards *Fusarium oxysporum*.

(Table 1 B) indicated that, the antifungal effect of control dose and different doses of diethyl ether and chloroform fractions of crude leaf extract was statistically measured by comparing each of them with the others by Post Hoc Test and the value of the mean difference was statistically significant.

(Table 2 A) indicated that, petroleum ether (60°-80°C) and diethyl ether fraction did not show any antifungal activity against the *Phytophthora infestans*. But chloroform fraction showed antifungal activity towards *Phytophthora infestans*.

(Table 2 B) indicated that, the antifungal effect of control dose and different doses of chloroform fraction of crude leaf extract was statistically measured by comparing each of them with the others by Post Hoc Test and the value of the mean difference was statistically significant ($F = 21.150$, $p < 0.005$).

(Table 3 A) indicated that, petroleum ether (60 - 80˚C), diethyl ether and chloroform fraction showed antifungal l activity towards *Curvularia lunata*.

(Table 3 B) indicated that, the antifungal effect of control dose and different doses of petroleum ether (60 -80˚C), diethyl ether and chloroform fraction of crude leaf extract was statistically measured by comparing each of them with the others by Post Hoc Test and the value of the mean difference was statistically significant.

(Table 4 A) illustrates the result of antifungal activities of Band I and II obtained from chromatographic separation of chloroform fraction. Antifungal activity was located in Band I against *Fusarium oxysporum*. The MIC was located at 10 mg ml^{-1}.

(Table 4 B) illustrates the antifungal effect of Band I obtained from chromatographic separation of chloroform fraction was statistically measured by comparing each of them with the others by Post Hoc Test and the value of the mean difference was statistically.

(Table 5 A) illustrates the result of antifungal activities of Band I and II obtained from chromatographic separation of chloroform fraction. Antifungal activity was located in Band I against *Phytophthora infestans*. The MIC was located at 10 mg ml^{-1}.

(Table 5 B) illustrates the antifungal effect of Band I obtained from chromatographic separation of chloroform fraction was statistically measured by comparing each of them with the others by Post Hoc Test and the value of the mean

difference was statistically significant F = 18.300 ($p < 0.005$).

(Table 6 A) illustrates the result of antifungal activities of Band I and II obtained from chromatographic separation of chloroform fraction. Antifungal activity was located in Band I against *Curvularia lunata*. The MIC was located at 10 mg ml^{-1}.

(Table 6 B) illustrates the antifungal effect of Band I obtained from chromatographic separation of chloroform fraction can be statistically analysed by comparing each of them with the others by Post Hoc Test and the value of the mean difference was statistically significant F= 15.779 ($p < 0.005$).

(Table 7 A) represents Sample was used in different concentrations (10 mg ml^{-1}, 30 mg ml^{-1} and 50 mg ml^{-1}) for screening the antifungal activity against *Fusarium oxysporum*, *Curvularia lunata* and *Phytophthora infestans*. The MIC was found to locate at 10 mg ml^{-1}in all cases.

(Table 7 B) represents the antifungal effect of control dose and different doses of fraction of chloroform extract was statistically measured by comparing each of them with the others by Post Hoc Test.

Statistical analysis: All data recorded in this chapter are statistically analyzed using ANOVA [11, 12, 13]

Chemical characterization of the bioactive compound by UV spectral analysis:

Ultraviolet spectrum of the sample in methanol shows absorption peaks (λ max, nm) at 252, 268, 307 which clearly indicates that the sample is flavonoid nature.

In continuation with the above inference, further UV spectral analysis of the sample was done with different reagents by the procedures put forwarded by [14].

UV spectral analysis of the sample in the presence of NaOMe:

Addition of NaOMe to flavones and flavonols in methanol usually produces bathochromic shift in all absorbtion bands. However, large bathochromic shift of Band I without decrease in intensity is diagnostic for presence of free 4 - hydroxyl group; also give a 50-60 nm bathochromic shift in Band I. There is usually a decrease in intensity of the peak. In these compounds the bathochromic shift results from free 3- hydroxyl group. A spectrum shows no such shift and hence confirmed absence of 3-and free 4′-hydroxyl group (**Figure 1a**).

UV spectral analysis of the sample in the presence of $AlCl_3$/HCl:

$AlCl_3$has been used as a diagnostic reagent for detection of ortho- hydroxyl group and also 3-5-hydroxyl groups. Hypsochromic shift of about 30-40 nm observed in Band I of $AlCl_3$ spectrum. On addition of acid results from the decomposition of the complex of the $AlCl_3$ with the ortho- hydroxyl group. Addition of acid to a methanolic solution of flavones and flavonols which already contains $AlCl_3$ decomposes complexes between $AlCl_3$ and ortho di hydroxyl group. Therefore any shift still remaining in Band I and Band II relative to the methanol spectrum will be due to the presence of free 3-& / or 5- hydroxyl groups in the flavonoids. So such shift in the spectra (**Figure 1b**) indicates the absence of ortho di hydroxyl groups, free 3-& / or 5- hydroxyl groups.

UV spectral analysis of the sample in the presence of NaOAc/H_3BO_3:

UV spectra of flavones and flavonols containing free 7-hydroxyl groups exhibit a diagnostic 5-20 nm bathochromic shift of Band II in the presence of NaOAc. A 14 nm shift of Band II in spectra (**Figure 1c**) in the sample indicates the presence of free 7- hydroxyl group. In the presence of NaOAc/H_3BO_3 flavones and flavonols containing B-ring ortho di hydroxyl group consistent 12-30 nm bathochromic shift of Band I. No such shift in spectra shows absence of ortho - hydroxyl group.

Hence, the sample was identified as 7-hydroxyflavone.

The plant *Cassia fistula* L. was examined with an aim to isolate and identify biologically active principles, particularly antifungal principles. Here, in the present work, the general methods to isolate phytochemicals, specially flavonoid was incorporated. Antifungal potentiality was tested by internationally accepted agar-cup bioassay test. The chemical nature of the antifungal compound was established by UV spectral analysis and the compound was identified as 7-hydroxyflavone.

Table 1 A: Screening of different solvent fractions obtained from crude leaf extract of *Cassia fistula* for fungicidal activity against *Fusarium oxysporum*

Fungus taken	Dose (mg ml^{-1})	Mean value of Diameter of inhibition zone (cm)			MIC (mg ml^{-1})		
		PE	DE	CHL	DE	PE	CHL
Fusarium oxysporum	Control	0	0	0			
	10	0	0	4.7			10
	30	0	1.2	6.5		30	
	50	0	2.5	8.0			

Table 1 B (i): Representation of the one way ANOVA to calculate the statistical significance among the antifungal effects of different doses (10 mg ml^{-1}, 30 mg ml^{-1} and 50 mg ml^{-1}) of diethyl ether and chloroform fraction of crude leaf extract with control

Analysis	F			Sig.
One way ANOVA among groups	DE		CHL	P<0.05
	2.19634		40.200	

Table 1 B (ii): Post Hoc Tests Multiple Comparisons LSD

Dependant variable	Other variables	Mean Difference		Standard Error		Sig.
		DE	CHL	DE	CHL	
Control (without treatment)	Treated with 10 mg ml^{-1}	0.00	4.70	0.0	0.77	p <0.05
	Treated with 30 mg ml^{-1}	1.20	6.50	0.0	0.77	p <0.05
	Treated with 50 mg ml^{-1}	2.50	8.00	0.0	0.77	p <0.05
Treated with 10 (mg ml^{-1})	Control (without treatment)	0.0	4.70	0.0	0.77	p <0.05
	Treated with 30 mg ml^{-1}	1.20	1.80	0.0	0.77	p <0.05
	Treated with 50 mg ml^{-1}	2.50	3.30	0.0	0.77	p <0.05
Treated with 30 (mg ml^{-1})	Control (without treatment)	1.20	6.50	0.0	0.77	p <0.05
	Treated with 10 mg ml^{-1}	1.20	1.80	0.0	0.77	p <0.05
	Treated with 50 mg ml^{-1}	1.30	1.50	0.0	0.77	p <0.05
Treated with 50 (mg ml^{-1})	Control (without treatment)	2.50	8.00	0.0	0.77	p <0.05
	Treated with 10 mg ml^{-1}	2.50	3.30	0.0	0.77	p <0.05
	Treated with 30 mg ml^{-1}	1.30	1.50	0.0	0.77	p <0.05

Table 2 A: Screening of different solvent fractions obtained from crude leaf extract of *Cassia fistula* for fungicidal activity against *Phytophthora infestans*

Fungus taken	Dose (mg ml^{-1})	Mean value of Diameter of inhibition zone (cm)			MIC (mg ml^{-1})		
		PE	DE	CHL	DE	PE	CHL
Phytophthora infestans	Control	0	0	0			
	10	0	0	1.8			10
	30	0	0	3.9			
	50	0	0	4.5			

Table 2 B (i): Representation of the one way ANOVA to calculate the statistical significance among the antifungal effects of different doses (10 mg ml^{-1}, 30 mg $ml^{-1}$and 50 mg ml^{-1}) of chloroform fraction of crude leaf extract with that of the control one

Analysis	F	Sig.
	CHL	
One way ANOVA among groups	21.150	P<0.05

Table 2 B (ii): Post Hoc Tests Multiple Comparisons LSD

Dependant variable	Other variables	Mean Difference	Standard Error	Sig.
		CHL		
Control (without treatment)	Treated with 10 mg ml^{-1}	1.80	0.63	p <0.05
	Treated with 30 mg ml^{-1}	3.90	0.63	p <0.05
	Treated with 50 mg ml^{-1}	4.50	0.63	p <0.05
Treated with 10 (mg ml^{-1})	Control (without treatment)	1.80	0.63	p <0.05
	Treated with 30 mg ml^{-1}	2.10	0.63	p <0.05
	Treated with 50 mg ml^{-1}	2.70	0.63	p <0.05
Treated with 30 (mg ml^{-1})	Control (without treatment)	3.90	0.63	p <0.05
	Treated with 10 mg ml^{-1}	2.10	0.63	p <0.05
	Treated with 50 mg ml^{-1}	0.60	0.63	p <0.05
Treated with 50 (mg ml^{-1})	Control (without treatment)	4.50	0.63	p <0.05
	Treated with 10 mg ml^{-1}	2.70	0.63	p <0.05
	Treated with 30 mg ml^{-1}	0.60	0.63	p <0.05

Table 3 A: Screening of different solvent fractions obtained from crude leaf extract of *Cassia fistula* for fungicidal activity against *Curvularia lunata*

Fungus taken	Dose (mg ml^{-1})	Mean value of Diameter of inhibition zone (cm)			MIC (mg ml^{-1})		
		PE	DE	CHL	DE	PE	CHL
Curvularia lunata	Control	0	0	0			
	10	0	0	2.0			10
	30	0	0	2.5			
	50	0	0	6.0	50	50	

Table 3 B (i): Representation of the one way ANOVA to calculate the statistical significance among the antifungal effects of different doses (10 mg ml^{-1}, 30 mg ml^{-1}and 50 mg ml^{-1}) of pet ether, diethyl ether and chloroform fraction of crude leaf extract with that of the control one

Analysis	F			Sig.
One way ANOVA among groups	PE	DE	CHL	p <0.05
	70.225	119.025	62.292	

Table 3 B (ii): Post Hoc Tests Multiple Comparisons LSD

Dependant variable	Other variables	Mean Difference			Standard Error			Sig.
		PE	DE	CHL	PE	DE	CHL	
Control (without treatment)	Treated with 10 mg ml^{-1}	0.00	0.00	2.00	0.44	0.44	0.44	p <0.05
	Treated with 30 mg ml^{-1}	0.00	0.00	2.50	0.44	0.44	0.44	p <0.05
	Treated with 50 mg ml^{-1}	5.30	6.90	6.00	0.44	0.44	0.44	p <0.05
Treated with 10 (mg ml^{-1})	Control (without treatment)	0.00	0.00	2.00	0.44	0.44	0.44	p <0.05
	Treated with 30 mg ml^{-1}	0.00	0.00	0.50	0.44	0.44	0.44	p <0.05
	Treated with 50 mg ml^{-1}	5.30	6.90	4.00	0.44	0.44	0.44	p <0.05
Treated with 30 (mg ml^{-1})	Control (without treatment)	0.00	0.00	2.50	0.44	0.44	0.44	p <0.05
	Treated with 10 mg ml^{-1}	0.00	0.00	0.50	0.44	0.44	0.44	p <0.05
	Treated with 50 mg ml^{-1}	5.30	6.90	3.50	0.44	0.44	0.44	p <0.05
Treated with 50 (mg ml^{-1})	Control (without treatment)	5.30	6.90	6.00	0.44	0.44	0.44	p <0.05
	Treated with 10 mg ml^{-1}	5.30	6.90	4.00	0.44	0.44	0.44	p <0.05
	Treated with 30 mg ml^{-1}	5.30	6.90	3.50	0.44	0.44	0.44	p <0.05

(PE- Petroleum ether, DE- Diethyl ether, CHL-Chloroform

Table 4 A: Antifungal screening of Band I and Band II fraction obtained from chromatographic separation of Chloroform fraction (CHL I) against *Fusarium oxysporum*

Dose (mg ml^{-1})	Mean value of diameter of inhibition zone (cm)		MIC mg ml^{-1}	
	Band I	Band II	Band I	Band II
Control	0	0		
10	1.2	0	10	0
30	3.2	0		
50	5.0	0		

Table 4 B (i): Representation of the one way ANOVA to calculate the statistical significance of Band I obtained from chromatographic separation of Chloroform fraction to study the antifungal efficiency at different doses (10 mg ml^{-1}, 30 mg ml^{-1}and 50 mg ml^{-1}) with that of the control one

Analysis	F	Sig.
One way ANOVA among groups	26.395	p <0.05

Table 4 B (ii): Post Hoc Tests Multiple Comparisons LSD

Dependant variable	Other variables	Mean Difference	Standard Error	Sig.
Control (without treatment)	Treated with 10 mg ml^{-1}	1.20	0.58	p <0.05
	Treated with 30 mg ml^{-1}	3.00	0.58	p <0.05
	Treated with 50 mg ml^{-1}	4.90	0.58	p <0.05
Treated with 10 (mg ml^{-1})	Control (without treatment)	1.20	0.58	p <0.05
	Treated with 30 mg ml^{-1}	1.80	0.58	p <0.05
	Treated with 50 mg ml^{-1}	3.70	0.58	p <0.05
Treated with 30 (mg ml^{-1})	Control (without treatment)	3.00	0.58	p <0.05
	Treated with 10 mg ml^{-1}	1.80	0.58	p <0.05
	Treated with 50 mg ml^{-1}	1.90	0.58	p <0.05
Treated with 50 (mg ml^{-1})	Control (without treatment)	4.90	0.58	p <0.05
	Treated with 10 mg ml^{-1}	3.70	0.58	p <0.05
	Treated with 30 mg ml^{-1}	1.90	0.58	p <0.05

Table 5 A: Antifungal screening of Band I and Band II fraction obtained from chromatographic separation of Chloroform fraction (CHL I) against *Phytophthora infestans*

Dose (mg ml^{-1})	Mean value of diameter of inhibition zone (cm)		MIC mg ml^{-1}	
	Band I	Band II	Band I	Band II
Control	0	0		
10	1.5	0	10	0
30	3.5	0		
50	4.2	0		

Table 5 B (i): Representation of the one way ANOVA to calculate the statistical significance of Band I obtained from chromatographic separation of Chloroform fraction to study the antifungal efficiency at different doses (10 mg ml^{-1}, 30 mg ml^{-1}and 50 mg ml^{-1}) with that of the control one

Analysis	F	Sig.
One way ANOVA among groups	18.300	p <0.05

Table 5 B (ii): Post Hoc Tests Multiple Comparisons LSD

Dependant variable	Other variables	Mean Difference	Standard Error	Sig.
Control (without treatment)	Treated with 10 mg ml^{-1}	1.50	0.63	p <0.05
	Treated with 30 mg ml^{-1}	3.50	0.63	p <0.05
	Treated with 50 mg ml^{-1}	4.20	0.63	p <0.05
Treated with 10 (mg ml^{-1})	Control (without treatment)	1.50	0.63	p <0.05
	Treated with 30 mg ml^{-1}	2.0	0.63	p <0.05
	Treated with 50 mg ml^{-1}	2.70	0.63	p <0.05
Treated with 30 (mg ml^{-1})	Control (without treatment)	3.50	0.63	p <0.05
	Treated with 10 mg ml^{-1}	2.00	0.63	p <0.05
	Treated with 50 mg ml^{-1}	0.70	0.63	p <0.05
Treated with 50 (mg ml^{-1})	Control (without treatment)	4.20	0.63	p <0.05
	Treated with 10 mg ml^{-1}	2.70	0.63	p <0.05
	Treated with 30 mg ml^{-1}	0.70	0.63	p <0.05

Table 6 A: Antifungal screening of Band I and Band II fraction obtained from chromatographic separation of Chloroform fraction (CHL I) against *Curvularia lunata*

Dose (mg ml^{-1})	Mean value of diameter of inhibition zone (cm)		MIC mg ml^{-1}	
	Band I	Band II	Band I	Band II
Control	0	0		
10	1.3	0	10	0
30	3.0	0		
50	4.0	0		

Table 6 B (i): Representation of the one way ANOVA to calculate the statistical significance of Band I obtained from chromatographic separation of Chloroform fraction to study the antifungal efficiency at different doses (10 mg ml^{-1}, 30 mg $ml^{-1}$and 50 mg ml^{-1}) with that of the control one

Analysis	F	Sig.
One way ANOVA among groups	15.779	p <0.05

Table 6 B (ii): Post Hoc Tests Multiple Comparisons LSD

Dependant variable	Other variables	Mean Difference	Standard Error	Sig.
Control (without treatment)	Treated with 10 mg ml^{-1}	1.30	0.63	p <0.05
	Treated with 30 mg ml^{-1}	3.00	0.63	p <0.05
	Treated with 50 mg ml^{-1}	4.00	0.63	p <0.05
Treated with 10 (mg ml^{-1})	Control (without treatment)	1.30	0.63	p <0.05
	Treated with 30 mg ml^{-1}	1.70	0.63	p <0.05
	Treated with 50 mg ml^{-1}	2.70	0.63	p <0.05
Treated with 30 (mg ml^{-1})	Control (without treatment)	3.00	0.63	p <0.05
	Treated with 10 mg ml^{-1}	1.70	0.63	p <0.05
	Treated with 50 mg ml^{-1}	1.00	0.63	p <0.05
Treated with 50 (mg ml^{-1})	Control (without treatment)	4.00	0.63	p <0.05
	Treated with 10 mg ml^{-1}	2.70	0.63	p <0.05
	Treated with 30 mg ml^{-1}	1.00	0.63	p <0.05

Table 7 A: Screening of Sample (Benzene: Chloroform – 2:1) for its antifungal property against *Fusarium oxysporum* (F.O), *Phytophthora infestans* (P.I) and *Curvularia lunata* (C.L) by Agar cup method

Doses of sample (mg ml^{-1})	Mean value of diameter of inhibition zone (cm)			MIC (mg ml^{-1})		
Control	F.O	C	P.H	F.O	C	P.H
	0	0	0			
10	2.5	2.0	2.0	10	10	10
30	3.5	3.1	3.0			
50	4.2	4.0	3.7			

Table 7 B (i): Representation the one way ANOVA to calculate the statistical significance of antifungal effects among the of different doses (10, 30, and 50 mg ml^{-1}) of Benzene: Chloroform – 2:1 fraction of chloroform extract, with that of the control one

Analysis	F			Sig.
One way ANOVA among groups	F.O	P.I	C.L	p <0.05
	16.883	14.846	12.946	

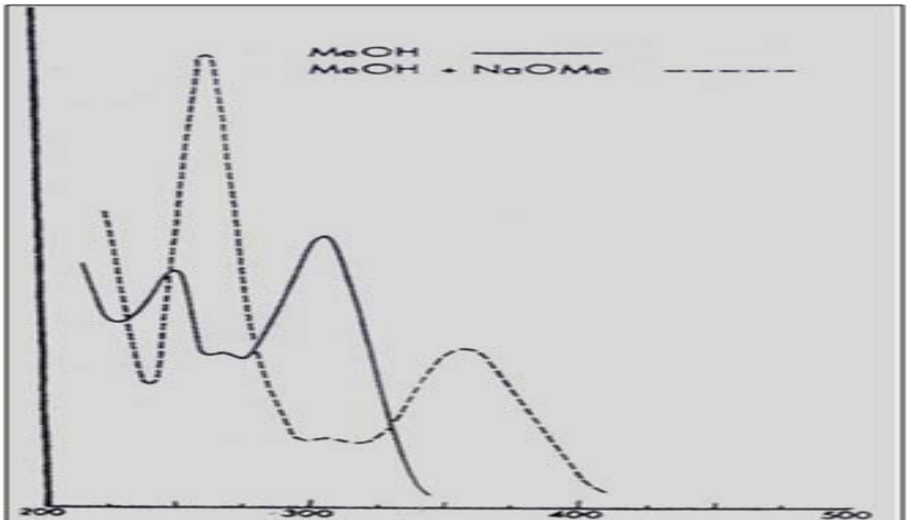

Figure 1(a): UV spectra of the sample in the Methanol (MeOH) and Sodium Methoxide (NaOMe)

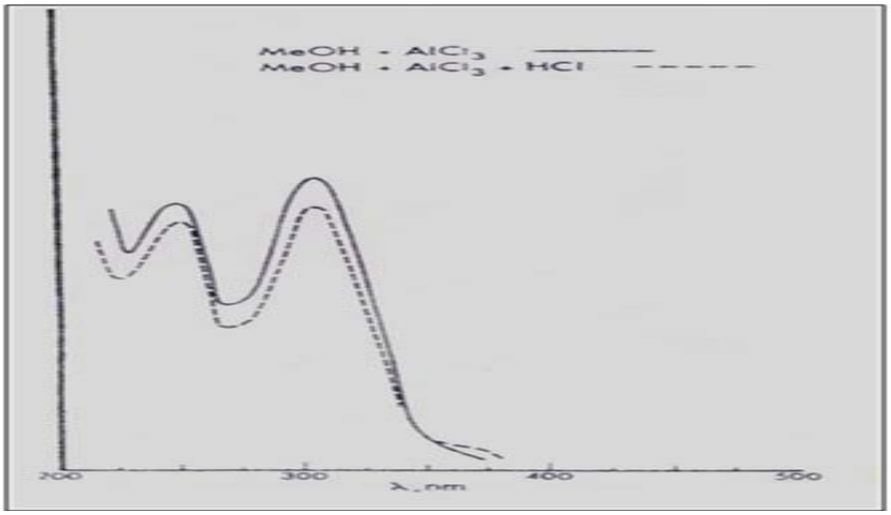

Figure 1(b): UV spectra of the sample in the presence of Aluminium Chloride (AlCl3) and of Aluminium Chloride /Hydrochloric acid (AlCl3/HCl)

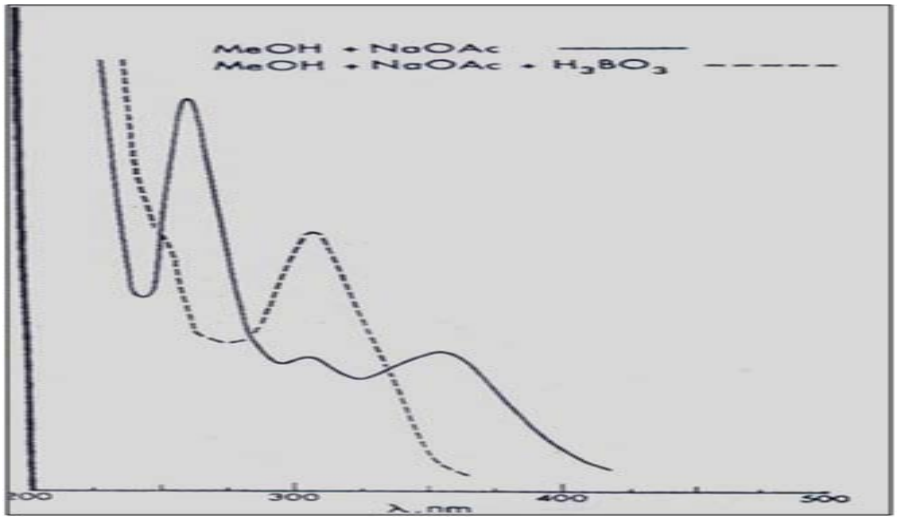

Figure 1(c): UV spectra of the sample in the presence of Sodiun Acetate (NaOAc) and Sodiun Acetate /Boric acid (NaOAc/H3BO3)

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ABBREVIATIONS AND NOMENCLATURE

PE - Petroleum ether fraction,
DE - Diethyl ether fraction,
CHL - Chloroform fraction
MIC- Minimum inhibitory concentration
Sig**.** - Significance
UV - Ultra violet
Nm – nanometre
NaOMe – Sodium methoxide
$AlCl_3$ – Aluminium chloride
HCl – Hydrochloric acid
NaOAc – Sodium acetate
H_3BO_3 – Boric acid
Rf - Retention factor
O.D. – Optical density
Cfu – Colony forming unit
Lit. – Litre
% - Percent
No. - Number
°C - Degree Celsius
µ - Micron
µg – Microgram
gm – Gram
ml – Mililitre
cm – centimeter
NH_3 – Ammonia
Mg – Miligram
λ – Lamda
Max – Maximum
F – variance of the group means / mean of the within group variances
P - Probability
Cassia fistula L.
HF-7-hydroxyflavone