International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
www.florajournal.com
IJHM 2020; 8(5): 134-142
Received: 25-07-2020
Accepted: 27-08-2020

Sandip S Kale
Assistant Professor,
Department of Biochemistry,
Shivaji University Kolhapur,
Maharashtra, India

Suresh Kale
Department of Botany, Sathaye College, Vile Parle, Mumbai,
Maharashtra, India

Padma Dandge
Department of Biochemistry,
Shivaji University, Kolhapur,
Maharashtra, India

Corresponding Author:
Sandip S Kale
Assistant Professor,
Department of Biochemistry,
Shivaji University Kolhapur,
Maharashtra, India

Structural identification of antibacterial components isolated from *Cassia fistula*

Sandip S Kale, Suresh Kale and Padma Dandge

Abstract
The present study was designed for isolation, purification, and chemical characterization of components from *Cassia fistula* responsible for antibacterial activity. The crude extract of leaves of *Cassia fistula* prepared using methanol as a solvent and phytochemical evaluation was done. An invitro antibacterial assay was conducted to evaluate the inhibitory activity of methanol extract against *E. coli* and *B. cereus*. The methanol extract was subjected to column chromatography with different mobile phases followed by TLC. HPLC of fractions showing maximum inhibitory activity against *E. coli* and *B. cereus* was performed. The chemical characterization of isolated compounds was achieved using FTIR and ^{1}H NMR spectroscopy. Screening of antibacterial activity of methanol extract showed marked activity against both gram-positive and gram-negative bacteria. FTIR and NMR analysis data show the presence of various functional groups that correspond to potential bioactive compounds from phenolics (Rhein, Catechin), triterpenes (Hopane), and Flavonoids (Amentoflavone). This plant formulation may thereby provide an alternative solution to the current problem associated with the use of antibiotics.

Keywords: *Cassia fistula*, phytochemicals, antibacterial, chromatography

1. Introduction
Plants produce a diverse range of bioactive molecules, making them a rich source of medicines. Higher plants, a source of a medicinal compound have continued to play a dominant role in the maintenance of human health since ancient times. Over 50% of modern clinical drugs are of natural plant origin and natural products play important role in drug development programs in the Pharmaceutical industry. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, flavonoids, and phenolic compounds that have important applications in the treatment of viral and bacterial diseases. In India, many plants extract use against many diseases in various areas of medicine like Ayurveda. Only a few of them have been scientifically explored. Pharmaceutical industries have produced several new antimicrobial drugs last year but resistance to these drugs by microbes has also increased. Interest in plants with antimicrobial property has revived as a result of the current problem associated with the use of antibiotics. *Cassia fistula* is traditionally used in Ayurveda medicine. They have various effects in combating inflammation, bleeding, GI disorder, etc. This plant is easily available as it grows in all climatic conditions. Although enough research has been conducted on the curative action of the bark, roots, and fruit. In the present work, we report phytoactive compounds from *Cassia fistula* leaves showing antibacterial activity [1-3].

1.1 General Information
***Cassia fistula* (Bahava)**
- Botanical name: *Cassia fistula*
- Common name: Bahava, golden shower, kallober
- Plant family: Fabaceae

Fig 1: *Cassia fistula* (PC- Suresh Kale)

Plant parts used: Leaves, seeds, Barks.
Cassia fistula (Fig. 1) is a flowering plant in the family *Fabaceae,* native to southern Asia from southern Pakistan east through India to Myanmar and South to Sri Lanka. It is widely grown as an ornamental plant. It blooms in late springs. In ayurvedic medicine, *C. fistula* is known as Argvadha (Disease killer).

1.1.1 Plant constituents: Laxative actions come from a group of documented compounds called Anthraquinones. Those are found in all *Cassia* plants in varying concentrations. Other compounds documented in the plant include Anthraquinones, glycosides, Methyl inositol, Luteolin, Pentacosane, Triacontane-1-ol, Fistulic acid, rhein, rheinglucoside, galactomannan, sennosides A and B, Tannin, phlobaphenes, oxyanthroquinones, emodine, chrysophanic acid, fistuacacidin, barbaloin, lupeol, beta–citosterol, hexacosanol, butanoic acid, 2-methyl-, Penthiophane (2H-Thiopyran, tetrahydro) and Isopropyl acetate (Acetic acid, 1-methyl ethyl ester) and phenolic compounds [4-13].

1.1.2 Medicinal use
Its fruit pulp is used as a mild laxative against fever, arthritis, nervous system diseases, bleeding such as hematemesis or hemorrhages as well as cardiac condition & stomach problems, etc. It is also used as a remedy for tumors of the abdomen, glands, liver, stomach, throat for burns, cancer, constipation, convulsions, diarrhea, dysurea, epilepsy, hematuria, pimples, etc. The leaves are used for malaria, rheumatism & ulcers. Roots are used for adenopathy, burning sensation leprosy skin diseases syphilis &tubercular glands. Roots are also considered a very strong purgative & self-medicinal or any use without medical supervision is strongly advised against Ayurveda texts [14-20].

2. Materials and Method

2.1 Preparation of leaf extract by solvent extraction: The leaves of *C. fistula* were collected, then shed dried for at room temperature and powdered by using a mechanical grinder. 10 g of powder was mixed in 150 ml methanol. The mixture was stirred well & left overnight for complete extraction. The suspension was then filtered & crude extract was obtained. The leaf extract was then concentrated using rotavapor at 42 ˚C till the maximum solvent was evaporated. The viscous extract was then taken on petriplates & evaporated completely. Powdered leaf extract was weighed and re-dissolved in methanol [21].

2.2 Phytochemical screening of extract
Methanolic extract was assessed for the existence of secondary metabolites such as flavonoids, tannins, saponins, alkaloids, steroids, and terpenoids [22-24].

2.2.1 Test for Flavonoids: 1 ml extract + few drops of conc. HCl +magnesium turnings, formation of Magenta red color within 3 min. indicate the presence of flavonoids [22-23].

2.2.2 Test for Tannins: 1 ml of leaf extract +2 ml FeCl3, formation of blue-black color indicate the presence of Tannins.

2.2.3 Test for Saponins: 1 ml leaf extract +2ml distilled water, shake vigorously, the appearance of Honeycomb broth indicates the presence of saponins**.**

2.2.4 Test for Alkaloids: 1 ml leaf extract + 2 ml of 2N HCl then incubate in a boiling water bath for 10 min, cooled then add few drops of Mayer reagent. The formation of precipitation or turbidity indicates the presence of Alkaloids.

2.2.5 Test for Steroids: 1ml leaf extract + 1ml chloroform +2ml acetic anhydride +conc. H2SO4, formation of blue-green color indicates the presence of steroids.

2.2.6 Test for Terpenoids: 1ml extract +1ml chloroform +1ml acetic acid +conc.H2SO4 dropwise from side of the tube. Formation of red ring at interphase indicates the presence of terpenoids.

2.3 Total protein by lowry's method: The total protein content was estimated by Lowry et al. (1951) using BSA as a standard. The blue color developed was read at 660 nm in a spectrophotometer. The total protein content was expressed in mg/ml of sample extract [25-26].

2.4 Total reducing sugar by DNSA method: The total reducing sugar content was estimated by DNSA method using glucose as a standard. The orange color developed was read at 530nm. The total reducing sugar content was expressed in mg/ml of sample extract [27-28].

2.5 Preparation of bacterial inoculums: The nutrient broth was prepared in 100ml volume & sterilized. After cooling, the pure culture of bacteria maintained on the slant was transferred to these broth flasks aseptically and incubate at 37 ˚C for 24 hrs. The broth culture obtained was used for the spread plate technique.

2.6 Spread plate technique: Spread plate method is used to get uniform growth on the agar plate. 100µl of bacterial inoculum is added to the plate. This inoculum is spread evenly on the plate using a sterile glass spreader to get uniform growth.

2.7 Well diffusion method: The antimicrobial activity was assessed using this method, where the wells are made on nutrient agar inoculated with the test bacteria, to get growth on incubation. The given drug is placed in the well which diffuses in the medium & inhibits bacterial growth around the well if it is effective this indicates the antibacterial activity of the drug. The larger the inhibition zone higher will be the activity. If the drug is not effective inhibition zone will not be observed.

2.8 Testing antibacterial activity of *C. fistula* leaf extract: Screening of *Cassia fistula* leaf extract was done using a well diffusion method. 24 hrs of bacterial cultures were used. The 100µl of test bacterial suspensions were spread over the nutrient agar plates using a sterile glass spreader. Wells were made in plates using 1 ml micro pipette's tip.100µl of leaf extract was added to the well. 100µl of antibiotic and 100µl of DMSO was added to the wells as a control. The plates were left for complete diffusion of the added sample. After that, they were kept in an incubator at 37 ˚C for 24 hrs. After the incubation period the inhibition zone was measured, studied were performed in duplicate, and the mean value was calculated. The negative control i.e. DMSO does not show any inhibition zone. Antibiotic Ampicillin shows an inhibition zone of 3.0 cm. The % RIZD (Relative Inhibition Zone Diameter) was calculated for the formula...

$$\%RIZD = \frac{(IZD\ sample - IZD\ negative\ control)}{IZD\ antibiotic\ control} X\ 100$$

It gives a comparative account of the antibacterial action of a given sample against that of standard antibiotic control.

2.9 Thin layer chromatography of *C. fistula* leaf extract: The extract was spotted on silica-coated aluminium plate till saturation was achieved. The plate then dipped in the solvent system (Hexane:ethyl acetate 2:1). After the solvent run completely, the plate was dried & developed using a suitable reagent, it was also visualized under the UV. Rf values of components were calculated as follow:

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

2.10 Column chromatography of *C. fistula* leaf extracts using silica gel

It was done using Hexane & Ethyl acetate solvent system in 2:1 ratio.30 ml column was taken having 2 cm diameter, cleaned with water &then with acetone. A piece of glass wool was placed at the bottom of the column using glass rode. The column was then filled with a given solvent system till the top. Silica gel (60-120 mesh size) was sprinkled into the column from the top to achieved proper packing of the gel. Care was taken that no air bubbles should trap inside.2 ml of concentrated extract (153 mg/ml) was loaded on top of the column & allowed to run down. The separation of components can be observed in the form of distinct color bands. The solvent system was added continuously maintaining the level above the gel. The 2 ml fractions were collected in Eppendorf tubes. These fractions were then subjected to antimicrobial analysis.

2.11 Identification of fractions showing antibacterial activity: The collected fractions were used for well diffusion. They were used directly in the same eluent system. The plates were incubated for 24 hrs at 37 ˚C. The zone of inhibition & RIZD value were measured. The fractions with maximum activity were given for HPLC to further fine separation of components in the fraction.

2.12 HPLC of individual fractions: HPLC (High performance liquid chromatography) is a technique used for the separation of individual components from the mixtures. It has more sensitivity and accuracy as compared to TLC. We used C18 column (250mm X 4.6 mm, 5 µm) Detection wavelength was 220 nm and 254nm. The mobile phase consists of Methanol (A) & deionized Water (B). Linear gradient :- 0 -40 min (85%A), 40-60 min (85%A- 95%A). Flow rate – 1.0 ml/min. The column temperature was maintained at 30 ^{0}C [29-32].

2.13 TLC of individual fractions: (13, 14, 25 & 28) Solvent system= methanol & water (70:30 ratio). Fraction 13 & 14 we get 3 bands which are similar in both. Fraction 25 & 28 we get a single band, then these fractions were given to FTIR and NMR. The fraction 13 was used for further separation by column chromatography using Methanol: water (70:30) solvent system. We used syringe column.10 fractions of 0.5ml were collected. Then similar color fractions were mixed, concentrated, and given to FTIR and NMR for further analysis.

2.14 Structural characterisation of individual component using FTIR and NMR

2.14.1 FTIR spectroscopy: Fourier Transform Infrared spectroscopy (FTIR spectroscopy) deals with the infrared region of the electromagnetic spectrum. Infrared spectroscopy exploits the fact that molecules have specific frequencies at which they rotate or vibrate corresponding to discrete energy levels (vibration mode). IR spectrum of the sample can be obtained by passing the IR rays through it. Examination of transmitted light shows much energy is absorbed in each wavelength from this transmittance or absorbance spectrum can be produced, showing at each I.R wavelength. The sample analysis of these absorption characteristics reveals details about the molecular structure of the sample. This technique works almost exclusively on samples with a covalent bond. Simple spectra are obtained from samples with few I.R active bonds and high levels of purity. The most complex molecular structure leads to more absorption bands and more complex spectra. The technique has been used for the characterization of very complex mixtures. Scanning range 400-4000 cm^{-1} with a resolution of 4^{-1} was used [33-38].

2.14.2 NMR: Nuclear magnetic resonance In NMR spectroscopy we study the interaction of the magnetic component of the electromagnetic radiation with the nuclei of certain atoms in the molecule. In NMR study, nuclei do not absorb at the same applied magnetic field and effective strength is different. For different nuclei, absorbance is measured at monochromatic radio frequency in a varying magnetic field. The spectra plot of energy absorbed vs. magnetic field strength applied. No. of singlet indicates different sets of equivalent protons where peak height or area under peak indicates no. of proton in each set [39].

3. Results

3.1 Phytochemical Tests

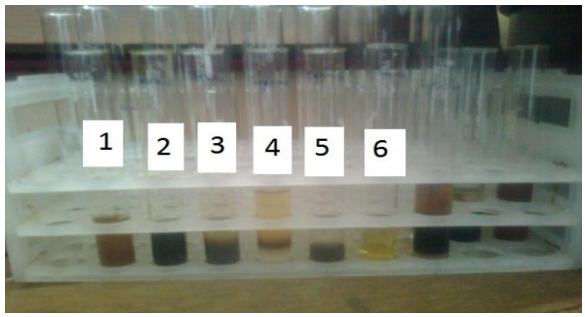


Fig 2: Phytochemical tests for 1) Alkaloids 2) Tannins 3) Flavanoids 4) Terpenoids 5) Steroids 6) Saponins.

The result shows the presence of all of the above phytochemicals except steroids.

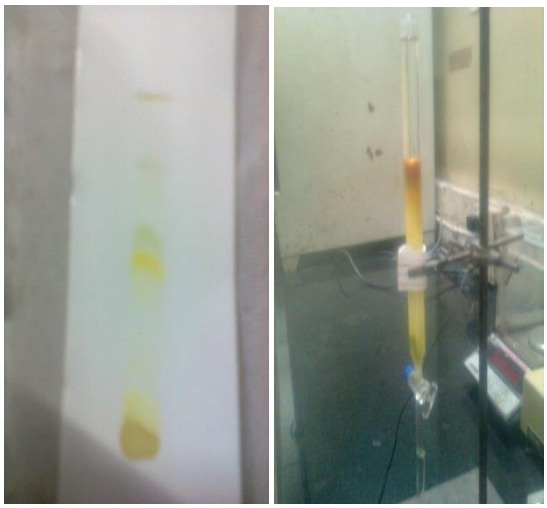
3.2 Total protein content by Lowry's method.

We got 5.52 mg/ml of protein in methanol extract.

3.3 Total reducing sugar by DNSA method.

We got 2.6 mg /ml of reducing sugar in methanol extract.

3.4 Thin layer chromatography of *Cassia fistula* leaf extract: TLC of crude extract performed using Haxane and Ethyl acetate (2:1) solvent systeme. (Rf= 0.50 to 0.90) (Fig. 3 A)



A B

Fig 3 A: Chromatogram Developed in Hexane Ethyl acetate solvent system(10 bands were observed, Better visualisation under UV). B: column chromatography of Bahava- Methanol extract using silica gel. Showing different colour during elution.

3.5 Column Chromatography of *C. fistula* leaf Extract

Different fractions were collected using Hexane Ethyl acetate solvent system by silica gel column (Fig. 3 B).

3.6 Identification of fractions showing Antibacterial activity.

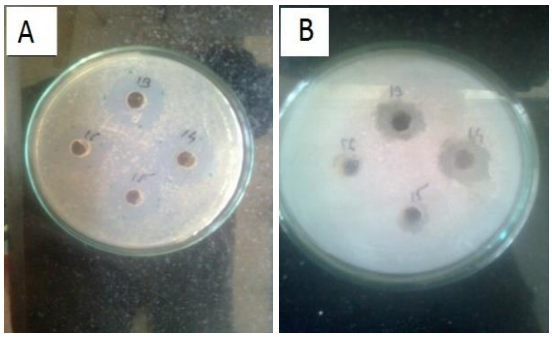


Fig 4: Shows maximum activity of fraction number 13 and 14 on A) *E. coli* and B) *B. cereus*.

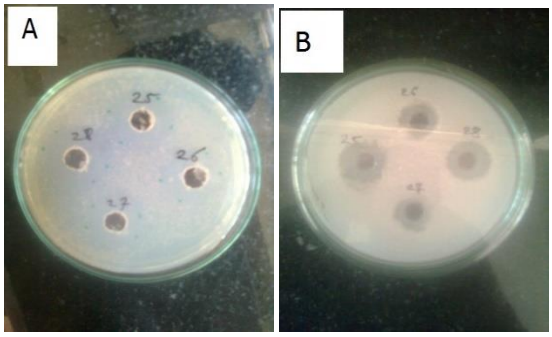


Fig 5: Shows maximum activity of fraction number 25 and 28 on A) *E. coli* and B) *B. cereus*

Results shows that the fraction no. 13&14 showing maximum inhibition zone against *E. Coli* & *B. cereus* (Fig 5 A). Fraction no. 25 & 28 showing maximum inhibition zone against *E. coli* & *B. cereus.* (Fig 5 B). Fraction no. 13,14,25 &28 collected from *C. fistula* leaf extract using Hexane Ethyl acetate solvent system shows significant antibacterial activity (Table no. 1). This indicates that these fractions contain the compounds having antibacterial activity. It can also possible that the antimicrobial activity is due to synergistic effects of compounds present in the fraction. These fractions further analysed using FTIR spectroscopy for functional group present in the compounds and HPLC is done for further fractionation of compounds.

Table 1: Antibacterial activity of different fractions against bacteria *E. coli* and *B. cereus*.

Fraction no.	Zone of inhibition in cm		%RIZD	
	E. coli	***B. cereus***	***E. coli***	***B. cereus***
13	2.5	2.0	83	66
14	2.5	2.0	83	66
25	2.3	2.2	76	73
28	2.5	2.1	83	70

3.7 TLC of individual fractions to know total no. of components in each fraction using different solvent system: Solvent system: -methanol and water (70:30).

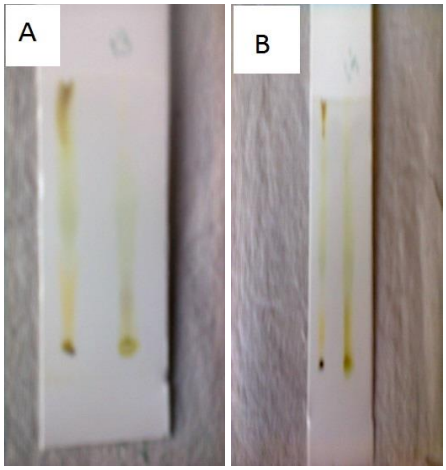


Fig 6: TLC of fractions with crude extract. Fraction no. 13 (A) and 14 (B) Fraction 13 & 14 shows 3 bands. Hence three components are responsible for antibacterial activity. Antibacterial activity may be due to synergistic effect of components.

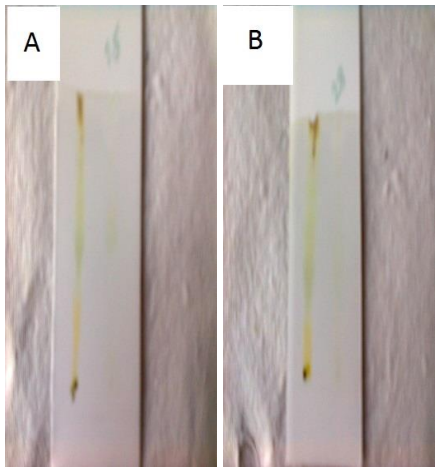


Fig 7: TLC of fractions with crude extract. Fraction no.25 (A) and 28 (B). Fraction 25 & 28 shows single band. Hence only one component is responsible for antibacterial activity. Then structural features of this component then analysed by IR and NMR.

3.8 HPLC Analysis: HPLC of crude extract shows 5 major peaks which indicate 5 compounds in the leaf extract. The peak at 23 & 24 min shows the presence of two compounds in a major amount. Another peak indicates other compounds in crude extract present in fewer amounts (Fig 8 A). HPLC of

fraction 13 shows one major peak at 23 min. indicates the presence of one component in a major amount. Other peaks at 41, 42 & 43 indicate the presence of other three compounds in less amount (Fig 8 B). HPLC of fraction 14 shows one major peak at 23 min. indicates the presence of one component in major amount (fig 8 C). HPLC of fraction 25 shows only one peak at 23 min (Fig 8 D). HPLC of fraction 28 shows only one peak at 23 min (Fig 8 E).

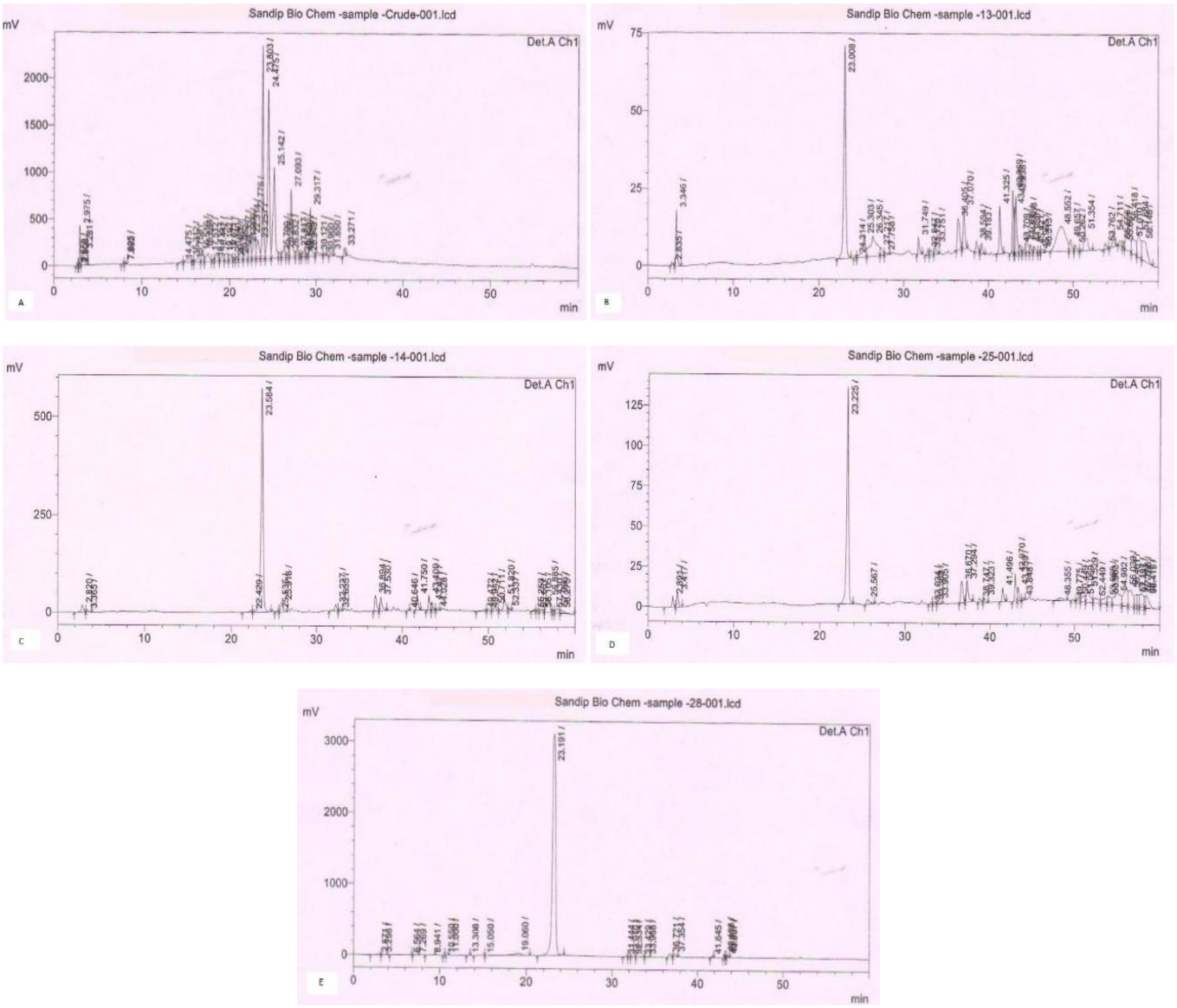


Fig 8: HPLC results of fractions A) crude sample B) Fraction no. 13 C) Fraction no. 14 D) Fraction no.25 E) Fraction no.28.

3.9 FTIR Analysis

Fraction 13 further separated by silica gel in a syringe column using methanol: water (70:30) solvent system. Four fractions were collected, concentrated & used for FTIR and NMR. Fraction 25 & 28 analyzed as it is by IR and NMR.

3.9.1 Fraction 1: Peak at 3384 indicates O-H stretching due to Alcohol or Phenol. The peak at 2922 indicates C-H stretching due to saturated alkanes. The peak of 1738 is due to Aldehyde. The peak at 1079 indicates C-O stretching due to Alcohol (Fig 9 A).

3.9.2 Fraction 2: Peak at 3374 indicates O-H stretching due to Alcohol or Phenol. The peak at 2922 indicates C-H stretching due to saturated alkanes. The peak of 1719 is due to Aldehyde. A peak at 1455 is due to NO_2 group. The peak at 1080 indicates C-O stretching due to Alcohol (Fig 9 B).

3.9. Fraction 3: Peak at 3355 indicates O-H stretching due to Alcohol or Phenol. The peak at 2922 indicates C-H stretching due to saturated alkanes. The peak of 1713 is due to Aldehyde. A peak at 1456 is due to NO_2 group. The peak at 1080 indicates C-O stretching due to Alcohol (Fig 9 C).

3.9.4 Fraction 4: Peak at 3387 indicates O-H stretching due to Alcohol or Phenol. The peak at 2922 indicates C-H stretching due to saturated alkanes. The peak of 1736 is due to Aldehyde or ester. A peak at 1078 indicates C-O stretching due to Alcohol (Fig 9 D).

3.9.5 Fraction 25: Peak at 2919 indicates C-H stretching due to saturated alkanes. The peak of 1708 is due to a carboxylic acid. A peak at 1459 is due to NO_2 group (Fig. 9 E).

3.9.6 Fraction 28: Peak at 2919 indicates C-H stretching due to saturated alkanes. The peak of 1706 is due to a carboxylic acid. The peak at 1459 is due to NO_2 group (Fig. 9 F).

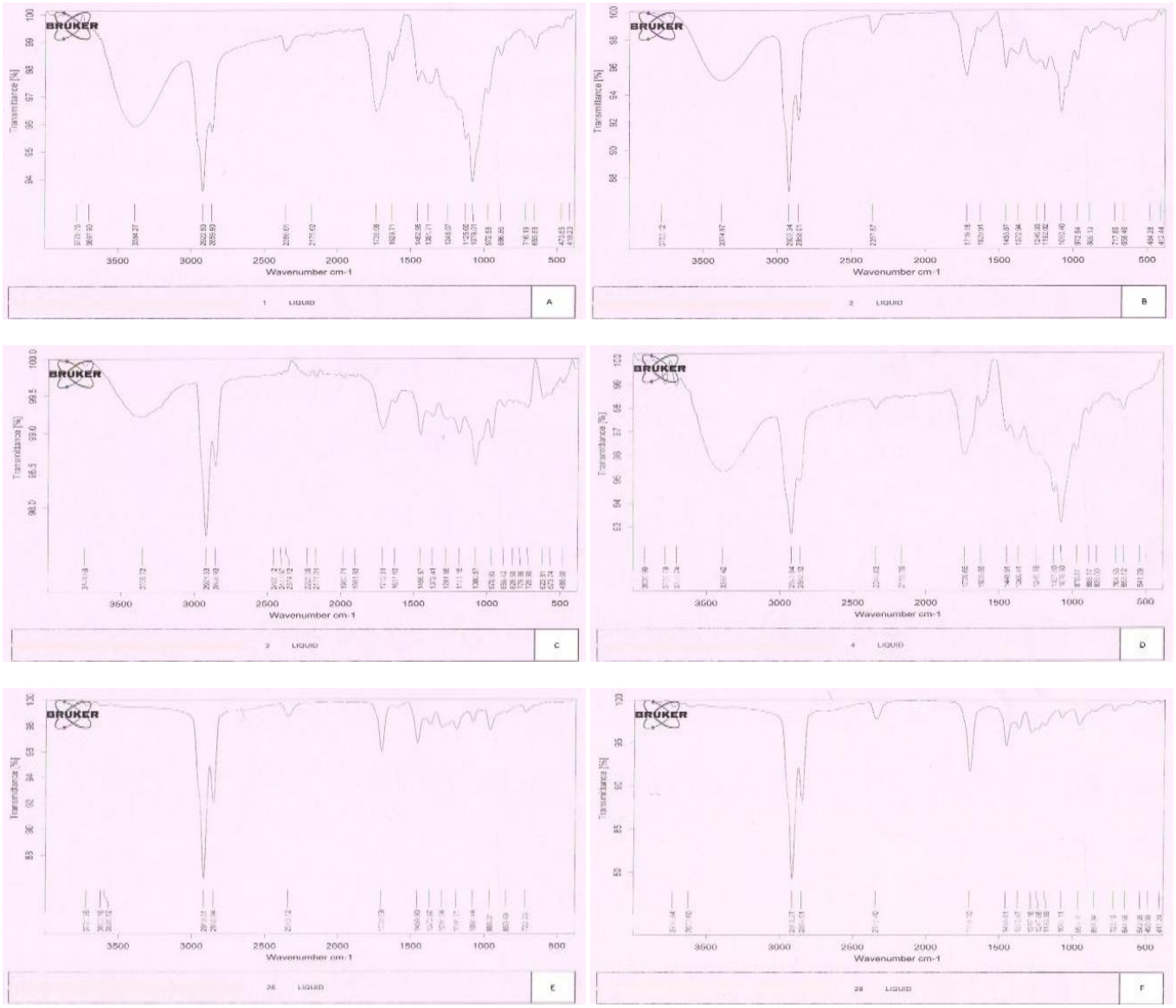


Fig 9: FTIR spectroscopy of fractions A) 1, B) 2, C) 3, D) 4, E) 25, F) 28.

3.10 NMR Analysis

3.10.1 Fraction no1: Peak at 1.4δ indicates the CH2-C group. The peak at 1.6δis of 3 protons of CH3-C=C. The peak at 7.3δ indicates the presence of H-Ar. The peak at 3.7δ indicates the presence of the CH-O- group (Fig. 10 A).

3.10.2 Fraction no2: Peak at 1.6δ is of 3 protons of CH3-C=C. The peak at 2.1δ indicates CH3-CO- group. The peak at 7.3δ indicates the presence of H-Ar. The peak at 3.7δ indicates the presence of the CH-O- group (Fig 10 B).

3.10.3 Fraction no3: Peak 0.9δ indicates the presence of CH3-C.The peak at 1.6δis of 3 protons of CH3-C=C. The peak at 2.1δ indicates CH3-CO- group. The peak at 7.3δ indicates the presence of H-Ar. The peak at 3.7δ indicates the presence of the CH-O- group (Fig. 10 C).

3.10.4 Fraction no4: Peak 0.9δ indicates the presence of CH3-C. The peak at 1.6δis of 3 protons of CH3-C=C. The peak at 2.1δ indicates CH3-CO- group. The peak at 7.3δ indicates the presence of H-Ar. The peak at 3.7δ indicates the presence of the CH-O- group (Fig. 10 D).

3.10.5 Fraction no25: Peak 0.9δ indicates the presence of CH3-C. Peak ranges from 1-3δ indicate the presence of the C-N group. The peak at 1.6δis of 3 protons of CH3-C=C. The peak at 2.1δ indicates CH3-CO- group. The peak at 7.3δ indicates the presence of H-Ar. The peak at 3.7δ indicates the presence of the CH-O- group (Fig. 10 E).

3.10.6 Fraction no28: Peak 0.9δ indicates the presence of CH3-C. Peak ranges from 1-3δ indicate the presence of the C-N group. The peak at 1.6δis of 3 protons of CH3-C=C. The peak at 2.1δ indicates CH3-CO- group. The peak at 7.3δ indicates the presence of H-Ar (Fig. 10 F).

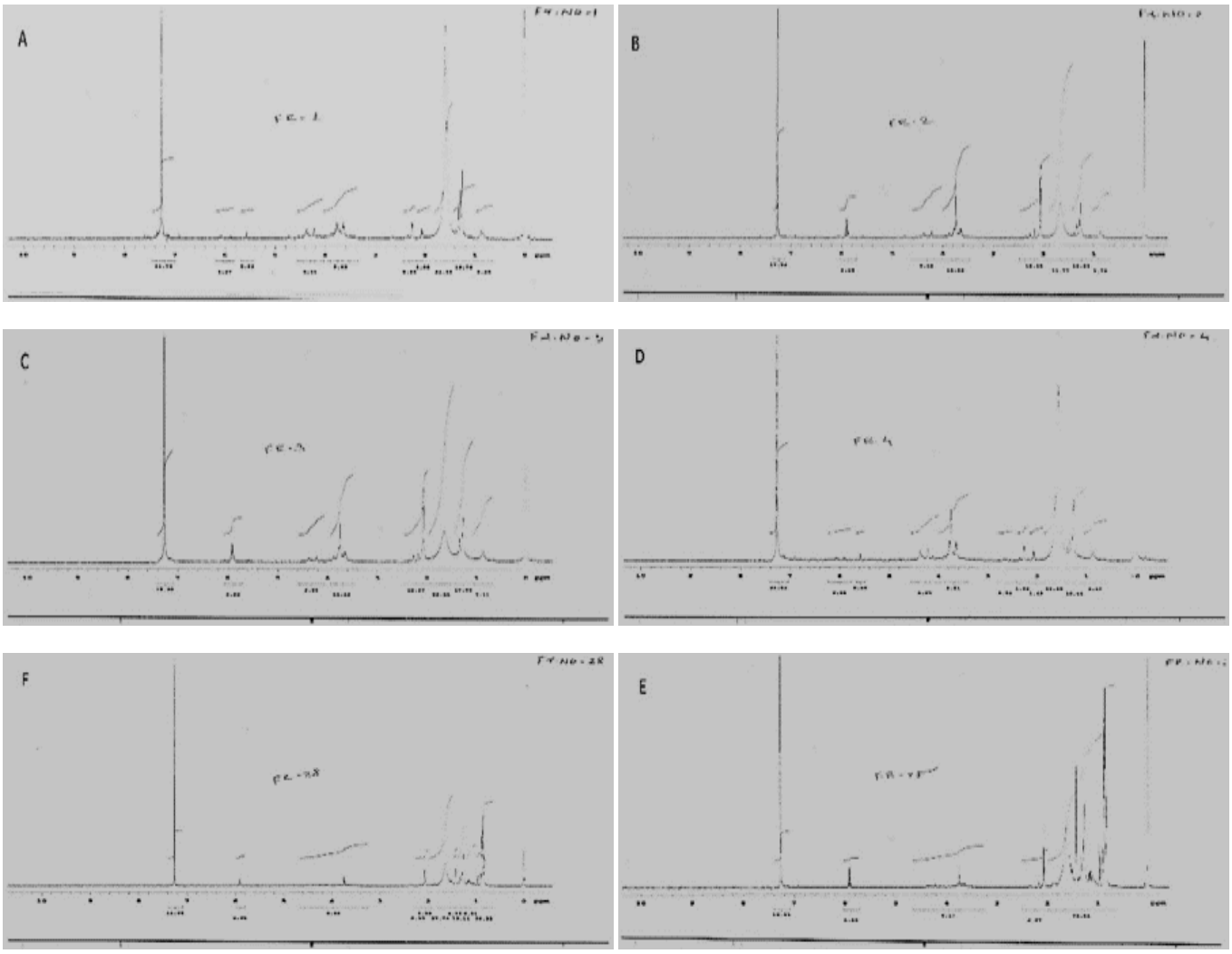


Fig 10: NMR spectroscopy of Fractions A) 1, B) 2, C) 3, D) 4, E) 25, F) 28.

4. Discussion

Screening of antibacterial activity of *C. fistula* leaf extract revealed that it has marked activity against both gram-positive and gram-negative bacteria. TLC of crude Extract shows better separation by Hexane: Ethyl Acetate (2:1) solvent system. Column chromatographic separation using the above solvent system fractions are collected which shows antibacterial activity. Fraction no. 13, 14, 12 &28 shows maximum activity. TLC of these fractions in methanol: water (70:30) solvent system shows 3 bands in 13&14 fraction and a single band in 25 &28 fractions. HPLC analysis of fraction 13 & 14 shows mainly three compounds responsible for antibacterial activity. And fraction 25 & 28 shows only one compound responsible for antibacterial activity. Fraction 13 further separated by methanol: water (70:30) solvent system. FTIR and NMR analysis data show various functional groups correspond to bioactive like Hopane (triterpene) [40], Rhein, and Catechin (phenolic) [41-42] Amentoflavone (flavonoid) [43].

5. Conclusions

This study showed that *Cassia fistula* (Bahava) has significant antimicrobial activity against gram-positive and gram-negative bacteria. They are thereby extremely effective as compared to standard antibiotics. Further research on this plant can lead to the formulation of unique drugs that can be used on various bacterial infections. This plant formulation may thereby provide an alternative solution to the current problem associated with the use of antibiotics. The FTIR and NMR analysis shows that triterpene, phenolic, and flavonoid compounds are responsible for antibacterial activity. Further studies will be included the incorporation of active compounds to ^{13}C NMR and GC-MS.

6. References

1. Nyeem MA, Haque MS, Hoque MA, Islam MM, Islam S. *Cassia fistula* (Bundaralati) Linn: Phytochemical and pharmacological studies: A review. International journal of advance scientific research 2017;2(1):25-30.
2. Sharma DK. Enumerations on phytochemical, pharmacological and ethnobotanical properties of *Cassia fistula* Linn: yellow shower. The Journal of Phytopharmacology 2017;6(5):300-306.
3. Seyyednejad SM, Motamedi H, Vafei M, Bakhtiari A. The Antibacterial Activity of *Cassia fistula* Organic Extracts. Jundishapur Journal of Microbiology 2014:7(1):e8921.
4. Antonisamy P, Agastian P, Kang CW, Kim NS, Kim JH. Anti-inflammatory activity of rhein isolated from the flowers of *Cassia fistula* L. and possible underlying mechanisms. Saudi Journal of Biological Sciences 2019;26:96-104.
5. Bahorun T, Neergheen VS, Aruoma IO. Phytochemical constituents of *Cassia fistula*. African Journal of Biotechnology 2005;4(13):1530-1540.
6. Gaikwad SA. Phytochemical investigation of bioactive Emodin and quercetin in *Cassia fistula* and *Cassia tora* plant parts by HPTLC. Journal of pharmacognosy and phytochemistry 2018;7(5):892-897.

7. Hada D, Sharma K. Isolation and Characterization of Chemical Compounds from Fruit Pulp of *Cassia Fistula* and Their Antimicrobial Activity. Journal of Drug Delivery & Therapeutics 2018;8(2):15-20.
8. Jadi S, Gorantla N, Nadendla S, Dange S. Isolation and chemical characterization of potential bioactive compounds from *Cassia uniflora.* International Journal of Pharmaceutical Sciences & Research 2019;10(12):5347-5361.
9. Jothy SL. Zakaria Z, Chen Y, Lau YL, Latha LY, Shin LN *et al.* Bioassay-Directed Isolation of Active Compounds with Antiyeast Activity from a *Cassia fistula* Seed Extract. Molecules 2011;16:7583-7592.
10. Krishnan P. Kruger NJ, Ratcliffe RG. Metabolite fingerprinting and profiling in plants using NMR. Journal of Experimental Botany 2004;56:255-265.
11. Ajay Kumar K, Satish S, Sayeed I, Hedge K. Therapeutic Uses of *Cassia fistula:* Review. International Journal of Pharma And Chemical Research 2017;3(1):38-42.
12. Verma K, Veerappapillai S, Karuppasamy R. Exploration of Plant Bioactive from *Cassia fistula* Leaves for The Treatment of Ovarian Cancer: An Integrative Approach. Asian Journal of Pharmaceutical & Clinical Research 2016;9(5):182-188.
13. HA Ibekwe. *In vitro* anthelmintic activities of aqueous crude extract of *Azadirachta indica* on *Paramphistomum cervi* and *Fasciola hepatica*. Int J Vet Sci Anim Husbandry 2019;4(1):14-18..
14. Chaerunisaa AY, Susilawati Y, Muhaimin M, Milanda T, Hendriani R, Subarnas A. Antibacterial activity and subchronic toxicity of *Cassia fistula* L. barks in rats. Toxicology Reports 2020;7:649-657.
15. Hanif MA, Bhatti HN, Nadeem R, Zia KM, Ali MA. *Cassia fistula* (Golden Shower): A Multipurpose Ornamental Tree. Global science books 2007;1(1):20-26.
16. Lavanya B, Narayanan N, Maheshwaran A, Suganya S, Sree YS, Arvindan SV *et al.* Basic Anatomical And Pharmacognostical Study of *Cassia fistula* Linn (Caesalpiniaceae). International Journal of Pharmacy & Biological Sciences 2016;6(3):105-120.
17. Pawar AV, Patil SJ, Killedar SG. Uses of *Cassia fistula* Linn as a Medicinal Plant. International Journal of Advance Research and Development 2017;2(3):85-91.
18. Wankhade RT, Bhabad PR, Gavane NA, Ade JS. Review Article on *Cassia fistula* Linn. International ayurvedic medical journal 2014;2(4):613-617.
19. Thirumal M, Surya S, Kishore G. *Cassia fistula* Linn - Pharmacognostical, Phytochemical and Pharmacological Review. Critical Review in Pharmaceutical Sciences 2012; 1(1):48-69.
20. Wulandari L, Retnaningtyas Y, Nuri, Lukman H. Analysis of Flavonoid in Medicinal Plant Extract Using Infrared Spectroscopy and Chemometrics. Journal of Analytical Methods in Chemistry 2016, 1-6.
21. Duraipandiyan V, Ignacimuthu S, Gabriel PM. Antifeedant and larvicidal activities of Rhein isolated from the flowers of *Cassia fistula* L. Saudi Journal of Biological Sciences. 2011; 18:129-133.
22. Bargah RK, Kushwaha PK. Extractions, Phytochemical Screening and *In-vitro* Antioxidant Activity of *Cassia fistula* Extracts. International journal of research in pharmacy and chemistry 2017;7(4):518-524.
23. Panda SK, Padhi LP, Mohanty G. Antibacterial activities and phytochemical analysis of *Cassia fistula* (Linn.) leaf. Journal of Advanced Pharmaceutical Technology & Research 2020;2(1):62-67.
24. Chinna V, Ratnampally SK. Quantitative Analysis of Phytochemicals in the Bark Extracts of Medicinally Important Plant *Cassia fistula*, Linn. International Journal of Current Microbiology & Applied Sciences 2017;6(4):1073-1079.
25. Hanne M, Lars D, Guro E, Edel E, Ida-Johanne J. Protein Determination—Method Matters. Foods 2018;7:5.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265.
27. Krivorotova T, Sereikaite J. Determination of fructanexohydrolase activity in the crude extracts of plants. Electronic Journal of Biotechnology 2014;17:329-333.
28. Saqib AN, Whitney PJ. Differential behavior of the dinitrosalicylic acid (DNS) reagent towards mono- and di-saccharide sugars. Biomass and Bioenergy 2011;35; 4748-4750.
29. Chewchinda S, Wuthi-udomlert M, Gritsanapan W. HPLC Quantitative Analysis of Rhein and Antidermatophytic Activity of *Cassia fistula* Pod Pulp Extracts of Various Storage Conditions. Bio Med Research International 2013, 1-5.
30. Numonov SR, Qureshi MN, Aisa HA. Development of HPLC Protocol and Simultaneous Quantification of Four Free Flavonoids from *Dracocephalum heterophyllum* Benth. International Journal of Analytical Chemistry 2015, 1–5.
31. Thirumal Y, Laavu S. HPLC Profile of Medicinal Plant Extracts and its Application in Aquaculture. Journal of Aquaculture Research & Development 2017;8:484.
32. Tripathi, I, Mishra P, Mahendra KR, Pardhi Y, Dwivedi A, Dwivedi N *et al*. HPLC Analysis of Methanolic Extract of Some Medicinal Plant Leaves of Myrtaceae Family. Internationale Pharmaceutica Sciencia 2012;2(3):49-53.
33. Ashokkumar R, Ramaswamy M. Phytochemical screening by FTIR spectroscopic analysis of leaf extracts of selected Indian Medicinal plants. International Journal of Current Microbiology and Applied Sciences 2014;3(1):395-406.
34. Hemmalakshmi S, Priyanga S, Devaki K. Fourier Transform Infra-Red Spectroscopy Analysis of *Erythrina variegata* L. Journal of Pharmaceutical Sciences and Research 2017;9(11):2062-2067.
35. Visveshwari M, Subbaiyan B, Thangapandian V. Phytochemical Analysis, Antibacterial Activity, FTIR and GCMS Analysis of *Ceropegia juncea* Roxb. International journal of pharmacognosy and phytochemical research 2017;9(7):914-920.
36. Pakkirisamy M, Kalakandan SK, Ravichandran K. Phytochemical Screening, GC-MS, FT-IR Analysis of Methanolic Extract of *Curcuma caesia* Roxb (Black Turmeric). Pharmacognosy Journal 2017;9(6):952-956.
37. Rajiv P, Deepa A, Vanathi P, Vidhya D. Screening for Phytochemicals and Ftir Analysis of *Myristica dactyloids* Fruit Extracts. International Journal of Pharmacy & Pharmaceutical Sciences 2016;9(1):315-318.
38. Seo JH, Kim JE, Shim JH, Yoon G, Bang MA, Bae CS *et al*. HPLC Analysis, Optimization of Extraction Conditions and Biological Evaluation of *Corylopsis coreana* Uyeki Flos. Molecules 2016;21:94.
39. Deborde C, Fontaine JX, Jacob D, Botana A, Nicaise V, Forget FR *et al.* Optimizing 1D 1H-NMR profiling of

plant samples for high throughput analysis: extract preparation, standardization, automation and spectra processing. Metabolomics 2019;15:28.
40. Lavanya B, Narayanan N. Isolation, Characterization And Formulation Of *Cassia Fistula*. Linn (Caesalpiniaceae). Indo American journal of pharmaceutical Research 2017, 7(1).
41. Duraipandiyan V, Ignacimuthu S. Antifungal activity of Rhein isolated from *Cassia fistula* L. flower. Webmed Central Pharmacology 2010;1(9); WMC00687.
42. Daisy P, Balasubramanian K, Rajalakshmi M, Eliza J, Selvaraj J. Insulin mimetic impact of Catechin isolated from *Cassia fistula* on the glucose oxidation and molecular mechanisms of glucose uptake on Streptozotocin - induced diabetic Wistar rats. Phytomedicine 2010;17:28-36.
43. Srividhya M, Hridya H, Shanthi V, Ramanathan K. Bioactive Amento flavone isolated from *Cassia fistula* L. leaves exhibits therapeutic efficacy. 3 Biotech 2017;7:33.
44. Martínez-Ávila GCG, Castro-López C, Rojas R. Screening of the *Cassia fistula* Phytochemical Constituents by UPLC-ESI-QTOF-MS2. Annals of Nutrition & Food Science 2018;2(2):1017.