EFFECT OF EXTRACTION SOLVENTS ON PHYTOCHEMICALS OF *CASSIA FISTULA* L. FLOWERS

Project submitted

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Submitted by

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CERTIFICATE

This is to certify that this project work entitled "**Effect of Extraction Solvents on Phytochemicals of *Cassia fistula* L. Flowers**" is a bonafide piece of project work done by **FATHIMA MEHEJABIN** (**Register No.: 210021022649), FATHIMA FARHANA K. S. (Register No.: 210021022666)** in the Department of Botany, Bharata Mata College, Thrikkakara under my guidance and supervision for the award of Degree of Bachelor of Science in Botany during the academic year 2021-2024. This work has not previously formed the basis for the award at any other similar title of any other university or board.

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DECLARATION

I hereby declare that this project entitled "**Effect of Extraction Solvents on Phytochemicals of *Cassia fistula* L. Flowers**" is the result of work carried out by me under the guidance of **Dr. Abin Kurian,** Department of Botany, Bharata Mata College, Thrikkakara. This work has not formed on the basis for the award at any other similar title of any other university of board.

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INTRODUCTION

Natural products are synthesized by various organisms, including plants, insects, fungi, algae, and prokaryotes, which coexist in ecosystems and interact chemically. These products, often biologically active, have served as traditional medicines. Among these organisms, plants are the primary source of natural products (Reynolds, 2007). The medicinal benefits of plant materials are largely due to the presence of phytochemicals, which are biologically active compounds found in fruits, vegetables, grains, nuts and seeds. These phytochemicals, including flavonoids, lignans, alkaloids, steroids, tannins, terpenoids, phenolic compounds, stilbenes and saponins, play a crucial role in promoting human health and preventing diseases. Scientific evidence supports their diverse biological properties, such as antioxidant activity, immune system stimulation, modulation of detoxification enzymes, antimicrobial effects, platelet aggregation reduction, hormone metabolism modulation and anticancer properties (Nyamai *et al*., 2016)

Plants synthesize two main categories of phytochemicals: primary and secondary metabolites. Primary metabolites, such as phytosterols, acyl lipids, amino acids, and organic acids, serve essential biological functions in all plant species, contributing to growth, development, and various metabolic processes. In contrast, secondary metabolites are not directly involved in plant metabolism but are derived from primary metabolites (Croteau *et al*., 2000; Irchhaiya *et al*., 2015). The study of medicinal plants begins with pre-extraction and extraction procedures, crucial steps for obtaining bioactive compounds. Traditional methods like maceration and Soxhlet extraction are often employed in small-scale research for this purpose (Azwanida, 2015).

Extraction is a crucial initial step in recovering and purifying active ingredients from plant materials. The goal of an extraction process is to achieve maximum yield of substances with high quality, including a high concentration of target compounds and antioxidant power of the extracts. Basic operations involve pre-washing, drying or freeze-drying, grinding for homogeneity, and improving extraction kinetics and sample-solvent contact. Care must be taken to prevent loss, distortion, or destruction of potential active constituents during extract preparation from plant sample (Spigno *et al*., 2007; Sasidharan *et al*., 2011). The choice of solvent for extraction depends on the nature of the targeted bioactive compound. Polar solvents like methanol, ethanol, or ethyl acetate are used for extracting hydrophilic compounds, while low polar solvents like hexane, petroleum ether is used for more lipophilic compounds

(Sasidharan *et al*., 2011; Cosa *et al*., 2006). The effectiveness of solvent extraction is influenced by various factors including solvent type, concentration, extraction time, temperature, pH, number of extraction steps, liquid-to-solid ratio, and plant material particle size (Cacace and Mazza, 2003). A variety of organic solvents are commonly used in phytochemical research to extract plant metabolites, each with its own elution effects. Different extraction techniques are also employed to prepare extracts, allowing for potential improvements in extraction methods. The selection of solvent depends on the intended use of the extract (George *et al*., 2001).

In isolating bioactive compounds, it is common to employ various separation techniques, including TLC, HPTLC, HPLC, flash chromatography, column chromatography and Sephadex chromatography to obtain pure compounds. These pure compounds are subsequently utilized to determine their structure and biological activity. Additionally, non-chromatographic techniques such as immunoassay, phytochemical screening assays, and FTIR can also aid in the identification of bioactive compounds (Sasidharan *et al*., 2011). TLC is a widely employed and effective method for detecting, evaluating, and isolating phytoconstituent molecules. Approximately 60% of global studies are predicted to be done using TLC. It is a simple, quick, and cost-effective approach that gives researchers with instant insights on the composition of a mixture. Additionally, TLC is performed to establish the identification of a component in a mixture by comparing its Rf value with that of a known compound. Other procedures include spraying phytochemical screening reagents, which create colour changes dependent on the phytochemicals contained in a plant extract, or inspecting the plate under UV light. These procedures are also used to validate the purity and authenticity of isolated items (Lade *et al*., 2014).

***Cassia fistula* L.** (Golden shower or Indian laburnum) a member of the Fabaceae family, is a medium-sized deciduous tree that may reach heights of up to 24 meters and girths of 1.8 meters. It is planted extensively over India and is one of the most prominent trees in Indian forests. The tree is commonly found in deciduous forests over most of India, reaching up to 1,220 meters in altitude in the sub-Himalayan and outer Himalayan areas. It is notably widespread in Central and Southern India. Cassia fistula is also often planted for decorative purposes in residential gardens and along roadsides (Pawar and Killedar, 2017). It has significant therapeutic qualities and rich in bioactive metabolites with varied biological activity. *C. fistula* is a decorative as well as a crucial component in the forest environment which blooms in summer and delivers an aesthetic view. All parts of the plant are medicinally effective in the treatment of fevers, heart illnesses, hemorrhages, ulcers, wounds, skin

problems, in traditional medicine. The plant is rich in flavonoids such as rhein, glycosides, phenolics such as fistulic acid, lignans, anthracene derivatives, catechins etc., derived from various portions. These phytochemicals contain pharmacological properties such as antioxidant, anti-inflammatory, hepatoprotective, antipyretic, antitussive action, antiulcer, central nervous system activity etc. (Bhakshu *et al*., 2023). The phytochemical components were extensively analyzed, particularly focusing on flavonoids, and their connection to various biological activities was explored (Zhao *et al*., 2016). Scientific evidence supports the therapeutic use of these compounds in health management, highlighting their antioxidant, anti-inflammatory, antidiabetic, and other beneficial biological effects (Rahmani, 2015).

All components of *C. fistula* such as leaves, bark, fruit, flowers, and seeds were beneficial in treating numerous ailments and established since ancient times. With the improvement in the scientific study there are numerous discoveries to locate the leads from this plant to show distinct medicinal claims like antioxidant, anti-inflammatory, hepatoprotective, antipyretic, antiulcer, larvicidal, laxative, anticancer, antidiabetic, antiepileptic, wound healing, antimicrobial, antifungal, antiviral and antibacterial activities (Rajagopal *et al*., 2013; Sanoria *et al*., 2020)

Solvent extraction is most frequently used technique for isolation of plant metabolites. However, the extract yields of the plant materials are strongly depended on the nature of extracting solvent, due to the different solubility of the chemical compounds present in it. Identification of most effective extraction solvents to increase the yield of raw materials is of great importance as it helps reducing the quantity required for medicine manufacture. Hence, preliminary phytochemical examination of the flowers of *C. fistula* along with determining the ideal solvent for the extraction was investigated in the current work.

OBJECTIVES OF THE STUDY

- Preparation of the different flower extracts of *Cassia fistula* for phytochemical analysis
- Preliminary phytochemical analysis of the flower extracts of *Cassia fistula*
- Standardization of the solvent systems for the chromatographic studies.
- Phytochemical investigation of the flower extracts of *Cassia fistula* using TLC.
- Comparative phytochemical analysis of the flower extracts to find the effect of different solvents.

REVIEW OF LITERATURE

Scientists and researchers have consistently been employing plant species for identification and extraction of phytocomponents with higher curative and therapeutic capacity. The plant extracts frequently exist as a mixture of multiple types of bioactive compounds or phytochemicals with varied polarity, their separation still remains a huge barrier for the process of identification and characterisation of bioactive compounds. In order to acquire these components in their purest form, they need to be separated from raw extract. The extracted crude samples are then needed to be further examined using analytical methods (Sasidharan *et al*., 2011; Lade *et al*., 2014).

The key processes for obtaining the biologically active component from plant resources are extraction, pharmacological screening, isolation and characterisation of bioactive compound, toxicological evaluation and clinical evaluation. Extraction procedures entail separation of medicinally active portions of plant tissue from inert components by employing selected solvents and extraction technologies. Solvents permeate into the solid plant tissues and solubilize molecules of comparable polarity. Quality of plant extract relies on plant material, choice of solvents and the extraction procedures (Das *et al*., 2010; Sasidharan *et al*., 2011). The pharmacopoeial criteria are not sufficient enough to assure the quality of plant materials since the materials received in the production facilities are not in a state where effective microscopic analysis can be done. Therefore, chemical methods instrumental methods and thin layer chromatographic analysis would establish the ideal quality of plant material. Non standardized processes of extraction may lead to the deterioration of the phytochemicals found in the plants and may lead to variations therefore leading to the lack of repeatability (Pandey and Tripathi, 2014)

Cassis fistula L. is a deciduous tree with grey bark and attractive yellow blooms. Its fruit has a cylindrical form and is filled with seeds. Seeds are separated by transverse walls. The diameter and length of fruit pod is in the range of 20- 27mm and 40-70 cm respectively. Fruit pod is somewhat bent and rounded at distal ends. It is attractive, medium sized and rapidly growing plant that shed its leaves at end of season (Danish *et al*., 2011). *C. fistula* grows natively in a variety of places around the world, including Pakistan, India, China, South Africa, Brazil, and the West Indies. C. *fistula* is found primarily on Caribbean islands and tropical forests in the West Indies (Duraipandiyan and Ignacimuthu, 2007). *C. fistula* has a long history of traditional use for treating fungal and nasal infections. Its fruit pulp acts as an antifungal and

has mild laxative effects. Across Asian countries, all parts of the plant are utilized for various medicinal purposes. It is known for its anti-inflammatory, cathartic, emollient and antipyretic properties, and is used to treat cardiovascular issues, respiratory tract infections and liver disorders. Additionally, Cassia fistula Linn exhibits a broad spectrum of antimicrobial activity and is effective against various skin diseases (Seasotiya *et al*., 2014).

2.1 Phytochemical evaluation

The primary and secondary metabolite composition of *C. fistula* extracts, which has been thoroughly investigated in a variety of plant parts including the seed, pollen, fruit, leaf, and pod, is primarily responsible for the biological effects of these extracts. The seeds have a high concentration of glycerides that consist mostly of linoleic, oleic, stearic, and palmitic acids. Additionally, there are small amounts of caprylic and myristic acids present. Lupeol, ß-sitosterol, and hexacosanol have been discovered as possible compounds derived from the stembark of *C. fistula.* The wild seeds of *C. fistula* have been discovered to contain around 31% crude proteins, mostly consisting of globulin and albumin, along with cephalin and lecithin phospholipids. In addition, the seeds contain a high amount of carbohydrates, including galactomannan, which is a complex carbohydrate made up of eight distinct kinds of sugar molecules (Bahorun *et al*., 2005). Ishita and Punita, (2018) carried out the phytochemical analysis of the various components of *C. fistula* (bark, seeds, pods, and leaves) and showed the existence of secondary metabolites, specifically alkaloids, tannins, anthraquinones, flavonoids, steroids, terpenoids, and glycosides. Additionally, the extractive values were also determined.

Various phytochemicals, including kaempferol-rhamnosyl-xyloside, apigenin-C-hexoside-O-pentoside, syringaresinol (lignan), quercetin-O-hexoside, apigenin-6,8-di-C-glycoside, apigenin-6-C-pentoside-8-C-hexoside (isomer 1,2,3), proanthocyanidin B, myricetin hexoside and 3,4-di-O-caffeoylquinic acid coumaric acid derivative, were identified in the leaves of *C. fistula* by Castro-Lopez *et al*., (2018). Rhein, a phytochemical marker, was highlighted for quality analysis in herbal laxatives and was extracted from the fruit pulp using ultrasonic-assisted extraction done by Yingngam *et al*., (2019). Fruit pulp decoction and its hydrolyzed solutions from Cassia fistula contain anthraquinone aglycones, which are effective against skin-infecting microbes, particularly fungal strains, with stable effectiveness over various storage periods (Chewchinda *et al*., 2013). Qualitative phytochemical analysis and identification of secondary metabolites in leaves and flowers of Cassia fistula were conducted by Bahorun *et al*. (2005) and Selvi *et al*. (2014).

2.2 Pharmacological activities

Sharma *et al*., (2010) investigated the antiviral activity of extracts from *Cassia fistula* pods and leaves against the infectious bovine rhinotracheitis (IBR) virus. The extracts were tested at various concentrations on MDBK cell lines. The pod extract showed a dose-dependent effect, inhibiting IBR virus proliferation, while the leaf extract did not show any significant effect. This suggests that *Cassia fistula* pod extract has potential antiviral properties against IBR virus. Laxmi *et al*., (2015) conducted study on the qualitative phytochemical tests and HPLC analysis on extracts obtained from *C.fistula* pods and leaves. The extracts demonstrated substantial efficacy against tested antimicrobial microorganisms including *E. coli, S. aureus, C. albicans*, and *Pasteurella multocida.* The leaf extracts were very efficient against *C. albicans*. These antibacterial activities were linked to the presence of quercetin dihydrate and kaempferol in the extracts (Irshad *et al*., 2013).

The pulp and seed extracts of Cassia fistula were found to have effective wormicidal properties against Pheretima posthuma, comparable to the standard drug Piperazine citrate. The seed extracts were particularly potent in their effects on the worms (Irshad *et al*., 2011). Similar results were observed by Satpute *et al*., (2017) with extracts from the stem bark and pods, with the ethanol extract being identified as a potent inhibitor. The ethanol extract of young bark and aqueous old bark showed significant anthelmintic activity in terms of paralysis and death time, compared to the control and standard drugs Albendazole and piperazine citrate. These findings suggest that extracts from the bark and pods could be used to treat chronic infections caused by parasitic worms. Munasinghe *et al*. (2001) reported on the antioxidant properties of C. fistula extracts compared to other plants in Sri Lanka. They found that ethanol extracts of leaves and methanol extracts of flowers, stem bark, and pulp showed antioxidant activity. Stem bark extracts exhibited particularly strong antioxidant effects, likely due to their high polyphenolic content.

Bhalodia *et al*. (2013) conducted qualitative and quantitative phytochemical analyses of the hydroalcoholic extract of Cassia fistula fruit pulp. They found various secondary metabolites and a significant amount of phenolic compounds. Despite this, the extract exhibited weak activity in quenching the DPPH radical. Ashraf *et al*. (2012) investigated the antidiabetic effects of an ethanolic extract from Cassia fistula pods in diabetic male Wistar rats. Over a 2-month period, diabetic rats were orally treated with three different concentrations of the extract. Glibenclamide was used as the standard drug control at a dose of 5 mg/kg body weight per day.

The results showed that the extract significantly reduced blood glucose levels, increased body weight, and raised glycogen levels in the liver tissues compared to diabetic control rats. Additionally, it improved oral glucose levels in diabetic rats.

MATERIALS AND METHODS

3.1 Collection and processing of plant material.

The dried flowers of *Cassia fistula* L. were used for the current study. The fresh flowers of *Cassia fistula* were collected from their natural localities of Ernakulam, Kerala and were authenticated. The fresh flowers obtained were shade dried for two weeks. After drying, the flowers were finely ground into powder using a mechanical blender and transferred into airtight glass containers at ambient temperature with proper labelling for further studies **(Plate: 1).**

3.2 Preparation of flower extracts

The flower extracts of *C. fistula* were prepared using three different solvents such as Hexane, Chloroform and Methanol. The cold extraction method was used to prepare both extracts. 5g of dried and powdered flower was taken in a conical flask (500ml) and 100 ml of hexane was added for the preparation of the hexane extract. Similarly, chloroform and methanol flower extracts of *C. fistula* were also prepared using the same methodology. The three conical flasks were kept in a rotary shaker for 3 days at room temperature. The extracts thus obtained were filtered using Whatman No. 1 filter paper. The extracts were later transferred to air tight glass containers, labelled and stored in refrigerator for the further analysis.

3.3 Qualitative preliminary phytochemical analysis.

To identify the presence of various classes of metabolites, different preliminary analysis was conducted.

3.3.1 Test for Alkaloids

3.3.1.1 Mayer's test: To a few ml of sample extract, two drops of Mayer's reagent are added along the sides of the tube. Appearance of white creamy precipitate indicates the presence of alkaloids (Tiwari *et al*., 2011).

3.3.1.2 Wagner's test: A few drops of Wagner's reagent are added to few ml of extract along the sides of the tube. A reddish-brown precipitate confirms the test as positive (Tiwari *et al*., 2011).

3.3.2 Test for Coumarins

Coumarins test: To two ml of sample extract, three ml 10% sodium hydroxide solution is added along the sides of the test tube. Appearance of yellow coloured solution indicates the presence of coumarins (Jayaprakash and Sangeetha, 2015).

3.3.3 Test for Flavonoids

3.3.3.1 Alkaline reagent test: 2 ml of 2% sodium hydroxide solution was mixed with plant crude extract, intensive yellow colour was formed, which turned colourless on addition of dilute acid (Jaradat *et al*., 2015).

3.3.3.2 Lead acetate test: To 2 ml of extract, few drops of lead acetate solution was mixed. Formation of yellow precipitate (Tiwari *et al*., 2011).

3.3.4 Test of Glycosides

Keller Killiani's test: To 2 ml extract glacial acetic acid is added along the sides of the test tubes and one drop of 5% ferric chloride solution added. Reddish brown colour appears at the junction of 2 layers and upper layer appears bluish green (Singh and Bag, 2013).

3.3.5 Test of Phenol

Ferric chloride test: To 2-3 ml of extract a few drops of 5% ferric chloride solution was added, presence of deep blue-black colour (Santhi and Sengottuvel, 2016).

3.3.6 Test for Quinones

Quinones test: To 2-3 ml of sample extract 3 ml hydrochloric acid is added. Appearance of yellow colour (Harborne, 1999).

3.3.7 Test for Steroids

Salkowski's test: To 2 ml of extract 2ml chloroform is added. 2 ml concentrated sulphuric acid is added along the sides of the test tube. Chloroform layer appears red colour and acid layer shows greenish yellow fluorescence (Joseph *et al*., 2013).

3.3.8 Test for Tannins

Braymer's test: 2-3 ml extract is diluted by adding 2 ml of distilled water. To which 2-3 drops of 5% ferric chloride is added. Appearance of black green or bluish colour (Rishikesh *et al*., 2013).

3.3.9 Test for Terpenes

Copper acetate test: 2 ml extract is dissolved in distilled water, to which 3-4 drops copper acetate solution is added and mixed, results in the production of emerald green (Morsy, 2014).

3.3.10 Tests for Carbohydrates

Molish's test: To 2 ml of plant sample extract, two drops of alcoholic solution of α- naphthol was added. The mixture is shaken well and few drops of concentrated sulphuric acid is added slowly along the sides of test tube. A violet ring indicates the presence of carbohydrates (Banu & Cathrine, 2015).

3.4 Solvent system standardisation

The solvent systems for the chromatographic analysis of hexane, chloroform and methanol were standardised by comparing the trials of different solvent system combinations.

3.4.1 Standardisation of Hexane and Chloroform

The solvent system required to be used for the TLC analysis of hexane and chloroform extracts of flower was standardised by conducting different TLC trials by 10 different ratios of low polar solvents such as Toluene and Ethyl acetate combinations. The different ratios of the solvent system were studied by increasing the ethyl acetate concentration from 0% to 90%. TLC trails were done using spot method.

Trials	Solvent system
T_1	100 % Toluene
T_2	Toluene : Ethyl acetate ; 9 : 1 ratio
T_3	Toluene : Ethyl acetate ; 8 : 2 ratio
T_4	Toluene : Ethyl acetate ; 7 : 3 ratio
T_5	Toluene : Ethyl acetate ; 6 : 4 ratio

T_6	Toluene : Ethyl acetate ; 5 : 5 ratio
T_7	Toluene : Ethyl acetate ; 4 : 6 ratio
T_8	Toluene : Ethyl acetate ; 3 : 7 ratio
T_9	Toluene : Ethyl acetate ; 2 : 8 ratio
T_{10}	Toluene : Ethyl acetate ; 1 : 9 ratio

3.4.2 Standardisation of Methanol

The methanol extract solvent system was standardised by 8 different TLC trials of Toluene, Ethyl acetate and Methanol combination ratios by increasing the ethyl acetate concentration. The polarity of the solvent system was increased for the standardisation of methanol sample.

Trials	**Solvent system**
T_1	Toluene : Ethyl acetate : Methanol ; 9:1:1 ratio
T_2	Toluene : Ethyl acetate : Methanol ; 8:2:1 ratio
T_3	Toluene : Ethyl acetate : Methanol ; 7:3:1 ratio
T_4	Toluene : Ethyl acetate : Methanol ; 6:4:1 ratio
T_5	Toluene : Ethyl acetate : Methanol ; 5:5:1 ratio
T_6	Toluene : Ethyl acetate : Methanol ; 4:6:1 ratio
T_7	Toluene : Ethyl acetate : Methanol ; 3:7:1 ratio
T_8	Toluene : Ethyl acetate : Methanol ; 2:8:1 ratio

3.5 Thin Layer Chromatographic studies

TLC analysis was performed on Aluminium backed pre-coated Merck silica gel plate 60 F_{254} plate (10 cm x 3 cm Merck, Germany). The origin was marked at 1cm from the bottom of the TLC plate. The TLC plates were activated and the test solutions was applied on to the TLC plate of uniform thickness of 0.2 mm in the form of bands with a width of 10 mm using a 2 mL capillary tube (Camag, Switzerland). The plates were then developed in their respective standardized solvent systems in a twin trough chamber previously saturated with solvent for

30 min to a distance of 9 cm. The plates were then treated at 100° C for 5 minutes before visualization.

3.5.1 Solvent system

Toluene: Ethyl acetate (9:1) was used as the solvent system for the hexane extract

Toluene: Ethyl acetate (8:2) was used as the solvent system for the chloroform extract.

Toluene: Ethyl acetate: Methanol (7:3:1) was used for the methanol extract.

3.5.2 Visualization

Observed the plates under UV light at 254 nm and 365 nm in the UV visualization chamber (Remi, India). TLC plates were also visualised in the visible light.

3.6 Chemicals and reagents used in the study

Solvents such as Hexane, Methanol, Chloroform, Toluene, Ethyl acetate was purchased from Merck and Nice was used. All other chemicals employed were of standard analytical grade from Merck, India.

Plate 1: A: Habit of *Cassia fistula*. **B:** Fresh flower of *C. fistula* **C:** Dried and powdered flower of *C. fistula*

RESULT AND DISCUSSION

4.1 Preliminary phytochemical analysis

The preliminary phytochemical analysis of the hexane, chloroform and methanol flower extracts of *C. fistula* was done using different phytochemical tests. The potential phytoconstituents present in the samples were analysed and characteristic differences were observed regarding the chemical composition of the three different extracts.

4.1.1 Hexane extract

The results of the preliminary phytochemical analysis of the Hexane extract using different phytochemical tests showed the presence of some major classes of phytoconstituents **(Table 1).**

Table-1 Preliminary phytochemical tests for Hexane extract

Sl. No.	Phytochemical constituents	Name of test	Inference
1	Alkaloids	Mayer's test Wagner's test	+ +++
2	Coumarins	Coumarins test	+
3	Flavonoids	Alkaline reagent test Lead acetate test	- -
4	Glycosides	Keller Killiani's test	+
5	Phenols	Ferric chloride test	-
6	Quinones	Quinones test	-
7	Steroids	Salkowski test	++
8	Tannins	Braymer's test	++
9	Terpenoids	Copper acetate test	+
10	Carbohydrates	Molish's test	+++

(+) Present, (-) Absent

In hexane extract, phytochemical constituents such as alkaloids, coumarins, glycosides, steroids, tannins, terpenoids and carbohydrates were present. The phytoconstituents belonging to the classes of flavonoids, phenols and quinones were not observed significantly in the phytochemical screening of the hexane extract.

4.1.2 Chloroform extract

The results of the phytochemical analysis of the Chloroform extract using different phytochemical tests showed the presence of different major classes of phytoconstituents **(Table 2).**

Table-2 Preliminary phytochemical tests for Chloroform extract

Sl. No.	Phytochemical constituents	Name of test	Inference
1	Alkaloids	Mayer's test Wagner's test	+ +++
2	Coumarins	Coumarins test	+
3	Flavonoids	Alkaline reagent test Lead acetate test	+++ +
4	Glycosides	Keller Killiani's test	+++
5	Phenols	Ferric chloride test	+
6	Quinones	Quinones test	-
7	Steroids	Salkowski test	++
8	Tannins	Braymer's test	+
9	Terpenoids	Copper acetate test	+
10	Carbohydrates	Molish's test	+++

(+) Present, (-) Absent

In the chloroform flower extract of *C. fistula*, majority of the phytochemical constituents such as alkaloids, coumarins, flavonoids, glycosides, phenols, steroids, tannins, terpenoids and carbohydrates were present. The preliminary phytochemical analysis showed the abundance of phytochemicals in the chloroform flower extracts of *C. fistula*. Whereas, only quinones were observed to be absent.

4.1.3 Methanol extract

The preliminary phytochemical analysis of the methanol extract to detect the presence of potential classes of phytoconstituents was done using different phytochemical tests **(Table 3).**

Table-3 Preliminary phytochemical tests for Methanol extract

Sl. No.	Phytochemical constituents	Name of test	Inference
1	Alkaloids	Mayer's test Wagner's test	+ +
2	Coumarins	Coumarins test	-
3	Flavonoids	Alkaline reagent test Lead acetate test	+ ++
4	Glycosides	Keller Killiani's test	+++
5	Phenols	Ferric chloride test	++
6	Quinones	Quinones test	+
7	Steroids	Salkowski test	+++
8	Tannins	Braymer's test	+++
9	Terpenoids	Copper acetate test	-
10	Carbohydrates	Molish's test	++

(+) Present, (-) Absent

In methanol extract, phytochemical constituents such as alkaloids, flavonoids, glycosides, phenols, carbohydrates, steroids, tannins and quinones are present. The phytoconstituents belonging to the classes of coumarins and terpenoids were not observed significantly in the phytochemical screening of the methanol extract. The glycosides, tannins and steroids were found to be most abundant among the different class of phytochemicals based on the results of the preliminary phytochemical analysis.

4.1.4 Comparative preliminary phytochemical analysis

The preliminary phytochemical analysis of hexane, chloroform extract and methanol extract confirmed the presence of various classes of phytochemicals. Seven different phytoconstituents were observed in the preliminary phytochemical analysis of hexane extract, nine different class of compounds were observed in the chloroform extract and eight classes were observed in methanol flower extracts of *C. fistula*. Alkaloids, glycosides, steroids, tannins and carbohydrates were found to be present in all three different solvent flower extracts. Alkaloids and carbohydrates had the highest intensities in hexane extract. All of the phytochemical tests conducted in the chloroform extract had the best results and the class of compounds such as, alkaloids, flavonoids, glycosides and carbohydrates could be considered as the major class of compounds in relation to their respective abundances. Glycosides, tannins and steroids were found be the characteristic class of compounds in the methanol flower extracts. The presence of only coumarins and terpenoids were not observed in the methanol extract as compared to the other solvent extracts. However, the presence of quinones was detected in the methanol flower extract alone.

The chloroform and methanol flower extracts of *C. fistula* alone showed the characteristic presence of two major class of phytochemicals such as phenols and flavonoids. Both class of compounds were absent in the hexane extract. The comparative analysis showed that the chloroform extract and the methanol extract observed the presence of maximum potential phytochemicals and the least was observed in the hexane extract.

Phytochemical analysis of the aqueous, methanol and petroleum ether extracts of dried parts of *C. fistula* was analysed by Kulkarni *et al.,* (2015) and revealed the presence of 11 important phytoconstituents *viz*., gums, amino, flavonoids, phenolic, compounds, glycosides, saponins, proteins, fats, acids, mucilages, anthraquinones, carbohydrates, alkaloids. The methanolic extract showed the presence all of phytochemicals tested, and tannins, flavonoids, alkaloids, phenolic compounds and saponins were present in high abundance. Khatak *et al.,* (2019) previously reported the preliminary phytochemical analysis of the methanolic flower extracts of *C. fistula* with the presence of flavonoids, carbohydrates, cardiac glycosides, steroids and phenols. Similarly, Bhalodia *et al*., (2011) found that the methanol extracts of *C. fistula* flowers contained a variety of compounds including tannins, anthraquinones, flavonoids, glycosides, saponins, proteins, triterpenoids, steroids, reducing sugars, carbohydrates and amino acids. In contrast, the chloroform extract of the same flowers showed

higher levels of phenolic compounds, anthraquinones, tannins, and glycosides. Panda *et al*., (2011) conducted similar studies on the leaf extracts of *C. fistula* using different solvents. The polar extracts like ethanol and methanol contained the majority of the constituents when compared to nonpolar extracts like petroleum ether and chloroform, indicating the presence of alkaloids, flavonoids, carbohydrates, glycosides, proteins, amino acids, saponins, and triterpenoids. However, tannins, phenols, proteins, amino acids, and flavonoids were present in every extract. The preliminary phytochemical analysis carried out by Bargah and Kushwaha, (2017) on the stem bark and leaves of *C. fistula* revealed the presence of various constituents such as flavonoids, alkaloids, terpenoids, reducing sugars, tannins, saponins, steroids, glycosides, phenolic compounds, proteins, and amino acids, with most of these being present in the polar extracts such as ethanolic and aqueous extracts.

Bhalodia and Shukla, (2011) discovered that the hydroalcoholic extracts of *Cassia fistula* leaves contain a variety of compounds, including anthraquinones, triterpenoids, tannins, flavonoids, saponins, steroids, carbohydrates, reducing sugars, proteins, glycosides and amino acids. Sakulpanich and Gritsanapan, (2009) revealed the presence of anthraquinone glycosides such as aloe-emodin, rhein, and chrysophanic acid on *Cassia fistula* leaves and pods, with rhein particularly noted for its potent laxative properties. Htun and Sann, (2021) indicated the existence of steroid, phenolic compound, alkaloid, saponin, glycoside, flavonoid, terpenoid, tannin, α-amino acid, reducing sugar, starch, carbohydrate and protein in the preliminary phytochemical analysis of the fruit pulp of *Cassia fistula*.

4.2 Chromatographic studies

The TLC profiling of the flower extracts of *C. fistula* using three different solvents were developed. The solvent systems for the hexane, chloroform and methanol extracts were standardized. Disparities were observed in terms of the number of bands and band intensity of the TLC profile developed for different solvent extracts. These disparities in turn show the qualitative and quantitative deviations in the phytochemical profile of the flower extracts of *C. fistula* using solvents of different polarities.

4.2.1 Hexane extract

TLC profile was developed for the hexane flower extracts of *C. fistula*. The best solvent system for the separation of hexane fraction was toluene: ethyl acetate mobile phase

combination in the ratio 9:1. The developed plate was visualized at different UV and visible wavelengths and Rf values are calculated.

4.2.1.1 At 254 nm

A total of three compounds were found to be present in the chloroform extract at 254 nm. Two bands were observed with an Rf value of 0.97 and 0.98 respectively. Both compounds were of relatively very low polarity in response to their observed high Rf values (**Table 4 and Plate 2).**

Table-4 TLC analysis of Hexane flower extracts of *C. fistula* at 254 nm

Sl. No.	Rf	Band colour
1	0.97	Light Blue
2	0.98	Pale Yellow

4.2.1.2 At 365 nm

The chloroform extract visualized at 365 nm had a total of six compounds. The compound with Rf value of 0.92 was found to be the prominent band with high intensity. The other major bands were observed with Rf values of 0.04, 0.78, 0.82 0.88 and 0.98. Four red coloured and 2 blue coloured UV active compounds were observed at 365 nm **(Table 5 and Plate 2).**

Table-5 TLC analysis of Hexane flower extracts of *C. fistula* at 365 nm

Sl. No.	Rf	Band colour
1	0.97	Blue
2	0.98	Red

4.2.1.3 At visible light

TLC plate of hexane extract at visible light had only a single light-yellow coloured compound with a Rf value of 0.98. No other characteristic bands were observed (**Table 6 and Plate 2).**

Table-6 TLC analysis of Hexane flower extracts of *C. fistula* at visible light

Sl. No.	Rf	Band colour
1	0.98	Light Yellow

4.2.2 Chloroform extract

TLC profile was developed for the chloroform flower extracts of *C. fistula.* The best solvent system for the separation of chloroform fraction was standardised as toluene: ethyl acetate mobile phase combination in the ratio 8:2. The developed plate was visualized at different UV and visible light wavelengths.

4.2.2.1 At 254 nm

A total of four compounds were found to be present in the chloroform flower extract at 254 nm. The bands were observed with an Rf value of 0.03, 0.12, 0.83 and 0.91 respectively (**Table 7 and Plate 2).**

Table-7 TLC analysis of Chloroform flower extracts of *C. fistula* at 254 nm

Sl. No.	Rf	Band colour
1	0.03	Black
2	0.12	Pale Black
3	0.83	Black
4	0.97	Black

4.2.2.2 At 365 nm

The chloroform extract visualized at 365 nm had a total of six compounds. The blue coloured band with Rf value of 0.79 was found to be the prominent compound with high

intensity. Similarly, a prominent yellow coloured band was observed at an Rf value of 0.06. The other major bands were observed with Rf values of 0.06, 0.38, 0.46, 0.81, 0.92 and 0.98. Diverse class of UV active compounds were observed in the TLC profiling with red-, blue- and yellow-coloured bands indicating potential phytochemicals. **(Table 8 and Plate 2).**

Table-8 TLC analysis of Chloroform flower extracts of *C. fistula* at 365 nm

Sl. No.	Rf	Band colour
1	0.04	Yellow
2	0.38	Pale Yellow
3	0.46	Blue
4	0.79	Fluorescent Blue
5	0.81	Light Red
6	0.92	Red
7	0.98	Light Blue

4.2.2.3 At visible light

TLC profile of chloroform extract at visible light had only a single compound with a Rf value of 0.06. No other characteristic bands were observed **(Table 9 and Plate 2).**

Table-9 TLC analysis of Chloroform flower extracts of *C. fistula* at visible light

Sl. No.	Rf	Band colour
1	0.05	Yellow

4.2.3 Methanol extract

TLC profile was developed for the methanol flower extracts of *C. fistula*. The best solvent system for the separation of methanol fraction was standardised as toluene: ethyl acetate: methanol mobile phase combination in the ratio 7:3:1. The developed plate was visualized at different wavelengths and Rf values are calculated.

4.2.3.1 At 254 nm

A total of three compounds were found to be present in the methanol extract at 254 nm. The major bands were observed with a calculated Rf value of 0.75 and 0.96 respectively and a light band at a Rf value of 0.75 **(Table 10 and Plate 3).**

Table-10 TLC analysis of Methanol flower extracts of *C. fistula* at 254 nm

S. No.	Rf	Band colour
1	0.68	Black
2	0.75	Light Black
3	0.96	Faded Blue

4.2.3.2 At 365 nm

The TLC profiling of the methanol extract visualized at 365 nm had a total of 10 different compounds. A fluorescent blue coloured band with a Rf value of 0.66 and a fluorescent red coloured band with a Rf value of 0.77 were found to be the two prominent bands with high intensity and band width. Along with that, there are compounds with Rf values of 0.21, 0.26, 0.35, 0.55, 0.62, 0.73, 0.90 and 0.98 observed in the TLC analysis of the methanol flower extract. The TLC profile of the methanol extract had the bands very well separated and could be easily distinguished from each other showing the efficacy of TLC profiling and the efficient solvent interaction **(Table 11 and Plate 3).**

Table-11 TLC analysis of Methanol flower extracts of *C. fistula* at 365 nm

S. No.	Rf	Band colour
1	0.21	Light Blue
2	0.26	Light Blue

3	0.35	Light Blue
4	0.55	Pale Blue
5	0.62	Light Red
6	0.66	Fluorescent Blue
7	0.73	Red
8	0.77	Fluorescent Red
9	0.90	Pale Blue
10	0.98	Blue

4.2.3.3 At visible light

TLC profiling of methanol extract at visible light had a total of 5 compounds. The bands were observed with a Rf values of 0.12,0.25,0.72,0.73 and 0.98 respectively **(Table 12 and Plate 3).**

Table-12 TLC analysis of Methanol flower extracts of *C. fistula* at visible light

S. No.	Rf	Band colour
1	0.12	Pale Yellow
2	0.25	Pale Yellow
3	0.72	Pale Yellow
4	0.73	Pale Yellow
5	0.98	Pale Yellow

4.2.4 Comparative TLC analysis of hexane, chloroform and methanol extracts.

The comparative analysis of the TLC profiles of the three solvents *viz*., hexane, chloroform and methanol extracts based on developed chromatographic plates showed that the Rf value of the bands which have the highest intensities in the developed chromatographic plates also possess a significant band width.

The comparative TLC analysis showed that a total of 35 distinct bands were observed among the three solvent extracts. The most efficient solvent that could be used for the isolation of phytochemicals from the flowers of *C. fistula* was found to be the methanol, having the maximum number of phytoconstituents. A total of 18 compounds were observed in the methanol flower extract of *C. fistula.* Thus, it further substantiates that majority of the phytochemicals in the flowers of *C. fistula* are having high polarity. TLC analysis of methanol flower extracts of *C. fistula* at 365 nm showed the highest number of bands (10) among all of the visualisation studies. Similarly, hexane flower extracts of *C. fistula* at visible light had the lowest number of bands (1). A total of 12 different phytoconstituents were present in the chloroform extract and the hexane extract had a total of 5 compounds. The comparative analysis showed that the phytoconstituents present in the flowers of *C. fistula* tends to be more on a medium to high polarity range as the majority of the bands with high intensities and band width were observed in the methanol extract. It further validates the findings of the preliminary phytochemical analysis studies of the flowers of *C. fistula.*

The effect of extraction solvents on the pulp and seed extracts of *C. fistula* using methanol and hexane was studied by Irshad *et al*., (2012) in relation to its antioxidant activities. *C. fistula* extract demonstrated the highest reducing power in the methanolic extract of pulp and seed as compared to the hexane extracts. It was concluded that the antioxidant activity of these extracts was directly proportional to the phenolic contents and the methanolic extracts had the abundance of phenolic compounds. The previous studies on the HPTLC analysis of the methanol leaf extracts of *C. fistula* showed five peaks under UV 366 nm, with the highest Rf values at 0.72 (43.51%), 0.63 (28.13%), and 0.86 (20.14%). The bands were identified as the major chemical groups in the leaves, confirming the presence of flavonoids and alkaloids as predominant compounds in the methanol extract (Chavan *et al*., 2016). Chewchinda *et al*., (2014) had developed a TLC image analysis and TLC densitometric method to quantify rhein content in *Cassia fistula* pod pulp aqueous extract. The HPTLC analysis of processed and unprocessed *C. fistula* fruit pulp revealed the presence of steroids, alkaloids, triterpenes glycosides, tannins, phenolic compounds, amino acids, carbohydrates and proteins in both types of samples (Agrawal *et al*., 2014). Verma *et al*., (2015) had developed an chromatographic method to find adulteration in *C. fistula* stem bark. The chromatographic fingerprint analysis of *C. fistula* stem bark and small branches showed distinct patterns that could effectively identify and differentiate between the two. The phytochemical profiles of

both parts were similar, suggesting that small branches could be used as a substitute for stem bark and to find adulterants.

The study by Khan *et al*., (2012) investigated the effects of four extracting solvents (ethanol, methanol, n-hexane, and petroleum ether) and two extraction techniques (simple maceration and hot percolation using a Soxhlet apparatus) on the antioxidant activity of pods, leaves, barks, and flowers of *C. fistula.* Similar to that of the current study the methanolic extract was found to be the most effective extraction method as compared to the other solvents for *C. fistula* in terms of its antioxidant activities. The high polar solvents like methanol or hydro-alcohol or even the aqueous extract could be more efficient in the extraction of the phytochemicals from *C. fistula.* The aqueous extract of the ripe pods of *C. fistula* was most effective in the quantification of glycosides and anthraquinones as reported by Sakulpanich and Gritsanapan, (2008).

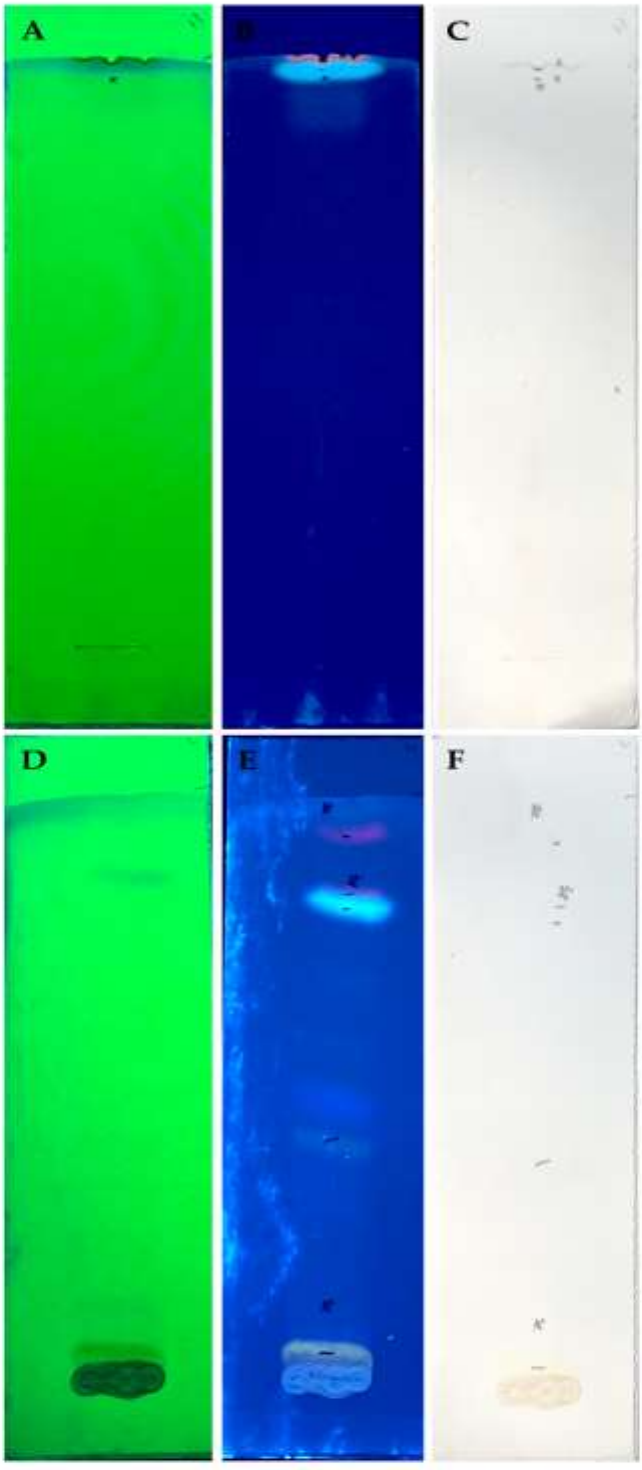

Plate 2: TLC profile of ***Cassia fistula*** **flower. A,B & C:** TLC chromatogram of the Hexane extract under 254nm, 365nm and visible light respectively. **D,E &F:** TLC chromatogram of Chloroform extract under 254nm, 365nm and visible light respectively

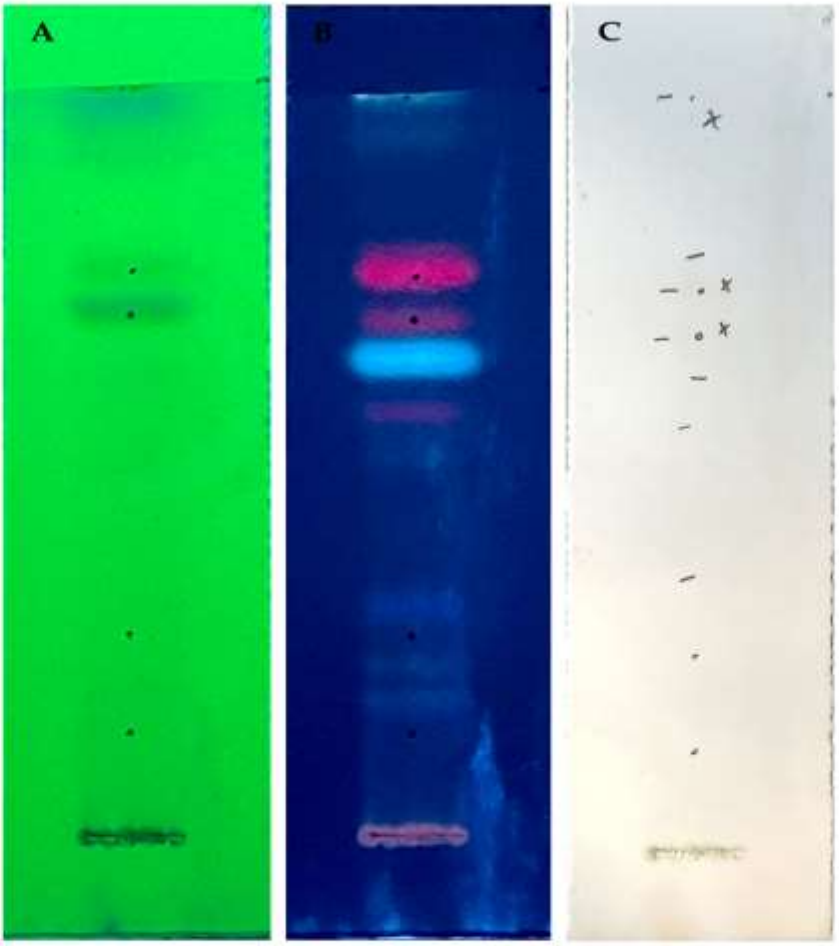

Plate 2: TLC profile of ***Cassia fistula*** **flower. A,B & C:** TLC chromatogram of the Methanol extract under 254nm, 365nm and visible light respectively.

SUMMARY AND CONCLUSION

Medicinal plants have been used across cultures for ages, serving as a vital source of medicine. Nature has long provided medicinal agents, with many modern drugs originating from natural sources. A significant number of these drugs were discovered by the process of isolating compounds from plants, guided by their traditional application in diverse medical systems. Traditional medicine, predominantly derived from plants, has served as a valuable reservoir for alternative remedies, novel pharmaceuticals, and healthcare products. Currently, a vast number of medicinal plant species are acknowledged for their curative attributes. The demand for Ayurvedic formulations is on the rise, both domestically and globally. However, the medicinal plant industry faces significant challenges due to the scarcity of genuine raw drugs, leading to the use of various substitutes or adulterants. In the coming years, many plant species may become scarce for industrial use due to overexploitation.

The chemical composition of medicinal plants varies due to factors such as species, plant part used, harvest time, and processing methods, which can result in differing pharmacological effects. Unlike conventional pharmaceuticals, herbal medicinal products can have varying compositions, leading regulatory agencies to emphasize standardization for ensuring safety and efficacy. It is essential to identify and assure the quality of herbal medicines to maintain consistency in their effectiveness. Solvent extraction is the most commonly used method for isolating plant metabolites. The yield of extracts from plant materials largely relies on the type of solvent used, which affects the solubility of the chemical compounds present. Polar solvents are often used to extract polyphenols from plant matrices. The flowers of *Cassia fistula,* one of the flowers of significant medical and cultural value was selected for the current study.

The preliminary phytochemical analysis of hexane, chloroform extract and methanol extract confirmed the presence of various classes of phytochemicals such as alkaloids, glycosides, steroids, tannins and carbohydrates in all extracts. Seven different phytoconstituents were observed hexane extract, nine in the chloroform extract and eight classes of phytochemicals were observed in the methanol flower extracts of *C. fistula*. Alkaloids and carbohydrates had the highest intensities in hexane extract. Glycosides, tannins and steroids were found be the characteristic class of compounds in the methanol flower extracts. The comparative analysis showed that the chloroform extract and the methanol extract

observed the presence of maximum potential phytochemicals and the least was observed in the hexane extract.

The TLC profiling of the flower extracts of *C. fistula* using the solvents such as hexane, chloroform and methanol in their increasing polarity observed a total of 35 distinct bands. The most efficient solvent that could be used for the isolation of phytochemicals from the flowers of *C. fistula* was found to be the methanol, having the maximum number of phytoconstituents. A total of 18 compounds were observed in the methanol flower extract of *C. fistula* as compared to a total of 12 different phytoconstituents in the chloroform extract and 5 compounds in the hexane extract. Thus, it further substantiates that majority of the phytochemicals in the flowers of *C. fistula* are having high polarity

In conclusion, the present work was an attempt to correlate the effect of extraction solvents on phytochemicals of *Cassia fistula* flowers using chromatographic techniques. The identification of most effective extraction solvents to increase the yield of raw materials is of great importance as it helps reducing the quantity required for medicine manufacture.