Asian Journal of Pharmaceutical Education and Research
Vol -7, Issue-4, October-December 2018
ISSN:2278 7496

RESEARCH ARTICLE Impact Factor: 5.019

EXTRACTION PHYTOCHEMICAL SCREENING AND QUANTITATIVE STUDY OF BIOACTIVE CONSTITUENTS PRESENT IN AQUEOUS EXTRACT OF *CASSIA FISTULA* L.

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Received 15 July 2018 Revised 19 July 2018; Accepted 19 July 2018, Available online 15 October 2018

ABSTRACT

The potential of higher plants as source for new drugs is still largely unexplored. Among the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller. *Cassia fistula* also known as the Golden Shower belongs to Fabiaceae family is one such higher plant whole of which is used to treat diarrhea, seeds, flowers and fruits are used to treat skin diseases, fever, abdominal pain and leprosy by traditional people. The present study was intended to explore the phytochemical constituents in aqueous extract of *Cassia fistula* L to assess its medicinal and therapeutic potential. The fine defatted powder leaves of *C. fistula* L were subjected to extraction with distilled water by soxhlation followed by concentration then tested for various phytochemical test, TLC analysis and estimation of total polyphenolic compound. The aqueous extract of leaves of *C. fistula* L were observed to be rich in glycosides, flavonoids, steroids, phenolics amino acids, proteins, saponins and diterpines. The TLC analysis indicated the presence of gallic acid equivalent polyphenols in aqueous extract in addition to presence of 6-7 unknown phytoconstituents. The aqueous leaf extract was reported contain the total polyphenolic compounds in in the range 8.09 mg per 100 mg equivalent to standard gallic acid. Since the aqueous extract are rich in gallic acid equivalent polyphenols indicates the prospects of this plant extract in medicinal and therapeutic significances due to the possible potential, anti-oxidant, anticancer, antimicrobial, antihelmithic, hepatoprotective, immunomodulation, and many other activities. The phetochemicals of this valuable plant could be prospected in development of valuable therapeutics upon further extensive investigation.

K**eywords:** *Cassia fistula* L, TLC, TPC, gallic acid.

INTRODUCTION

The 80% of the world's population is dependent on the traditional therapies involves the use of plant extracts or their active constituents. The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries[1]. From about 60,000 years ago humankind has been using plants as ethnomedicine around the world [2, 3]. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs[4].

In recent years the plant-derived substances have been gaining significance again due to their versatile applications. The potential of higher plants as source for new drugs is still largely unexplored. Among the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller[5].

Cassia fistula also known as the Golden Shower belongs to Fabiaceae family is one such higher plant though studied but still keeps potential for further exploration[6, 7]. It is a semi-wild Indian Labernum distributed in several countries including Asia, South Africa, Mexico, China, West Indies, East Africa and Brazil. It is an ornamental tree with beautiful bunches of yellow flowers. *C. fistula* L. exhibited significant antifungal activity and showed properties that support folkloric use in the treatment of some diseases as broad-spectrum antifungal agent[8]. The whole plant is used to treat diarrhea; seeds, flowers and fruits are used to treat skin diseases, fever, abdominal pain and leprosy by traditional people[9].

This plant has a strong tendency to contain anthraquinone derivatives. From the genus Cassia many quinone derivatives such as Kaempferol have also been isolated; a proanthocyanidin has been isolated from the acetone extract of the flower[10]. A bianthraquinone glycoside, fistulin, together with kaempferol and rhein has been isolated from ethanol extracts of its flowers[11]. Besides phenols and their derivatives, a certain amount of alkaloids have also been reported in the flowers; traces of triterpenes have been observed in both flowers and fruits[12]. The present study was intended to explore the phytochemical constituents in aqueous extract of *Cassia fistula* L to assess its medicinal and therapeutic potential.

MATERIALS AND METHODS

Collection of plant material

The leaves of *Cassia fistula* L., were collected from the well identified plants from the road sides with the Bhopal city. The identification of the complete plant and its parts were done by Dr. Madhuri Modak, Professor at Department of Botany, Government College, Ashta, Madhya Pradesh, India.

Extraction of Phytochemicals

The leaves of *Cassia fistula* L., were washed, cleaned and dried at room temperature for 2-3 days then grounded into fine powder using electric grinder. This powder is then subjected to defatting in petroleum ether overnight. 100 gm of defatted & dried leaf powder of the *C. fistula* L., were extracted exhaustively using distilled water in a 250 ml capacity Soxhlet apparatus (Borosil, India) for 16 hours to obtain the aqueous extract of leaves these plants[13, 14]. The extract was concentrated in water bath and subjected to phytochemical analysis both qualitatively and quantitatively.

Qualitative phytochemical analysis

Various type of phytoconstituents present in the aqueous extract of leaves of *C. fistula* L., was determined by the methods suggest by Khandelwal, (2005) and Kokate, (1994) which is described ahead[15, 16].

Preliminary chemical analysis:

The preliminary phytochemical test were conducted to check the present of, alkaloids, steroids, carbohydrates, glycosides, flavonoids, tannin, triterpenoids, saponins, proteins & amino acids, resins, fixed oils, fats and waxes. The tests conducted are discussed as follows;

i. Test for Alkaloids

a) **Dragendorff's Test**: Approx. 1ml of stock extract is diluted with 5 ml distilled water followed by addition of few drops of 1N HCl; To this 1 ml of dragendorff's reagent was added, development of an redish orange or brown precipitate indicates the presence of alkaloids.

b) **Wagner's Test**: Approx. 1ml of stock extract diluted in 5 ml distilled water is acidified HCl then few drop of Wagner's reagent (iodine potassium iodide solution) was added to it. Any reddish brown precipitate indicates the presence of alkaloids.

c) **Mayer's Test**: 2-3 drops Mayer's reagent (potassium mercuric iodide solution) was added in 2 ml of extract solution, if a dull white precipitate forms, it indicates the presence of alkaloid.

d) **Hager's Test**: 3 ml of Hager's reagent (saturated solution of picric acid) when added to extract solution and a yellow precipitate develops, this again confirms the presence of alkaloids in any extract.

ii. Test for Steroids and Sterols

a) **Liebermann's Burchard Reaction**: The test extract solution was dissolved in 2 ml of chloroform in a dry test tube. Now 10 drops of acetic anhydride and 2 drops of concentrated sulfuric acid were added. The solution became red, then blue and finally bluish green in color.

b) **Salkowsky Test**: The extract of test solution dissolved in chloroform and equal volume of conc. sulphuric acid was added. Bluish red cherry, red and purple color was noted in chloroform layer, whereas acid assumes marked green fluorescence.

iii. Test for Carbohydrates

a) **Molisch's Test:** To 1-2 ml of phytochemical extract stock 2 drop of freshly prepared 20% alcoholic α-napthol solution was added and to this concentrated sulfuric acid along the sides of the test tube. Purple

color or reddish violet color at the junction between two liquids will be an indication for the presence of carbohydrates.

b) **Benedict's Test:** Upon adding benedict's solution to the diluted plant extract in a test tube followed by boiling & shaking vigorously for 2 minutes and then bringing down to room temperature. The presence of carbohydrates will be confirmed if red precipitate appears.

c) **Barfoed's Test:** To 0.5 ml of plant extract solution, barfoed's solution is added and heated to boil. Red precipitate will indicate the presence of carbohydrates.

d) **Anthrone Test:** When a green or blue colour forms upon adding 2 ml of anthrone test solution, to plant extract solution confirms the presence of carbohydrate.

iv. Test for Glycosides

a) **Legal's Test**: The solution of extract is mixed with pyridine then sodium nitroprusside solution was added to it and made alkaline. The formation of redish pink colour will indicate the presence of glycosides.

b) **Keller Kiliani test**: diluted extract stock prepared in methanol was dissolved in glacial acetic acid contained the trace of ferric chloride the 1 ml concentrated sulphuric acid was carefully added by the side of the test tube. At the junction blue colour in the acetic acid layer and red colour of the two liquid indicates the presence of glycosides.

v. Test for Flavonoids

a) **Shinoda Test**: To the diluted extract stock 5-10 drops of dil. HCl was added followed by putting a small piece of magnesium in that test tube. The formation of pink, reddish pink or brown colour will be the indication for the present of flavonoids.

b) **Lead Acetate Test**: To the 2 ml of diluted extract, 2-3 drops of 10% lead acetated solution were added followed by gentle warming of solution, this solution when cools down dirty white or yellow precipitate forms at the bottom indicates the present of flavonoids

vi. Test for Tannins

a) To the diluted plant extract, few drops of ferric chloride solution was added. Formation of dark blue or greenish black colour in the solution indicate the presence of tannins.

b) To the sample of extract, potassium dichromate solution was added, yellow precipitate was produced.

vii. Test for Triterpenoids

a) 2-3 granules of tin were taken in test tube, and were dissolved in 2 ml of thionyl chloride solution. To this solution diluted plant extract was added. Formation of pink colour indicates the presence of triterpenoids in this solution.

b) To approx. 1 ml of diluted extracts 2ml chloroform ($CHCl_3$) was added and concentrated H_2SO_4 (3ml) was carefully added from the side wall of test tube to form a layer. A reddish brown coloration of the interface formed indicating the presence of terpenoids.

viii. Test of Saponins

a) **Froth Test**: 1 ml of extract stock is diluted upto 5 ml with distilled water in a test tube. The test tube is the vigorously shake putting thumb on the mouth of test tube for 30 seconds. The formation of froth above the solution that stays more than 1 to 5 minutes is the indication for the presence of saponins.

ix. Test for Protein and Amino acid

a) Biuret's Test: To 2 - 3 ml of the plant extract stock 1 ml of 40 % sodium hydroxide solutions and 2 drops of 1 % copper sulphate solution was added, a purpl-violet or pinkish-violet colour indicates the presence of proteins.

b) Ninhydrin's Test: Two drops of freshly prepared 0.2 % ninhydrin reagent was added to the extract and heated to boiling for 1 - 2 min. and allowed cooling. Development of blue colour indicates the presence of proteins, peptides or amino acids.

c) Xanthoprotein Test: Upon adding concentrated nitric acid to diluted extract stock leads to formation of a white precipitate which upon heating turns yellow. After carefully cooling down this solution adding 20 % of NaOH solution in excess gives orange colour indicates presence of aromatic amino acid.

d) Millon's Test: 5 - 6 drop of Millon's reagent was added to the diluted extract. Formation of a white precipitate which turns red on heating indicates the presence of proteins.

x. Test of Resins

Dissolved the extract in the acetone and pore the solution in the distilled water. Turbidity indicated the presence of resin.

xi. Test of fats or fixed oils

a) **Using sodium hydroxide:** The extract was mixed in one ml 1 % of copper sulphate solution then added 10 % sodium hydroxide solution a clear blue solution was obtain which shows the presence of glycerin in sample.

b) **Using sodium hydrogen sulphate:** The extract was taken in test tube added a pinch of sodium hydrogen sulphate if a pungent odour forms, glycerin is present.

c) **Saponification:** Four ml of 2 % sodium carbonate solution was taken and the extract was added. Agitated vigorously and boiled. A clean soapy solution was formed cooled and added few drops of concentrated HCl and observed that fatty separate out and float up.

TLC analysis

Thin layer chromatography profiling of aqueous extract of *C. fistula* L., leaves was performed according to methods suggested by Biradar & Rachetti (2013) with suitable modifications[17]. Ready to use silica gel TLC plates 60G F_{254} DC Kieselgel 60 plates from Merck were used to perform the thin layer chromatography. The plates were activated by drying in hot air oven at 110°C for 30 mins then cooled to ambient temperature at dry place. Sample were prepared by diluting the crude extracts in sterile distilled water then applied to TLC plates with the help of fine glass capillary 1 cm above its bottom. The plates were allowed to develop in TLC glass chamber containing solvent system Toluene: Ethyl acetate: Formic acid in 7:5:1 ratio. Gallic acid was used as standard polyphenol to compare the presence of standard compound under white light, long & short UV light.

Quantitative analysis

Total Phenolic Content Estimation:

As a part of quantitative estimation of phyto-constituents in plant extracts, total phenolic content (TPC) in extract was determined by the modified Folin-Ciocalteu method[18, 19, 14].

Preparation of Standard: Gallic acid (GA) (HiMedia Laboratories Pvt Ltd India) standard solution was prepared by dissolving 50 mg of GA in 50 ml methanol, various aliquots of 10- 50µg/ml was prepared in methanol.

Procedure: 1 ml of suitably diluted aqueous extract of plant or standard was mixed with 5 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 4 ml (75g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 30 min at 40°C for colour development. The absorbance was measured at 765 nm using a spectrophotometer. Using gallic acid as standard total

phenolic content (standard curve was prepared using concentrations 10- 50µg/ml) was expressed as mg GA equivalent/100 mg of extract.

RESULTS & DISCUSSION

Preliminary phytochemicals analysis

Out of the 100 grams of defatted leaf powder of plant, 25 grams of leaf powder was subjected to aqueous extraction yielded 1.025 grams *C. fistula* L. dry leaf extract that amount to be 4.1% in terms of percentage yield of extraction. The aqueous extracts of plants *C. fistula* L., subjected to test for detection of various phytoconstituents was observed to be rich in various types of phytochemical groups that are depicted in table 1. There were total 10 types of phytochemical groups were tested in aqueous extract of the *C. fistula* L. leaves out of which only alkaloids and carbohydrates were reported to be absent from the extract in present study. The aqueous extract to the plant was observed to be rich in glycosides, flavonoids, steroids, phenolics amino acids, proteins, saponins and diterpines.

Table 1: Result of phytochemical screening of aqueous extract of leaves of *C. fistula* L.

S.N.	Constituents tested	Observation
1.	Alkaloids	–ve
2.	Glycosides	+ve
3.	Flavonoids	+ve
4.	Steroids	+ve
5.	Phenolics	+ve
6.	Amino Acids	+ve
7.	Carbohydrate	–ve
8.	Proteins	+ve
9.	Saponins	+ve
10.	Diterpines	+ve

Detection gallic acid equivalent polyphenol in TLC profile

In present study the TLC analysis with sample extract was done to check out the number of unknown components present aqueous leaf extracts and to detect the presence of gallic acid equivalent phenolics compound in the aqueous extracts on TLC plate. From the results of preliminary phytochemical analysis of aqueous extract of leaves *C. fistula* L., was positive of the presence of phenolics.

With reference to figure 1, it is observed that the standard gallic acid like compounds were present in aqueous extracts obtained from *C. fistula* L., when the spots sample was compared at a region on of where R_f values of gallic acid standard falls though they were feeble in visualization.

Referring to table 2 it has been observed that the samples A (aq. Extract) shows 7 spots in short UV light while 6 spots in long UV light which indicates that aqueous leaf extract of *C. fistula* L., contains 6 to 7 different types of chemical constituents which could be further separated investigated in future studies.

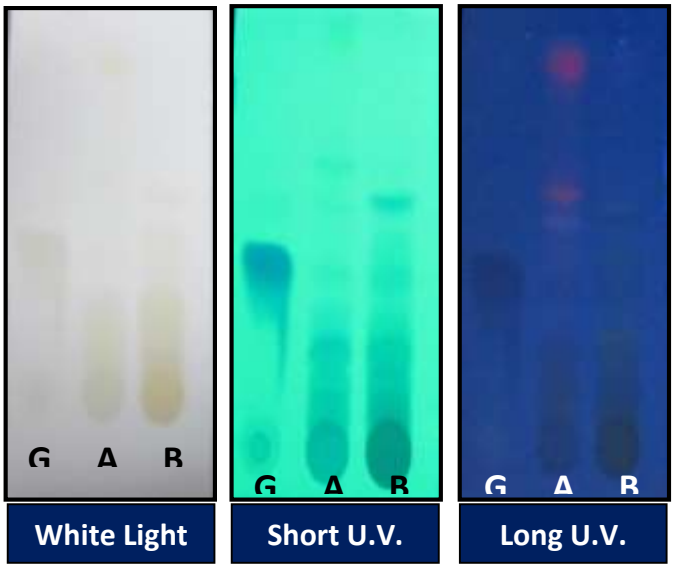

Figure 1: TLC profile of aqueous extracts of *C. fistula* L., (A) and Standard Gallic acid (G)

Table 2: TLC profile of aqueous extract (A) of *C. fistula* L., and detection of phenolic components running in solvent system Toluene: Ethyl acetate: Formic acid (7:5:1)

S.N	Samples	Number of developed Spots		Phenolics
		In Short UV Light	In Long UV Light	
1.	**G**	1	1	+ve
2.	**A**	7	6	+ve

Quantitative Analysis

Total Phenolic content estimation (TPC)

The content of total phenolic compounds (TPC) was expressed as mg/100mg of Gallic acid equivalent of dry extract sample with the help of equation obtained from the calibration curve:

$$\mathbf{Y = 0.011X+0.011,\ R^2= 0.998}$$

Where: *X is the absorbance and Y is the phenolic equivalent (GAE).*

Table 3: Preparation of calibration curve of Gallic acid

S.N	Gallic acid Conc. (in µg)	Absorbance at 765 nm
1	10	0.135
2	20	0.247
3	30	0.364
4	40	0.474
5	50	0.581

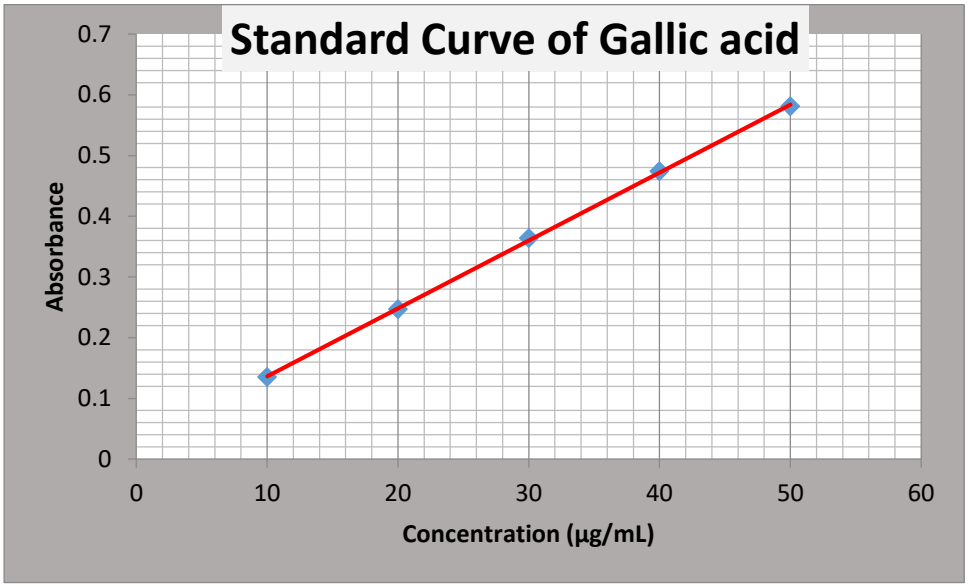

Figure 2: Standard Curve of Gallic acid for Estimation of Total Phenolic Content at 765 nm by Folin-Ciocalteu method.

The results of total phenolic content was generated by comparing the figures of absorbance so obtained from the Folin-Ciocalteu test with standard curve of known concentration of Gallic acid (refer table 3 and figure 2). It was observed that the TPC of *Cassia fistula* L. aqueous leaf extract was reported to be in the range 8.09 mg per 100 mg equivalent to standard Gallic acid considered to be enough to bear medicinal and therapeutic properties including antimicrobial activity, immunomodulation, hepatoprotective and anticancer activities. The extracts rich in TPC also possess high free radical scavenging activity.

Rathi, (2015)[20] reported the presence of flavonoids, phenolic compounds, anthraquinone, glycosides, saponins, steroids, alkaloids, and tannins carbohydrate, protein, fats, and free rhein, sennosides A and B in the leaves of *C. fistula*. Earlier Panda, *et al*., (2011) [21] also reported the presence of similar phytochemical components in aqueous extracts of the leaves of *C. fistula* while doing its antibacterial activities, but they reported a percentage yield of 27.28% which is much higher compared to present study which is 4.1%. Nagpal *et al*., (2011) [22] also considered to work with leaves of *Cassia fistula* (Linn.). With reference to literature water soluble flavonoids & phenolics does not pose any antimicrobial

significance, but the antioxidant potential[23]. Khan *et al*., (2017) [24] reported flavonoids in fresh juice of pods while tannin, and saponin in methanolic extracts whereas mentioned the presence of glycosides and alkaloid in its aqueous extract.

CONCLUSIONS

The aqueous extract of leaves of *Cassia fistula* L. was observed to be rich in various types of phytochemicals due reference to phytochemical analysis both qualitatively and quantitatively. The aqueous extract are rich in gallic acid equivalent polyphenols indicates the prospects of this plant extract in medicinal and therapeutic significances due to the possible potential, anti-oxidant, anticancer, antimicrobial, antihelmithic, hepatoprotective and many other activities. The phetochemicals of this valuable plant could be prospected in development of valuable therapeutics upon further extensive investigation.

Acknowledgement

The authors would like to pay their sincere thanks to Dr. Madhuri Modak, Professor, Department of Botany, Govt. College, Ashta, M.P., India for valuable input in plant identification. Authors would also like to acknowledge Scan Research Laboratory and Lenience Biotech Lab, Bhopal for necessary laboratory facilitation, constant help and moral support in this work.

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