Research Article

Pharmacognostic Investigation, Acute Toxicity Studies and Isolation of Steroidal Compound from the Leaves of *Cassia fistula* Linn.

Tejendra Bhakta[1*], Prashanta Kr. Deb[2], Kaushik Nath Bhaumik[2], Joydip Saha[1]
[1]Regional Institute of Pharmaceutical Science & Technology, Abhoynagar, Agartala, Tripura (W), India.
[2]Department of Pharmacy, Tripura University (A Central University), Suryamaninagar, Agartala, Tripura (W), India.
***Corresponding author's E-mail:** deboam2012@gmail.com

Accepted on: 07-01-2014; **Finalized on:** 28-02-2014.

ABSTRACT

Pharmacognostic, phytochemical and acute toxicity studies of *Cassia fistula* leaves were performed which led to the isolation of a steroidal compound (β-sitosterol) and reported for the first time from this plant. The structure of the compound established on the basis of extensive spectroscopic analysis including UV spectra, IR spectra, ^{1}H- NMR spectra, Mass spectral data. All the pharmacognostic experimental evidences conclusively prove the identity and quality of the leaves of *Cassia fistula* Linn. The acute toxicity of different extracts on animal models has also been studied to access the minimum lethal dose (MLD). All the spectroscopic data were compared with the β-sitosterol and found matched.

Keywords: Acute-toxicity study, *Cassia fistula* leaves, Pharmacognostic parameters, Phytochemical investigation, β-sitosterol.

INTRODUCTION

A plant may be considered as a biosynthetic laboratory, not only for the chemical compounds such as carbohydrates, proteins and lipids that are utilized as food by man but also for a multitude of compounds like glycosides, alkaloids, steroids, volatile oils, tannins etc. that exert a physiological effect. The compounds that are responsible for the therapeutic effect are usually the secondary metabolites. A systematic study of a crude drug embraces thorough consideration of both primary and secondary metabolites derived as a result of plant metabolism. The plant material may be subjected to preliminary phyto-chemical screening for the detection of various plant constituents.[1]

Cassia fistula Linn. (Synonym: *Purging fistula,* Fam. Leguminosae) commonly known as Sundali (Bengali: Banor lathi), is a deciduous middle-sized tree, indigenous to India and often cultivated as an ornamental plant in many parts of India.[2,3] This plant is used by traditional medical practitioners for the treatment of various diseases; leaves are used in ringworm and as a purgative.[2-4] Many rural people of the North-Eastern region of India used pods and leaves of this plant as anti-allergic, purgative, wound healing, antidiabetic, antipyretic, anti-inflammatory and as hepatoprotective.[5-7] In Ayurvedic system of medicine this plant is used in haematemesis, pruritis, leucoderma, diabetes and many other ailments.[2,3] Earlier investigations revealed the presence of fistucacidin, rhein and its glucosides, sennosides A and B, methyleugenol, lupeol, hexacosanol, giberellic acid, kaempferol, leucopelargonidin, barbaolin, aloe-emodin etc. from the different parts of this plant .[8,9]

The present study was undertaken with a view to investigate the pharmacognostic, phytochemical and acute toxicity profile including the isolation of active steroidal compound from the leaves of *Cassia fistula* Linn.

MATERIALS AND METHODS

Instruments & Chemicals

MP, Kofler type melting point apparatus and are uncorrected; IR, Perkin Elmer FTIR-100 spectrometer; UV, Perkin Elmer Lambda 25; NMR, JEOL/FT/NMR/FX 100; MASS, Jeol JMS-HX 110 mass spectrometer; UV-lamp (Unicon, India); Alumina- Neutral, Grade-I (Sigma–Aldrich, India) and TLC, silica gel G (Merck, India). All the chemicals and reagents obtained from Rankem Labs. (Okhla, Delhi) were analytical grade and used without further purification.

Plant Material

Fresh *Cassia fistula* leaves were collected from the Tripura University (A Central University), Agartala, Tripura; in the month of January 2013 and identified by the renowned Taxonomist Prof. B. K. Datta, Dept. of Botany (Plant Taxonomy & Biodiversity Research Lab.), Tripura University. The fresh leaves were dried in shaded floor and powdered in hand mill.

Pharmacognostic Studies[10-15]

Pharmacognostic studies of the leaves including determination of extractive values, ash value, powder analysis with different reagents and fluorescence properties under UV lamp were performed. Preliminary proximate phytochemical screening also has been performed for the identification of chemical groups was performed according to the established procedure.

Determination of MLD of different extracts[16-18]

Animals Used

Swiss albino mice (20-25 gm) were used for the toxicity study. The animals were maintained on the suitable nutritional and environmental condition throughout the

experiment. The animals were housed in polypropylene cages with paddy house bedding under standard laboratory condition for an acclimatization periods of 7 days prior to performing the experiment. The animals had access to laboratory chow and water *ad libitum*. The experimental protocols were approved and a written permission from Institutional Animal Ethical Committee (Regd. No.-1006/ac/06/CPCSEA, from Ministry of Environment & Forests, Govt. of India) has been taken to carry out and complete this study.

Method of Toxicity Study

The method followed for toxicity study was as per the method of Litchfield *et al.* (1949). Different doses of each extract ranging 0.5-3.5 gm/kg was administered to the animals. Each group contains six (6) animals and in every case there was a control group which received normal saline solution (1 ml/kg). All the treatments were given orally. The range of doses administered to the mice followed by the method of Lorke *et al.* (1983). After administration of extract, the animals were observed under open field condition for 72 hours and the number of death and signs of clinical toxicity were recorded.

Isolation and structural elucidation of steroidal compound (β-sitosterol) [19-21]

The shade dried coarse powdered *Cassia fistula* leaves (500 gm) were extracted with 1500 ml of MeOH (90%) in a Soxhlet apparatus for 4 hours. The solvent was removed by distillation under reduced pressure and a deep greenish coloured semi-solid mass was obtained (yield 12.20%). The MeOH extract (15gm) was dissolved in 150 ml of water and 20% NaOH solution (8-10 ml) was added to it. The mixture was refluxed for 2 hours to hydrolyze the materials present in the extract. The hydrolyzed product thus obtained was extracted several times with C_6H_6. The C_6H_6 fraction washed with distilled water to remove alkali and concentrated to 10 ml under reduced pressure. 10 gm of C_6H_6 fraction (obtained from MeOH extract) was subjected to column chromatography on Alumina column. The column was slowly eluted with Heptane at a rate of 15 drops/min. When about 500 ml of elute was allowed to pass through the column, a yellow colored band was separated out from the top layer. The TLC was carried out with yellow colored eluting solvent using the solvent system CCl_4: $CHCl_3$ (5:1). One bluish spot was viewed under UV light with R_f 42. The yellow colour liquid has evaporated to dryness. The dried material was recrystalized from EtOH. Fine needle shaped colorless crystals were obtained. 1.02 gm of white crystals, m.p.140-142^{0}C, were obtained.

RESULTS AND DISCUSSION

Organoleptic standardization confirms the identity of plant as *Cassia fistula* L. The results of different pharmacognostic parameters including physical constants, powder analysis, fluorescence in the Table: 1, 2 and 3 respectively. Form the chemical group tests it was observed that the crude MeOH extract contains alkaloids, reducing sugar, tannins, flavonoids, steroids, saponins and anthraquinones.

Table 1: Physical constant values of *Cassia fistula* Linn. Leaves

Parameters	Percentage (w/w)
Total Ash	9.350
Acid insoluble Ash	0.497
Sulphated Ash	12.396
Extractive Values	
Methanol extract	12.20
Pet. Ether (40-60) extract	3.10
Benzene extract	2.67
Chloroform extract	1.85
Aqueous extract	13.80

Table 2: Powder analysis of *Cassia fistula* Linn. Leaves

Reagents	Behavior of Powder
Powder+ Picric acid	Yellowish
Powder+ Nitric Acid	Reddish brown
Powder+ HCl	Black
Powder + H_2SO_4	Greenish black
Powder + Glacial acidic acid	Dark brown
Powder + Aq. NaOH	Reddish yellow
Powder + I_2 Solution	Yellowish brown
Powder + 5% $FeCl_3$ Solution	Yellowish brown
Powder + Antimony trichloride	Greenish brown

Table 3: Fluorescence characters of the powdered leaves of *C. fistula* under UV light.

Reagents	Behavior of Powder
Powder mounted with nitrocellulose	Greyish white
Powder treated with NaOH in Methanol	Greenish black
Powder treated with NaOH in Methanol and mounted with nitrocellulose	Yellowish green
Powder treated with HCl	Bluish black
Powder treated with HCl and mounted with nitrocellulose	Greenish yellow
Powder treated with NaOH in water	Violet
Powder treated with NaOH in water and mounted with nitrocellulose	Greenish red
Powder treated with HNO_3 diluted with equal volume of water	Grey

Oral acute toxicity study on Swiss albino mice exhibited that all the extracts were found safe upto dose 3 gm/kg (p.o). All the extracts showed more than half of the

population mortality above dose 3 gm/kg. Hence, minimum lethal dose (MLD) found at 3 gm/kg (p.o). Results of oral acute toxicity been tabulated below in table 4.

Table 4: Determination of Minimum Lethal Dose (MLD) of various extracts

Drug/Extracts	Dose (gm/kg)	No. of Animals	No. of Death	MLD (gm/kg)
Methanol Extract	0.5	6	0	>3
	1.0	6	0	
	1.5	6	0	
	2.0	6	0	
	2.5	6	0	
	3.0	6	0	
	3.5	6	4	
Pet. ether Extract	0.5	6	0	>3
	1.0	6	0	
	1.5	6	0	
	2.0	6	0	
	2.5	6	0	
	3.0	6	0	
	3.5	6	6	
Aqueous Extract	0.5	6	0	>3
	1.0	6	0	
	1.5	6	0	
	2.0	6	0	
	2.5	6	0	
	3.0	6	0	
	3.5	6	4	
Control (Saline)	1ml/kg	6	0	---

Isolation and structural elucidation of the steroidal compound (β-sitosterol)

The isolated compound was white crystalline-solid, odourless, practically insoluble in water, slightly soluble in EtOH, freely soluble in $CHCl_3$ and CS_2. The isolated compound melts at 142 °C. A mixture of an equal amount of isolated compound and β- sitosterol also melts at 142 °C without showing any depression. The crystalline material isolated showed positive tests for steroid (Liebermann Burchard test and Salwaski test) with colour reactions. The EtOH solution of isolated compound exhibits absorption maxima (λ_{max}) at 265 nm. IR spectra (Figure 1) of the isolated compound in KBr discs in the region 4000 cm^{-1} to 1110 cm^{-1} which are 1420, 1340, 1050, 1020.

The NMR spectrum (Figure 2) of the isolated compound was done in JEOL/FT/NMR/FX 100 by taking the sample in $CDCl_3$. The absorption of the angular methyl groups were found at 220 and 239 cps. The isopropyl doublet (26-, 27-hydrogens) was at about 227 and 233 cps and was approximately superimposed on the doublet from the 21-hydrogens. The 29-hydrogens was observed as an irregular triplet at 223, 228, 234 cps (Figure 3). The isolated compound exhibited principle peaks at M/e: 414, 412, 298, 270, and 255 (Figure 4). Fragmentation occurs at M/e – 412 (414-2, loss of mass-2 due to loss of 2H ion), M/e - 298 (412-14, loss of Mass -114 due to loss of side chain at double bond), M/e - 270 (298-28, loss of mass-28 due to loss of CH_3 molecule), M/e - 255 (270-15, loss of mass-15 due to loss of CH_3 molecule).

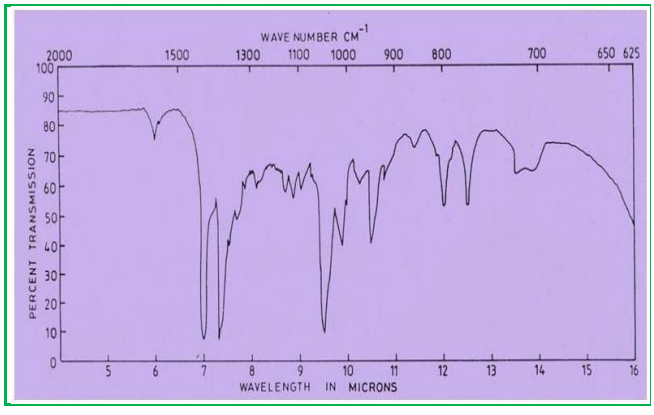

Figure 1: IR spectra of the isolated compound

The intense peak with highest mass number was shown at M/e 414 which is due to the parent molecular ion. This provides the molecular weight of the compound was 414, which exactly as same as that of β-sitosterol. Different

peaks of low intensities at lower value of M/e correspond to complicated and random fragmentation of the molecule. The elemental analysis of the compound dried under high vacuum at 60 °C. Following result was obtained as analytical: C_{29} H_{52} O (Mol. Wt. 416.71), Calculated: C = 83.61%, H = 12.63%, O = 3.86%, Found: C = 83.58% H = 12.58%, O = 3.80%.

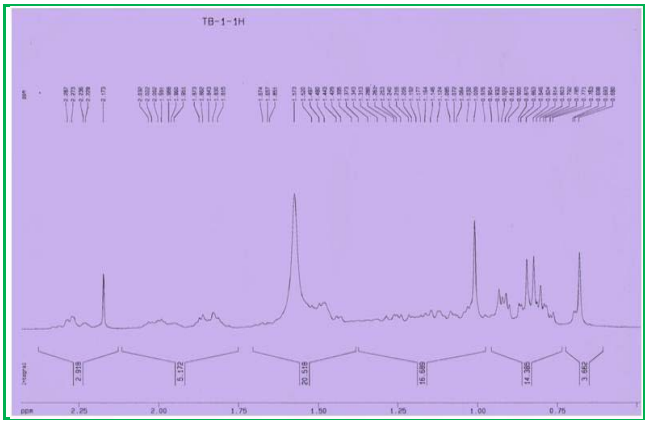

Figure 2: NMR spectra of the isolated compound

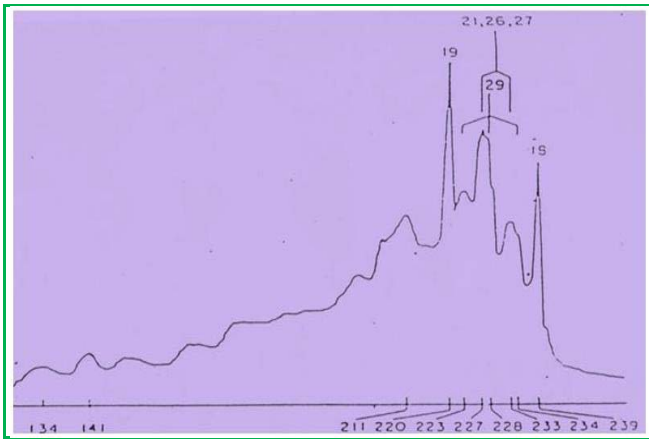

Figure 3: NMR spectra of the isolated compound (Aliphatic portion)

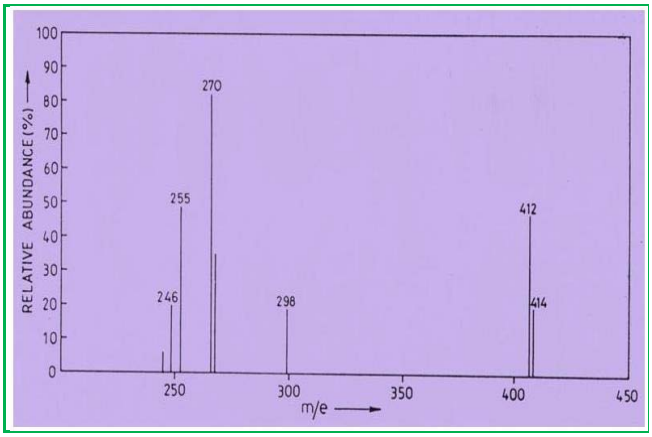

Figure 4: Mass spectra of the isolated compound

The UV, IR, NMR and Mass spectra of the isolated compound as described earlier resembles with that of authentic sample of β- sitosterol (Figure 5).

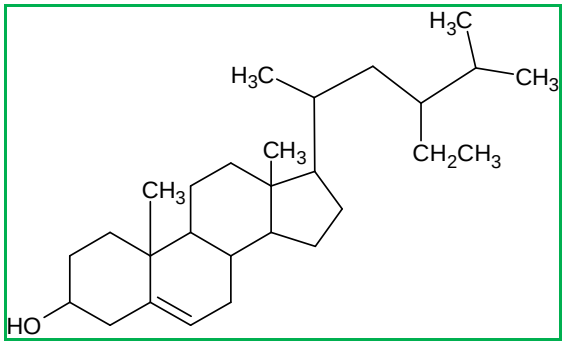

Figure 5: Structure of the isolated compound (β-sitosterol)

CONCLUSION

All the pharmacognostic parameters studied satisfies the quality standards of *Cassia fistula* L. According to the study extracts are non-toxic and experimental evidences as explained previously (UV, IR, NMR, MASS, Elemental analysis, melting point and mixed melting point) conclusively proves the identity of the isolated compound from the leaves of *Cassia fistula* as β-sitosterol.

Acknowledgement: The authors are grateful to Prof. B.K. Datta, Department of Botany (Plant Taxonomy & Biodiversity Research Lab.), Tripura University for identification of the plant material and Dept. of Pharmacology, TMC & Dr. BRAM Teaching Hospital, Hapania, Tripura (W), for facilitating the animal experimentation.

REFERENCES

1. Kokate CK, Pourohit AP, Gokhale SB, Pharmacognosy, Nirali Prakashani, Pune, 34, 2006, 106.
2. Kirtikar KR, Basu BD, Indian Medicinal Plants, International Book Publication Distribution, Dehradun, India, 2, 1975, 856-860.
3. Khare CP, Indian medicinal plants, Springer, Delhi, 2007, 128.
4. Gupta RK, Medicinal & Aromatic plants, CBS publishers & distributors, New Delhi, 1, 2010, 116-117.
5. Kushawaha M, Agrawal RC, Biological activity of medicinal plant Cassia fistula – A review, Journal of Scientific Research in Pharmacy, 1(3), 2012, 7-11.
6. Bhakta T, Banerjee S, Mandal SC**,** Maity TK, Saha BP, Pal M, Hepatoprotective activity of Cassia fistula leaf extract, Phytomedicine, 8(3), 2001, 220-224.
7. BhaktaT, Mukherjee PK, Saha K, Pal M, Saha BP, Evaluation of Anti-inflammatory effects of Cassia fistula (Leguminosae) Leaf extracts o rats. Journal of Herbs, Spices & Medicinal Plants, 6(4), 1999, 67-72.
8. Misra TR, Singh RS, Pandey HS, Singh BK, A new diterpene from *Cassia fistula* pods, Fitoterapia, 58, 1997, 375–377.
9. Misra TR, Singh RS, Pandey HS, Pandev RP, Chemical constituents of hexane fraction of *Cassia fistula* pods, Fitoterapia, 57, 1996, 173-174.
10. Trease GE, Evans WC, Pharmacognosy, Alden Press, Oxford, 12, 1983, 344, 539.

11. Wallis TE, Text Book of Pharmacognosy, CBS Publishers & Distributors, Delhi, 5, 1985, 252-253.
12. Kokate CK, Practical Pharmacognosy, Vallabh Prakashan, Delhi, 2001, 114-121.
13. Srinivasa B, Kumar A, Prabhakarn V, Lakshman K, Nandeesh R, Subramanyam P et al., Pharmacognostical studies of Portulaca oleracea Linn. Brazilian Journal of Pharmacognosy, 18(4), 2008, 527-531.
14. Subha S, Vijayakumar B, Prudhviraj K, Anandhi MV, Shankar M, Nishanthi M, Pharmacognostic and Preliminary Phytochemical Investigation on Leaf Extracts of Myristica dactyloides Gaertn, International Journal of Phytopharmacology, 4(1), 2013, 18-23.
15. Deb PK, Das S, Bhaumik KN, Ghosh R, Ghosh TK, Bhakta T, Pharmacognostic & Preliminary Phytochemical Investigations of Neptunia prostrata L, Journal of Pharmacognosy and Phytochemistry, 2(3), 2013, 5-11.
16. OECD Guideline 425, Acute Oral Toxicity up and-Down Procedure in OECD Guidelines for the Testing of Chemicals. Organization for Economic Cooperation and Development, Paris, OECD, 2001.
17. Okon J, Emmanuel E, Godwin E, Nse U, Phytochemical Screening, Analgesic and Anti-inflammatory Properties and Median Lethal Dose of Ethanol Leaf Extract of Wild Species of Eryngium foetidum L. on Albino Rats, International Journal of Modern Biology and Medicine, 3(2), 2013, 69-77.
18. Prabu PC, Panchapakesan S, Raj CD, Acute and Sub-Acute Oral Toxicity Assessment of the Hydroalcoholic Extract of Withania somnifera Roots in Wistar Rats, Phytother. Res., 27, 2013, 1169–1178.
19. Stahl E, Thin Layer Chromatography, Springer Verlag, New York, 2, 1969, 313-357.
20. Harborne JB, Phytochemical Methods (A guide to modern techniques of plant analysis), Chapman and Hall, London, 1, 1973, 109-117.
21. Morales MA, Aliphatic alcohols, β-sitosterols and alkaloids in Cassia jahanii, Phytochemistry, 10, 1971, 2255-2256.

Source of Support: Nil, **Conflict of Interest:** None.

About Corresponding Author: Dr. T. Bhakta

Dr. T. Bhakta was post graduated from Department of Pharmaceutical Technology, Jadavpur University, Kolkata, in Pharmaceutical Chemistry. He was awarded PhD in Pharmaceutical Sciences (Natural Product Chemistry) in the year 1999 from Jadavpur University. Currently he is working as Assistant Professor in Pharmaceutical Chemistry at Regional Institute of Pharmaceutical Science & Technology, Agartala, Tripura, India. He has published one book entitled “Common Vegetables of the Tribals of Tripura” and several research articles in reputed journals.