Journal of Applied Pharmaceutical Science Vol. 5 (03), pp. 073-079, March, 2015
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2015.50312
ISSN 2231-3354 

HPTLC finger printing profile and evaluation of *in vitro* antidiabetic potential of medicinally important plant, *Cassia obtusa* L. (Caesalpiniaceae)

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ARTICLE INFO

Article history:
Received on: 17/12/2014
Revised on: 10/01/2015
Accepted on: 22/01/2015
Available online: 28/03/2015

Key words:
Cassia obtusa, antidiabetic activity, HPTLC fingerprint profile, alkaloids, flavonoids.

ABSTRACT

The aim of the study was to establish the finger printing profile and evaluation of *in vitro* antidiabetic potential of *C. obtusa. In vitro* antidiabetic activity was carried out according to the method adopted by Miller, 1959. HPTLC studies were carried out using CAMAG HPTLC system equipped with Linomat 5 applicator, TLC scanner 3 and Win cats-4 software for the active fractionation of aqueous-methanolic leaf extracts of *C. obtusa*. Among the various plant parts analyzed, leaf exhibited efficient inhibitory activity against α-amylase and α-glucosidase enzymes. Therefore, the leaf extract was further fractionated using various solvent systems petroleum ether, chloroform, ethylacetate, butanol and water and were subjected to *in vitro* antidiabetic activity. Among the fractions analyzed chloroform fraction exhibited remarkable antidiabetic activity by inhibiting α-amylase and α-glucosidase enzymes. Furthermore, the active leaf fractions were analyzed with HPTLC to develop fingerprint profiles and these fractions revealed the presence of 13 and 22 major spots of alkaloids and flavonoids respectively with different Rf values. The results of the present study thus claim the folkloric usage of the plant in diabetic related maladies.

INTRODUCTION

Diabetes is a metabolic disorder caused by an absolute or relative lack of insulin and/or reduced insulin activity which results in hyperglycemia and abnormalities in carbohydrates, fat and protein metabolites (Bayens, 1991; Bhatensa and Velasquez, 2002). The main cause of this problem is aging, urbanization and increasing privilege leading to obesity and physical inactivity. Management of diabetes without any side effects is still a major challenge for researchers; however, perfect glycemic control is rarely achieved. For quite some time, diabetes has been treated orally with various medicinal plants or their extracts based on indigenous medicine. Several species/genera of plants have also been described in the scientific and popular literature as having hypoglycemic property. Therefore, search for more effective and safer hypoglycemic agents has continued to be an important area of active research.

Cassia obtusa is a perennial herb occurring in the arid, subtropical open sandy places. They are used in various indigenous systems of medicine and are popular among the various ethnic groups for the treatment of various ailments such as influenza, gout, rheumatism and other parasitic disorders. They are assigned as laxative and are used as an excellent source of remedy for diabetes mellitus and other inflammatory disorders (Jeyapal *et al.*, 2008). In some parts of the world, the seeds are eaten as food and are added to native beer. They are used in indigenous medicine as purgative and exhibit a wide range of phytochemical constituents such as sennosides, anthroquinones, aloe-emodin, emodin, saponins, alkaloids, flavonoids, anthrancene derivatives, sterols, triterpenes and tannins (Sharma *et al.*, 1982a, b; Anonymous, 1992; EI-Sayed *et al.*, 1992; Jain *et al.*, 1996, 1997). Despite several ethnobotanical claims, laboratory data on their bioactivities still in paucity.

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To address these lacuna, the present study was intended to evaluate the *in-vitro* antidiabetic potential of *C. obtusa* accompanied with HPTLC fingerprint to identify active fractions.

MATERIALS AND METHODS

Plant collection and authentication

Fresh leaves, stem, root, flower and pods of *C. obtusa* L. were collected from Perundurai, Erode district, Tamil Nadu, India. The selected plant species was authenticated at Botanical Survey of India, Southern Circle, Coimbatore (vide No. BSI/SC/5/23/10-11/Tech-535) and deposited in the Botany Department herbarium. The plant materials were cleaned, washed with copious amounts of distilled water, shade dried, chopped into bits, and coarsely powdered in a Willy mill to 60mesh size (Nippon Electricals, Chennai, India) for extraction.

Extraction and fractionation

Dried and powdered plant materials were subjected to extraction with aqueous methanol (30/70 v/v) using round bottom flask with an attached reflux condenser for 3h at a controlled temperature and the extract (8.7 w/w percentage yield) was further dissolved in water and partitioned between petroleum ether-water (1:1) (1.5 w/w percentage yield), chloroform-water (1:1) (2.5 w/w percentage yield), ethylacetate-water (1:1) (2.4 w/w percentage yield), and n-butanol-water (1:1) (1.4 w/w percentage yield) to obtain the respective solvent soluble fraction. The extracts were filtered and concentrated to dryness under reduced pressure using rotary vacuum evaporator (RE300; Yamato, Japan), lyophilized (4KBTXL-75; Vir Tis Benchtop K, New York, USA) to remove traces of water molecules and stored at -20°C for further use.

In vitro antidiabetic activity

α-amylase inhibition assay

α-amylase inhibition assay was carried out according to the method developed by Miller, (1959). Briefly, various concentrations of the extracts (100-500µg/mL) and 500µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing porcine pancreatic α-amylase (EC 3.2.1.1) (0.5 mg/mL) were incubated at 25 °C for 10 min. Then, 500µL of 1% starch solution in 0.02M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to the reaction mixture. Thereafter, it was incubated at 37°C for 5min and 2.0mL of 3,5-dinitrosalicylic acid (DNSA) was added. Then the reaction was stopped by incubating in a boiling water bath for 15min at 100 °C and later cooled to room temperature. The reaction mixture was then diluted by adding 10mL of distilled water in an ice bath, and absorbance was measured at 540 nm. A system devoid of test sample served as reference sample. The α-amylase inhibitory activity was expressed as percentage inhibition.

$$\% \text{ Inhibition} = [(Abs_{ref} - Abs_{sample})/Abs_{ref}] \times 100 \text{---(1)}$$

where Abs_{ref} = absorbance of the reference; Abs_{sample} = absorbance of the test samples.

α-glucosidase inhibition assay

α-glucosidase inhibition assay was made by Miller (1959). Various concentrations of the extracts (100-500µg/mL) and 100µL of α-glucosidase (EC 3.2.1.20) (0.5 mg/mL) in 0.1M phosphate buffer (pH 6.9) solution were incubated at 25°C for 10 min. Then, 50µL of 3mM p-nitrophenyl- α-D-glucopyranoside in 0.1 M phosphate buffer (pH 6.9) solution was added. The mixtures were incubated at 37°C for 30min and the reaction was terminated by addition of 2mL of sodium carbonate and the absorbance was read at 400nm. A system devoid of test sample served as reference sample. The α-glucosidase inhibitory activity was expressed as percentage inhibition (formula 1).

HPTLC fingerprinting analysis

A densitometric HPTLC analysis was performed for the development of characteristic finger printing profile. *C. obtusa* leaf fractions were dissolved with HPTLC grade methanol. TLC aluminium plates precoated with silica gel 60 F_{254} (10×10 cm, 250 µm thickness). Samples were applied as bands 8 mm wide and 8 mm apart by use of a CAMAG Linomat 5 ["Linomat 5_170147" S/N 170147 (1.00.12)] sample applicator, equipped with 100 µL Hamilton syringe and the constant application rate was 150nl/s. The sample loaded plate was kept in a TLC twin trough 20 × 10cm developing glass chamber [after saturation with solvent vapour for 15 min at room temperature (30 °C)]. Good separation and well-developed peaks were obtained using ethylacetate : water : formic acid (85:5:5) for alkaloids and ethylacetate : formicacid : glacial acetic acid : water (10:0.5:0.5:1.3) for flavonoids as a mobile phase. The chromatogram was run over a distance of 70 mm. After development, the plates were dried in an oven for 5 min at 60°C. Densitometric scanning was performed with a TLC scanner equipped with win CATS 1.4.2 software ("Scanner_170418" S/N 170418) in reflectance absorbance mode with slit dimension (6.00×0.45mm, micro), scanning speed 20mm/s, data resolution 100µm/step, optical filter (second order), and filter factor (Stavitsky-golay 7), and the images were captured at UV 254 and 366nm. The peak table, display and densitogram were identified.

Statistical analysis

For *in vitro* antidiabetic activity of the extracts, the results were recorded as mean ± standard deviation (SD) (n=3) and subjected to one-way analysis of variance (ANOVA) using SPSS (Version 9, SPSS Inc., Chicago, USA). $P<0.05$ was chosen as the criterion of statistical significance.

RESULTS

In vitro antidiabetic activity

α-amylase inhibition assay

The α-amylase inhibitory potential for aqueous-methanolic extracts *C. obtusa* plant parts and various fractions of leaf were determined and presented in Table 1. However, among the plant parts analyzed, the aqueous methanolic extract of leaf (276.78 µg/mL) unveiled higher activity than the other plant parts.

Therefore, leaf extract was further used for fractionation. Among the fractions determined the chloroform (87.82µg/mL) and ethylacetate extracts (89.28µg/mL) of *C. obtusa* leaf manifested fairly outstanding α-amylase inhibitory activity. On the other hand, petroleum ether, butanol and water fractions of *C. obtusa* leaf exhibited very least activity.

Table 1: Inhibition of α-amylase and α-glucosidase activities of various parts and different leaf fractions of *Cassia obtusa*.

Sample*	IC_{50} (µg/mL)	
	α-amylase inhibition activity	α-glucosidase inhibition activity
CO-Leaf	276.78 ± 10.71b	194.11 ± 5.36a
CO-Stem	355.83 ± 4.50c	345.05 ± 3.25c
CO-Root	329.16 ± 0.00bc	406.69 ± 9.09d
CO-flower	295.20 ± 8.32b	224.82 ± 4.90b
CO-Pod	387.15 ± 5.41d	263.82 ± 5.03bc
CO-Leaf - petroleum ether	232.64 ± 1.93b	527.59 ± 7.49e
CO-Leaf - chloroform	87.82 ± 0.73a	203.95 ± 2.27b
CO-Leaf - ethyl acetate	89.28 ± 0.57a	550.34 ± 8.29e
CO-Leaf - n-butanol	318.36 ± 3.65bc	487.31 ± 6.49d
CO-Leaf - water	479.45 ± 16.12e	345.11 ± 6.39c

*CO-*Cassia obtusa*. Values are means of three independent analysis of the extract ± standard deviation (n = 3). Mean values followed by different superscript in a column are significantly different (P<0.05).

α-glucosidase inhibition assay

In the present study, the calculated IC_{50} values obtained from the plots of concentration dependent inhibition of α-glucosidase for aqueous methanolic extracts of *C. obtusa* plant parts and various fractions of leaf were presented in Table 1. Among the samples examined, leaf extract exhibited plausible α-glucosidase inhibitory activity with IC_{50} value of 194.11µg/mL, followed by flower (224.82µg/mL) and pod (263.82µg/mL). In contrast, *C. obtusa* stem and root parts determined significantly very weak inhibitory concentration of 345.05 and 406.69µg/mL, respectively. Similarly, among the fractions determined the chloroform extracts of *C. obtusa* leaf registered markedly higher inhibitory activity of 203.95µg/mL. The decreasing order of α-glucosidase enzyme inhibitory activity for the various fractions of *C. obtusa* leaf were in the order of water>butanol>petroleum ether>ethylacetate.

HPTLC profiles of active fractions of aqueous methanolic leaf extract of *Cassia obtusa*

Alkaloid profiles of various fractions of *C. obtusa* leaf was recorded and depicted in Table 2 and Figures 1a and 2a. Presence of brown coloured zones at day light mode in the sample tract observed in the chromatogram after derivatization, confirmed the presence of alkaloids in the given sample extract. In the present study, a total of 13 bands of alkaloids from various solvent extracts were obtained. Out of which 2 bands of each for petroleum ether and butanol fractions, and similarly, 3 and 6 bands for chloroform and ethyl acetate extracts, respectively were determined. Nevertheless, no bands were seen in water extract. Best solvent system to view the above separation is ethylacetate : water : formic acid (85:5:5). For detection of flavonoids HPTLC chromatogram can be best visualized under fluorescence (254nm and 366nm) before and after derivatization (Table 3 and Figures 1b and 2b). At UV 366nm, the presence of yellow coloured zones in the given sample tract, after derivatization, confirms the presence of flavonoids in the given sample extracts. About 22 different types of flavonoids bands were determined before derivatization with different Rf values. However, best solvent system to scrutinize the above partition is ethylacetate : formic acid : glacial acetic acid : water (10:0.5:0.5:1.3).

Table 2: HPTLC alkaloid profiles of various fractions (petroleum ether, chloroform, butanol, ethylacetate and water) of *Cassia obtusa* leaf.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
Petroleum ether extract										
1	0.01	0.1	0.04	47.9	30.86	0.05	1.5	651.2	27.46	Unknown
2	0.08	0.1	0.11	18.2	11.70	0.14	0.2	350.8	14.79	Alkaloid 1
3	0.59	0.3	0.61	27.6	17.76	0.64	2.5	301.3	12.71	Alkaloid 2
4	0.78	3.0	0.81	19.7	12.67	0.82	15.7	233.3	9.84	Unknown
5	0.82	15.7	0.84	22.6	14.53	0.87	10.6	582.9	24.58	Unknown
6	0.87	10.9	0.89	19.4	12.48	0.91	3.8	251.7	10.62	Unknown
Chloroform extract										
1	0.01	0.9	0.05	68.4	25.83	0.06	57.4	1638.1	27.00	Unknown
2	0.07	57.5	0.08	67.6	25.53	0.10	58.0	1453.2	23.95	Unknown
3	0.10	58.1	0.11	63.3	23.90	0.15	0.1	1173.9	19.35	Alkaloid 3
4	0.29	6.9	0.31	16.0	6.03	0.35	7.9	478.5	7.89	Alkaloid 4
5	0.61	6.5	0.64	19.8	7.47	0.67	6.9	500.7	8.25	Alkaloid 5
6	0.91	4.3	0.96	29.7	11.23	0.99	4.7	822.6	13.56	Unknown
n -Butanol extract										
1	0.01	69.3	0.01	71.5	29.26	0.04	0.0	316.4	7.72	Unknown
2	0.05	3.2	0.10	158.9	65.03	0.13	0.2	3667.8	89.54	Alkaloid 6
3	0.31	7.1	0.31	13.9	5.70	0.33	2.1	112.0	2.73	Alkaloid 7
Ethyl acetate extract										
1	0.00	19.0	0.02	127.8	17.07	0.04	1.3	1663.1	7.04	Unknown
2	0.06	0.0	0.14	466.1	62.26	0.18	0.4	17997.3	76.23	Alkaloid 8
3	0.38	10.2	0.43	31.5	4.20	0.47	3.9	1138.1	4.82	Alkaloid 9
4	0.61	2.7	0.66	15.3	2.05	0.69	4.4	409.9	1.74	Alkaloid 10
5	0.69	4.5	0.74	32.3	4.32	0.77	10.4	826.3	3.50	Alkaloid 11
6	0.79	12.1	0.82	26.3	3.51	0.83	22.7	424.2	1.80	Alkaloid 12
7	0.90	21.2	0.94	49.2	6.58	0.98	0.9	1151.0	4.88	Alkaloid 13

Table 3: HPTLC flavonoid profiles of various fractions (petroleum ether, chloroform, butanol, ethylacetate and water) of *Cassia obtusa* leaf.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
Petroleum ether extract										
1	0.74	5.7	0.76	26.4	8.17	0.79	0.4	383.5	7.84	Flavonoid 1
2	0.95	22.3	1.00	297.4	91.83	1.02	101.9	4507.2	92.16	Flavonoid 2
Chloroform extract										
1	0.06	0.0	0.09	21.3	1.40	0.11	9.3	467.0	1.11	Flavonoid 3
2	0.12	9.3	0.13	10.5	0.69	0.15	0.1	158.9	0.38	Flavonoid 4
3	0.46	1.9	0.54	101.4	6.67	0.60	9.5	3742.5	8.93	Flavonoid 5
4	0.66	18.5	0.74	128.1	8.43	0.78	30.9	4251.7	10.14	Flavonoid 6
5	0.86	36.9	0.98	563.3	37.04	1.01	236.1	17094.4	40.77	Flavonoid 7
6	1.01	236.6	1.06	696.0	45.77	1.07	2.3	16217.3	38.68	Unknown
n- Butanol extract										
1	0.01	16.8	0.02	22.3	2.11	0.03	1.5	181.0	0.97	Unknown
2	0.03	1.0	0.05	26.7	2.53	0.07	0.9	409.1	2.20	Unknown
3	0.08	0.4	0.12	89.5	8.47	0.15	0.8	1892.1	10.17	Flavonoid 8
4	0.20	1.8	0.26	41.2	3.90	0.28	11.2	1116.2	6.00	Flavonoid 9
5	0.32	9.7	0.36	41.4	3.92	0.40	19.2	1295.1	6.96	Flavonoid 10
6	0.42	18.6	0.48	124.2	11.76	0.54	2.6	4426.1	23.78	Flavonoid 11
7	0.67	5.7	0.73	42.7	4.05	0.75	1.8	777.9	4.18	Flavonoid 12
8	0.91	9.7	0.97	186.4	17.65	1.00	20.8	2679.1	14.39	Flavonoid 13
9	1.01	21.0	1.06	481.7	45.62	1.08	0.5	5834.6	31.35	Unknown
Ethyl acetate extract										
1	0.01	5.6	0.14	109.5	5.19	0.19	41.6	7160.4	9.32	Unknown
2	0.22	45.0	0.27	71.7	3.40	0.30	50.7	3113.9	4.05	Flavonoid 14
3	0.34	49.4	0.39	94.6	4.48	0.42	60.5	3484.3	4.54	Flavonoid 15
4	0.42	61.5	0.54	460.8	21.83	0.61	53.0	26352.7	34.31	Flavonoid 16
5	0.63	59.1	0.72	275.1	13.03	0.76	61.8	10159.5	13.23	Flavonoid 17
6	0.84	72.6	0.97	549.7	26.04	1.00	68.6	18019.5	23.46	Flavonoid 18
7	1.00	69.6	1.05	549.3	26.02	1.07	2.6	8527.9	11.10	Unknown
Water extract										
1	0.06	0.9	0.12	32.3	4.24	0.15	4.6	957.4	9.12	Unknown
2	0.59	1.0	0.60	12.0	1.57	0.60	0.3	72.9	0.69	Flavonoid 19
3	0.64	3.0	0.66	14.1	1.85	0.68	5.6	213.1	2.03	Flavonoid 20
4	0.85	5.2	0.87	19.4	2.55	0.89	9.9	278.6	2.65	Flavonoid 21
5	0.91	13.0	0.95	152.3	20.02	0.98	34.0	2160.6	20.58	Flavonoid 22
6	1.00	31.6	1.04	531.0	69.77	1.08	1.0	6817.3	64.93	Unknown

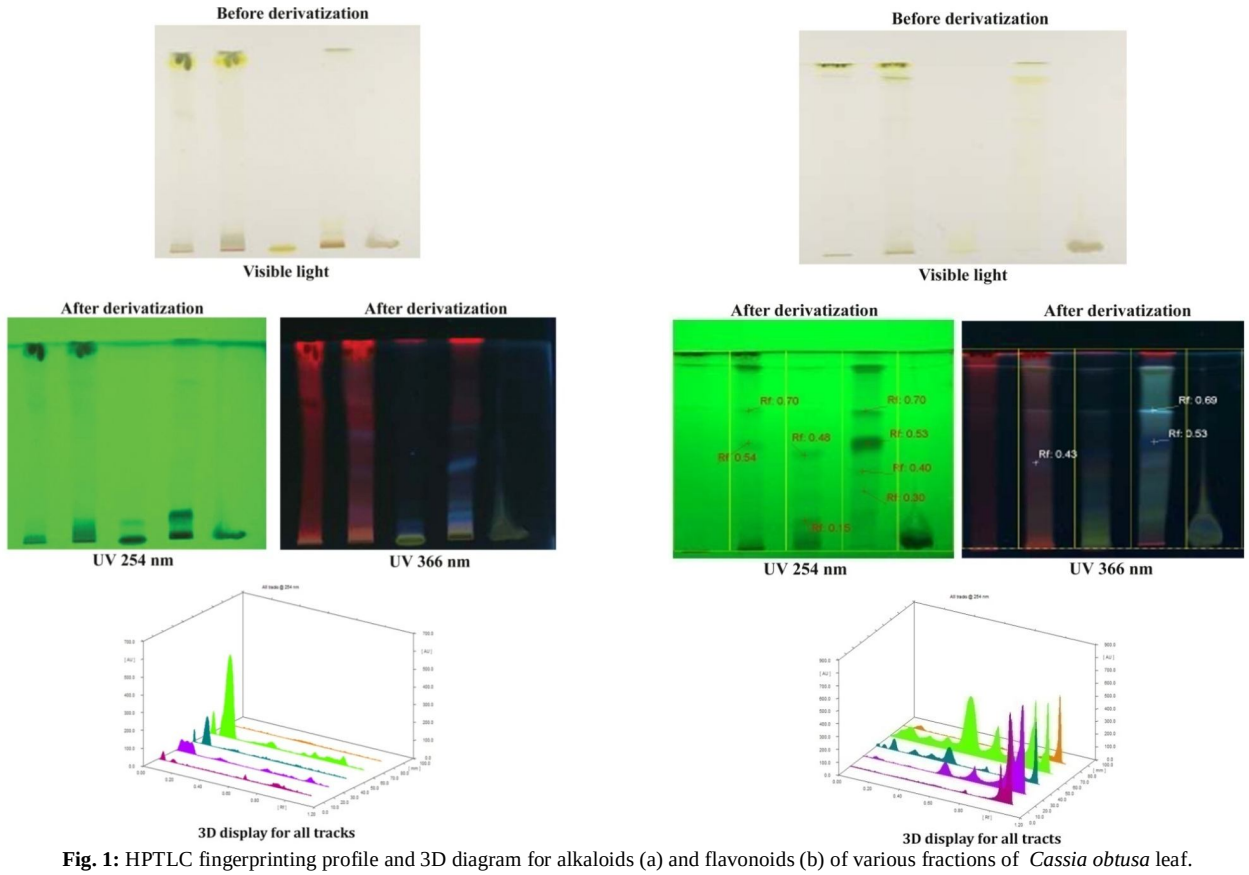

Fig. 1: HPTLC fingerprinting profile and 3D diagram for alkaloids (a) and flavonoids (b) of various fractions of *Cassia obtusa* leaf.

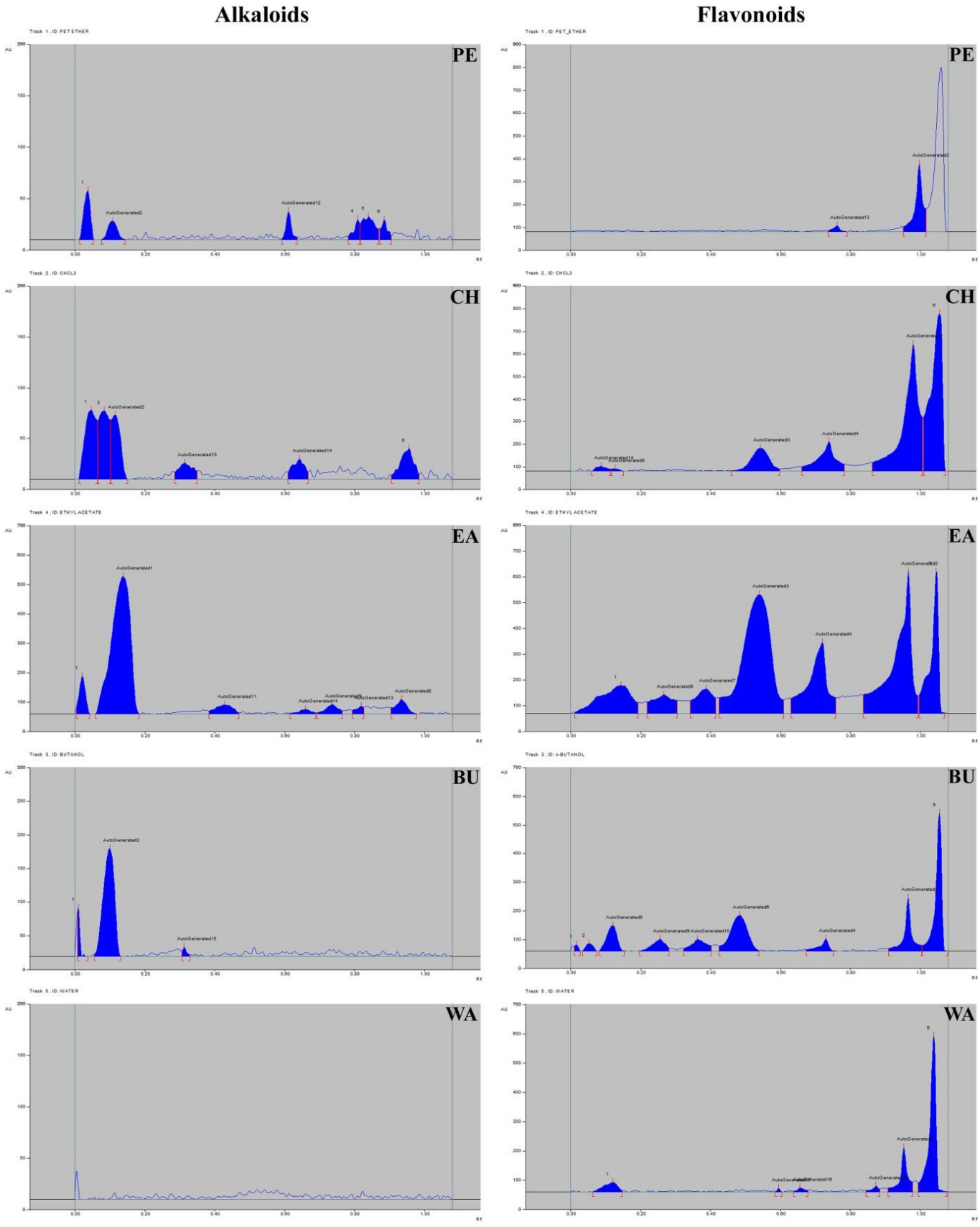

Fig. 2: HPTLC densitogram for alkaloids (a) and flavonoids (b) of various fractions of *Cassia obtusa* leaf.

DISCUSSION

Research conducted over decades, and the on-going studies have shown that plants and plant-based therapies may be the potent source of controlling and treating diabetes and its complications. The qualitative determination of HPTLC profiles from medicinal plants is a fast expanding field of research to identify the chemical profiles with curative properties. In the view of the up surging interest in medicinal plants, we examined the *in vitro* antidiabetic activity of aqueous methanolic extracts of different parts (leaf, stem, root, flower and pod) and various fractions of *C. obtusa* leaf. Further, the active fractions of *C. obtusa* leaf were further evaluated through HPTLC analysis for the detection of active constituents responsible for the activity.

Therapeutic approach for the management of diabetes is by controlling postprandial hyperglycemia. This can be achieved by the inhibition of carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase present in the gastrointestinal tract (Shim *et al.,* 2003). Inhibitors of these enzymes slowdown carbohydrate digestion thus, prolonging the overall digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (Habasa-Lhoret and Chiasson, 2004). Acarbose (the first dual inhibitor), miglitol, metformin and voglibose are commercially available enzyme inhibitors for type II diabetes. However, these drugs are reported to cause various side effects such as abdominal distention, flatulence and possibly diarrhoea (Ranilla *et al.,* 2008). Therefore, search for safer and effective inhibitors from natural sources is of emerging interest. In the present study, among the samples investigated, the aqueous methanolic leaf extract possessed significant antidiabetic properties than the other plant parts studied. Therefore, they were further fractionated with petroleum ether, chloroform, ethyl acetate, n-butanol and water for *in vitro* antidiabetic activity. Among the fractions examined, chloroform fractions exhibited remarkable activity by inhibiting the α-amylase and α-glucosidase enzymes (Table 1). Furthermore, in HPTLC finger print analysis, at the given concentration of 10µL of the sample applications, these fractions exposed the existence of 13 and 22 alkaloids and flavonoids spots respectively with different Rf values, indicating the diverse composition of the extract.

Overall results confirm that among the samples investigated, *C. obtusa* leaf may be considered as potential dietary antidiabetic agents for the control of postprandial hyperglycemia thus offers a prospective therapeutic approach for the management of type 2 diabetes mellitus. It is presumed that the presence of considerable amount of water soluble active components such as flavonoids and alkaloids in these fractions are known to directly interact with proteins and thus inhibit enzyme activities (Tables 2 and 3, Figs. 1 and 2) (Dawra *et al.,* 1988; Suryanarayana *et al.,* 2014). Such outcomes are similar to those reported by McCue *et al.* (2004). There are also convincing evidences to suggest that several flavonoids are reported to have antidiabetic activities (Abdulrahman and Bushra, 2013; Chitra *et al.,* 2014). Babu *et al.,* (2003) have also reported the presence of relatively high concentration of coumarins, terpenoids and flavonoids in the ethylacetate fractions of *Cassia kleinii* leaf which are said to possess effective antidiabetic activity.

Since it is the first phytochemical report for this valuable traditional medicinal plant of Tamil Nadu, India, the data obtained from the present investigation can be used as baseline information for further studies. Therefore from the present findings, it is emphasized that owing to its efficient protective features, the leaf extract of *C. obtusa* holds a greater promise in antidiabetic research since their bioactive potentiality could be tapped for the development of excellent drug for diabetic mellitus. In this regard, this plant can be used as a potential, inexpensive, natural nutraceutical source in the management of diabetes disorders. The presence of rich contents of flavonoids and alkaloids reported in this study might contribute a big deal for rejuvenating radical mediated diseases including diabetes mellitus. In addition, the observed *in vitro* activities suggest that all the investigated plant extract could exert protective effects also in *in vivo* studies against oxidative and free radical injuries occurring in different pathological conditions. Ongoing and further studies will explore the mechanism of action of *C. obtusa* and will attempt to isolate, identify and characterize the active principles.

ACKNOWLEDGEMENT

The authors are graciously acknowledge the financial support given by University Grants Commission, Government of India, New Delhi [Grant No. 42-938/2013(SR)] to carry out the work.

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How to cite this article:

K. Thenmozhi, S. Jamuna, K. Karthika, S. Manian, S. Paulsamy, C. Chitravadivu. HPTLC finger printing profile and evaluation of *in vitro* antidiabetic potential of medicinally important plant, *Cassia obtusa* L. (Caesalpiniaceae). J App Pharm Sci, 2015; 5 (03): 073-079.