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The hydroalcoholic extract of *Cassia alata* (Linn.) leaves and its major compound rhein exhibits antiallergic activity *via* mast cell stabilization and lipoxygenase inhibition

Baljinder Singh^{a,d,*}, Janhavi R. Nadkarni^b, Ram A. Vishwakarma^a, Sandip B. Bharate^{a,**},
Manish Nivsarkar^c, Sheetal Anandjiwala^d

^a Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu 180001, India

^b Padmashree Dr. D.Y. Patil Institute of Biotechnology and Bioinformatics, CBD Belapur, Navi Mumbai 400614, India

^c B.V. Patel Pharmaceutical Education and Research Development Center, Ahmedabad 380054, Gujarat, India

^d National Institute of Pharmaceutical Education and Research, Ahmedabad 380054, Gujarat, India

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ABSTRACT

Ethnopharmacological relevance: Leaves of *Cassia alata* (family: Caesalpiniaceae) are ethnomedically claimed as anti-asthmatic. In the current study we aimed to investigate the anti-allergic activities of hydro-methanolic extract of *Cassia alata* (Linn.) and its constituents rhein and kaempferol on triple antigen/sheep serum-induced mast-cell degranulation in rats.

Materials and methods: Antiallergic activity of hydroalcoholic extract of *Cassia alata* along with its two components rhein and kaempferol was evaluated using *in vivo* mast cell stabilization assay. Inhibitory effect on lipoxygenase (LOX) enzyme was also evaluated *in vitro*. Further chemical standardization of *Cassia alata* extract was done using rhein and kaempferol by HPTLC-densitometric method.

Results: The hydroalcoholic extract of *Cassia alata* significantly inhibited mast cell degranulation at 200 mg/kg dose. Both chemical constituents rhein and kaempferol also showed potent (>76%) inhibition of mast-cell degranulation at 5 mg/kg. Extract and rhein inhibited LOX enzyme with IC₅₀ values of 90.2 and 3.9 µg/mL, respectively, whereas kaempferol was inactive.

Conclusion: Our results suggest that *Cassia alata* exhibit anti-allergic activity through mast cell stabilization and LOX inhibition. Thus, *Cassia alata* or its active constituents could be potential alternative treatment for allergic diseases.

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1. Introduction

The utilization of medicinal plants in modern medicine suffers from the fact that, although plants are traditionally being used to prevent or cure disease, scientific evidence in terms of modern medicine is lacking in many cases. However, with the development of scientific knowledge in pharmacology, it has become necessary to provide scientific basis as to whether or not it is justified to use a plant for specific disease (Balunas and Kinghorn, 2005). Allergic

diseases are the major cause of illness among the people of all ages and are a major problem all over the world because of their diverse manifestations. Allergic diseases are second most frequent complaint in the population which significantly affects the quality of life. Amongst these, asthma is one of the most common allergic disease accounting for significant morbidity and mortality in both children and adults (Canonic and Ciprandi, 1989).

Several medicinal plants are used ethnomedically in the treatment of allergy/asthma but there is a need to conduct pharmacological studies to ascertain their therapeutic values. One of such plants is *Cassia alata* Linn. (synonym: *Senna alata*; family: Caesalpiniaceae) which occurs in Andhra-Pradesh State of India (commonly called as Simavisi), is used as a traditional medicine to treat bronchitis and asthma (Savithramma et al., 2007). It is an important medicinal tree as well as an ornamental flowering plant and is commonly known as ‘candle-tree’. This shrub also grows in the tropical climate of Philippines (locally called as ‘Aka-pulko’) and has been used in the form of herbal tea, lotion and ointment for different purposes. The herbal tea (decoction of leaves

Abbreviations: LOX, lipoxygenase; HPTLC, high-performance thin-layer chromatography; UV, ultraviolet; LOD, limit of detection; LOQ, limit of quantitation; RSD, relative standard deviation.

* Corresponding author at: Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu 180001, India. Tel.: +91 191 2569000; fax: +91 191 2569333.

** Corresponding author. Tel.: +91 191 2569000; fax: +91 191 2569333.

E-mail addresses: singh0306@gmail.com (B. Singh), sbharate@iiim.ac.in (S.B. Bharate).

and flowers) of *Cassia alata* is used as expectorant and to alleviate the symptoms of asthma attacks. The herbal lotion prepared from leaves is used for variety of skin diseases such as tinea infections, insect bites, ringworms, scabies, herpes, blotch, eczema and mycosis and also used as purgative (Khare, 2007). *Cassia alata* leaves are also reported to possess anti-inflammatory, antimutagenic, analgesic and antimicrobial properties (Khan et al., 2001; Villasenor et al., 2002; Somchit et al., 2003). Further *Cassia alata* extract showed anti-cryptococcus activity when used in combination with *Ocimum sanctum* extract (Ranganathan and Balajee, 2000). Chemical constituents of *Cassia alata* includes phenolics (chrysaphanol, emodin, rhein, aloe-emodin, kaempferol and their glycosides), fatty acids (palmitic, oleic, linoleic acids), terpenoids (β -sitosterol, stigmasterol, campesterol) and anthraquinones (alatonal, alatinone) (Khare, 2007; Liu et al., 2009). Anthraquinones and flavonoids of *Cassia alata* are known to possess diverse range of biological activities (Castiglione et al., 1993, 1990; Borderie et al., 2001; Ackland et al., 2005; Chang et al., 2006; Bandyopadhyay et al., 2008; Choi and Ahn, 2008; Olszanecki et al., 2008; Aviello et al., 2009; Fernand et al., 2011).

Mast cells are important in the numerous physiological processes of homeostasis and disease. Degranulated mast cells release a number of constituents and *de novo* synthesized mediators which include histamine, leukotrienes and prostaglandins (Slater et al., 1999). Therefore, mast cell stabilization is a key factor in controlling the occurrence of asthma. The antiallergic/asthmatic effect of *Cassia alata* leaves has not been significantly evaluated despite its wide use by tribals in the treatment of asthma. The present study was therefore, designed to evaluate the antiallergic potential of *Cassia alata* leaves and its main chemical constituent (s) using *in vivo* mast cell stabilization model. Herein, we also report a HPTLC-densitometric simultaneous quantification method for rhein and kaempferol in hydro-methanolic extract of *Cassia alata* leaves.

2. Materials and methods

2.1. Materials

Leaves of *C. alata* were collected from local herbal nursery, authenticated by our botanists and a voucher specimen was preserved in the Department of Natural Products, NIPER-Ahmedabad (Specimen no. NIPER-A/NP/0908/04). The plant material was dried in vacuum oven at 40–45 °C, stored in airtight glass bottle at 30 °C and powdered to mesh size #40 when required. All solvents were purchased from Qualigens Fine Chemicals Mumbai, India and were of analytical grade. Reference standards of rhein (purity 98%) and kaempferol (purity 97%) were purchased from Natural Remedies (Bangalore, India) and Shaanxi Huike Botanical Development Co. Ltd. (Shanghai, China), respectively. Other chemicals such as ketotifen fumarate (Torrent Pharmaceuticals, Ahmedabad, India), triple antigen (Serum Institute of India, Pune, India), phenidone, linoleic acid and lipoxygenase enzyme (MP Biomedicals, USA), toluidine blue (Merck, Germany) were also procured from commercial sources.

2.2. Animals

Healthy male adult Albino (Sprague–Dawley) rats, weighing between 200 and 250 g were housed in standard environmental condition and were maintained on chow diet and water *ad libitum* throughout the course of study. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. All animal experimental

protocols were approved by the Institutional Animal Ethics Committee (IAEC/CA/1).

2.3. Methods

2.3.1. Preparation of hydro-methanolic extract

Accurately weighed 250 g of the powdered drug was extracted with hydro-methanol (70%) (250 ml $\times$ 4) under reflux on a water bath. Extracts were filtered through a Whatman I filter paper, pooled and concentrated under vacuum to dryness (yield 26%, w/w). The extract was stored at 4 °C. Doses of the extract for *in vivo* models: 50, 100 and 200 mg/kg body weight of rat were prepared using 0.1% (w/v) of agar solution as the vehicle. For *in vitro* lipoxygenase assay, the dried hydro-methanolic extract was re-dissolved in methanol to make required concentrations (50, 100, 150, 200, 250, 300, 400 and 500 μ g/ml).

2.3.2. Mast cell stabilization assay

The mast cell stabilization activity was carried out as reported previously (Harish et al., 2001). Rats were sensitized by injecting subcutaneously 0.5 ml sheep serum along with 0.5 ml of triple antigen. Animals were divided into 12 groups with 6 animals in each group. Group I served as a control (non-sensitized) and received vehicle (0.1% agar solution), Group II (sensitized control group received 0.1% agar solution), Group III (ketotifen fumarate 1 mg/kg), Group IV (*Cassia alata* 50 mg/kg), Group V (*Cassia alata* 100 mg/kg), Group VI (*Cassia alata* 200 mg/kg), Group VII (rhein 1 mg/kg), Group VIII (rhein 2 mg/kg), Group IX (rhein 5 mg/kg), Group X (kaempferol 1 mg/kg), Group XI (kaempferol 2 mg/kg) and Group XII (kaempferol 5 mg/kg). Treatment was lasted for 14 days. On the 14th day, 2 h after the assigned treatment, rats were sacrificed and intestinal mesenteries were taken out. The mesenteric fans were fixed, dried, stained with toluidine blue and examined microscopically for the number of intact and degranulated mast cells in at least twenty randomly selected high power fields. The percentage inhibition of the mast cell degranulation was calculated by using formula:

%Inhibition of mast cell degranulation

$$= \frac{100 - \text{number of degranulated mast cells}}{\text{total number of mast cells}} \times 100$$

Inhibition values were presented as mean $\pm$ SEM. ANOVA test was used to determine the significance of data sets. Probability values of <0.05 were considered as significant.

2.3.3. Lipoxygenase assay

The lipoxygenase assay was carried out as reported previously (Sircas et al., 1983; Gaffney, 1996; Mahesha et al., 2007) with some modifications. The *Cassia alata* extract was screened at eight different concentrations (50, 100, 150, 200, 250, 300, 400 and 500 μ g/ml) along with compounds rhein and kaempferol at concentrations of 0.1, 0.5, 1, 2, 5, 10, 20 and 50 μ g/ml. After obtaining the active concentration range, extract and two compounds were tested for dose dependent inhibition. Absorbance readings were collected at 254 nm. Phenidone was used as a positive control. The percentage inhibition of lipoxygenase was calculated using formula: Percentage inhibition = [(absorbance of blank – absorbance of test)/absorbance of test] $\times$ 100. The IC₅₀ values were calculated by using Finney's probit analysis software.

2.3.4. Chemical standardization of *Cassia alata* extract

2.3.4.1. Chemical fingerprinting of *Cassia alata* extract. Accurately weighed 1 g quantity of powdered drug was extracted with hydro-methanol (70%) (25 ml $\times$ 4) under reflux on a water bath. The

hydro-methanolic extract was filtered through Whatman I filter paper and filtrate was concentrated on vacuo rotavapor. The residue was dissolved in 50 ml methanol in a volumetric flask. This extract was used for TLC fingerprinting and co-chromatography with rhein and kaempferol. Stock solutions of rhein and kaempferol (200 µg/ml) were prepared. Protocol used for chemical fingerprinting is as follows: 10 µl each of extract solution along with rhein and kaempferol solutions were applied on a TLC plate and the plate was developed in petroleum ether:ethyl acetate:formic acid (7.5:3.5:0.1) solvent system to a distance of 8 cm. The plate was observed under UV at 254 and 366 nm. The R_f and the colour of resolved bands were observed.

2.3.4.2. Simultaneous quantification of rhein and kaempferol. Calibration curves for rhein and kaempferol were prepared using six different concentrations viz. 4, 8, 12, 16, 20 and 24 µg/ml. The protocol used is as follows: 10 µL each of the standard solutions of rhein (40–240 ng per respective spot) or kaempferol (120–280 ng per respective spot) were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicate on a TLC plate using automatic sample spotter (Linomat V). The plate was developed in a twin trough chamber (20 cm × 10 cm) up to a distance of 8 cm using a mobile phase of petroleum ether:ethyl acetate:formic acid (7.5:3.5:0.1) at 25 ± 2 °C temperature and 40% relative humidity. The plate was dried at room temperature and scanned at 260 nm in absorbance mode using deuterium lamp. Peak areas were recorded and calibration curves were obtained by plotting peak areas vs applied concentrations of rhein/kaempferol. For simultaneous quantification of rhein and kaempferol in the *Cassia alata* extract, 10 µL of suitably diluted extract solution was applied in triplicate on a TLC plate. The plate was developed and scanned as mentioned above at 260 nm. Peak areas and absorption spectra were recorded and the amount of rhein and kaempferol were calculated using their respective calibration curves. Identity of the bands of rhein and kaempferol in the sample extract was checked by overlaying their UV absorption spectra with that of the respective reference standards using CAMAG TLC Scanner with winCATS software. Purity of the band in the sample extract track was checked by comparing the absorption spectra recorded at start, middle and end positions of the band. The method was validated for precision, repeatability and accuracy as per ICH guidelines (Singh et al., 2009).

3. Results and discussion

The toxicity data for hydro-alcoholic extract of *Cassia alata* in albino rats has been already known. The reported LD₅₀ value is 18.5 g/kg, indicating the safe use of *Cassia alata* extract (Pieme et al., 2006). The dose selection criteria for mast-cell stabilization assay was mainly based on two parameters, firstly based on known LD₅₀ value for the extract; and secondly, using literature information of doses of other plant extracts used in this assay (50–500 mg/kg) (Chandrashekhar et al., 2011). Thus, three doses viz. 50 (low), 100 (medium) and 200 mg/kg (high) of hydromethanolic extract of *Cassia alata* were selected. Results of the mast cell stabilization assay indicated that there was significant protection from mast cell degranulation on treatment with *Cassia alata* extract (75.67 ± 3.77% protection at 200 mg/kg) in a dose-dependent pattern. This result was comparable to the reference standard ketotifen fumarate (77.67 ± 3.77% of protection at 1 mg/kg) with significance of $p < 0.001$. However, medium (100 mg/kg) and lower (50 mg/kg) dose of *Cassia alata* extract have not shown significant mast cell stabilization activity (<35% protection). The mast cell stabilizing potential of two chemical constituents of *Cassia alata* viz. rhein and kaempferol was then evaluated. The selection of doses for these constituents was also based on their known LD₅₀ values (rhein,

Table 1

Mast cell stabilization effect of the *Cassia alata* extract, rhein and kaempferol.

Group	Treatment	Dose (mg/kg)	%Inhibition of mast cell degranulation ^a
I	Vehicle (non-sensitized)	–	^b
II	Vehicle (sensitized)	–	23.67 ± 2.43
III	Ketotifen fumarate	1	*77.67 ± 3.77
IV	<i>Cassia alata</i> extract	50	30.64 ± 2.34
V	<i>Cassia alata</i> extract	100	34.40 ± 3.61
VI	<i>Cassia alata</i> extract	200	*75.67 ± 3.77
VII	Rhein	1	28.97 ± 1.98
VIII	Rhein	2	42.76 ± 2.01
IX	Rhein	5	*76.88 ± 2.35
X	Kaempferol	1	34.79 ± 2.16
XI	Kaempferol	2	66.84 ± 1.93
XII	Kaempferol	5	*80.22 ± 1.64

^a Data are expressed as mean ± SEM, N=6 rats in each group.

^b Group-I received vehicle and was not sensitized with antigen. Percentage of stable mast cells found in this group were, 95.74 ± 1.69%.

* Significant values at $P < 0.05$ when compared to the sensitized control group.

LD₅₀ 3.2 g/kg (Li, 2001); flavonoids LD₅₀ 2–10 g/animal (Tapas et al., 2008) and literature information on doses of pure compounds used in this assay (0.5–10 mg/kg) (Chandrashekhar et al., 2011)). Thus rhein and kaempferol were tested at three doses 1, 2 and 5 mg/kg. Results indicated that both compounds showed >75% inhibition of mast cell degranulation at 5 mg/kg dose, indicating a contribution of these components in the antiallergic activity of crude extract. Results are summarized in Table 1.

It has been reported that compounds inhibiting lipoxigenases can be employed in the treatment of allergy/asthma (Sircas et al., 1983). Therefore, *Cassia alata* extract along with rhein and kaempferol were also evaluated for their ability to inhibit lipoxigenase enzyme. The hydro-methanolic extract of *Cassia alata* was screened at eight different concentrations viz. 20, 50, 100, 150, 200, 250, 300 and 500 µg/ml and compounds rhein and kaempferol at concentrations of 0.1, 0.5, 1, 2, 5, 10, 20 and 50 µg/ml. The plant extract and rhein showed promising activity with IC₅₀ value of 90.18 and 3.9 µg/ml, respectively, whereas kaempferol was found to be inactive (only 10% inhibition at 50 µg/ml). The IC₅₀ value for positive control phenidone was 0.55 µg/ml.

Cassia alata is one of the most commonly used herb in traditional medicine in various forms. Its therapeutic benefits are largely based on folklore rather than scientific evidences. This study has substantiated that *Cassia alata* has significant antiallergic properties and thus its wide traditional use in asthma has now been scientifically validated. Based on our findings, the antiallergic activity of *Cassia alata* can be attributed to its ability to inhibit mast-cell degranulation and its ability to inhibit lipoxigenase enzyme. Further two main chemical constituents of *Cassia alata* showed significant activity in mast-cell stabilization model suggesting their contribution to the antiallergic activity of the herb. In addition, rhein exhibited lipoxigenase inhibitory activity, further justifying its key role in purported bioactivity of *Cassia alata*.

Due to promising biological profile of *Cassia alata* and its two active principles, a hydro-methanolic extract was standardized using these chemical constituents. HPTLC based densitometric method was used for standardization. A HPTLC based method for simultaneous quantification of these two components in the *Cassia alata* extract was also established. Firstly, the HPTLC solvent system was optimized by trial and error taking into consideration, markers to be well resolved. Different combinations of petroleum ether and ethyl acetate were tested and finally, petroleum ether: ethyl acetate (7.5:3.5) system was found to be optimum. To avoid the tailing effect of marker compounds, 0.1% formic acid was added to the solvent system, making final solvent system

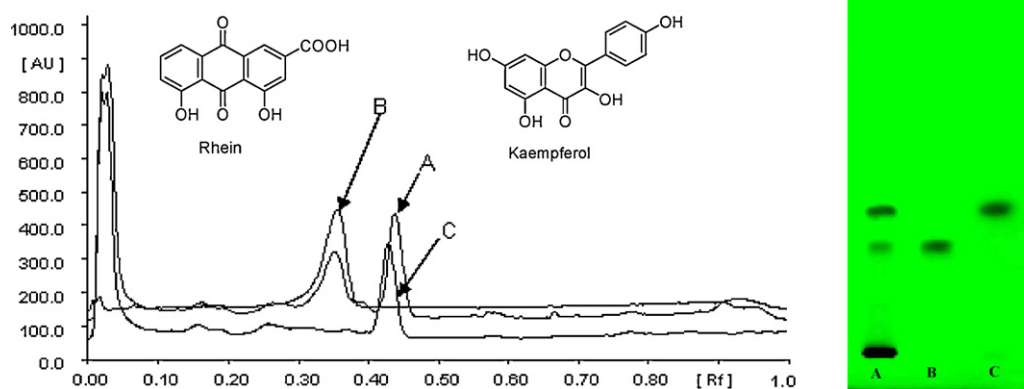


Fig. 1. TLC densitometric chromatogram (at 260 nm) and TLC photograph (at 254 nm) of sample solution of *Cassia alata* leaves along with markers. (A) Hydro-methanolic extract; (B) kaempferol standard; (C) rhein standard.

as, Petroleum ether:ethyl acetate:formic acid (7.3:3.5:0.1) with R_f values of 0.42 and 0.35 for rhein and kaempferol, respectively (Fig. 1). The HPTLC densitometric overlay chromatogram of hydro-methanolic extract of *C. alata* clearly showed presence of these two marker components with R_f values of respective peaks matching with reference standards within $\pm 5\%$ (Indrayanto, 2011). Fig. 1 also shows the TLC photograph of marker compounds and their comparison with hydro-methanolic extract of *Cassia alata*. Identity of the bands of rhein and kaempferol (Fig. S1 in supplementary data) in the sample extract was confirmed by overlaying their respective UV absorption spectra with that of rhein and kaempferol standard respectively. Purity of the bands of rhein and kaempferol (Fig. S2 in supplementary data) in the sample extract tracks were also confirmed by comparing their respective absorption spectra recorded at start, middle and end positions of the bands. Amounts of these two components in extract were found to be 0.38 and 0.08%, w/w, respectively. TLC densitometric method was validated in terms of precision, repeatability, and accuracy. The linearity ranges for rhein and kaempferol were found to be 40–240 and 120–280 ng/spot with correlation coefficients (r values) of 0.998 and 0.999, respectively (Table S1 in supplementary data).

The developed method was found to be precise with %RSD values >1.28 for both intra-day and inter-day experiments for different concentrations of rhein and kaempferol (Table S2 in supplementary data), indicating reproducibility of the method. LOD/LOQ values for rhein and kaempferol were found to be 20/40 and 40/120 ng, respectively (Table S1). Recovery experiments also showed good results with average recoveries of $>99.39\%$ (Table S3 in supplementary data). Thus the developed HPTLC based simultaneous estimation method is simple, faster, accurate and precise and could be useful for analysis of these marker components in various plant extracts, polyphenolic rich extracts or herbal formulations.

4. Conclusions

The hydro-alcoholic extract of *Cassia alata* leaves exhibits antiallergic activity via mast cell stabilization and lipoyxygenase inhibition. Further our results suggest that the observed antiallergic activity of crude extract can be attributed to the presence of anthraquinones and flavonoids. These results completely justify the folkloric use of *Cassia alata* to treat allergic diseases. Active components of *Cassia alata* could be potential alternative treatment for allergic diseases.

Appendix A. Supplementary data

Overlay UV absorption and purity spectra of marker compounds; and validation parameters of HPTLC densitometric method are provided.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2012.03.012.

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