STRUCTURAL DIVERSITY IN MEDICINAL PLANTS: IDENTIFICATION AND QUANTITATIVE ANALYSIS OF SECONDARY METABOLITES USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY AND LIQUID CHROMATOGRAPHY MASS SPECTROMETRY FOR DETERMINING ITS ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

Objective: This study involves identification and quantitative studies of various metabolites present in various medicinal plants by means of high performance thin layer chromatography (HPTLC) and liquid chromatography mass spectrometry (LC-MS). In addition, aqueous leaves extract of these medicinal plants could be evaluated for its anti-inflammatory activity.

Methods: In this study, our group focused on various medicinal plants, that is, *Prosopis spicigera, Anthocephalus cadamba, Mangifera indica,* and *Madhuca indica* to determine its secondary metabolites that are present in the aqueous leaves extract. In addition, these medicinal plants also determined its anti-inflammatory activity using non-specific antigen (Concanavalin A, Con A) on human whole blood.

Results: The results of these studies showed that these secondary metabolites showed wide variation in phytochemicals qualitatively and quantitatively using HPTLC and also studied by LC-MS as well. In addition, variable concentration of aqueous leaves extract when exposed to lysed human whole blood in the presence of Concanavalin (Con)-A, and the results showed that aqueous leaves extract showed anti-inflammatory activity against Con A at higher doses.

Conclusion: Overall, the results showed that these medicinal plants (*P. spicigera, A. cadamba, M. indica* and *M. indica*) timing with complex and architecturally challenging structures of various molecules which could be the reason of anti-inflammatory activity.

Keywords: Inflammation, Medicinal plants, Secondary metabolites, High-performance thin layer chromatography.

INTRODUCTION

As per Ayurveda, use of medicinal plant products in traditional health care system is a heritage of India. Numerous diseases are reported being cured using these medicinal plants by administering either in the form of raw plant parts or some formulations. The therapeutic activity of these plants is due to the secondary metabolites present in it [1,2]. These secondary metabolites are organic compounds which are not directly involved in the survival of plants, unlike primary metabolites. They produce some products which help plants in their normal growth and development. Secondary metabolites involve compound of class Terpenes, Alkaloids, Phenols, etc. These are non-essential component for survival but rather are required for the interaction of plants with their environment, survival in hostile conditions, disease protection mechanism, etc. These are structurally diverse and so can be diagnostic in chemotaxonomic studies.

The chemical structure of a molecule contains the features responsible for its physical, chemical and biological properties [3]. Biological activity (mutagenicity, toxicity, teratogenicity, etc.) of substances is governed by their properties, which in turn are determined by their chemical structure. So to speak, medicinal plants produce various complex and architecturally unique molecules as secondary metabolites, proved its mettle, by having been used in traditional health care system for thousands of years. Nature provides us plethora of active molecules, which need to be identified and derivatized if necessary to have better activity. Computer aided structure-activity relationship prediction have provided quick and precise mode and helped in creating a database of such compounds.

The absence of worldwide acceptance to traditional health care system due to lack of standardized methods for preparation of such formulations is one of the major drawbacks. Modern analytical techniques, for example, liquid chromatography-mass spectrometry (LC-MS), high-performance thin layer chromatography (HPTLC), GC-MS aided analysis of traditional drugs could be achieved, and database could be created to help in the calibration and standardization of the drugs [4-6]. The utmost separation efficiency and use of state-of-the-art instrumentation are salient features of these techniques. HPTLC offers widely standardized methodology based on scientific facts and use of well-established methods for qualitative and quantitative analysis. As a result, HPTLC including LC-MS stands important techniques to meet all the criteria related to quality requirements of today's analytical laboratories and due to which it is in great demand among scientific fraternity. The study focuses at the quantitative phytochemical study of *Prosopis spicigera, Anthocephalus cadamba, Mangifera indica,* and *Madhuca indica.* HPTLC analysis was performed along with identification of some of the medicinally and biologically important molecules on the basis of LC-MS data and determined its anti-inflammatory activity.

METHODS

HPTLC analysis for various phytoconstituents

Densitometric HPTLC analysis was performed for the development of fingerprinting profile of various medicinal plants (sample collection from the garden of Vidya Pratishthan). The samples (aqueous extract) were loaded in the form of band length on precoated silica gel $60F_{254}$ TLC plate [Merck] using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturation with solvent vapor) with respective mobile phase [7]. The developed plate was dried using hair drier to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) for documentation.

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Finally, the plates were fixed in scanner stage and scanned at multiple wavelengths (CAMAG SCANNER 3). The winCats 1.2.3. Version software used for HPTLC. The Peak table, Peak display, and Peak densitograms were identified.

Preparation of extract for LC-MS

Freshly harvested plant leaves were washed with tap water. Leaves were air-dried, cut into small pieces and macerated to prepare fine powder. A total of 2 g of leaves powder of these medicinal plants were macerated in 20 ml PBS (phosphate buffered saline; pH 7.2) simultaneously using mortar and pestle. The aqueous extract was filtered after high speed centrifuging and filtrate was collected and kept in refrigerator at 4°C. All the extracts were subjected to LC-MS analysis [7].

LC-MS analysis

All MS acquisitions were performed in the positive electrospray ionization mode. The capillary voltage, cone voltage, fragmentor voltage were 4 kV, 45 V, and 170 V, respectively whereas gas temperature was set at 325°C. Data were acquired at scan rate of 3 Hz in mass range 100-100 m/z. Further data were analyzed with Mass hunter qualitative software and METLIN database [7].

Proliferation assay

In this study, lysed human whole blood (100 µl) were cultured with variable doses of aqueous leaves extract of *P. spicigera, A. cadamba, M. indica* and *M. indica* (6.25-25 mg/ml; 50 µl). Incubate the sample for 2 h at room temperature. Add FACS lysing solution (1 X solution) and incubate the sample for another 5 minutes. Centrifuging and washing the sample with PBS. Finally, cells were analyzed through flow cytometer (BD, FACS Calibur) [8].

RESULTS AND DISCUSSION

Qualitative as well as the quantitative phytochemical investigation was carried out for *P. spicigera, A. cadamba, M. indica, and M. indica,* and confirmed the presence of flavonoids, phenolics, saponins, terpenoids (Table 1). The study was carried out according to the standard procedures. Aluminum chloride colorimetric method was used for the total flavonoid determination and Gallic acid, for total phenol content. The study revealed that all the medicinal plants contain total flavonoid content as 42.5, 71.25, 95, 65 mg/g, total phenolic content as 291.4, 277.71, 217.39, 212.10 mg/g, and total terpenoid content as 22, 11.6, 9, 8 mg/g, respectively, whereas total alkaloid content for *P. spicigera* was found to be 9.5 µg/g. It is concluded that the given plant is rich with major phytoconstituents and ascertains the claim of being medicinally important plant.

The medicinal plant extracts were screened for saponins, terpenoids, phenolics, glycosides, and alkaloids using HPTLC analysis. The densitometry HPTLC fingerprint profile of these medicinal plants ascertains the presence of various phytochemicals in the extracts. The solvent systems were optimized according to the type of analysis, and then screening was done as shown in Fig. 1. There are total 3 plants rich with various saponins, whereas all medicinal plants under investigation found to have appreciable number of terpenoids. The *P. spicigera* is only candidate where detectable glycosides and alkaloids can be found, as shown in figure.

The aqueous extracts of *P. spicigera, A. cadamba, M. indica,* and *M. indica* were subjected to LC-MS analysis, and samples were characterized

based on their m/z ratio. The identification was done by comparison of fragmentation pattern and precursor ion with reference molecules reported in the literature. Representative examples of identified phytochemicals of medicinally important drugs are shown in Fig. 2. *P. spicigera* contains Oxethazaine identified at rt 10.32-10.63 minutes and [M+H]⁺ ionat m/z 472.29, which is a potent local anesthetic. It is also used in the ointments for the relief of pain associated with hemorrhoids [9]. Tamoxifen, with rt 10.34-10.86 minutes and [M+H]⁺ ionat m/z 376.20, a drug prescribed for breast cancer in women and men as well as other types of cancers too. It stood potent medication for Albright syndrome [10,11]. Fungal infections of the fingernails and toenails are effectively treated with amorolfine which is obtained at rt 10.68-10.86 minutes and [M+H]⁺ ionat m/z 335.30.

A macrolide antibiotic 10-deoxymethymycin also found at rt 12.27-12.75 minutes and [M+H]⁺ ionat m/z 279.19 in the given plant [12]. Oxymetazoline, a drug used for relieving nasal congestion rendered by common cold, sinus infection and other types of allergies. It may be effective for treatment of nosebleed [13,14]. It got separated along the chromatogram at rt 12.33-12.87 minutes and [M+H]⁺ ionat m/z 281.14. *A. cadamba* having Mesoporphyrin IX found at rt 8.63-8.92 minutes and [M+H]⁺ ionat m/z 567.29, is used in constructing efficient dye-sensitized solar cells. The Sn derivative of Mesoporphyrin dramatically enhances the ability of the metalloporphyrin to inhibit *in vivo* heme catabolism [15]. Lasalocid is yet another antibacterial agent which is also added in feed additives [16]. It is detected at rt 10.38-10.88 minutes and [M+H]⁺ ionat m/z 573.37. Digoxin and its derivative, dihydrodigoxin are found in *A. cadamba* known for the treatment of cardiac insufficiencies such as atrial fibrillation, atrial flutter and sometimes heart failure [17], separation of which is obtained at rt 11.45-11.83 minutes and [M+H]⁺ ionat m/z 765.44. Cloprostenol, at rt 18.89-19.20 minutes and [M+H]⁺ ionat m/z 445.11 is used in the termination of pregnancy and the expulsion of mummified fetus in cattle [18].

An architecturally complex molecule rifaximin, suggested for Traveler's diarrhea, irritable bowel syndrome and hepatic encephalopathy [19,20]. Separation is achieved at rt 22.25-22.43 minutes and [M+H]⁺ ionat m/z 808.34. The presence of macrolide antibiotic dirithromycin which can be seen at rt 23.34-23.78 minute and [M+H]⁺ ionat m/z 817.54, also confirmed using LC-MS. Ophiobolin A, a fungal metabolite and a phytotoxin was found in *M. indica* know to be a potent inhibitor of calmodulin-activated cyclic nucleotide phosphodiesterase [21]. A drug *N*-desalkylfurzaepam having rt 11.61-11.96 minutes and [M+H]⁺ ionat m/z 327.00, contains benzodiazepine core, well known to be a psychoactive drug [22] found in *M. indica*. The secondary metabolite crustecdysone is identified at rt 18.55-19.04 minutes and [M+H]⁺ ionat m/z 485.28, is the main hormone found in brown blowfly calliphora stygia is the only molting hormone which can be detected in significant amounts [23]. Notable phytoconsituents which are having complex structures containing either bicyclic ring, macrocyclic ring, or labile functional groups are presented in Fig. 3. The complexity of these molecules gives selectivity to their mode of action and target oriented specific cure to the disease is possible.

One of the complex pathophysiological process, that is, inflammation (acute or chronic), which is mediated by a variety of signaling molecules. Lot of anti-inflammatory drugs (synthetic based origin) that

Table 1: Quantitative phytochemical screening of medicinal plants

Botanical name	Phytoconstituents			
	Flavonoids mg/g	Alkaloids µg/g	Phenolics mg/g	Terpenoids mg/g
Prosopis spicigera	42.5	9.5	291.4	22
Anthocephalus cadamba	71.25		277.71	11.6
Mangifera indica	95		217.39	9
Madhuca indica	65		212.10	8

M. indica: Madhuca indica, M. indica: Mangifera indica, A. cadamba: Anthocephalus cadamba, P. spicigera: Prosopis spicigera

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are available but some of them are no longer use due to its side effects. Recently, people relied on various medicinal plant products, especially leaves pertaining to secondary metabolites. For anti-inflammatory studies, lysed human whole blood were cultured with Con A in the presence of aqueous leaves extract of medicinal plants (*P. spicigera, A. cadamba, M. indica* and *M. indica*) and showed declined in Con A proliferation at higher doses (Table 2) as compared to control. In other

words, these medicinal plants showed anti-inflammatory activity in lysed human whole blood against non-specific antigen.

In nutshell, qualitative and quantitative phytochemical analysis of *P. spicigera, A. cadamba, M. indica* and *M. indica* have been investigated. Standard methods have been used for quantitative estimation of phytoconstituents such as terpenoids, flavonoids, saponins
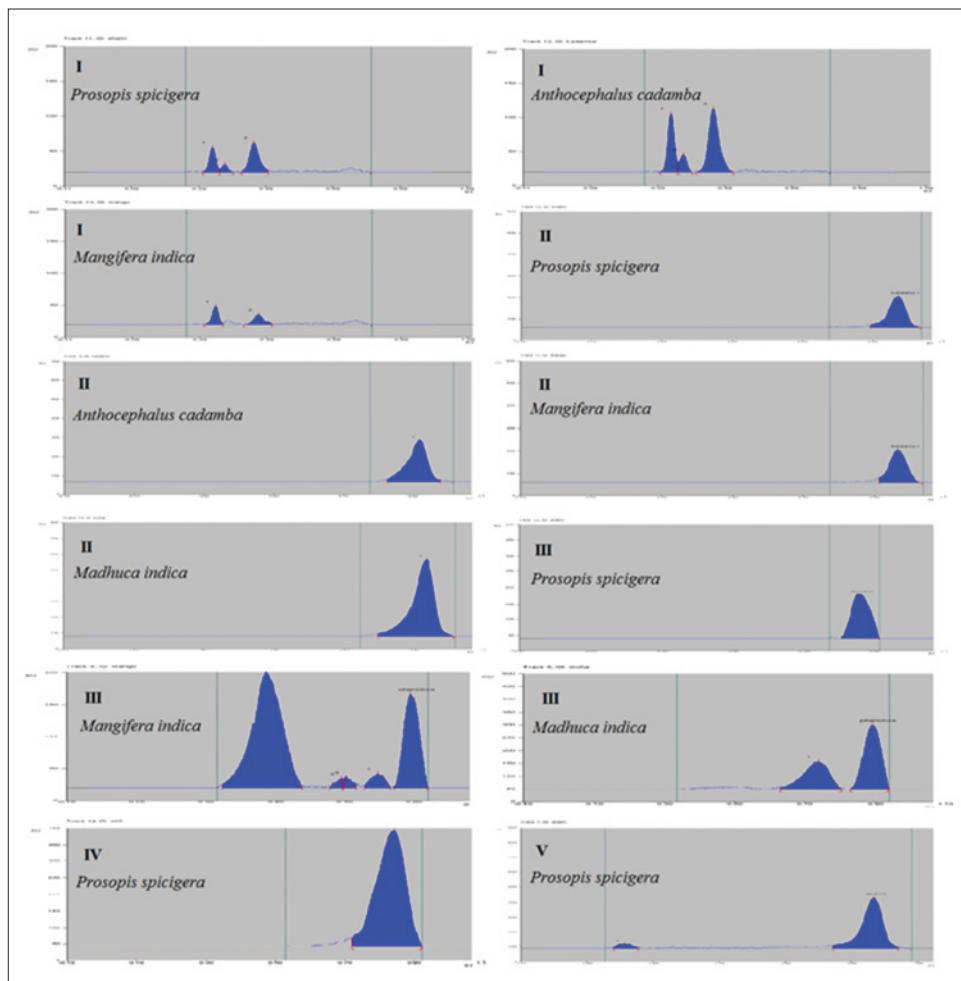

Fig. 1: Typical high performance thin layer chromatography chromatogram for *Prosopis spicigera, Anthocephalus cadamba, Mangifera indica* and *Madhuca indica* of, I. saponins, II. terpenoids, III. phenolics, IV. glycosides and V. alkaloids

Table 2: Effect of variable doses of aqueous leaves extract of medicinal plants on Con A stimulated lysed human whole blood

Plant material	Doses (mg/ml; 50 µl)	OD at 570 nm (Mean±S.E.)	% stimulation/suppression
Prosopis spicigera	Control PBS	0.435±0.06	
	Con A, 10 µg/ml; 10 µl	0.921±0.14	111.72 ↑
	6.25	0.631±0.08	45.05 ↑
	12.5	0.478±0.02**	9.88 ↑
	25	0.348±0.01***	20 ↓
Anthocephalus cadamba			
	6.25	0.712±0.14	63.67 ↑
	12.5	0.642±0.06	47.58 ↑
	25	0.332±0.02***	23.67 ↓
Mangifera indica			
	6.25	0.688±0.10	58.16 ↑
	12.5	0.412±0.04*	5.28 ↓
	25	0.378±0.04***	13.10 ↓
Madhuca indica	6.25	0.814±0.12	87.12 ↑
	12.5	0.373±0.08	14.25 ↓
	25	0.312±0.06***	28.27 ↓

Values are expressed as mean±S.E. The difference between control and variable doses of aqueous extract is controlled by one way ANOVA test (Bonferroni multiple comparison test). *$p<0.05$; **$p<0.01$ and ***$p<0.001$, *M. indica*: *Madhuca indica, M. indica*: *Mangifera indica*, *A. cadamba*: *Anthocephalus cadamba*, *P. spicigera*: *Prosopis spicigera*
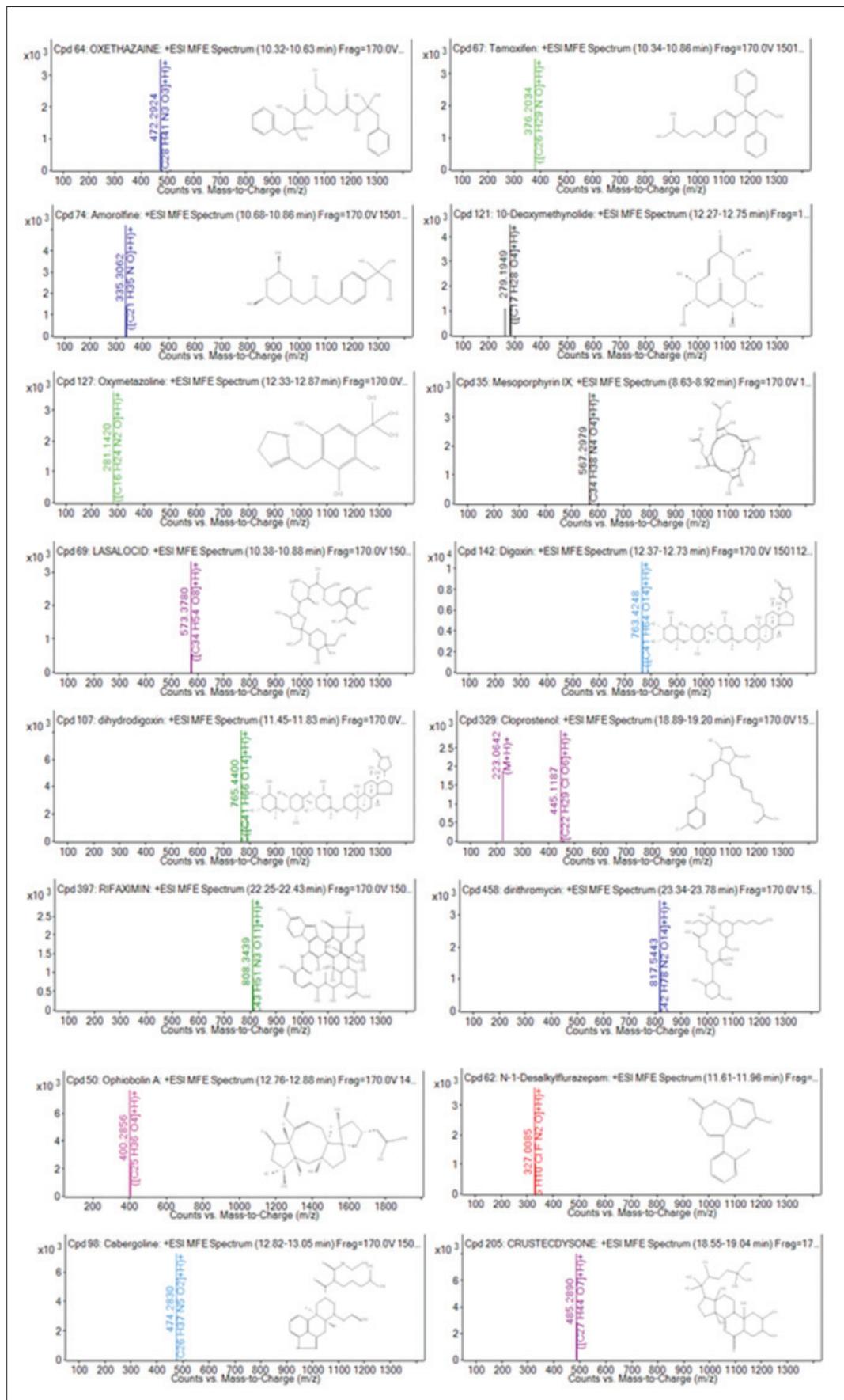
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Fig 2: Mass spectrometry spectra for representative bioactive phytochemicals identified in *Prosopis spicigera, Anthocephalus cadamba, Mangifera indica* and *Madhuca indica*

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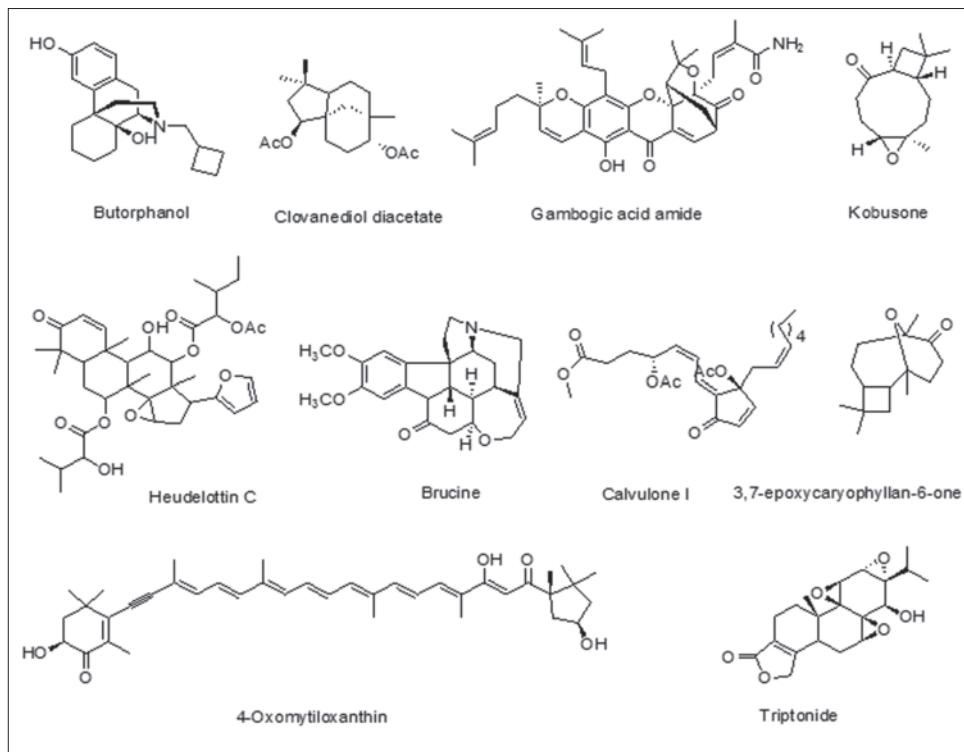
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Fig. 3: Notable structurally complex bioactive phytochemicals identified in *Prosopis spicigera, Anthocephalus cadamba, Mangifera indica* and *Madhuca indica*

and glycosides and phenolics. The study inferred the presence of terpenoids, flavonoids in all medicinal plants with optimum availability. The study also highlights the richness of secondary metabolites present in medicinal plants. HPTLC analysis also confirms the saponins, terpenoids, and glycosides present in the samples whereas glycosides and alkaloids are present only in *P. spicigera*. The identification of bioactive phytoconstituents was done based on LC-MS observations based on their m/z ratio and fragmentation patterns.

CONCLUSION

From these studies, it is confirmed that medicinal plants especially *P. spicigera, A. cadamba, M. indica* and *M. indica* contained more potent molecules related to biological and medicinal interest such as Oxethazaine, Ophiobolin A, Lasalocid, Cloprostenol, Rifaximin, Dirithromycine, Crustecdysone, etc. Taking into an account of structural diversity and complexity, undoubtedly these molecules stands ideal and potent anti-inflammatory candidates for site-specific cure to the diseases.

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