Chemical studies on antidiabetic botanical drug: \textit{Cassia auriculata}

Girme AS, Gaikar NV, Saste GB and Kunkulol RR

Abstract

\textit{Cassia auriculata} Linn (vernacular name: Avartaki,) is a traditional medicine used in the treatment of Diabetes mellitus. The present study is aimed to find out the phytochemical evaluation of \textit{Cassia auriculata} Linn seeds using qualitative and quantitative tests on extracts. The chemical studies of different extracts were screened for the presence of phytochemicals. Fourier Transform Infra-Red studies were done for functional group identification. The quantitative yield and its determination of various phytochemicals such as polyphenols were confirmed. Also a rapid and reverse phase Ultra High Performance Liquid Chromatography (RP-UHPLC) method developed with help of UV detector for six polyphenolic compounds namely gallic acid, caffeic acid, ferulic acid, ellagic acid, epicatechin and epigallocatechin.

Keywords: \textit{Cassia auriculata}, phytochemical, chemical studies, polyphenols, rp-UHPLC

Introduction

Botanical drugs have been used in patients with Type-I and Type-II diabetes mellitus (DM) with other complications like diabetic retinopathy, diabetic peripheral neuropathy, etc. Compounds with different structure with different chemical moieties are responsible for the therapeutic activity in the treatment of DM. Its chemical studies can lead to further isolation and identification of potential lead towards its traditional claim \cite{1}. Several phytoconstituents such as alkaloids, glycosides, flavonoids, saponins, dietary fibres, polysaccharides, glycolipids, peptidoglycans, amino acids and others obtained from various plant sources that have been reported as potent hypoglycaemic agents \cite{2}. The validated botanical drugs are available now days in market with scientific studies and chemical standardisation. However, still potent traditional drugs like \textit{Cassia auriculata} (CA) have been not studied for chemical studies for complete understanding of its phytochemistry.

Materials and methods

Collection and identification of plant material

Seeds were collected from District-Ahmednagar, Maharashtra. The plant material was authenticated at BSI-Pune by comparing morphological features (twigs arrangement, flower etc.). The herbarium of the plant specimen has been deposited at BSI-Pune. The dried seeds were cleaned, coarsely powder and used for chemical and phytochemical analysis.

Correspondence

Girme AS
Department of Pharmacology, Pravara Institute of Medical Sciences, A/P: Loni Dist: Ahmednagar, Maharashtra, India

Fig 1: Flowering plant and pods with different maturity of \textit{Cassia auriculata}
Botanical Description
Scientific Name: Cassia auriculata Linn
Common Names: Tanner’s Cassia (English), Tarvar Taroda (Hindi), Taroda, Taravada (Marathi); Avaqtaki, Vibhandi, Carmaranga (Sanskrit); Tangedu (Kannada); Avarai (Tamil); Aval, Awal (Gujarati); Synonyms: Senna auriculata; Family: Caesalpiniaeae.
Description: The leaves are alternate, stipulate, paripinnate compound, very numerous, closely placed, rachis 8.8-12.5 cm long, its flowers are irregular, bisexual, bright yellow and large (nearly 5 cm across), the pedicels glabrous and 2.5 cm long. The fruit is a short legume, 7.5–11 cm long, 1.5 cm broad, oblong, obtuse, pale brown. 12-20 seeds per fruit are carried each in its separate cavity.

Preparation of seed extracts
Seeds were collected from Dist. - Ahmednagar, Maharashtra. Seeds cleaned off adhering dust and unwanted plant material, shade dried, cut and pulverized (powdered). Further Seeds (500 g) were extracted with successive extraction at room temperature, filtered and concentrated under reduced pressure on rotary evaporator. The dried extract was successively fractionated in Petroleum Ether (40.5 gm) [CA-PE], n-butanol, (5.8g) [CA-NB], acetone: methanol 1:1 (26.8g) [CA-AM] and methanol: water 1:1 (21.23g) [CA-MW]. Also, separately seeds extracted with methanol by Soxhlet extraction at 60 °C [CA-TS]. The solvents were chosen for larger delivery of bioactive compounds which are polar and mid-polar.

Phytochemical screening
The preliminary screening analysis of petroleum ether extract [CA-PE], n-butanol extract[CA-NB], acetone: methanol extract[CA-AM], methanol: water extract [CA-MW] was screened for the presence of phytochemicals like Steroids, Triterpenoids, alkaloids, saponins, glycosides, sugars, flavonoid, tannins, Protein, Amino acid by standard methods [3].

1. Detection of steroids
Salkowski Test: Few mg of extract was taken in 2.0 ml of chloroform and 2.0ml of concentrated Sulphuric acid was added from the side of test tube. It was shaken for few min. The development of red colour in the chloroform layer indicated the presence of steroids.
Liebmann Burchard Test: Few mg of extract in a test tube, few ml of acetic anhydride was added and heated gently. Few drops of concentrated sulphuric acid were added to it appearance of blue colour gave evidence for presence of steroids.

2. Detection of Glycosides
Keller-Killani test- To 2 ml of extract, glacial acetic acid, one drop 5% ferric chloride and concentrated sulphuric acid were added. Appearance of reddish-brown colour at the junction of the two liquid layers indicates the presence of cardiac glycosides.

3. Detection of Alkaloids
Few mg of each extract was taken separately in dilute hydrochloride acid and filtered.
Chemical studies

The chemical studies were done to determine quantitative analyses of phytoconstituent found in preliminary phytochemical testing as shown in fig.2. All the results have been expressed as mean ± standard deviation (S.D).

Determination of Total polyphenols content
Polyphenols are secondary metabolites of plants and are generally involved in defence against ultraviolet radiation or aggression by pathogens. Polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and based on structural elements that bind these rings to one another. The main classes include phenolic acids, flavonoids, stilbenes and lignans. The antidiabetic polyphenols play major role in diabetes because of their antioxidant nature [5].

Folin-Ciocalteu assay method was used for the determination of the total polyphenol content. 100mg of the plant extract was used to determine total polyphenols content [6]. Set of three test tubes each containing 1ml of the standard solution, sample solution and water (as blank). 5ml Folin-Ciocalteu reagent was added into each tube and mixed well. 4.0mL of sodium carbonate solution into each tube was added after 8 minutes and mixed well. The reaction mixture could stand at room temperature in dark condition for 1 hour. The absorbance was measured at 765 nm against a blank solution. The content of total polyphenolics was extrapolated from the standard curve for Gallic acid and was expressed as mg Gallic acid equivalents (GAE) per gram of dry mass of extracts [7].

Determination of total flavonoid content
Structurally, flavonoids consist of two aromatic rings (A and B rings) linked by a 3-carbon chain that forms an oxygenated heterocyclic ring (C ring). There are six subclasses of flavonoids including flavones, flavonols, flavanones, flavanols, isoflavones and anthocyanidins based on differences in generic structure of the C ring, functional groups on the rings and the position at which the B ring is attached to the C ring. Within each subclass, individual compounds are characterized by specific hydroxylation and conjugation patterns. Flavonoids that can scavenge free radicals and chelate metals [8], Quercetin, Diosmin, Fisetin, Quercetin-3-O-rutinoside, Luteolin and Naringenin [9]. Are some of well-known anti-diabetic flavonoids [10]. The total flavonoids contents quantitatively estimated by UV Spectrophotometer method? 10mg of the sample was used to determine total flavonoids content. Test sample solution was prepared in test tube by adding 1ml of sample stock solution along with 3ml methanol, 0.2ml aluminium chloride, 0.2ml potassium acetate solution and 5.6ml distilled water and mix well in test tube. Sample blank was prepared in same way by replacing aluminium chloride with distilled water. Prepared solutions were filtered through Whatman filter paper and their absorbance was measured at 415nm against a blank solution. The content of total flavonoids contents was extrapolated from the standard curve for Quercetin [11].
Determination of total Alkaloids content
Alkaloids are organic compounds with nitrogen. The nitrogen atom will be at heterocyclic ring, with some exceptions. The alkaloids are mainly classified as true alkaloids and pseudo-alkaloids. The true alkaloids are derived from α-amino acid precursors. Other alkaloids, such as terpenes and steroids, are called pseudo-alkaloids because a relatively late amination process occurs in a transamination reaction by donating a nitrogen atom from an amino acid source. Alkaloids like vindoline I, vindoline II, vindoline III and vindoline IV from catharanthus species [12]. Arecaidine [13], Aegeline [14], Berberine [15]. Have been shown their potential in DM. So, quantitative determination of total alkaloids was performed by gravimetric analysis. 1gm of sample substance was poured into the conical flask. 100mL mixture of solvent ether; methanol [80:20] was added along with 5ml ammonia solution. Flask was frequently shake for one hour and solution was filtered. The residue was wash with mixture of ether; methanol [80:20] in 5 lots of 5mL each. 30mL of 1N sulphuric acid was added to total ether methanol solution in separating funnel. Acidic layer was collected and washed with of 20mL mixture of 0.5N sulphuric acid: methanol until complete extraction of the alkaloids is affected. Mixed acid solution was wash with 10mL chloroform. Acidic layer was made alkaline with dilute ammonia solution to the pH 7. Basified portion was partitioned with chloroform. All collected fractions were mixed together. Pooled chloroform fractions were evaporated to dryness [16]. Calculate the percent of alkaloids.

Determination of Saponin content
Saponins are naturally occurring surface-active glycosides. Saponins consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, glycosidically linked to a hydrophobic aglycone (sapogenin) which may be triterpenoid or steroid in nature [17]. There have been several reports for saponin working as hypoglycemic agents [18, 19, 20, 21]. One gram of dry powder was refluxed with 50mL of 90% v/v methanol in flask. Residue was washed with 90% v/v methanol and all collected fractions were mixed together and evaporate on water bath to obtain a thick paste like residue. It was filtered, and the above process was repeated twice. Methanol was then evaporated to obtain a thick paste approximate 5mL volume and dissolved in 25 mL of hot water completely. The extract was fractionated in a separating funnel with petroleum ether in separating funnel. Further petroleum ether layer was fractionated with Ethyl acetate thrice. All collected fractions were mixed together. Pooled ethyl acetate fractions were evaporated to dryness. Calculate the percent of total bitter [29].

Total Terpenoid content
Terpenes and terpenoids are the primary constituents of the essential oil compounds including terpenes, diterpenes and sesquiterpenes. They have unsaturated molecules composed of linked isoprene units, generally having the formula (C₅H₈)n. The compounds belong to the isoprenoid group. The triterpenoids [30]. And compounds like Limonene [31], Mahanimbine [32], Myrcene [33], 5α-Cucurbitane [34]. from this class have been reported as anti-diabetic. So, the protocol developed for analysis of total terpenoids from CA as a Linalool [35].

Ultra High Performance Liquid chromatography (UHPLC) analysis
Chemicals &Reagents- Methanol and all other reagents of HPLC Grade were obtained from Ran Kemp, India. All standards such as Gallic acid, Caffeic acid, Ferulic acid, Ellagic acid, Epicatechin and Epigallocatechin were procured from Sigma-Aldrich Company, USA. Double distilled water was used during analysis.

Instrument
The UHPLC system consists of Shimadzu corporation series with SIL-30AC auto sampler, LC-30AD quaternary solvent delivery unit, CTO-20AC column oven and SPD-20A UV detector with lab solution data software used. The analysis was performed on Reverse phase (RP) Phenomenex Luna-C18 column (250x4.6mm, 5µm).

Chromatographic conditions
The composition of the mobile phase was optimised by
varying the percentage of methanol, acetonitrile and water in reverse phase column. The optimization was done by different solvent system and operating system. Mobile phase Acetonitrile: 0.1% formic acid in water with gradient elution from 90% to 45% in 60 min, flow rate was 0.8ml/min, column temperature was 35°C, Detector wavelength 272nm and 320nm. The GA, EA, EC and EGC were analysed at 272 nm and CF, FA analysed at 320 nm.

Sample Preparation
Test sample solution 10mg/ml was prepared in methanol.

Preparation of the Standard Solutions
Standard stock solution 0.1mg/ml of GA, CA, FA, EC and EGC were prepared separately in methanol and 0.1mg/ml of EA in double distilled water.

Results
Cassia auriculata (CA) seeds, family Caesalpiniaeeae collected from Ahmednagar area, MH, India was authenticated by BSI-Pune (BSI/WRC/IDEN.CER/2016/53). The extractive yield for different extracts such as CA-PE, CA-NB, CA-AM, CA-MW, CA-TS was 9.2%, 8.15%, 11%, 9.65%, 13% respectively. Different chemical tests were performed on the extracts to find out nature of chemical constituents. The preliminary phytochemical investigation of different CA extract revealed the presence of sterols, triterpenes, flavonoids, tannins, glycosides and alkaloids as tabulated in Table1.

Table 1: Phytochemical results for various extracts

<table>
<thead>
<tr>
<th>S.no</th>
<th>Phytochemical components</th>
<th>Chemical test</th>
<th>PE extract</th>
<th>NB extract</th>
<th>AM extract</th>
<th>MW extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Steroids</td>
<td>Liebermann- Burchard Test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Glycosides</td>
<td>Keller-killeri test</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Alkaloids</td>
<td>Dragendorff’s Test</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>4</td>
<td>Triterpenoids</td>
<td>Liebermann- Burchard Test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Flavanoids</td>
<td>Shinoda Test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Tannins</td>
<td>Ferric chloride Test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Proteins</td>
<td>Biuret test Millon’s test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Amino acid</td>
<td>Ninhydrin test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Saponins</td>
<td>Foam Test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Sugars</td>
<td>Fehlings Test</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+ =Positive, - =Negative)

CA-PE: Petroleum ether extract, CA-NB: n-Butanol extract, CA-AM: Acetone Methanol extract, CA-MW: Methanol water extract

As per FTIR spectra (fig.5) of Cassia auriculata test sample extract, the functional groups of the components were separated based on their peak ratios. The result confirmed the presence of characteristic band at 3007.55 cm\(^{-1}\), 2922.45 cm\(^{-1}\),2853 cm\(^{-1}\) shows the C-H stretching, at 2378.10 cm\(^{-1}\), 2310.22 shows -P-H stretching, at 1742 cm\(^{-1}\) shows the C=O stretch carboxylic acid / Ketone group, at 3397.87cm\(^{-1}\) shows O-H stretch group, at 1457.46 cm\(^{-1}\) shows C-H bending, at 1055.21 cm\(^{-1}\), 1032.84cm\(^{-1}\), 1097cm\(^{-1}\) shows C-C stretch group.

Fig 5: FTIR spectra of Cassia auriculata extract

Table 2: Results of chemical studies

<table>
<thead>
<tr>
<th>S.no</th>
<th>Name of extracts</th>
<th>Terpenoids</th>
<th>Polyphenols</th>
<th>Flavonoids</th>
<th>Alkaloids</th>
<th>Tannins</th>
<th>Saponins</th>
<th>Polysaccharides</th>
<th>Bitters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CA-PE</td>
<td>17.54</td>
<td>14.87</td>
<td>10.51</td>
<td>--</td>
<td>2.31</td>
<td>2.35</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>CA-AM</td>
<td>18.31</td>
<td>28.04</td>
<td>13.51</td>
<td>5.45</td>
<td>5.62</td>
<td>6.54</td>
<td>25.21</td>
<td>1.89</td>
</tr>
<tr>
<td>4</td>
<td>CA-MW</td>
<td>19.59</td>
<td>34.41</td>
<td>12.78</td>
<td>6.12</td>
<td>10.15</td>
<td>8.42</td>
<td>21.24</td>
<td>2.14</td>
</tr>
<tr>
<td>5</td>
<td>CA-TS</td>
<td>31.59</td>
<td>38.23</td>
<td>15.27</td>
<td>6.23</td>
<td>15.12</td>
<td>11.41</td>
<td>24.15</td>
<td>2.51</td>
</tr>
</tbody>
</table>

Results are expressed as n=3 (%w/w) Mean ± S.D.

CA-PE: Petroleum ether extract, CA-NB: n-Butanol extract, CA-AM: Acetone Methanol extract, CA-MW: Methanol water extract CA-TS: Test sample
Results of chemicals studies as per Table 2 indicates that, CA-TS fraction showed higher content of terpenoids, polyphenols, flavonoids, alkaloids, tannins, saponins, bitters as compared to other extracts whereas CA-AM extracts shown maximum content of Polysaccharides. As polyphenols were the major group present quantitatively in different extracts of CA, a rapid and Reverse phase Ultra High Performance Liquid Chromatography (RP-UHPLC) method was developed with help of UV detector. The chromatographic conditions for CA test sample and standard during analysis applied was same. The chromatogram of CA test sample scanned at 320nm and 270nm showed different peaks with retention time (fig.6 to 7). The identification of the peaks in chromatogram through eluted compounds were identified through comparison of retention time with standards (fig.8 to 10). The retention time of GA, EGC, EP, EA at 272nm were 5.7 min, 10.6 min, 18.1 min, 37.2 min and of CF, FA at 320nm were 17.9 min, 32.4 min respectively. Quantification of all markers in µg/mL from CA test sample as tabulated in Table 3 was performed.

![Fig 6: Test sample (TS) Chromatogram at 320 nm](image)

![Fig 7: Test sample (TS) Chromatogram at 272 nm](image)

**Standard chromatograms**

![Fig 8: Standard Gallic acid (GA), Ellagic acid (EA), Epicatechin (EP), and Epigallocatechin (EGC) chromatogram at 272 nm](image)

![Fig 9: Standard Ferulic acid (FA) chromatogram at 320 nm](image)
FIG 10: Standard Caffeic acid (CF) chromatogram at 320 nm

Table 3: Quantification of markers in (%w/w) from Test sample (TS)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name</th>
<th>Gallic acid</th>
<th>Caffeic acid</th>
<th>Ferulic acid</th>
<th>Ellagic acid</th>
<th>Epicatechin</th>
<th>Epigallocatechin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CA-TS</td>
<td>0.8</td>
<td>0.05</td>
<td>0.02</td>
<td>0.005</td>
<td>0.01</td>
<td>0.02</td>
</tr>
</tbody>
</table>

CA-TS: Test sample

Discussion

The detailed chemical investigation involves the process from collection and authentication of plant material to its extraction by different solvent and its evaluation by qualitative and quantitative methods for phytochemical evaluation of crude drugs.

The present work represents the systematic study of plant material with qualitative and quantitative analysis of preliminary phytochemical investigation to fingerprinting of functional group with FTIR spectra, further laboratory experiment of quantitative determination of chemical groups supporting claims to sophisticated analytical techniques for the optimization, development and identification of phytoconstituent in *Cassia auriculata*.

*Cassia auriculata* (CA) is a traditional ayurvedic medicine used for DM. Phytochemical screening showed presence of different phytochemicals such as polyphenols, flavanoids, terpenoids, tannins, polysaccharides, saponins. Chemicals studies were carried out on different extracts for quantitatively determination of polyphenol, terpenoids, flavonoids, alkaloids, polysaccharides, saponins, bitters and tannins.

Reverse phase UHPLC method was optimised and developed for the detection of polyphenols in different extracts of CA. The common phenolic markers such as CF, FA, GA, EGC, EP, EA chosen for standardisation of CA different extracts including test sample. The identification done through method development by retention time in comparison to standard chromatogram. The developed method was found well resolved for this quantification.

Conclusion

Chemical studies on the seeds of *Cassia auriculata* showed the presence of different phytochemicals mainly polyphenols. Similarly quantitative analysis on chemical studies showed polyphenols content was maximum in CA test sample as 8.43%/w/w. Further RP-UHPLC method was developed for quantification and identification of polyphenols markers such as CF, FA, GA, EGC, EP, EA in *Cassia auriculata* test sample.

List of Abbreviations

<table>
<thead>
<tr>
<th>S. No</th>
<th>Abbreviation</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CA</td>
<td><em>Cassia auriculata</em></td>
</tr>
<tr>
<td>2</td>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>3</td>
<td>RP-UHPLC</td>
<td>Reverse phase Ultra High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>4</td>
<td>CA-PE</td>
<td>Petroleum Ether extract</td>
</tr>
<tr>
<td>5</td>
<td>CA-NB</td>
<td>n-Butanol extract</td>
</tr>
<tr>
<td>6</td>
<td>CA-AM</td>
<td>Acetone Methanol extract</td>
</tr>
<tr>
<td>7</td>
<td>CA-MW</td>
<td>Methanol water extract</td>
</tr>
<tr>
<td>8</td>
<td>CA-TS</td>
<td>Test sample</td>
</tr>
<tr>
<td>9</td>
<td>FTIR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>10</td>
<td>GA</td>
<td>Gallic acid</td>
</tr>
<tr>
<td>11</td>
<td>CF</td>
<td>Caffeic acid</td>
</tr>
<tr>
<td>12</td>
<td>FA</td>
<td>Ferulic acid</td>
</tr>
<tr>
<td>13</td>
<td>EA</td>
<td>Ellagic acid</td>
</tr>
<tr>
<td>14</td>
<td>EC</td>
<td>Epicatechin</td>
</tr>
<tr>
<td>15</td>
<td>EGC</td>
<td>Epigallocatechin</td>
</tr>
</tbody>
</table>

Acknowledgement

We would like to acknowledge Dr. Lal Hingorani (Pharmanza Herbal Pvt Ltd, Anand, India) and Dr. Bhushan Patwardhan (Professor, Savitribai Phule Pune University) for the technical support required in this research.

References


25. Corzo J, Leon-Barrios M, hernando-Rico V, Gutierrez-Navarro AM. Precipitation of metallic cations by the acidic Exopolysaccharides from *Bradyrhizobium japonicum* and *Bradyrhizobium (Chamaecyticus)* strain BGA-1, Applied and environmental microbiology. 1994; 60(12):4531-4536


