Research article

Evaluation for substitution of stem bark with small branches of Cassia fistula Linn for traditional medicinal uses: A comparative chemical profiling studies by HPLC, LC-MS, GC-MS

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ABSTRACT

Background: The Aim of the present research article is to proposing a conservative approach for the Cassia fistula by using of small branches instead of stem bark because of plant has many important chemical constituents which show different medicinal activity so consumption of plant is high. We studied here Comparative preliminary phytochemical screening test of the ethanol extract and aqueous extract of the stem bark and small branches of Cassia fistula obtained by cold maceration process. Physicochemical analysis of Cassia fistula was done to ascertain the quality of the raw material used in the study. Successive soxhlet extraction method used for the successive extraction of stem bark and small branches with different solvents for comparative chemical profile study by HPLC, LC-MS, and GCMS. Molecular Docking Interaction of Abundant Medicinal Phytochemicals in the Liquid Chromatography–Mass Spectrometry (LC–MS) Analysis Data of C. fistula with the L. donovani Drug Target Proteins and Pancreatic lipase colipase target protein.

Result: The pH of the small branches was found slightly higher as compared to stem bark and the percentage of other parameters like total ash content, acid insoluble ash, loss on drying at 105 °C, water soluble extractive and alcohol soluble extractive values were found fewer in the small branches as compare to stem bark of the plant. It was observed that the number of peaks in stem bark and small branches of the plant sample were almost similar and the retention time of each peak in stem bark was coincide with the retention of small branches of the sample. Therefore, similarity was observed in stem bark and small branches of the Cassia fistula plant in HPLC, LC-MS and GC-MS. The results obtained from HPLC analysis shows that stem bark contains 0.0084% and small branches having 0.0257% of rhein in Cassia fistula. Compounds 3, 9 and 12 are present in Stem bark as well as small branches of C. fistula and Compounds 22, 32 and 37 are present in small branches only. All the compounds have very good binding energy (Kcal/mol) with the respective target proteins.

Conclusion: The small branches have more active chemical constituents than stem bark against particular target proteins.

1. Introduction

Cassia fistula Linn. commonly known as the Golden Shower belongs to the family Fabaceae. It is a deciduous tree with greenish grey bark, compound leaves, leaflets are each 5–12 cm long pairs. A semi-wild tree known for its beautiful bunches of yellow flowers and also used in traditional medicine for several indications. A fruit is cylindrical pod and seeds many in black, sweet pulp separated by transverse partitions. The long pods which are green, when unripe, turn black on ripening after flowers shed [1]. Pulp is dark brown in Colour, sticky, sweet and mucilaginous, odour characteristic, and somewhat disagreeable [2, 3]. Drug occurs in flat or curved thick pieces; outer surface smooth to rough with warty patches; greenish grey to red; inner surface rough, reddish with parallel striations; fracture, laminated; odour, sweet and characteristic; taste, astringent [4]. It is a medium size tree which is native of tropical Asia. It is widely cultivated in South Africa, East Africa, Brazil, India,
West Indies, China, Mexico, etc. All parts (see stem bark and small branches of plants in Figure 1) of the plant have medicinal properties so it is a very valuable medicinal plant which is utilized in the traditional system of medicine.

1.1. Taxonomic classification

**Kingdom** - **Plantae**
**Subkingdom** – **Tracheobionta**
**Super Division** - **Spermatophyta**
**Division** - **Magnoliophyta**
**Class** – **Magnoliopsida**
**Sub Class** - **Rosidae**
**Order** - **Fabales**
**Family** - **Fabaceae**
**Genus** - **Cassia**
**Species** – **fistula**

1.2. Chemical constituents

**Cassia fistula** was reported to have important classes of phyto constituents like Anthraquinone glycosides, cardiac glycosides, phenolic compounds, carbohydrate, protein, fats, alkaloids, tannins, saponins, steroids, ter-penoids and phloba-tannins, linoleic acid, oleic acid, stearic acid, rhein glycosides, sennosides A, B, anthraquinones, flavanoid-3-olderivatives, ceryl alcohol, kaempferol, bianthraquinone glycosides, fistulin, essential oils, volatile components, phyto (16.1%), 2-hexadecanone (12%), crystals and 4-hydroxy benzoic acids hydrate etc. [5, 6, 7, 8].

Lupeol, β-sitosterol, hexacosanol, 5,7,3,4′-tetrahydroxy-6, 8-dimethoxy flavone-3-O-α-arabinopyranoside, 5,7,4′-trihydroxy-6,8,3′-trimethoxy flavone-3-O-α-L-rhamnosyl (1→2)-O-β-D-glucopyranoside and 1,8-dihydroxy-3, 7-dimethoxy anthraquinone-4-O-α-L-rhamnosyl (1→2)-O-β-D-glucopyranoside are present in the stem bark of the plant (see chemical structure in Figure 2). It is a major bioactive compound reported in **Cassia fistula** for many therapeutic activities [4, 9, 10, 11, 12, 13, 14].

The aim of the present study was to recommend the suitable substituent for those drugs which are uses in huge amount. Stem bark or root of plant like Aragvadha (**Cassia fistula**) stem bark is mentioned as ingredient in some ayurvedic formulations. It is difficult to get huge amounts of roots from the big trees without uprooting. Removal of the stem bark from the trunk of the tree makes the plant weak and susceptible to damage by insects and natural elements. The usage of roots and barks of the trunk is therefore forbidden with an aim to conserve and protect the medicinal plants from extinction and make them available for future generation.

2. Material and methods

**Cassia fistula** Linn. stem bark and small branches were procured from Regional Ayurveda Research Institute, Central Council for Research in Ayurvedic Sciences, Ministry of AYUSH, Government of India, Gwalior Road, Jhansi, Uttar Pradesh.

2.1. Molecular docking studies

AutoDock Vina was used for the virtual screening of the phytochemical compounds and target proteins of **L. donovani** [15] and pancreatic lipase target protein. The target protein was changed into a macromolecule, which converted the atomic coordinates into pdbqt format. The grid box was selected around the crystal structure while other parameters were left as default for molecular docking by AutoDock Vina [16]. The binding affinity was used to analyze the results of molecular docking, and then all possible docked conformations were generated for different constituents. The detailed interactions, including their types such as hydrogen bonding, van der Waals, alkyl, pi-alkyl, and halogen interactions, between different constituents and the target proteins were analyzed by BIOVIA Discovery Studio [17]. The most favorable binding poses of the compounds were analyzed by choosing the lowest free energy of binding (ΔG).
Figure 2. A) Chemical Structure of chemical constituents present in both stem bark and small branches of *Cassia fistula*. 1. Betaine, 2. Nicotinic acid, 3. Butein, 4. 4-Methoxycinnamic acid, 5. 4-Hydroxycoumarin, 6. Caffeic acid, 7. (E)-parinaric acid, 8. Oleanolic acid, 9. Lup-20(29)-en-28-al, 3beta-hydroxy, 10. (22E)-Stigmasta-5,22-dien3-ol, 11. Erucamide, 12. Betulin. B) Chemical Structure of chemical constituents present only in stem bark of *Cassia fistula*. 13. Abietic acid, 14. Nervonic acid, 15. Epicatechin, 16. Aloin A, 17. Quercetin, 18. Lateolin, 19. Rhamnetin, 20. (-)-Epigallocatechin. C) Chemical Structure of chemical constituents present only in small branches of *Cassia fistula*. 21. Apigenin, 22. Kaempferol, 23. 4-Piperidone, 24. Vanillin, 25. Quinine, 26. b-Asarone, 27. (E)-Ferulic acid, 28. 3-Hydroxypyridine, 29. 6-Gingerol, 30. 10-Gingerol, 31. 8-Hydroxyquinoline, 32. (±)-Naringenin, 33. 7-Ethoxycoumarin, 34. (±/-)-Methoprene, 35. (E)-4-Methoxycinnamic acid, 36. Asiatic acid, 37. Lupa-12,20(29)-dien-3-one, 38. Aloe-emodin, 39. (±)-ar-Turmerone, 40. Adipic acid.
2.2. Receptor and ligand preparation

The crystal structure of Nucleoside hydrolase, Sterol 24-c-methyl transferase and Pancreatic lipase were downloaded from PDB (ID: 5TSQ, 5WP4 and 1LPB) [18, 19]. The proteins were finally prepared by Discovery Studio keeping all of the parameters at default. The X, Y and Z coordinates in the 5TSQ proteins were 10.14 Å, 31.63 Å and 18.52 Å respectively. The X, Y and Z coordinates in the 5WP4 proteins were 10.23 Å, -1.58 Å and 33.49 Å respectively. The X, Y and Z coordinates in the 1LPB proteins were 9.82 Å, 23.49 Å and 50.87 Å respectively. The critical residues of the binding pockets were identified from the native catalytic pockets of the available crystal structure of proteins and Discovery Studio. The 3D structure of the constituents of the Stem bark and Small branches extract of C. fistula was retrieved from the PubChem database in SDF format [20]. Aloin A, Erucomide, and (22E)-Stigmasta-5,22-dien-3-ol were sketched by Chemdraw 16.0. The atomic coordinates of all of the ligands were changed to pdbqt setup using Open Babel GUI, an open-source chemical toolbox for the interconversion of chemical structures. MMFF94 was used for energy minimization.

2.3. Preliminary phytochemical analysis

Preliminary phytochemical screening results showed the presence or absence of certain phytochemicals in the Cassia fistula sample. 4g of the sample was taken in a glass stoppered 250 ml flask. 100 ml of absolute ethanol was added. The flasks were shaken occasionally for 6 hours and allowed to stand for 18 hours. The extract was filtered and evaporated to dryness. The same procedure was followed for aqueous extraction. The extracts were collected, dried, weighed and stored separately for pre-

2.4. Physicochemical parameters

Physicochemical analysis was done to ascertain the quality of the raw material used in the study. Various type of physicochemical parameters performed like loss on drying, total ash content, acid insoluble ash, water extractive value, alcohol soluble extractive value and pH (10% w/v aqueous solution) [24, 25].

2.5. HPLC, LCMS and GCMS chemical profile

The chromatographic profiling of Cassia fistula was performed using three different techniques (HPLC, LC-MS & GC-MS) for the comparison between stem bark and small branches of the plant. The dried powdered stem bark and small branches of Cassia fistula were successively extracted with 200 ml of each solvent in the increasing order of polarity i.e. hexane, chloroform, ethyl acetate and ethanol by using soxhlet apparatus for 24 hrs. The extracts were evaporated to dryness under reduced pressure. The same procedure was followed for total ethanol extraction. The obtained extracts were collected, dried, weighed and stored separately for further studies. The different extracts of the Cassia fistula stem bark and small branches were weighed and dissolved in appropriate solvents and filtered through 0.22 μm membrane filters and used for HPLC profiling and compared under the same chromatographic conditions like Column type: ZORBAX Eclipse XBD- C8, Mobile Phase: Acetonitrile: Water (75:25), Injection Volume: 10 μL in each analysis. For the LCMS analysis the test solution was prepared by dissolving
|          | A. Butein (3) | B. Lup-20(29)-en-28-al, 3beta-hydroxy (9) | C. Betulin (12) |
|----------|--------------|------------------------------------------|-----------------|
|          | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
|          | D. (±)-Naringenin (22) | E. Kaempferol (32) | F. Lupa-12,20(29)-dien-3-one (37) |
|          | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |

Figure 3. Binding pattern of *C. fistula* major chemical constituents with the Pancreatic lipase colipase. Two-dimensional (2D) and its significant interactions with (A) Butein, (B) Lup-20(29)-en-28-al, 3beta-hydroxy, (C) Betulin (D) (±) Naringenin, (E) Kaempferol, (F) Lupa-12,20(29)-dien-3-one.
Figure 4. Binding pattern of *C. fistula* major chemical constituents with the Sterol 24-C-methyltransferase (SMT). Two-dimensional (2D) and its significant interactions with (A) Butein, (B) Lup-20(29)-en-28-al, 3beta-hydroxy, (C) Betulin, (D) (+)-Naringenin, (E) Kaempferol, (F) Lupa-12,20(29)-dien-3-one.
|   | A. Butein | B. Lup-20(29)-en-28-al, 3beta-hydroxy | C. Betulin |
|---|----------|--------------------------------------|-----------|
| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| D. (±)-Naringenin | E. Kaempferol | F. Lupa-12,20(29)-dien-3-one |
| ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |

**Figure 5.** Binding pattern of *C. fistula* major chemical constituents with the Nucleoside hydrolase (NH). Two-dimensional (2D) and its significant interactions with (A) Butein, (B) Lup-20(29)-en-28-al, 3beta-hydroxy, (C) Betulin (D) (±) Naringenin, (E) Kaempferol, (F) Lupa-12,20(29)-dien-3-one.
10 mg of each ethanol extracts of *Cassia fistula* stem bark and small branches in 2 ml methanol. It was filtered and sent for LC-MS analysis. For the GCMS analysis the volatile content of *Cassia fistula* stem bark and small branches were dissolved in 2 ml chloroform. It was filtered and sent for GC-MS analysis.

3. Result and discussion

3.1. Molecular docking studies

Compounds 3, 9, 12, 22, 32 and 37 have very good binding energy with the particular targets. The comparative molecular docking results of compounds 3, 9, 12, 22, 32 and 37 of *Cassia fistula* have more binding energy (kcal/mol) with target protein are tabulated in Table 1 and interaction tabulated in Figures 3, 4, and 5.

3.2. Physicochemical parameters

The comparative analysis results of physicochemical parameters for *Cassia fistula* stem bark and small branches are tabulated in Table 2. The results of all the parameters of stem bark comply with the Ayurvedic Pharmacopoeia of India (API) standards.

The pH of the small branches was found slightly higher as compared to stem bark and the percentage of other parameters like total ash content, acid insoluble ash, loss on drying at 105 °C, water soluble extractive and alcohol soluble extractive values were found fewer in the small branches as compare to stem bark of the *Cassia fistula* plant [24, 25].

3.2.1. Preliminary phytochemicals screening

The comparative preliminary phytochemicals screening results of aqueous and ethanol extracts of *Cassia fistula* stem bark and small branches are tabulated in Table 3. The results reveal the presence of similar phytochemicals in stem bark and small branches except flavonoids which were present in both extracts of stem bark and absent in both extracts of small branches [21, 22, 23].

3.2.2. HPLC chromatographic profiling of *Cassia fistula*

The obtained residue weights and extractive values of extraction are given in Table 4. While comparing the HPLC chromatographic profiling of successive extracts of *Cassia fistula*, it was observed that 19 peaks in stem bark and 16 peaks in small branches of the samples were detected in hexane extracts, 10 peaks in stem bark and 10 peaks in small branches of the samples were detected in chloroform extracts, 06 peaks in stem bark and

### Table 2. Physicochemical parameters of *Cassia fistula* stem bark and small branches.

| S. No. | Test parameter | Results | Stem bark | Small branches |
|--------|----------------|---------|-----------|---------------|
| 1.     | pH (10% w/v aqueous solution) | 6.90 | 7.60 |
| 2.     | Total ash content (% w/w) | 7.46 | 2.69 |
| 3.     | Acid insoluble ash (% w/w) | 0.26 | 0.01 |
| 4.     | Water soluble extractive (% w/w) | 19.29 | 5.68 |
| 5.     | Alcohol soluble extractive (% w/w) | 20.14 | 1.55 |
| 6.     | Loss on drying at 105 °C (% w/w) | 8.56 | 8.14 |

### Table 3. Preliminary phytochemicals screening tests of *Cassia fistula* stem bark and small branches.

| S. No. | Phytochemical constituents | Results | Stem bark | Alcohol extract | Small branches | Alcohol extract |
|--------|---------------------------|---------|-----------|-----------------|---------------|----------------|
| 1.     | Alkaloids                 | +       | +         |                  | -             | -              |
| 2.     | Coumarins                 | -       | ++        |                  | -             | +              |
| 3.     | Flavonoids                | +       | ++        |                  | -             | -              |
| 4.     | Furanoidoids              | +++     | +++       |                  | -             | +              |
| 5.     | Phenols                   | +       | +         |                  | +             | +              |
| 6.     | Quinones                  | +++     | +         | +                | +             | ++             |
| 7.     | Reducing sugars           | +       | +++       | +                | +             | +              |
| 8.     | Saponins                  | +++     | +         |+++              | +             | +              |
| 9.     | Sugar (Carbohydrate)      | -       | +         |+++              | +             | +              |
| 10.    | Tannins                   | +       | ++        |+++              | +             | +              |
| 11.    | Triterpenoids             | -       | +++       |                  | +             | +              |

### Table 4. Extractive values of *Cassia fistula* stem bark and small branches.

| S. No. | Name of the solvent for extraction | Stem bark | | | Small branches | | |
|--------|---------------------------------|-----------|-----------|-----------|-------------|-----------|-----------|
|        |                                 | Weight of sample (g) | Weight of extract (g) | Percentage of extract | Weight of sample (g) | Weight of extract (g) | Percentage of extract |
| 1.     | Successive extraction           | Hexane    | 10.0108   | 0.0546   | 0.55       | 10.0128   | 0.0539   | 0.54       |
|        |                                 | Chloroform| 0.0469    | 0.47     |            | 0.0883    | 0.88     |            |
|        |                                 | Ethyl acetate| 1.1537   | 11.52    |            | 0.0808    | 0.81     |            |
|        |                                 | Ethanol   | 1.2442    | 12.43    |            | 0.3595    | 3.59     |            |
| 2.     | Total ethanol                   | 10.0217   | 2.4911    | 24.86    |            | 10.0172   | 0.3387   | 3.38       |
Figure 6. HPLC profiling chromatogram of successive hexane extracts of *Cassia fistula* stem bark and small branches.

| Chromatographic Conditions | Stem bark | Small branches |
|---------------------------|-----------|---------------|
| Column type               | ZORBAX Eclipse XBD- C<sub>18</sub> | | |
|                          | (4.6 mm x 150 mm), 5μm particle size | | |
| Mobile Phase              | Acetonitrile: Water (67:33) | | |
| Detection                 | VWD Detector @ 254nm | | |
| Flow Rate                 | 0.5 mL/min | | |
| Injection Volume          | 10 μl | | |
Figure 7. HPLC profiling chromatogram of successive chloroform extracts of *Cassia fistula* stem bark and small branches.
Figure 8. HPLC profiling chromatogram of successive ethyl acetate extracts of *Cassia fistula* stem bark and small branches.
### Chromatographic conditions

- **Column type**: ZORBAX Eclipse XBD- C$_{18}$
  
  (4.6 mm x 150 mm), 5μm particle size

- **Mobile Phase**: Acetonitrile: Phosphate Buffer (50:50)

- **Detection**: DAD Detector @ 254nm

- **Flow Rate**: 1.0 mL/min

- **Injection Volume**: 10 μl

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#### Stem bark

![HPLC chromatogram of stem bark](chart1)

#### Small branches

![HPLC chromatogram of small branches](chart2)

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*Figure 9. HPLC profiling chromatogram of successive ethanol extracts of *Cassia fistula* stem bark and small branches.*
| Chromatographic conditions | ➢ Column type          : ZORBAX Eclipse XBD- C₁₈  
|                           | (4.6 mm x 150 mm), 5µm particle size |
|                           | ➢ Mobile Phase         : Acetonitrile: Water (60:40) |
|                           | ➢ Detection            : DAD Detector @ 254nm |
|                           | ➢ Flow Rate            : 1.0 mL/min |
|                           | ➢ Injection Volume     : 10 µl |

**Figure 10.** HPLC profiling chromatogram of total ethanol extracts of *Cassia fistula* stem bark and small branches.
### Table 5. HPLC peaks details of successive hexane extracts of *Cassia fistula* stem bark and small branches.

| Peak No. | Ret. Time [min] | Area [mAU*s] | Area % | Peak No. | Ret. Time [min] | Area [mAU*s] | Area % |
|----------|-----------------|--------------|--------|----------|-----------------|--------------|--------|
| 1        | 1.589           | 149.50922    | 0.8897 | 1        | 1.580           | 103.48606    | 0.7698 |
| 2        | 1.718           | 139.68979    | 0.8311 | 2        | 1.708           | 90.54295     | 0.6735 |
| 3        | 1.882           | 200.25746    | 1.1917 | 3        | 1.847           | 184.42491    | 1.3718 |
| 4        | 2.247           | 1444.48193   | 8.5960 | 4        |                |              |        |
| 5        | 2.291           | 426.46875    | 2.5022 | 5        |                |              |        |
| 6        | 2.349           | 1856.65857   | 11.0488| 6        |                |              |        |
| 7        | 2.784           | 457.82662    | 2.7244 | 7        |                |              |        |
| 8        | 2.938           | 333.25076    | 1.9831 | 8        |                |              |        |
| 9        |                |              |        | 9        |                |              |        |
| 10       | 4.936           | 2209.57007   | 13.1489| 10       |                |              |        |
| 11       | 5.973           | 1354.29077   | 8.0593 | 11       |                |              |        |
| 12       | 6.334           | 2420.41626   | 14.4037| 12       |                |              |        |
| 13       | 7.053           | 1200.93823   | 7.1467 | 13       |                |              |        |
| 14       | 7.942           | 340.91782    | 2.0288 | 14       |                |              |        |
| 15       | 8.561           | 227.86253    | 1.3560 | 15       |                |              |        |
| 16       | 8.965           | 558.20044    | 3.3218 | 16       |                |              |        |
| 17       | 9.698           | 173.10760    | 1.0301 | 17       |                |              |        |
| 18       | 10.759          | 749.83698    | 4.4622 | 18       |                |              |        |
| 19       | 11.473          | 532.97852    | 3.1717 | 19       |                |              |        |
| **Total**|                | 1.680424     | 100.0000 | **Total**|                | 1.344374     | 100.0000 |

### Table 6. HPLC peaks details of successive chloroform extracts of *Cassia fistula* stem bark and small branches.

| Peak No. | Ret. Time [min] | Area [mAU*s] | Area % | Peak No. | Ret. Time [min] | Area [mAU*s] | Area % |
|----------|-----------------|--------------|--------|----------|-----------------|--------------|--------|
| 1        | 2.686           | 1413.94092   | 17.5177| 1        | 2.604           | 2169.96729   | 15.5223 |
| 2        | 2.870           | 2256.06689   | 27.9510| 2        | 2.873           | 4167.65039   | 29.8122 |
| 3        | 3.068           | 3059.15527   | 37.9006| 3        | 3.095           | 3431.93970   | 24.5495 |
| 4        |                |              |        | 4        |                |              |        |
| 5        | 4.203           | 606.60406    | 7.5154 | 5        |                |              |        |
| 6        |                |              |        | 6        |                |              |        |
| 7        |                |              |        | 7        |                |              |        |
| 8        |                |              |        | 8        |                |              |        |
| 9        | 6.099           | 104.24109    | 1.2915 | 9        | 6.072           | 60.75570     | 0.4346 |
| 10       | 6.822           | 167.93848    | 2.0806 | 10       | 6.798           | 200.31689    | 1.4239 |
| 11       | 7.181           | 225.29892    | 2.7913 | 11       | 7.147           | 134.73520    | 0.9638 |
| 12       | 8.284           | 82.74257     | 1.0251 | 12       |                |              |        |
| 13       | 9.296           | 92.94195     | 1.1515 | 13       |                |              |        |
| 14       | 9.871           | 62.58374     | 0.7754 | 14       |                |              |        |
| **Total**|                | 8071.51389   | 100.0000 | **Total**|                | 1.344374     | 100.0000 |

### Table 7. HPLC peaks details of successive ethyl acetate extracts of *Cassia fistula* stem bark and small branches.

| Peak No. | Ret. Time [min] | Area [mAU*s] | Area % | Peak No. | Ret. Time [min] | Area [mAU*s] | Area % |
|----------|-----------------|--------------|--------|----------|-----------------|--------------|--------|
| 1        | 1.247           | 7609.18701   | 25.0800| 1        | 1.252           | 1533.26746   | 14.2287 |
| 2        | 1.331           | 5945.04541   | 19.5949| 2        | 1.355           | 1782.12549   | 16.3581 |
| 3        | 1.419           | 4751.75391   | 15.6618| 3        | 1.425           | 1718.84949   | 15.9059 |
| 4        | 1.587           | 7001.36768   | 23.0766| 4        | 1.631           | 1884.67175   | 17.4897 |
| 5        | 1.728           | 5003.45557   | 16.4914| 5        | 1.734           | 2621.03247   | 24.3232 |
| 6        |                |              |        | 6        |                |              |        |
| 7        | 2.622           | 28.91096     | 0.0953 | 7        |                |              |        |
| 8        |                |              |        | 8        |                |              |        |
| 9        |                |              |        | 9        |                |              |        |
| 10       |                |              |        | 10       |                |              |        |
| **Total**|                | 3.033974     | 100.0000 | **Total**|                | 1.077594     | 100.0000 |
10 peaks in small branches of the samples were detected in ethyl acetate extracts, 07 peaks in stem bark and 09 peaks in small branches of the samples were detected in ethanol extracts. In the comparison of total ethanol extracts, 07 peaks in stem bark and 09 peaks in small branches of the samples were detected.

It was observed that the number of peaks in stem bark and small branches of the plant sample were almost similar and the retention time of each peak in stem bark was coincide with the retention of small branches of the sample. Therefore, similarity was observed in stem bark and small branches of the *Cassia fistula* plant. The detailed peak identification and peak area results are shown in Figures 6, 7, 8, 9, and 10 and Tables 5, 6, 7, 8, and 9.

### 3.2.3. LC-MS chromatographic profiling of *Cassia fistula*

The LC-MS profiling chromatograms of *Cassia fistula* stem bark and small branches are given in Figure 11 and retention time, name of compound, molecular formula, molecular weight and maximum peak area are given in Table 10 (see chemical structure in Figure 2). From the report of LC-MS, it was observed that, the LC-MS analysis of active compounds showed similarity in the both extracts of stem bark and small branches of the plant [26, 27].

### 3.2.4. GC-MS chromatographic profiling of *Cassia fistula*

The detailed peak identification shown in Figure 12 and retention time, compound name, molecular weight and maximum peak area, are given in Table 11. The significant similarities have been observed in the GC-MS chromatographic profiling of volatile content of the *Cassia fistula* stem bark and small branches [26, 27].

| Peak No. | Ret. Time [min] | Area [mAU*s] | Area % |
|----------|-----------------|--------------|--------|
| 1        | 1.150           | 1.41661e4    | 34.8629|
| 2        | 1.250           | 818.31155    | 20.1943|
| 3        | 1.536           | 1.09232e4    | 26.8623|
| 4        | 1.665           | 678.29297    | 16.6913|
| 5        | 2.104           | 345.49762    | 0.8503 |
| 6        | 2.462           | 151.43039    | 0.3727 |
| 7        | 2.929           | 83.77605     | 0.2062 |
| 8        | 3.416           | 3.416        | 0.0930 |
| 9        | 3.674           | 3.674        | 0.0930 |
| 10       | 4.823           | 4.823        | 0.1276 |

### 3.2.5. Quantitative estimation of rhein biomarker compound in *Cassia fistula* stem bark and small branches by HPLC

#### (i) Test solution:
The residues obtained from ethanol extracts of stem bark and small branches of *Cassia fistula* were accurately weighed in triplicate and dissolved in ethanol extract. The residues were accurately weighed and dissolved in HPLC grade methanol using 5 ml volumetric standard flasks, filtered through 0.22 μm membrane filters and used for HPLC analysis.

#### (ii) Standard solution:
1.0 mg of rhein reference standard was accurately weighed and dissolved in HPLC grade methanol and the volume was made up to 5 ml to obtain 0.20 mg/ml rhein stock solution.

#### (iii) Chromatographic conditions:
- **Column:** ZORBAX Eclipse XBD-C18 (4.6 mm × 150 mm), 5μm particle size
- **Detection:** VWD Detector at 247 nm
- **Mobile phase:** Acetonitrile: Phosphate Buffer (55:45)
- **Flow rate:** 1.2 ml/min
- **Injection volume:** 10 μl
- **Retention time:** 3.076
- **Mode of operation:** Isocratic elution

#### (iv) Calibration curve:
0.20 mg/ml rhein stock solution was appropriately diluted further to obtain a concentration of 0.05, 0.025, 0.0125, 0.00625 mg/ml of rhein. Each of the standard solution was run through HPLC system and used in the construction of the calibration curve.
Figure 11. LC-MS Chromatogram of ethanol extracts of \textit{Cassia fistula} stem bark and small branches.
Table 10. LC-MS Peak details of ethanol extracts of *Cassia fistula* stem bark and small branches.

| Peak No. | Ret. Time | Name of the compound | Molecular Formula | Molecular Weight | Area Maximum |
|----------|-----------|----------------------|-------------------|------------------|--------------|
| **Stem bark** | | | | | |
| 1 | 1.072 | Betaine | C₅H₁₁NO₂ | 117.0787 | 15260766.1 |
| 2 | 1.191 | Nicotinic acid | C₆H₅NO₂ | 123.0318 | 393452.2136 |
| 3 | 2.509 | (–)-Epigallocatechin | C₁₅H₁₄O₇ | 306.073 | 464997.9638 |
| 4 | 7.388 | Epicatechin | C₁₅H₁₄O₆ | 290.0781 | 2571077.704 |
| 5 | 11.094 | Butein | C₁₅H₁₂O₅ | 272.0676 | 411811.0544 |
| 6 | 13.95 | 4-Methoxycinnamic acid | | 178.0627 | 722746.2581 |
| 7 | 14.321 | Aloin A | C₂₁H₂₂O₉ | 418.1255 | 969916.0451 |
| 8 | 14.432 | Quercetin | C₁₅H₁₀O₇ | 302.0421 | 265656.2337 |
| 9 | 14.855 | Luteolin | C₁₅H₁₀O₆ | 286.047 | 745514.2985 |
| 10 | 20.072 | 4-Hydroxycoumarin | | 162.0313 | 860981.3235 |
| 11 | 20.11 | Caffeic acid | C₉H₈O₄ | 180.0418 | 2391754.538 |
| 12 | 20.295 | (E)-parinaric acid | | 276.2083 | 250512.54 |
| 13 | 21.633 | Abietic acid | C₉H₈O₄ | 302.2238 | 194720.7413 |
| 14 | 22.935 | Oleanolic acid | C₁₅H₂₀O₂ | 438.3488 | 231825.8614 |
| 15 | 23.856 | Lup-20(29)-en-28-al, 3beta-hydroxy- | | 440.3647 | 194223.063 |
| 16 | 24.262 | Nervonic acid | C₁₅H₂₀O₂ | 366.3489 | 155724.8206 |
| 17 | 24.488 | Erucaamide | C₁₅H₂₀NO | 337.3335 | 1517686.26 |
| 18 | 26.046 | Betulin | C₁₅H₂₀O₂ | 442.3803 | 399168.5256 |
| 19 | 26.662 | (22E)-Stigmasta-5,22-dien3-ol | | 412.3692 | 26940935.26 |
| **Small branches** | | | | | |
| 1 | 1.01 | 3-Hydroxypyridine | C₅H₅NO | 95.03695 | 301739.2746 |
| 2 | 1.036 | Betaine | C₅H₁₁NO₂ | 117.07878 | 72844002.46 |
| 3 | 1.19 | Nicotinic acid | C₆H₅NO₂ | 123.03192 | 2574509.917 |
| 4 | 2.504 | 4-Piperidone | C₅H₉NO | 99.06827 | 2786020.512 |
| 5 | 6.456 | 8-Hydroxyquinoline | C₉H₇NO | 145.05255 | 845239.8029 |
| 6 | 7.575 | Adipic acid | C₆H₁₀O₄ | 146.05771 | 673583.4342 |
| 7 | 9.276 | Vanillin | C₁₅H₂₀O₄ | 152.04697 | 1048031.784 |
| 8 | 10.336 | Quinine | C₁₅H₂₄N₂O₂ | 324.18236 | 401047.9444 |
| 9 | 10.473 | β-Asarone | C₁₅H₂₀O₄ | 208.10939 | 740911.8038 |
| 10 | 10.893 | (E)-Ferulic acid | C₁₅H₂₀O₄ | 194.0576 | 836774.0259 |
| 11 | 11.431 | Butein | C₁₅H₂₀O₂ | 272.06804 | 660531.6233 |
| 12 | 12.455 | (–)-Naringenin | C₁₅H₂₀O₂ | 272.06804 | 136131.3751 |
| 13 | 13.517 | 7-Ethoxycoumarin | C₁₅H₂₀O₂ | 190.06275 | 4982798.85 |
| 14 | 13.605 | Apigenin | C₁₅H₂₀O₂ | 270.05223 | 266768.5365 |
| 15 | 13.945 | 4-Methoxycinnamic acid | C₁₅H₂₀O₂ | 178.06282 | 1108157.784 |
| 16 | 14.678 | (E)-4-Methoxycinnamic acid | C₁₅H₂₀O₂ | 178.06282 | 106125.6885 |
| 17 | 15.588 | Kaempferol | C₁₅H₂₀O₆ | 286.04717 | 320185.0805 |
| 18 | 16.847 | Aloe-emodin | C₁₅H₂₀O₅ | 270.05223 | 181000.967 |
| 19 | 18.293 | (+)-(6)-Gingerol | C₁₇H₂₆O₄ | 294.18236 | 274825.6524 |
| 20 | 19.728 | (+)-ar-Turmerone | C₁₅H₂₀O₄ | 216.15097 | 168543.0028 |
| 21 | 19.947 | 6-Gingerol | C₁₇H₂₆O₄ | 294.18236 | 451217.3484 |
| 22 | 20.038 | 10-Gingerol | C₁₇H₂₆O₄ | 350.24452 | 212893.1025 |
| 23 | 20.152 | Caffeic acid | C₁₅H₂₀O₄ | 180.04178 | 1885849.78 |
| 24 | 20.153 | 4-Hydroxycoumarin | C₁₅H₂₀O₃ | 162.03131 | 99683.3751 |
| 25 | 20.572 | (E)-parinaric acid | C₁₇H₂₆O₄ | 276.20818 | 7802595.386 |
| 26 | 22.208 | (+/-)-Methoprene | C₁₅H₂₀O₃ | 310.24982 | 3161195.753 |
| 27 | 23.708 | Oleanolic acid | C₁₅H₂₀O₃ | 438.34877 | 1783708.336 |
| 28 | 23.812 | Lup-20(29)-en-28-al, 3beta-hydroxy- | | 440.36464 | 802911.4302 |
| 29 | 23.833 | Astatic acid | C₁₅H₂₀O₃ | 448.34922 | 335493.9399 |
| 30 | 23.851 | Lup-12,20(29)-dien-3-one | C₁₅H₂₆O₄ | 422.35384 | 874681.9728 |
| 31 | 24.482 | Erucaamide | C₁₅H₂₆NO | 337.33344 | 2353754.824 |
| 32 | 26.037 | Betulin | C₁₅H₂₀O₂ | 442.37984 | 1024698.489 |
| 33 | 26.663 | (22E)-Stigmasta-5,22-dien3-ol | C₁₇H₂₆O₄ | 412.36937 | 130035124.4 |
recorded the respective peak areas. Calibration curve was established for peak area vs concentration of rhein applied shown in Figure 13.

(v) Estimation of Rhein:

Injected 10 μl each of the test solution to HPLC system. Record the chromatogram and determine the area of the peak of the test solution corresponding to that rhein as described above from the calibration curve. Calculated the amount of rhein present in the residues extracted in ethanol for each test sample of *Cassia fistula* stem bark and small branches is given in Figure 14 and Table 12.

The results obtained from HPLC analysis shows that stem bark contains 0.0084% and small branches having 0.0257% of rhein in *Cassia fistula*.
Figure 13. HPLC Chromatogram of Rhein Standard and Calibration curve.
Figure 14. Estimation of Rhein in Ethanol extracts of Cassia fistula stem bark and small branches.
Table 12. Estimation of Rhein in ethanol extracts of Cassia fistula stem bark and small branches.

| S. No. | Name of extract | Rhein (% w/w) | Stem bark | Small branches | Results | Mean | Result | Mean |
|--------|----------------|---------------|-----------|----------------|---------|------|--------|------|
| 1.     | Ethanol extract | 0.0096        | 0.0084%   | 0.0252         | 0.0052  | 0.0257% |
|        |                | 0.0077        | 0.0059    |                |         |       |        |      |
|        |                | 0.0080        | 0.0061    |                |         |       |        |      |

*Percentage of results was given from the means of triplicates for stem bark and small branches samples of optimized extracts of ethanol.

4. Conclusion

The results obtained from HPLC analysis shows that stem bark contains 0.0084% and small branches having 0.0257% of rhein in Cassia fistula.

Compounds 3, 9, 12, 22, 32 and 37 gave excellent interaction with 5WP4, STSQ and 1LPB target protein in molecular docking. Compounds 3, 9 and 12 obtained in stem bark and small branches of the plant while compounds 22, 32 and 37 were present in small branches only. So small branches are more efficiently work as Antileishmanial drug as well as Pancreatic lipase inhibitor than stem bark on the basis of molecular docking. Similarities in different chromatographic profiles, phytochemical analysis of various extracts of stem bark and small branches and quantitative estimation of rhein suggests that, the small branches may have almost similar active chemical constituents like stem bark. Hence, the study provides the base for further study to recommend small branches in place of stem bark and vice-versa after comparison and confirmation of the same for pharmacological activities.

Declarations

Author contribution statement

Ajay Kumar Meena: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
R. llavarasan; Ravindra Singh; N. Srikanth; K. S. Dhiman: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.
Vikas Ojha: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Ayyam Perumal: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest’s statement

The authors declare no conflict of interest.

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No additional information is available for this paper.