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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, LCMS, 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, leaf lets 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, laminate; 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,

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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 – *Tracheobinota*
Super Division - *Spermatophyta*
Division - *Mangoliophyta*
Class – *Magnoliopsida*
Sub Class - *Rosidae*
Order - *Fabales*
Family - *Fabacae*
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 fistulic acids, sennosides A, B, anthraquinones, flavanoid-3-olderivatives, ceryl alcohol, kaempferol, bianthraquinone glycosides, fistulin, essential oils, volatile components, phytol (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-dimethoxyflavone-3-O-α-arabinopyranoside,5,7,4′-trihydroxy-6,8,3′-trimethoxyflavone-3-O-α-L-rhamnosyl (1→2) -O-β-D- glucopyranoside and 1,8-dihydroxy-3, 7-dimethoxyxanthone-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).

A major anthraquinone derivative called rhein and 1,8-dihydroxy-3-anthraquinone found in fruit pulp of the plant. Fistucacidin, an optically inactive leucoanthocyanidin which is a phenolic compound from the heart wood of the plant. A bianthoquinone glycoside, fistulin together with kaempferol and rhein found in the flowers of the plant. A major carbohydrate called galactomannan consisting of 8 different type of sugar moieties are reported from the seeds of the plant. The anthraquinones like Rhein, Chrysophanol and Physcion, 9-(-)-epiafzelechin, 3-O-B-D-Glucopyranoside, 7 bioflavonoids and two triflavonoids together with (-) – epiafzelechin, (-)-epicatechin and procyanidin B-2 from the leaves of the plant. 3B-hydroxy-17-norpimar- 8(9)-en-15-one, 3-formyl-1-hydroxy-8-methoxy anthraquinone and fistulic acid from pods of the plant. The young and old leaves of the plant contain highest amount of phenolic, flavonoid and proanthocyanidin contents. Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is a lipophilic anthraquinone extensively found in medicinal plants (*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 1. Raw Plant material of *Cassia fistula* Linn.

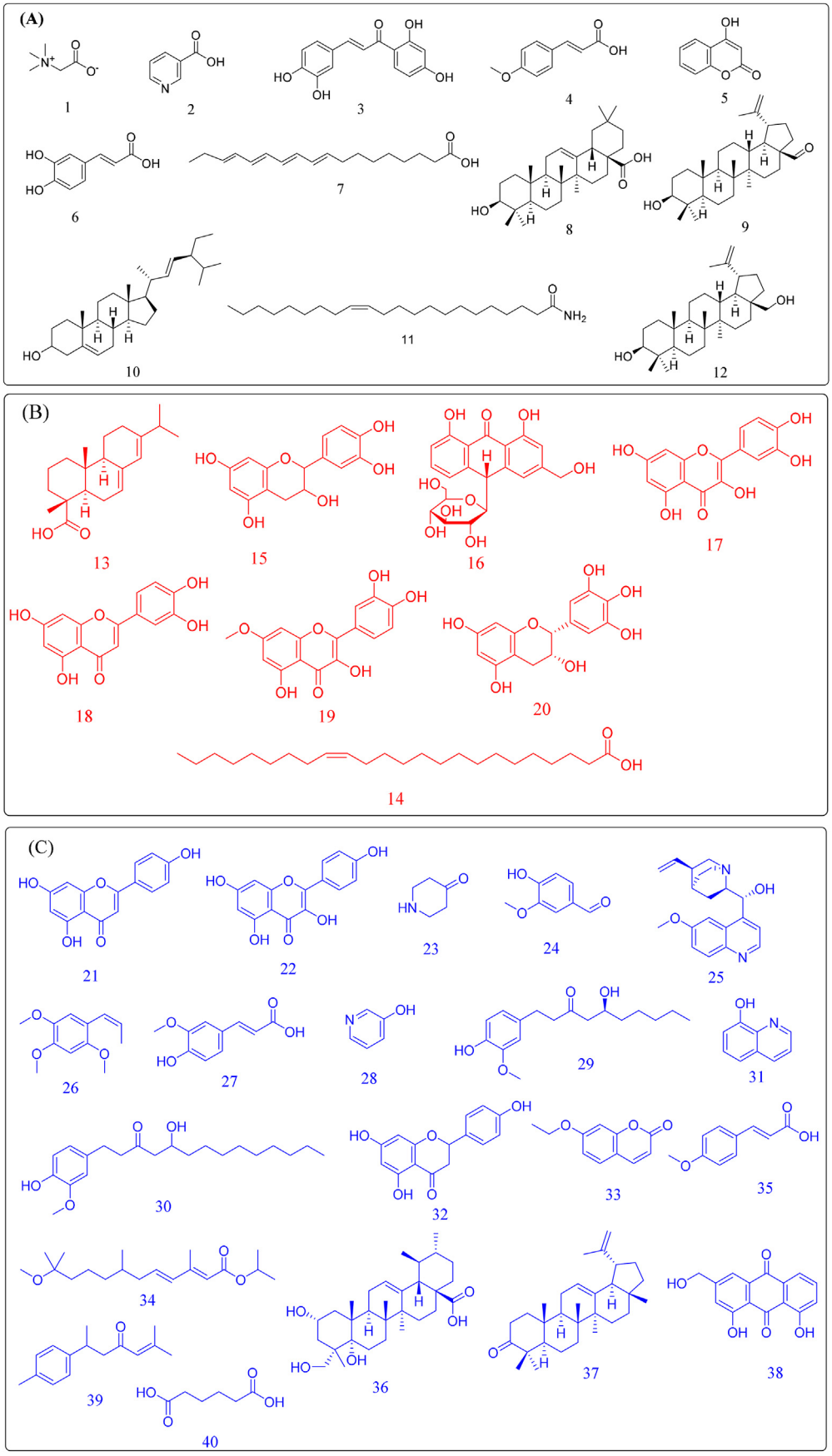


Figure 2. A) Chemical Structure of chemical constituents present in both stem bark and small branches of *Cassia fistula.* 1. Betaine, 2. Nicotinic acid, 3. Butein, 4. 4-Methoxycinnamic acid, 5. 4-Hydroxycoumarin, 6. Caffeic acid, 7. (E)-parinaric acid, 8. Oleanolic acid, 9. Lup-20(29)-en-28-al, 3beta-hydroxy, 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. Luteolin, 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.

Table 1. Molecular Docking Interaction of Abundant Medicinal Phytochemicals of *C. fistula* with the *L. donovani* Drug Target Proteins and Pancreatic lipase colipase.

S.No.	Proteins	Ligands	Binding energy kcal/mol	Interacting residues
1	Sterol 24-C-methyltransferase (PDB ID 5WP4)	S-Adenosyl-L-Homocysteine	10.1	Ala 106, Arg 66, Asn 126, Asp 82, Asp 107, Gly 61, Leu 128, Phe 83, Trp 16, Tyr 131, Val 108
		3	10.6	Arg 9, Asp 82, Leu 128, Phe 83, Val 87, Trp 16
		9	13.1	Asp 82, Met 28, Phe 83, Trp 16
		12	11.5	Ala 106, Gly 61, Leu 128, Met 28, Phe 83, Trp 16, Trp 127, Tyr 131, Val 108
		22	10.7	Arg 9, Asp 82, Leu 128, Phe 83, Trp 16, Trp 127
		32	11.2	Asp 82, Leu 128, Phe 83, Trp 16, Val 108
		37	11.4	Asp 82, Leu 128, Met 28, Phe 83, Trp 16
2	Nucleoside hydrolase (PDB ID 5TSQ)	β-D-ribofuranose	6.4	Asn 39, Asn 168, Asp 10, Asp 14, His 240, Tyr 225
		3	9.3	Ala 78, Ala 161, Arg 233, Asn 168, Asp 14, Asp 241, Gln 80, His 240, Met 152, Phe 167, Tyr 225
		9	16.8	Ala 78, Asp 10, Asp 14, Gln 80, His 82, His 240, Phe 167
		12	16.0	Ala 78, Gln 80, His 82, His 240, Ile 81, Phe 167, Tyr 225
		22	8.9	Ala 78, Ala 161, Arg 233, Asn 39, Asn 168, Ile 81, His 82, His240, Phe 167, Tyr 225
		32	8.5	Ala 78, Ala 161, Arg 233, Asn39, Asn 168, Ile 81, Phe 167, His 82, His 240, Tyr 225
		37	11.5	Ala 78, Gln 80, Asp 241, Arg 233, Glu 166
3	Pancreatic Lipase Colipase (PDB ID 1LPB)	Methoxyundecylphosphinic Acid	6.2	Ala 178, Arg 256, Asp 79, His 263, Phe 215, Phe 77, Ile 209, Pro 180, Tyr 114
		3	9.0	Asp 79, His 263, Ile 78, Phe 215, Pro 180, Ser 152, Tyr 114
		9	9.4	Phe 215
		12	8.9	Phe 215, Tyr 114
		22	9.4	Ala 260, Asp 79, Arg 256, Leu 264, His 263, Phe 77, Ser 152, Tyr 114
		32	9.5	Asp 79, His 151, His 263, Phe 215, Tyr 114
		37	16.8	Ile 209, Pro 180, Tyr 114

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 A°, 31.63 A° and 18.52 A° and radius was 5.0 A°. The X, Y and Z coordinates in the 5WP4 proteins were 10.23 A°, -1.58 A° and 33.49 A° and radius was 8.89 A°. The X, Y and Z coordinates in the 1LPB proteins were 9.82 A°, 23.49 A° and 50.87 A° and radius was 10 A°. 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, Erucamide, and (22E)-Stigmasta-5,22-dien3-ol were sketched by Chem draw 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 preliminary phytochemicals screening. The tests performed using alcoholic extract and aqueous extract to different types of qualitative test for the identification of phytoconstituents present in the stem bark and small branches of *Cassia fistula* [21, 22, 23].

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 µ membrane filters and used for HPLC profiling and compared under the same chromatographic conditions like Column type: ZORBAX Eclipse XBD- C_{18} (4.6 mm × 150 mm), 5µm particle size, Mobile Phase: Acetonitrile: Water (67:33), Acetonitrile: Phosphate Buffer (75:25), Acetonitrile: Phosphate Buffer (60:40), Acetonitrile: Phosphate Buffer (50:50), Acetonitrile: Water (60:40) used in the analysis of hexane, chloroform, ethyl acetate, ethanol, total ethanol extract, VWD Detector @ 254nm used in the detection of hexane, chloroform, ethyl acetate extract peak, DAD Detector @ 254nm used in the detection of ethanol extract and total ethanol extract peak, Flow Rate: 0.5 mL/min in the hexane and chloroform extract analysis and 1.0 mL/min in the ethyl acetate, ethanol and total ethanol extract analysis, Injection Volume: 10 µl in each analysis. For the LCMS analysis the test solution was prepared by dissolving

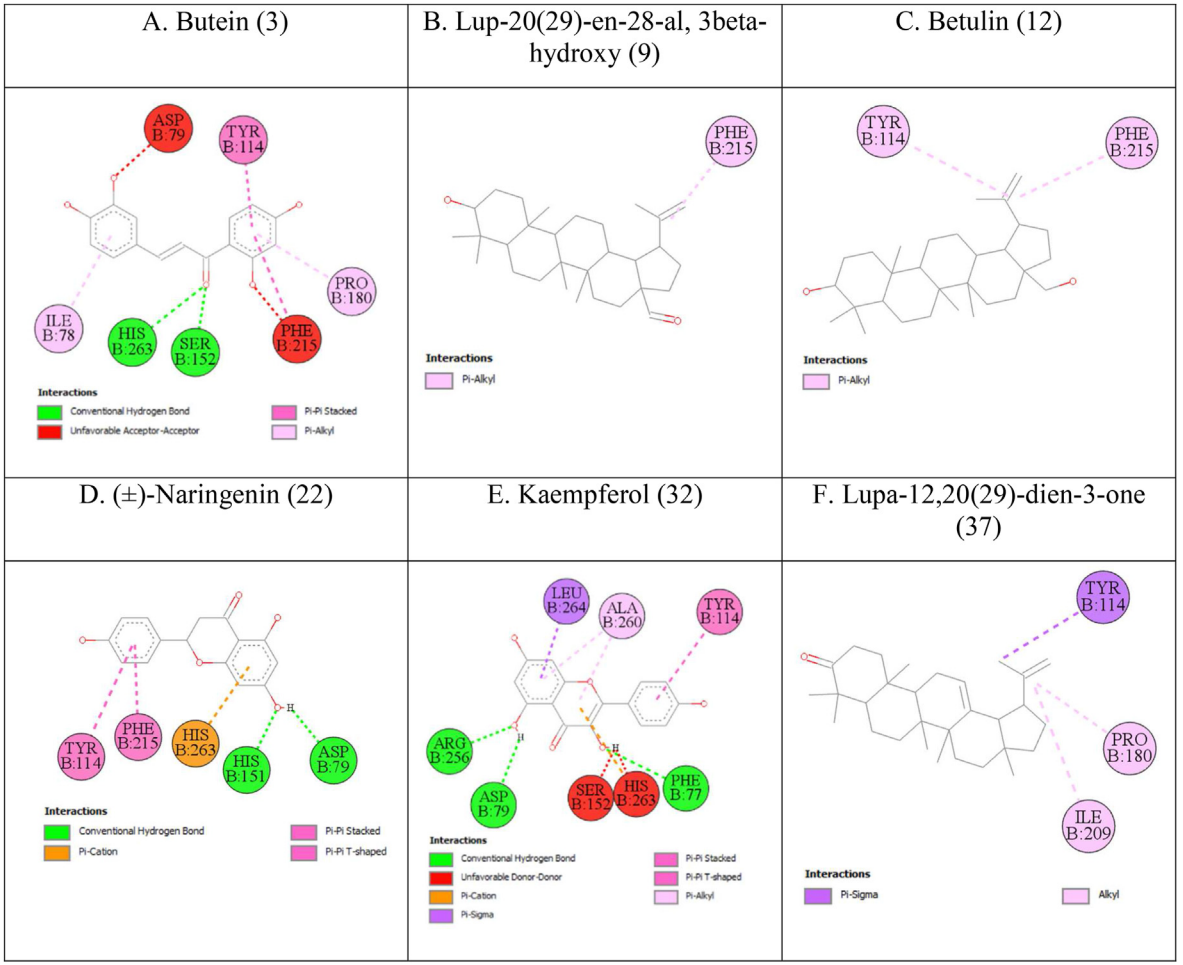


Figure 3. Binding pattern of *C. fistula* major chemical constituents with the Pancreatic lipase colipase. Two-dimensional (2D) and its significant interactions with (A) Butein, (B) Lup-20(29)-en-28-al, 3beta-hydroxy, (C) Betulin (D) (±) Naringenin, (E) Kaempferol, (F) Lupa-12,20(29)-dien-3-one.

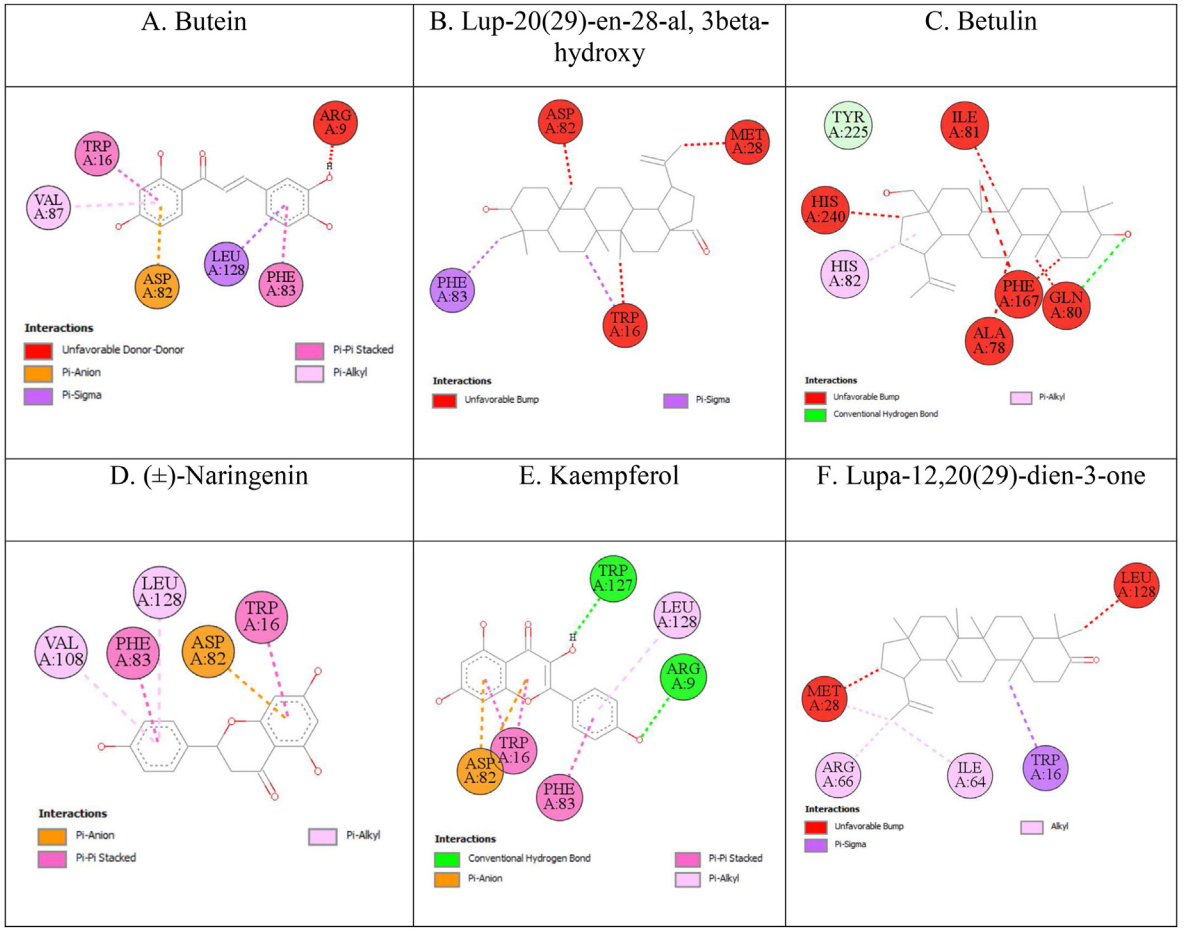


Figure 4. Binding pattern of *C. fistula* major chemical constituents with the Sterol 24-C-methyltransferase (SMT). Two-dimensional (2D) and its significant interactions with (A) Butein, (B) Lup-20(29)-en-28-al, 3beta-hydroxy, (C) Betulin (D) (±) Naringenin, (E) Kaempferol, (F) Lupa-12,20(29)-dien-3-one.

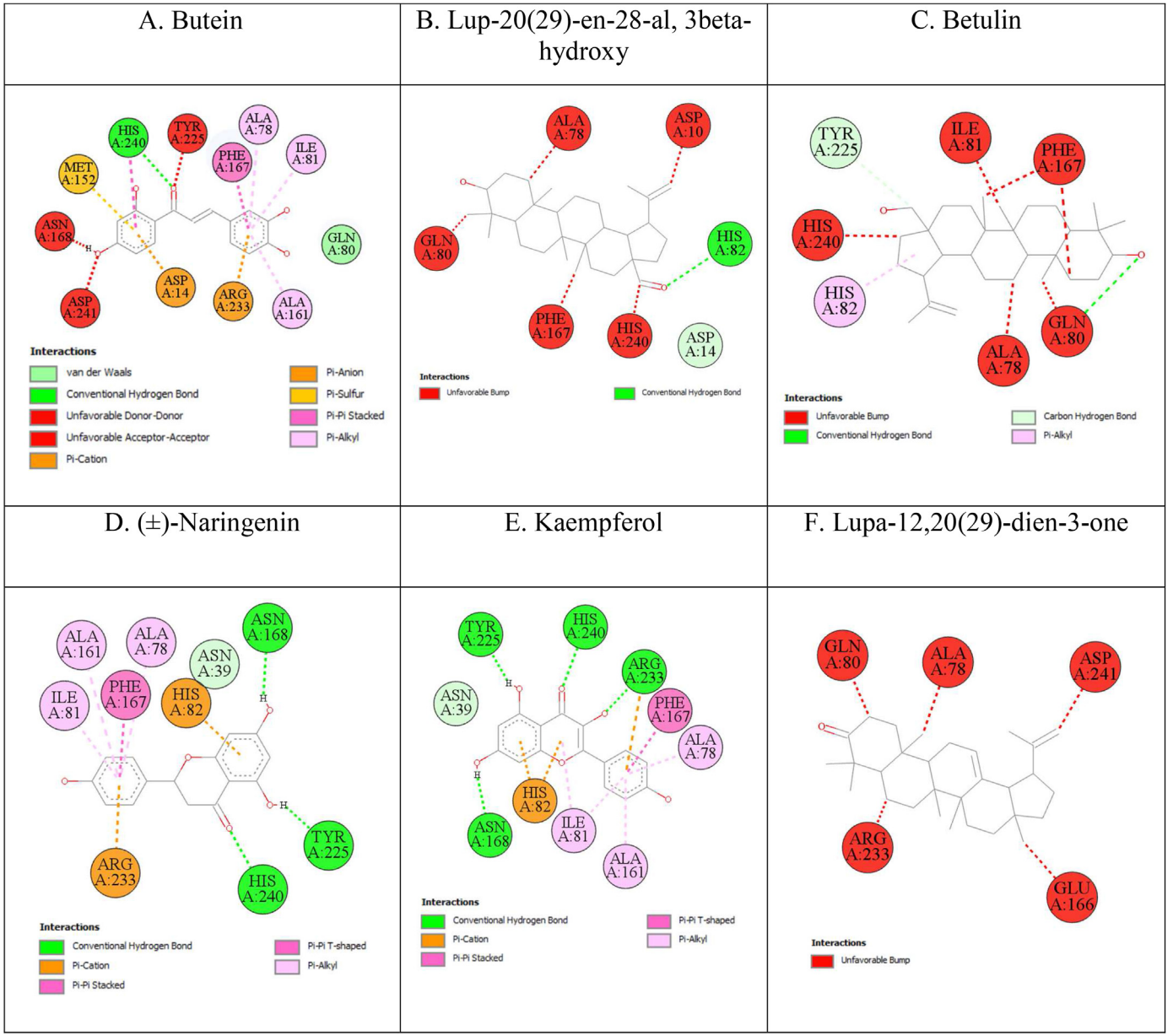


Figure 5. Binding pattern of *C. fistula* major chemical constituents with the Nucleoside hydrolase (NH). Two-dimensional (2D) and its significant interactions with (A) Butein, (B) Lup-20(29)-en-28-al, 3beta-hydroxy, (C) Betulin (D) (±) Naringenin, (E) Kaempferol, (F) Lupa-12,20(29)-dien-3-one.

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		Small branches	
		Aqueous extract	Alcohol extract	Aqueous extract	Alcohol extract
1.	Alkaloids	+	-	+	-
2.	Coumarins	-	++	-	+
3.	Flavonoids	+	+++	-	-
4.	Furanoids	+++	+++	-	+
5.	Phenols	++	+++	+	+
6.	Quinones	+++	+++	+	++
7.	Reducing sugars	++	+++	+	-
8.	Saponins	+++	-	+++	-
9.	Sugars (Carbohydrate)	-	+	+++	++
10.	Tannins	++	++	++	+
11.	Triterpenoids	-	+++	-	+++

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 Pharmacopeia of India (API) standards.

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 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

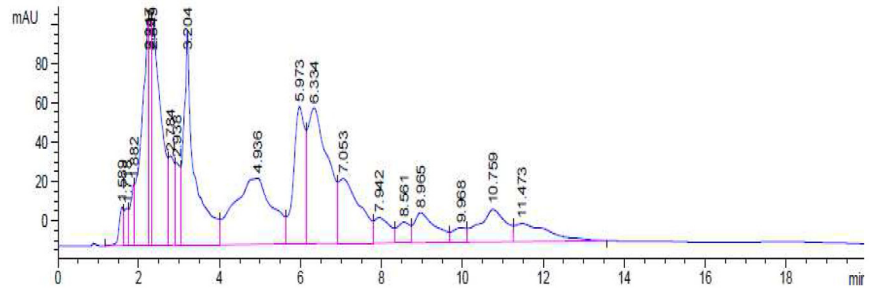
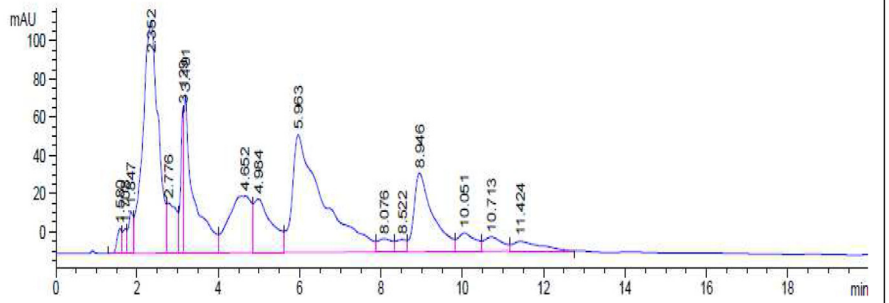
Chromatographic conditions	➢ Column type : ZORBAX Eclipse XBD- C_{18} (4.6 mm x 150 mm), 5µm particle size ➢ Mobile Phase : Acetonitrile: Water (67:33) ➢ Detection : VWD Detector @ 254nm ➢ Flow Rate : 0.5 mL/min ➢ Injection Volume : 10 µl
Stem bark	
Small branches	

Figure 6. HPLC profiling chromatogram of successive hexane extracts of *Cassia fistula* stem bark and small branches.

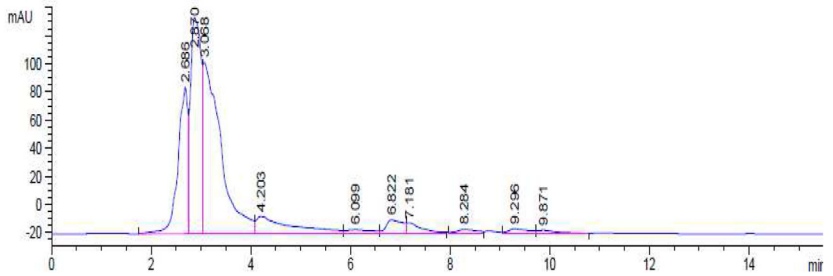
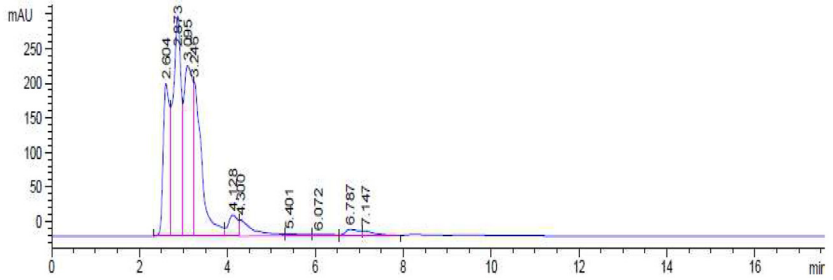
Chromatographic conditions	➢ Column type : ZORBAX Eclipse XBD- C_{18} (4.6 mm x 150 mm), 5µm particle size ➢ Mobile Phase : Acetonitrile: Phosphate Buffer (75:25) ➢ Detection : VWD Detector @ 254nm ➢ Flow Rate : 0.5 mL/min ➢ Injection Volume : 10 µl
Stem bark	
Small branches	

Figure 7. HPLC profiling chromatogram of successive chloroform extracts of *Cassia fistula* stem bark and small branches.

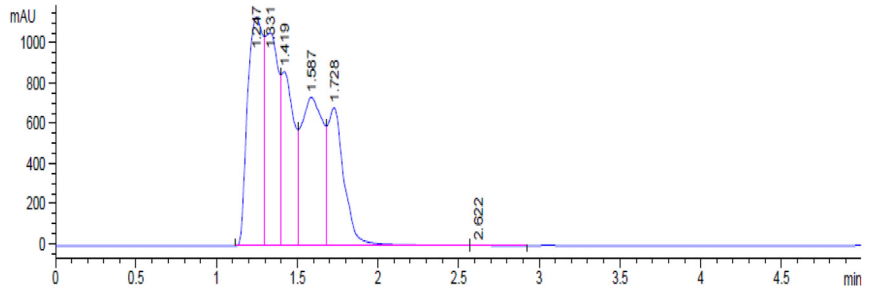
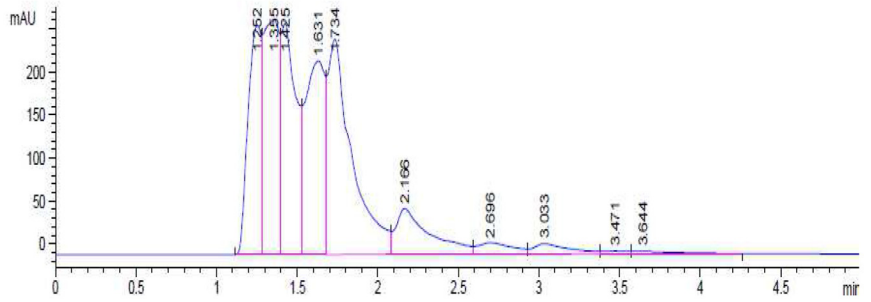
Chromatographic conditions	➢ Column type : ZORBAX Eclipse XBD- C_{18} (4.6 mm x 150 mm), 5µm particle size ➢ Mobile Phase : Acetonitrile: Phosphate Buffer (60:40) ➢ Detection : VWD Detector @ 254nm ➢ Flow Rate : 1.0 mL/min ➢ Injection Volume : 10 µl
Stem bark	
Small branches	

Figure 8. HPLC profiling chromatogram of successive ethyl acetate extracts of *Cassia fistula* stem bark and small branches.

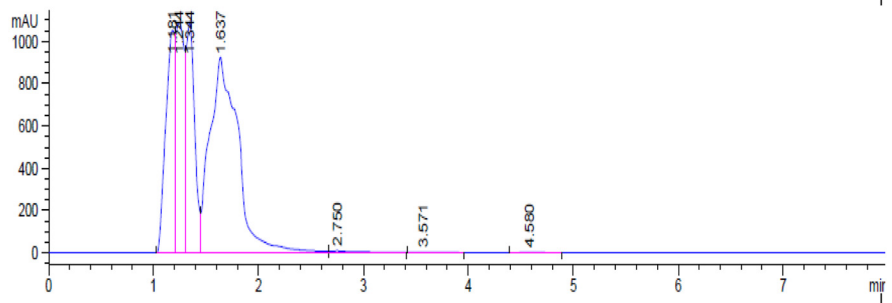
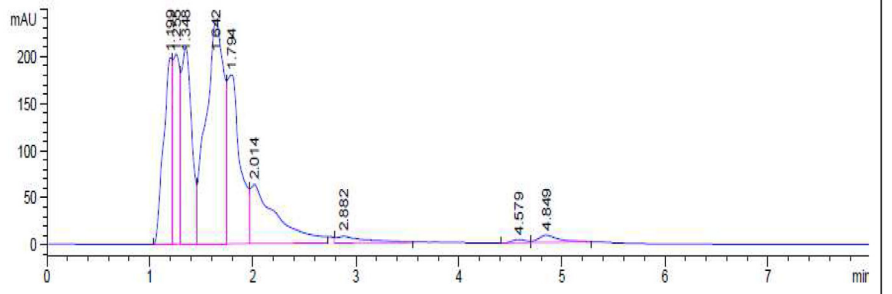
Chromatographic conditions	➢ Column type : ZORBAX Eclipse XBD- C_{18} (4.6 mm x 150 mm), 5µm particle size ➢ Mobile Phase : Acetonitrile: Phosphate Buffer (50:50) ➢ Detection : DAD Detector @ 254nm ➢ Flow Rate : 1.0 mL/min ➢ Injection Volume : 10 µl
Stem bark	
Small branches	

Figure 9. HPLC profiling chromatogram of successive ethanol extracts of *Cassia fistula* stem bark and small branches.

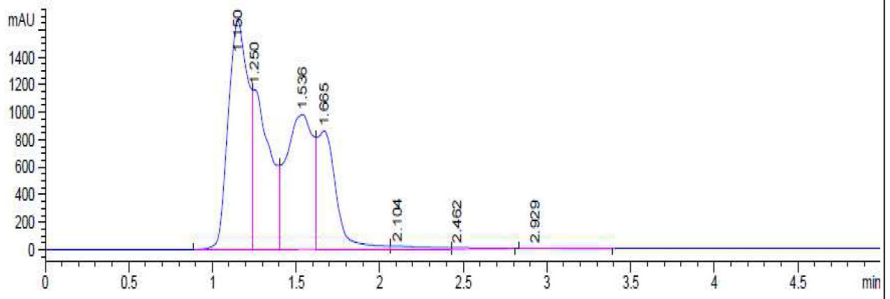
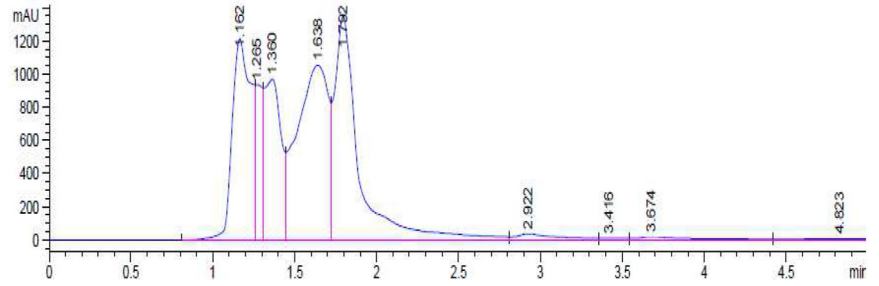
Chromatographic conditions	➢ Column type : ZORBAX Eclipse XBD- C_{18} (4.6 mm x 150 mm), 5µm particle size ➢ Mobile Phase : Acetonitrile: Water (60:40) ➢ Detection : DAD Detector @ 254nm ➢ Flow Rate : 1.0 mL/min ➢ Injection Volume : 10 µl
Stem bark	
Small branches	

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.

Stem bark				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	0.8313	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	-	-	-	-
5	2.291	420.46875	2.5022	-	-	-	-
6	2.349	1856.65857	11.0488	4	2.352	3193.80786	23.7569
7	2.784	457.82062	2.7244	5	2.776	420.07623	3.1247
8	2.938	333.25076	1.9831	-	-	-	-
-	-	-	-	6	3.129	406.59952	3.0245
9	3.204	2033.92200	12.1037	7	3.191	1360.34729	10.1188
-	-	-	-	8	4.652	1159.55225	8.6252
10	4.936	2209.57007	13.1489	9	4.984	863.45996	6.4228
11	5.973	1354.29077	8.0593	10	5.963	3289.10571	24.4657
12	6.334	2420.41626	14.4037	-	-	-	-
13	7.053	1200.93823	7.1467	-	-	-	-
14	7.942	340.91782	2.0288	11	8.076	167.28749	1.2444
15	8.561	227.86253	1.3560	12	8.522	115.64956	0.8602
16	8.965	558.20044	3.3218	13	8.946	1330.86389	9.8995
17	9.968	173.10760	1.0301	14	10.051	290.90503	2.1639
18	10.759	749.83698	4.4622	15	10.713	233.54915	1.7372
19	11.473	532.97852	3.1717	16	11.424	234.07089	1.7411
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.

Stem bark				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	3.246	2771.26514	19.8235
4	4.203	606.60406	7.5154	5	4.128	445.39230	3.1860
-	-	-	-	6	4.300	518.91888	3.7120
-	-	-	-	7	5.401	78.72145	0.5631
5	6.099	104.24109	1.2915	8	6.072	60.75570	0.4346
6	6.822	225.29892	2.7913	9	6.787	200.31689	1.4329
7	7.181	167.93848	2.0806	10	7.147	134.73520	0.9638
8	8.284	82.74257	1.0251	-	-	-	-
9	9.296	92.94195	1.1515	-	-	-	-
10	9.871	62.58374	0.7754	-	-	-	-
Total		**8071.51389**	**100.0000**	**Total**		**1.39797e4**	**100.0000**

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

Stem bark				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	16.5381
3	1.419	4751.75391	15.6618	3	1.425	1718.84949	15.9509
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	2.166	774.16815	7.1843
6	2.622	28.91096	0.0953	7	2.696	197.91785	1.8367
-	-	-	-	8	3.033	183.10434	1.6992
-	-	-	-	9	3.471	32.90925	0.3054
-	-	-	-	10	3.644	47.82133	0.4438
Total		**3.033974**	**100.0000**	**Total**		**1.077594**	**100.0000**

Table 8. HPLC peaks details of successive ethanol extracts of *Cassia fistula* stem bark and small branches.

Stem bark				Small branches			
Peak No.	Ret. Time [min]	Area [mAU*s]	Area %	Peak No.	Ret. Time [min]	Area [mAU*s]	Area %
1	1.181	6042.50293	16.8212	1	1.199	1168.21667	12.4974
2	1.244	6150.87988	17.1229	2	1.255	920.84351	9.8510
3	1.344	6271.53662	17.4587	3	1.348	1409.40515	15.0775
4	1.637	1.72378e4	47.9868	4	1.642	2877.07544	30.7784
-	-	-	-	5	1.794	1562.69836	16.7174
-	-	-	-	6	2.014	1131.03308	12.0996
5	2.750	163.38998	0.4548	7	2.882	136.38690	1.4590
6	3.571	28.72318	0.0800	-	-	-	-
7	4.580	27.19285	0.0757	8	4.579	31.10106	0.3327
-	-	-	-	9	4.849	110.95047	1.1869
Total		**3.592204**	**100.0000**	**Total**		**9347.71065**	**100.0000**

Table 9. HPLC peaks details of total ethanol extracts of *Cassia fistula* stem bark and small branches.

Stem bark				Small branches			
Peak No.	Ret. Time [min]	Area [mAU*s]	Area %	Peak No.	Ret. Time [min]	Area [mAU*s]	Area %
1	1.150	1.41661e4	34.8629	1	1.162	9786.41602	19.8888
2	1.250	8181.31152	20.1343	2	1.265	2775.98022	5.6416
-	-	-	-	3	1.360	6830.32959	13.8812
3	1.536	1.09232e4	26.8823	-	-	-	-
4	1.665	6782.29297	16.6913	4	1.638	1.38504e4	28.1480
-	-	-	-	5	1.792	1.41270e4	28.7100
5	2.104	345.49762	0.8503	-	-	-	-
6	2.462	151.43039	0.3727	-	-	-	-
7	2.929	83.77605	0.2062	6	2.922	741.33044	1.5066
-	-	-	-	7	3.416	149.57335	0.3040
-	-	-	-	8	3.674	637.74231	1.2961
-	-	-	-	9	4.823	306.90524	0.6237
Total		**4.063364**	**100.0000**	**Total**		**4.920564**	**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].

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 HPLC grade methanol using 5 ml volumetric standard flasks, filtered through 0.22 μ 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- C_{18} (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 obtained 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

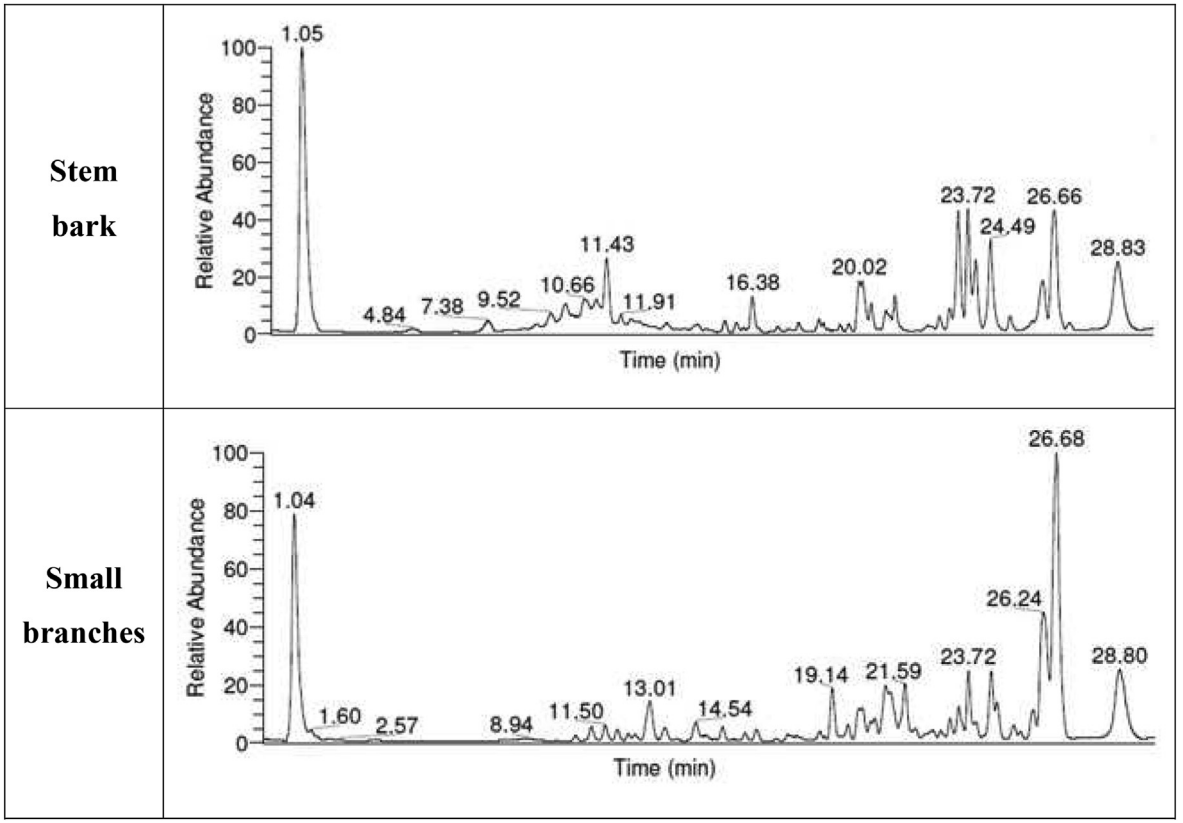


Figure 11. LC-MS Chromatogram of ethanol extracts of *Cassia fistula* stem bark and small branches.

Table 10. LC-MS Peak details of ethanol extracts of *Cassia fistula* stem bark and small branches.

Stem bark					
Peak No.	Ret. Time	Name of the compound	Molecular Formula	Molecular Weight	Area Maximum
1	1.072	Betaine	$C_5H_{11}NO_2$	117.0787	15260766.1
2	1.191	Nicotinic acid	$C_6H_5NO_2$	123.0318	393452.2136
3	2.509	(-)-Epigallocatechin	$C_{15}H_{14}O_7$	306.073	464997.9638
4	7.388	Epicatechin	$C_{15}H_{14}O_6$	290.0781	2571077.704
5	11.094	Butein	$C_{15}H_{12}O_5$	272.0676	411811.0544
6	13.95	4-Methoxycinnamic acid	$C_{10}H_{10}O_3$	178.0627	722746.2581
7	14.321	Aloin A	$C_{21}H_{22}O_9$	418.1255	969916.0451
8	14.432	Quercetin	$C_{15}H_{10}O_7$	302.0421	265656.2337
9	14.855	Luteolin	$C_{15}H_{10}O_6$	286.047	745514.2985
10	15.004	Rhamnetin	$C_{16}H_{12}O_7$	316.0576	299669.8381
11	20.072	4-Hydroxycoumarin	$C_9H_6O_3$	162.0313	860891.3235
12	20.11	Caffeic acid	$C_9H_8O_4$	180.0418	2391754.538
13	20.925	(E)-parinaric acid	$C_{18}H_{28}O_2$	276.2083	250512.54
14	21.633	Abietic acid	$C_{20}H_{30}O_2$	302.2238	194720.7413
15	22.935	Oleanolic acid	$C_{30}H_{48}O_3$	438.3488	231825.8614
16	23.856	Lup-20(29)-en-28-al, 3beta-hydroxy-	$C_{30}H_{48}O_2$	440.3647	194223.063
17	24.326	Nervonic acid	$C_{24}H_{46}O_2$	366.3489	155724.8206
18	24.488	Erucamide	$C_{22}H_{43}NO$	337.3335	15176586.26
19	26.046	Betulin	$C_{30}H_{50}O_2$	442.3803	399168.5256
20	26.662	(22E)-Stigmasta-5,22-dien3-ol	$C_{29}H_{48}O$	412.3692	26940935.26
Small branches					
Peak No.	**Ret. Time**	**Name of the compound**	**Molecular Formula**	**Molecular Weight**	**Area Maximum**
1	1.01	3-Hydroxypyridine	C_5H_5NO	95.03695	301739.2746
2	1.036	Betaine	$C_5H_{11}NO_2$	117.07878	72844002.46
3	1.19	Nicotinic acid	$C_6H_5NO_2$	123.03192	2574509.917
4	2.504	4-Piperidone	C_5H_9NO	99.06827	2786020.512
5	6.456	8-Hydroxyquinoline	C_9H_7NO	145.05255	845239.8029
6	7.575	Adipic acid	$C_6H_{10}O_4$	146.05771	673583.4342
7	9.276	Vanillin	$C_8H_8O_3$	152.04697	1048031.784
8	10.336	Quinine	$C_{20}H_{24}N_2O_2$	324.18294	401047.9444
9	10.473	β-Asarone	$C_{12}H_{16}O_3$	208.10939	740911.8038
10	10.893	(E)-Ferulic acid	$C_{10}H_{10}O_4$	194.0576	836774.0259
11	11.431	Butein	$C_{15}H_{12}O_5$	272.06804	660531.6233
12	12.455	(±)-Naringenin	$C_{15}H_{12}O_5$	272.06804	136131.3751
13	13.517	7-Ethoxycoumarin	$C_{11}H_{10}O_3$	190.06275	4987298.85
14	13.605	Apigenin	$C_{15}H_{10}O_5$	270.05223	266768.5365
15	13.945	4-Methoxycinnamic acid	$C_{10}H_{10}O_3$	178.06282	1101857.784
16	14.678	(E)-4-Methoxycinnamic acid	$C_{10}H_{10}O_3$	178.06282	106125.6885
17	15.588	Kaempferol	$C_{15}H_{10}O_6$	286.04717	320138.5085
18	16.847	Aloe-emodin	$C_{15}H_{10}O_5$	270.05223	181000.967
19	18.293	(+)-[6]-Gingerol	$C_{17}H_{26}O_4$	294.18236	274825.6524
20	19.738	(+)-ar-Turmerone	$C_{15}H_{20}O$	216.15097	168543.0028
21	19.947	6-Gingerol	$C_{17}H_{26}O_4$	294.18236	451219.7348
22	20.028	10-Gingerol	$C_{21}H_{34}O_4$	350.24452	212893.1035
23	20.152	Caffeic acid	$C_9H_8O_4$	180.04178	1885849.78
24	20.153	4-Hydroxycoumarin	$C_9H_6O_3$	162.03131	998683.3751
25	20.572	(E)-parinaric acid	$C_{18}H_{28}O_2$	276.20818	7882595.386
26	22.208	(+/-)-Methoprene	$C_{19}H_{34}O_3$	310.24982	3161195.753
27	23.708	Oleanolic acid	$C_{30}H_{48}O_3$	438.34877	1783708.336
28	23.812	Lup-20(29)-en-28-al, 3beta-hydroxy-	$C_{30}H_{48}O_2$	440.36464	802911.4302
29	23.833	Asiatic acid	$C_{30}H_{48}O_5$	488.34922	335493.9399
30	23.851	Lupa-12,20(29)-dien-3-one	$C_{30}H_{46}O$	422.35384	874681.9728
31	24.482	Erucamide	$C_{22}H_{43}NO$	337.33344	23537542.84
32	26.037	Betulin	$C_{30}H_{50}O_2$	442.37984	1024698.489
33	26.663	(22E)-Stigmasta-5,22-dien3-ol	$C_{29}H_{48}O$	412.36937	130035124.4

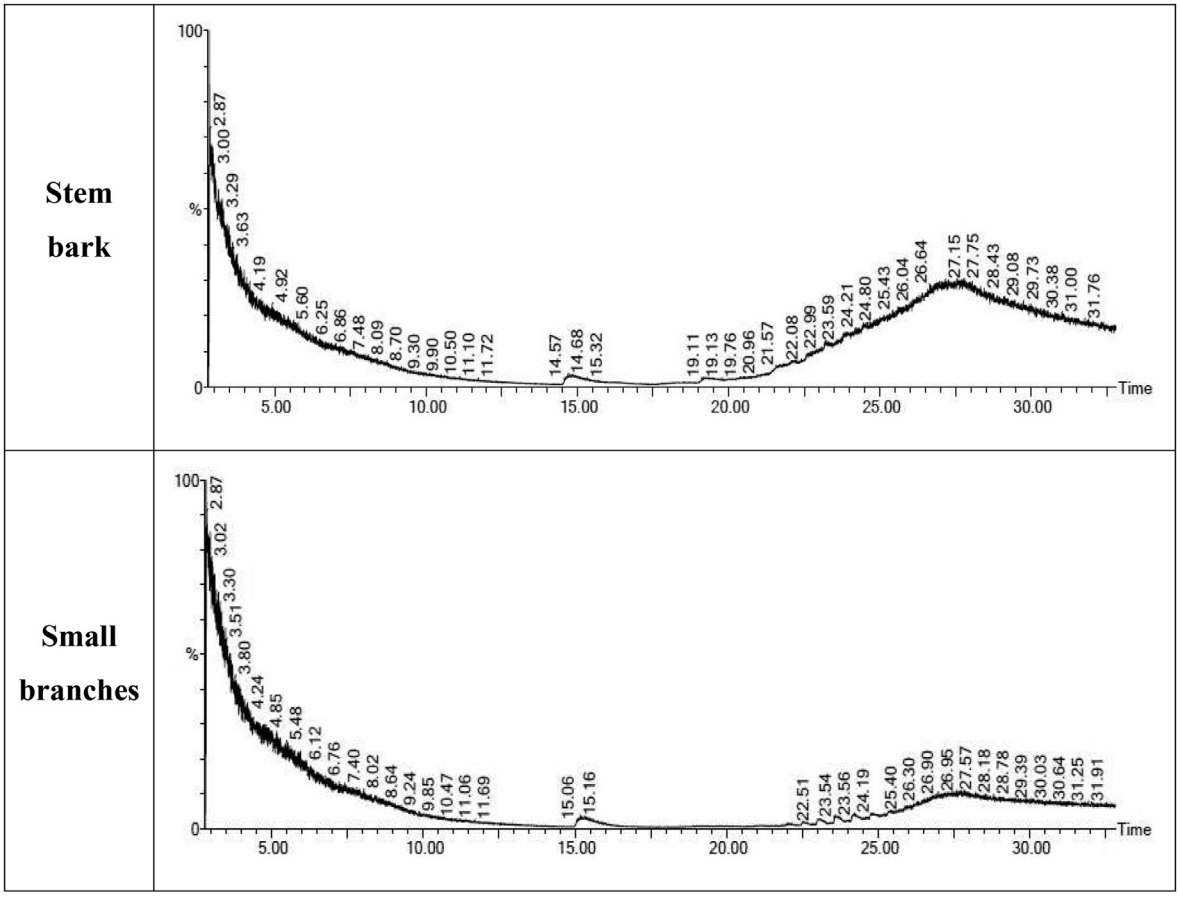


Figure 12. GC-MS Chromatogram of volatile content of *Cassia fistula* stem bark and small branches.

Table 11. GC-MS Peak details of volatile content of *Cassia fistula* stem bark and small branches.

Stem bark					
Peak No.	Ret. Time	Name of the compound	Molecular Formula	Molecular Weight	Area Maximum
1	14.683	Azulene	$C_{10}H_8$	128	1,084,225.0
2	14.753	Naphthalene	$C_{10}H_8$	128	521,754.2
3	14.853	Azulene	$C_{10}H_8$	128	738,852.2
4	14.913	1h-Indene, 1-Methylene	$C_{10}H_8$	128	688,024.4
5	26.393	2-Nitro-5-Aminobenzoic acid	$C_7H_6O_4N_2$	182	580,434.5
6	26.923	6-Methylquinolinic acid diamide	$C_8H_9O_2N_3$	179	260,495.9
7	26.968	Benzoic acid, 4-(acetylamino)-2-nitro	$C_9H_8O_5N_2$	224	304,433.7
Small branches					
Peak No.	**Ret. Time**	**Name of the compound**	**Molecular Formula**	**Molecular Weight**	**Area Maximum**
1	15.103	Naphthalene	$C_{10}H_8$	128	464,276.4
2	15.203	Naphthalene	$C_{10}H_8$	128	1,027,875.1
3	15.313	Naphthalene	$C_{10}H_8$	128	378,814.3
4	15.363	Azulene	$C_{10}H_8$	128	339,622.5
5	23.066	1-Butanol, 4-Butoxy	$C_8H_{18}O_2$	146	617,018.8
6	23.111	Sulfurous acid, 2-propyl tridecyl ester	$C_{16}H_{34}O_3S$	306	272,625.6
7	23.162	(2s,3s)-(-)-3-Propyloxiranemethanol	$C_6H_{12}O_2$	116	261,251.5
8	23.592	1-Butanol, 4-Butoxy	$C_8H_{18}O_2$	146	421,100.4
9	24.242	Di-N-Decylsulfone	$C_{20}H_{42}O_2S$	346	1,948,454.4

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.*

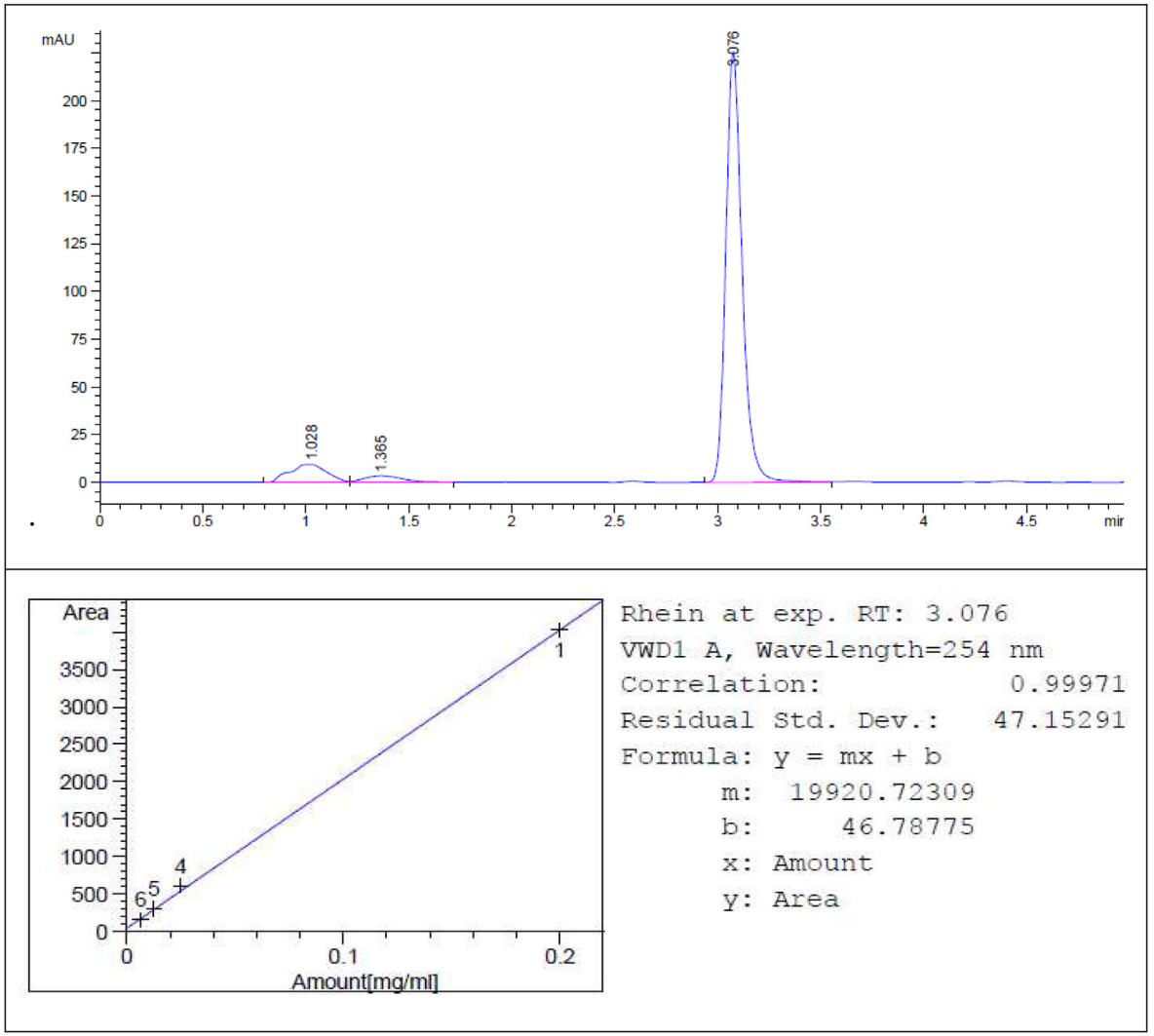


Figure 13. HPLC Chromatogram of Rhein Standard and Calibration curve.

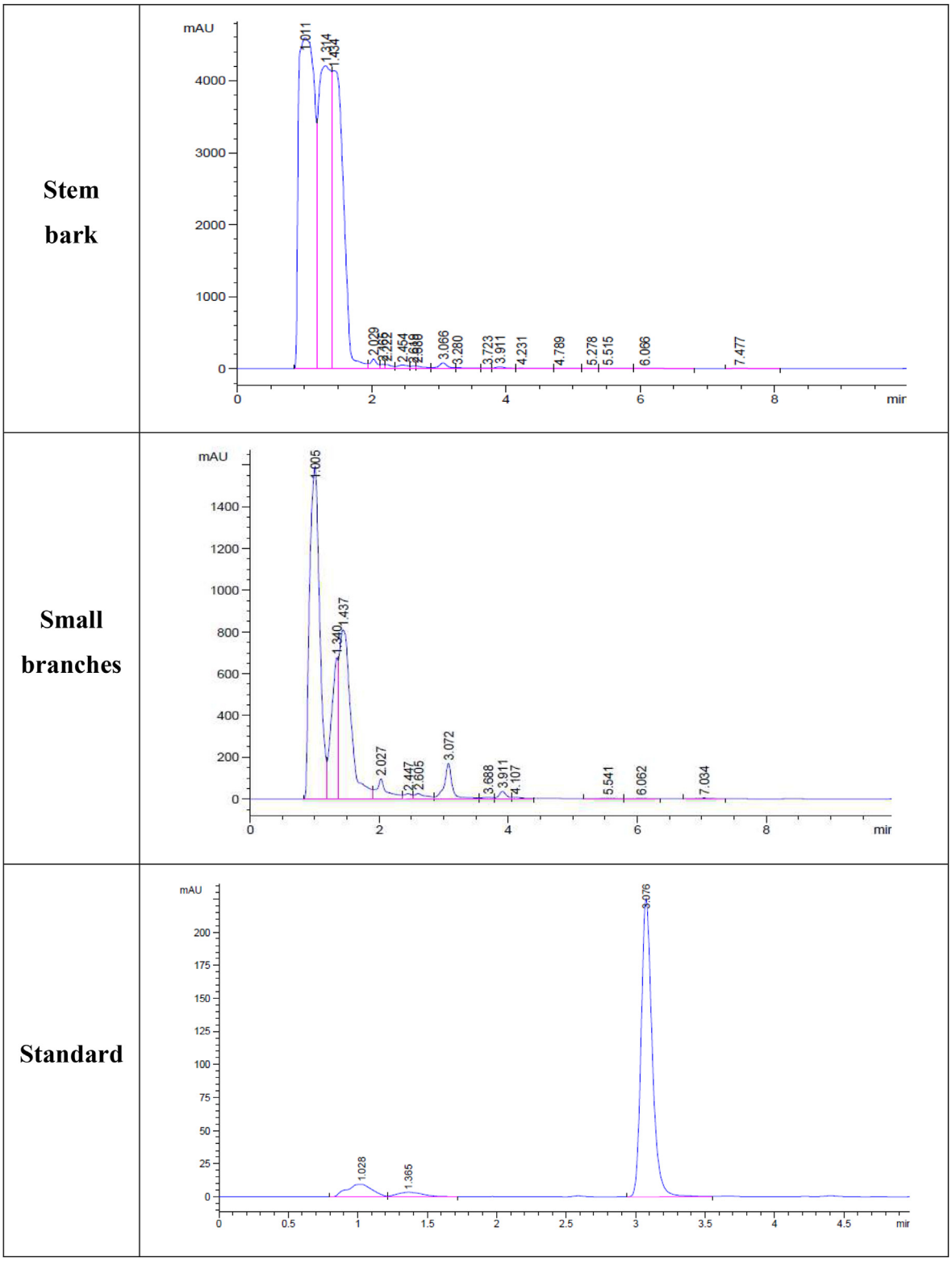


Figure 14. Estimation of Rhein in Ethanol extracts of *Cassia fistula* stem bark and small branches.

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	0.0257%
		0.0077		0.0259	
		0.0080		0.0261	

*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, 5TSQ 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. Ilavarasan; 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.

Additional information

No additional information is available for this paper.

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