Published online in http://ijam.co.in ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 13 (1), 41-50

Simultaneous estimation of epigallocatechin, fisetin, and quercetin in *Acacia suma* and its potential against postprandial hyperglycemia

Research Article

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Abstract

Anti-diabetic activity of the plants may follow one or the other mechanism such as their ability to restore the pancreatic tissue function or increase in insulin output or inhibiting the intestinal absorption of glucose or inhibiting the pancreatic enzymes like alpha-amylase, alpha-glucosidase, etc. The alpha-amylase and alpha-glucosidase inhibition assays were performed to analyze the anti-diabetic potential of *Acacia suma.* RP-HPLC method was developed and validated for simultaneous estimation of Epigallocatechin (EGC), Fisetin (FT), and Quercetin (QT) in hydroalcoholic extract (HAE) of *Acacia suma* and formulations. The simultaneous estimation was performed using the Luna C18 column with 20µl sample injection volume. Whereas, acetonitrile and phosphate buffer (0.1%, 30:70 v/v, pH 2.7) was used for resolution as mobile phase with column temperature 40 ^{o}C and flow rate 1 ml/min. The IC_{50} for alpha-amylase inhibition and alpha-glucosidase inhibition activity was found to be 12.5µg/ml and 93.79µg/ml respectively was compared with standards. The developed method was found simple, specific, precise and linear with regression coefficient (R^2 = 0.999) over the selected range of concentration (2.5-160 µg/mL) having detection limits 0.076, 0.012, 0.010 µg/mL and quantification limits 0.231, 0.038, 0.031 µg/mL for EGC, FT and QT respectively. In-vitro alpha-amylase and alpha-glucosidase inhibition assay support the traditional claim of the plant in the treatment of metabolic disorders. The established RP-HPLC method demonstrates the precise and easy determination of EGC, FT, and QT and is effectively studied by a gradient elution system.

Key Words: *Acacia suma,* Anti-diabetic, Epigallocatechin, Fisetin, RP-HPLC, Quercetin.

Introduction

Diabetic Mellitus is rapidly increasing worldwide. The deficiency of insulin leads to people suffering from diabetes and causes an elevated level of glucose in the blood. Anti-diabetic activity of the plants may follow one or the other mechanism such as their ability to restore the pancreatic tissue function or increase in insulin output or inhibiting the intestinal absorption of glucose or inhibiting the pancreatic enzymes like alpha-amylase, alpha-glucosidase, etc. (1).

The polyphenolic compounds and their estimation can be helpful in the exploration of the pharmacological effects of medicinal plants. Phenols are the major group of nutritional compounds of plants and are classified as secondary metabolites such as tannins and flavonoids. Compounds like catechins, resveratrol, quercetin, procyanidins, and anthocyanins are taken into consideration by researchers for investigating their potentials against inflammation, metabolic disorder, viruses, cancer, and other ailments (2, 3). The characterization of medicinal plants and derived medicines utilizing marker compounds or biochemical profiling of bioactive has become essential as some of which displays significant pharmacological actions. This phenomenon has directed to a faster and improved evaluation of the quality of plant products (4). One of the tools playing an essential part towards characterizing the plant material quality used in medicine is the simultaneous determination of marker compounds using HPLC.

Accurate identification and analyte quantification are particularly linked to the separation of the analyte. Because of this phenomenon, the RP-HPLC method is previously recognized to separate and measure the active constituents from complex matrices such as food products, beverages, plant extracts, and its formulations (5, 6). To ensure the presence of polyphenols in *Acacia suma* and Ayurvedic formulations, we aim to develop the RP-HPLC method for simultaneous determination of Epigallocatechin (EGC), Fisetin (FT), and Quercetin (QT).

Genus *Acacia* is known for the most prominent source of polyphenols, belongs to the family Leguminosae (Fabaceae) and approximately 800 species are documented globally (7). The heartwood of *Acacia suma* (Roxb) Buch.-Ham., commonly known as

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'*Shwet khadir*' or '*White catechu*' has a broad range of significance in the treatment of obesity and diabetics (8). Previously we have reported anti-lipase activity for *Acacia suma* where EGC, FT, and QT were predicted for significant anti-lipase activity with IC_{50} 46.07 µg/ml (9).

Some of the studies have been previously established for simultaneous estimation of polyphenols. A study by Fernando CD *et al*. on tea polyphenols - gallic acid, caffeine, epicatechin, and epigallocatechin gallate (EGCG) were simultaneously estimated utilizing an isocratic elution system and suggested that EGCG with more retention time i.e. 15.49 min (10). Another study by Zuo Y *et al*. has described simultaneous estimation of catechins, caffeine, and gallic acids in four tea verities and suggested 10 min retention time for epigallocatechin at 280 nm (11). Furthermore, a study by Dimcheva V. *et al.* indicated the simultaneous determination of nine polyphenols: (Epicatechin gallate, catechin, epigallocatechin, rutin, quercetin, myricetin, kaempferol, gallic acid, and vanillic acid); among them, EGC and QT displayed 17 and 23 min retention time respectively (12) even though there are many studies on simultaneous estimation of polyphenols (13) none has been performed for isosbestic point-based simultaneous estimation of EGC, FT and QT in *Acacia suma* and Ayurvedic drugs.

Therefore, we aim to investigate the anti-diabetic potential of hydroalcoholic extract of *Acacia suma* and standardization of plant by isosbestic point-based RP-HPLC method. The developed method was validated with system suitability, intraday and interday precision, accuracy, repeatability, linearity, recovery, and robustness/ruggedness parameters. In addition, a forced degradation assay was performed to confirm the suitability of the developed method. Furthermore, the applicability of the developed method was analyzed in Ayurvedic drugs.

Material and Methods

α- amylase inhibition assay

Phosphate buffer solution (1ml) was mixed with 0.5 ml of different concentrations (12.5, 25, 50, 100 & 200 µg/ml) of samples with the standard solution and 200µl of 0.5mg/ml α-amylase was added followed by 200µl of 5mg/ml starch solution and incubated for 10 minutes at room temperature. Starch was taken as control. Then the reaction was stopped by adding 400µl of DNS solution boiled for 5 min and cooled. The absorbance was measured at 540 nm. The percent of enzyme inhibition was calculated using the following formula:

$$\% \text{ of } \alpha\text{-amylase inhibition} = [(Ac - As)/Ac] \times 100$$

Where, Ac and As is absorbance of control and sample, respectively. Metformin was used as standard (14).

α- glucosidase inhibition assay

The α-glucosidase inhibition activity was performed as described by Li et al., 2005. 3 mM p-nitrophenyl α-D-glucopyranoside (PNPG) was used as substrate and prepared by dissolving in 50 mM phosphate buffer (pH 6.5). Samples were prepared at 50, 100, 150, 200 and 250 µg/ml concentration in 5% DMSO. 30 µL of each concentration was added with 36 µL phosphate buffer pH 6.8 and 17 µL p-nitrophenyl-α-D-glucopyranose (5 mM). The mixture was incubated for 5 min at 37°C. To this solution, 17 µL of α-glucosidase 0.15 unit/mL was added again incubated for 15 min at 37°C. Once the second incubation was finished, 100 µL of Na_2CO_3 (267 mM) was added to the solution to stop the enzymatic reaction. The absorbance was measured at 405 nm. Acarbose was tested as a standard drug. The concentration of samples that inhibited α-glucosidase activity by 50% was defined as the IC_{50} value. The percentage inhibition was calculated by following the formula (15).

Inhibition Percentage =
OD of Blank-OD of Sample/OD of Blank X 100.

RP-HPLC Method development and validation

Reagents and solvents

EGC was procured from Yarrow Pharma, Mumbai, India. FT was purchased from Sigma-Aldrich; and QT was obtained from ACTIN Pharmaceuticals, China. HPLC-grade acetonitrile (ACN) and orthophosphoric acid (OPA) were purchased from Fisher Scientific Mumbai, India. HPLC analytical-grade water (Merck, Mumbai, India) was used. All the other solvents or reagents used were of pharmaceutical or analytical grade. Ayurvedic marketed formulations were procured from KLE's BMK Ayurveda Pharmacy, Belagavi, India.

RP-HPLC instrument and conditions

Shimadzu HPLC prominence instrument system (LC-20AD, Japan) equipped with SPD-M20A prominence diode array detector (PDA), LC- 20AD pump, a SIL-20AC HT auto sampler, DGU-20A5 online degasser, rheodyne injection value with 20 µL loop, and CTO-10AS VP column oven. The data interpretation and analysis were done using the software Shimadzu LC solution (version 1.25). Chromatographic separation and analysis were carried out using Phenomenex Luna C18 analytical column C-18(2) 100 A (250×4.60 mm internal diameter, 5 µm particle size, Phenomenex Inc., Canada, USA) at a column temperature of 40 °C. The optimized mobile phase ACN: Phosphate buffer (0.1% OPA) 30:70 v/v ratio was adjusted to pH 2.7 with sodium hydroxide and pumped through the column at a flow rate of 1 mL/min. Before use, the mobile phase was filtered through PVDF filter membrane (0.45 µm; Millex HV®, Millipore, USA) and ultrasonically degassed. The injection volume was 20 µL for the sample analysis.

Preparation of standards

Stock solutions of EGC, FT, and QT were individually prepared at a concentration of 1 mg/mL in methanol. The standard preparation of EGC, FT, and QT were prepared in the concentration range of 2.5-160 µg/mL, by the dilution of stock solution using the HPLC grade water. All standard preparations were stored in the amber-colour and tightly-stopper volumetric flask at 4 °C, before the analysis.

Preparations of hydroalcoholic extract (HAE)

The plant material was made in the coarse powder form [100 g] for the preparation of hydroalcoholic extract using ethanol: water [70:30 v/v] by cold maceration method. Further, the liquid extract was filtered and concentrated under a vacuum at 45ºC using a rotary evaporator. The extract was lyophilized to obtain a fine powder of crude extract.

Preparation of test sample

Hydroalcoholic extract of *Acacia suma* (10 mg) was transferred in 10 ml of distilled water and sonicated for five min to develop better dissolution.

Khadiradivati (100 mg) and Khadirarista (1 ml) were dissolved in 10 ml of distilled water followed by filtration through filter paper to avoid un-dissolved particles and re-filtered through syringe filters before sample injection. 20 µl of both the samples were injected for better peak resolutions.

Method development

The development of the method for simultaneous analysis of EGC, FT, and QT was done by using different mobile phase ratios, concentration and pH values of OPA, flow rates, and column oven temperature (16, 17).

Method validation

The specificity of the method was validated according to ICH guidelines to ensure that the developed method is acceptable and reliable for the proposed work. The method was validated with different validation parameters such as system suitability, linearity, accuracy, precision, LOD, LOQ, robustness, and ruggedness (18, 19).

System suitability

The developed method ensured the resolution and reproducibility by system suitability study. The percent RSD of different parameters such as peak area, retention time (tR), theoretical plates, and tailing factor were determined. The percent RSD of peak area (<2), tailing factor (<2), and theoretical plates (N>2000) were within the acceptable limits. The peaks obtained for EGC, FT, and QT were found to be sharp, well separated, and with high resolution.

Linearity

The linearity of the method was checked with EGC, FT, and QT standards with the calibrated curve of seven different concentrations in six replicate with the range of concentrations 2.5-160 µg/mL. The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the standard deviation and slope of the calibrated curve using the formula 3.3*σ/s and 10*σ/s respectively. σ - standard deviation of intercept and s - slope.

Accuracy

Preanalysed standards EGC, FT, and QT were spiked with a low, medium, and high concentration in the calibration range. The assay was repeated three times to check the recovery of the injected standards.

Precision

The measures of repeatability of each analytical standard were validated by intraday and interday precision. Three concentrations of each standard were verified by three times on the same day (Intraday) and the three different days (Interday).

Robustness and Ruggedness

The susceptibility of the optimized method was determined with the small changes in the mobile phase composition, temperature, flow rate, pH, and concentration of OPA (20, 21). At each condition, the mixture of standard EGC, FT, and QT was administered to the chromatographic system in triplicates, and % RSD of the peak area was calculated for each standard. Further, ruggedness was measured for intermediate precision using pre-determined conditions by considering similar measurement procedures, same optimized system, on the same location, and replicate measurements on the same objects over a prolonged period by preparing three individual samples.

Forced degradation study

A forced degradation study was performed using various stress conditions. Each standard was employed for acidic, alkaline, temperature, oxidative, and photolytic degradation states in individual and mixture form. Hydrolytic degradation was performed by using 1 N HCl and 1 N NaOH, oxidative degradation was done by 30% hydrogen peroxide, 80ºC temperature was maintained for thermal degradation and photolytic degradation was by exposing the samples to sunlight for 1 hr. The 1 ml of the final sample was prepared using mobile phase and 20 µl of injection was run through a chromatographic system (22).

Results

α-amylase inhibition assay

The IC_{50} values of the *Acacia suma* for alpha-amylase activity were found to be 12.5µg/ml and for standard alpha-amylase inhibition i.e. Metformin was 52.09µg/ml depicted in (Fig.1.A).

α-glucosidase inhibition assay

The IC_{50} values of the *Acacia suma* for alpha-glucosidase activity were found as 93.79µg/ml and for standard alpha-glucosidase inhibition i.e. Acarbose was 79.74µg/ml depicted in (Fig.1.B).

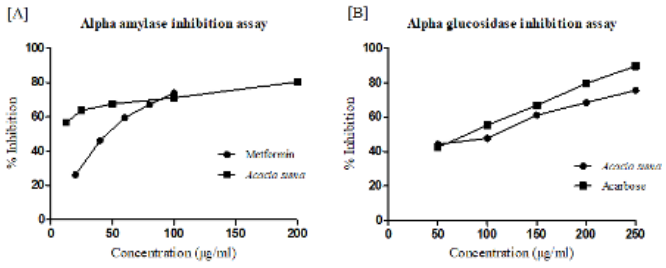


Figure 1: Enzyme inhibition assays for *Acacia suma* extract [A] alpha-amylase inhibition [B] Alpha-glucosidase inhibition.

Method development and optimization

The method optimization was initiated with various mobile phase systems with their different compositions. The stock solution for each standard compound was prepared in methanol (1 mg/ml) and further dilutions were prepared using the mobile phase. Good selectivity was achieved by modification in mobile phase composition with different solvents; column temperature was set at 40^{0}C and 45^{0}C, flow rate as 1 ml/min and 0.9 ml/min, and pH of orthophosphoric acid at 2.7 and 6 was tested. Finally, the composition of ACN and OPA (0.1%) in the ratio 30:70 was fixed with pH 2.7 and temperature 40^{0}C to achieve better resolution.

Method validation

The developed method was validated to ensure the acceptability and reliability of the developed RP-HPLC method. System suitability study, linearity, accuracy, precision, LOD, LOQ, robustness, and ruggedness are the parameters to validate the RP-HPLC method as per ICH guidelines.

System suitability

To assure the specificity of the optimized method system suitability was performed. The resolution and reproducibility of the HPLC technique, 50 µg/mL mixture concentrations of EGC, FT, and QT were injected, % RSD was evaluated by considering peak area, retention time, theoretical plates, and tailing factors. The peak obtained for EGC, FT and QT were sharp; the number of theoretical plates is >2000, tailing factor is <2. The % RSD of each parameter was found <2. The system suitability of each analyte is represented in (Table 1).

Table 1: System suitability

Parameters	EGC		FT		QT	
	Mean ± SD	% RSD	Mean ± SD	% RSD	Mean ± SD	% RSD
tR (min.)	3.51 ± 0.002	0.04	6.98 ± 0.001	0.06	12.1 ± 0.03	0.24
Peak area	195410 ± 3020.6	1.55	4935729 ± 39946	0.81	3179821 ± 7201	0.23
Plate count	4531 ± 60.5	1.33	7620 ± 78.4	1.03	8293.5 ± 79.01	0.95
Tailing factor	1.33 ± 0.01	0.79	1.31 ± 0.00	0.18	1.3 ± 0	0.15

SD: Standard deviation, tR: Retention time, RSD: relative standard deviation

Linearity

The targeted standards were found linear in the concentration range 2.5-160 µg/mL. Calibration curves were calculated for slope/intercept and correlation coefficient (R^2), represented in (Table 2). Further, the LOD of the method was evaluated considering the analyte concentration having *S/N* value 3; the LOQ represents the analyte concentration having *S/N* value 10. The LOD and LOQ values of targeted analytes are illustrated (Table 2).

Table 2: Linear regression data and sensitivity parameters of the RP-HPLC method

Standard compounds	Concentration Range (µg/mL)	Slope	Intercept	R^2	LOD (µg/mL)	LOQ (µg/mL)
EGC	2.5-160	6802	35670	0.999	0.076	0.231
FT	2.5-160	143642	276370	0.999	0.012	0.038
QT	2.5-160	90074	158303	0.999	0.010	0.031
At Isosbestic Point 287 nm						
EGC	2.5-160	1605	28455	0.999	0.054	0.162
FT	2.5-160	38239	54749	0.999	0.008	0.025
QT	2.5-160	29016	36200	0.999	0.007	0.021

R^2: Regression coefficient, LOD: limit of detection, LOQ: limit of quantification

Precision

The precision of the optimized chromatographic system for the selected standard was studied by the mean of intraday and interday multiple injections of sample mixture than checking the % RSD value obtained from found concentration is listed in the (Table 3).

Table 3: Precision intraday and interday

Standards	Active content (µg/mL)	Intraday (n=3)		Interday (n=3)					
				1st day		2nd day		3rd day	
		Found ± SD (µg/mL)	% RSD	Found ± SD (µg/mL)	% RSD	Found ± SD (µg/mL)	% RSD	Found ± SD (µg/mL)	% RSD
EGC	5	4.97 ± 0.02	0.48	4.98 ± 0.09	1.82	4.95 ± 0.01	0.20	4.89 ± 0.08	1.72
	10	10.06 ± 0.11	1.06	10.15 ± 0.10	0.97	9.75 ± 0.10	0.99	9.86 ± 0.11	1.07
	20	20.10 ± 0.18	0.91	20.03 ± 0.02	0.10	20.05 ± 0.24	0.69	20.08 ± 0.12	0.58
FT	5	4.93 ± 0.01	0.27	5.06 ± 0.07	1.43	5.03 ± 0.02	0.3	4.99 ± 0.04	0.88
	10	10.04 ± 0.06	0.60	9.83 ± 0.11	1.12	9.69 ± 0.18	1.83	10.01 ± 0.19	1.93
	20	20.06 ± 0.06	0.31	19.94 ± 0.04	0.19	19.96 ± 0.05	0.23	19.90 ± 0.07	0.35
QT	5	5.02 ± 0.10	1.90	4.53 ± 0.02	0.51	4.54 ± 0.02	0.44	4.57 ± 0.40	0.88
	10	10.07 ± 0.03	0.58	10.18 ± 0.11	1.12	10.32 ± 0.10	0.97	10.26 ± 0.14	1.33
	20	20.12 ± 0.13	0.63	19.76 ± 0.08	0.38	19.93 ± 0.16	0.79	19.89 ± 0.10	0.51

RSD: relative standard deviation, SD: Standard deviation

Accuracy

The recovery study was performed to check the accuracy of the optimized method for all three standards in triplicates. The known quantity of each standard was spiked with three concentrations of the standard compound of the linearity range and the percentage of recovered quantity was calculated. The recovery values obtained were close to 98.20 - 102.8 % for all three standards presented in (Table 4). Therefore, the developed RP-HPLC method can be considered accurate.

Table 4: Recovery studies

Standards	Active content (µg/mL)	Level (%)	Spiked quantity (µg/mL)	Recovered quantity (µg/mL)	Recovery (%)	RSD (%)
EGC	10	50	6.23 ± 0.08	6.12 ± 0.10	98.20	0.67
	10	100	8.53 ± 0.12	8.51 ± 0.06	99.80	1.19
	10	150	11.25 ± 0.08	11.10 ± 0.02	98.40	0.82
FT	10	50	7.40 ± 0.09	7.55 ± 0.01	101.75	0.54
	10	100	9.52 ± 0.10	9.85 ± 0.00	102.8	1.05
	10	150	12.37 ± 0.06	12.63 ± 0.06	101.81	0.92
QT	10	50	7.40 ± 0.04	7.52 ± 0.06	101.72	1.19
	10	100	9.67 ± 0.01	9.65 ± 0.05	99.72	0.43
	10	150	12.40 ± 0.02	12.54 ± 0.10	101.13	0.69

Experiments were performed in triplicates (n=3), % RSD: Percent relative standard deviation

Robustness and ruggedness

The susceptibility and intermediate precision were evaluated by robustness and ruggedness assay. Minor modifications were made in optimized HPLC parameters and influences of those changes in the developed method are described (Table 5). Hence, the developed RP-HPLC method confirms the robustness of the developed method for EGC, FT, and QT.

Table 5: Robustness and ruggedness

Parameters	Changes made	Epigallocatechin		Fisetin		Quercetin	
		Retention time ± SD	RSD (%)	Retention time ± SD	RSD (%)	Retention time ± SD	RSD (%)
Mobile phase Composition [ACN:OPA 0.01%]	30:70	3.35 ± 0.029	0.86	6.98 ± 0.009	0.14	12.05 ± 0.02	0.17
	32:68	3.31 ± 0.008	0.23	6.96 ± 0.020	0.28	11.91 ± 0.13	1.06
Concentration of OPA	0.1%	3.35 ± 0.029	0.86	6.98 ± 0.009	0.14	12.05 ± 0.02	0.17
	0.01%	3.28 ± 0.003	0.00	6.95 ± 0.004	0.06	11.93 ± 0.01	0.10
Flow rate	1 ml/min	3.35 ± 0.029	0.86	6.98 ± 0.009	0.14	12.05 ± 0.02	0.17
	0.9 ml/ml	3.30 ± 0.010	0.24	6.96 ± 0.020	0.28	11.96 ± 0.05	0.42
Column oven temperature	40^{0}C	3.35 ± 0.029	0.86	6.98 ± 0.009	0.14	12.05 ± 0.02	0.17
	45^{0}C	3.28 ± 0.020	0.46	6.87 ± 0.060	0.95	11.57 ± 0.06	0.48
pH of OPA	2.76	3.35 ± 0.029	0.86	6.98 ± 0.009	0.14	12.05 ± 0.02	0.17
		3.30 ± 0.002	0.07	6.94 ± 0.000	0.008	11.89 ± 0.006	0.05
Wavelength (λ max) nm at Isosbestic point	285	3.31 ± 0.003	0.11	6.98 ± 0.008	0.12	12.11 ± 0.056	0.46
	287	3.31 ± 0.004	0.11	6.97 ± 0.011	0.16	12.10 ± 0.036	0.30
	289	3.31 ± 0.004	0.11	6.95 ± 0.009	0.13	12.11 ± 0.015	0.13

% RSD: Percent relative standard deviation, SD: Standard deviation

Isosbestic point determination

The isosbestic point was determined for the mixture of EGC, FT, and QT by UV spectrophotometer as well as on HPLC and the isosbestic point was confirmed at 287 nm. The UV spectrophotometer graph and chromatogram of isosbestic point are described in (Fig. 2) and (Fig. 3) respectively.

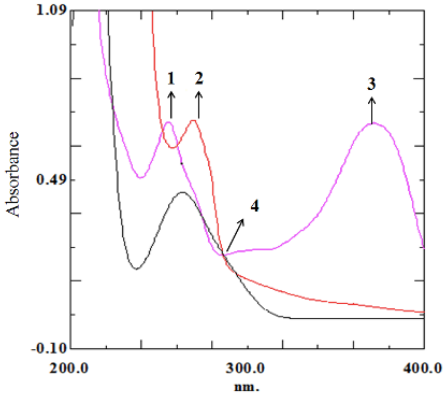
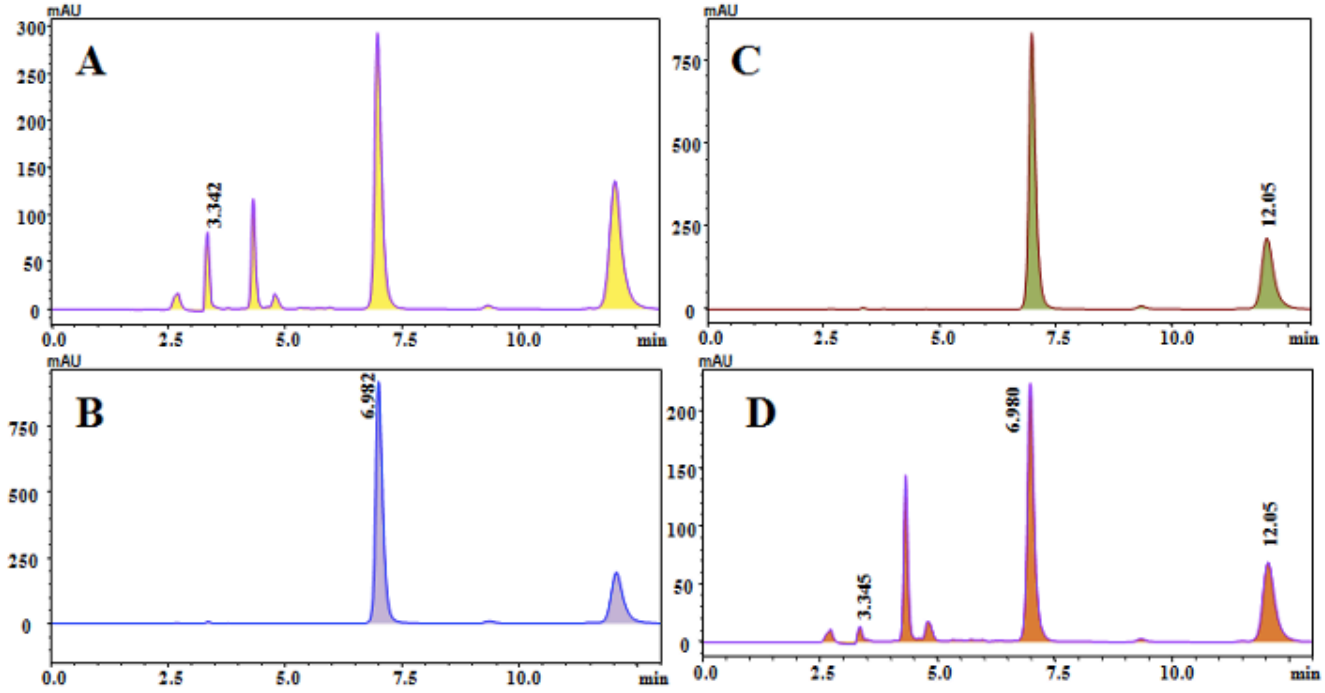


Figure 2: Isosbestic point on UV spectrophotometer. 1) EGC, 2) FT, 3) QT and 4) Isosbestic point.

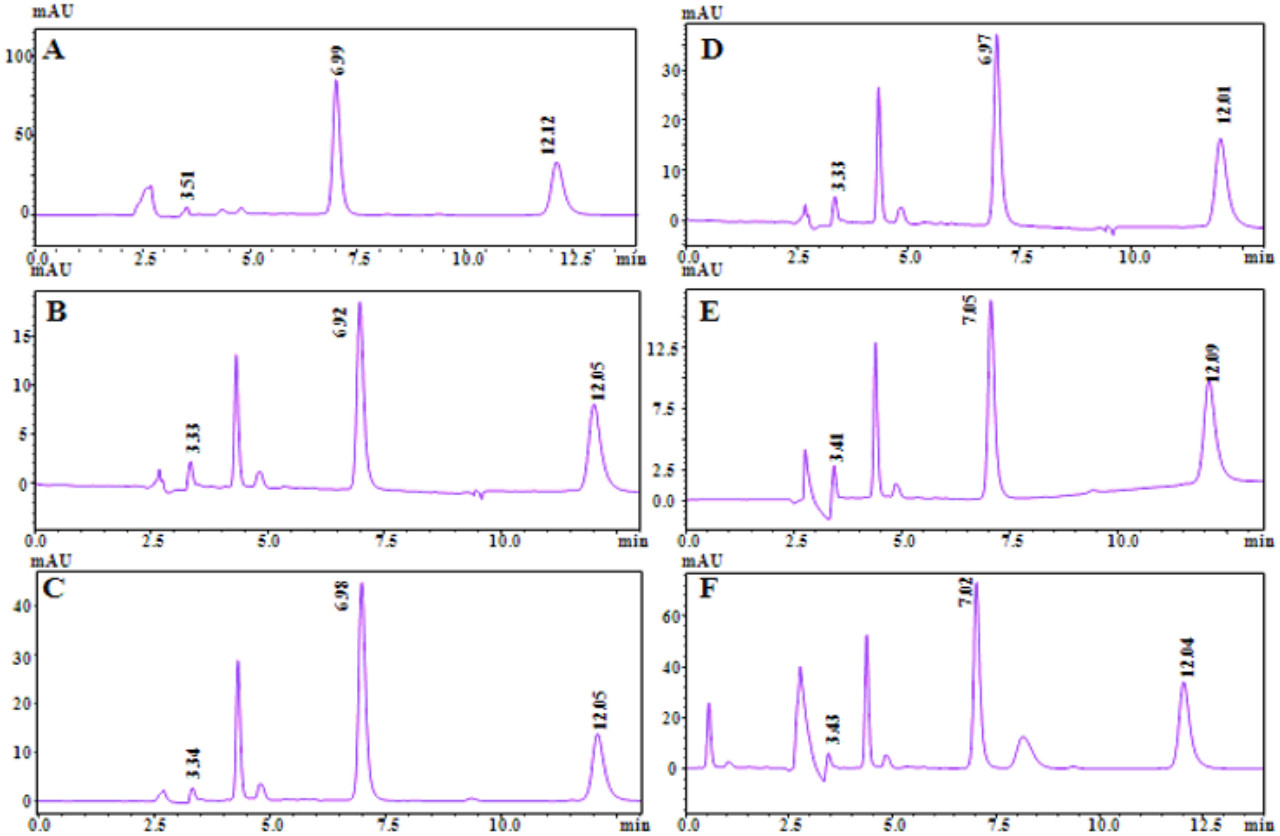
Figure 3: HPLC chromatograms. A) EGC (269 nm), B) FT (359 nm), C) QT (370 nm), D) Isosbestic point (287 nm)



Forced degradation study

The standards were further tested for forced degradation; acidic, alkaline, thermal, sunlight, and oxidative stressed conditions in individual and mixture forms of the compounds. The percent degradation of EGC, FT, and QT was observed with higher photolytic degradation i.e. 24.81 ± 12.09, 38.72 ± 5.4, and 39.82 ± 6.16 respectively since all three selected marker compounds are light-sensitive they had shown drug degradation and their chromatograms are represented in (Fig. 4).

Figure 4: HPLC Chromatogram of EGC, FT, and QT in forced degradation study A) Normal B) Sunlight C) Oxidative D) Basic E) Thermal and F) Acidic stress conditions.



Applicability of optimized method on HAE of Acacia suma and Ayurvedic formulations

The HPLC chromatograms were obtained for *Acacia suma* extract (Fig. 5) and Ayurvedic marketed formulations such as Khadirarista (Fig. 6) and Khadiradivati (Fig. 7) revealed the retention time of EGC, FT, and QT at 3.26 min; 6.93 min; and 11.85 min. respectively. The developed method was able to quantify EGC, FT, and QT in *Acacia suma* extract and Ayurvedic formulations. It has been observed with a similar retention time as standard compounds. The quantitative estimation of EGC, FT, and QT was calculated based on the peak area (Table 6).

Figure 5: HPLC Chromatogram of *Acacia suma*: A) EGC; B) FT; C) QT; D) Isosbestic point

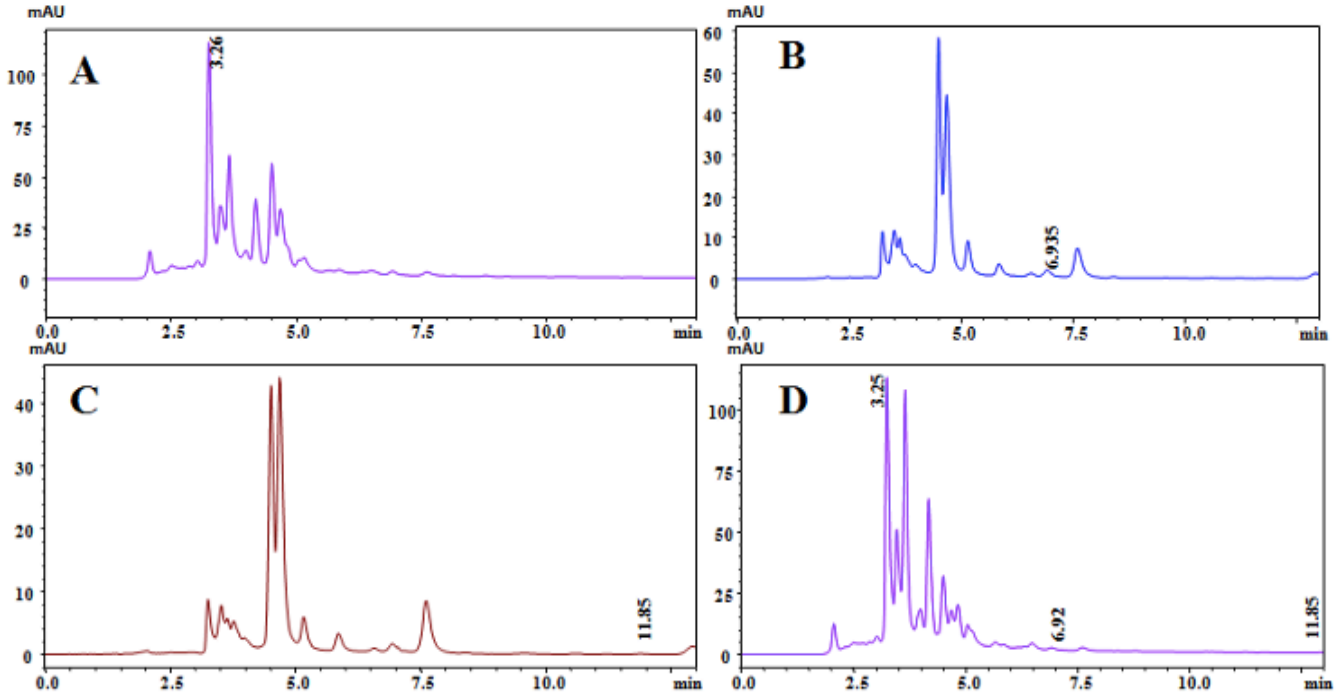


Figure 6: HPLC Chromatogram of Khadirarista: A) EGC; B) FT; C) QT; D) Isosbestic point

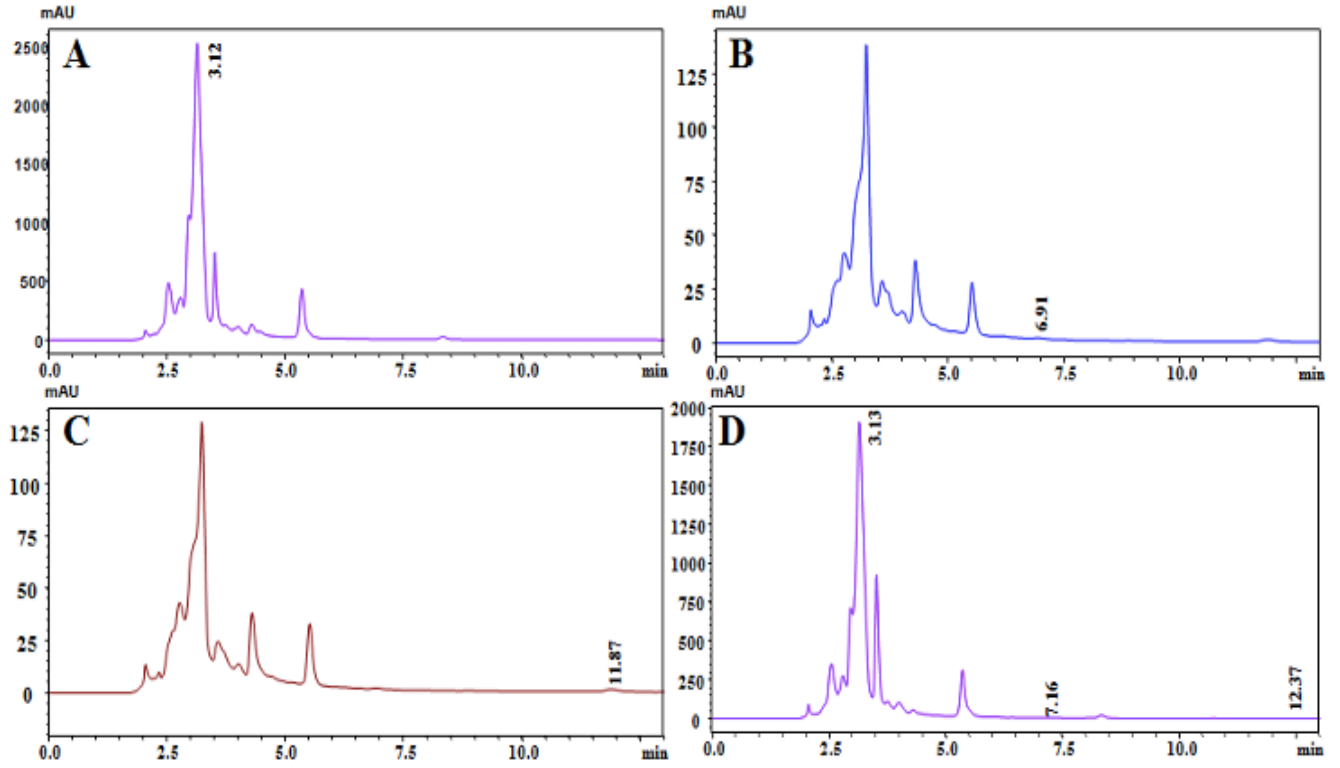


Figure 7: HPLC Chromatogram of Khadiradivati: A) EGC; B) FT; C) QT; D) Isosbestic point.

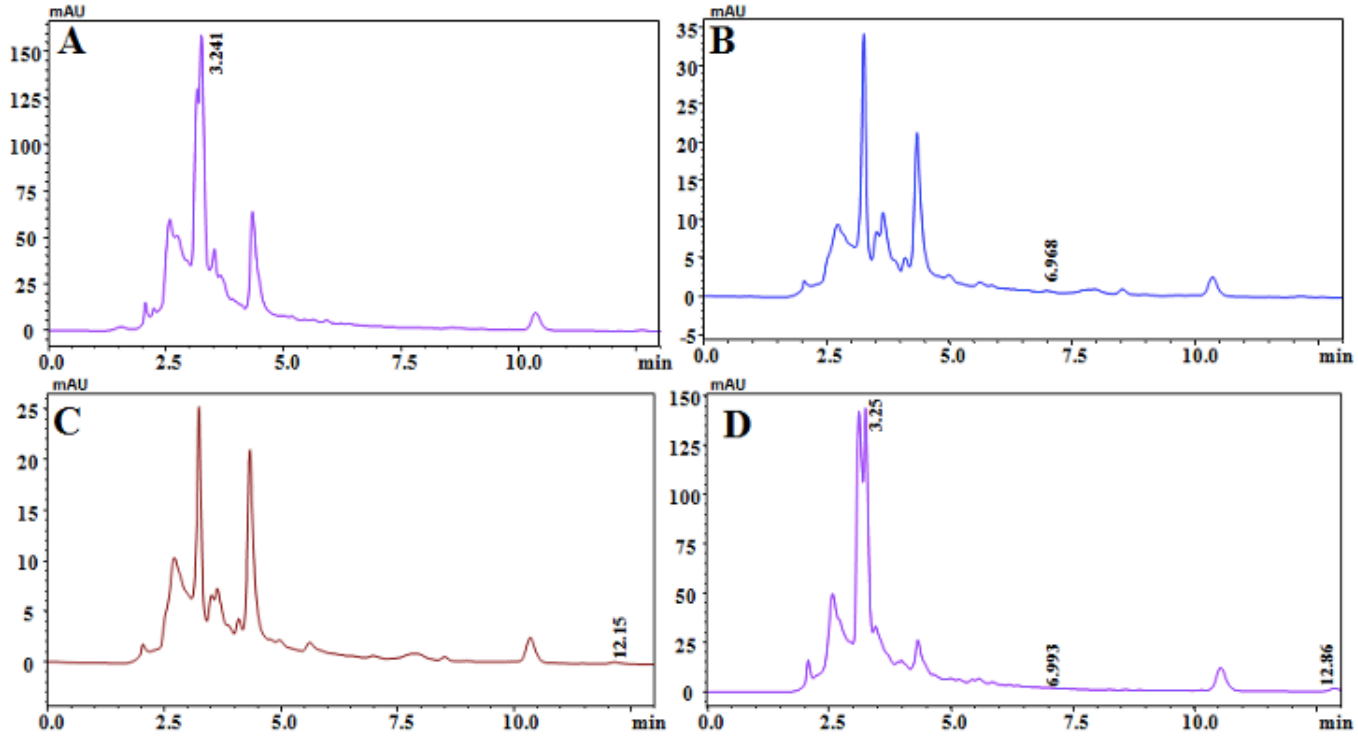


Table 6: Drug content estimation

Standards	HAE of *A. suma (µg/mL)*	Khadiradivati (µg/mL)	Khadirarista (µg/mL)
EGC	107.2 ± 0.22	459.5 ± 0.22	161.1 ± 2.2
FT	1.71 ± 0.03	1.30 ± 0.02	1.69 ± 0.05
QT	1.72 ± 0.07	1.46 ± 0.01	1.70 ± 0.02

Discussion

Diabetes mellitus (DM) is categorized by defects in insulin secretion, impaired insulin action, or both by metabolic dysregulation primarily of carbohydrate metabolism manifested by hyperglycemia. DM can lead to complications in the vascular system, eyes, nerves, and kidneys leading to peripheral vascular disease, nephropathy, neuropathy, and retinopathy (23). Oral anti-diabetic agent metformin comes underclass of biguanides and is used in type 2 diabetics, also can be used in combination with any other oral anti-diabetic drug. In the case of the mechanism of action, this drug works by its hepatic sensitivity to insulin secretion thereby reducing gluconeogenesis as well as glycogenolysis, leading to reduce plasma glucose. The α-glucosidase inhibitors act on glucosidase enzymes in the intestine. Acarbose is the glucosidase inhibitor it has a beneficial effect in reducing postprandial hyperglycemia by reducing insulin secretion (24). In the present study hydroalcoholic extract of *Acacia suma* had shown significant alpha-amylase and alpha-glucosidase inhibition and was compared with metformin and acarbose as standard anti-diabetic agents. In Ayurvedic Pharmacopoeia of India, the heartwood of *Acacia suma* is duly mentioned for the treatment of metabolic disorders (25).

The marker phytochemicals selected in this study are previously reported for anti-diabetic potential. Quercetin is a flavonoid with antioxidant properties that helps in the regeneration of the pancreatic cells and increases insulin release in streptozocin-induced diabetic rats it has beneficial anti-diabetic potential (26). Fisetin enhances the enzymatic activities of hexokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase whereas restraining the activities of lactate dehydrogenase, G6Pase, fructose-1,6-biphosphatase in hepatic and renal tissues of STZ-diabetic rats. Oral administration of fisetin reduces blood glucose levels and increases plasma insulin concentrations (27). Further, in the oral glucose tolerance test (OGTT) epigallocatechin had high glucose tolerance ability it suppresses the b-cell destruction by inhibition of NF-kB activation in RINm5F cells (28).

Natural products are the starting point for future bioactive product development. Herbal extracts involve a large number of phytochemicals and few may present in less concentration. Retention of solute is based on the nature of the compounds, the components with high affinity towards mobile phase elute faster whereas low-affinity compounds take more time for retention (29). The main advantage of this optimized method is that it takes a short retention time for the resolution of all three marker compounds also instead of using three different wavelengths only one wavelength i.e. 287 nm as an isosbestic point can be used to confirm the presence of EGC, FT, and QT in the herbal extracts. The applicability of the optimized method was evaluated for the presence of selected standards in the Ayurvedic formulation and HAE of *Acacia suma* (30, 31) and was identified with retention time.

Further, in the degradation study, FT and QT were shown degradation in presence of alkaline and thermal

stress conditions. FT and QT are well-known flavonoids having 4 and 5 hydroxyl groups respectively. The presence of hydroxyl groups leads to flavonoids being less stable and therefore, study findings indicate instability of FT and QT in alkaline and thermal conditions (32) on the other hand, EGC was observed with degradation in presence of oxidation and sunlight (33). The developed RP-HPLC method justifies concerning specificity, sensitivity, and efficiency. The usage of EGC, FT, and QT are considered for disease management such as anti-cancer, anti-diabetic, anti-inflammatory, hepatoprotective, anti-obesity, anti-oxidant, anti-microbial, cardio-protective, and anti-viral activities as documented previously (34-36).

The selected marker compounds have been predicted for their anti-lipase activity by the in-silico method and extract had shown lipase inhibition potential by the in-vitro method. The study had predicted the presence of EGC, FT, and QT in *Acacia suma* as lipase inhibitors with the highest docking score hence it could be helpful to explore the anti-diabetic potential of *Acacia suma* along with standardization of the plant by RP-HPLC method to confirm the presence of these phytomarkers in the extract.

From the perspective of this study, herein we suggest performing the network pharmacology and molecular docking of *Acacia suma* in tracing the protein-phytoconstituents interaction as detailed previously for various traditional medicinal plants (37- 40) and tracing the lead hit to assess the gene ontology of the lead bioactives (epigallocatechin, fisetin, and quercetin) as demonstrated previously (41).

Conclusion

The study findings revealed the significant anti-diabetic potential of the extract by in-vitro alpha-amylase and alpha-glucosidase inhibition assays, which support the traditional claim of the plant in the treatment of metabolic disorders. The established RP-HPLC method demonstrates the precise and easy determination of EGC, FT, and QT and is effectively studied by a gradient elution system. The developed method is simple and specific for the estimation and quantification of polyphenols from herbal extract having the presence of EGC, FT, and QT. The method was validated to ensure acceptable and reliable considerations such as system suitability, linearity, accuracy, precision, LOD, LOQ, and robustness/ruggedness. Further, the applicability of the developed method was analyzed on HAE of *Acacia suma* and in Ayurvedic formulations. Hence, this study concludes that extract had the presence of EGC, FT, and QT along with its anti-diabetic potential.

Acknowledgement

The authors are thankful to the director of the Analytical laboratory at KAHER's BSRC, Belagavi 590010, Karnataka, India, for providing facilities to complete this study. I'm thankful to my research guide Late **Dr. Sanjay Mishra** for his kind guidance and suggestions.

Disclosure statement

The authors have no conflict of interest.

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