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Identification and Quantification of Polyphenolic Secondary Metabolites in Stem Bark of *Ficus religiosa* (Moraceae) Using UPLC-HRMS and RP-HPLC-PDA

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Abstract: *F. religiosa* bark has been extensively used in traditional medicinal systems, such as Ayurveda, for its health benefits. The aim of this study was to investigate the secondary metabolites (phenolics and flavonoids) of the hydroalcoholic stem-bark extract from *F. religiosa* because this plant has been proven to have a beneficial effect on health disorders. Therefore, a pilot study was conducted for the identification and quantification of polyphenolic compounds in *F. religiosa* bark using sophisticated chromatographical techniques such as UPLC-HRMS and RP-HPLC-PDA. Additionally, total flavonoids, total phenolics and the scavenging profile of the bark were studied using a UV spectrophotometer. A total of 23 compounds identified with UPLC-HRMS were mainly phenolic acids, polyphenolics, and flavonoids (flavanols and proanthocyanidins). Among the identified compounds, gallic acid, catechin, epicatechin, epigallocatechin gallate, and ellagic acid were simultaneously quantified (0.031–0.380%) using RP-HPLC-PDA. Thereafter, the study complied by evaluating the total flavonoids (109.15 ± 1.2 mg RuE/g and 33.78 ± 0.86 mg CaE/g), total phenolics (4.81 ± 1.01 mg GaE/g), and scavenging profiles (IC_{50} 13.75 ± 0.12 µg/mL) of the *F. religiosa* bark. This is the first report on the chemical profiling of *F. religiosa* bark, which is a necessary step to evaluate its nutraceutical properties, paving the way for possible food application.

Keywords: *F. religiosa*; flavonoids; polyphenolics; phytochemical; stem bark; UPLC-HRMS

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1. Introduction

F. religiosa (family *Moraceae*) is native to the Indian subcontinent and has received mythological, religious, and medicinal importance in Indian and Southeast Asian culture. Its medicinal properties are mentioned in ancient Hindu texts, such as *Atharvaveda*, and many more [1]. Its stem bark, in powder and decoctions forms, are traditionally used in a wide range of ailments, including endocrine (diabetes), gastrointestinal (diarrhea and ulcers), reproductive, respiratory (asthma) [2–6]. It has been extensively used for topical disorders (intrinsic and extrinsic topical disorders) [7]. It has been used in combination to achieve greater nutrotherapeutic effects, e.g., fresh stem-bark juice along with black pepper is used for liver diseases [8]. Various formulations mentioned in Ayurvedic formulary of India, such as *Nayagrodhadi kvatha curna*, *Sarivadyasava*, and *Arasam pattai powder*, contain *F. religiosa* bark and are used as combinatorics for bleeding disorders, syphilis, digestive impairments, and urinary obstructions [9,10]. Its indigenous uses and recent pharmacological studies reports show great therapeutic potential against many diseases [11].

There is a need to explore and validate the traditional claims, which can be endorsed by its phytochemical exploration. Only a few natural products, such as triterpenes and phytosterols (Lupeol, α-amyrin, β-amyrin, and β-sitosterol) [12], furano coumarins (bergapten and bergaptol) [13], and phenolics and flavonoids (inositol, ellagic acid, N-isobutyl-ecosa-trans-4-dienamide) [14,15] have also been reported from the bark. Other investigations concerning the phytochemical diversity of *Ficus* species have been investigated, and various flavonoids, glycosides, and polyphenolics have been reported as antimicrobial agents. Previously, authors have identified kaempferol glycosides and other *c*-glycosides in the leaf. Recently, antioxidant phenolics have also been investigated using liquid chromatography–mass spectrometry (LCMS) in leaves [16]. In another study, colorimetric studies of the *F. religiosa* revealed that stem bark has highest levels of bioactive compounds (total phenolics, terpenoids, and flavonoids) as compared to fruit and leaf extracts and exhibit significant free-radical scavenging activities [17].

An updated phytochemical profiling with the sophisticated analytical techniques, such as ultraperformance liquid chromatography–high-resolution mass spectrometry (UPLC-HRMS) and further, proper secondary metabolite quantification with chromatographic techniques, such as reverse-phase–high-performance liquid chromatography–photodiode array (RP-HPLC-PDA), can further endorse the existing therapeutical potential of the *F. religiosa* bark and hence provide convincing support for its further use in modern medicine [18]. Such studies can also be useful in optimizing the quality of the plant material and to standardize the finished products with respect to the phytoconstituents. Thus, there is a need to explore other bioactive polyphenolics in the bark; hence, the present investigation of chemical content was undertaken.

The current work describes a complete investigation into the secondary metabolite profile of *F. religiosa* bark, particularly polyphenolic phytoconstituents. This study has been carried out starting with the UPLC-HRMS analysis for phytoconstituents investigation of the hydroalcoholic extract of the *F. religiosa* bark. Further, identified polyphenolics were then analyzed with the RP-HPLC-PDA for the quantification of the five polyphenolic compounds. A new RP-HPLC-PDA method has been developed for simultaneous estimation of five phenolic compounds of the bark. A wavelength of 280 nm was applied during the analysis, and a gradient chromatographic method is preferred over the isocratic for the better resolution of peaks. Better-resolved, more easily identified peaks, a shorter runtime, and good reproducibility ensures the identification and quantification of secondary metabolites. Additionally, total flavonoids, such as rutin and catechin equivalent, total phenolics, such as gallic acid equivalent, and the free-radical scavenging profiles of the *F. religiosa* bark have also been evaluated to compile the study. Overall, the implementation of these types of studies can lead to better quality standardization of plant parts (*F. religiosa* bark) and their finished products, ultimately benefiting both the industry and the consumers. As the herbal medicine industry continues to grow, there are many other herbal-analysis methods that could obtain the same benefits from better standardization and adaptation to sophisticated chromatographic techniques.

2. Material and Methods

2.1. Plant Material

The stem bark of *F. religiosa* was collected in November 2021 from the Rupnagar district in Punjab. The botanical identity of the plant material was carefully verified, and specimens were deposited at the herbarium at the Department of Natural Products, NIPER Mohali, with accession number NIP-H-481. A specimen voucher with the number SU-PHD-481 was submitted in School of Pharmaceutical Sciences, Shoolini University. Authenticated bark samples were then cleaned, shade-dried, and coarsely ground, and then passed through a sieve (#20 mesh). Powder was preserved in clean plastic containers and stored away from light, heat, and moisture until used for the study.

2.2. Chemicals and Reagents

The standard marker compounds gallic acid, catechin, epicatechin, epigallocatechin gallate, ellagic acid, and rutin were purchased from Sigma Aldrich (St. Louis, MO, USA). Analytical-grade acetic acid, DPPH, chloroform, ethanol, aluminum chloride, sodium carbonate, sodium nitrite, sodium hydroxide, ascorbic acid, and acetonitrile (HPLC grade) were purchased from the Rankem (Haryana, India). Purified HPLC water was obtained from Millipore (Burlington, NJ, USA), and Folin–Ciocalteu's phenol reagent was from Merck (Darmstadt, Germany). All other chemicals and solvents used were of the highest analytical grades available.

2.3. Extraction of Samples

About 5 g of coarsely powdered *F. religiosa* stem-bark sample was defatted twice with 60 mL chloroform via ultrasonication for 15 min. Ultrasonication technique was preferred for better and fast extraction [19]. After defatting, the remaining powder was then extracted 2 times with 50 mL of hydroalcoholic solvent (ethanol and water in 50:50 ratio) via ultrasonicator for 15 min. The extract was filtered through filter paper, and the filtrates were collected. The filtrate is further filtered with a 0.45 µ syringe filter and then used for the RP-HPLC-PDA and UPLC-HRMS. The filtrate was concentrated using a rotary evaporator to avoid degradation of the constituents and to calculate the extraction yield. All of the extract was stored at −4 °C before estimation of total phenolic content, total flavonoid content, and free-radical scavenging activity.

2.4. General Instrumentation

Solvents were concentrated using a vacuum rotary evaporator (Buchi R-300, Flawil, Switzerland). For weighing purposes, an analytical-balance Sartorius-CP-225D (Goettingen, Germany) was used. For ultrasonication and UV spectroscopy, these were carried out in a PCi-analytics ultrasonicator and a UV-1700 Pharmaspec-SHIMADZU spectrophotometer, respectively. For recording mass spectra of the extract, the UPLC-HRMS system of Waters-USA, Synapt XS HDMS was used. The RP-HPLC-PDA system (WATERS-USA) binary pump 515 with PDA 2996 detector and Empower 3.0 controlling software were used for quantification of marker compounds.

2.5. UPLC-HRMS Conditions and Identification of Secondary Metabolites

The UPLC-HRMS (Waters-USA) equipped with LC Column C18 (Waters-USA) Acquity BEH 2.1 × 100 mm 1.7 µm was used for separation at a flow rate of 0.35 mL/min. The injection volume was kept at 5 µL. Mass measurement was carried out in electrospray positive mode ES^+. For the mass spectrometer, the following parameters were considered: desolvation gas: 900 Lts/Hr, cone gas: 30 Lts/Hr, desolvation temperature: 450 °C, source temperature: 120 °C, capillary voltage: 3.22 keV, cone voltage: 50 V, collision energy 4 ev. Gases used were N_2 and argon, with pressure supply of N_2: 6–7 bar, argon: 5–6 bar. A binary gradient mobile phase was used for elution: A (0.1% acetic acid in water), solvent B (acetonitrile and solvent A in 90:10 ratio). The elution gradient program was as follows: 0–2 min, 90% A; 2–5 min, 80% A; 5–10 min, 70% A; 10–12 min, 50% A; 12–14 min, 90% A; 5 min equilibration time. Chromatographical profiles were observed at 280 nm [20,21]. UV absorption at selected wavelengths and values of molecular ion base peaks with other fragment ions was mostly used to facilitate the identification of constituents. Mass data were also compared with available standard compounds obtained from Sigma-Aldrich (ellagic acid (98%), catechin (98%), gallic acid (98%), epicatechin (98%), and ECGC (98%)) and the reported literature.

2.6. RP-HPLC-PDA Conditions and Quantification of Polyphenolics

Simultaneously, five polyphenolic compounds, i.e., gallic acid, catechin, epicatechin, ECGC, and ellagic acid were quantified using RP-HPLC-PDA (WATERS, binary pump 515 with PDA 2996 detector, Milford, MA, USA). The data were acquired using the Empower

3.0 controlling software. Separation was obtained on the Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 µ). Retention time and UV spectra of the peaks in the sample were compared with those of authentic reference markers. The method of external standards was applied for the quantitation of each compound, and data were recorded at 280 nm (Figure 1). The developed method for quantitation was validated for linearity, recovery, accuracy, LOD, and LOQ. The precision of data under repeatability conditions, i.e., intra- and interday, were evaluated. Combination of two solvent systems, i.e., solvent A (0.1% acetic acid in water) and solvent B (acetonitrile and solvent A in 90:10 ratio), was used with the following gradient program: 0–5 min, 88% A; 5–10 min, 85% A; 10–15 min, 85% A; 15–20 min, 80% A; 20–25 min, 80% A; 25–30 min, 70% A; 30–40 min, 50% A; 40–45 min, 95% A; 5 min equilibration time. Flow rate was kept at 0.70 mL/min with injection volume of 20 µL. The column temperature was set to 26 °C.

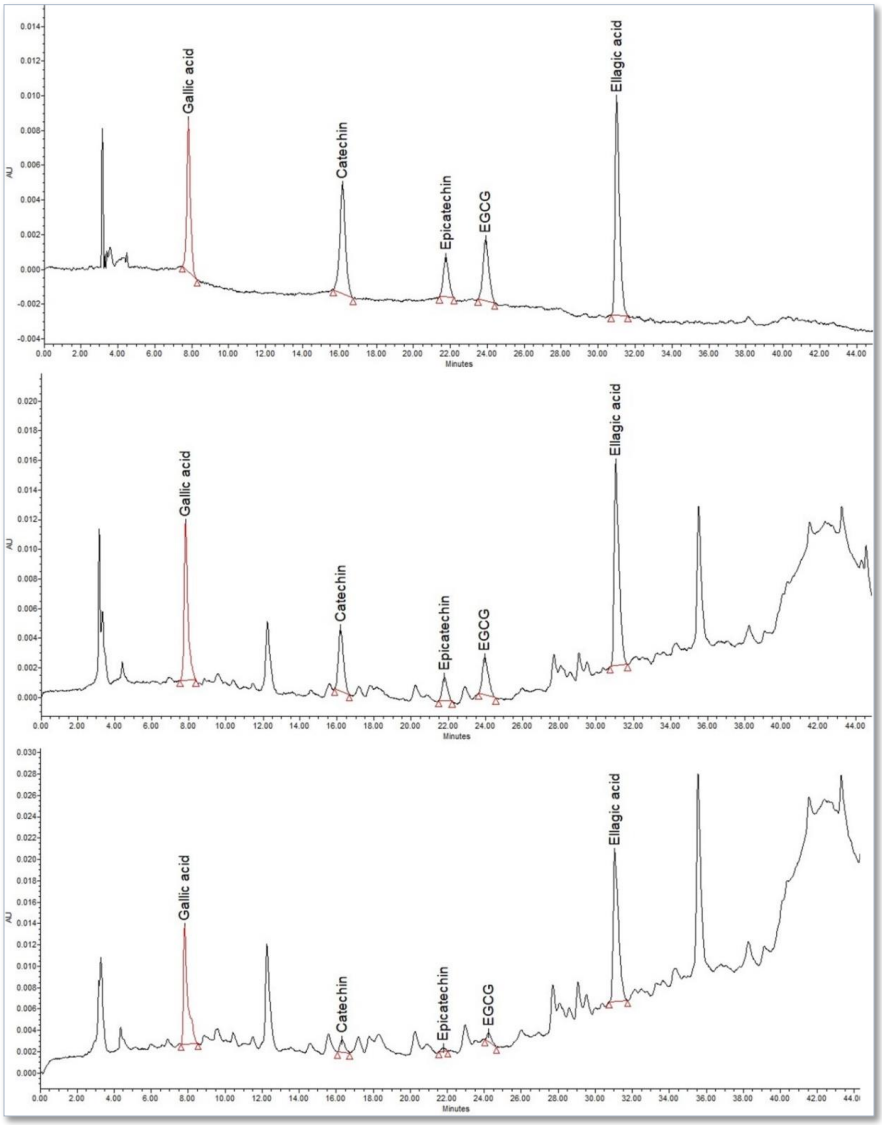

Figure 1. HPLC-PDA chromatograms of simultaneous quantitation of gallic acid, catechin, epicatechin, epigallocatechin gallate (EGCG) and ellagic acid. (**A**) Standard chromatogram at 280 nm; gallic acid (2.56 µg/mL), catechin (9.20 µg/mL), epicatechin (4.0 µg/mL), epigallocatechin gallate (EGCG) (3.52 µg/mL), and ellagic acid (4.0 µg/mL). (**B**) Hydroalcoholic extract of F. religiosa bark sample spiked with standards at 280 nm. (**C**) Hydroalcoholic extract of F. religiosa bark sample (1.40 mg/mL) with marked phenolics at 280 nm.

2.7. Determination of Total Flavonoid Content

Total flavonoid content was determined according to the aluminum chloride colorimetric method [22,23]. An amount of 2.0 mL of the bark extract (68.0 µg/mL) and standard samples were added to 4 mL of distilled water. Then, 0.3 mL of 5% sodium nitrite was added. After 5 min, 0.3 mL of 10% aluminum chloride was added. After 6 min, 2 mL of 1 M sodium hydroxide was added to the mixture. Volume was increased to 10 mL with distilled water and vortexed. The absorbance was determined at 510 nm against the blank. Catechin (8.0 µg/mL to 24 µg/mL) and rutin (50.0 µg/mL to 100 µg/mL) concentrations were used as the standards for the calibration curve (Figure 2A,B). Total flavonoid content was expressed as mg catechin and rutin equivalent per gram of the bark sample (mg/g). All tests were carried out in triplicate.

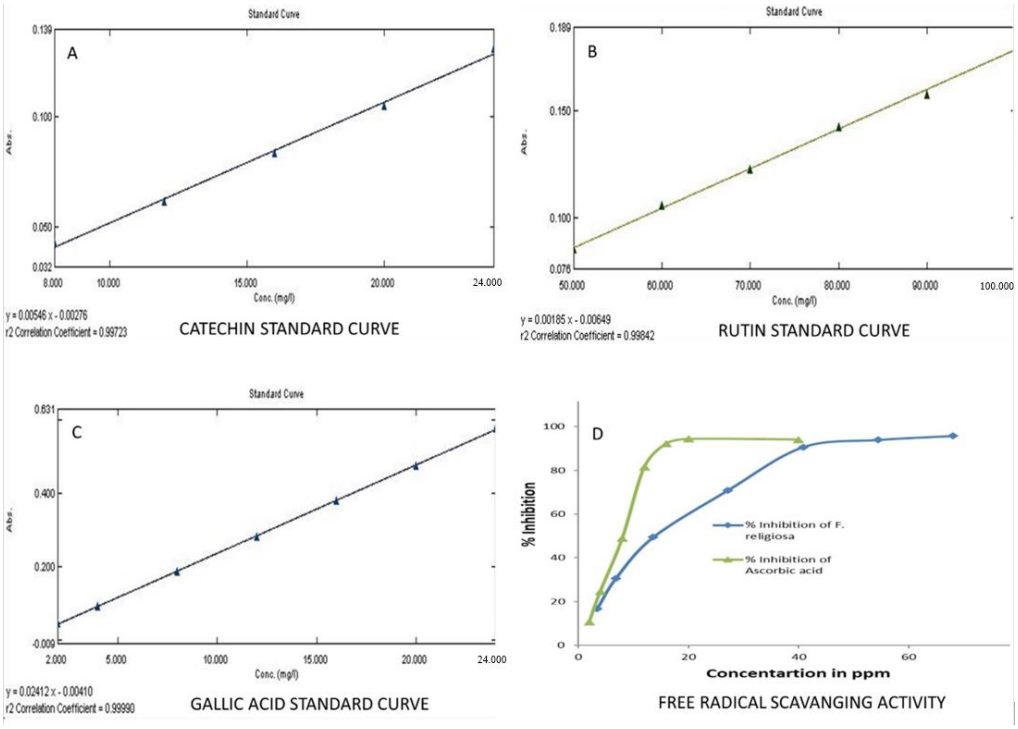

Figure 2. (**A**,**B**) Calibration curve of total flavonoid content assay as catechin and rutin equivalent. (**C**) Calibration curve of total phenolic content assay as gallic acid equivalent. (**D**) Antioxidant activity (DPPH radical scavenging) and IC_{50} values with respect to ascorbic acid equivalent against the bark hydroalcoholic extract.

2.8. Determination of Total Polyphenolic Content

Total polyphenolic content was determined in bark extract using the Folin–Ciocalteu method [24], using gallic acid as the reference standard. An amount of 2.0 mL of the bark-extract sample (10.8 µg/mL) was mixed with 1 mL of 1 N Folin–Ciocalteu reagent. The solution was kept at 25 °C for 5–8 min before adding 5 mL of 20% sodium carbonate solution. After 1 h, absorbance was measured at 725 nm. Gallic acid concentrations from 2 µg/mL to 24 µg/mL were used for the calibration curve (Figure 2C). Total phenolic content was expressed as mg gallic acid equivalent per gram of the bark sample (mg/g). All tests were carried out in triplicate.

2.9. Free-Radical Scavenging Assay

The antioxidant activity was expressed as free-radical scavenging activity of bark extract, estimated using the DPPH free-radical assay [25]. A total of 1 mL of DPPH solution (40 µg/mL) was added to 1 mL of solvent and 0.5 mL of bark-extract sample ranging from 3.4 µg/mL to 81.6 µg/mL. The reaction was allowed for 30 min, and absorbance was

measured at 517 nm using a UV spectrophotometer. All tests were carried out in triplicate. A blank was also prepared similarly by replacing the extract with ethanol. A calibration curve of the standard ascorbic acid concentration (ranging from 10 µg/mL to 160 µg/mL) was prepared against the percent inhibition, and the results were expressed as the IC_{50} value. The concentration of sample required to scavenge 50% inhibition of DPPH free radical was calculated from the plotted graph of radical scavenging activity against the concentration of extracts (Figure 2D). A low IC_{50} value indicates strong antioxidant activity, meaning that low concentrations of antioxidant substances in the extract can inhibit 50% of the radical reaction.

Formula used:

$$\text{DPPH inhibition (\%)} = \{(A_0 - A_1)/A_0\} \times 100$$

where A_0 is the absorbance of control, and A_1 is the absorbance of the test.

2.10. Statistical Analysis

All determinations were carried out at least in triplicate, and values were averaged and given with the standard deviation (±SD). The correlation was established using simple linear regression analysis. Data were processed using Microsoft Excel (2021).

3. Results and Discussion

The stem bark of *F. religiosa* was subjected to successive extraction via ultrasonication using chloroform and 50% ethanol. Hydroalcoholic extract was preferred for better extractability of the polyphenolics and also to co-relate the identified secondary metabolites with traditional biological claims [9,26,27]. Furthermore, traditional formulations of *F. religiosa* bark, mainly indicated for skin-related disorders and gastrointestinal disorders, are based on hydroalcoholic extracts [28,29].

An analysis study was performed via UPLC-HRMS with an electrospray ionization source in the positive ion mode. Overall, 23 compounds were detected and identified, which belong mainly to the classes of phenolic acids, polyphenols, and flavonoids (Table 1). Among polyphenols, epicatechin gallate, epigallocatechin gallate (EGCG), chicoric acid, chlorogenic acid, methyl ellagic acid glycoside, mono-galloyl glucose, di-galloyl glucose, and methyl ellagic acid were identified [30–32]. Mass spectra also confirmed the presence of the flavonoids quercetin, hyperoside, kaempferol glycoside, rutin, and taxifolin [33–35]. Procyanidin A-type dimers and trimers were also observed at retention times 9.659 min and 9.284 min, with masses of 576.2134 $(M + H)^+$ and 865.3522 (M^+) [36,37]. The other type of procyanidin was ruled out based on mass [38–41]. Catechin derivatives, flavanols, galloyl derivatives, procyanidin, and phenolic acids have proven pharmacological reports for the mentioned indications [42,43].

A large number of studies have revealed that catechins, such as epicatechin, gallic acylated catechins, and their derivatives, have significant antibacterial, anti-inflammatory, antioxidant, antitumor, and antiaging health functions, which have received wide attention [44]. EGCG has shown wide pharmacological activities, such as when given in combination with chemotherapeutic agents such as cisplatin or doxorubicin; it enhances antitumor activity and reduce their side effects, such as gastrointestinal disorders [45].

Gallic acid is a naturally occurring phenolic compound that belongs to a hydroxyl–benzoic acid class of phenolic acids. It has pharmacological effects such as hepatoprotective, neuroprotective, renal protective, and antidiabetic activities [46]. Recent studies have revealed that gallic acid has protective activity against oxidative stress, inflammation, and apoptosis in Alzheimer's disease. Further, phenolic compounds, such as ellagic acid, have shown anticancerous activity in vitro. Ellagic acid and gallotannins have shown good antidiabetic activity in clinical and preclinical studies [47,48].

Table 1. Tentative identification of natural compounds in *F. religiosa* bark extract using UPLC-HRMS in positive mode.

S. no.	Compound Name	RT (min)	MW	MS Positive Ions (*m*/*z*)	
				HRMS of Parent Ion (*m*/*z*)	Other Characteristic Ions (*m*/*z*)
1	Ferulic acid	2.65	194	194.1288 (M^+)	179[(M + H)-CH_3], 178 (M^+-CH_3)
2	Gallic acid	5.763	170	171.1494 $(M + H)^+$	171
3	Caffeic acid	3.388	180	181.0496 $(M + H)^+$	149, 139
4	Quercetin	20.527	302	302.1449 (M^+)	277, 231, 195, 171,149
5	Kaempferol glycoside	12.18	448	449.0270 $(M + H)^+$	285, 263, 233, 149
6	Quercetin glycoside (hyperoside)	12.930	464	464.0913 (M^+)	301, 149, 195, 249
7	Catechin glucoside	5.43	452	453.2711 $(M + H)^+$	149, 195, 251, 241
8	Catechin gallate	6.60	443	445.1485 (M + 23)	359, 249, 123
9	Epicatechin gallate	8.59	442	443.1899 $(M + H)^+$	249, 149
10	Epigallocatechin gallate	14.327	458	458.1517 (M^+)	269, 247, 359
11	Chlorogenic acid	2.780	354	354.28 (M^+)	178, 195, 149, 212
12	Procyanidin dimer	9.659	575	576.2136 $(M + H)^+$	473, 249, 149
13	Procyanidin dimer	9.991	575	576.2134 $(M + H)^+$	473, 401, 287, 265, 249
14	Procyanidin trimer	9.284	865	865.3522 (M^+)	725, 476, 443, 249
15	Ellagic acid	13.843	301	302.1452 $(M + H)^+$	218, 195, 171, 149
16	Rutin	11.371	610	611.2104 $(M + H)^+$	447, 301,
17	Taxifolin	36.839	304	305.0810 $(M + H)^+$	149, 195, 287
18	Oleuropein	5.76	540	541.2260 $(M + H)^+$	149, 279, 207
19	Protocatechuic acid	5.25	154	155.0720 $(M + H)^+$	155
20	Methyl ellagic acid	29.24	316	317.0810 $(M + H)^+$	301, 195, 160, 149
21	Catechin	5.763	290	291.0859 $(M + H)^+$	171, 139
22	Chicoric acid	8.29	474	475.1942 $(M + H)^+$	354, 195, 149
23	Caffeoyl quinic acid	3.388	678	679.1487 $(M + H)^+$	515, 351, 263, 195, 178, 149

RT—retention time; MW—molecular weight; HRMS—high-resolution mass spectrometry.

Quercetin is the 3,5,7, 3′4′ penta hydroxy flavone and other flavone, has been found to be cardioprotective, antidepressive, and anticancerous in numerous studies. [49,50] Recently, the generally recognized as safe (GRAS) status has been granted to quercetin. Quercetin shows positive results against rheumatic arthritis. As a powerful antihistaminic, it acts as a good antiallergic compound, and showing good activities in asthmatic cases and atopic dermatitis [50].

The presence of catechin derivatives and procyanidins has been reported for the first time in the *F. religiosa* stem bark. A few other species of *Ficus* have also reported this class of compounds. Detected compounds, with their corresponding observed mass, are shown in Table 1 and Figure S1 (in supplementary data). Contrary to a previous report [51], bergapten was not detected in the bark with UPLC-HRMS. Five of the detected phytoconstituents, gallic acid, catechin, epicatechin, EGCG, and ellagic acid, were then quantified simultaneously using the RP-HPLC-PDA system. Their identification and quantification were based on the co-chromatography of the reference standards with respect to peak area and UV spectra.

3.1. Quantification with RP-HPLC-PDA

A simultaneous gradient RP-HPLC-PDA method was developed and used for the quantification of five identified phenolic compounds (Figure 1). The constituents present in the extract did not interfere with any of the five markers, indicating the specificity of the method. The method was precise and had low relative standard deviation (RSD) values, i.e., %RSD with respect to area (1.9–3.9%) and with respect to retention time ($\leq$1.8%).

The linearity range of the responses to the collective standards was determined on five concentration levels, with three injections for each level. Calibration graphs for HPLC were recorded, with sample amounts ranging from 2.56 µg/mL to 7.68 µg/mL (gallic acid), 9.2 µg/mL to 27.6 µg/mL (catechin), 4.0 µg/mL to 12.0 µg/mL (epicatechin), 3.52 µg/mL to

10.56 µg/mL (ECGC), and 4.0 µg/mL to 12.0 µg/mL (ellagic acid). These calibration curves have regression coefficients ranging from 0.996 to 0.999. It showed a linear relationship between peak area and concentration over range for all five compounds. The simultaneous method was well-validated and has an %RSD of the retention times of less than 1.8% for each estimated polyphenol. Limit of detection (LOD) and limit of quantification (LOQ) were also performed for each polyphenol through this simultaneous method, with the following results: LOD: 0.0066 µg/mL and LOQ: 0.02 µg/mL (gallic acid); LOD: 0.028 µg/mL and LOQ: 0.084 µg/mL (catechin); LOD: 0.012 µg/mL and LOQ: 0.036 µg/mL (epicatechin); LOD: 0.009 µg/mL and LOQ: 0.027 µg/mL (ECGC); and LOD: 0.018 µg/mL and LOQ: 0.054 µg/mL (ellagic acid). High intraday repeatability was observed, with RSD values ranging from 1.8 to 2.9%. Accuracy (expressed as recovery) of the method was determined by analyzing the percentage recovery of the added markers. The high recovery values (from 95.89 to 98.54%) indicated satisfactory accuracy. Finally, the robustness of the method was studied by changing the mobile phase; minor changes in the mobile phase (solvent B from 70:30, 80:20 to 90:10) showed no effect on peak resolution. The results of the quantitation of the predominant polyphenols (EGCG) and monomeric phenols (gallic acid, ellagic acid, catechin, and epicatechin) found to be present in the majority of *F. religiosa* bark extracts are summarized in Table 2.

Table 2. Parameters of RP-HPLC-PDA method validation and quantified values of five markers.

Marker	Linearity Range (µg/mL)	Area (%RSD)	LOD (µg/mL)	LOQ (µg/mL)	Intraday Precision (%RSD)	Accuracy (%)	Quantitative Results (% *w*/*w* Extract of Bark)
Gallic acid	2.56–7.68	3.6	0.007	0.02	2.1	96.81	0.270 ± 0.020
Catechin	9.20–27.60	1.9	0.028	0.084	1.8	97.47	0.110 ± 0.008
Epicatechin	4.0–12.0	3.8	0.012	0.036	2.5	96.58	0.031 ± 0.009
ECGC	3.52–10.56	3.8	0.009	0027	2.9	95.89	0.047 ± 0.007
Ellagic acid	4.0–12.0	1.5	0.018	0.054	1.9	98.54	0.380 ± 0.012

R^2 regression coefficient ranging from 0.996 to 0.999 respectively; %RSD of the retention times ≤ 1.8%; LOD—limit of detection; LOQ—limit of quantification; RSD—relative standard deviation.

The developed method for the quantitation of the mentioned phytoconstituents will help in determining these *F. religiosa* bark markers in their traditional formulations, thus defining their therapeutic efficacy. It is also worth mentioning here that the traditional liquid formulations are rich in phenolics and flavonoids, as revealed by the RP-HPLC analysis by Lal et al. [52].

3.2. Total Phenolic Content and Total Flavonoid Content of the Bark Extract

The total phenolic content (TPC) of the *F. religiosa* bark extract was determined as the gallic acid equivalents (GaE) per g of the dry extract, whereas the total flavonoid content (TFC) was determined as the rutin equivalent (RuE) per g of dry extract and as the catechin equivalent (CaE) per g of dry extract (Figure 2A–C). The hydroalcoholic extract of the bark showed a good TPC value of 4.81 ± 1.01 mg GaE/g of dried bark extract, whereas the TFC values were 109.15 ± 1.2 mg RuE/g of dried bark extract and 33.78 ± 0.86 mg CaE/g of dried bark extract.

3.3. DPPH Radical Scavenging Capacity of the Bark Extract

Antioxidant activity of the *F. religiosa* bark extract using the DPPH method [25,53] was reported after 30 min of reaction time. The parameter used to measure the radical scavenging activity of the extract was evaluated in terms of the IC_{50} value in µg/mL of the bark extract as ascorbic acid equivalent (Figure 2D). The IC_{50} value of bark extract was 13.75 ± 0.12 µg/mL, which was around the IC_{50} (8.18 µg/mL) of ascorbic acid.

4. Conclusions

The present work demonstrates the presence of polyphenolic compounds in the hydroalcoholic extract of the stem bark of *F. religiosa*. The compounds identified through UPLC-HRMS were mainly phenolic acids (ferulic acid, gallic acid, caffeic acid, catechin, epicatechin, and ellagic acid), polyphenolics (epicatechin gallate, epigallocatechin gallate, chicoric acid, chlorogenic acid, methyl ellagic acid glycoside, mono-galloyl glucose, di-galloyl glucose, and methyl ellagic acid), and flavonoids (quercetin, hyperoside, kaempferol glycoside, rutin, and taxifolin). Procyanidin A-type dimers and trimers were also observed. Using the developed reliable method of RP-HPLC-PDA, the five quantified phytoconstituents can be potentially used as markers for the quality control and authentication of bark and its extracts. These identified compounds have beneficial pharmacological effect and are a promising source of antioxidants for the development of food and nutraceutical products. The undertaking of this should be considered in future works.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10060338/s1, Figure S1: Mass spectrum of (a) ferulic acid, (b) gallic acid, (c) caffeic acid, (d) quercetin, (e) kaempferol glycoside, (f) quercetin glycoside (hyperoside), (g) catechin glucoside, (h) catechin gallate, (i) epicatechin gallate, (j) epigallocatechin gallate, (k) chlorogenic acid, (l) procyanidin dimer, (m) procyanidin dimer, (n) procyanidin trimer, (o) ellagic acid, (p) rutin, (q) taxifolin, (r) oleuropein, (s) protocatechuic acid, (t) methyl ellagic acid, (u) catechin, (v) chicoric acid and (w) caffeoylquinic acid.

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Abbreviations

EGCG	Epigallocatechin gallate
RP-HPLC-PDA	Reverse-phase–high-performance liquid chromatography–photodiode array
UPLC-HRMS	Ultraperformance liquid chromatography–high-resolution mass spectrometry
LCMS	Liquid chromatography mass spectrometry
TPC	Total phenolic content
TFC	Total flavonoid content
RSD	Relative standard deviation
LOD	Limit of detection
LOQ	Limit of quantification
DPPH	2,2-diphenyl-1-picrylhydrazyl
IC_{50}	Half-maximal inhibitory concentration

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