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Research Article

Determination of Flavonoids in Stem Bark of *Manilkara hexandra* by Simultaneous and Validated HPLC Methods

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ABSTRACT

Manilkara hexandra Roxb. Dubard, another name is *Mimusops hexandra* is a tall, glabrous evergreen tree belonging to the Sapotaceae family. Ethnopharmacological uses of *M. hexandra* in toothache, tonic, dysentery, diarrhea, alimentary disorders, infertility and veterinary use by tribal people of different regions in India. Research articles revealed immunostimulant, inhibitory effect on the SARS-CoV-2 protease enzyme, antiulcer and antioxidant activity of stem bark. A simple, accurate and reproducible reversed-phase liquid chromatographic method was developed for the qualitative and quantitative determination of four bioactive flavonoids (quercetin, luteolin, kaempferol and apigenin) from the stem bark extract of *M. hexandra*. Chromatographic separation was performed on a C18 column (5 µm C18, 4.6 × 250 mm) with a mobile phase consisting of 0.5% orthophosphoric acid and 100% methanol (40:60 %v/v), at a flow rate of 1.0 mL/min. The analysis was performed using a UV detector at different wavelengths. The method was validated in terms of selectivity, linearity, accuracy, precision, robustness and recovery. Good linearity was observed over the investigated concentration range of 5-30 µg/mL for quercetin and luteolin; 2-64 µg/mL of kaempferol and 2-12 µg/mL of apigenin with correlation coefficient (r^2) values greater than 0.998. The intra- and inter-day precision over the concentration range was <0.57% (relative standard deviation) and the accuracy was between 98.06% and 100.65%. The %RSD of recovery for all the analytes was 0.49%-0.81%. This method was successfully applied in the quantity assessment of bioactive flavonoids in the stem bark extract of *M. hexandra*.

Keywords: HPLC; HPTLC; *Manilkara hexandra*; Quercetin; Luteolin; Apigenin; Kaempferol

INTRODUCTION

Medicinal plants, with diverse applications in healthcare, provide valuable chemical compounds. Whether used whole or in specific parts, such as roots, stems, leaves, barks, fruits or seeds, they contribute to disease control and treatment. Their bioactive substances highlight their importance in traditional and modern medicine [1].

Manilkara hexandra (Roxb.) Dubard (Synonym: *Mimusops hexandra* (Roxb.) Dubard) commonly known as 'Khirni or Rayan', is found in central India and the Deccan peninsula and cultivated throughout the greater parts of India. Ethnomedicinally, stem bark is popularly used as an astringent, aphrodisiac, stomatitis, fever, jaundice, asthma, diseases of gum and teeth as well as vitiated conditions of pitta, contains approximately 10% tannin, rendering it valuable for tanning purposes.

The plant is reported to exhibit antioxidant, anti-inflammatory, antihyperglycemic, antiviral, antimicrobial, antiulcer, immunostimulant and antidiabetic activities. Also revealed methanolic extract of stem bark showed the presence of significant amounts of phenolic contents, tannins, alkaloids, flavonoids, terpenoids, phlobatannins, anthraquinones, saponins, reducing sugars, steroids, glycosides, carbohydrates. Chemically diverse groups of substances, flavonoids are one of the most widely occurring groups, playing pivotal roles in a spectrum of health-promoting properties and finding indispensable applications in pharmaceuticals, cosmetics, nutraceuticals and

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medicinal realms. Flavonols quercetin and kaempferol possess diverse functionalities within plant organisms and as potent natural antioxidants, show promise for intestinal health. With high lipid affinity, both exert anti-inflammatory properties, help manage obesity and regulate intestinal permeability. Additionally, kaempferol may prevent colorectal cancer and impact anti-obesity mechanisms by modulating gut microbiota. Luteolin, a flavone is a viable option for treating viral infections and related diseases due to its ability to regulate the expression of inflammatory factors and promote the repair of cells damaged by antiviral response molecules. Further, it exerts antifibrosis, anti-inflammatory and antioxidant effects and reduces lipid accumulation. Apigenin most common monomeric flavone acts as an antineoplastic agent by induction of autophagy in leukemia cells. Since it is lipophilic can survive in the gastrointestinal tract of the host under a low acidic environment, reducing cholesterol levels, fatty acid production levels and obesity [2].

A dig in the literature regarding the qualitative and quantitative analysis of phytoconstituents in the stem bark of *M. hexandra* indicated a dearth of HPLC methodology. It is one of the most accurate modern analytical methods that enables the separation of complex mixtures, especially herbal extracts.

This study addresses this knowledge gap by developing the first ever concurrent HPLC method that is easy, rapid and accurate, as per the International Council for Harmonisation (ICH) guidelines for the analysis of quercetin, kaempferol, luteolin and apigenin and confirms their presence and quantifies the amount of four flavonoids in *M. hexandra* stem bark (Figure 1) [3].

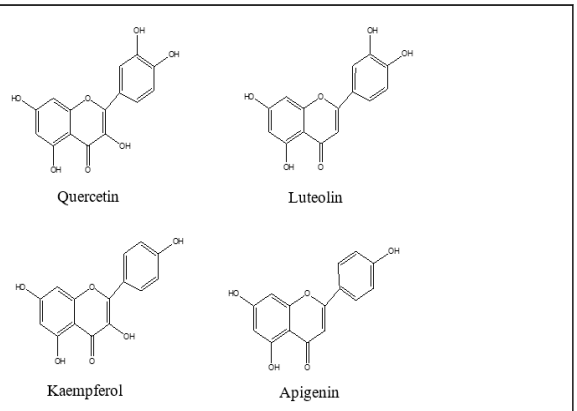

Figure 1: Structures of flavonoids.

MATERIALS AND METHODS

Plant material collection and authentication

Stem bark was freshly gathered during the monsoon season in July from the medicinal garden at L. M. College in Ahmedabad, Gujarat. The authenticity of the samples was confirmed by Dr. Hitesh Solanki, professor, department of botany, Gujarat university, Ahmedabad. A voucher specimen LMCP-PS/19102016/01 was deposited in the department of pharmacognosy and phytochemistry, L. M. College of Pharmacy, Ahmedabad. Fresh stem bark was cleaned, dried in a hot air oven to maintain a 60°C temperature and powdered to 80 # used for further study.

Chemical and reagents

Quercetin, kaempferol, luteolin and apigenin were purchased from sigma-aldrich chemicals (Bengaluru, India). Water and methanol were HPLC grade and orthophosphoric acid (analytical grade) was purchased from Finar Limited (Gujrat, India) [4].

Extraction procedure

Precisely weighed 100 g of powder was defatted using petroleum ether. Subsequently, the defatted material was refluxed for 8 hours in a Soxhlet apparatus using methanol and dried to yield 26.2 g of extract. Tannins were removed from 20 g of this extract by redissolving in methanol to get a saturated solution followed by adding 10% poly vinyl pyrrolidone solution. The resulting tannin precipitate was filtered and the extract was dried under vacuum to yield 6.64 g of reddish-brown semisolid consistency that was dissolved in ethyl acetate. The ethyl acetate layer was collected by filtering and evaporated under vacuum and yield was noted as 0.37 g (ESB).

Preparation of standard stock solution: Accurately weighed 1 mg of standards, quercetin, kaempferol, luteolin and apigenin were transferred to a separate 10 ml volumetric flask and dissolved in methanol. Volume in each case was made with methanol to obtain standard stock solutions of concentration 100 µg/ml for each standard. These stock solutions were further diluted for the studies as required.

Preparation of test solution: Precisely weighed at 50 mg Ethyl Acetate Stem Bark extract (ESB) was dissolved in methanol within a 10 ml volumetric flask. Methanol was then added to achieve a sample stock solution with a concentration of 5 µg/ml. Subsequently, these stock solutions were subjected to further studies [5].

Instrumentation and chromatographic conditions

Shimadzu HPLC system equipped with an SPD-40V Ultraviolent (UV) detector, SIL-40C autosampler, CTO-10ASVP column oven and LC-20AD pump using lab solutions software version 6.110. The chromatographic separation for the HPLC method was achieved using a Shimadzu shim-pack solar column (5 µm C18, 4.6 × 250 mm), with column oven temperature maintained at 25°C throughout the analysis. The mobile phase consisted of 0.5% orthophosphoric acid (solvent A) and 100% methanol (solvent B) (40:60, v/v). The mobile phase flow rate was 1.0 mL/min with isocratic elution. The injection volume was 20 µL, and samples were run for a total of 20 min. The detector is configured specifically for the detection of various flavonoids quercetin was detected at 370 nm and kaempferol at 367 nm, while luteolin and apigenin exhibited absorption maxima at 350 nm and 340 nm, respectively.

Analytical method validation

The RP-HPLC method underwent validation following ICH guidelines, encompassing assessments of system suitability, linearity, limits of quantitation and detection, precision, accuracy and robustness.

System suitability studies

System suitability was ensured by conducting six replicate injections of a standard solution containing quercetin, kaempferol, luteolin and apigenin. The % Relative Standard Deviation (% RSD) of peak areas, Tailing factor (T) and theoretical plate Number (N) were subsequently determined.

Calibration curve (linearity)

The contents of the markers were determined using a calibration curve established with six dilutions of each standard, at concentrations ranging from 5-30 µg/mL mg/ml of quercetin and luteolin; 2-64 µg/mL kaempferol and 2-12 µg/mL of apigenin. Each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentrations of the markers injected. Peak identification was achieved by comparison of both the Retention Time (RT) and UV absorption spectrum with those obtained for standards [6].

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ values were derived from the calibration curves using the formula k SD/b, where k equals 3 for LOD and 10 for LOQ. Here, SD represents the standard deviation of the response of the minimum detectable drug concentration, while b denotes the slope of the calibration curve.

Accuracy (recovery)

Accuracy could refer to how well a system can retrieve lost or corrupted data without errors or loss of integrity. The method involves spiking samples at three distinct levels (50%, 100% and 150%) and conducting triplicate analysis. Recovery is then computed by determining the disparity between the spiked and unspiked samples for each recovery level.

Precision (repeatability)

Intra-day precision was determined by conducting three analyses of the standard on the same day. Inter-day precision, on the other hand, was determined by carrying out the same analysis every day for three consecutive days, selecting low, medium and high concentrations within the range and conducting triplicate analysis.

Robustness

To demonstrate the robustness of the method, intentional variations were made to the chromatographic conditions. This included adjusting the flow rate of the mobile phase from 1.0 to 0.9 mL/min and from 1.0 to 1.1 mL/min. Additionally, alterations were made to the composition of the mobile phase from 60:40 (methanol: 0.5% orthophosphoric acid) to 65:35 (methanol: 0.1% orthophosphoric acid) and from 60:40 (methanol: 0.1% orthophosphoric acid) to 55:45 (methanol: 0.1% orthophosphoric acid), representing a 5% change. Furthermore, variations in the temperature of the column oven from 25°C to 30°C to 25°C to 20°C (*i.e.*, 25 ± 5°C) were introduced. The sample solution for the robustness study was applied to the column in triplicate and the resulting responses were determined [7].

Quantification

The developed analytical method was applied to the simultaneous determination of the four flavonoids in the ESB samples. An aliquot of 20 µL of sample solution (5 µg mL^{-1}) was run along with a range of standard solutions: Quercetin and luteolin ranging from 5 to 30 µg mL^{-1}, kaempferol ranging from 2 to 64 µg mL^{-1} and apigenin ranging from 2 to 12 µg mL^{-1} on the HPLC system. The peak areas were noted and quantification of flavonoids in the ESB sample was performed using linear regression equations of the respective compound.

RESULTS AND DISCUSSION

Chromatography

A favorable separation was achieved using a mobile phase composed of methanol and 0.5% Orthophosphoric Acid (OPA) (60:40, v/v) with a flow rate set at 1 ml^{-1}. The initial analysis of the sample extract involved the setup of a UV detector based on recommendations from for details on the spectral maximum of flavonols and flavones. The High-Performance Liquid Chromatography (HPLC) conditions for *M. hexandra* stem bark extract was fine-tuned based on literature data and the corresponding flavonoid reference standards. A typical chromatogram of the mixture of standards and a sample chromatogram is shown in Figure 2. Quercetin, luteolin, kaempferol and apigenin were eluted at retention times of 9, 10.6, 13.7 and 15.2 min, respectively. All analytes exhibited satisfactory absorbance at their respective wavelengths, ensuring well-resolved peaks with baseline separation [8].

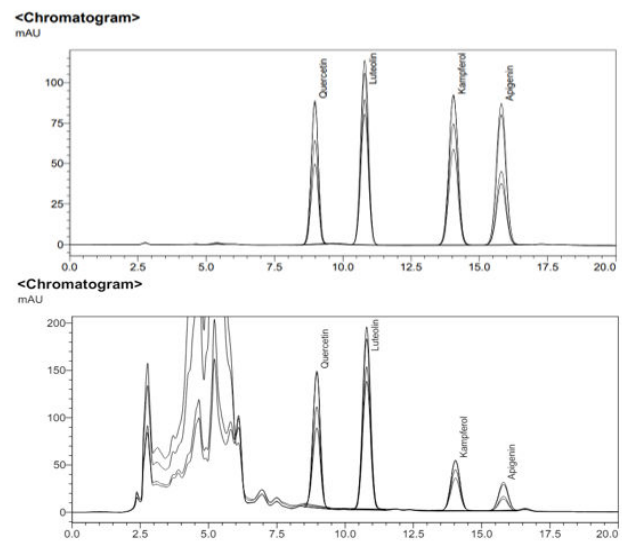

Figure 2: Representative HPLC chromatograms of the standard mixture and sample of stem bark of M. *hexandra*.

Analytical method validation

The selectivity of the method was determined by comparing certain parameters of the chromatographic profile, such as retention time, structure of the UV spectrum and λ_{max} of the reference standards and the plant extract sample. The chromatographic profiles of the standard mixture and the extracted sample were identical concerning the parameters mentioned above. In addition, no interference was observed at the retention times of any analytes in the chromatogram of the ESB sample [9].

The ranges of all the calibration curves shown in Figure 3 were adequate for the simultaneous analysis of the flavonoids in the ESB samples. The linear correlation from the standard solution was found to be a good relation for all flavonoids (correlation coefficient>0.99), highly reliable and accurate for quantification purposes. The LOD and LOQ of the analytes were measured by analyses of serially diluted standard solutions. All flavonoids were in the ranges of 0.1-0.4 and 0.4-1.3 µg/injection respectively. This indicates that this method can be used to detect trace amounts of these flavonoids and quantify them in crude extracts of M. *hexandra* stem bark and formulation products containing the extract (Table 1).

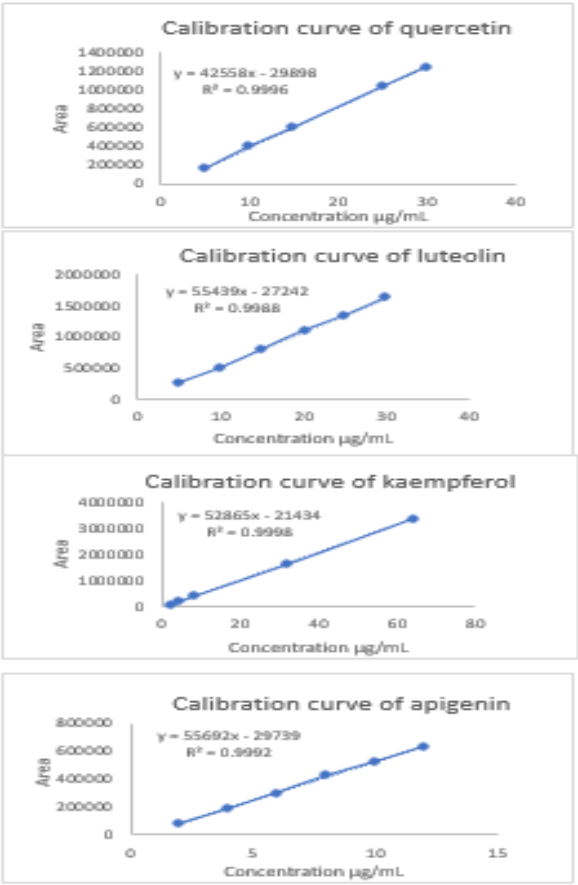

Figure 3: Calibration curve of quercetin, luteolin, kaempferol and apigenin.

Table 1: Calibration curve data for quercetin, luteolin, kaempferol and apigenin.

Standards	Calibration curve	Correlation coefficient (r^2)	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Quercetin	y=42558x-29898	0.9996	5-30	0.3128	0.948
Luteolin	y=55439x-27242	0.9988	5-30	0.4589	1.3908
Kaempferol	y=52865x-21434	0.9998	2-64	0.4077	1.2356
Apigenin	y=55692x-29739	0.9992	2-12	0.1494	0.453

System suitability was established by injecting six replicate injections (50 µL mL^{-1}) of standard solution the % Relative Standard Deviation (%RSD) of retention time, tailing factor and theoretical plates were determined (Table 2).

Table 2: Result data of system suitability study.

Standards	Area (Mean ± SD)	% RSD	Retention time	Theoretical plate number (N)	Tailing factor (T)
Quercetin	1846526 ± 2380.79	0.128	8.862 ± 0.050	5507	1.064
Luteolin	2608635 ± 2318.71	0.123	10.538 ± 0.042	6815	1.066
Kaempferol	2704874 ± 839.40	0.031	13.695 ± 0.048	8559	1.051
Apigenin	2430387 ± 3491.12	0.143	15.259 ± 0.094	9730	1.019

Intra-assay precision was assessed by analyzing five sets of samples, independently prepared at low, middle and high concentrations. Inter-assay precision and accuracy were tested over 3 days, with the samples prepared each day. The % RSD values of both intra-assay and inter-assay precision were below 0.579% (Table 3).

Table 3: Result data of intra and inter-day precision.

Standards	Concentration (µg/mL)	Intra-day (n=3)		Inter-day (n=3)	
		Area mean ± SD	RSD (%)	Area mean ± SD	RSD (%)
Quercetin	5	167084 ± 514.84	0.308	175047 ± 657.80	0.375
	15	604048 ± 699.65	0.115	614816 ± 1396.35	0.227
	30	1234915 ± 2268.08	0.184	1249951 ± 2978.26	0.237
Luteolin	5	267806 ± 2377.77	0.887	270701 ± 1479.44	0.546
	15	787428 ± 2492.57	0.316	807954 ± 7193.37	0.29
	30	1642146 ± 8721.91	0.531	1639852 ± 3304.33	0.201
Kaempferol	2	97536 ± 894.76	0.917	98579 ± 169.77	0.172
	8	413034 ± 1750.00	0.423	414433 ± 595.52	0.143
	64	3363221 ± 33148.09	0.985	3344987 ± 4073.69	0.121
Apigenin	2	80564 ± 511.76	0.635	81094 ± 353.32	0.435
	6	303551 ± 438.90	0.144	305066 ± 1766.81	0.579
	12	524550 ± 841.04	0.16	524848 ± 1091.02	0.207

The recovery remained between 98.06%-100.65%. The results of how accurately recovered from the extract were satisfactory for the quantitative analysis of the flavonoids (Table 4).

Table 4: Analytical recovery of quercetin, luteolin, kaempferol and apigenin standard solution added to a known concentration of ESB sample.

Standard	Level of recovery %	Amount of extract (µg/mL)	Amount (Std.) added (µg/mL)	Amount found (n=3) (µg/ml)	%Recovery	%RSD
Quercetin	50	18.4213	10	28.5034	100.82 ± 1.40	0.89
	100	18.4213	20	38.3364	99.57 ± 0.65	
	150	18.4213	30	48.1465	99.08 ± 1.02	
Luteolin	50	18.4545	10	28.3393	100.65 ± 0.74	0.41

	100	18.4545	20	38.2393	99.82 ± 0.65	
	150	18.4545	30	48.3283	100.18 ± 1.02	
Kaempferol	50	6.5019	3	9.5093	100.24 ± 1.43	0.68
	100	6.5019	6	12.4358	98.89 ± 1.06	
	150	6.5019	10	16.4718	99.69 ± 0.98	
Apigenin	50	3.8438	2	5.9823	98.8469 ± 0.71	0.54
	100	3.8438	4	7.9689	99.0869 ± 0.83	
	150	3.8438	6	9.8891	98.0622 ± 0.65	

Deliberate changes in different parameters like flow rate (1.0 mL ± 0.1 mL), mobile phase (60:40 ± 5) and column oven temperature (25 ± 5°C) were done while injecting and % RSD of peak area were observed to indicate that the method was robust (Table 5).

Table 5: Result data of the robustness study.

Standards	Parameters	Area mean ± S.D.	% RSD
Flow rate mL/min			
Quercetin (100 µg/mL)	0.9	4256811 ± 30900.20	0.725
	1.1	3508035 ± 5475	0.156
Luteolin (100 µg/mL)	0.9	5205570 ± 23395.41	0.449
	1.1	4031637 ± 12744.07	0.316
Kaempferol (100 µg/mL)	0.9	5256684 ± 20516.59	0.39
	1.1	4340756 ± 5796.78	0.133
Apigenin (100 µg/mL)	0.9	5436149 ± 6228.7	0.114
	1.1	4473496 ± 9659.55	0.215
Column oven temperature (°C)			
Quercetin (100 µg/mL)	20°C	3838663 ± 4058.48	0.105
	30°C	3835790 ± 22565.41	0.588
Luteolin (75 µg/mL)	20°C	3549095 ± 9274.13	0.278
	30°C	3549095 ± 14806.80	0.416
Kaempferol (100 µg/mL)	20°C	4761063 ± 12949.52	0.271
	30°C	4717078 ± 35750.07	0.757
Apigenin (75 µg/mL)	20°C	3719263 ± 6617.29	0.177
	30°C	3678650 ± 24851.36	0.675
Mobile phase			

Quercetin (100 µg/mL)	55:45	3819504 ± 5440.73	0.177
	65:35	3850773 ± 5008.41	0.675
Luteolin (75 µg/mL)	55:45	3333617 ± 9274.63	0.278
	65:35	3552428 ± 14806.36	0.416
Kaempferol (100 µg/mL)	55:45	4761063 ± 12949.52	0.271
	65:35	4717078 ± 35750.07	0.757
Apigenin (75 µg/mL)	55:45	3719263 ± 6617.29	0.17792
	65:35	3678650±24851.36	0.675557

Note: *SD: Standard deviation; RSD: Relative Standard Deviation

Quantification

The ethyl acetate Extract of Stem Bark (ESB) was found to contain 0.8928% w/w of quercetin, 0.8944% w/w of luteolin, 0.3115% w/w of kaempferol and 0.1863% w/w of apigenin. Quercetin and luteolin were found to be the most abundant flavonoids [10].

CONCLUSION

This study aimed to establish and validate an HPLC method that is capable of simultaneously identifying and quantifying four predominant flavonoids (quercetin, luteolin, kaempferol and apigenin) in *M. hexandra*. The validation outcomes demonstrated the method's sensitivity, accuracy and reproducibility. Subsequently, the developed method was effectively employed to determine the flavonoid content in the ESB sample obtained through different extraction methods. As a result, this method holds promise for the quality assessment of formulation products containing *M. hexandra* extract.

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REFERENCES

1. Ahad B, Shahri W, Rasool H, Reshi ZA, Rasool S, Hussain T. Medicinal plants and herbal drugs: An overview. Med Aromat Plants Healthcare Ind Appl. 2021:1-40.
2. Durazzo A, Lucarini M, Souto EB, Cicala C, Caiazzo E, Izzo AA, et al. Polyphenols: A concise overview on the chemistry, occurrence and human health. Phytother Res. 2019;33(9):2221-2243.
3. Singh P, Arif Y, Bajguz A, Hayat S. The role of quercetin in plants. Plant Physiol Biochem. 2021;166:10-19.
4. Chen J, Zhong H, Huang Z, Chen X, You J, Zou T. A critical review of kaempferol in intestinal health and diseases. Antioxidants. 2023;12(8):1642.
5. Yao C, Dai S, Wang C, Fu K, Wu R, Zhao X, et al. Luteolin as a potential hepatoprotective drug: Molecular mechanisms and treatment strategies. Biomed Pharmacother. 2023;167:115464.
6. Lu P, Zhang T, Ren Y, Rao H, Lei J, Zhao G, et al. A literature review on the antiviral mechanism of luteolin. Nat Prod Commun. 2023;18(4):1934578X231171521.
7. Maria do Socorro S, Behrens MD, Moragas-Tellis CJ, Penedo GX, Silva AR, Gonçalves-de-Albuquerque CF. Flavonols and flavones as potential anti-Inflammatory, antioxidant and antibacterial compounds. Oxid Med Cell Longev. 2022;2022:9966750.
8. Ali AH. High-Performance Liquid Chromatography (HPLC): A review. Ann Adv Chem. 2022;6(1):10-20.
9. Abd El-Mordy FM, El-Hamouly MM, Ibrahim MT, Abd El-Rheem G, Aly OM, Abd El-Kader AM, et al. Inhibition of SARS-CoV-2 main protease by phenolic compounds from *Manilkara hexandra* (Roxb.) Dubard assisted by metabolite profiling and *in silico* virtual screening. RSC Adv. 2020;10(53):32148-32155.
10. Patel K, Ali AK, Nair N, Kothari V. *In vitro* antibacterial activity of *Manilkara hexandra* (Sapotaceae) seed extracts and violacein against multidrug resistant Streptococcus mutans. J Nat Remedies. 2015;15(1):1-11.