



Received on 21 February 2023; received in revised form, 06 July 2023; accepted 15 July 2023; published 01 October 2023

## QUANTITATIVE SIMULTANEOUS RP-HPLC ESTIMATION OF GALLIC ACID, QUERCETIN AND GLYCYRRHIZIN IN THE METHANOLIC EXTRACTS OF *ABRUS PRECATORIUS* AND *CORDIA WALLICHI* LEAVES

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### Keywords:

Simultaneous quantification, RP-HPLC, Biomarkers, *Abrus precatorius*, *Cordia wallichi*

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**ABSTRACT: Background:** Important mistletoe species include *Abrus precatorius* (Linn.) and *Cordia wallichi* (Don). According to reports, the leaves of *Abrus precatorius* (L.) have neuromuscular, antiepileptic, anti-diabetic, and many other properties. Leaves are traditionally administered to cuts, swellings, and mouth ulcers as a nerve tonic. Less studies have been recorded on the species of *wallichii*, compared to other species of the genus. According to reports, both plants' leaves include alkaloids, flavonoids, tannins, terpenoids, saponins, steroids, and glycyrrhizin (*Abrus*). **Objective:** A simple, accurate and reproducible RP-HPLC method has been developed for the simultaneous quantification of Gallic acid, Quercetin and Glycyrrhizin equivalents in the methanolic extracts of *Abrus precatorius* (MEAP) and *Cordia wallichi* leaves (MECW). **Material and Methods:** The RP-HPLC method was carried out in reverse phase C18 column, (5  $\mu$ m, 250mm x 4.6mm i.d) Dionex Ultimate 3000 liquid chromatography and detection was carried out at 254nm, using a mobile phase of Water: Acetonitrile: Methanol (pH 3.5) (15:15:70 v/v/v) with Isocratic reverse phase technique. **Results:** The experimental results showed the amount of Gallic acid in the methanolic extracts of *A. precatorius* and *C. wallichi* leaves (0.63% and 0.36% respectively), Quercetin (0.16% and 0.24% respectively), and Glycyrrhizin 0.11% in the extract of *A. precatorius* only. The high percentage of recovery (96-103%), low coefficient of variation ( $R^2 > 0.99$ ) and low limit of detection (LOD), and limit of quantitation (LOQ) confirm the suitability of the method for simultaneous quantification of these three biomarkers in the two plants under investigation. **Conclusion:** This RP-HPLC may be useful for quantitative estimation of the chemical constituents present in the herbal product.

**INTRODUCTION:** Medicinal plants are also major raw materials for the pharmaceutical industry and thus have gained importance in the global drug market. However, the unavailability of documentation and rigorous quality control acts as a key hindrance in the approval of herbal products as alternative medicine in developed countries.

*Abrus precatorius* (L.) (Family Fabaceae) is a perennial climber, native to India and found throughout the tropical regions of the world. It is used in traditional medicine for the treatment of a wide range of ailments.

The leaves are used as sweeteners and also have therapeutic effects in treating fever, cough and cold while the seeds have been used in the treatment of worm infection, vitiligo patches, baldness<sup>1</sup>, aphrodisiac, anti-diabetic, anti-cancer, anti-oxidative, anti-inflammatory, anti-microbial, anti-fertility in males, abortifacient in females<sup>2, 3, 4</sup>. The seed kernels are found to contain flavonoids, abrectorin and glycoside semethoxycentaureidin 7-

	<b>QUICK RESPONSE CODE</b> <b>DOI:</b> 10.13040/IJPSR.0975-8232.14(10).4929-36
	This article can be accessed online on <a href="http://www.ijpsr.com">www.ijpsr.com</a>
DOI link: <a href="https://doi.org/10.13040/IJPSR.0975-8232.14(10).4929-36">https://doi.org/10.13040/IJPSR.0975-8232.14(10).4929-36</a>	

O-rutinoside<sup>5</sup>, 8-C glucosylscutellarine<sup>6</sup>, 7-dimethyl ether, 2-O-apioside flavones C-glycoside, alkaloids, methyl ester of N-N dimethyl-tryptophanmethylation and precatorine<sup>6</sup>, indole derivatives, anthocyanins, sterols, terpenes. Despite many reports on the medicinal properties of *Abrus precatorius* and *Cordia wallichii* leaves, no paper has reported the simultaneous quantification of Gallic acid, Quercetin and Glycyrrhizin in the selected plants using RP-HPLC. Consequently, present study was focused on the quantitative estimation in the methanolic extracts of *Abrus precatorius* and *Cordia wallichii* leaves for the identification of Gallic acid, Quercetin and

Glycyrrhizin equivalents by Reverse phase high performance thin layer chromatography. Three major chromatographic peaks were detected, attributed to, Gallic acid, Quercetin and Glycyrrhizin which have all been reported as major phytoconstituents for healing many disorders. This article presents a simple, accurate, reproducible and thoroughly validated RP-HPLC-based method for qualitative and quantitative analysis of these three phytoconstituents, as part of the quality assessment of products containing methanolic extracts of *Abrus precatoriu s*(MEAP) and *Cordia wallichii* leaves (MECW).

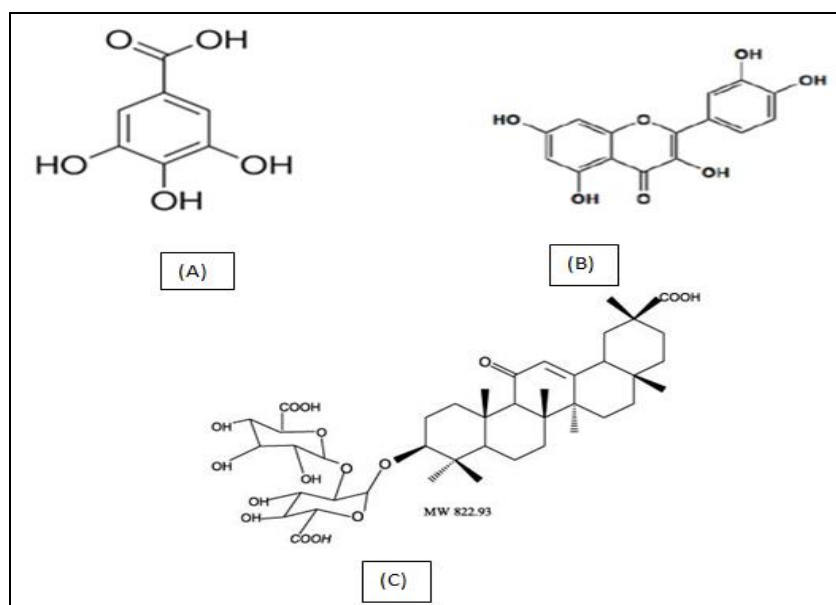


FIG. 1: THE CHEMICAL STRUCTURES (A) GALLIC ACID, (B) QUERCETIN AND (C) GLYCYRRHIZIN

## MATERIALS AND METHODS:

**Collection and Extraction of Plants:** The authentic plant materials of *Abrus precatorius* and *Cordia wallichii* leaves were collected from Kamrup district, Assam and identified and authenticated by Dr. T. G. Gohil, taxonomist and HOD of Botany, Botanist in B.K.M Science College, Valsad (Gujarat). The voucher specimens Ref. no. (BKM/Bio/37/2018) were deposited Botany Department of BKM Science College, Valsad, Gujarat for further reference. Both the plant leaves were washed with water to remove any dust particles, shade dried, powdered and then sieved through BSS mesh size 85 and stored at 25°C in an airtight container for extraction. The dried plant materials were pulverized by using a mechanical grinder to make a coarse powder. Then 940 gm of powder was soaked with 95% methanol at room

temperature (25°C) for successive extraction. The whole extract was collected, and filtered and the solvent was evaporated to dryness under reduced pressure and temperature (45 °C) by using DOLPHIN Rotary Evaporator (Mumbai, India). The yield of methanolic extracts of *Abrus precatorius* and *Cordia wallichii* were found to be 12.8% w/w and 5.2% w/w respectively. Dried extracts were stored at 4 °C for further use<sup>7,8,9</sup>.

**Chemicals:** Reference standards of Gallic acid (GA), Quercetin (QUE) and glycyrrhizin (GLY) were purchased from Sigma-Aldrich GmbH, Germany. All other solvents and chemicals were of the highest analytical grade. The HPLC-grade solvents such as methanol, acetonitrile and water were purchased from Merck (Germany).

**RP-HPLC Instrumentation and Chromatographic Conditions:** HPLC analyses were performed with Shimadzu LC2010 CHT integrated assembly, equipped with a UV detector, quaternary pump and autosampler, a manual sample injection valve equipped with a 20 $\mu$ L loop and LC solution software: Version: 1.25 as data processor. The separation was achieved by a reversed-phase Phenomenex Gemini C18 column, (5  $\mu$ m, 250 mm x 4.6mm i.d). The mobile phase Acetonitrile: Methanol: Water (pH 3.5 with orthophosphoric acid) (15:15:70 v/v/v) finally adopted with a flow rate of 1  $\mu$ L min<sup>-1</sup> and column compartment temperature of 25°C. The detection wavelength was 254 nm, followed by washing and reconditioning the column. The chromatographic peaks of the analytes

were confirmed by their retention times and UV spectra with those of the reference standards. Working standard solutions were injected into the HPLC, and peak area responses obtained. Standard graphs were prepared by plotting concentration ( $\mu$ g mL<sup>-1</sup>) versus peak area. Gallic acid, quercetin and glycyrrhizin were quantified by an external standard method.

**Selection of Wavelength:** A UV spectrum of Gallic acid, Quercetin and Glycyrrhizin in methanol was noted by scanning the solution in the range of 200-400 nm, Gallic acid, Quercetin, and Glycyrrhizin were showing significant absorption at 248 nm. Thus, 248 nm was selected as the wavelength for analysis.

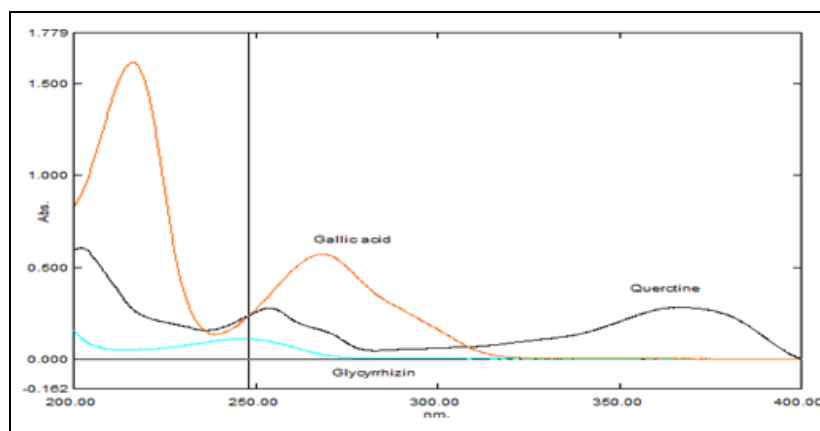


FIG. 2: AN UV OVERLAY SPECTRA OF QUERCETIN, GALLIC ACID AND GLYCYRRHIZIN IN METHANOL (248NM)

#### Preparation of Standard and Sample Solutions:

A standard solution of gallic acid, quercetin and glycyrrhizin were prepared in methanol (1 mg/mL. It was further diluted to 100 $\mu$ g/ml in methanol. Calibration samples were prepared in the range of 40-200  $\mu$ g/ml. The plant extracts, 1 gm powder extract with 25 ml methanol. The process was repeated three times, combining the methanolic extracts and volume was made up to 100 ml with methanol both the standard and sample solutions were filtered through Whatman NYL 0.45 mm syringe filter. The resulting solution was injected to find the amount of components present in the powder. The responses were measured as peak areas vs concentration.

**Preparation of Mobile Phase:** Mobile phase was prepared by using Acetonitrile: Methanol: Water (15:15:70 v/v/v). The pH of water was adjusted 3.5 with orthophosphoric acid. The orthophosphoric

acid and all the solvents were filtered through 0.45 mm Millipore membrane filter followed by ultrasonication to de-gas the solvent system.

**Method Validation:** The method was validated for linearity, specificity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision according to ICH guidelines<sup>10</sup>.

**Linearity:** The linearity range of oleanolic acid was analyzed (n=6) of the standard solutions containing gallic acid, quercetin and glycyrrhizin of 40-200  $\mu$ g/ml in the optimized chromatographic conditions. The calibration curve was made by plotting the main peak area in Y-axis vs the concentration in X-axis and linearity was determined by the linear regression analysis.

**Specificity:** The specificity of the method was determined by comparing the retention time of the

standard and test samples. This mainly ensures the identity and the purity of the analyte and to minimize the error of the result.

**Limit of Detection (LOD) and Limit of Quantification (LOQ):** The LOD and LOQ were calculated based on the ICH guideline by determining the SD of the response ( $\sigma$ ) and the slope of the linear equation (S). The LOD and LOQ were calculated by the following equation;

$$\text{LOD} = 3.3 \sigma / S$$

$$\text{LOQ} = 10 \sigma / S$$

**Accuracy and Precision:** Intra-day and inter-day assay accuracy and precision for each analyte were determined at Low quality control (LQC), Medium quality control (MQC) and High quality control (HQC). Both data were assessed by comparing the data within one run ( $n = 6$ ). Accuracy of the method was determined by standard addition technique and expressed in terms of % RSD. The precision of the method was analyzed by performing intra-day and inter-day variation, assessed by injecting six replicates at three different concentrations of the reference compounds. The values were represented as % RSD.

**Robustness:** Robustness study was performed by changing different mobile phase composition, flow

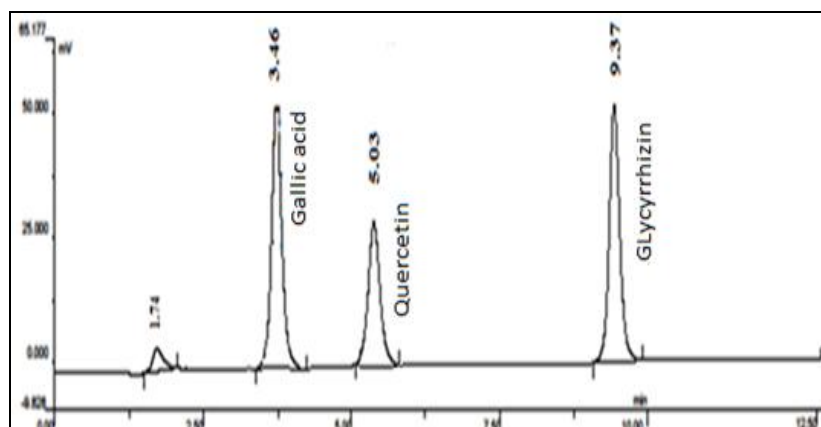
rate and detection of wave length to determine their influence on the retention time. Statistical analysis was performed using the Graph Pad Prism Version 5.0 and results are represented as the mean  $\pm$  % RSD.

## RESULTS:

**Optimization of Chromatographic Conditions:** The chromatographic separation was determined through Isocratic reversed-phase technique. The separation was achieved by a reversed-phase Phenomenex Gemini C18 column, (5  $\mu\text{m}$ , 250mm x 4.6mm i.d). The mobile phase Acetonitrile: Methanol: Water (pH 3.5 with orthophosphoric acid) (15:15:70 v/v/v) finally adopted with a flow rate of 1  $\mu\text{L min}^{-1}$  and column compartment temperature of 25°C. The detection wavelength was 248 nm. Sharp, symmetrical and with high resolution were obtained at Retention Time (Rt) 3.46 $\pm$ 0.01, 5.03 $\pm$ 0.02 and 9.37 $\pm$ 0.03 for gallic acid, quercetin and glycyrrhizin respectively. Quantification of the selected three markers in the selected plants was done with respect to a linear regression equation. The results of the quantification of active markers in the MEAP and MECW are summarized in **Table 1**. **Fig. 3, 4, 5** represents the chromatograms for standard markers as well as in MEAP and MECW at optimum chromatographical conditions,

**TABLE 1: ASSAY RESULTS IN THE EXTRACTS**

Methanolic extracts	Peak area of Standard (AUC)			Peak Area of the sample (AUC)			% Content		
	GAE	QE	GE	GAE	QE	GE	GAE	QE	GE
<i>Abrus precatorius</i>	29947 $\pm$	21596 $\pm$	24295 $\pm$	9475 $\pm$	1699 $\pm$	1305 $\pm$	0.63	0.16	0.11
	09.51	71.81	22.52	09.50	08.91	10.01			
<i>Cordia wallichii</i>	29947 $\pm$	21596 $\pm$	ND	5458 $\pm$	2598 $\pm$	ND	0.36	0.24	ND
	09.51	71.81		11.01	09.52				



**FIG. 3: CHROMATOGRAM OF STANDARDS USING MOBILE PHASE WATER: ACETONITRILE: METHANOL (15: 15: 70V/V/V)**

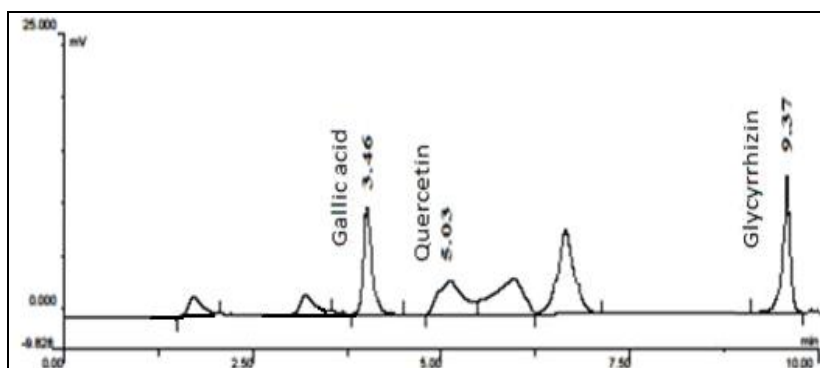


FIG. 4: CHROMATOGRAMS OF METHANOLIC EXTRACTS OF *ABRUS PRECATORIUS* LEAVES (MEAP) USING MOBILE PHASE WATER: ACETONITRILE: METHANOL (15:15:70V/V/V)

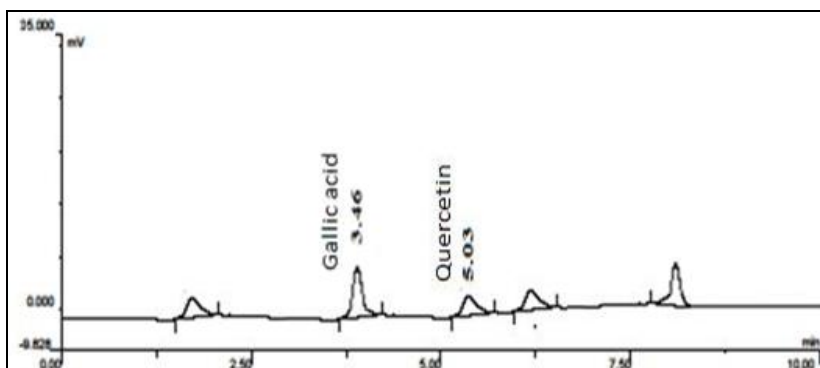


FIG. 5: CHROMATOGRAMS OF METHANOLIC EXTRACTS OF *CORDIA WALLICHI* LEAVES (MECW) USING MOBILE PHASE WATER: ACETONITRILE: METHANOL (15:15:70V/V/V)

**Linearity:** The calibration range of for gallic acid, quercetin and glycyrrhizin were found to be 40-200 µg/mL. The coefficient of determinants ( $r^2$ ) of for gallic acid, quercetin and glycyrrhizin were found

to be 0.996 with linear equation  $Y=524562X-116219$ ,  $Y= 9470X+26811$  and  $Y=11063X+17379$  respectively.

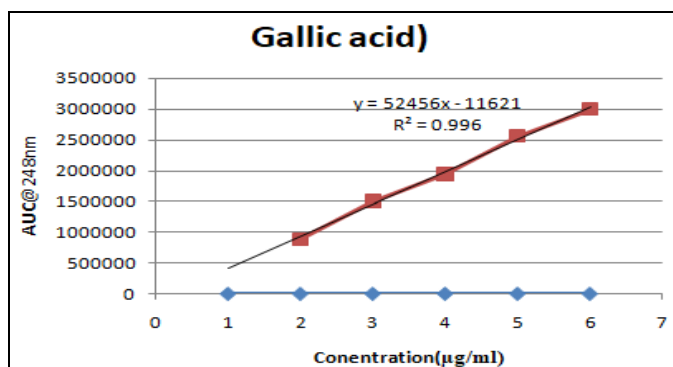


FIG. 6: GRAPH REPRESENTING THE CALIBRATION CURVE OF GALLIC ACID

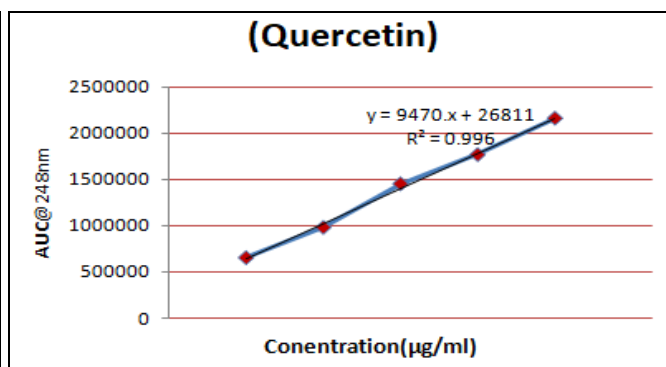


FIG. 7: GRAPH REPRESENTING THE CALIBRATION CURVE OF QUERCETIN

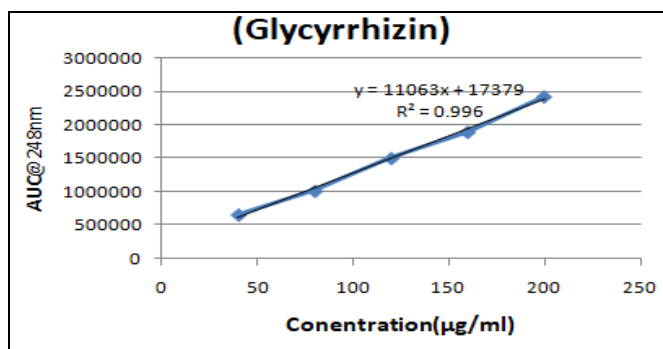


FIG. 8: GRAPH REPRESENTING THE CALIBRATION CURVE OF GLYCYRRHIZIN

**Specificity:** By the specificity test, the well-shaped peak indicated that other constituent present in the methanolic extracts of the leaves of *Abrus precatorius* and *Cordia wallichii* does not interfere with the main peak of gallic acid, quercetin and glycyrrhizin.

**Limit of Detection (LOD) and Limit of Quantification (LOQ):** The LOD and LOQ of gallic acid, quercetin and glycyrrhizin were found to be  $14.64 \pm 0.63$  and  $44.37 \pm 0.87$   $\mu\text{g}/\text{mL}$ ,  $13.45 \pm$

$0.92$  and  $40.75 \pm 0.43$  and  $15.01 \pm 0.22$  and  $45.47 \pm 0.82$  respectively.

**Accuracy:** High recoveries were obtained by the fortification of the sample at three QC levels for GA, QUE and GLY. It is evident from the results that the percent recoveries for three markers after sample processing and applying were in the range of 98.23-99.69% (gallic acid), 98.79-99.57% (quercetin) and 99.01-99.51% (glycyrrhizin) as shown in **Table 2**.

**TABLE 2: RECOVERY STUDIES FOR DETERMINATION OF GALLIC ACID, QUERCETIN AND GLYCYRRHIZIN IN ABRUS PRECATORIUS**

Biomarker	Amount added	Sample concentration ( $\mu\text{g}/\text{mL}$ )	Theoretical concentration ( $\text{mg}/\text{mL}$ )	Actual concentration ( $\text{mg}/\text{mL}$ )	Percentage recovery
Gallic acid	50	63	113	111	98.23
	100	63	116	162.5	99.69
	150	63	213	209.9	98.54
Quercetin	50	16	66	65.2	98.79
	100	16	116	115.5	99.57
	150	16	166	165.2	99.52
Glycyrrhizin	50	11	61	60.7	99.51
	100	11	111	109.9	99.01
	150	11	161	159.95	99.35

The % RSD of intra-day and inter-day precision was found to be  $<2\%$ , which confirms high repeatability of the method. Results are presented in **Table 3**.

**TABLE 3: INTRA-DAY AND INTER-DAY PRECISION OF FOR GALLIC ACID, QUERCETIN AND GLYCYRRHIZIN BY USING HPLC METHOD**

Gallic acid							
Intra Day(n=6)				Inter Day(n=6)			
Rt(Min)		Response(AU)		Rt(Min)		Response(AU)	
Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
3.46	0.242	893354	0.01	3.45	0.508	893357.33	0.01
3.43	0.160	1947737	0.002	3.44	0.622	1947563.7	0.021
3.45	0.351	2994690	0.007	3.45	0.367	2994763.3	0.001
Quercetin							
Intra Day(n=6)				Inter Day(n=6)			
Rt(Min)		Response(AU)		Rt(Min)		Response(AU)	
Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
5.05	0.583	657824	0.011	5.05	0.593	657610.83	0.062
5.07	0.738	1452992	0.033	5.06	0.537	1452532.3	0.041
5.06	0.570	2159476	0.018	5.05	0.560	2159624.2	0.004
Glycyrrhizin							
Intra Day(n=6)				Inter Day(n=6)			
Rt(Min)		Response(AU)		Rt(Min)		Response(AU)	
Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
9.35	0.416	658117.8	0.015	9.35	0.410	658307.83	0.048
9.35	0.468	1505401	0.003	9.33	0.491	1505420.3	0.004
9.34	0.397	2429046	0.034	9.34	0.382	2429543	0.002

**Robustness:** The robustness was evaluated by analyzing (n= 6) the standard solution of gallic acid, quercetin and glycyrrhizin ( $120\mu\text{g}/\text{mL}$ ) under the small changes ( $\pm 2$ ) in the optimum conditions such as column temperature, flow rate, detection of

wavelength and pH. But no significant changes were observed in the retention time, peak area, and recovery study.

### Content of Gallic Acid, Quercetin and Glycyrrhizin Methanolic Extracts of *Abrus precatorius* (MEAP) and *Cordia wallichii* Leaves (MECW):

The mean retention time of gallic acid, quercetin and glycyrrhizin were observed  $3.46 \pm 0.02$ ,  $5.03 \pm 0.04$  and  $9.37 \pm 0.02$ . The experimental results showed the amount of Gallic acid in the methanolic extracts of *A. precatorius* (L.) and *C. wallichii* (D.) leaves ( $0.63\%$  w/w and  $0.36\%$  w/w respectively), Quercetin ( $0.16\%$  w/w and  $0.24\%$  w/w respectively) and Glycyrrhizin  $0.11\%$  w/w in the extract of *A. precatorius* only.

**DISCUSSION:** Chemical marker plays an important role to ensure the quality of medicinal plants and their products. The limited evidence of chemical marker remains a major problem for the quality control of herbal medicines. Keeping in view, our present study dealt with the simultaneous estimation of gallic acid, quercetin and glycyrrhizin in HPLC and RP-HPLC method development in the methanolic extracts of the leaves of selected plants to ensure the content of active Phyto molecule responsible for physiological activity<sup>11, 12</sup>.

A validated RP-HPLC method has been developed for the simultaneous determination of gallic acid, quercetin in the methanolic extracts of *Abrus precatorius* and *Cordia wallichii* leaves. Thus, it is utmost essential to determine most of the phytochemicals of medicinal plant products in order to ensure the reliability and repeatability of pharmacological research to ensure the quality of the medicinal plant products<sup>13</sup>. Our present study dealt with the RP-HPLC method validation of leaves of *Abrus precatorius* and *Cordia wallichii* to ensure the content of active Phyto molecules as well as reproducibility of the developed method<sup>14</sup>. The developed method was validated to quantify the amount of gallic acid, quercetin in the methanolic extracts of *Abrus precatorius* and *Cordia wallichii* leaves. This method was also found accurate, specific, precise, robust and reproducible with a narrow linearity range<sup>15-18</sup>.

**CONCLUSION:** HPLC fingerprinting, estimation, and method validation of the methanolic extract of leaves of the selected plants were performed which confirmed the presence of the flavonoid quercetin, gallic acid, and glycyrrhizin equivalents in the

extracts, which will definitely help future researchers for quantification of plant phytoconstituents.

**ACKNOWLEDGEMENTS:** Authors wish to acknowledge, Entire team of Emami Limited Masat, Quality Control Department, Dadra and Nagar Haveli (U.T) India, for providing excellent facilities to carry out HPLC work. We also would like to extend our sincere thanks to Dr. Prateek Patel, Quality Control Head, Emami Limited, Vapi (Gujarat) Plant for his unprecedented support and encouragement for development and providing HPLC facilities for this research work.

**CONFLICTS OF INTEREST:** We declare that we have no conflict of interest.

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**How to cite this article:**

Sengupta R: "Quantitative simultaneous RP-HPLC estimation of gallic acid, quercetin and glycyrrhizin in the methanolic extracts of *Abrus precatorius* and *Cordia wallichii* leaves". *Int J Pharm Sci & Res* 2023; 14(10): 4929-36. doi: 10.13040/IJPSR.0975-8232.14(10).4929-36.

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