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SIMULTANEOUS QUANTIFICATION OF THREE NAPHTHOQUINONES FROM *IMPATIENS BALSAMINA* L. LEAVES USING VALIDATED RP-HPLC METHOD

A. A. Sonandkar *, P. N. Agrawal, D. M. Madrewar, S. M. Labana and A. P. Jain

Prin. K.M. Kundnani College of Pharmacy, Cuffe Parade, Mumbai - 400005, Maharashtra, India.

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Correspondence to Author:
Abhijeet A. Sonandkar

M. Pharm Pharmaceutical Analysis, TF-5, Laxminarayan Apt., S.S. Bazar, Solapur - 413003, Maharashtra, India.

E-mail: jeet_as@rediffmail.com

ABSTRACT: *Impatiens balsamina* belonging to family Balsaminaceae is commonly known as terda. The plant has an extensive range of phyto-constituents like naphthoquinones, coumarins, glycosides, phenolic acids, flavonoids, anthocyanidins and steroids which have effective antimicrobial, anti-anaphylaxis, anti-allergic, antipruritic, and anti-inflammatory activity. Potent naphthoquinones like lawsone, methylene-3, 3'-bilawsone and lawsone methyl ether are present in the leaves of the plant that exhibits was proved to possess an intensive antitumor activity. Taking into account these ethnobotanical, nutritional, medicinal and commercial values of *I. balsamina*, a simple, rapid and sensitive High-Performance Liquid Chromatography method for quantification of three naphthoquinones in is developed. Jasco CrestPak C8 (4.6×250mm, 5µm) RP-HPLC column was used as the stationary phase. Sodium acetate (pH3.2): ACN (50:50% v/v) was used as mobile phase with flow rate 1ml/min and detection at a wavelength of 280nm using PDA multi-wavelength Detector. The developed method was validated for linearity, accuracy, precision, and system suitability. LOD for naphthoquinones was found to be 200ng/mL, whereas LOQ for the same was found to be 600ng/mL. The method was found to be linear over the range 5 µg/mL to 15 µg/mL with the coefficient of regression 0.997, 0.990 and 0.998 for lawsone, methylene-3, 3'-bilawsone and lawsone methyl ether respectively. The result indicated that the developed HPLC method could be successfully used for quantitative estimation of naphthoquinones from *Impatiens balsamina*.

INTRODUCTION: *Impatiens balsamina*, a traditional plant has gained popularity recently due to the expanding scope of biological activities. The groups of compounds commonly found in this plant are naphthoquinones, coumarins, phenolic acids, flavonoids, anthocyanidins, and steroids. Flavonoids and naphthoquinones have strong antimicrobial [1], anti-anaphylaxis [2], anti-allergic [2,] and anti-inflammatory [3] activities.

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Lawsone and lawsone methyl ether **Fig. 1**, has been reported as the active constituents exhibiting antifungal and antibacterial activities [4, 5].

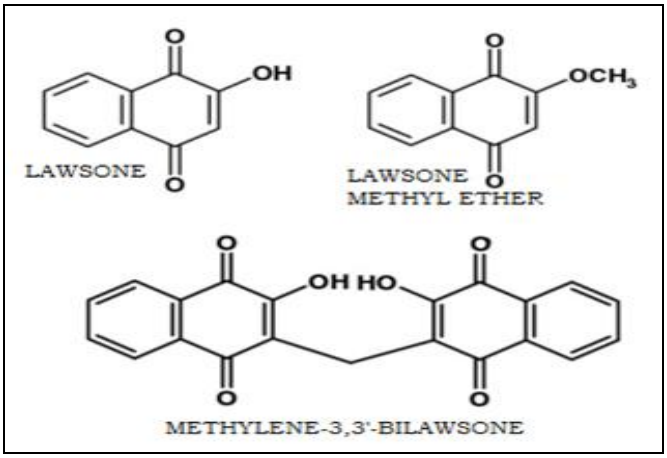


FIG. 1

Also, lawsone methyl ether exhibited an intensive anti-tumor activity against HepG2 cells [6]. Methylene-3, 3'- bilawsone **Fig. 1** has been reported as a naphthoquinone found in *I. balsamina*, which exhibited antipruritic activity [3].

Due to an intensive anti-tumor activity of these naphthoquinones, the economic importance of this plant has increased substantially, which focuses on an apt standardization method development using sophisticated instruments and techniques. Literature survey indicates few methods have been reported for estimation of naphthoquinones by HPLC[7] from *I. Balsamina.* However, the reported methods suffered from drawbacks such as less sensitivity, time-consuming, tedious, and laborious.

Hence, an attempt had been made to develop a new method to standardize *Impatiens balsamina L.* by simultaneous determination of three naphthoquinones which is easy, fast and more sensitive using chromatographic Technique like HPLC.

MATERIALS AND METHODS:

Working Standard: The working standard lawsone was purchased from Sigma-Aldrich, Steinheim, Germany. The other working standards lawsone methyl ether and methylene-3,3'-bilawsone were synthesized from procured lawsone.

Synthesis of Lawsone Methyl Ether: Lawsone methyl ether was prepared by methylation of lawsone under acid conditions. Briefly, lawsone (1.0 g) was dissolved in absolute methanol (50mL), and concentrated hydrochloric acid (0.8 mL) was added.

The mixture was heated under reflux conditions for 4 h. The mixture was then cooled to room temperature. The resulting yellow precipitate was recrystallised in a mixture of ethyl acetate and methanol, to give yellow needles of lawsone methyl ether.

Synthesis of methylene-3, 3'-bilawsone: Methylene-3, 3'-bilawsone was prepared by Mannich reaction between lawsone and p-nitroaniline. Briefly, lawsone (1.74 g) and p-nitroaniline (1.52 g) were dissolved in absolute ethanol (50 mL) at 25 °C, and 37% formaldehyde (1mL) was added to the solution. The yellow solid soon began to separate, and after 30 min, the mixture was boiled for 5 min and filtered. The yellow residue of m.p. 248-250 °C was identified as methylene-3, 3'-bilawsone. Both compounds were characterized by techniques like Mass Spectroscopy, NMR-Spectroscopy, IR finger printing, and UV Spectroscopy.

Plant material: The material, *i.e.* leaves of I. balsamina were collected from Keshav Shrushti plant nursery, Mumbai. The samples obtained were authenticated at Blatter Herbarium, St Xavier's college, Mumbai. They were tested for heavy metals, trace elements, and microbial contamination [8-9].

Equipment: The HPLC system employed for method development and validation was Jasco PU 2089 Plus Quaternary Gradient HPLC pump and Jasco MD-2018 Plus Photo Diode Array Detector with ChromNav software as data integrator. Jasco CrestPak C8 (4.6 × 250 mm, 5µm) column was used.

Chemicals and Solvents: All the chemicals and solvents used were of HPLC grade. Acetonitrile (Merck), Methanol (Merck), Sodium Acetate (Loba chemie), Glacial Acetic Acid (SD Fine) and Millipore system is used to make distilled water (Milli-Q) of HPLC grade.

EXPERIMENTAL WORK:

Preparation of Standards: 10 mg of lawsone, lawsone methyl ether and methylene-3,3'-bilawsone was dissolved in 10 ml of methanol separately to yield solutions of 1000 µg/mL of solutions.

These solutions were further diluted equimolarly to give a stock solution of 100 µg/mL of individual naphthoquinones concentration. Further dilutions were made as required with methanol.

Preparation of Sample: 100 gm of dried leaves powder of *I. balsamina* was extracted with 500 mL of methanol by soxhlet at 60 °C for 8 h. The extract was filtered under vacuum, and the pooled filtrate was concentrated under reduced pressure on Rotavapor at 40 ºC. This extract was diluted to get solution of 1 mg/mL and was used for further studies.

RP-HPLC Conditions: The mobile phase used was 50: 50 (% v/v) mixture of sodium acetate buffer (pH-3.2, adjusted with glacial acetic acid): Acetonitrile. The flow rate was adjusted to 1 ml/min. The column was equilibrated for 20 min with mobile phase before injection. The injection volume was 20µl, and detection was carried out at 280 nm.

Preparation of Mobile Phase: 8.203g of sodium acetate was dissolved in 1000ml of HPLC grade water (pH adjusted with glacial acetic acid to 3.2) to make a buffer. This was then mixed with acetonitrile in 50:50 ratio and filtered through the 45µ filter under vacuum filtration. It was then degassed in an ultrasonic bath for 15 min before use.

RESULTS AND DISCUSSION:

Optimization of Chromatographic Conditions: The main aim of this study was to develop an apt method of quantification of naphthoquinones from *I. balsamina*. The largest obstacle in this was that all three naphthoquinones were derivatives of lawsone and thus chemically very similar, making it difficult to get sufficient resolution by LC. Two columns, C8 and C18, were tested for separation. C18 column being more hydrophobic as compared to C8, retention of drug on C18 column was a problem. Hence, C8 column was selected. C8, also known as octylsilane, contains octyl chain in the periphery which comes in contact with the sample. This helps to retain relatively polar molecule more efficiently, as compared to C18.

Several solvent combinations without buffers were initially tried for as mobile phase. However, the peaks obtained were broad, and resolution in the extract was a problem. This was solved with the use of a buffer. Initially, Ammonium Acetate having buffering capacity at required pH range, *i.e.* pH 3.8-5.8 was tried. However, peak tailing was above 2. This indicated that the mobile phase was not strong enough to elute naphthoquinones.

Hence, Acetate buffer having a pH range 2.8-5.6 was tried. Low pH helped suppress silanol ionization, which helped reducing peak tailing problem. At lower pH values, the acid was present as a neutral molecule and was retained on the column. The mobile phase consisting of Sodium acetate buffer (pH 3.2): ACN (50:50) was used. This mobile phase composition resulted in symmetric peak with acceptable tailing factor (≤ 2).

Method Validation [10]:

Limit of Detection and Limit of Quantification: LOD and LOQ were determined by injecting progressively low concentration of standard solutions and were found to be 0.2µg/ml and 0.6µg/ml respectively. The LOD and LOQ values reveal that the developed method shows very good sensitivity.

Linearity and Range: Each set of linearity solutions were injected with optimized chromatographic conditions for five times. The calibration plot was found to be linear in the range 5µg/mL to 15µg/mL with a correlation coefficient 0.997, 0.990 and 0.998 for lawsone, methylene-3, 3'-bilawsone and lawsone methyl ether respectively. Linearity data and curve are given in **Table 1** and **Fig. 2**, respectively.

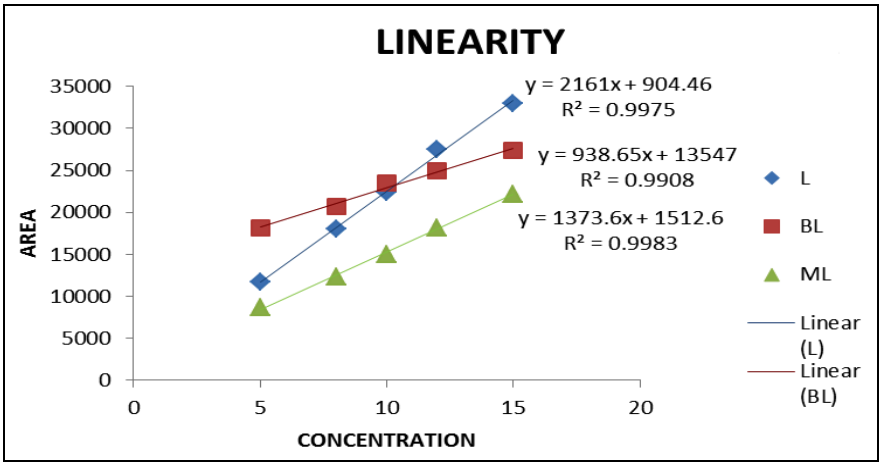


FIG. 2: LINEARITY

TABLE 1: DATA FOR LINEARITY OF NAPHTHOQUINONES

Conc. (µg/mL)	Lawsone	Methylene-3,3'-bilawsone	Lawsone methyl ether
	Mean area ± S.D; %CV		
5	11655.8 ± 183.53; 1.57	18190 ± 238.38; 1.31	8614.6 ± 105.35; 1.22
8	18065.4 ± 247.72; 1.52	20704.8 ± 91.83; 0.44	12277.2 ± 91.16; 0.74
10	22374.4 ± 288.46; 1.23	23470.6 ± 280.38; 1.19	15010.4 ± 66.19; 0.44
12	27542.8 ± 146.34; 0.53	24906.2 ± 88.43; 0.36	18135 ± 293.8; 1.62
15	32932 ± 310.52; 0.94	27397.8 329.82; 1.2	22205 ± 250.49; 1.13

Precision and Accuracy: Precision and accuracy studies were carried out at three different concentrations, namely 8, 10, 12 µg/ml. Precision was reported in terms of coefficient of variance (% CV) over the range of quantization for a single experiment in which standards were assayed in replicate (Intraday) and for a series of experiments in which standards were assayed in over several experiments (Interday). The % RSD was found to be within the specified limits (not more than 2) for intra and inter-day precision. The results in **Table 2** stand for the true goodness of method.

TABLE 2: DATA FOR ACCURACY AND PRECISION {INTER-DAY AND INTRA-DAY}

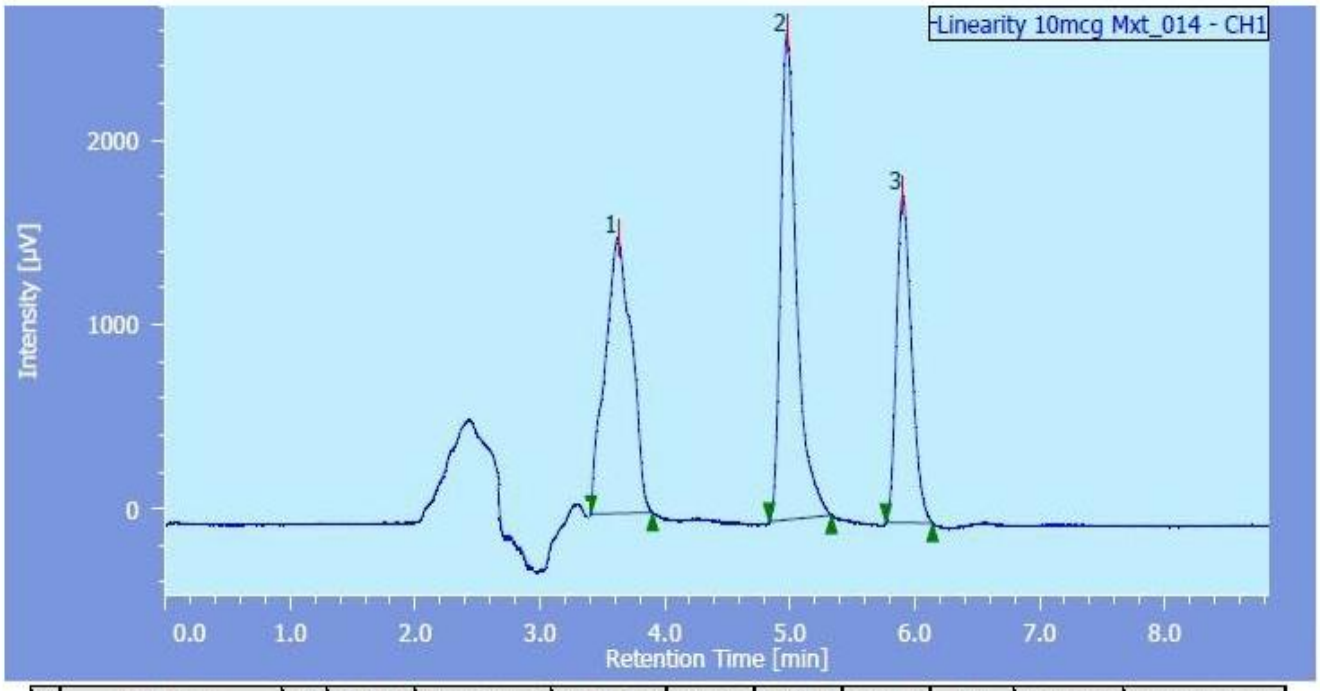
Lawsone				
Actual Concentration (µg/mL)	Precision		Accuracy	
	Measured Concentration (µg/mL), ± S.D; %CV			
	Intra-day	Inter-day	Intra-day	Inter-day
8	7.96 ± 0.06; 0.81	7.97 ± 0.05; 0.63	99.55	99.58
10	9.97 ± 0.11; 1.06	10.03 ± 0.11; 1.14	99.69	100.29
12	12.02 ± 0.04; 0.32	12.01 ± 0.04; 0.33	100.19	100.08
Lawsone methyl ether				
Actual Concentration (µg/mL)	Precision		Accuracy	
	Measured Concentration (µg/mL), ± S.D; %CV			
	Intra-day	Inter-day	Intra-day	Inter-day
8	8.00 ± 0.05; 0.58	7.99 ± 0.04; 0.49	99.97	99.85
10	10.01 ± 0.05; 0.53	10.01 ± 0.05; 0.45	100.06	100.15
12	11.99 ± 0.03; 0.27	12.01 ± 0.05; 0.38	99.91	100.04
methylene-3,3'-bilawsone				
Actual Concentration (µg/mL)	Precision		Accuracy	
	Measured Concentration (µg/mL), ± S.D; %CV			
	Intra-day	Inter-day	Intra-day	Inter-day
8	7.94 ± 0.08; 1.05	7.92 ± 0.09; 1.14	99.19	99.05
10	10.06 ± 0.10; 1.03	10.05 ± 0.08; 0.80	100.64	100.47
12	11.99 ± 0.03; 0.29	12.01 ± 0.05; 0.38	99.95	100.9

System Suitability: The working standard solution was prepared and injected 6 times into the HPLC system, and system suitability parameters were evaluated. Tailing factor, theoretical plates, RSD of retention time, and peak area is given in **Table 3**. All these parameters were found to be well within limits.

TABLE 3: RESULTS FOR SYSTEM SUITABILITY TESTING

Concentration (µg/mL)	Mean ± S.D; %CV		
	Lawsone	Methylene-3,3'-bilawsone	Lawsone methyl ether
Area	32989.17 ± 311.04; 0.94	22222.33 ± 228.03; 1.03	27405.33 ± 295.58; 1.08
NTP	2242.67 ± 40.27; 1.80	11744.70 ± 47.52; 0.40	9065 ± 142.25; 1.57
Tailing Factor	0.96 ± 0.01; 0.93	1.33 ± 0.02; 1.29	1.54 ± 0.01; 0.58

Specificity: Fig. 3 shows the specificity of the method for the quantitative Analysis of naphthoquinones in the plant extract. The method was quite selective for Naphthoquinones since there was no other interfering peak around the retention time of Naphthoquinones. The baseline did not show any significant peak.



#	Peak Name	CH	tR [min]	Area [µV·sec]	Height [µV]	Area%	Height%	Quantity	NTP	Resolution	Symmetry Factor
1	LAWSONE	1	3.625	22097	1494	36.265	25.276	N/A	2151	4.176	1.022
2	BILAWSONE	1	4.975	23755	2641	38.985	44.663	N/A	8114	4.169	1.673
3	METHYL LAWSONE	1	5.900	15082	1777	24.751	30.061	N/A	11096	N/A	1.353

FIG. 3: REPRESENTATIVE CHROMATOGRAM OF 10µg/ml SOLUTION

Solution Stability: The stability studies were evaluated for three different concentration, *i.e.* 8 µg/mL, 10 µg/mL and 12 µg/mL for stock solution stability, solutions corresponding to the above three concentrations were stored at refrigerated temperature (2-8 ºC) for a week. Stability was calculated by comparing the results of six replicate injections of stored solutions with fresh samples. Results are given in **Table 4**.

TABLE 4: DATA FOR THE STABILITY SOLUTIONS PARAMETER

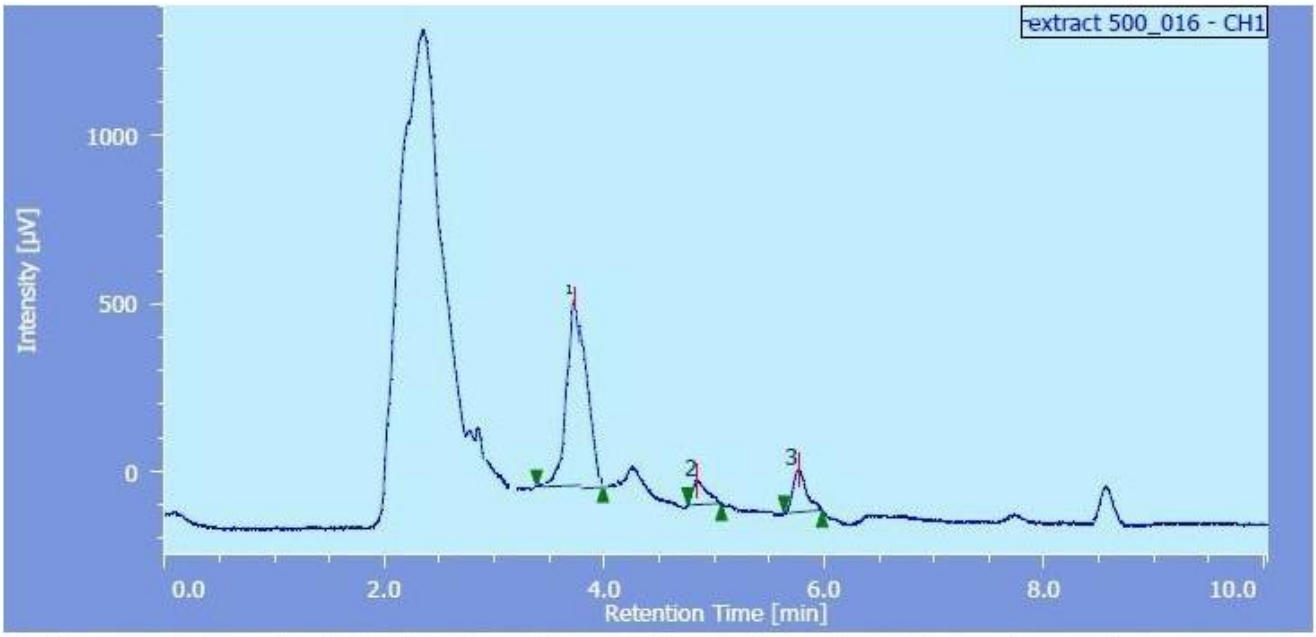
Lawsone				
Actual Concentration (µg/mL)	**Measured Concentration (µg/mL), ± S.D; %CV**		**% Stability**	
	Conc. of Fresh Solution	**Conc. of Stability Solution**	**Fresh solution**	**Stability solution**
8	7.88 ± 0.09; 1.18	7.96 ± 0.06; 0.81	98.54	99.55
10	9.91 ± 0.09; 0.88	9.97 ± 0.11; 1.06	99.1	99.69
12	12.02 ± 0.10; 0.79	12.02 ± 0.04; 0.32	100.14	100.19
Lawsone methyl ether				
Actual Concentration (µg/mL)	**Measured Concentration (µg/mL), ± S.D. ; %CV**		**% Stability**	
	Conc. of Fresh Solution	**Conc. of Stability Solution**	**Fresh solution**	**Stability solution**
8	7.97 ± 0.11; 1.33	8.00 ± 0.05; 0.58	99.65	99.97
10	10.00 ± 0.09; 0.86	10.01 ± 0.05; 0.53	99.98	100.06
12	12.03 ± 0.09; 0.75	11.99 ± 0.03; 0.27	100.21	99.91
methylene-3,3'-bilawsone				
Actual Concentration (µg/mL)	**Measured Concentration (µg/mL), ± S.D; %CV**		**% Stability**	
	Conc. of Fresh Solution	**Conc. of Stability Solution**	**Fresh solution**	**Stability solution**
8	8.00 ± 0.09; 1.10	7.94 ± 0.08; 1.05	100	99.19
10	10.00 ± 0.11; 1.13	10.06 ± 0.10; 1.03	99.97	100.64
12	12.01 ± 0.10; 0.80	11.99 ± 0.03; 0.29	100.04	99.95

Quantification of Naphthoquinones in Leaf Extract: All sample solutions (10 µg/mL) of different extract prepared in methanol was injected under the same chromatographic conditions, and the chromatograms were recorded in triplicate. The amount of naphthoquinones present in the extract was determined by comparing the peak area from the standard.

The results are furnished in **Table 5**. No interfering peaks were found in the chromatogram indicating that the determination of the naphthoquinones content was free from interference by other constituents present in the extract **Fig. 4**.

TABLE 5: CONTENT DETERMINED IN THE PLANT MATERIAL

Component	Content (% w/w)
Lawsone	1.03
Lawsone methyl ether	0.99
Methylene-3,3'-bilawsone	0.67



#	Peak Name	CH	tR [min]	Area [µV·sec]	Height [µV]	Area%	Height%	Quantity	NTP	Resolution	Symmetry Factor
1	LAWSONE	1	3.667	23634	854	93.172	81.170	N/A	2243	1.992	0.736
2	BILAWSONE	1	4.850	657	72	2.590	6.798	N/A	5980	3.994	1.656
3	METHYL LAWSONE	1	5.767	1075	127	4.238	12.032	N/A	12136	N/A	1.427

FIG. 4: REPRESENTATIVE CHROMATOGRAM OF EXTRACT SOLUTION

CONCLUSION: The proposed RP-HPLC method is simple, rapid, accurate, reproducible, selective and economic and can be used for routine quality control analysis of *Impatiens balsamina* L. (Leaf) powder and quantitative determination of Naphthoquinones in leaf powder.

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CONFLICT OF INTEREST: Nil

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