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RESEARCH ARTICLE

RP-HPLC and MS Methods for Simultaneous Estimation of Arbutin and Quercetin from *Origanum majorana*

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Abstract

Background: Arbutin (ARB) and quercetin (QUER) are two important phytoconstituents with proven anti-cancer effects. In vitro cytotoxic actions against a variety of tumor cell lines or antitumor-promoting properties have also been associated with extracts from *Origanum majorana*. **Aim:** To develop an easy, rapid, accurate, precise and reliable novel analytical method for the simultaneous estimation of quercetin and arbutin from *Origanum majorana* extracts and its marketed formulation. **Method:** The ideal chromatographic conditions for estimating arbutin and quercetin simultaneously using the RP-HPLC method are a stationary phase LichroCART C18 column (250mm × 4.0mm, 5µm), a mobile phase that contains 1% orthophosphoric acid and methanol in a 50:50% v/v ratio, a flow rate of 1 ml/min, ambient temperature operation, and a detection wavelength of 287 nm. The presence of arbutin and quercetin were qualitatively analyzed using mass spectroscopy. **Results:** The new RP-HPLC technique yielded a correlation value of 0.9999 and was demonstrated to be linear in the 0.5-2.5µg/ml range. Arbutin and quercetin were shown to have retention periods of 2.24 and 13.5 minutes, respectively. The amount of arbutin and quercetin in formulation were found to be 0.6484 µg and 0.1420 µg, respectively. The technique was validated using criteria such as specificity, accuracy, LOD, LOQ, precision, repeatability, stability, and system adaptability. Arbutin and quercetin were also confirmed to be present in the extract and formulation using mass spectroscopy (273 and 303 m/z values). **Conclusion:** Simple, fast, accurate, reliable, and economical, the suggested RP-HPLC and mass spectroscopy techniques are useful for consistently identifying arbutin and quercetin in both their prescription dose form and bulk.

Major Findings: For arbutin and quercetin, the linearity was found to be between 0.5 and 2.5µg/ml, respectively. Among the extracts chloroform extract was found with separation of many components. The amount of arbutin and quercetin was determined to be 0.6484 µg and 0.1420 µg in the formulation.

Keywords: Anti-infective, Antitumour, Antioxidant, Diuretic, ICH

1. Introduction

Since a medicinal herb is made up of many different chemical entities, such as alkaloids, glycosides, resins, oleoresins, sesquiterpene, saponins, flavonoids, lactones, and oils, it can be compared to a chemical factory[1]. There are many ways to use plants, ranging from extracts to powders. Recent years have seen a rise in the use of herbal medicine systems. Quality assurance and control are of crucial significance in the increasing demand for herbal drugs. Standardized herbal extracts are regarded to be a more scientifically sound alternative to unrefined medications. The preparation known as the standardized herbal extract

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has a fixed amount of the active ingredient in it. The important aspect of standardization is to ensure the presence of one or more active constituents and their levels. Herbal medications are increasingly favoured because they have a lower potential for adverse effects, work well for treating chronic illnesses, are more widely available, less expensive, and offer a wide variety of therapeutic applications in addition to encouraging the body to repair itself[2]. *O. majorana* contains 3% volatile oil, glycosylated hydroquinone, sterols and flavonoids[3]. Among these flavonoids and hydroquinones have antitumor-promoting or cytotoxic activity[4]. Arbutin (ARB) (Figure 1) and quercetin (QUER) (Figure 2) are the important phytoconstituents which are proven to have anti-cancer activity[5-7].

The lack of research on *O. majorana* is the primary reason for the study's selection. ARB is a naturally occurring substance that can be found in foods, herbal dietary supplements, and cosmetic skin-lightening products. It is used in medicine as a diuretic and an anti-infective for the urinary system[8]. In addition, it is present in compositions used to treat skin cancer as a skin-lightening agent and an inhibitor of melanin formation. QUER, a substance belonging to the flavonoid family, has several biological properties, such as anti-inflammatory, anti-cancer, and cardiovascular protective effects. According to a review of the literature, no analytical technique has been described for estimating ARB and QUER (anti-cancer agents) from *O. majorana* simultaneously utilizing reference standards for phytochemicals. By ICH recommendations, this study details the development and validation of a new RP-HPLC method for the simultaneous assessment of ARB and QUER.

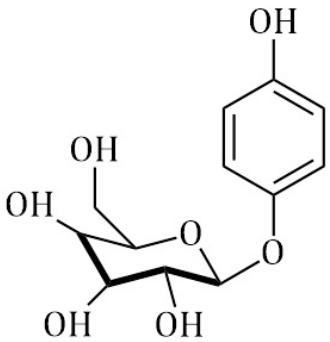


Figure 1. Chemical structure of Arbutin - $C_{12}H_{16}O_7$.

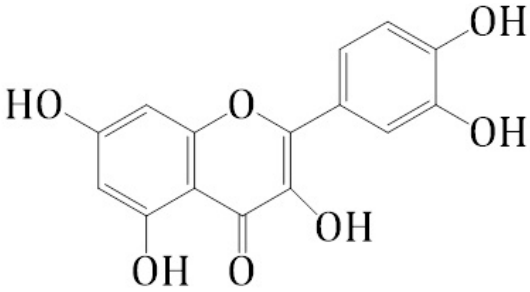


Figure 2. Chemical structure of Quercetin - $C_{15}H_{10}O_7$.

2. Materials and Methods

2.1 Chemicals and Reagents

Pure drug samples of ARB and QUER (phytochemical reference standard) were purchased from Sigma-Aldrich, Bangalore and Himedia Laboratories (Mumbai, India). Three Indian companies—S.D. Fine Chemicals Ltd., Mumbai, Qualigens Fine Chemicals Ltd., and Ranbaxy Chemicals Ltd., New Delhi—supplied all the chemicals and solvents used.

2.2 Equipment

Shimadzu Prominence UFLC equipped with LC-20 AD pump, SPD-M20A diode array detector, DGU-20A3 degasser, SIL-20 AC auto sampler, CTO-10ASVP column oven and chromatographic software, version 3.41.324. For this investigation, a Leela Sonic ultra sonicator and an Elico LI 127 pH meter were utilized.

2.3 Formulation Used

The formulation used in this study is *O. majorana* 30C manufactured by Boiron Homeopathics USA. It is a homoeopathic formulation–containing pellets composed of *O. majorana* (0.443 mg), lactose and sucrose, used in the treatment of headaches (especially migraine).

2.4 Authentication

The plant collected was submitted for authentication to a botanical survey of India, Southern Circle Herbarium – Coimbatore and was identified as *O. majorana* belonging to the family *Lamiaceae*.

2.5 Extraction

The fresh plant collected was washed with water to eliminate the natural weeds and dried in shadows. For later use, the plant's aerial portion was pulverized

and kept in an airtight container. The preliminary step for the simultaneous estimation of ARB and QUER from *O. majorana* is the extraction of these two active constituents from the plant by single Step/successive extractions[9]. Methanol, ethanol, butyl acetate, ethyl acetate, petroleum ether, Chloroform, cyclohexane and water were used for single extraction. Petroleum ether, chloroform, ethyl acetate and methanol were used for successive extraction[10].

2.6 Chromatographic Conditions

2.6.1 Optimized Chromatographic Conditions

The ideal chromatographic conditions were identified for the RP-HPLC method's simultaneous quantification of arbutin and quercetin. (Table 1).

2.6.2 Preparation of Standard Stock Solution

A concentration of 1 mg/ml was achieved by adding methanol to two distinct 100 ml standard flasks containing approximately 100 mg each of arbutin and quercetin.

2.6.3 For Simultaneous Estimation

To obtain a concentration of 10µg/ml of arbutin and quercetin, respectively, 1 ml of each substance was transferred from the stock solution into a 100 ml standard flask along with additional methanol to fill the volume.

3. Results

3.1 Individual Estimation

A volume of 10µg/ml of arbutin and quercetin, respectively, was obtained by taking about 0.1 ml of each compound from the stock solution and adding it separately to a 10 ml standard flask. Add methanol to get the final concentration of arbutin and quercetin. About 10µl of this solution was injected into the instrument to record the individual component chromatogram (Figure 3). A comparison is made between the obtained spectrum and the UV spectrum.

Table 1. Optimized chromatographic conditions

Stationary phase	LichroCART, C18 column (250mm×4.0mm, 5µm)
Mobile phase	1% Orthophosphoric acid: Methanol
Ratio	50: 50
Flow rate	1ml/min
Operating temperature	Room temperature
Detection wavelength	287nm

3.2 Preparation of Standard Graph for Simultaneous Estimation

To record the chromatogram and measure the peak regions, 10µl of each solution was injected into the system. Approximately 0.5–5 ml of the stock solution, which had a concentration of 10µg/ml, was taken and placed in a series of 10 ml standard flasks. The volume was then increased to 10 ml using the mobile phase to achieve a concentration of 0.5–2.5 µg/ml (Figure 4).

Peak areas of arbutin and quercetin were plotted against concentration on the calibration graph (Figure 5). It was discovered from the graph that there was linearity between the concentrations of 0.5 to 2.5µg/ml (Table 2).

3.3 Analysis of the Extract

From 25ml of the extract solution obtained by single and successive extractions about 1ml of the solution was taken and is diluted to 10ml with mobile phase. To record the chromatogram, the system was injected with 10**µl** of each solution. The peak area was then measured.

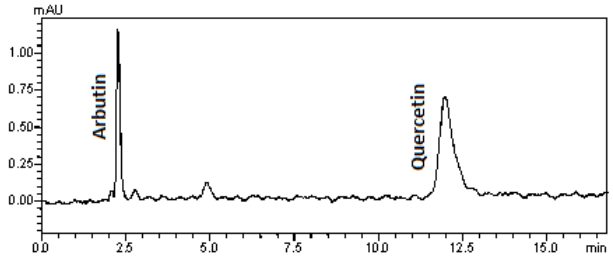


Figure 3. Chromatogram of arbutin and quercetin.

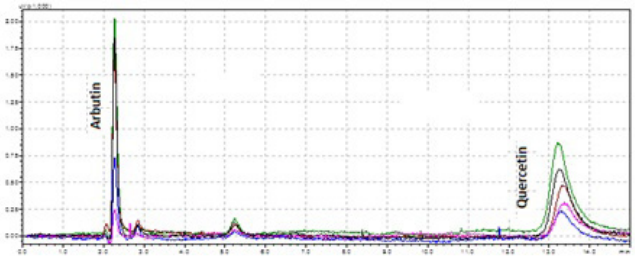


Figure 4. Chromatogram of simultaneous analysis of arbutin and quercetin.

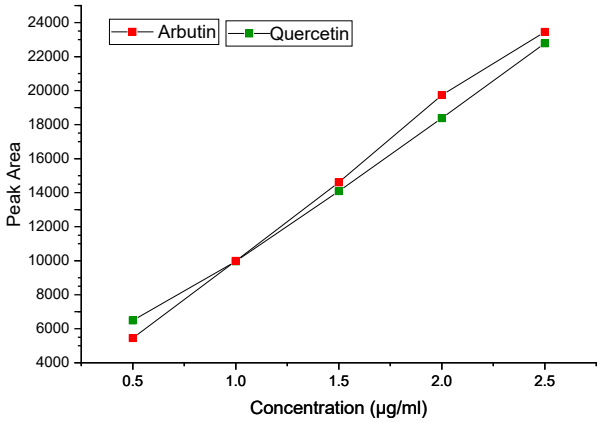


Figure 5. Calibration graph of Arbutin and Quercetin – concentration range of 0.5 to 2.5µg/ml.

Table 2. Calibration data of arbutin and quercetin

S. No	Concentration (µg/ml)	Peak areas	
		Arbutin	Quercetin
1.	0.5	3660	5450
2.	1	5975	9987
3.	1.5	8726	14607
4.	2	11586	19740
5.	2.5	14520	23448

The amount of arbutin and quercetin was calculated from the peak areas obtained (Figure 6) (Table 3) the extracts and the dilution factor.

3.4 Analysis of Formulation

As part of the standard addition technique analysis, a known concentration of the standard is added to the formulation. After being sonicated for about ten pellets in ten millilitres of mobile phase, the pellets were filtered out. To 5ml of solution (or 2µg of the standard), add approximately 5ml of the filtrate. After injecting 10**µl** of the solution into the system, the amount of arbutin and quercetin was quantified (Table 3) and the peak areas obtained (Figure 7) were used to calculate the unknown concentration of the analytes.

3.5 Specificity

Although no new peaks were discovered, there were minor variations in the retention time, peak shape, and peak areas due to adjustments made to the optimized chromatographic conditions, such as the mobile phase ratio, flow rate, and ionic strength. It was discovered that the peak purity indexes of quercetin and arbutin were, respectively, 0.999 and 0.999. The values are near the peak purity of quercetin and arbutin.

3.6 Accuracy

The accuracy of the developed method is evaluated through recovery studies, which involve injecting 10µl of the solution into the system and adding a known quantity of the pure drug at 100% level to the pre-analysed formulation. The % RSD and recovery were computed (Table 4).

3.7 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated using the individual calibration graphs for quercetin and arbutin. The residual standard deviation approach was utilized to ascertain the LOD and LOQ. The algorithm below was used to get the LOQ and LOD values (Table 5).

LOD = 3.3 * (SD/S).

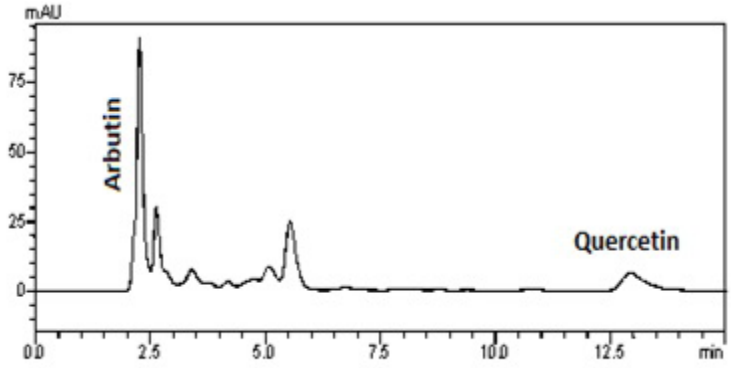


Figure 6. Chromatogram of methanol extract.

Table 3. The peak area of arbutin and quercetin in extracts

S. No.	*Extract*	*Peak Area*		Amount found in mg	
		Arbutin	**Quercetin**	**Arbutin**	**Quercetin**
1	*Water*	*928939*	6969	68	0.371
2	*Methanol*	*270648*	-	20	-
3	*Ethanol*	*428865*	1072	31.6	0.058
4	Ethyl acetate	373756	-	27.5	-
5	Butyl acetate	42048	4023	3.2	0.22
6	Chloroform	2461	-	0.20	-
7	Cyclohexane	4242	-	0.33	-
8	Petroleum ether	2096	-	-	-
Successive extraction					
1	Petroleum ether	2096	-	-	-
2	Chloroform	4820	-	0.41	-
3	Ethyl acetate	26979	10388	2.3	-
4	Methanol	705900	114757	60	6.11
Formulation analysis					
Amount of desired active constituents found from the formulation in µg					
				Arbutin	**Quercetin**
				0.6484	0.1420

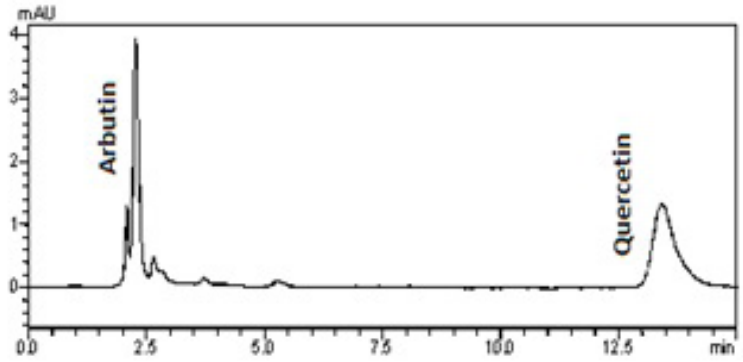


Figure 7. Chromatogram of formulation.

Table 4. Accuracy

Level	% Recovery		%RSD*	
	Arbutin	Quercetin	Arbutin	Quercetin
100%	99.9	99.86	0.2854	0.3497

Table 5. LOD and LOQ

Parameters	Arbutin	Quercetin
LOD (µg/ml)	0.4188	0.33
LOQ (µg/ml)	1.2	1.006

LOQ = 10 * (SD/S).

Where SD- standard deviation of intercept: S-Slope of calibration graph

3.8 Precision

The precision of the developed RP-HPLC method was determined by Inter-day precision, intra-day precision and repeatability of injection. The % RSD was calculated and reported (Table 6).

3.9 Stability

Both room temperature and refrigerator conditions were used to test the standard solution's stability. By observing changes in the solution's retention time, resolution, peak area, and peak shape over time, the stability of the solution was ascertained. The chromatogram of a recently made standard solution was compared to the data. Researchers found that the solution held its stability for 48 hours in a refrigerator and 24 hours at ambient temperature.

3.10 System Suitability Studies

The system suitability study was checked (Table 7) with parameters like the number of theoretical plates (N), peak asymmetry factor (As), resolution (Rs) etc.

3.11 Robustness

By intentionally varying the optimized chromatographic conditions, such as the flow rate by ±0.1 ml/min, ionic strength by ±0.1 unit, and organic composition of the mobile phase by ±1%, the developed RP-HPLC method's robustness was demonstrated. The modifications obtained under different conditions were found to be nearly identical to those of the standard solution under optimal conditions (Table 8). It was discovered that the developed method was reliable.

4. Mass Spectroscopic Analysis

The mass spectroscopic analysis was carried out in the scan and SIM mode for individual standard compounds arbutin, and quercetin separately followed by the analysis of the mixture containing arbutin and quercetin in a 1:1 ratio. The measurement of the standard mass spectrum of arbutin and quercetin in the SIM mode was carried out with the input values of m/z of arbutin and quercetin[11]. The scan mode was carried out in the range of 200-400 m/z. In the same way, the mixture, extracts and the formulation were analysed. The mass spectrum of the mixture, extracts and formulation were compared with the mass spectrum of the individual standard mass spectrum of

Table 6. Precision and results

Precision	Concentration in µg/ml	Mean Peak area		%RSD*	
		Arbutin	Quercetin	Arbutin	Quercetin
Intra day	1.5	8785	14695	0.493	0.553
Inter day	2	11691	19695	0.802	0.640
Repeatability	2	11633	20063	0.586	0.573

*-mean of six determinations

Table 7. System suitability studies

Drugs	R_s	N	A_s	Tailing factor	Capacity factor k'
Arbutin	12.775	3981	1.299	1.416	4.888
Quercetin		5179	1.333	1.385	

Table 8. Robustness

Chromatographic condition		Peak area	
		Arbutin	Quercetin
Mobile phase ratio (50:50)	49:51	1384	56053
	51:49	1346	53228
The ionic strength of H_3PO_4	0.9%	1379	53675
	1.1%	1750	54153
Flow rate	0.9	1490	53727
	1.1	1402	52874

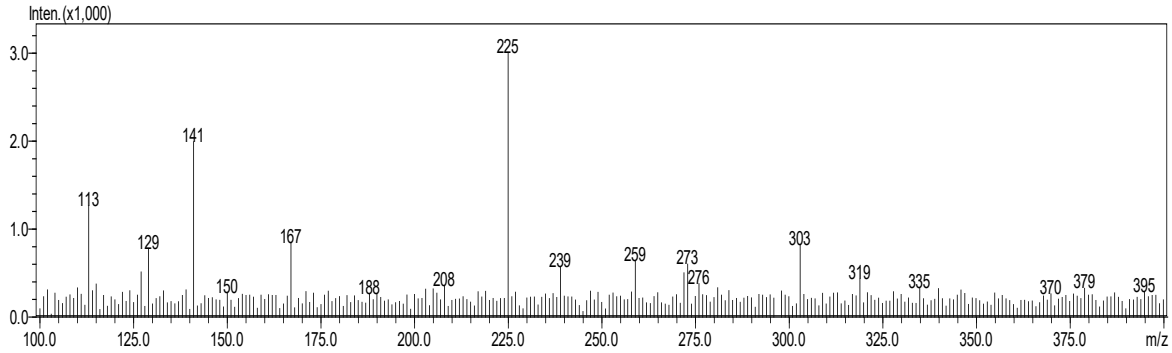


Figure 8. Mass spectrum of arbutin and quercetin mixture.

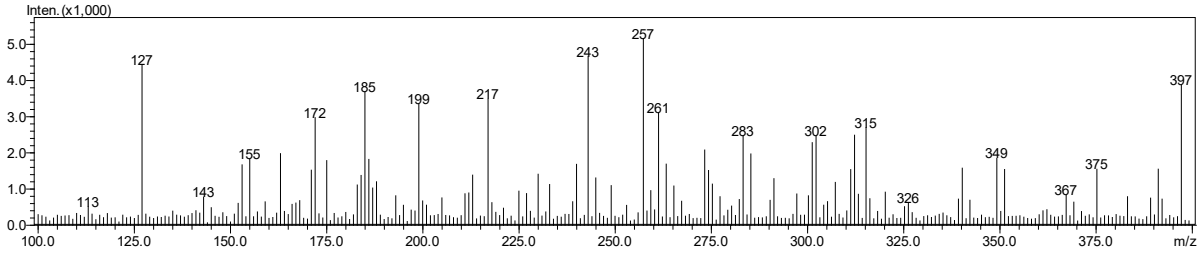


Figure 9. Mass spectrum of methanol and aqueous extract (overlay).

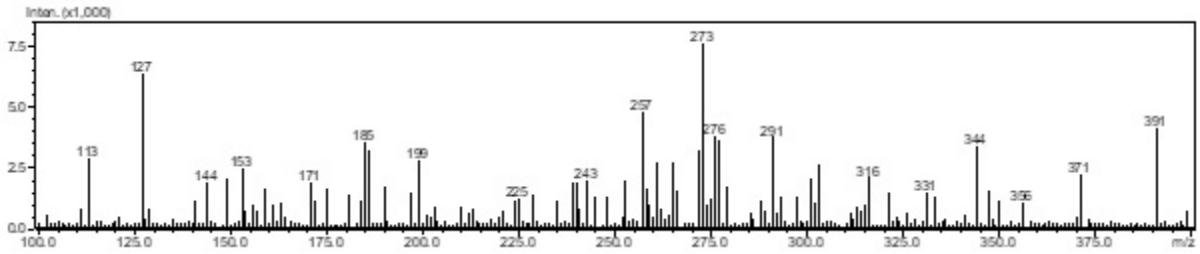


Figure 10. Mass spectrum of formulation.

arbutin and quercetin. The m/z values of arbutin and quercetin were found to be 273 and 303 respectively.

4.1 Analysis of the Standard

The analysis of the standard solution was made by injecting 10µl of the solution into the system and was analysed both in scan and SIM modes (Figure 8).

4.2 Analysis of Extracts

The extracts were analysed in the same way as the standard drug analysis. About 1ml from the extract (25ml) was transferred into a 10ml standard flask and the volume was made up with the mobile phase and the mass spectroscopic analysis was made (Figure 9).

4.3 Analysis of Formulation

The analysis of formulation was made by dissolving 10 pellets in 10ml of the mobile phase and 10µl of the solution was injected into the system for analysis and was analysed as like that of the standard (Figure 10).

5. Discussion

For the simultaneous estimation of arbutin and quercetin the HPLC method was carried out in the reverse phase since arbutin, and quercetin are polar components. For arbutin and quercetin, the linearity was found to be between 0.5 and 2.5µg/ml, respectively. Although the peak forms were high, the extracts were analyzed by diluting 1 ml of the extract with 10 ml of methanol. So, further dilution was made with the mobile phase and injected into the system. Among the extracts chloroform extract was found with separation of many components. The formulation was analysed by dissolving 10 pellets in 10ml of mobile phase and it was found that arbutin was detected in very low concentration, but quercetin was not detected. So, for the simultaneous estimation, the standard addition method was followed. By the simultaneous method, the amount of arbutin and quercetin was estimated with the peak areas obtained. The amount of arbutin and quercetin was determined to be 0.6484 µg and 0.1420 µg in the formulation. Specificity, accuracy, repeatability, stability, LOQ, precision, and system adaptability were the metrics used to verify the approach. Mass spectroscopic analysis was also carried out to confirm the presence of Arbutin and Quercetin in the formulation and the extract using methanol as the solvent and 1 µg /ml of standard solution (ARB and QUER) as reference. The m/z values are found to be 273 and 303 respectively.

6. Conclusion

RP-HPLC method and mass spectroscopic analysis are more precise and reliable due to their sensitivity and specificity than the already existing analytical methods. Standardization of arbutin and quercetin using the novel analytical methods from *O. majorana* made an additional contribution to the mainstream in the development of new herbal anticancer agents. Therefore, it can be concluded that the established validated process may be used for both the pellet formulation and the simultaneous standardization of ARB and QUER from *O. majorana*.

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8. Author Contribution

Arun NT: designed and drafted the manuscript; Suganthi A: drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

9. References

1. Assaf MH, Ali AA, Makboul MA, Beck JP, Anton R. Preliminary study of phenolic glycosides from *Origanum majorana*, quantitative estimation of arbutin; cytotoxic activity of hydroquinone. Planta Med. 1987; 53(4):343-345. http://dx.doi.org/10.1055/s-2006-962734. PMID: 3671554.
2. Qari SH. *In vitro,* evaluation of the anti-mutagenic effect of *Origanum majorana* extract on the meristemetic root cells of *Vicia faba*. J Taibah Univ Sci. 2008; 1:6-10. http://dx.doi.org/10.1016/s1658-3655(12)60028-4
3. Hossain MB, Camphuis G, Aguilo-Aguayo I, Gangopadhyay N, Rai DK. Antioxidant activity guided the separation of major polyphenols of marjoram (*Origanum majorana* L.) using flash chromatography and their identification by liquid chromatography coupled with electrospray ionization tandem mass spectrometry. J Sep Sci. 2014; 37(22):3205-3213. http://dx.doi.org/10.1002/jssc.201400597. PMID: 25161079.
4. Lukas B, Schmiderer C, Mitteregger U, Novak J. Arbutin in marjoram and oregano. Food Chem. 2010; 121(1):185-190. https://doi.org/10.1016/j.foodchem.2009.12.028
5. Bouyahya A, Chamkhi I, Benali T, Guaouguaou FE, Balahbib A, El Omari N, *et al.* Traditional use, phytochemistry, toxicology, and pharmacology of *Origanum majorana* L. J Ethnopharmacol. 2021; 265:113318. https://doi.org/10.1016/j.jep.2020.113318. PMID: 32882360.
6. Nahar L, Al-Groshi A, Kumar A, Sarker SD. Arbutin: Occurrence in plants, and its potential as an anticancer

agent. Molecules. 2022; 27(24):8786. https://doi.org/10.3390/molecules27248786. PMID: 36557918; PMCID: PMC9787540.
7. Charles DJ. Antioxidant properties of spices, herbs and other sources. Springer Science and Business Media. 2012. https://doi.org/10.1007/978-1-4614-4310-0_37
8. Hossain MB, Rai DK, Brunton NP, Martin-Diana AB, Barry-Ryan C. Characterization of phenolic composition in Lamiaceae spices by LC-ESI-MS/MS. J Agric Food Chem. 2010; 58(19):10576-10581. https://doi.org/10.1021/jf102042g. PMID: 20825192.
9. Erenler R, Sen O, Aksit H, Demirtas I, Yaglioglu AS, Elmastas M, *et al.* Isolation and identification of chemical constituents from *Origanum majorana* and investigation of antiproliferative and antioxidant activities. J Sci Food Agric. 2016; 96(3):822-836. https://doi.org/10.1002/jsfa.7155. PMID: 25721137.
10. Celik SE, Tufan AN, Bekdeser B, Ozyurek M, Guclu K, Apak R. Identification and determination of phenolics in Lamiaceae species by UPLC-DAD-ESI-MS/MS. J Chromatogr Sci. 2017; 55(3):291-300. https://doi.org/10.1093/chromsci/bmw184. PMID: 27884875.
11. Dursunoglu B, Hafize YU, Guvenalp Z, Gozcu S, Yilmaz B. Simultaneous determination of arbutin and hydroquinone in different herbal slimming products using gas chromatography-mass spectrometry. Turk J Pharm Sci. 2018; 15(3):298. https://doi.org/10.4274/tjps.38258. PMID: 32454673; PMCID: PMC7227836.