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Simultaneous Standardization of Arbutin and Quercetin from Origanum Majorana by Novel HPTLC Technique

A. Suganthi[1]*, Arun NT[2], T. K. Ravi[1]

1.Department of Pharmaceutical Analysis, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, 395, Sarojini Naidu Road, Coimbatore-641 044

2. Department of Pharmaceutical Chemistry, Paavai College of Pharmacy and Research, Pachal- 637 018.

ABSTRACT

Arbutin and quercetin are anti-cancer agents, extracted from leaves and flowers of Origanum majorana L which can be simultaneously estimated using a rapid, specific and sensitive high – performance thin layer chromatographic technique using silica gel G60F254 as the stationary phase and butyl acetate: methanol: formic acid: toluene (8: 1.5: 5: 0.5) as mobile phase for the separation. The method was validated for linearity, precision and recovery. Linearity was established in the range 300-900 ng/spot for arbutin and 150-450 ng/spot for quercetin, respectively. The % RSD values of repeatability of application, repeatability of measurement, intra-day and inter day precision studies of arbutin and quercetin were found to be below 1 for 500 and 250 ng/spot of arbutin and quercetin, respectively. The recovery of the drugs were found to be 99.8 and 100.2%, proves that the developed method was highly accurate. Hence the proposed validated method could be applied for the simultaneous analysis of arbutin and quercetin in methanolic extract of Origanum majorana L as well as for its marketed formulation.

Keywords: Arbutin, quercetin, Origanum majorana, validation, HPTLC

*Corresponding Author Email: suganlemu@gmail.com

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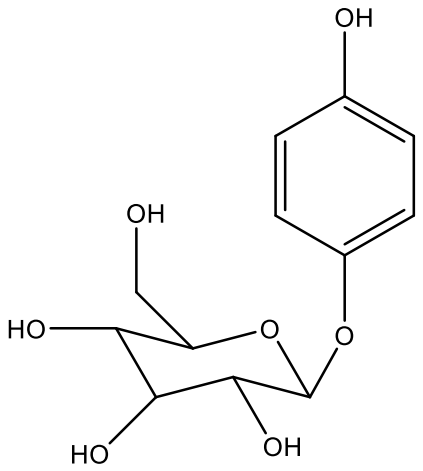
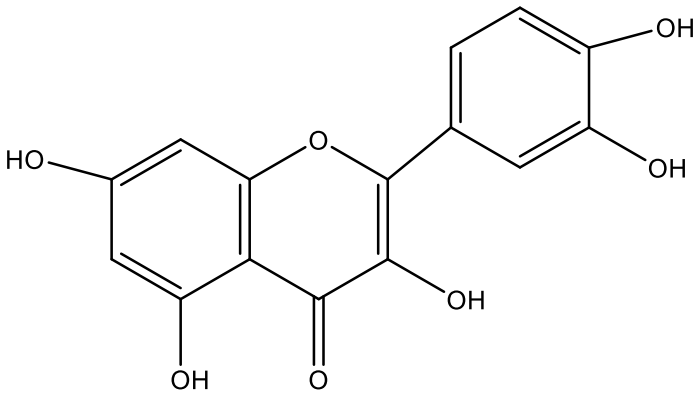
INTRODUCTION

A medicinal herb is equivalent to a chemical factory as it is composed of numerous chemical entities like alkaloids, glycosides, resins, oleoresins, sesquiterpene, saponins, flavonoids, lactones, and oils[1]. Plants are used in different forms varying from powders to extracts. Herbal systems of medicine have become increasingly popular in recent years. In light of the growing demand of herbal drugs, quality control and assurance is primarily important. The standardized herbal extracts are considered to be more scientific than crude drugs. The standardized herbal extract is a preparation, which contains certain fixed proportion of the active constituent. The important aspect of standardization is to ensure the presence of one or more active constituents and their levels present. Herbal drugs are preferred more as they have reduced risk of side effects, being effective with chronic conditions, lower cost with widespread availability along with natural factor, wide range of healing and their ability to encourage the body to heal itself [2]. *Origanum 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) and quercetin (QUER) are the important phytoconstituents which are proved to have anti-cancer activity [5]. Not much studies has been carried out in *Origanum majorana* which is the main reason for the selection of study. ARB is a natural product found in foods, herbal dietary supplements, and cosmetic skin-lightening products. Therapeutically, it is used as an anti-infective for the urinary system as well as a diuretic[6]. It is also an inhibitor of melanin formation and a skin-lightening agent that is included in compositions used for treating skin cancer [7,8]. Whereas QUER is a part of flavonoid family of compounds, members of which display a variety of biological activities, including cardiovascular protection, anti-cancer activity and anti-inflammation[9].

For assurance of quality, safety and efficacy of herbal drugs and pharmaceuticals is quite important because of their use not only as health care products but also lifesaving substances. Literature survey revealed that there is no analytical method reported for the simultaneous estimation of ARB and QUER *(anti-cancer agents)* from *Origanum majorana* using phytomarkers. Hence this paper reports the development of a novel HPTLC method for simultaneous determination of ARB and QUER and validated in compliance with ICH guidelines[10,11].



ARBUTIN



QUERCETIN

MATERIALS AND METHODS

Pure drug samples of ARB and QUER (phytochemical reference standard) were purchased from Sigma-Aldrich, Bangalore and Himedia Laboratories (Mumbai, India). All the chemicals and solvents used were supplied by S.D. Fine Chemicals Ltd., India, Qualigens Fine Chemicals Ltd., Mumbai, India and Ranbaxy Chemicals Ltd., New Delhi, India. Precoated silica gel G60 F_{254} plates supplied by E.Merck. CAMAG Linomat V sample applicator and TLC scanner III controlled by win CATS-planar chromatography manager, version: 1.2.6. Millipore Milli-Q Academic water purifier, Elico LI 127 pH meter and LeelaSonic Ultrasonicator were used for this study.

AUTHENTICATION

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

EXTRACTION[12]

The fresh plant collected was washed with water in order to eliminate the natural weeds and dried in shadows. The aerial part of the plant was powdered and stored in an air tight container for further use. The preliminary step for the simultaneous estimation of ARB and QUER from *Origanum majorana* is the extraction of these two active constituents from the plant by single step/successive extractions. 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.

Selection of stationary phase and mobile phase:

Silica gel G60 F_{254} was selected as the stationary phase and butyl acetate: methanol: formic acid:

toluene (8: 1.5: 5: 0.5) was used as mobile phase for the separation. The drugs were dissolved in methanol and the solution was scanned in the UV- region and the detection wavelength of 290nm was selected for the simultaneous determination.

Preparation of stock solution:

Stock solutions containing 1mg / ml were prepared, from these 20µg/ml of ARB and 10µg/ml QUER were also prepared as working standard. From the working standard solution about 0.5 to 5 µl was spotted on the TLC plate, followed by development and scanning of spots. The chromatograms were recorded and peak areas were measured.

Preparation of calibration curves for ARB and QUER:

About 100mg of arbutin and quercetin were taken separately in two different100ml standard flask and the volume was made up with methanol to get a concentration of 1mg/ml. From the stock solution about 2ml of arbutin and 1ml of quercetin were transferred together into another 100ml standard flask and the volume was made up with methanol to get a concentration of 20µg/ml of arbutin and 10µg/ml quercetin, respectively. From the working standard solution about 0.5 to 5 µl was spotted on the TLC plate, followed by development and scanning of spots at 290 nm. The peak areas were recorded.

Analysis of the extracts:

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 methanol. Then the solution is applied onto the plate, developed and scanned at 290nm.The amount of ARB and QUER found in the extracts was determined from the peak areas obtained.

Analysis of marketed formulation:

Analysis of formulation involves the standard addition method in which about 10 pellets of the formulation were accurately weighed, powdered, dissolved in 10ml of methanol and filtered. To 5ml of the filtrate about 5ml of standard mixture, equivalent to 3µg/ml was added and it is spotted on the plate. The plate was developed under optimal chromatographic condition and scanned at 290nm. From the peak area obtained the unknown concentration of ARB and QUER in the formulation was calculated.

Method validation:

The method of analysis was validated as per the recommendations of ICH[11] and WHO for the parameters like linearity, limit of detection and limit of quantification, accuracy, precision, specificity and stability studies. The limit of detection and limit of quantification was determined by applying the least concentration of the standard onto the plate in triplicate. Accuracy of the

developed method was determined by recovery study which was done by the addition of a known quantity of the pure drug into the preanalyzed formulation at 100% level. The percentage recovery and %RSD were calculated.

Intraday and interday precision was determined by the analysis of the standard drug solution in 500 and 250 ng/spot within the linearity range for three times / three days on a day / three different days and the %RSD was calculated. Interday precision was found out by the analysis of two different concentrations of the standard solution within the linearity range for three days and the %RSD was calculated. Repeatability of sample application was assessed by the analysis of a concentration of a standard solution of concentration 400 and 200 ng/spot within the linearity range which is applied six times on the precoated TLC-plate, developed and the spots were scanned. The %RSD was calculated from the peak areas. Repeatability of measurement was determined by measuring the peak area of concentration of standard solution 400 and 200 ng/spot) within the linearity range for six times on the pre-coated TLC plate and the plate was scanned without changing the position of the plate and the %RSD was calculated

RESULTS AND DISCUSSION

Based on the solubility and polarity nature of the analytes different solvent systems (ethyl acetate: methanol: formic acid: cyclohexane, butyl acetate: methanol: formic acid: cyclohexane, butyl acetate: methanol: formic acid: chloroform, butyl acetate: methanol: glacial acetic acid: chloroform, ethyl acetate: methanol: formic acid: cyclohexane, butyl acetate: methanol: formic acid: toluene) with different ratio were tried for the separation. Among this butyl acetate: methanol: formic acid: toluene, at a ratio of 8:1.5:5:0.5 gave a good separation and peak shape for ARB and QUER. The R_f values for ARB and QUER were found to be 0.55 and 0.88, respectively at 290 nm under optimized chromatographic condition (Fig. 1 and Table 1). The linearity was observed in the range of 300-900 ng/spot for ARB and 150-450 ng/spot for QUER (Fig. 2 & 3).

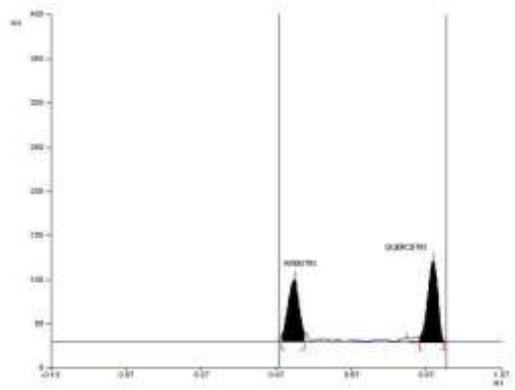


Fig.1: Standard Chromatogram of Arbutin (800ng/spot) and Quercetin (400ng/spot)

Table 1: Optimized Chromatographic Conditions

Parameters studied	Optimized conditions	
Stationary phase	Silica gel G60 F_{254}, 20x10cm TLC plate	
Mobile phase	Butyl acetate: methanol: formic acid: toluene	
Ratio	8: 1.5: 5: 0.5	
Chamber used	Camag twin trough glass chamber	
Chamber saturation time	15min	
Separation technique	Ascending technique	
Distance of solvent front	90mm	
Band width	6mm	
Slit dimension	5.00 x 0.45mm, micro	
Source of radiation	D_2 and W	
Detection wavelength	290nm	
R_f value	Arbutin	Quercetin
	0.55	0.88

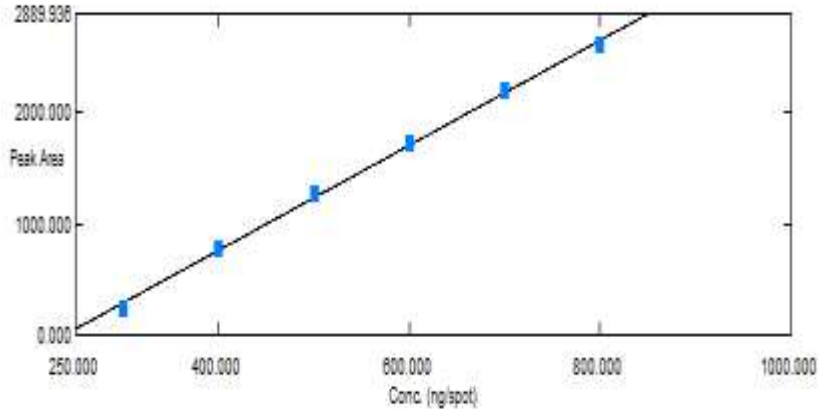


Figure. 2: **Calibration graph of Arbutin**

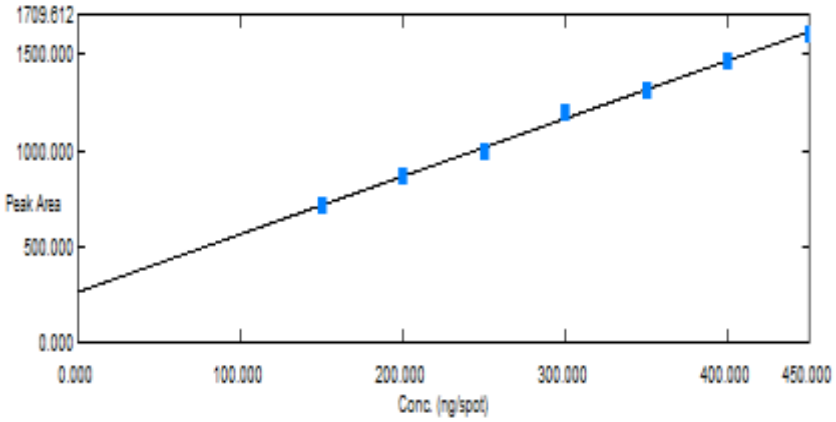


Figure 3: **Calibration graph of Quercetin**

The proposed method was successfully applied for the determination of ARB and QUER in various extracts as well as in herbal formulation. It was found that methanolic extract showed good fingerprinting when compared to other extracts which are mentioned earlier (Fig 4). The developed

method was found to be more specific since the chromatogram of formulation showed only two peaks (Fig. 5) corresponds to ARB and QUER, respectively.

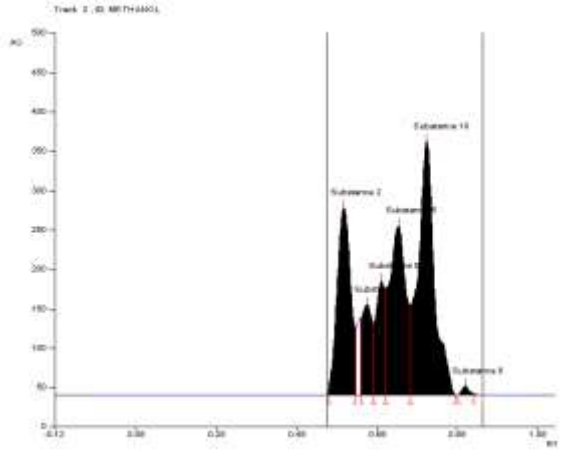


Figure. 4: Chromatogram of methanolic extract

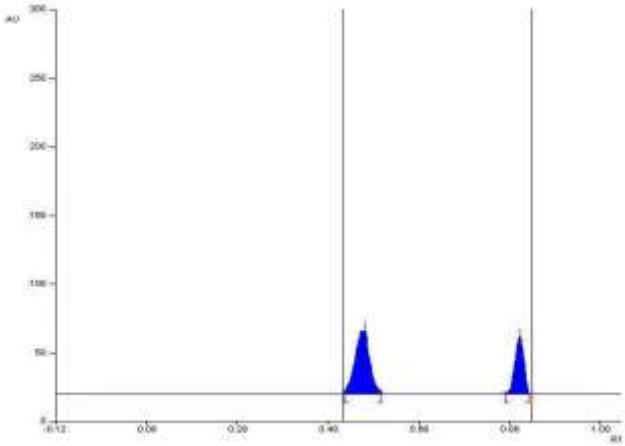


Figure. 5: **Chromatogram of marketed herbal formulation**

The results of precision, accuracy, all validation parameters are summarized in table 2 and the result of analysis of formulation is given in table 3.

Table 2: Summary of Validation Parameters

Parameters	Arbutin	Quercetin
Linearity (ng/spot)	300-900	150-450
Correlation coefficient	0.9987±0.0022	0.9994±0.0012
LOD (ng/spot)	200	100
LOQ (ng/spot)	300	150
Percentage Recovery	99.8	100.5
Precision (%RSD*)	0.5223	0.7177
Robustness	Robust	Robust
Specificity	Specific	Specific

*RSD: Relative standard deviation shows each result is an average of six observations, LOD: limit of detection LOQ: limit of quantification

Table 3: Analysis of formulation

Amount of desired active constituents found from formulation in µg	
Arbutin	**Quercetin**
0.5438	0.075

Stability studies:

When the developed chromatographic plate was exposed to atmosphere, the analytes are likely to decompose. Hence the stability study was conducted in order to assess the stability of the individual components on the plate. The stability of the individual components on the plate was studied at different time intervals and the peak areas were compared with the peak area of the standard solution. Both QUER and ARB were found be to stable on the plate for about 8 hours.

CONCLUSION

In the present study, HPTLC method was developed and validated as per ICH guidelines for the simultaneous determination of ARB and QUER. Statistical analysis proved that the method was specific, sensitive, precise and accurate. The method was also successfully applied to the marketed formulation, showed 0.5438 µg of ARB and 0.075 µg of QUER. Hence it is concluded that the developed validated method can be applied for the simultaneous standardization of ARB and QUER from *Origanum majorana* as well as its pellet formulation. It also made an additional contribution to the main stream in the development and analysis of new herbal anticancer agents. Hence in future the work may be extended with research focused on authentication of pharmacokinetics, adverse interactions, quality control and preclinical investigations, adjuvant therapy for specific cancers, and prevention of toxicity from anticancer therapies.

ACKNOWLEDGEMENT

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