DEVELOPMENT AND VALIDATION OF NOVEL GREEN ANALYTICAL SPECTROSCOPIC METHODS FOR SIMULTANEOUS ESTIMATION OF ARBUTIN AND QUERCETIN FROM *ORIGANUM MAJORANA*

Arun NT*[1], M Karthikeyan[1], A. Suganthi [2]

1- PSG College of Pharmacy, Coimbatore
2- College of Pharmacy – SRIPMS, Coimbatore

***** Corresponding author

ARUN NT,
Assistant professor,
PSG College of Pharmacy,
Coimbatore – 04
Email: arunngt@psgpharma.ac.in
Mobile: 9894438742

ABSTRACT

Arbutin and quercetin are anticancer agents, extracted from leaves and flowers of *Origanum majorana L*. The present study was to develop a simple, precise, accurate method for simultaneous estimation of Arbutin and quercetin by UV Spectroscopic method and also to perform the heavy metal analysis in the plant extract and formulation by Atomic absorption Spectroscopy (AAS). Methanol was selected as the solvent since the solubility and stability were found good. The simultaneous estimation depends on the λmax and the isobestic points 257nm and 287nm were selected. The calibration graph was obtained in a range of 3-30µg/ml and 2-20µg/ml. The amount of Arbutin and Quercetin were found to be 1.870 µg and 0.799 µg in the formulation respectively. The method was validated as per ICH guidelines and Green Chemistry Principles. Hence the method can be conveniently adopted for the routine analysis of Arbutin and Quercetin in bulk and pharmaceutical dosage form.

Key words: Arbutin, Quercetin, Origanum majorana, UV, AAS, Green Chemistry.

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 quality assurance are 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 have been carried out in *Origanum majorana* which is the main reason the selection of the current 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. 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 UV method for simultaneous determination of ARB and QUER and validated in compliance with ICH guidelines and also performs atomic absorption spectroscopy for heavy metal analysis. Green analytical method tries to accomplish greenness by lessening or ruling out possible hazards related with chemical procedures [14,15].

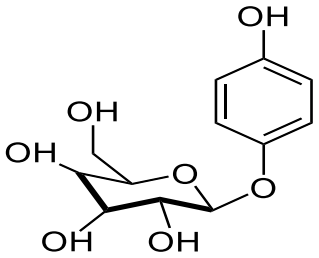


Fig.1: Arbutin

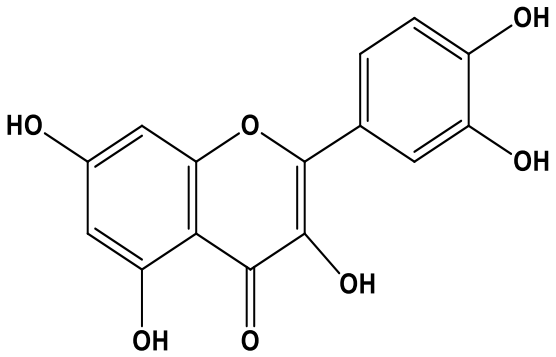


Fig.2: Quercetin

MATERIALS AND METHODS

Chemicals and Reagents

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.

Equipments

Jasco V-630 double-beam UV–visible spectrophotometer, ShimadzuAA-7000 Series Atomic Absorption Spectrophotometer Millipore Milli-Q Academic water purifier, Elico LI 127 pH meter and Leela Sonic 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

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.

UV

Selection of solvent:

By trials the solubility of arbutin and quercetin was checked in various solvents like water, methanol, ethanol, 1N HCl and 1N NaOH. The drugs showed good spectrum and good stability in methanol. So, methanol was selected as the solvent for further estimation.

Fig.3: Arbutin in different solvents

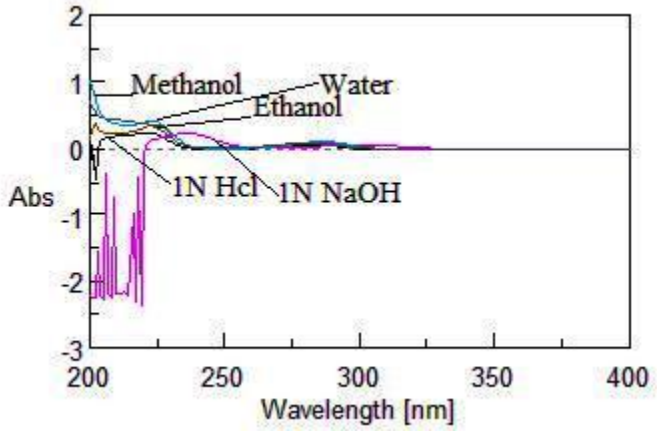
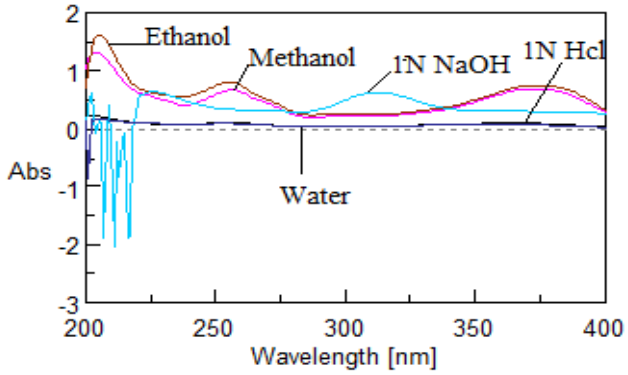


Fig.4: Quercetin in different solvents



Preparation of standard stock solution:

About 100mg of arbutin and 100mg of quercetin were dissolved in 100 ml of methanol separately to get a stock solution of concentration 1000µg/ml.

Preparation of working standard solution:

For individual estimation:

From the stock solution of about 1ml of arbutin and quercetin were taken and transferred to two different 10ml standard flasks and the volume was made up with methanol to get a concentration of 100µg/ml.

For simultaneous estimation:

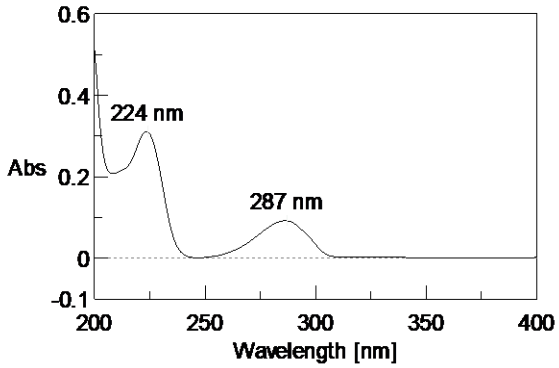
Stock solution for simultaneous estimation was prepared by means of adding about 30ml of arbutin and 20ml of quercetin (from the standard stock solution prepared) into a 100ml standard flask and made upto the volume with methanol to get a concentration of 300µg/ml of arbutin and 200µg/ml of quercetin.

Selection of λ_{max} of individual components:

Arbutin

From the working standard solution about 1ml of arbutin was taken and diluted to 10ml with methanol to get a concentration of 10µg/ml arbutin. The solution was scanned in the UV-region from 200-400 nm and the wavelength of maximum absorbance was found to be at 224nm and 287nm.

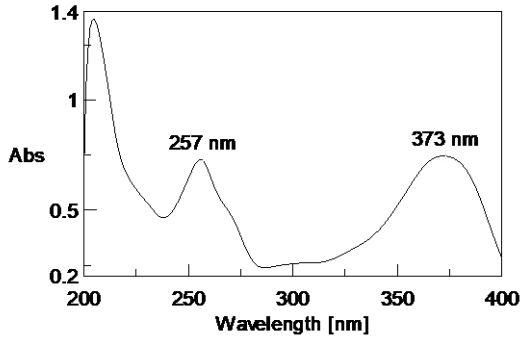
Fig.5: UV spectrum of Arbutin (10µg/ml) in methanol



Quercetin

From the working standard solution about 1ml of quercetin was taken and diluted to 10ml with methanol to get a concentration of 10µg/ml quercetin. The solution was scanned in the UV-region from 200-400 nm and the wavelength of maximum absorbance was found to be at 257nm and 373nm.

Fig.6: UV spectrum of Quercetin (10µg/ml) in methanol



For simultaneous estimation:

For the simultaneous estimation about 3ml of arbutin and 2ml of quercetin from working standard solution were taken and diluted to 10ml to get a concentration of 30µg/ml and 20µg/ml of arbutin and quercetin respectively. The solution was scanned in the UV-region from 200-400 nm and the wavelength of 257 and 287nm were selected for the study.

Fig.7: Overlay spectrum of arbutin quercetin (20µg/ml) in methanol

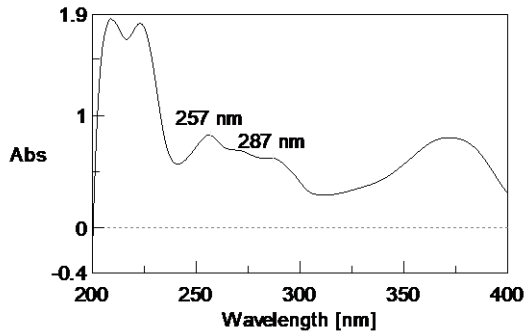
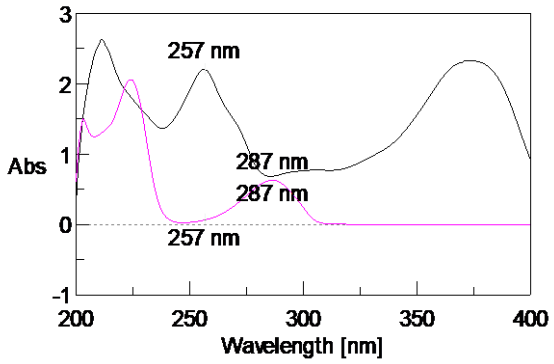


Fig.8: Mixture of arbutin (30µg/ml) and quercetin(20µg/ml) in methanol



Preparation of calibration of graph:

From the stock solution (mixture) containing 300µg/ml of arbutin and 200µg/ml about 0.1-1ml was transferred into series of 10ml standard flask and the volume was made up with methanol to get a concentration of 3-30µg/ml of arbutin and 2-20µg/ml of quercetin and the absorbance was measured at 287nm and 257nm respectively. The calibration graph was plotted between absorbance Vs concentration.

Fig.9: Linear graph at 257nm

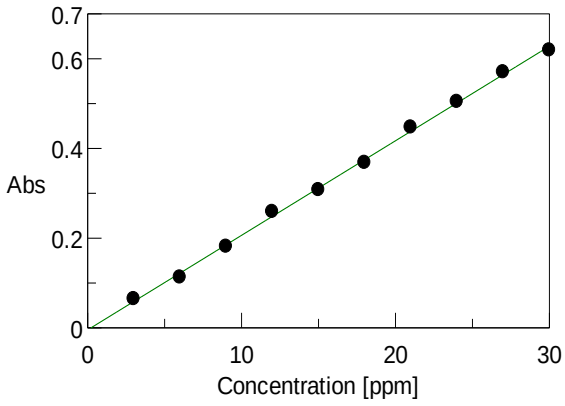


Fig.10: Linear graph at 287nm

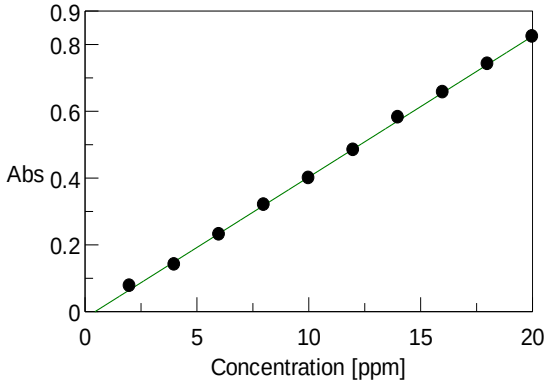


Fig.11: Overlay spectra of the linearity range

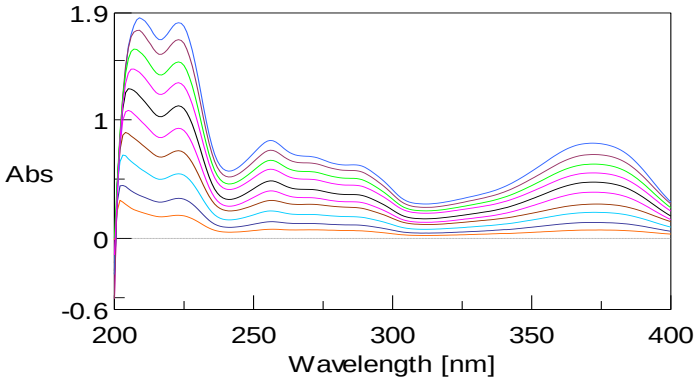


Table 1: Absorbance and absorptivity of Arbutin (3-30µg/ml) and Quercetin (2-20µg/ml) at 257 and 287nm

Absorbance	Absorptivity	Avg. absorptivity	Absorbance	Absorptivity	Avg. absorptivity
At 257nm			At 287nm		
0.0768	0.0256	0.02379	0.0644	0.0322	0.0310
0.1405	0.0234		0.1126	0.0281	
0.2305	0.0256		0.1812	0.0302	
0.3195	0.0266		0.2589	0.0323	
0.3995	0.0266		0.3075	0.0307	
0.4835	0.0268		0.3684	0.0307	
0.5810	0.0276		0.4469	0.0319	
0.6564	0.0273		0.5041	0.0315	
0.7411	0.0274		0.5702	0.0316	
0.8229	0.0276		0.6190	0.0309	

Estimation of Arbutin and Quercetin from different extracts:

From the 25ml extract solution about 0.1ml of the solution was diluted to 10ml with methanol. The spectrum of different extract solutions was recorded and the absorbance at 257nm and 287nm was noted and the amount of arbutin and quercetin was calculated. The results were given in the table:

Fig.12: Overlain spectra of different extracts

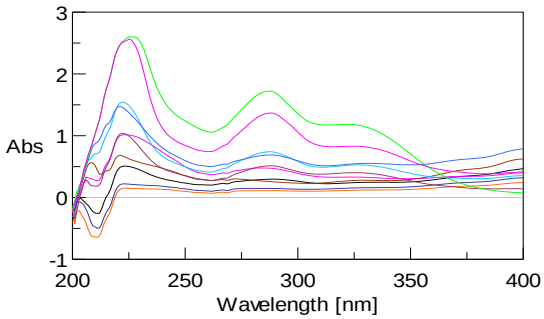


Table.2: Quantification of extract

S.No	Extracts	Amount found in mg	
		Arbutin	Quercetin
01.	Water	41.5	13.7
02.	Methanol	12.5	3.7
03.	Ethanol	18.05	5
04.	Ethyl acetate	7	2.5
05.	Butyl acetate	16.5	6.4
06.	Chloroform	2.67	0.97
07.	Cyclohexane	3.55	1.4
08.	Petroleum ether	6	3.6
Successive extraction			
09.	Petroleum ether	6	3.6
10.	Chloroform	4.24	3.14
11.	Ethyl acetate	11.58	4.9
12.	Methanol	33	9.4

Analysis of formulation:

Standard addition method:

About 10 pellets of the formulation was dissolved in 10ml of methanol by means of sonication and filtered off. To 5ml of the filtrate 5ml of standard mixture solution equivalent to 18µg/ml of arbutin and 12µg/ml of quercetin were added and the absorbance of the resulting solution was measured. The concentration of arbutin and quercetin in the formulation was determined.

Fig.13: Spectrum of formulation along with known concentration of standard

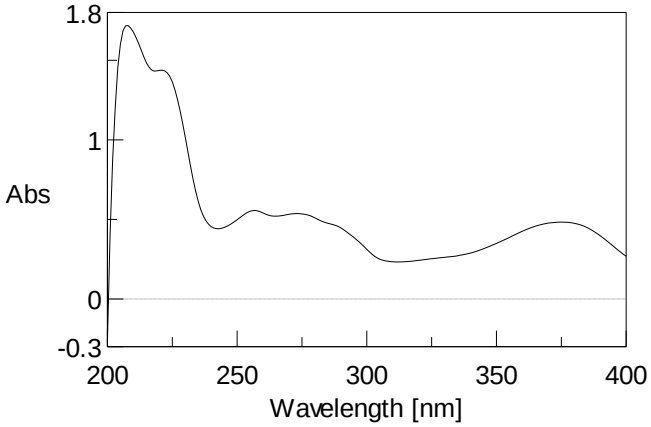


Table.3: Analysis of formulation.

Amount of desired active constituents found from formulation in µg	
Arbutin	**Quercetin**
1.870	0.799

VALIDATION OF THE METHOD

Specificity:

The specificity of the proposed method was assessed by their application to the analysis of laboratory prepared mixtures containing different ratios of intact arbutin and quercetin. Satisfactory results were obtained confirming that each of the cited drugs could be successfully determined without interference from the other.

Linearity:

The linearity of the developed method was evaluated by measuring the absorbance of different concentrations of the solution at 257nm and 287nm. The linearity was found at the level of 3-30µg/ml for arbutin and 2-20µg/ml for quercetin. The value of correlation coefficient was close to unity indicating good linearity.

Table.4: Linearity and validation parameters

S.No	Parameters	Arbutin	Quercetin
1.	Linearity	3-30µg/ml	2-20 µg/ml
2.	Correlation coefficient	0.9997	0.9998
3.	Slope	0.08434	0.0632
4.	Intercept	0.01873	0.0044

Accuracy:

Accuracy of the developed method was determined by recovery study which is 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.

Table.5: Accuracy

Level	% Recovery		%RSD*	
	Arbutin	Quercetin	Arbutin	Quercetin
100%	99.8	100.5	0.2761	0.3172

***-mean of six determinations.**

Precision:

Precision of the method was determined by inter-day and intra-day precision.

Intraday precision:

Intraday precision was performed by analyzing the standard solution for two different concentrations within the linearity range for six times a day and %RSD was calculated.

Table.6: Intraday precision

S. No	Arbutin concentration	Mean Absorbance	%RSD	Quercetin concentration	Mean Absorbance	%RSD*
1.	9	0.2312	0.6473	6	0.1822	0.6629
2.	21	0.5798	0.4815	14	0.4452	0.3593

***-mean of six determinations.**

Interday precision:

Interday precision was performed by analyzing the standard solution for two different concentrations within the linearity range for three days over a period of one week and %RSD was calculated.

Table.7: Interday precision

Arbutin concentration	Day	Mean Absorbance	%RSD*	Quercetin concentration	Mean Absorbance	%RSD*
12	1 2 3	0.3195 0.3163 0.3134	0.9481	8	0.2589 0.2565 0.2548	0.7791
24	1 2 3	0.6564 0.6478 0.6468	0.5365	16	0.5041 0.5008 0.4994	0.4786

***-mean of six determinations.**

Stability:

The stability of the standard solution was analysed in different time intervals in both normal and refrigerated conditions. The absorbance of the solution was compared with the absorbance of the freshly prepared standard solution. The solution was found stable for 36 hours under room temperature and 48 hours under refrigerated condition.

Table.8: Stability studies

Time in hours	Arbutin		Quercetin	
	Concentration (µg/ml)	Absorbance at 287nm	Concentration (µg/ml)	Absorbance at 257nm
0		0.5062		0.6558
1	24	0.5041	16	0.6564
4		0.5036		0.6524
8		0.5021		0.6508
12		0.4994		0.6489
16		0.4981		0.6472
20		0.4894		0.6442
24		0.4824		0.6410
36		0.4461		0.6398
48		0.4124		0.6280

EVALUATION OF GREENESS:

In the recent years, the assessment of analytical procedures for their greenness has become vital which ultimately made way for ranking greenness profile.

To evaluate the greenness profile of the developed the following parameters are checked.

i) AGREE

ii) Analytical Eco Scale

AGREE – METRIC TOOL

This technique is based on a novel software tool (AGREE) for assessing the greenness profile. The output of AGREE software is a clock-wise circular diagram with numbers from 1 to 12 around the edge that indicated 12 ideologies of green analytical chemistry. The results of each segment if the 12 principles were given on the aggregate scale from 0 to 1 with the inputs provided along with their weightage. The outcome of all the 12 principles and the core of the AGREE diagram depicts the score.

The colour range from red-yellow-green is based on the value of results obtained from 0 to 1. The colour is dark green when the score values are close to 1 white it is red colour if the score values are close to 0. The output on the AGREE metrics software was 0.68. hence the developed method was eco-friendly.

Fig.14: Greenness AGREE score for UV.

ANALYTICAL ECO SCALE

Analytical eco scale is another tool for the assessment of greenness profile. The penalty points are assigned for each of the selected analytical parameters like the amount of reagents, hazards, energy and waste generated. The penalty points for the entire procedure are added and combined in the eco scale calculation as indicated by the following formula.

Analytical Eco scale = 100 – Total penalty points.

The value of the eco scale adjacent to 100 indicates a greener analytical method. The score above 75 are regarded outstanding green, white the score 50-75 are considered average. The UV spectrophotometry method received a score 87. Based on the results the developed method is more environmentally friendly.

Table.9: Eco scale assessment

S. No	Parameters	Penalty points
1.	Methanol	2
2.	Instrument	3
3.	Energy used	0
4.	Waste	5
5.	Occupational hazard	3
	Total	**13**

Analytical eco scale = 100 – 13

= 87

AAS -HEAVY METAL ANALYSIS

Sample preparation:

The sample preparation for heavy metal analysis involves the digestion of 1.124gm of the residue obtained from the successive extraction using four different solvents (petroleum ether, chloroform, ethyl acetate, and methanol) in 25ml of Milli-Q water and filtered through Whatmann filter paper

Sample Analysis

The amount of heavy metals present is expressed as ppm or mg/kg. The amount of each heavy metal estimated was interpreted with the limits prescribed by WHO and European pharmacopoeia.

Table.10: Heavy metal analysis of extracts and their limits

Heavy metals	Amount present in plant powder (ppm)	Limits prescribed by WHO (ppm)
Arsenic	0.202	10
Lead	ND_1	10
Mercury	0.9705	0.1
Cadmium	ND_2	0.3
Iron	0.5815	MPL 1mg

*ND1- Not Detected upto 0.05mg/litre *ND2- Not Detected upto 0.005mg/litre

*MPL-Maximum Permissible Level per day

The Food and Nutrition Board (FNB) of the Institute of Medicine (IM) recommended dietary allowance of 7 -10 mg/day (700 – 1000 μg/day) for children, 8 mg/day (800 μg/day) for adults and 27mg/day (27,000 μg/day) during pregnancy for mothers.

RESULT AND DISCUSSION

Under UV spectral analysis, the absorption maxima (λmax) of Arbutin 257nm and Quercetin 287 nm was observed and showed in Figure 5 & 6. Obeyance to beer's law was confirmed by the linearity of the calibration curve of Arbutin and Quercetin, which represented in figure-12. Arbutin showed linearity in the concentration range of 3-30µg/ml and Quercetin 2-20µg/ml and are given in figure -9&10. The quantitative estimation was carried out in pellet formulation by taking concentrations of 18µg/ml of arbutin and 12µg/ml of quercetin and tabulated in table-3. The validation of the studies, the proposed method was further confirmed by recovery studies. The recovery data is given in table-6. The recovery values vary for ARB 99.8% and QUER 100.5%. This serves as a good index of accuracy of the method. The heavy metal analysis shows that the plant material contains arsenic, lead, cadmium and iron within the permissible limit but the amount of mercury does not lie within the limit. So, when preparing the formulation using the plant it has to be considered and necessary steps should be taken to control the level of mercury. With the use of green analytical chemistry valuation tool AGREE and Analytical Eco scale the method was found ecofriendly with the score of 0.68 (AGREE) and 87 (Analytical Eco Scale).

CONCLUSION

In the present study, UV 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 1.870 µg of ARB and 0.799 µg of QUER. Hence it is concluded that the developed validated method can be applied for the simultaneous estimation of ARB and QUER from *Origanum majorana* as well as its pellet formulation. The results of heavy metals analysis by atomic absorption spectroscopy clearly indicated the presence of heavy metals within the limits except mercury, which have to be considered while making the formulation in future. It also made an additional contribution to the main stream in the development and analysis of new herbal anticancer agents. On the basis of our experimental results, we conclude that the UV Spectrophotometric method developed for the simultaneous estimation of ARB and QUER was found to be rapid, precise, accurate and cost effective. Hence this method can be used for the routine analysis of ARB and QUER in bulk and pharmaceutical dosage forms. The validated method was also found eco-friendly with the use of Green Chemistry Principles and Tools.

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