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Development and validation of LC-MS/MS Method for the Simultaneous Determination of Arjunic Acid, Arjungenin and Arjunetin in *Terminalia arjuna (Roxb.) Wight & Arn.*

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Abstract: ***Arjunic acid, Arjungenin and Arjunetin are one of the three major triterpenoids in the bark of Terminalia arjuna (Roxb.) Wight & Arn. A simple, precise and accurate LC-MS/MS method has been developed for simultaneous determination of these three triterpenoids. MRM (Multiple Reaction Monitoring) transitions 487.20>425.30, 503.20>409.30, and 695.30>393.40 were optimized on Shimadzu triple quadrupole mass spectrometer instrument (Model: LCMS-8040) for quantification of Arjunic acid, Arjungenin, and Arjunetin respectively. Chromatographic method development was carried out using Shimadzu shimpack-XR C18 column (75mm x 3.0mm x 2.2 µ) with mobile phase containing 0.1% formic in water & acetonitrile and it was run with a gradient time program.***
The proposed method was validated for linearity, accuracy, precision, recovery, limit of detection (LOD) and limit of quantitation (LOQ). The validated LC-MS/MS method can be used for a routine quality control analysis and simultaneous quantitation of triterpenoids viz. Arjunic acid, Arjungenin, and Arjunetin in Terminalia arjuna formulation.

Keywords: *Terminalia arjuna*, LC-MS/MS, Arjunic acid, Arjungenin and Arjunetin

1. Introduction

Medicinal plants play an essential role in health care and are the major raw materials for both traditional and conventional medicine preparations; still most of the people choose herbal medicines than conventional medicines.[1] They expanded attention due to their effectiveness, lack of current medical alternatives, increasing cost of modern medicines and cultural preferences.[2,3]Ethnobotanical studies are most important to expose the ancient times and current culture about plants in the world and reserving original knowledge of medicinal plants. The evaluation of new drugs, especially the phytochemical obtained materials has opened a vast area for research and helpful in making a transition from traditional to modern medicine in India.

Terminalia arjuna (Roxb.) Wight & Arn. (*Terminalia arjuna*) (family: Combretaceae) is a large tree distributed throughout India. It is a commonly occurring medicinal plant growing as a 20-30m high tree. It is also well recognized in Ayurveda for its various therapeutic values[4,5]. Chemical constituents of different classes such as hydrolysable tannins[6], triterpenoid acids and their glycosides[7,8], flavonoids[9], Phenolics[10], phytosterol[11], were reported from stem bark portion of *Terminalia arjuna* species. Additionally, Arjunglucoside I-III, arjunic acid, arjunetin, arjunolic acid, and terminoic acid also form group of important constituents of the bark[12]. A number of previously published papers reports the therapeutic properties for *Terminalia arjuna*[13-15].

Hence, the principle of the study was to develop a simple, economic, rapid, precise LC-MS/MS method for simultaneous quantitation of three triterpenoid acids viz. Arjunic acid, Arjungenin, Arjunetin which is available in herbs and herbal formulation of *Terminalia arjuna.*

2. Materials and Methods

2.1 Plant material and sample preparation

Terminalia arjuna was collected from thane district of Maharashtra. Herbarium of *Terminalia arjuna* was prepared and authenticated from MS University (Vadodara), India. The bark collected were washed under running tap water. The plant material was kept for drying in oven at temperature 40±2°C. The dried material was powdered and sieved through ASTM sieve (85/BS sieve) separately and was kept in separate airtight containers.

500 mg of *Terminalia arjuna* bark powder was extracted with 10 mL of acidic methanol. The mixture was vortexed for 5 mins and it was put for overnight extraction. Extract was filtered through 0.2micron syringe filter and then it was subjected to LC-MS/MS analysis.

2.2 Chemicals and standard solutions Preparation

All the chemicals used in the experiments were of LCMS grade. Arjunic acid (88.0% purity), Arjungenin (95.0% purity), and Arjunetin (85.0% purity), were procured from sigma Aldrich chemie (steinheim Germany). The stock solutions (1mg/mL) of each were prepared separately in methanol. From this individual stocks mix working stock solution of 10ug/mL of each standard were prepared in methanol. Standard solutions were prepared by dilution of the mixed working stock solutions.

2.3 Instrumentation and Chromatographic Conditions

Chromatographic development was performed on Shimadzu Nexera, UHPLC (Ultra High Performance Liquid Chromatograph) system with LC-30AD pumps, SIL-30A autosampler and CTO-20AC as column oven. LABsolutions software was used for operating the instrument. Shimadzu LCMS-8040 model (Triple Quadrupole Mass Spectrometer) was used for optimization of MRM transitions 487.20>425.30, 503.20>409.30, and 695.30>393.40 for Arjunic acid, Arjungenin and Arjunetin respectively. Arjunetin was found to give formate ion adduct 695 [M - H + 46], hence precursor 695 was selected for MRM optimization of Arjunetin. Analysis was performed on Shimadzu, shimapack-XR, C18 column (75mm x3.0mm, 2.2 µm). The mobile phase comprising of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile was filtered through a 0.2 µm membrane filter (Millipore) and degassed by sonication. Gradient method was optimized for better chromatographic separation. Optimized gradient program is given in below table1.

Table 1: HPLC gradient program

Time	*% Mobile phase A*	*% Mobile phase B*
1.00	70	30
4.00	10	90
7.00	10	90
7.50	70	30
10.00	STOP	STOP

Flow rate 0.5mL/min was used for analysis. Column oven was set at 40°C. Analysis was performed using ESI (Electro Spray Ionization) interface at negative mode. Other MS parameters; Nebulizing Gas flow: 2L/min, Drying Gas flow : 15 L/min ,DL temperature: 250°C and Heating Block : 400°C were used for the analysis.

3. Results

Overlay mass chromatograms of Arjunic acid, Arjungenin and Arjunetin standard mixture is given in figure 1.

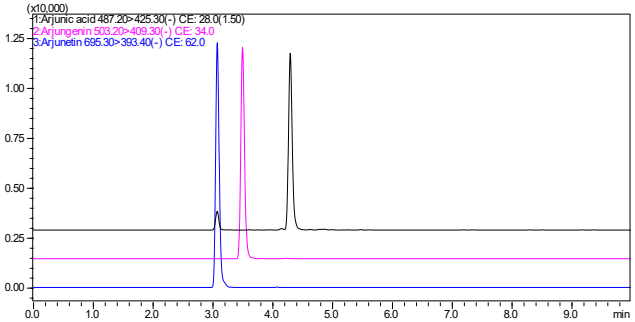
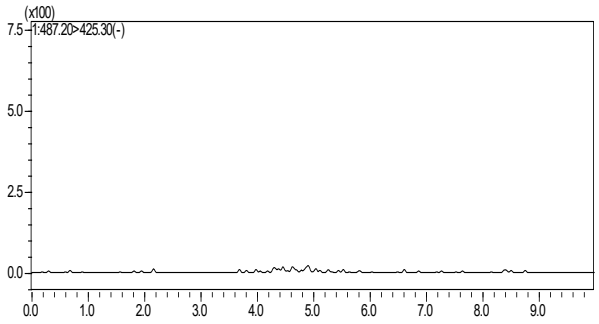
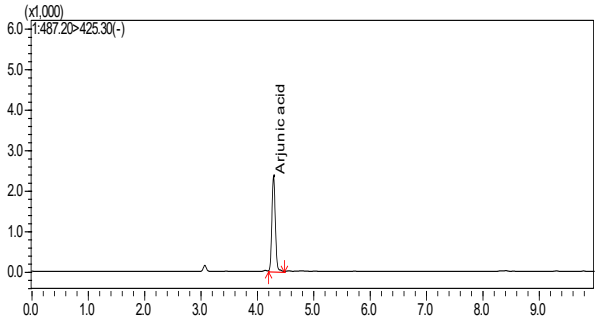


Figure1: Overlay mass chromatograms of Arjunic acid, Arjungenin and Arjunetin standard mixture

Detail linearity results for Arjunic acid, Arjungenin and Arjunetin is given in figure 2, 3 and 4.Chromatogram of *Terminalia arjuna* bark extract is given in figure 5.

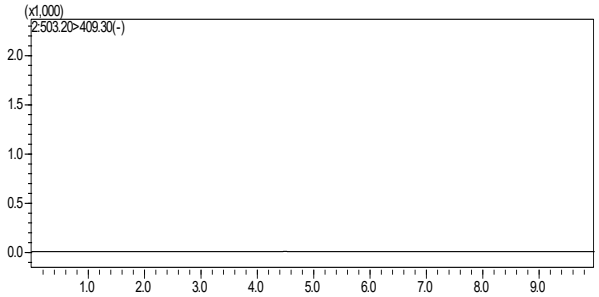


2-a: Arjunic acid _blank run

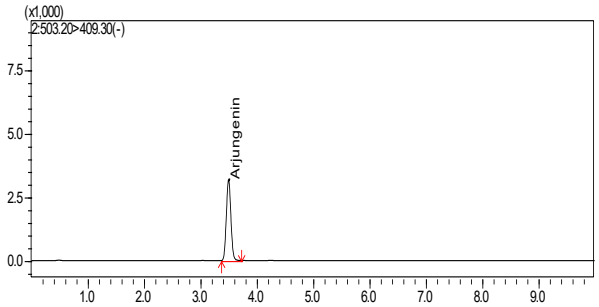


2-b: Arjunic acid _ 20 ng mL-1

Figure 2: Arjunic acid mass chromatograms and linearity graph

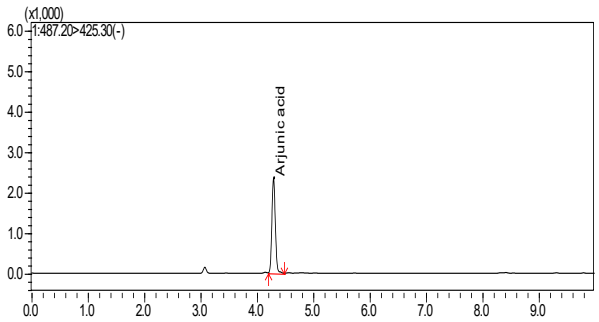


3-a: Arjungenin _blank run

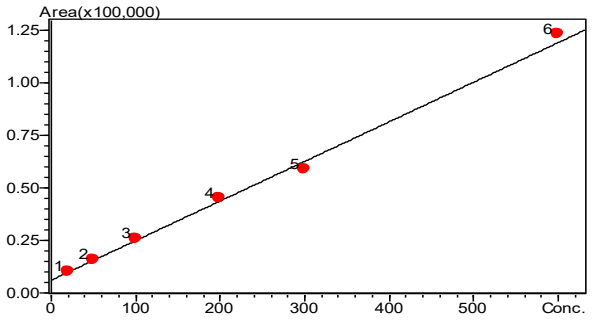


3-b: Arjungenin _15 ng mL-1

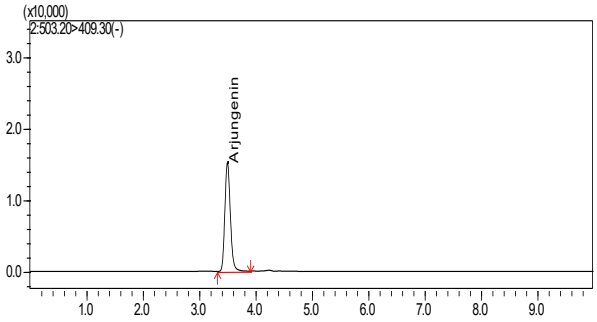
Figure 3: Arjungenin mass chromatograms and linearity graph



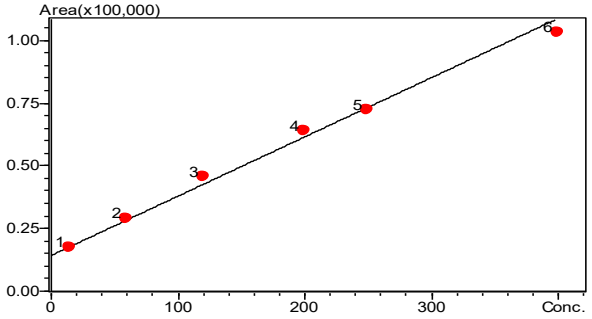
2-c: Arjunic acid _600 ng mL-1



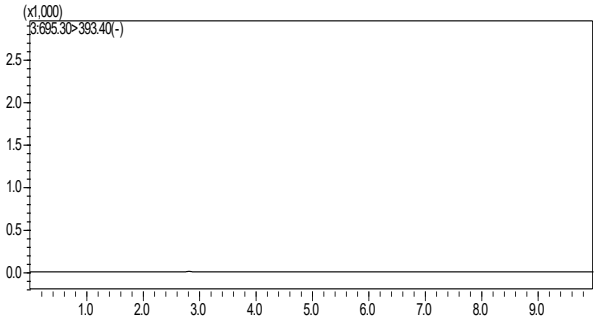
2-d: Linearity_ (20-600 ng mL-1)



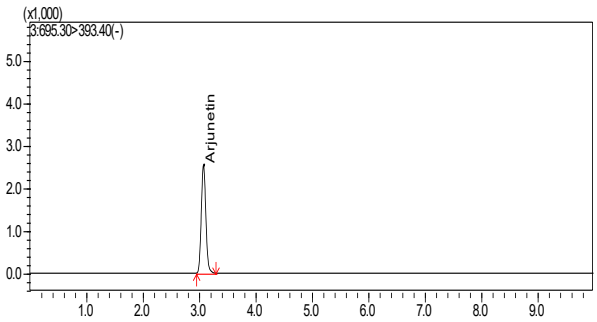
3-c: Arjungenin _400 ng mL-1



3-d: Linearity_ (15-400 ng mL-1)

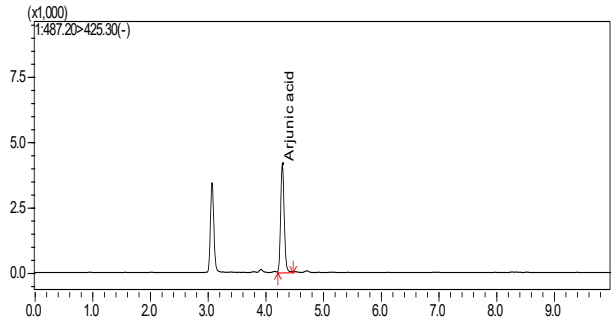


4-a: Arjunetin_blank



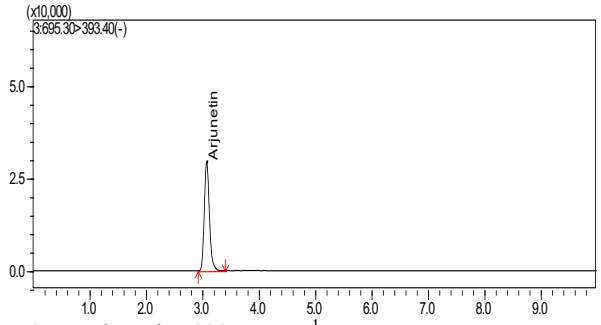
4-b: Arjunetin_25 ng mL^{-1}

Figure 4: Arjunetin mass chromatograms and linearity graph

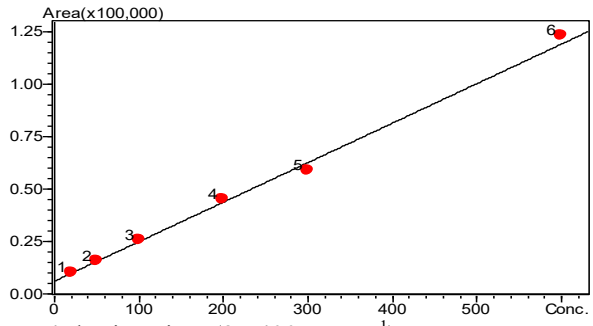


5-a: Arjunic acid

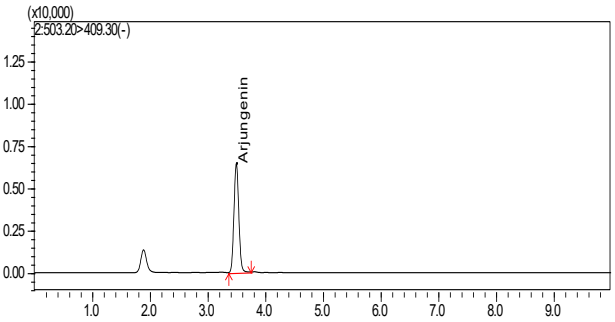
Figure 5: Chromatograms of *Terminalia arjuna* bark extract.



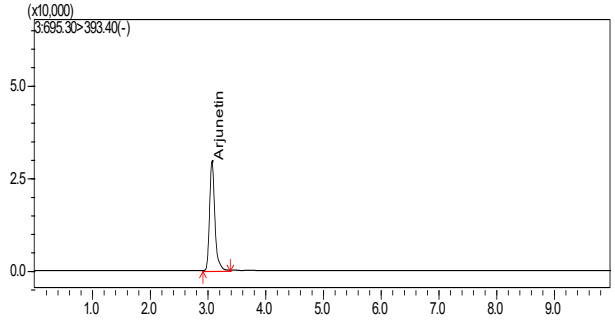
4-c: Arjunetin_400 ng mL^{-1}



4-d: Linearity_ (25-400 ng mL^{-1})



5-b: Arjungenin



5-c: Arjunetin

4. Method Validation Summary

4.1 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The signal-to-noise ratio of 3:1 and 10:1 was used to establish LOD and LOQ, respectively. The LOD and LOQ of Arjunic acid was 0.7 ng mL^{-1} and 1.0 ng mL^{-1}, Arjungenin was 0.8 ng mL^{-1} and 2.50 ng mL^{-1}, Arjunetin was 0.3 ng mL^{-1} and 1.0 ng mL^{-1} respectively.

4.2 Linearity

The experiment was performed five times and the mean was used for the calculations. The data was analyzed by linear regression least squares fitting. The statistical data obtained is given in Table 2.

Table 2

Parameters	1	2	3
Linearity range [ng mL^{-1}]	20-600	15-400	25-400
Slope [m][1]	188.365	236.313	447.680
Intercept [c][1]	6092.61	14289.6	3678.73
Correlation Coefficient [R^2]	0.9968	0.9939	0.9963
LOD [ng mL^{-1}] [2]	0.7	0.8	0.3
LOQ [ng mL^{-1}] [2]	2.0	2.5	1.0
Intraday precision(n=5 COV)	0.62	0.56	0.67
Inter day precision (n=5 COV)	0.72	0.84	0.78

[1] $y = mx + c$ equation, where y is peak area, m is the slope, x is the concentration, and c is the intercept.

[2] LOD (Limit of Detection) and LOQ (Limit of Quantitation) were calculated based on S/N ratio using LABSolutions software, Shimadzu.

4.3 Recovery

Three replicates at 100 ng mL^{-1}, 200 ng mL^{-1}, and 300 ng mL^{-1} concentration for Arjunic acid, Arjungenin and Arjunetin were prepared for recovery determination. The mean recovery for Arjunic acid, Arjungenin and Arjunetin were 97%, 98% and 94% respectively.

4.4 Assay

The developed HPLC method was used for simultaneous determination of Arjunic acid, Arjungenin and Arjunetin from bark powder of *Terminalia arjuna*. The sample working solution (5 µL) was injected and the area of these three triterpenoids were measured and quantitated against the calibration curve. The retention time Arjunic acid, Arjungenin and Arjunetin in sample solution was 4.306mins, 3.491mins and 3.102mins. The mean assay value of Arjunic acid was found to be 0.595 ug per 500 mg of plant powder with % RSD as 1.121, mean assay value of Arjungenin was found to be 1.0108 ug per 500 mg of plant powder with % RSD as 0.927 and mean assay value of Arjunetin was found to be 4.161 ug per 500 mg of plant powder with % RSD as 1.337.

4.4 Precision and Accuracy

The intra-day and inter-day precision was used to study the variability of the method. The % RSD for intra-day and inter-day precision for Arjunic acid were 0.62 and 0.72%, respectively, Arjungenin were 0.56 and 0.84 % respectively and Arjunetin were 0.67 and 0.78 % respectively

5. Conclusion

The application of a simple, rapid and accurate LC-MS/MS method for the simultaneous quantitation of triterpenoid acids viz. Arjunic acid, Arjungenin and Arjunetin in bark powder of *Terminalia arjuna*. The method was validated to track the active principles in the complex mixture of herbal ingredients. The method could be extended for the marker-based standardization of other herbal product containing triterpenoids viz. Arjunic acid, Arjungenin and Arjunetin.

The method was found to be simple, precise, accurate, specific and sensitive and can be used for routine quality control of herbal raw materials and for the quantification of these compounds in plant materials.

6. Acknowledgement

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