Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 6, 2014

Original Article

# SIMULTANEOUS DETERMINATION OF QUERCITIN AND AMENTOFLAVONE IN METHANOLIC LEAF EXTRACT OF SEMECARPUS ANACARDIUM (LINN.F.) BY REVERSE PHASE LIQUID CHROMATOGRAPHY

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*Received: 18 Mar 2014 Revised and Accepted: 27 Apr 2014*

**ABSTRACT**

**Objective:** To develop a sensitive, simple, rapid and reliable RP-HPLC method that can simultaneously determine Quercitin and Amentoflavone markers in methanolic leaf extract of *Semecarpus anacardium* (Linn.f.). In this regard HPLC method has been developed and validated as per ICH guidelines.

**Methods:** The reverse phase chromatographic separation was achieved using a Surveyor Agilent 1100 series HPLC system equipped with U.V.detector. The column used for method development and validation was Kromasil 100-5C18 (250x 4.6, 5 µm) as stationary phase with a mobile phase comprising of Water of pH 3(adjusted with O-phosphoric acid): Acetonitrile (55:45 v/v) at a flow rate of 1.0 ml $min^{-1}$. The injection volume of the sample was 20µl with U.V. detection at 280 nm. The run time of the sample was 10.0 min & the column oven temperature was 25$^0$C.

**Results:** The proposed RP-HPLC method has been validated as per ICH guidelines for its system suitability, linearity, LOD & LOQ, precision (Intra-day & Inter-day), accuracy & robustness.

**Conclusion:** The developed RP-HPLC method holds potential for detection and quantification of Quercitin and Amentoflavone markers in methanolic leaf extract of *Semecarpus anacardium* (Linn.f.).

**Keywords:** RP-HPLC, Quercitin, Amentoflavone, *Semecarpus anacardium* (Linn.f.).

## INTRODUCTION

*Semecarpus anacardium* is a deciduous tree belonging to Anacardiaceae family, growing in tropical and temperate regions of south east asian countries. Anacardiaceae family contains 700 species distributed among 60 genera. The nuts are used for treatment of variety of disorders in Ayurveda. It has been used therapeutically in neurological disorders, ulcers [1], corns [2] leprosy, leucoderma [3]. The plant is rich in secondary metabolites and mineral compositions which can be a potential source of useful therapeutic drugs [4-7].

Amentoflavone is naturally occurring biflavonoid that is found in a number of plants. It has many pharmacological activities. Investigation studies on the anti-inflammatory activities of amentoflavone and its mechanism of action has been done recently [8]. It exerts antibacterial, anti-inflammatory and antioxidative activities [9-13]. It has exhibited strong anticancer activities against MCF-7 and HeLa cancer cells through activation of hPPARγ, which is regulated by PTEN activation [14]. Amentoflavone, the main biflavonoid obtained from the genus *Calophyllum*, has been shown to inhibit HIV-reverse transcriptase, prevent histamine release from mast cells and cause mice to enhance their production of interferon [15]. Amentoflavone from *Selaginella*, has various biological and pharmacological effects, including antioxidant [16-19], anticancer [20-22], anti-inflammatory [23-25], antimicrobial [26], antivirus such as influenza (A, B), hepatitis (B), human immunodeficiency virus (HIV-1), herpes (HSV-1, HSV-2), herpes zoster (VZV), measles [27-32], respiratory syncytial virus (RSV) [33], vasorelaxation [34], antiurcerogenic [10], antistomachic-ache [11], antidepressant [23], anxiolytic [35,36], analgesic [37] and antiangiogenesis agent [38].

Quercetin has an antioxidative capacity [39]. Hydroxyl radical scavenging activity was detected in quercetin [40]. A human study showed topical and oral administration of quercetin to reduce skin damage during radiotherapy in patients with head and neck cancers [41]. It has a history of use by nutritional physicians as an anti-inflammatory and antiallergy agent [42]. It is found in a broad range of foods like grape skins, red onions, green tea and tomatoes. Quercetin is attracting intense scientific interest for its unique antiaging and immune boosting activities [43]. Laboratory studies demonstrate that quercetin traps developing cancer cells in the early phases of their replication cycle, effectively preventing further malignant development and promoting cancer cell death [44].

## MATERIALS AND METHODS

### Collection and Authentification of the plant

The leaves of *Semecarpus anacardium* were collected from Mumbai, Maharashtra. The identification of the plant was done at the Blatter Herbarium, St. Xavier's College, Mumbai. The plant specimen matches with the Blatter Herbarium specimen no.T- 472 of S. C. Tavakari.

### Chemicals and Standards

HPLC grade Acetonitrile from Qualigens, Mumbai were used. Ultra pure water, generated by use of a Milli-Q System (Millipore), was used for preparation of mobile phase. Standard compounds Quercitin and Amentoflavone were from Sigma Aldrich. O-phosphoric acid of Merck specialities Pvt. Ltd., Mumbai were used in study.

### Sample preparation

The leaf powder of *Semecarpus anacardium* (20 gm) was extracted with 250 ml methanol by soxhlet extraction for 8 hrs. The extract was concentrated on water bath at 60$^o$C. The obtained dark brown thick liquid was stored in a glass vial in refrigerator.

### Selection of Mobile Phase

Sample and standard solubility, stability and suitability were considered while choosing from different mobile phase compositions and proportions to get good chromatographic separations. From the various mobile phases, Water of pH3: Acetonitrile (55:45 %v/v) was selected. Water of pH3 was adjusted with O-phosphoric acid. The detection wavelength of 280nm was used throughout.

**Selection of Diluent**

The Distilled water was used as diluent.

**Preparation of Standard Stock Solution**

An accurately weighed quantity of Quercitin and Amentoflavone was transferred to a volumetric flask, dissolved and diluted to the mark with distilled water to obtain standard stock solution of 1000µg $ml^{-1}$.

**Apparatus and Chromatographic Parameters**

The optimized parameters which were used as a final method for the simultaneous estimation of Quercitin and Amentoflavone is represented in the Table 1.

**Method validation**

The optimized chromatographic method was completely validated according to the procedures described in ICH guidelines Q2 (R1) for the validation of analytical methods [45].

**System Suitability**

20µl of mixed standard solution of Quercitin and Amentoflavone (5 µg/ml) was injected (n=4) under optimized chromatographic conditions to evaluate the suitability of system. Values with % RSD < 2 were accepted (Table 2).

**Specificity**

Specificity of the HPLC method was demonstrated by the separation of the analytes from other potential components such as impurities, degradants or excipients. A volume of 20µl of individual standards, mixed stock of standards and extract solution were injected and the chromatogram was recorded (n=3). Hence, the proposed method was specific for Quercitin and Amentoflavone (Figure 1).

**Linearity**

The linearity of calibration curve of Quercitin and Amentoflavone over the concentration range of 0.25–20 µg/ml (n=3) through proposed HPLC method was carried out. Correlation coefficient (r) was found to be 0.9949 for Quercitin & 0.9964 for Amentoflavone respectively (Table 3 & Figure 2 & 3).

**Limit of Detection and Limits of Quantitation**

Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined by using the formula based on the standard deviation of the response area of blank and the slope of calibration curve. LOD and LOQ were calculated by using equations,

LOD= 3.3 x σ/slope & LOQ= 10 x σ/slope,

Where, σ = Standard deviation of Blank area (n=3), Slope = Slope of the calibration curve.

By these equations LOD was found to be 5.690 x $10^{-3}$ µg/ml for Quercitin & 6.729 x $10^{-5}$ µg/ml for Amentoflavone and LOQ for Quercitin 0.017 µg/ml & 2.039 x $10^{-4}$ µg/ml for Amentoflavone.

**Precision**

**Intra-day precision**

Intra–day precision was determined by injecting 3 different concentrations (80%, 100%, 120%) of the target concentration (n=4) times in the same day. Peak area was measured and %RSD was calculated **(**Table 4).

**Inter-day precision**

Inter-day precision was determined by injecting 3 different concentrations (80%, 100%, 120%) of the target concentration (n=4) times for 3 consecutive days. Peak area was measured and %RSD was calculated (Table 5-7).

**Accuracy**

For the accuracy of proposed method (n=3), recovery studies were performed by Standard addition method at 3 different levels 50%, 80% and 100% of the target concentration i.e. Quercitin (15 µg/ml) & Amentoflavone (9.49 µg/ml). Results of Recovery studies were found to be satisfactory (Table 8).

**Table 1: Optimized chromatographic conditions for Quercitin and Amentoflavone.**

| | |
|---|---|
| **Equipment** | Surveyor Agilent 1100 series HPLC system with G1379A Degasser, G1311A Quaternary pump, G1329A Autosampler, G1330B Thermostat ALS Therm, G1316A Column oven (COLCOM), G1314A U.V. visible detector |
| **Column** | Kromasil 100-5C18 (250x 4.6, 5 µm) |
| **Mobile Phase** | Water of pH3: Acetonitrile (55:45 %v/v) |
| **pH of Mobile Phase** | pH 3.00 adjusted with OPA |
| **Flow rate** | 1.0 ml $min^{-1}$ |
| **Injection volume** | 20 µl |
| **Column oven** | 25$^0$C |
| **Wavelength** | 280 nm |
| **Run time** | 10.0 min |
| **Mode of Operation** | Isocratic elution |

**Table 2: System Suitability of Quercitin and Amentoflavone (n=4).**

| Compound | Parameter | Acceptance | Average | %RSD |
|---|---|---|---|---|
| **Quercitin** | Retention Time (min) | % RSD < 2 | 4.55 | 0.03 |
| **5 µg/ml** | Peak area | % RSD < 2 | 112.8 | 1.45 |
| | Theoretical plates per meter | > 3500 | 11,252.64 | 1.69 |
| | Symmetry factor / Tailing factor | < 2 | 0.63 | 1.62 |
| | Resolution | >2 | 9.59 | 0.54 |
| **Amentoflavone 5 µg/ml** | Retention Time (min) | % RSD < 2 | 6.29 | 0.04 |
| | Peak area | % RSD < 2 | 175.05 | 0.48 |
| | Theoretical plates per meter | > 3500 | 17,437.52 | 0.45 |
| | Symmetry factor / Tailing factor | < 2 | 0.74 | 0.68 |
| | Resolution | >2 | 9.59 | 0.54 |

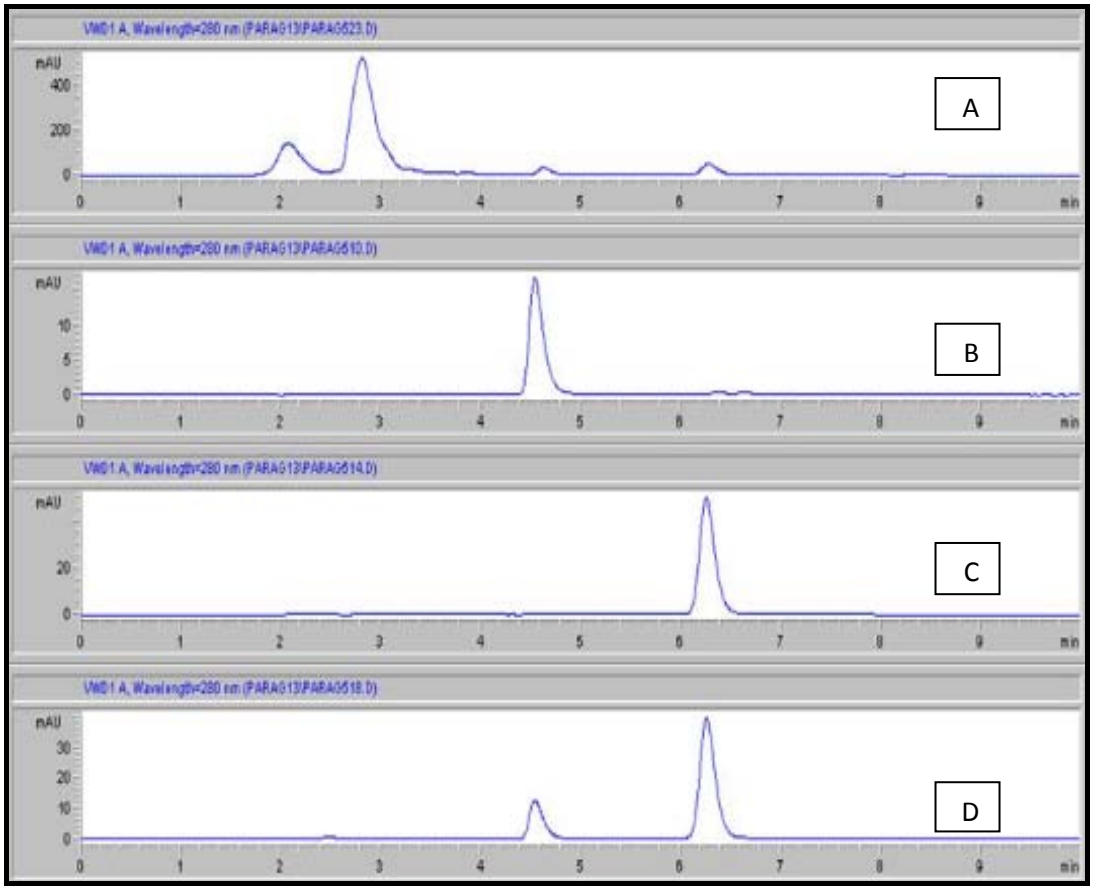


**Fig. 1: Chromatograms of A: Methanolic soxhlet leaf extract of *Semecarpus anacardium*, B: Quercitin 10 µg/ml, C: Amentoflavone 10µg/ml D: Quercitin & Amentoflavone 10 µg/ml.**

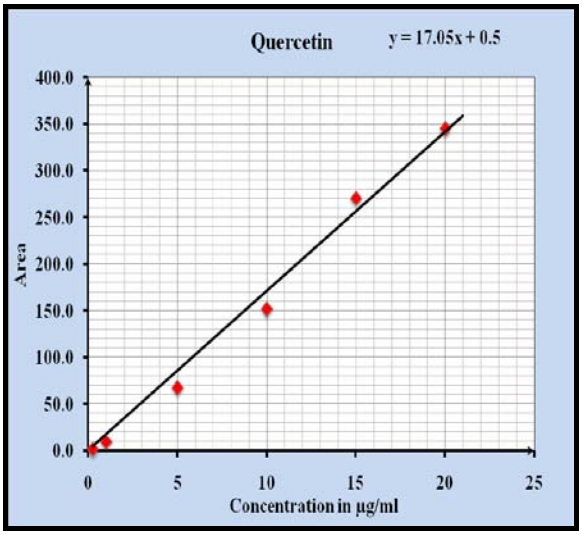


**Fig. 2: Linearity studies of Quercitin**

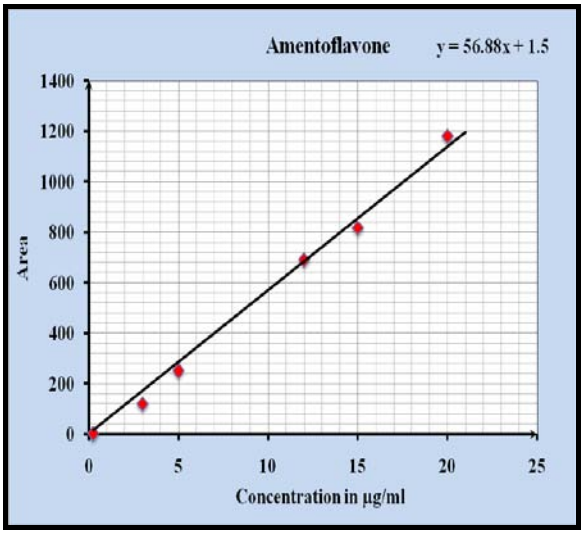


**Fig. 3: Linearity studies of Amentoflavone**

**Table 3: Linear regression data for Calibration curves (n= 3).**

| Parameters | Quercitin | Amentoflavone |
|---|---|---|
| **Detection wavelength** | 280 nm | 280 nm |
| **Linearity range** | 0.25 µg/ml – 20 µg/ml | 0.25 µg/ml - 20 µg/ml |
| **Correlation coefficient (r)** | 0.9949 | 0.9964 |
| **Regression equation** | y = 17.05x + 0.5 | y = 56.88x + 1.5 |
| **LOD** | $5.690 \times 10^{-3}$ µg/ml | $6.729 \times 10^{-5}$ µg/ml |
| **LOQ** | 0.017 µg/ml | $2.039 \times 10^{-4}$ µg/ml |

**Table 4: Intra-day precision of Quercitin and Amentoflavone (n=4).**

| **Quercitin** | **Intra-day** | | | |
|---|---|---|---|---|
| | **Retention time** | | **Peak Area** | |
| | **Mean** | **%RSD** | **Mean** | **%RSD** |
| 12 µg/ml | 4.53 | 0.08 | 254.68 | 0.23 |
| 15 µg/ml | 4.54 | 0.05 | 329.08 | 0.07 |
| 18 µg/ml | 4.54 | 0.04 | 389.6 | 0.10 |
| **Amentoflavone** | **Intra-day** | | | |
| | **Retention time** | | **Peak Area** | |
| | **Mean** | **%RSD** | **Mean** | **%RSD** |
| 7.59 µg/ml | 6.25 | 0.07 | 466.75 | 0.12 |
| 9.49 µg/ml | 6.26 | 0.08 | 593.5 | 0.03 |
| 11.39 µg/ml | 6.25 | 0.10 | 721.8 | 0.09 |

**Table 5: 1st Inter-day precision of Quercitin and Amentoflavone (n=4).**

| **Quercitin** | **1st Inter-day** | | | |
|---|---|---|---|---|
| | **Retention time** | | **Peak Area** | |
| | **Mean** | **%RSD** | **Mean** | **%RSD** |
| 12 µg/ml | 4.55 | 0.18 | 263.38 | 0.13 |
| 15 µg/ml | 4.54 | 0.12 | 331.58 | 0.08 |
| 18 µg/ml | 4.56 | 0.07 | 373.63 | 0.06 |
| **Amentoflavone** | **1st Inter-day** | | | |
| | **Retention time** | | **Peak Area** | |
| | **Mean** | **%RSD** | **Mean** | **%RSD** |
| 7.59 µg/ml | 6.26 | 0.17 | 412.25 | 0.03 |
| 9.49 µg/ml | 6.25 | 0.15 | 547.40 | 0.13 |
| 11.39 µg/ml | 6.29 | 0.14 | 590.53 | 0.05 |

**Table 6: 2nd Inter-day precision of Quercitin and Amentoflavone (n=4).**

| **Quercitin** | **2nd Inter-day** | | | |
|---|---|---|---|---|
| | **Retention time** | | **Peak Area** | |
| | **Mean** | **%RSD** | **Mean** | **%RSD** |
| 12 µg/ml | 4.54 | 0.08 | 254.78 | 0.07 |
| 15 µg/ml | 4.55 | 0.10 | 318.58 | 0.03 |
| 18 µg/ml | 4.55 | 0.04 | 372.83 | 0.06 |
| **Amentoflavone** | **2nd Inter-day** | | | |
| | **Retention time** | | **Peak Area** | |
| | **Mean** | **%RSD** | **Mean** | **%RSD** |
| 7.59 µg/ml | 6.23 | 0.18 | 384.45 | 0.02 |
| 9.49 µg/ml | 6.26 | 0.16 | 504.40 | 0.07 |
| 11.39 µg/ml | 6.27 | 0.06 | 506.43 | 0.04 |

**Table 7: 3rd Inter-day precision of Quercitin and Amentoflavone (n=4).**

| **Quercitin** | **3rd Inter-day** | | | |
|---|---|---|---|---|
| | **Retention time** | | **Peak Area** | |
| | **Mean** | **%RSD** | **Mean** | **%RSD** |
| 12 µg/ml | 4.55 | 0.07 | 247.45 | 0.19 |
| 15 µg/ml | 4.55 | 0.04 | 322.65 | 0.04 |
| 18 µg/ml | 4.55 | 0.07 | 374.63 | 0.07 |
| **Amentoflavone** | **3rd Inter-day** | | | |
| | **Retention time** | | **Peak Area** | |
| | **Mean** | **%RSD** | **Mean** | **%RSD** |
| 7.59 µg/ml | 6.26 | 0.19 | 363.45 | 0.07 |
| 9.49 µg/ml | 6.26 | 0.04 | 489.10 | 0.02 |
| 11.39 µg/ml | 6.27 | 0.12 | 471.75 | 0.03 |

**Robustness**

The Robustness of the HPLC method (n=3) was evaluated by analysing the parameters after varying the flow rate (± 20%), temperature (±5 $^0$C) and wavelength (± 5nm). Changes in % RSD of peak area w.r.t Quercitin and Amentoflavone standards and *Semecarpus anacardium* methanolic leaf extract are given below in Table 9.

**Table 8: Accuracy study (n=3).**

| Standard | Level | Preanalysed sample (µg ml$^{-1}$) | Amount of standard added to preanalysed sample (µg ml$^{-1}$) | Total amount Recovered/ Found in sample (µg ml$^{-1}$) | SD | % RSD | % Recovery |
|---|---|---|---|---|---|---|---|
| **Quercitin** | 0% | 15.00 | 0 | 0 | 0 | 0 | 0 |
| | 50% | 15.00 | 7.50 | 21.88 | 0.01 | 0.06 | 91.69 |
| | 80% | 15.00 | 12.00 | 27.89 | 0.14 | 0.49 | 107.43 |
| | 100% | 15.00 | 15.00 | 29.51 | 0.05 | 0.16 | 96.72 |
| **Amentoflavone** | 0% | 9.49 | 0 | 0 | 0 | 0 | 0 |
| | 50% | 9.49 | 4.75 | 13.56 | 0.01 | 0.08 | 85.79 |
| | 80% | 9.49 | 7.59 | 16.96 | 0.01 | 0.06 | 98.35 |
| | 100% | 9.49 | 9.49 | 18.07 | 0.01 | 0.07 | 90.43 |

**Table 9: Robustness study (n=3).**

| Quercitin and Amentoflavone Standards | | | | |
|---|---|---|---|---|
| **Changing Parameter** | **Actual Parameter** | **Modification** | **% RSD of Peak areas** | |
| | | | **Quercitin** | **Amentoflavone** |
| **Flow rate** | 1 ml/min | 0.8ml/min | 0.68 | 0.05 |
| | | 1.2ml/min | 0.60 | 0.32 |
| **Wavelength** | 280nm | 275nm | 0.40 | 0.50 |
| | | 285nm | 0.61 | 0.15 |
| **Temperature** | 25$^0$C | 20$^0$C | 1.02 | 0.09 |
| | | 30$^0$C | 1.80 | 0.33 |
| ***Semecarpus anacardium* methanolic leaf extract** | | | | |
| **Flow rate** | 1 ml/min | 0.8ml/min | 0.06 | 0.06 |
| | | 1.2ml/min | 0.22 | 0.11 |
| **Wavelength** | 280nm | 275nm | 0.29 | 0.03 |
| | | 285nm | 0.21 | 0.14 |
| **Temperature** | 25$^0$C | 20$^0$C | 0.06 | 0.06 |
| | | 30$^0$C | 0.32 | 0.13 |

## RESULTS

A satisfactory separation and good peak symmetry was obtained with Kromasil 100-5C18 (250x 4.6, 5 µm) column and mobile phase comprising of Water of pH 3: Acetonitrile (55:45 v/v).

The pH of water was adjusted at 3.00 using O-phosphoric acid. The flow rate was 1.0 ml/min to get better reproducibility and repeatability. Quantification was achieved with UV detection at 280nm based on peak area. The retention time was found to be 4.55 min for Quercitin and 6.29 min for Amentoflavone. Correlation coefficient (r) was found to be 0.9949 for Quercitin & 0.9964 for Amentoflavone respectively. The optimised method was validated as per ICH guidelines.

## CONCLUSION

A specific, precise, accurate, sensitive, rapid and reliable Reverse phase HPLC method has been developed and validated. It has runtime of 10 min and retention time 4.55 min for Quercitin and 6.29 min for Amentoflavone. Short run time allows analysis of large number of samples in a less period of time. So this RP-HPLC method can be used in the quality control department for estimation of Quercitin and Amentoflavone.

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