QUERCETIN-3-*O*-*D*-GLUCURONIDE, A NEW FLAVONOL FROM THE LEAVES OF *ANNONA MURICATA*

Nguyen Trong Khoi[1], Nguyen Quach Khanh Linh[1], Vo Hoang My Duyen[1], Huynh Thi My Duyen[1], Pham Doan Vi[2], Do Chau Minh Vinh Tho[1*]

1. Can Tho University of Medicine and Pharmacy

2. Tay Do University

**Corresponding author: dcmvtho@ctump.edu.vn*

ABSTRACT

Background: *Soursop (Annona muricata L., Annonaceae) is commonly known as a fruit tree in Vietnam. Nowadays, there are more and more functional foods from them with biological effects, especially antioxidant use. However, there have not been many studies in Vietnam on bioactive substances from this species, particularly flavonoids. Therefore, partly making it difficult to control the content of bioactive substances.* ***Objectives:*** *Extraction, isolation, and elucidation of the structure of flavonoids from Annona muricata L. leaves.* ***Materials and methods:*** *Flavonoids were extracted from 1.5 kg of soursop leaves powder with 70% ethanol by percolation. The extraction was then partitioned with n-hexane and extracted with ethyl acetate after adjusted pH to 1-2. The fractions obtained from the above procedure were subjected to chromatographic columns using Sephadex LH-20 as stationary phases and then followed by removing impurities with a suitable solvent to achieve pure flavonoids. The purity of the isolated compound was determined by TLC and HPLC-DAD techniques and the structure was deduced from its UV, MS, and NMR spectroscopic data, and comparison with published data.* ***Results:*** *FAM-1 compound was identified as quercetin-3-O-D-glucuronide by its UV, MS, and 1D and 2D-NMR data in comparison with those of published data and its purity was 91.24%. by HPLC/PDA.* ***Conclusions:*** *From Annona muricata L. leaves, a new flavonol was isolated (quercetin-3-O-D-glucuronide, 39.5mg) and its purity is 91.24%. This is the first time in Vietnam quercetin-3-O-D-glucuronide has been identified in Annona muricata leaves.*

Keywords: *Flavonoid, Annona muricata L, sephadex LH-20, mass spectroscopic.*

I. INTRODUCTION

Annona muricata L., Annonaceae is a familiar fruit tree species in Vietnam, especially in the southwestern provinces. In addition to the economic benefits, many studies also mention the effective pharmacological effects of this plant, including the leaves that have antibacterial, anti-inflammatory, and blood sugar improvement properties and prevent oxidation [1], [2]. Many compounds have been identified and isolated from Soursop leaves and of interested constituents are flavonoids, which are substances capable of antioxidants to prevent the development of cardiovascular disease, cancer, arthritis, Parkinson's, Alzheimer's [6], [7]. However, most of the uses are in the form of total extracts or whole extracts, and control of the content of biologically active flavonoids is almost hardly done. Therefore, the isolation of new compounds of biological significance will be used as a reference standard as well as enriching the source material for future scientific studies.

II. MATERIAL AND METHODS

2.1. Reagents and materials:

Annona muricata fresh leaves were collected at Can Tho city in July 2018. The leaves were washed, dried at 50- 60^{o}C until the humidity was less than 10%, and then pulverized into coarse powder for extraction. Ethanol for extraction was food-grade. The reagents and other solvents used for separation were of analytical grade (Shanghai Chemical

Reagent Co. Ltd., Shanghai, China). Solvents for HPLC analysis were of chromatographic grade, and TLC on Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany).

2.2. Apparatus

The HPLC Hitachi Elite L-2000 system including L-2130 Pump, L-2455 DAD, autosampler L-2000 and column compartment L-2300, EZChrome HPLC workstation (Hitachi Technologies, Japan). MS measurement was performed on a Xevo-TQD (Waters). The NMR measurement was recorded on a Brucker Avance 500 MHz NMR system (Brucker, Germany).

2.3. Methods

The 70% ethanol solvent was used for extraction. Alcoholic extract was evaporated under reduced pressure to give a concentrated solution, then diluted by adding water and subjected to liquid-liquid extraction with *n*-hexane to remove a nonpolar compound. The aqueous solution was made acid to pH 1-2 by H_2SO_4 5% and subjected to liquid-liquid extraction with ethyl acetate to yield the total ethyl acetate extract and then concentrated under reduced pressure to form a syrup. The syrup was milled in water (500 mL x 3) by sonication and then centrifuged at 5000 rpm for 10 min to achieve dried crude flavonoid fractions, which were subjected to a Sephadex LH-20 column with 100% methanol as eluent to give twenty-four fractions. Among them, the S-5 fraction contained a flavonoid with high intensity and 2 impurities with very low contents, which was dissolved in 500 mL water and then adjusted to pH 9 by 5% sodium hydroxide and subjected to a liquid-liquid extraction with chloroform (500 mL x 3) to give flavonoid fraction in aqueous layer and two impurities in the chloroform layer. Consequently, a pure flavonoid (FAM-1) was obtained by crystallizing in chloroform.

The purity of the FAM-1 was determined by TLC and HPLC-DAD methods, and the structure was elucidated by its UV, MS, and NMR data in comparison with those of published data.

III. RESULTS

The FAM-1 was a light-yellow amorphous powder, soluble in MeOH and slightly soluble in water, ethyl acetate. It had two λmax in MeOH at 256 nm and 354 nm, and then FAM-1 was checked for impurity by TLC technique with threes of different polarized solvent systems (**Figure 1**). Additionally, the impurity of FAM-1 was determined by 3D spectrum and total peak purity evaluation by HPLC-PDA method with the optimum chromatographic conditions: Phenomenex Synergi Fusion (250 mm, 4.6 mm, 4 µm) column, mobile phase: ACN and water containing 0.1% acid formic (25:75) in isocratic mode, the flow rate of 1.0 mL/min, detection wavelength of 354 nm, injection volume 20 µL (**Figure 2**).

ESI (-) mass spectrum of FAM-1 gave an $[M-H]^-$ precursor ion at *m/z* 477.36 and daughters-scan mode obtained specific fragment ions at *m/z* 301, 255, 179, 151, 113 (voltage cone at 50eV and collision cell energy at 60eV) of parent ion corresponding to the molecular weight of quercetin-3-*O*-*D*-glucuronide ($C_{21}H_{18}O_{13}$) [3], [5] (**Figure 3**).

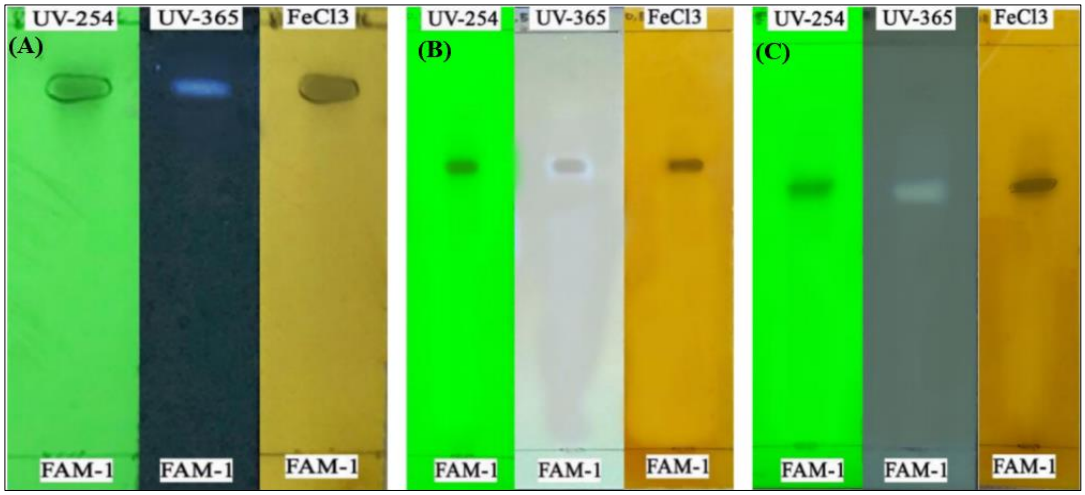

Figure 1. TLC of FAM-1 with three solvent systems: (A) MeOH-acetone-H_2O (18:1:1); (B) $CHCl_3$-MeOH-HCOOH (2:8:0.5); (C) EtOH-HCOOH (100:1)

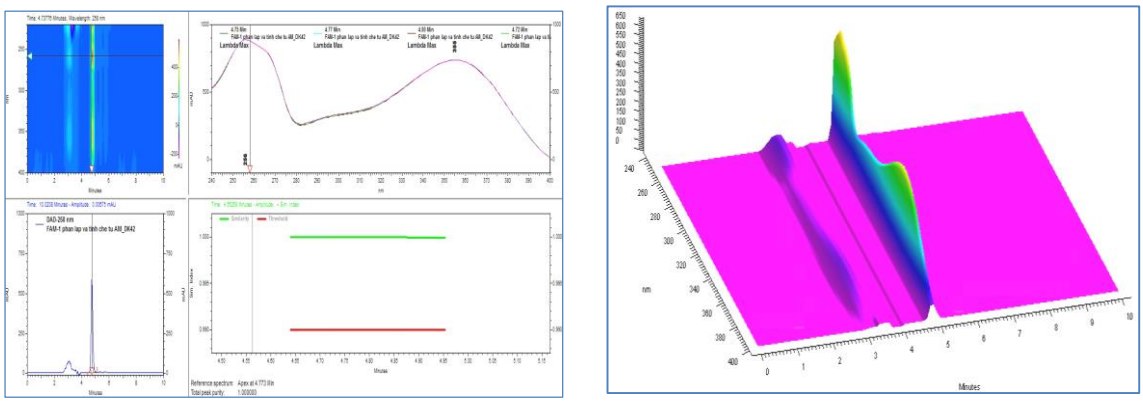

Figure 2. The purity of FAM-1 by HPLC/PDA

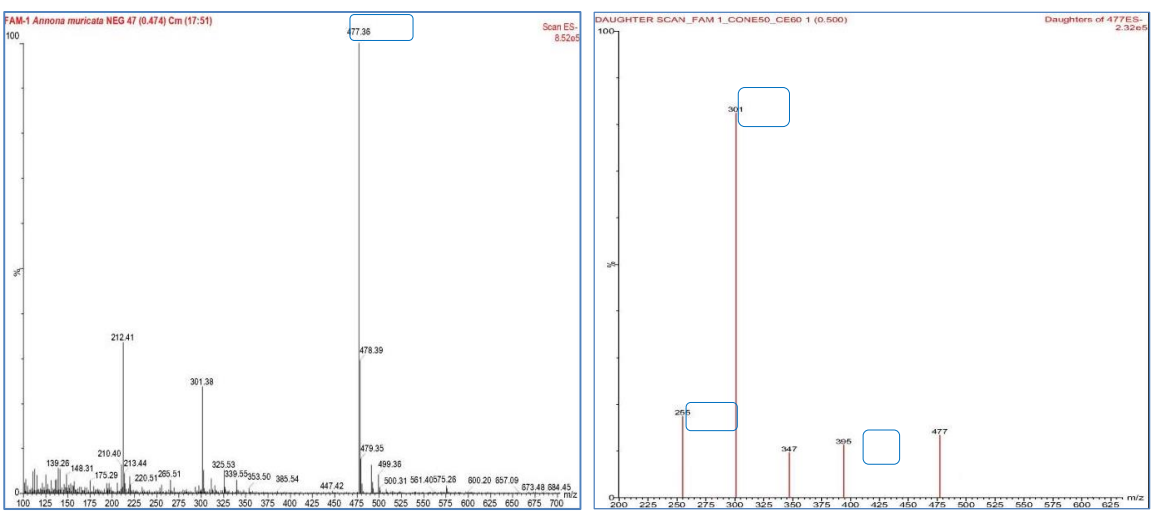

Figure 3. MS1-scan and daughters-scan mode of FAM-1

Moreover, the structure of FAM-1 was elucidated by ^{13}C-CPD, ^{13}C-DEPT, ^{1}H, COSY, HSQC, HMBC data in comparison with those of quercetin-3-*O*-*D*-glucuronide published in the literature [1]. As the result in **Table 1**, NMR data of FAM-1 were in good agreement with those of flavonoid quercetin-3-*O*-*D*-glucuronide (**Figure 4**). Therefore, the compound FAM-1 was identified as quercetin-3-*O*-*D*-glucuronide with the molecular formula $C_{21}H_{18}O_{13}$, one of the main flavonoids of soursop leaves.

Table 1. NMR data of FAM-1 and quercetin-3-*O*-*D*-Gln.

FAM-1 (MeOD, 500MHz)					**Quercetin-3-*O*-*D*-Gln** (MeOD, 500MHz) [3],[4]	
C	**δ_C (ppm)**	**δ_H (ppm), J (Hz)**	**HMBC**	**COSY**	**δ_C (ppm), J (Hz)**	**δ_H (ppm)**
2	159.3, *s*				157.0, *s*	
3	135.8, *s*				133.5, *s*	
4	179.5, *s*				177.6, *s*	
5	163.0, *s*				161.6, *s*	
6	99.9, *d*	6.2 (*d*, 2Hz)	C_8; C_{10}; C_5; C_7	H_8	99.2, *d*	6.2 (*d*, 2Hz)
7	166.1, *d*				164.7, *d*	
8	94.8, *d*	6.4 (*d*, 2Hz)	C_6; C_7; C_9; C_{10}	H_6	94.1, *d*	6.4 (*d*, 2Hz)
9	158.5, *s*				156.7, s	
10	105.8, *s*				104.3, s	
1’	122.7, *s*				121.8, s	
2’	116.2, *d*	6.9 (*d*, 8.5Hz)	C_2; $C_{4'}$; $C_{3'}$; $C_{6'}$		116.5, *d*	7.53 (*br s*)
3’	145.9, *s*				145.4, *s*	
4’	149.9, *s*				149.1, *s*	
5’	118.2, *d*	7.98(*d*, 2Hz)	$C_{4'}$; $C_{3'}$; $C_{6'}$; $C_{1'}$	$H_{6'}$	115.7, *d*	6.8(*d*; 9Hz)
6’	122.8, *d*	7.5 (*dd*, 2, 8.5Hz)	C_2; $C_{4'}$; $C_{5'}$	$H_{5'}$; $H_{2'}$	122.2, *d*	7.6 (*dd*, 2.5 9Hz)
1”	104.4, *d*	5.4 (*d*, 7.5Hz)	$C_{3'}$	$H_{2''}$	101.5, *d*	5.5 (*d*, 7.5Hz)
2”	77.6, *d*	3.56, *m*	$C_{3''}$; $C_{4''}$	$H_{3''}$	74.2, *d*	3.56, *m*
3”	75.7, *d*		$C_{6''}$; $C_{2''}$; $C_{1''}$	$H_{2''}$	76.3, *d*	
4”	73.4, *d*				72.0, *d*	
5”	78.1, *d*		$C_{6''}$; $C_{2''}$; $C_{1'}$	$H_{3''}$; $H_{4''}$	76.4, *d*	
6”	176.2, *s*	3.66, 3.58, *m*	$C_{5''}$; $C_{4''}$	$H_{4''}$; $H_{5''}$	175.3, *s*	

s: singlet, dd: doulet, td, triplet, m: multilet

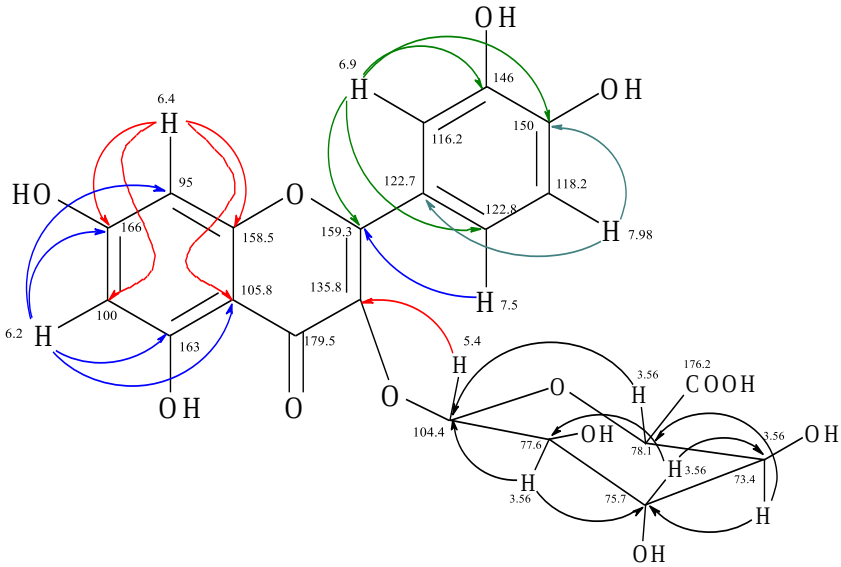

Figure 4. The structure of FAM-1 (quercetin-3-O-D-glucuronide) and HMBC correlations

IV. DISCUSSION

The total flavonoid content of *Annona muricata* L. leaves were announced at 0.025% [4] comparing to this result about 0.021% including quercetin-3-O-D-glucuronide, one of the main flavonoids of *Annona muricata.*

The total peak purity of the isolated FAM-1 was determined by comparing the similarity of the UV spectrum of any points on the peak with the average UV spectrum taken from 3 points, 01 point at the peak, 02 points on the left, and the right at 30 % height of the peak curve with the UV spectrum similarity threshold over 990 points. As a consequence, the peak of FAM-1 at minute 4.77 obtained very good total peak purity with UV spectrum similarity at 1000 points. Additionally, to calculate the % purity of FAM-1, the method refers to the sum of 100% of the peak area at max plot mode was used. The chromatographic result showed that there were FAM-1 peaks with the largest peak area, three impurities peaks, and the interference peaks from the solvents. The percentage of FAM-1 purity (91.24%) was obtained by the ratio of peak area of FAM-1 (21,461.946) and the peak area of impurities (2,060.670) adding to FAM-1.

According to the EC-657/2002 guidelines, the identification of a substance by mass spectrometry requires 5.5 IP points. Accordingly, the results of mass spectrometry analysis for FAM-1 received initial molecular ion with m/z 477.36 corresponding to 1 IP point and in daughters-scan mode obtained threes specific fragments with m/z respectively 301.38, 178.98, 151.19 corresponding to 4.5 IP points. Moreover, precursor ion and these fragments are in agreement with (merge: 999) the publication in the European mass spectrometry library [8].

Furthermore, comparing the chemical shift spectrum ^{13}C-CPD, ^{13}C-DEPT 90 & 135, ^{1}H, COSY, HSQC, HMBC to published data, the HMBC correlations were deduced in **Figure 4**. It indicated that the structure of FAM-1 met full agreement with quercetin-3-*O*-*D*-glucuronide.

V. CONCLUSIONS

A new flavanol was isolated and identified from *Annona muricata* L. leaves by modern chromatographic and spectrum techniques, namely quercetin-3-*O*-*D*-glucuronide, and the percentage of purity was determined by HPLC/PDA method of 91.24%. To our best knowledge, this is the first time, quercetin-3-*O*-*D*-glucuronide is identified in soursop leaves of *Annona muricata* L. in Vietnam. This compound could be used as references for quantitative and qualitative analysis of raw materials and herbal preparations containing this herb, as well as for the investigation of its pharmacological activities in further studies.

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