ISOLATION AND STRUCTURAL ELUCIDATION OF ALKALOIDS FROM LEAVES OF *ANNONA MURICATA*

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ABSTRACT

Background: *Annona muricata L., a member of Annonaceae is a fruit tree with a high valuable medicinal herb. A. muricata, also known as soursop that is mostly distributed in tropical and subtropical regions of the world. However, most of the formulations used are in the form of total concentrates or extracts, and control of the content of biologically active alkaloids is almost impossible. One of the reasons is the lack of standardization and isolation studies of the main alkaloids as reference standards.* ***Objectives:*** *To isolate and determine purity and elucidate the structure of alkaloids from Annona muricata L. leaves extract.* ***Materials and methods:*** *Alkaloids were extracted from 1.5 kg leaves powder of Annona muricata L. with 70% ethanol by percolation. The fractions obtained from liquid-liquid distribution based on pH were subjected to chromatographic columns using Silica gel and Sephadex LH-20 as stationary phases and then liquid-liquid distribution to obtain TP-3A-3 alkaloid. The purity of the isolated compound was determined by the HPLC-DAD method. The structure of TP-3A-3 was elucidated by UV, MS, and NMR data in comparison with those of published data.* ***Results:*** *TP-3A-3 was identified Annonamine by its UV, MS, and 1D and 2D-NMR data in comparison with those of published data and its purity was 96.2% by HPLC/PDA.* ***Conclusions:*** *From Annona muricata L. leaves, one alkaloid was isolated (annonamine, 14.5mg) and its purity was 96.2%. This is the first time in Vietnam anonamine has been identified in Annona muricata L in Viet Nam*

Keywords: *Annona muricata L., soursop, alkaloid, annonamine.*

I. INTRODUCTION

Soursop (*Annona muricata* L.) is familiar fruit tree in Vietnam. Soursop offers many economic benefits, but the other parts of the plant are barely utilized. In Vietnam the soursop leaves are widely used for inhibiting the growth of cancer cells [2],[3], supporting cancer treatment [4],[9], antioxidant potentials [3],[7]. Pharmacological studies have revealed that alkaloid composition is among bioactive compounds of soursop leaves. Numbers of studies on its alkaloid constituents have been conducted in the world [1],[5],[6]. However, the chemical constituents and pharmacological activities of Vietnamese soursop leaves remain little known, so far. In the course of chemical and pharmacological studies on *A. muricata* L. This paper will report the extraction, isolation, and structure elucidation of alkaloid from the soursop leaves growing in Vietnam. The isolated compound will be used as references in quality controls of these crude drugs and herbal products, as well as for pharmacological studies.

II. MATERIALS AND METHODS

2.1. Reagents and materials

Soursop leaves were collected at Can Tho city in July, 2018. The leaves were washed, dried at 50 - 60°C until the humidity was less than 10%, and then pulverized into coarse powder for extraction.

Alcohol for extraction was of food-grade and 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 $60F_{254}$ plates (Merck, Darmstadt, Germany)

2.2. Apparatus

The HPLC analyses were run on 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 leaves powder was extracted with 70% ethanol by percolation. The extract was filtered, and the solvent was evaporated in a rotary evaporator under reduced pressure to discard most of the ethanol and then partitioned with *n*-hexane to remove nonpolar compounds. The lower phase was evaporated in a rotary evaporator under reduced pressure to give total extraction (TE). TE was acidified with sulfuric acid 5 % to pH 2-3 and subjected to liquid-liquid extraction with ethyl acetate to obtain acidic layer, continue to basify to pH 10 with NaOH 10%, and subjected to liquid-liquid extraction with chloroform to obtain pH 10 crude alkaloid fraction (TP). 15g TP was subjected to vacuum liquid chromatography (VLC) using *n*-hexane – $CHCl_3$ (90:10); *n*-hexane – $CHCl_3$ (80:20); *n*-hexane – $CHCl_3$ (85:15); *n*-hexane – $CHCl_3$ (75:25) for elution to give three fractions (TP-1, TP-2, TP-3). After the TP-3 fraction was eliminated impurities by using the pH gradient with liquid-liquid distribution, the TP-3 fraction was subjected to a Sephadex LH-20 column with 100% methanol as eluent to obtain pure alkaloid fraction TP-3A-3. The purity of the isolated compound was determined by TLC and HPLC-DAD methods. The chemical structure of the isolated compound was elucidated by its UV, MS, and NMR data in comparison with those of published data.

III. RESULTS

From total alkaloid fraction (TP), TP-3A-3 was obtained and recrystallized in $CHCl_3$ to give colorless needles crystal (14.5 mg) with λ_{max} at 282 nm and 254 nm in MeOH (**Figure 2**). TP-3A-3 was checked for impurity by TLC technique with threes of different polarized solvent systems (**Figure 1**), 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 (60:40) in isocratic mode, the flow rate of 1.0 ml/min, detection wavelength of 258 nm, injection volume 20 µl. The impurity was determined by 3D spectrum and total peak purity check, as the result, the purity of TP-3A-3 was 96.2% (**Figure 2**).

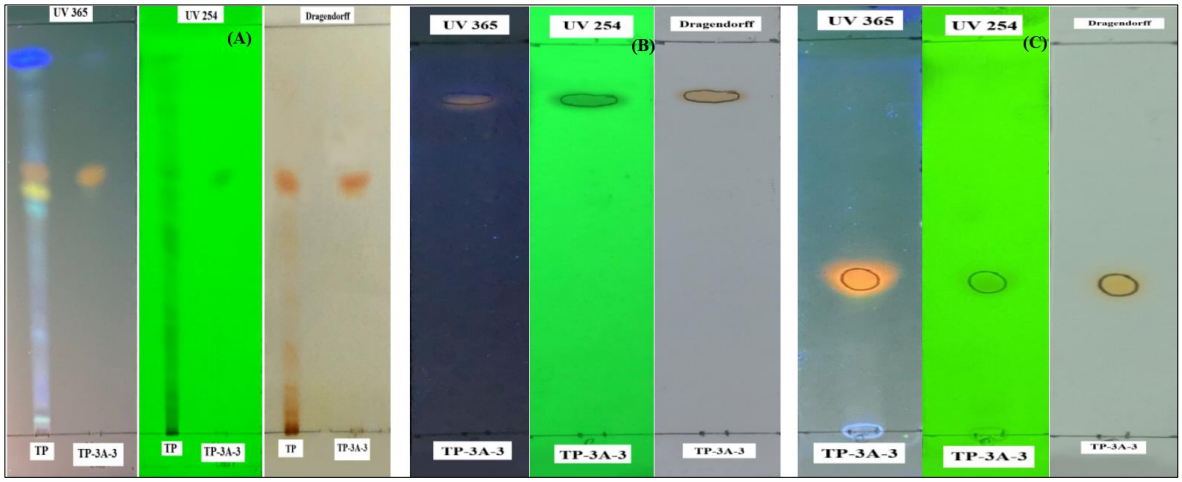

Figure 1. TLC of TP-3A-3 with three different mobile phases:(A) $CHCl_3$ – MeOH (98:2); (B) *n*-hexane – $CHCl_3$ (70:30); (C) $CHCl_3$ – acetone (70:30)

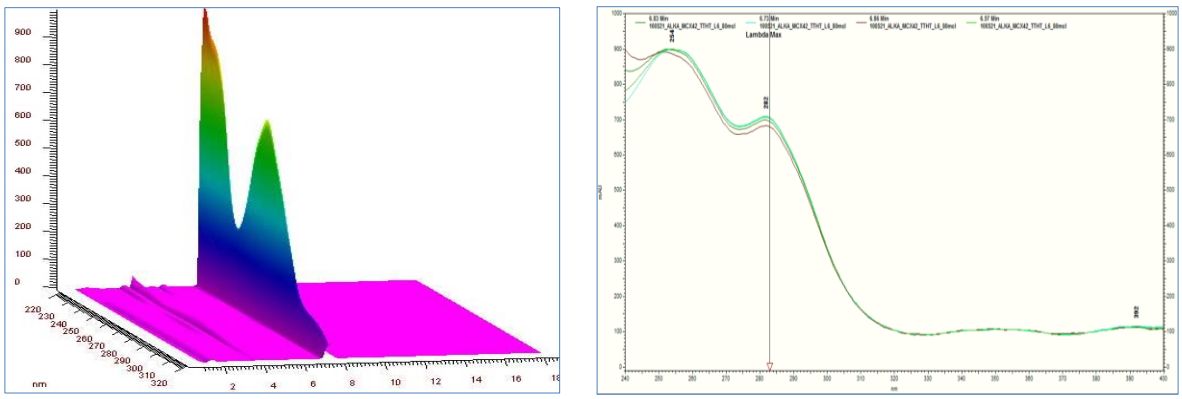

Figure 2. The purity of TP-3A-3 by HPLC/PDA

ESI (+) mass spectrum of TP-3A-3 gave a $[M+H]^+$ precursor ion at m/z 297 and daughters-scan mode obtained specific fragment ions m/z 249, m/z 191, and m/z 205 of parent ion corresponding to the molecular weight of 296 of annonamine [1],[2] (**Figure 3**).

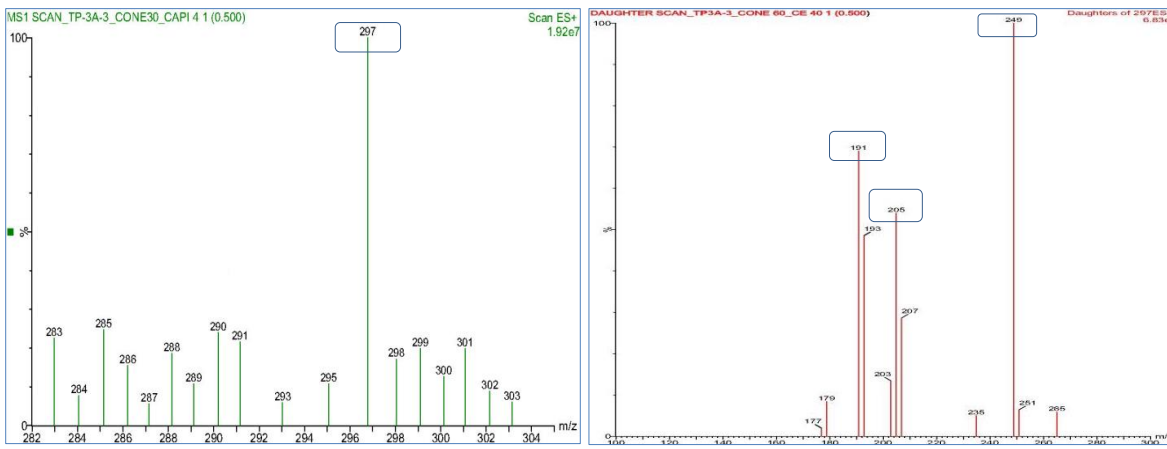

Figure 3. MS1-scan and daughters-scan mode of the TP-3A-3.

Moreover, the structure of TP-3A-3 was elucidated by ^{13}C-NMR, ^{1}H-NMR, COSY, HSQC, HMBC data in comparison with those of annonamine published in the literature [1].

As to be seen in **Table 1**, the NMR data of TP-3A-3 were in good agreement with those of annonamine [1], [8]. Hence, the compound TP-3A-3 was identified as annonamine (**Figure 4**) with the molecular formula $C_{19}H_{22}NO_2^+$, one of the main alkaloids of soursop leaves.

Table 1. NMR data of TP-3A-3 and annonamine

TP-3A-3 ($CDCl_3$, 500 MHz)				**annoamine** ($CDCl_3$, 600 MHz) [1]	
C	**DEPT**	**δ_c ppm**	**δ_H ppm, J, (Hz)**	**δ_c ppm**	**δ_H ppm, J, (Hz)**
C-1	C	146.2		146.9	
C-1a	C	126.9		128.6	
C-1b	C	120.0		120.2	
C-2	C	152.0		152.9	
C-3	CH	116,3	6.78 s	116.5	6.78 s
C-3a	C	126,9		126.2	
C-4	CH_2	24,7	3.16 m	24.7	3.34 m
C-5	CH_2	62,8	3.73 ddd (13,7,1)	62.8	3.79 ddd (13,7,1)
C-6a	CH	70,3	4.50 dd (14,4)	70.8	4.56 dd (14,4)
C-7	CH_2	29,8	3.25 m	30.9	3.37 m
C-7a	C	134,2		133.5	
C-8	CH	129,7	7.38 d (7)	129.4	7.40 d (7)
C-9	CH	129,2	7.28 td (7,1)	129.5	7.31 td (7,1)
C-10	CH	128,5	7.21 br t (8)	129.2	7.31 br t (8)
C-11	CH	127,3	8.32 dd (8,1)	129.1	8.32 dd (8,1)
C-11a	C	132,2		132.5	
O-Me	CH_3	60,2	3.62 (3H,s)	60.8	3.62 (3H,s)
N-Me α*	CH_3	43,9	3.08 (3H,s)	43.8	3.08 (3H,s)
N-Me β*	CH_3	55,8	3.42 (3H,s)	54.2	3.42 (3H,s)

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

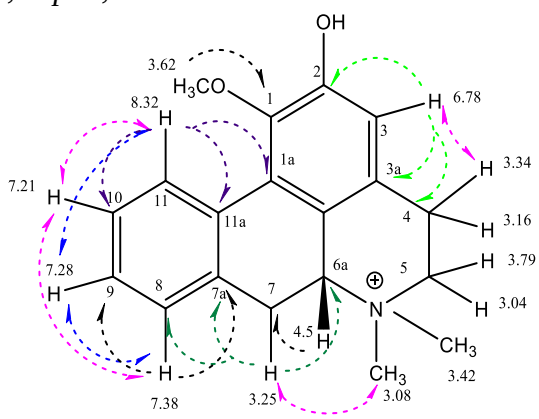

Figure 4. Chemical structure of TP-3A-3 (annonamine) and 2D-NMR spectral interaction

IV. DISCUSSION

In this study, we used the alcohol solvent for the extraction so that both free and salt alkaloids can be suited. As the result, the hydrophilic impurities such as polysaccharides, protein…, and hydrophobic impurities are extracted. Therefore, we used a liquid-liquid partition with n-hexane to remove nonpolar compounds and the aqueous phase was partitioned with the pH solution changes from 2 to 10 to remove the impurities. Consequently, the total alkaloid fraction (TP) with fewer impurities at pH 10 is the optimum condition for alkaloid extracting from soursop leaves.

The total extract obtained from this process, which is promising for enhanced extraction and isolation, is relatively clean. Theoretically, the total alkaloid content of Annona muricata L. leaves to be announced just only 0,0125% comparing to the result is about 0,04% including identified annonamine, one of the main alkaloids of *Annona muricata* L. The chemical structure of the isolated compound was elucidated by its UV, MS, and NMR data in comparison with those of published data, and the purity of determined by HPLC-DAD with 96.2%. These results demonstrate the use of the studied procedure to extract and isolate alkaloids from soursop leaves.

V. CONCLUSIONS

From *Annona muricata* L. leaves, one alkaloid was isolated, and its purity is 96.2%. This is the first time anonamine 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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