QUALITATIVE AND QUANTITATIVE ANALYSIS OF FLAVONOIDS IN *ARTOCARPUS TONKINENSIS* LEAVES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY (HPLC-MS)

PHÂN TÍCH ĐỊNH TÍNH CÁC HỢP CHẤT FLAVONOID VÀ ĐỊNH LƯỢNG CHẤT CHÍNH TRONG LÁ CHAY BẮC BỘ (*ARTOCARPUS TONKINENSIS)* BẰNG SẮC KÝ LỎNG HIỆU NĂNG CAO GẮN KHỐI PHỔ (HPLC-MS)

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ABSTRACT

HPLC-ESI-MS method was used for the identification of the flavonoids and quantitative determination of the flavonoid constituents in the water extract of *Artocarpus tonkinensis* leaves. Seven flavonoids were unambiguously identified as catechin (**1**), alphitonin 4-O-β-D-glucopyranoside (**2**), maesopsin 4-O-β-D-glucopyranoside (**3**), quercetin 3-O-β-D-glucopyranoside (**4**), kaempferol-3-O-β-D-glucoside (**5**), quercetin (**6**) and kaempferol (**7**), by comparing their retention times, UV and ESI-MS spectra with those of the authentic isolated compounds. Additionally, the main compounds were **2** and **3**, with hight yield (2.86 and 2.45 mg/g, respectively) in this plant leaves.

***Keywords*:** *Artocarpus tonkinensis, flavonoids, maesopsin 4-O-β-D-glucopyranoside, HPLC-MS.*

TÓM TẮT

Trong nghiên cứu này phương pháp sắc ký lỏng hiệu năng cao gắn khối phổ (HPLC-ESI-MS) được sử dụng để định tính flavonoid và định lượng chất chính trong dịch chiết nước của lá Chay Bắc bộ (*Artocarpus tonkinensis* (A.Chev. ex Gagnep)). Bảy flavonoid được định tính từ dịch chiết nước này là catechin (**1**), alphitonin 4-O-β-D-glucopyranoside (**2**), maesopsin 4-O-β-D-glucopyranoside (**3**), quercetin 3-O-β-D-glucopyranoside (**4**), kaempferol-3-O-β-D-glucoside (**5**), quercetin (**6**) and kaempferol (**7**) bằng cách so sánh thời gian lưu, phổ UV và phổ ESI-MS của chúng với các hợp chất đã phân lập từ cây này. Ngoài ra, hàm lượng chất **3** (maesopsin 4-*O*-β-D-glucopyranosid) được xác định là 24,5mg/g.

Từ khóa: *Lá Chay, flavonoids, maesopsin 4-O-β-D-glucopyranoside, sắc ký lỏng hiệu năng cao gắn khối phổ.*

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Received: 10/8/2020

Revised: 15/9/2020

Accepted: 21/10/2020

1. INTRODUCTION

Natural products as a rich source of raw materials for the pharmaceutical industry have been actively investigated with with encouraging results. According to statistics, more than 80% of the world population in developing countries use herbal medicines for basic healthcare needs [1]. *Artocarpus* species belong to the Moraceae family, which were used in folk medicines to treat inflammation, malarial fever, diarrhea, diabetes and tape worm infection. As previous report, the primary constituents of *Artocarpus* species are phenolic compounds, including flavonoids, stilbenoids, arylbenzofurons and some other compounds. They showed anti-mycobacterial, anti-malarial, and anti-inflammatory effects as well as cyclooxygenase- and tyrosine's-inhibitory activities [2-4]. *A. tonkinensis* A. Chev. ex Gagnep grows in northern Vietnam. Its dried leaf decoction is used in traditional medicine for the treatment of backache and rheumatic joint disease [5]. Our previous studies demonstrated that flavonoids in *A. tonkinensis* leaves (Arto-flavonoid, ATF) possess significant biological activities [5-10]. In the published studies, these flavonoids have been proven as potential antioxidant, anti-inflammatory agents [2-4], inhibited the hepatocellular carcinoma (SMMC-7721), gastric carcinoma (SGC-7901 and BGC-823) cell lines [3]. In particular, the main compound maesopsin 4-*O*-β-D-glucoside (**3**) showed antiproliferative effects on acute myeloid leukemia cells (OCI-ALM3) and modulated expression of cancer-related 19 genes encoding proteins such as heme oxygenase-1, sulfiredoxin 1 homolog, and breast carcinoma amplified sequence 3 [6], and exhibited *in vivo* anti-cancer effects [9]. Our recent study showed that mixture ATF in its decotion significantly alleviated the signs and symptoms of CIA and inhibited function of Th17 cells, highlighting its potent anti-inflammatory activity [11].

Although, a number of investigations on chemical constituents of *A. tonkinensis* leaves have been reported in

recent years, there is no report to give a complete analysis of the flavonoids in this plant material [12]. Moreover, its decoction significantly alleviated the signs and inhibited the development and function of Th17 cells, highlighting its potent anti-inflammatory activity [11]. Thus, the intention of this work was to identify and determine the flavonoid composition in water extract of *A. tonkinensis* leaves on the basis of high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC–MS) methods.

2. EXPERIMENTAL

2.1. Plant materials

The *A. tonkinensis* leaves were collected from the village outside of Ha Noi city, Viet Nam (October 2018) and identified by the Mr. Nguyen The Anh, Institute of Chemistry, Vietnam Academy of Science and Technology (ICH, VAST). The voucher specimen (Nr. AT-2018) is maintained in the same Institute (ICH, VAST) for further reference.

2.2. Standards and chemicals

The reference flavonoids of catechin (**1**) [8, 10], alphitonin 4-O-β-D-glucopyranoside (**2**), maesopsin 4-O-β-D-glucopyranoside (**3**) [5], quercetin 3-O-β-D-glucopyranoside (**4**), kaempferol-3-O-β-D-glucoside (**5**), quercetin (**6**) and kaempferol (**7**) [8, 10] were isolated from *A. tonkinensis* leaves by repeated column chromatography. Their structures were confirmed by comparison of their MS, ^{1}H and ^{13}CNMR spectral data with those reported in previous papers [5, 8, 10]. Their purities were established at over 96 % by HPLC analysis.

Methanol, acetonitrile and double distilled water for HPLC were obtained from Fisher Scientific (USA). Acetic acid (glacial) 100% of HPLC grade was purchased from Merck (Germany).

2.3. Instruments and chromatographic conditions

Ultrasonic tank Elmasonic S100H (Elma, Germany), micropipette of 200, 1000µL (Eppendorf, Germany) and plastic cylinder of 5 mL were serviced for experimental process. A Spectra System HPLC system (Thermo Separation, San Jose, CA, USA.), fitted with a quaternary pump module (P4000), an online degasser, and a diode array detector (DAD) SpectroSystem UV 6000lp (Thermo Separation.) was used. Analytes were separated using a reversed-phase Agilent Zorbax ODS column (5-µm particle size, 3.0 x 150mm i.d.; Agilent Technologies, Milan, Italy), coupled to a 20 x 4.6mm C18 guard column. Gradient elution with a flow rate of 1mL/min was used. The mobile phase consisted of the following: (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The initial mobile phase composition was 95% A. The percentage of B was linearly increased to 20% at 30 min and to 55% at 50 min. Finally, the percentage of B was reduced to 5% and the column re-equilibrated to the initial conditions for 7 min. The injection volume was 20µL. Detection was performed online using DAD in the range from 200 to 700nm. Chromatograms were acquired and data were handled using Xcalibur software version 1.2 (Finnigan Corporation 1998-2000, San Jose, CA, USA).

MS analysis condition used electro spray ionization (ESI) in a negative mode with molecular ionic peak chosen as in the Table 1. HPLC/MS system was connected with Agilent Open LAB Control Panel software. The nitrogen gas flow rate was 5 L/min at a pressure of 40 psi with drying temperature of 250^{0}C. UV spectrum was recorded from 190nm to 400nm. Detection was carried over 45 min at a flow rate of 0.5mL/min at 290 and 360nm. The injection volume was 5µL. HPLC-ESI-MS (negative ion), fullscan (*m/z* from 100 to 1300), confirmed by comparison to authentic standards and reffences (Table 1).

2.4. Preparation of samples and standard solutions

Dried powdered leaves of *A. tonkinensis* (5g) were ultrasonically extracted with boiled water (25ml x 3 times). The extracts were combined and transferred into a 100mL volumetric flask (Glassco, USA) and diluted to a volume of 100mL with water. A standard solution containing compounds **1**-**7** were isolated and used to identify and quantify the analytes [5, 8, 10].

For MS analysis, the positive and negative ion modes of ESI were compared, and the negative mode of ESI provided more extensive information of flavonoids. All standard solutions and sample extraction for HPLC–MS were filtered through 0.45µm membranes prior to HPLC analysis. Calibration curves were generated using three injections at different concentrations ranging from 1.5µg/mL to 120.0µg/mL. The content of compounds **1**-**7** were determined by using the regression equation ($y = ax \pm b$, where y is the analyte area and x is the concentration µg/ml). The peak area of compounds were plotted against the corresponding concentrations.

3. RESULTS AND DISCUSSION

3.1. Qualitative analysis of flavonoids

Under the optimal qualitative conditions, a water extract was analyzed by the HPLC-ESI/MS method. The identification of flavonoid peaks in the chromatogram was achieved in comparison of their retention time, UV and MS profiles with authentic compounds.

The absorption and mass spectral data flavonoids in water extract, seven compounds **1**-**7** were identified (Table 1, Fig. 1). The negative ESI–MS mode of compounds **1** -**7** clearly showed the characteristic diagnostic ion (Fig. 2). Peaks **1**-**7** were positively identified as catechin (**1**, *m/z* 289.0 $[M-H]^-$, 325.0 $[M+Cl]^-$), alphitonin 4-*O*-β-D-glucopyranoside (**2**, *m/z* 465.0 $[M-H]^-$), maesopsin 4-*O*-β-D-glucopyranoside (**3**, *m/z* 449.0 $[M-H]^-$)based on comparison of those retention time and mass spectra with an authentic standards [4, 8]. Other individual flavonoids (peaks **4**, **5**, **6** and **7**) were identified on the same finding as **2** and **3**, namely, quercetin 3-O-β-D-glucopyranoside (**4**), kaempferol-3-*O*-β-D-glucoside (**5**), quercetin (**6**) and

kaempferol (**7**) by comparison their retention time and MS data matched with those of spectral data **4** (*m/z* 463.0 [M-H]$^-$), **5** (*m/z* 447.0 [M-H]$^-$), **6** (*m/z* 301.0 [M-H]$^-$), and **7** (*m/z* 285.0 [M-H]$^-$), respectively (Fig. 2, Table 1).

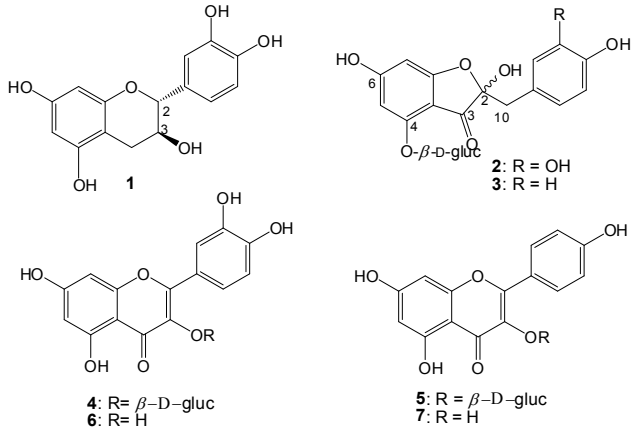


Figure 1. Chemical structures of flavonoids in the water extract of A. tonkinensis leaves by HPLC-ESI-MS

Table 1. Mass spectral data for identification of flavonoids in water extract of A. tonkinensis leaves (ATF)

Peak [a]	Compound[b]	*m/z*	Molecular Formula	References
1	Catechin	325.0 [M+Cl]$^-$ 289.0 [M-H]$^-$	$C_{15}H_{14}O_6$	[8]
2	Alphitonin 4-O-*β*-D-glucopyranoside	465.0 [M-H]$^-$	$C_{21}H_{22}O_{12}$	[5]
3	Maesopsin 4-*O*-*β*-D-glucopyranosid	449.0 [M-H]$^-$	$C_{21}H_{22}O_{11}$	[5, 9]
4	Quercetin 3-O-*β*-D-glucopyranoside	463.0 [M-H]$^-$	$C_{21}H_{20}O_{12}$	[10]
5	Kaempferol-3-*O*- *β*-D-glucosid	447.0 [M-H]$^-$	$C_{21}H_{20}O_{11}$	[10]
6	Quercetin	301.0 [M-H]$^-$	$C_{15}H_{10}O_7$	[10]
7	Kaempferol	285.0 [M-H]$^-$	$C_{15}H_{10}O_6$	[8]

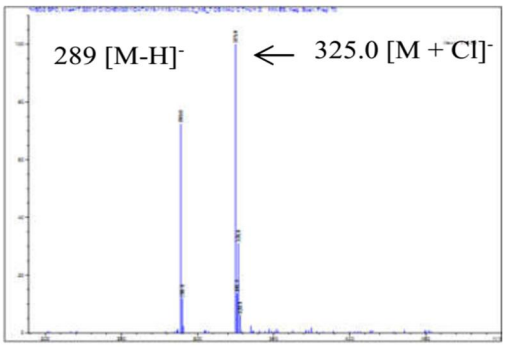
[a]Peak number corresponded to the chromatogram in Fig. 3

[b]Compounds were identified by comparison to authentic compounds (retention time, t_R; and MS spectra data)

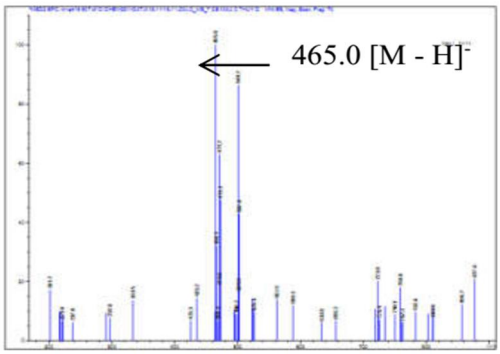
3.2. Quantification of flavonoids

To determine quantitatively of flavonoids ATF, three independent HPLC experiments were performed. Due to the flavonoids **1-7** were powerfull active, and higher amounts, therefore compounds **1**-**7** were further quantitatively determinated [5-7]. Compounds **1**-**7** were quantified based on response factors obtained from their calibration curves using the internal standard method. The quantities of each compound were consistent among the three experiments (Fig. 3). The main compounds were **2** and **3,** with no significant difference (142.8 ± 17.0µg/mL and 122.5 ± 16.5µg/mL, respectively, p = 0.29 (Fig. 3, Table 2). Thus, quality monitoring showed that *Atocarpus* water extract composition was quite constant among experiments.

It is interesting that compounds **2** and **3** belong to the rare auronol glucosides group, but they are found with high yield (2.86 and 2.45mg/g), respectively in this plant leaves. Hitherto, compound **2** was found only in this plant, additional compound **3** was found in *Hovenia trichocarea, Ribes rubrum* and *Sonneratia ovate* species [4, 11].



A)



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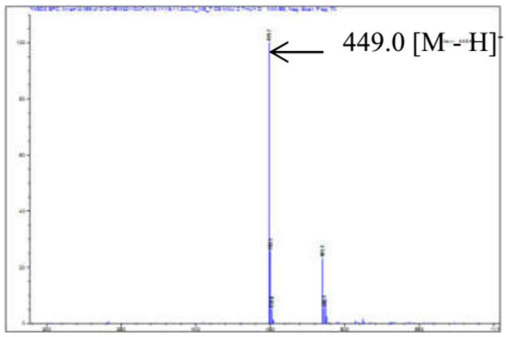
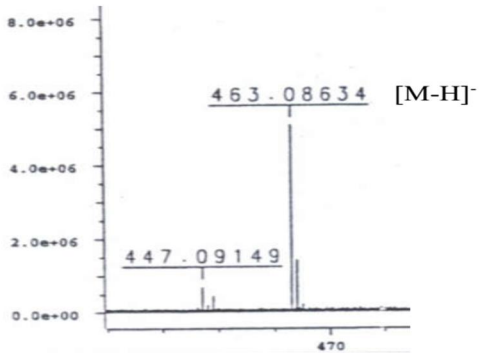


C)



D)

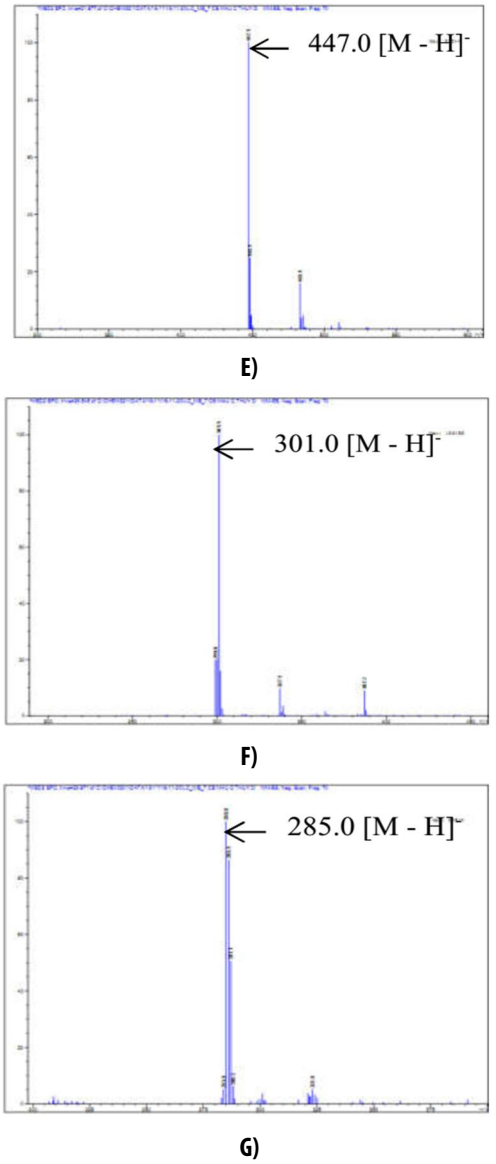


Figure 2. ESI-MS spectra in negative ion mode of compounds 1–7

A **1:** m/z 325.0 $[M+Cl]^-$ B **2:** m/z 465.0 $[M-H]^-$

C **3:** m/z 449.0 $[M-H]^-$ D **4:** m/z 463.0 $[M-H]^-$

E **5:** m/z 447.0 $[M-H]^-$ F **6:** m/z 301.0 $[M-H]^-$

G **7:** m/z 285.0 $[M-H]^-$

Table 2. Peak assignments in HPLC-ESI-MS for identification of flavonoids in water extract of A. tonkinensis leaves

Peak No.	Compounds	Concentrations (µg/ml)		
		I^0 experiment	II^0 experiment	III^0 experiment
1	Catechin	52.07	53.82	53.31
2	Alphitonin 4-*O*-β-D-glucopyranosid (TAT-6)	166.68	133.80	127.94
3	Maesopsin 4-*O*-β-D-glucopyranosid (TAT-2)	145.77	111.94	109.72
4	Quercetin-3-β-D-glucoside	9.92	9.27	8.14
5	Kaempferol-3-*O*-β-D-glucosid	47.4	40.44	39.25
6	Quercetin	4.8	4.45	4.31
7	Kaempferol	3.43	3.11	3.19

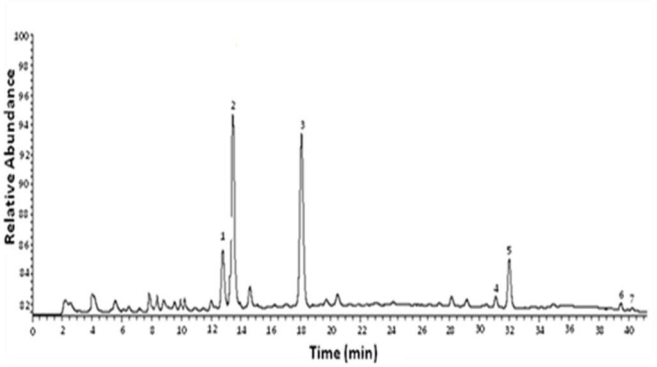


Figure 3. HPLC profile of flavonoids (ATF) from A. tonkinensis leaf water extraction. Identified compounds: **1**. catechin, **2**. aphitonin-4-O-glucoside (TAT-6), **3**.maesopsin-4-O-β-glucoside (TAT-2), **4**. quercetin-3-β-D-glucoside, **5**. kaempferol-3-O-glucoside, **6**. Quercetin, **7**. kaempferol.

4. CONCLUSIONS

In summary, the HPLC-ESI/MS method was applied for the qualitative and quantitative analysis of flavonoids in the water extract of *A. tonkinensis* leaves. Seven flavonoids, catechin (**1**), alphitonin 4-O-*β*-D-glucopyranoside (**2**), maesopsin 4-O-*β*-D-glucopyranoside (**3**), quercetin 3-O-*β*-D-glucopyranoside (**4**), kaempferol-3-O-*β*-D-glucoside (**5**), quercetin (**6**) and kaempferol (**7**), were identified by comparing their retention times, UV and ESI-MS spectra with those of the authentic isolated compounds.

ACKNOWLEDGEMENTS

This research was supported by a grant (NDT.33.ITA/17) from Vietnam Ministry of Science and Technology and the Italian Minister for Foreign Affairs of International Cooperation (Ministero degli Affari Esteri e della Cooperazione Internazionale, MAECI).

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THÔNG TIN TÁC GIẢ

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