ANTIOXIDATIVE ACTIVITY OF SOME ISOLATED FLAVONOIDS FROM *Vernonia amygdalina* Del.

Đến tòa soạn 26-08-2022

Le Thi Bach[1], Nguyen Trong Tuan[1], Lien Thao Van[1], Tran Khanh Tien[1], Doan Minh Trung[1], Dang Trung Hieu[1], Truong Huynh Kim Ngoc[1], Quach Thi Hong Dung[*1], Pham Thi Kim Phuong[2]

1. Department of Chemistry, College of Natural Sciences, Can Tho University

2. An Giang University, Vietnam National University – Ho Chi Minh City

Email: ltbach@ctu.edu.vn; qthdung@ctu.edu.vn

TÓM TẮT

HOẠT TÍNH KHÁNG OXI HÓA CỦA MỘT SỐ HỢP CHẤT FLAVONOID PHÂN LẬP ĐƯỢC CỦA LÁ CÂY MẬT GẤU (*Vernonia amygdalina* Del.)

Nghiên cứu này nhằm mục đích đánh giá hoạt tính kháng oxi hóa của một số hợp chất phân lập được của lá cây Mật gấu (Vernonia amygdalina Del.). Kết quả khảo sát thành phần hóa học cho thấy, từ cao phân đoạn ethyl acetate của lá cây Mật gấu, ba hợp chất flavonoid đã được phân lập và nhận danh là luteolin (1), luteolin-7-O-β-D-glucopyranoside (2), và kaempferol (3). Cấu trúc các hợp chất này được xác định bằng phương pháp phổ NMR và so sánh với các tài liệu đã công bố. Thêm vào đó, hoạt tính kháng oxi hóa của cả ba hợp chất này đã được khảo sát theo hai phương pháp DPPH và $ABTS^{\bullet+}$ và cho thấy hiệu quả kháng oxi hóa tốt. Những kết quả này cho thấy lá cây Mật gấu là một dược liệu tiềm năng chứa các hợp chất flavonoid thể hiện hoạt tính kháng oxi hóa tốt.

Từ khóa: *kháng oxi hóa, hợp chất flavonoid, cây mật gấu.*

1. INTRODUCTION

Natural products possessing antioxidant properties play a very crucial role in ameliorating deleterious effects of reactive oxygen species. Many plants in Vietnamese traditional medicine have been proved to possess medicinal functions, especially the antioxidant activity. *Vernonia amygdalina* Del. (commonly called "bitter leaf" because of its bitter taste), a species belonging to the Asteraceae family, has been found pharmacologically useful [1]. The health-promoting ability of this plant species might be related to the antioxidative effect of its constituents. Phytochemicals such as saponins and alkaloids, terpenes, steroids, coumarins, flavonoids, phenolic acids, lignans, xanthones, anthraquinones, edotides and sesquiterpenes have been extracted and isolated from *Vernonia amygdalina* [2-6]. These bioactive compounds made them possess different pharmacological properties like antimicrobial, antimalarial, antioxidant, anti-diabetic, anticancer, anti-fungi, antibacterial, and among others [7-9].

Therefore, in this study, we investigated the chemical components as well as the antioxidant activity of three isolated flavonoids from *Vernonia amygdalina* Del.

2. EXPERIMENTAL

2.1. Chemicals and reagents

NMR spectra were recorded on a Bruker AM600, AM500 FTNMR spectrometer (Bruker, Karlsruhe, Germany) using TMS as an internal standard, Institute of Chemistry - Vietnam Academy of Science and Technology,

Hanoi, Vietnam. TLC was performed on silica gel 60 F_{254} (0.063–0.200mm, Merck, Germany). The zones were detected using UV at 254 or 365 nm or a solution of $FeCl_3$/EtOH or H_2SO_4/EtOH. Column chromatography was performed on silica gel (240-430 mesh, Merck, Germany), ODS (70-230 mesh, Merck, Germany).

Solvents utilized including *n*-hexane, chloroform, ethyl acetate, *n*-butanol, methanol (purity ≥ 99.0%), and ethanol 96% were purchased from Chemsol company (Vietnam).

2.2. Sample treatment and preparation

The leaves of *Vernonia amygdalina* Del. were collected on August 2021 from Can Tho city and authenticated by Dr. Dang Minh Quan. A voucher specimen is kept at the Department of Biology, School of Education, Can Tho University, under the number: VaD210920.

The sample was then washed away from muds and dust; the rotten and damaged parts were also discarded. The raw materials were left to dry in the shade at room temperature for some days and then dried in an oven at about 50°C until well-dried.

2.3. Extraction and isolation

The well-dried plant was ground into powder (3.8 Kg) which was then soaked in 96% ethanol at room temperature for four times (20 L/time) and filtered. The filtrate was concentrated under reduced pressure to give brown residue as crude ethanol extract (360 g). This crude extract was then extracted on flash column chromatography successively with *n*-hexane, ethyl acetate, *n*-butanol, and methanol, respectively to yield the corresponding extracts of *n*-hexane (120 g), ethyl acetate (55 g), *n*-butanol (65g), and methanol extract (70 g).

The ethyl acetate extract was subjected to flash column chromatography (CC) on silica gel and eluted with various proportions of *n*-hexane and ethyl acetate (from 100:0 to 0:100, v/v) to obtain 11 fractions (EE1-11).

Fraction EE7 was further separated on a silica gel column, eluted with $CHCl_3$: MeOH (from 90:1 to 10:1, v/v) to yield six subfractions (EE7.1-6). Finally, compound **1** (40 mg) was obtained from subfraction EE7.3.

Fraction EE8 was separated by a silica gel column and eluted with $CHCl_3$:MeOH (from 50:1 to 5:1, v/v) to yield 12 subfractions (EE8.1-12). Subfraction EE8.3 was further chromatographed on silica gel CC, eluted with $CHCl_3$: MeOH (from 10:1 to 1:1, v/v) to obtain eight subfractions (EE8.3.1-8). At last, compound **2** (30 mg) was obtained from subfraction EE8.3.2. And the subfractions EE8.4 then was repeatedly purified by silica gel CC to obtain compound **3** (18 mg).

2.4. Antioxidant activity

2.4.1. DPPH Assay

In a 96-well microtiter plate, 50 µL of each sample was added to 6 $\times 10^{-5}$ M methanol solution of DPPH. After mixing with a vortex mixer, the mixture was incubated for 30 min at room temperature and the absorbance was measured at 517 nm. The DPPH radical scavenging activity was recorded as a percentage in comparison to the control. Vitamin C was used as positive standard [10].

2.4.2. ABTS $^{*+}$ Assay

The free radical 2,2′-azinobis 3-ethyl benzothiazoline-6-sulfonic acid ($ABTS^{+*}$) was produced by reacting ABTS solution in methanol (7 mM) with aqueous potassium persulfate solution (2.45 mM). The resulting mixture was allowed to stand in the dark for 12-16 hours before use. For aqueous extract ABTS was diluted with PBS (7.4 pH) to an absorbance of 0.700 ± 0.002 at 734 nm and Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl2-carboxylic acid) was used as standard for calibration curve. Activity was expressed in terms of µmol TEAC (Trolox Equivalent Antioxidant Capacity) value [11].

2.5. Statistical analysis

The variation in a set of data has been estimated by performing one way analysis of variance (ANOVA). Results were calculated from three independent experiments and are shown as mean ± SD, n=3. Results were considered as statistical significant when p value was < 0.05.

3. RESULTS and DISCUSSION

3.1. Structural elucidation

The structures of isolated compounds were characterized NMR spectra and by comparison with literature data.

3.1.1. Compound 1

Compound **1** was obtained as yellow needles, m.p. 303-305°C

^{1}H-NMR (600 MHz, CD_3OD**)**, δ_H (ppm): 7.39 (1H, *m*, H-2′); 7.38 (1H, *m*, H-6′); 6.92 (1H, *d*, *J* = 9.0 Hz, H-5′); 6.54 (1H, *s*, H-3); 6.45 (1H, *d*, *J* = 1.8 Hz, H-8); 6.22 (1H, *d*, *J* = 1.8 Hz, H-6).

^{13}C-NMR (150 MHz, CD_3OD), δ_C (ppm): 183.9 (C-4); 166.3 (C-7); 166.0 (C-2); 163.2 (C-5); 159.4 (C-9); 151.0 (C-4′); 147.0 (C-3′); 123.7 (C-1′); 120.3 (C-6′); 116.8 (C-5′); 114.2 (C-2′); 105.3 (C-10); 103.9 (C-3); 100.1 (C-6); 95.0 (C-8).

3.1.2. Compound 2

Compound **2** was obtained as light yellow powder, m.p. 189-191°C

^{1}H-NMR (500 MHz, CD_3OD**)**, δ_H (ppm): 7.42 (1H, *dd*, *J* = 8.4 and 1.8 Hz, H-6′); 7.41 (1H, *d*, *J* = 1.8 Hz, H-2′); 6.91 (1H, *d*, *J* = 8.4 Hz, H-5′); 6.80 (1H, *d*, *J* = 2.4 Hz, H-8); 6.61 (1H, *s*, H-3); 6.50 (1H, *d*, *J* = 2.4 Hz, H-6); 5.07 (1H, *d*, *J* = 7.2 Hz, H-1′′); 3.93 (1H, *dd*, *J* = 12.0 and 2.4 Hz, H-6′′); 3.72 (1H, *dd*, *J* = 12.0 and 6.0 Hz, H-6′′).

^{13}C-NMR (125 MHz, CD_3OD), δ_C (ppm): 181.8 (C-4); 164.4 (C-2); 162.9 (C-7); 161.1 (C-5); 156.9 (C-9); 149.8 (C-4′); 145.7 (C-3′); 121.3 (C-1′); 119.1 (C-6′); 115.9 (C-5′); 113.5 (C-2′); 99.9 (C-1′′); 99.5 (C-6); 94.6 (C-8); 77.1 (C-5′′); 76.3 (C-3′′); 73.0 (C-2′′); 69.5 (C-4′′); 60.6 (C-6′′).

3.1.3 Compound 3

Compound **3** was obtained as light yellow powder, m.p. 275-277°C.

^{1}H-NMR (500 MHz, CD_3OD**)**, δ_H (ppm): 8.10 (2H, *d*, *J* = 9.0 Hz, H-2′, 6′); 6.93 (2H, *d*, *J* = 9.0 Hz, H-3′, 5′); 6.41 (1H, *d*, *J* = 2.5 Hz, H-8); 6.20 (1H, *d*, *J* = 2.0 Hz, H-6).

^{13}C-NMR (125 MHz, CD_3OD), δ_C (ppm): 177.4 (C-4); 165.6 (C-7); 162.5 (C-5); 160.6 (C-4′); 158.3 (C-9); 148.1 (C-2); 137.1(C-3); 130.7 (C2′, 6′); 123.8 (C-1′); 116.3 (C3′, 5′); 104.6 (C-10); 99.3 (C-6); 94.5 (C-8).

The ^{1}H-NMR data of compound **1** revealed the presence of a pair of *meta*-coupled aromatic protons at δ_H [6.45 (1H, *d*, *J* = 1.5 Hz, H-8) and 6.22 (1H, *d*, *J* = 1.5 Hz, H-6)], an aromatic singlet (δ_H 6.54). In combination with the ^{13}C-NMR data, which showed signals for a carbonyl group at δ_C 183.9 (C-4), six oxygenated quaternary carbons (δ_C 147.0, 151.0, 159.4, 163.2, 166.0, and 166.3), two sp^2 quaternary carbons (δ_C 105.3 and 123.7), and six sp^2 tertiary carbons (δ_C 95.0, 100.1, 103.9, 114.2, 116.8, and 120.3). From these evidences and by comparison with literature data [12], we were able to identify the molecules of compound **1** as luteolin.

The ^{1}H-NMR and ^{13}C-NMR spectra of compound **2** showed 22 carbon resonances, including 15 C signals of luteolin aglycone. The ^{13}C-NMR spectrum of compound **2** also showed the existence of glycoside (δ_C 99.9). There are 5 sugar ring atoms between δ 60.6 and 77.1, and the signal at position δ 5.07 is the end group H signal of glucose, which indicated that the configuration of the glucose is *β* type. Meanwhile, the HSQC data indicated that H-1′′ (δ 5.07) correlated with C-1′′. HMBC data showed that H-1′′ (δ 5.07) had a correlation to C-7 (δ 162.9), which suggested the presence of glycosides attached to C-7. All the spectroscopic data confirmed that the structure of compound **2** was luteolin-7-*O*-*β*-D-glucopyranoside [13].

Compound **3** was isolated as yellow amorphous powder, m.p. 275-277°C. The ^{1}H-NMR spectrum of compound **3** appeared four signals of six aromatic protons in which there were two couples of chemical shift equivalent protons at δ_H 8.10 (2H, *d*, *J* = 9.0 Hz) and 6.93 (2H, *d*, *J* = 9.0 Hz); two *meta*-coupling signals at 6.41 ppm (1H, *d*, *J* = 2.5 Hz) and 6.20 ppm (1H, *d*, *J* = 2.0 Hz). ^{13}C-NMR and DEPT spectra also exhibited signals of total 15

carbons of a flavone backbone. These carbons consisted of two signals of two couples of chemical shift equivalent carbons at δ_C 116.3 (2C) and 130.7 (2C), related to two couples of chemical shift equivalent protons in its ^{1}H-NMR spectrum. It proved that the four-hydroxyl substituted flavone had a symmetric aromatic ring. Moreover, the 1D-NMR spectral data of compound **3** were similar to those of kaempferol notified in the literature [14]. From these evidences, compound **3** was determined as 3,5,7,4′-tetrahydroxyflavone or kaempferol.

Three compounds **1-3** were isolated and identified from the leaves of *Vernonia amygdalina* Del., including luteolin (**1**), luteolin-7-*O*-*β*-D-glucopyranoside (**2**), and kaempferol (**3**) by analysis of their NMR spectra and comparison with literature data (Figure **1**).

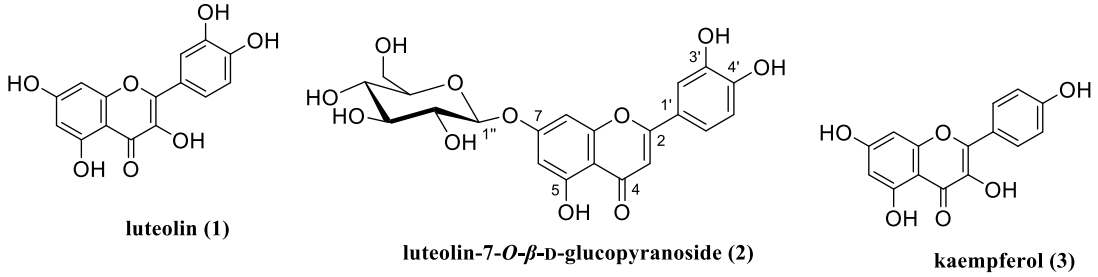

Figure 1. Chemical structures of compounds 1–3.

3.2. *In vitro* antioxidant activity results

Table 1. IC_{50} values of isolated flavonoid compounds

Isolated flavonoids	IC_{50} (μg/mL)	
	DPPH	ABTS
Luteolin	8.89 ± 1.05	3.35 ± 0.82
Luteolin-7-*O*-*β*-D-glucopyranoside	14.86 ± 9.68	11.035± 4.95
Kaempferol	15.74 ± 11.29	13.09 ± 5.87
Positive Control		
Vitamin C	3.88 ± 0.74	
Trolox		3.17 ± 0.07

The free radical scavenging activities of isolated flavonoids were measured by DPPH and ABTS methods. The results are presented in Table **1**. All of the three compounds exhibited a good ability of scavenging DPPH and ABTS radicals. Luteolin exhibited high scavenging potential with IC_{50} = 8.89 μg/mL and 3.35 μg/mL for both DPPH and ABTS, respectively.

Iwalokun *et al.* reported the antioxidant effects of an aqueous extract of *Vernonia amygdalina* leaves against acetaminophen-induced hepatotoxicity and oxidative stress in mice. Pre-administration of *Vernonia amygdalina* resulted in a dose-dependent reversal of acetaminophen-induced alterations of all the liver function parameters and suppressed acetaminophen-induced lipid peroxidation and oxidative stress. The study suggested that *Vernonia amygdalina* protected against acetaminopheninduced hepatic damage in mice by antioxidant mechanisms [15]. The antioxidant mechanism of *Vernonia amygdalina* has been justified by the recent studies of Adesanoye and Farombi [16]. In this study, *Vernonia amygdalina* protected against carbon tetrachloride-induced liver injury by inducing antioxidant and phase 2 enzymes. The antioxidant activity of *Vernonia amygdalina* has been attributed to the presence of flavonoids, as reported by Igile *et al* [17]. Using spectroscopic techniques, the study isolated and characterized the flavonoids occurring in *Vernonia amygdalina*. Three flavones were identified with chemical and spectroscopic techniques namely: luteolin, luteolin-7-*O*-*β*-D-glucopyranoside, and

kaempferol. Determination of the antioxidant activity of the three flavones had shown that luteolin showed greater activity than the other two. Since flavonoids are established as possessing antioxidant activity [18-20]. It can be speculated that the antioxidant properties of *Vernonia amygdalina* can be attributed to the presence of these flavonoids.

The production of reactive oxygen species during metabolism and the downregulation of anti-oxidant defense systems in the human body leads to oxidative stress and cellular dysfunction, resulting in chronic disorders such as cancer, cardiovascular diseases, and diabetes, among others. Therefore, reducing the oxidative stress is important for the anti-oxidant defense system. Plants have been known to contain natural anti-oxidants, such as flavonoids, which have well-known anti-oxidant properties. This particular structure of flavonoids, combined with the position of its hydroxyl groups and the polarity of its glycoside derivatives, contributes to its anti-oxidant properties. The present study revealed that the *Vernonia amygdalina* Del. has potent antioxidant properties and might be a good candidate for development as a novel natural antioxidant.

4. CONCLUSION

The results of this study revealed that from the leaves of *Vernonia amygdalina* Del., grown in Can Tho city, we have isolated and identified three flavonoid compounds: luteolin (**1**), luteolin-7-*O*-*β*-D-glucopyranoside (**2**), and kaempferol (**3**). The structures of these compounds have been elucidated by the spectroscopic method NMR and in comparison with the literature data. Furthermore, the antioxidant potentials of three isolated flavonoids were also evaluated through the DPPH and ABTS assays. The results indicated that all three isolated compounds exhibited the ability of scavenging DPPH and ABTS radicals.

ACKNOWLEDGEMENT

This study is funded in part by the Can Tho University, Code: TSV2022-43.

REFERENCES

[1] Adesanoye, O. A., Adekunle, A. E., Adewale, O. B., Mbagwu, A. E., Delima, A. A., Adefegha, S. A., & Farombi, E. O. (2016). Chemoprotective effect of *Vernonia amygdalina* Del. (Astereacea) against 2-acetylaminofluorene-induced hepatotoxicity in rats. *Toxicology and Industrial Health*, 32(1), 47-58.

[2] Izevbigie, E. B. (2003). Discovery of water-soluble anticancer agents (Edotides) from a vegetable found in Benin City, Nigeria. *Experimental Biology and Medicine*, 228 (3), 293-298.

[3] Tona, L., Cimanga, R. K., Mesia, K., Musuamba, C. T., De Bruyne, T., Apers, S., & Vlietinck, A. J. (2004). *In vitro* antiplasmodial activity of extracts and fractions from seven medicinal plants used in the Democratic Republic of Congo. *Journal of Ethnopharmacology*, 93(1), 27-32.

[4] Muraina, I. A., Adaudi, A. O., Mamman, M., Kazeem, H. M., Picard, J., McGaw, L. J., & Eloff, J. N. (2010). Antimycoplasmal activity of some plant species from northern Nigeria compared to the currently used therapeutic agent. *Pharmaceutical Biology*, 48(10), 1103-1107.

[5] Hoang Le Tuan Anh, Le Thi Lien, Pham Viet Cuong, Masayoshi Arai, Tran Phuong Ha, Ton That Huu Dat and Le Canh Viet Cuong, (2018). Sterols and flavone from the leaves of *Vernonia amygdalina* growing in Thua Thien Hue, Vietnam. *Journal of Science and Technology*, 56 (6), 681-687.

[6] Hoang Le Tuan Anh, Le Ba Vinh, Le Canh Viet Cuong and Young Ho Kim, (2019). *In vitro* study on *α*-amylase and *α*-glucosidase inhibitory activities of a new stigmastane-type steroid saponin from the leaves of *Vernonia amygdalina. Natural Product Research*, 35(5), 873-879.

[7] Moundipa, P. F., Flore, K. G. M., Bilong, C. F., & Bruchhaus, I. (2005). *In vitro* amoebicidal activity of some medicinal plants

of the Bamun region (Cameroon). *African Journal of Traditional, Complementary and Alternative Medicines*, 2(2), 113-121.

[8] Krief, S., Hladik, C. M., & Haxaire, C. (2005). Ethnomedicinal and bioactive properties of plants ingested by wild chimpanzees in Uganda. *Journal of Ethnopharmacology*, 101(1-3), 1-15.

[9] Nguyễn Thị Hồng Hạnh, Đoàn Thanh Hiếu và Trần Thị Hồng (2020). Tác dụng hạ Glucose huyết của dịch chiết Lá đắng *Vernonia amygdalina* Del. trên chuột nhắt trắng, *Tạp chí khoa học và Công nghệ Đại học Thái Nguyên*, 225(01), 144-149.

[10] Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry*, 113(4), 1202-1205.

[11] Nenadis, N., Wang, L. F., Tsimidou, M., & Zhang, H. Y. (2004). Estimation of scavenging activity of phenolic compounds using the ABTS•+ assay. Journal of Agricultural and Food Chemistry, 52(15), 4669-4674.

[12] Nguyen Thi Kim Thoa, Nguyen Thi Thu Hien, Le Thi Phuong Thao, Vu Thi Minh Hong and Pham Tien Dung, (2019). Isolation of some flavonoids from *Vitex trifolia* in Bachma National Park, Thua Thien Hue, Vietnam. *HNUE Journal of Science*, 64(6), 130-135.

[13] Lin, L. C., Pai, Y. F., & Tsai, T. H. (2015). Isolation of luteolin and luteolin-7-O-glucoside from *Dendranthema morifolium* Ramat Tzvel and their pharmacokinetics in rats. *Journal of Agricultural and Food Chemistry*, 63(35), 7700-7706.

[14] Vân, N. T. (2019). Nghiên cứu qui trình phân lập kaempferol và xác định hàm lượng kaempferol trong các chế phẩm từ lá Bạch quả. *Journal of Science and Technology*, 2(3), 16-21.

[15] Iwalokun, B. A., Efedede, B. U., Alabi-Sofunde, J. A., Oduala, T., Magbagbeola, O. A., & Akinwande, A. I. (2006). Hepatoprotective and antioxidant activities of *Vernonia amygdalina* on acetaminophen-induced hepatic damage in mice. *Journal of Medicinal Food*, 9(4), 524-530.

[16] Adesanoye, O. A., & Farombi, E. O. (2010). Hepatoprotective effects of *Vernonia amygdalina* (astereaceae) in rats treated with carbon tetrachloride. *Experimental and Toxicologic Pathology*, 62(2), 197-206.

[17] Igile, G. O., Oleszek, W., Jurzysta, M., Burda, S., Fafunso, M., & Fasanmade, A. A. (1994). Flavonoids from *Vernonia amygdalina* and their antioxidant activities. *Journal of Agricultural and Food Chemistry*, 42(11), 2445-2448.

[18] Mathew, J., Latha, P. G., & George, V. (2004). Antioxidant flavonoids from *Vitex peduncularis*. *Journal of Tropical Medicinal Plants*, 5, 217-221.

[19] Schuldt, E. Z., Farias, M. R., Ribeiro-do-Valle, R. M., & Ckless, K. (2004). Comparative study of radical scavenger activities of crude extract and fractions from *Cuphea carthagenensis* leaves. *Phytomedicine*, 11(6), 523-529.

[20] Prabhakar, K. R., Veeresh, V. P., Vipan, K., Sudheer, M., Priyadarsini, K. I., Satish, R. B. S. S., & Unnikrishnan, M. K. (2006). Bioactivity-guided fractionation of *Coronopus didymus:* A free radical scavenging perspective. *Phytomedicine*, 13(8), 591-595.