DOI: 10.31276/VJSTE.64(3).19-23

Curcuminoids from the Vietnamese *Curcuma longa*: Phytochemical analysis and biological activities

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Received 16 September 2021; accepted 11 November 2021

Abstract:

A phytochemical investigation on the ethanolic (EtOH) extract of the Vietnamese *Curcuma longa* (*C. longa*) rhizomes has led to the isolation of four curcuminoids including curcumin (1), demethoxycurcumin (2), bisdemethoxycurcumin (3), and cyclocurcumin (4). The chemical structure of compounds 1-4 was elucidated by Nuclear magnetic resonance (NMR) and Mass spectrometry (MS) spectral data. Based on High-performance liquid chromatography (HPLC) quantitative analysis, the amounts of three major compounds 1-3 in the *C. longa* extract were calculated and reached 7.215±0.101, 3.927±0.031, and 2.255±0.049 mg/g, respectively. Curcuminoids 1-4 have induced IC_{50} values of 9.23-14.6 µg/ml in a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as compared with that of the positive control resveratrol (IC_{50} 11.5 µg/ml). Compounds 1-4 with IC_{50} values ranging between 8.7-15.54 µg/ml were better than the positive control acarbose (IC_{50} 169.14 µg/ml) in α-glucosidase inhibitory examination. In addition, the EtOH extract and compounds 1-4 were also responsible for inhibitions against enzyme acetylcholinesterase and four cancer cell lines including including epidermoid carcinoma (KB), hepatocellular carcinoma (HepG2), lung cancer (SK-LU-1), and breast cancer (MCF7).

Keywords: ***Curcuma longa*, curcuminoids, cytotoxicity, DPPH radical scavenging, HPLC analysis, α-glucosidase inhibition.**

Classification number: **2.2**

Introduction

Curcuma longa L. (the Zingiberaceae family) is a perennial herb, which is distributed throughout the world and widely cultivated in Asian countries [1]. Its rhizomes have features like oblong, ovate, pyriform, and are often shorty branched [1, 2]. The powders derived from rhizomes have been in continuous use in food preparations [3]. Current users of traditional medicine claim the application of its rhizome powders has antioxidant, antibacterial, anticancer, anti-inflammatory, antimutagenic, antidiabetic, and hepatoprotective activities [4-6].

The extracts of the *C. longa* species react with alkalis to create red-brown salts. *C. longa* extracts are soluble in alkalis, ethanol, ketone, acetic acid, and chloroform [1]. This phenomenon is mainly due to the presence of curcumin and its derivatives [1]. Chromatographic HPLC is a powerful and robust technique for both qualitative and quantitative analysis of curcumin and its derivatives [7-10]. In the current paper, we report the phytochemical and HPLC quantitative procedures for identifying curcuminoids from the Vietnamese *C. longa* rhizomes together with their DPPH radical scavenging, α-glucosidase, acetylcholinesterase (AChE), and cytotoxic assays.

Materials and methods

General procedures

The Bruker Avance 500 MHz was used to measure 1D and 2D-NMR with TMS as an internal standard. A Thermo Scientific LTQ Orbitrap XL instrument was used to collect ESI-MS data. Silica gel (40-63 µm mesh, Sigma) and Sephadex LH-20 (75-150 µm, Bio-Science, Sweden) were used for column chromatography (CC). TLC examination was performed on plates that had been precoated with silica gel 60 F_{254} (Merck, Germany). Compounds were seen using a UV lamp with wavelengths of 254 and 365 nm, as well as spraying with indicators (5% H_2SO_4 and vanillin).

Solvents for the HPLC analysis were purchased from Merck, Germany. HPLC-DAD data were obtained using a ZORBAX Eclipse XDB C18 column (150x4.6 nm, 5 m) coupled with a ZORBAX Eclipse XDB guard C18 column (12.5x4.6 mm, 5 m) on an Agilent Series 1260 (Agilent Technologies, USA) system, which included a vacuum degasser, a quaternary mixing pump, an auto-sampler, a column oven, and a diode-array detector (DAD) (Agilent Technologies, USA).

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Plant material

The rhizomes of *C. longa* were collected in Bac Giang, Vietnam in December 2020 and were identified by the taxonomist Nguyen The Cuong of the Institute of Ecology and Biological Resources. A voucher specimen labelled CL-2020 was deposited in the Department of Applied Biochemistry, Institute of Chemistry, Vietnam Academy of Science and Technology.

Extraction and isolation

C. longa dried rhizomes (0.5 kg) were extracted with EtOH (4 l x 3 times) under reflux for 3.5 h. The crude EtOH extract (120 g) was obtained by evaporating the mixed extract under reduced pressure.

The EtOH extract was subjected to silica gel CC and eluted with *n*-hexane (1 l), CH_2Cl_2 (1.5 l), and EtOAc (1.2 l) to afford 7 fractions (CL1-CL7). Fraction CL2 (15.1 g) was chromatographed on silica gel CC [*n*-hexane-acetone (3:1, v/v)] to yield 7 fractions (CL21-CL27). The fraction CL22 (2.2 g) was subjected to sephadex LH-20 CC [MeOH/CH_2Cl_2 (9:1, v/v)] to give compound **2** (8.0 mg). The fraction CL3 (9.7 g) was separated on sephadex LH-20 column [MeOH (100%)] to yield 6 fractions (CL31-CL36). The fraction CL33 (1.2 g) was separated by sephadex LH-20 CC eluted with MeOH (100%) to provide 3 fractions (CL331-CL333). Compound **4** (1.5 mg) was isolated from the fraction CL332 (0.2 g) by using preparative TLC [CH_2Cl_2/EtOAc (6:1, v/v)]. Silica gel CC [CH_2Cl_2-EtOAC, 9:1 to 1:1, v/v)] was utilized for fraction CL24 (8.9 g) to produce 8 fractions (CL241-CL248). Both compound **1** (9.1 mg) and compound **3** (12.1 mg) were derived from the fraction CL242 (1.5 g) by using silica gel CC [CH_2Cl_2/CH_3COCH_3 (3:1, v/v)].

Curcumin (**1**): Yellow powder; ESI-MS (+): *m/z* 369.1 $[M+H]^+$ (calcd for $C_{21}H_{21}O_6$, 369.0); ^{1}H-NMR (500 MHz, CD_3OD, δ_H ppm): 7.60 (2H, d, 16.0 Hz, H-4), 7.23 (2H, d, 1.5 Hz, H-6), 7.13 (2H, dd, 1.5, 8.0 Hz, H-10), 6.85 (2H, d, 8.0 Hz, H-9), 6.62 (2H, d, 16.0 Hz, H-3), 5.99 (1H, s, H-1), 3.93 (6H, s, 7-OCH_3); ^{13}C-NMR (125 MHz, CD_3OD, δ_C ppm): 184.8 (C-2), 150.5 (C-8), 149.5 (C-7), 142.1 (C-4), 128.6 (C-5), 124.1 (C-3), 122.3 (C-10), 116.6 (C-9), 111.8 (C-6), 101.4 (C-1), 56.5 (7-OCH_3).

Demethoxycurcumin (**2**): Yellow powder; ESI-MS (+): *m/z* 339.2 $[M+H]^+$ (calcd for $C_{20}H_{19}O_5$, 339.0); ^{1}H-NMR (500 MHz, DMSO-d_6, δ_H ppm): 10.10 (1H, s, 8'-OH), 9.72 (1H, s, 8-OH), 7.61 (3H, m, H-4', H-6', H-10'), 7.57 (1H, d, 15.0 Hz, H-4), 7.20 (1H, d, 10.0 Hz, H-10), 7.32 (1H, s, H-6), 6.87 (3H, d, 10.0 Hz, H-9, H-7', H-9'), 6.79 (1H, d, 15.0 Hz, H-3), 6.74 (1H, d, 15.0 Hz, H-3'), 3.90 (3H, s, 7-OCH_3); ^{13}C-NMR (125 MHz, DMSO-d_6, δ_C ppm): 184.1 (C-2'), 183.8 (C-2), 160.1 (C-8'), 148.7 (C-7), 148.5 (C-8), 141.5 (C-4), 141.0 (C-4'), 131.0 (C-7', C-9'), 126.8 (C-5), 126.1 (C-5'), 123.9 (C-3), 121.5 (C-3'), 115.2 (C-6', C-10'), 111.9 (C-6), 101.4 (C-1), 56.4 (7-OCH_3).

Bisdemethoxycurcumin (**3**): Yellow powder; ESI-MS (+): *m/z* 308.1 $[M+H]^+$ (calcd for $C_{19}H_{17}O_4$, 308.0); ^{1}H-NMR (500 MHz, DMSO-d_6, δ_H ppm): 10.03 (2H, s, 8-OH), 7.55 (6H, m, H-4, H-6, H-10), 6.82 (4H, d, 8.5 Hz, H-7, H-9), 6.70 (2H, d, 15.5 Hz, H-3), 6.04 (1H, s, H-1); ^{13}C-NMR (125 MHz DMSO-d_6, δ_C ppm): 184.8 (C-2), 161.1 (C-8), 141.8 (C-4), 131.1 (C-7, C-9), 126.0 (C-5), 122.0 (C-3), 116.9 (C-6, C-10), 101.5 (C-1).

Cyclocurcumin (**4**): Yellow powder; ESI-MS (+): *m/z* 369.2 $[M+H]^+$ (calcd for $C_{21}H_{21}O_6$, 369.0); ^{1}H-NMR (500 MHz, $CDCl_3$, δ_H ppm): 7.07 (1H, d, 10.0 Hz, H-9), 7.03 (4H, m, H-6, H-15, H-18, H-19), 6.95 (1H, d, 10.0 Hz, H-10), 6.50 (1H, d, 14.5 Hz, H-12), 7.33 (1H, d, 1.4.5 Hz, H-13), 5.60 (1H, s, H-3), 5.40 (1H, dd, 1.5, 14.5 Hz, H-4), 3.99 (3H, s, 7-OCH_3), 3.94 (3H, s, 16-OCH_3), 3.04 (1H, dd, 15.0, 20.0 Hz, H-2a), 2.90 (1H, d, 1.5, 14.5 Hz, H-2b); ^{13}C-NMR (125 MHz, $CDCl_3$, δ_C ppm): 194.1 (C-1), 170.0 (C-11), 147.7 (C-17), 147.0 (C-16), 146.8 (C-7), 146.1 (C-8), 146.5 (C-7), 137.9 (C-13), 130.4 (C-5), 127.7 (C-14), 123.0 (C-19), 120.1 (C-10), 118.1 (C-12), 114.1 (C-18), 113.9 (C-9), 109.2 (C-6), 108.9 (C-15), 105.7 (C-3), 80.9 (C-4), 43.5 (C-2), 56.5 (16-OCH_3), 56.1 (16-OCH_3).

HPLC quantitative analysis

Quantitative analysis by HPLC has been applied to identify the amounts of curcuminoids in the EtOH extract of the Vietnamese *C. longa* rhizomes. Due to a material shortage of compound **4,** the analysis was focused on compounds **1-3**. The HPLC method has been carefully described in a previous publication [11]. Briefly, three isolated compounds were used as standard compounds in which their calibration solutions were prepared in a concentration range of 10-400 µg/ml by MeOH dilution. Linearity was evaluated in this range three times. The extract of *C. longa* was also diluted by MeOH and filtered before use. The mobile phase was made up of 0.1% acetic acid in water (mobile phase A) and ACN (mobile phase B). The HPLC running conditions were the same for standard compounds and extracts, which include sample injection (2 µl), flow rate (1.6 ml/min), column temperature (40°C), UV detector (λ=425 nm) for 0 min (75% A, 25% B), 0-20.0 min (30% A, 70% B), and 20-25 min (100% B).

The limit of detection (LOD) is the lowest concentration of the standard substance that can be detected. Similarly, the limit of quantitation (LOQ) shows the lowest concentration of analyte that can be quantified. Both LOD and LOQ were calculated using a calibration curve based on the standard deviation (SD or σ) of the data response and the slope of the calibration curve (*a*) using the following equations: LOD=3.3 σ/a and LOQ=10 σ/a.

Biological assays

Antioxidative assay: Our earlier reports have detailed descriptions of the DPPH antioxidant assay [12-14]. In brief, DPPH (0.1 mM) was diluted in MeOH. DPPH (200 µl) was

then continually added to 1.3 µl of various concentrations of samples in Dimethyl sulfoxide (DMSO) (128, 25.6, 5.12, and 1.024 µg/ml). A 96-well plate was used to mix the ingredients at 25°C for 30 min. The mixture was carried out by a 96-well plate at 25°C and 30 min. An Elisa reader was used to determine the absorbance at 517 nm.

The inhibitory rate (%)=[(OD_o-OD_1)/OD_o]x100%, where OD_o (optical density) stands for reaction absorbance of the control and OD_1 is the absorbance of the sample. Each experiment was repeated three times with resveratrol serving as the positive control. The IC_{50} value, commonly known as the concentration of tested sample with half-maximal reaction, was determined using Excel linear regression of serial scavenging percent values vs concentrations.

α-Glucosidase assay: The inhibitory assay for α-glucosidase reaction was carried out similarly to our prior protocol [15, 16]. The sample was dissolved in DMSO to produce a stock of 400 mg/ml. In a 100-mM phosphate buffer (pH 6.8), a series of dilutions to final concentrations of 256, 64, 16, and 4 µg/ml were made for each sample. A reaction mixture containing sample, 100-mM phosphate buffer (pH 6.8), and α-glucosidase (0.4 U/ml) was pre-incubated for 10 min at 37°C in a 96-well plate. As a substrate, 2.5 mM of pNPG (p-nitrophenyl-α-D-glucopyranoside) solution was added to the combination. The reaction was halted by adding Na_2CO_3 0.2 M after 30 min of incubation at 37°C. The number of released p-nitrophenol from pNPG was calculated at 410 nm by an Elisa reader. Acarbose was used as a positive control, and each assay was carried out three times.

Similar to above, the IC_{50} value stands for the concentration of compound exhibiting 50% inhibition of α-glucosidase activity under the assay condition.

AChE assay: The AChE inhibitory assay was performed based on the modified Ellman's method [17]. The sample was dissolved in DMSO (20 mg/ml) and sodium phosphate buffer to final concentrations of 128.0, 32.0, 8.0, 2.0, and 0.5 mg/ml. In a 96-well plate, the mixture of 25 ml of the prepared sample as described above was added to 25 mL PBS, 25 ml of 0.22 U/ml AChE, and 125 ml of 3 mM DTNB (5-5'-dithiobis-2-nitrobenzoic) and pre-incubated for 15 min at 25°C. The substrate acetylthiocholine iodide (ACTI) (25 ml of 15 mM ATCI) was then added. Hydrolytic reaction of ATCI catalysed by AChE was carried out at 412 nm by an Elisa reader. Each experience was repeated three times and donepezil was used as a positive control.

Cytotoxic assay: The MTT experiment was used to assess the cytotoxic activity of EtOH extract and isolated compounds **1-4** against human cancer cell lines KB, LU-1, Hep-G2, and MCF-7 [18]. Cells were grown in DMEM (Dulbecco's Modified Eagle Medium) medium containing 10% fetal bovine serum (FBS), 1% Penicillin and Streptomycin, and 1% L-glutamine at 37°C in a humidified environment of 5% CO_2. Samples were dissolved in DMSO to produce a stock of 20 mg/ml and each compound with final concentrations of 256, 64, 16 and 4 µg/ml was then prepared from this stock.

On 96-well plates, cells were separated with trypsin, planted with $3x10^4$ cells/ml in each well, then treated with varying doses of material. The controls were cells that had not been treated. After 72 h of treatment, each well was treated with an MTT solution (10 µl, 5 mg/ml) of phosphate buffer for 4 h until intracellular purple formazan crystals were evident. The MTT was removed and replaced with a 100-µl DMSO solution. An Elisa reader at 540 nm was used to determine the OD of the solution. ODs from the three duplicate experiments were used to calculate the inhibition ratio. Ellipticine was used as a positive control.

Results and discussion

The phytochemical investigation of the EtOH extract of *C. longa* rhizomes, collected in Bac Giang, Vietnam, has led to the isolation of four compounds **1-4**. Based on NMR and MS spectral analyses, together with a comparison with literature, the chemical structures of these compounds were elucidated as curcumin (**1**), demethoxycurcumin (**2**), bisdemethoxycurcumin (**3**), and cyclocurcumin (**4**) (Fig. 1) [19].

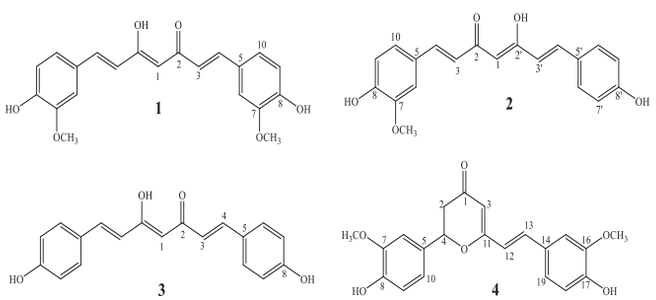

Fig. 1. The chemical structures of the isolated compounds 1-4.

Regarding quantitative analysis, an optimized strategy of the HPLC-DAD system was carried out to obtain the three separated peaks with R_t of 14.390 min (compound **1**), 13.760 min (compound **2**), and 13.117 (compound **3**) on the chromatogram, as compared with those of *C. longa* extract (Fig. 2). The calibration curve of each compound was established from at least six appropriate concentrations in triplicate by plotting the peak areas versus the concentration and good linearity was found in the test ranges, which ranged from 0.9986 to 0.9990 (Table 1). The LODs and LOQs were in the range of 0.7108-0.9265 mg/mg and 2.1538-2.8075 mg/mg, respectively. Most importantly, the contents of compounds **1-3** found in the *C. longa* extract was identified to be 7.215±0.101, 3.927±0.031, and 2.255±0.049 mg/g, respectively. As mentioned above, pharmacological values of *C. longa* are becoming more and more involved in the presence of curcuminoids. As compared to

literature, compounds **1-3** are dominate in Vietnamese *C. longa* extract, and higher than those of Indonesian *C. aeruginosa*, *C. heyneana*, *C. manga*, and *C. soloensis* extracts [20] as well as Indian *C. longa* extract [21].

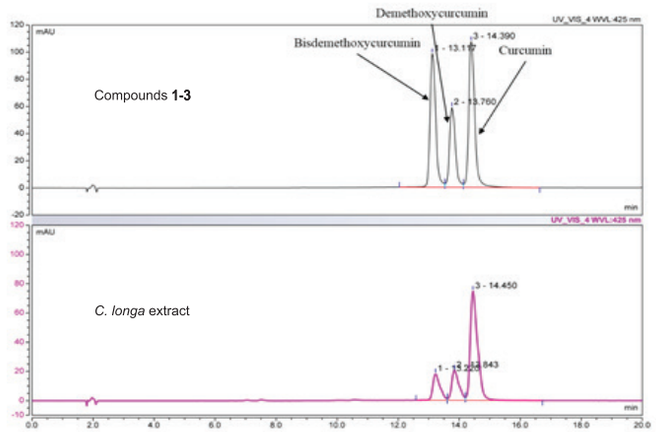

Fig. 2. The representative chromatograms at UV 254 nm of a mixture of compounds 1-3 and *C. longa* extract.

Table 1. Retention time, regression equation, R^2, LOQ, LOD, and content of compounds 1-3.

No	R_t (min)	Regression equation	R^2	LOD (µg/g)	LOQ (µg/g)	Content in extract (mg/g)
1	14.390	y=0.170x-0.242	0.9986	0.9095	2.7562	7.215±0.101
2	13.760	y=0.091x-0.184	0.9989	0.7108	2.1538	3.927±0.031
3	13.117	y=0.152x-0.512	0.9990	0.9265	2.8075	2.255±0.049

The EtOH extract and its isolated curcuminoids **1-4** were further subjected to antioxidative and α-glucosidase inhibitory assays (Table 2). Regarding the antioxidative assay, the EtOH extract showed free radical scavenging capability with an IC_{50} value of 25.6±1.15 µg/ml. Curcuminoids **1-4** are now considered promising antioxidants since they possess remarkable free DPPH radical scavenging. Their IC_{50} values ranged from 9.23±0.2 to 14.6±0.3 µg/ml as compared with that of the positive control resveratrol (IC_{50} 11.5±0.09 µg/ml). The deletion of one methoxy group has caused a larger IC_{50} value when compared between compounds **1** and **2**. However, the deletion of two methoxy groups in compound **3** would help to reduce the IC_{50} value.

Considering the α-glucosidase inhibitory assay, the EtOH extract exhibited inhibitory activity with an IC_{50} value of 45.8±2.1 µg/ml. Curcuminoids **1-4** also induced significant activity. The IC_{50} values of compounds **1-4** were 8.7±0.2, 14.91±0.23, 10.46±0.3, and 15.54±0.32 µg/ml, respectively. Notably, these values are much less than that of the positive control acarbose (IC_{50} 169.14±3.2 µg/ml). However, in contrast to antioxidative activity, compound **1** with the lowest IC_{50} value showed better activity than compounds **2** and **3** due to the number of methoxy groups.

Regarding the AChE assay, the EtOH extract showed activity with an IC_{50} value of 46.45±3.9 µg/ml (Table 2). As compared to donepezil (IC_{50} 0.035±0.01 µg/ml), the IC_{50} values of the isolated compounds occurred in following the order: **3** (IC_{50} 9.32±0.42 µg/ml) < **2** (IC_{50} 16.52±1.81 µg/ml) < **1** (IC_{50} 29.27±2.1 µg/ml) < **4** (IC_{50} 38.12±3.45 µg/ml). Therefore, it can be concluded that methoxylation is the main cause for the IC_{50} increase.

Table 2. DPPH radical scavenging, α-glucosidase, and AChE inhibitory activities.

No.	IC_{50} values (µg/m)		
	DPPH radical scavenging	*α-Glucosidase inhibition*	*AChE inhibition*
1	9.23±0.2	8.7±0.2	29.27±2.1
2	10.78±0.25	14.91±0.23	16.52±1.81
3	8.09±0.15	10.46±0.3	9.32±0.42
4	14.6±0.3	15.54±0.32	38.12±3.45
EtOH extract	25.6±1.15	45.8±2.1	46.45±3.9
References	Resveratrol: 11.5±0.09	Acarbose: 169.14±3.2	Donepezil: 0.035±0.01

The search for cytotoxic agents from natural plants is one of the most important components of drug development. In this regard, we applied an MTT assay to reveal potential cytotoxicity of the EtOH extract of the Vietnamese *C. longa* and its isolated compounds **1-4**. The EtOH extract was associated with IC_{50} values ranging from 22.73±1.08 to 77.71±2.64 µg/ml against four cancer cell lines KB, Hep, Lu, and MCF7 (Table 3). Curcuminoids **1-4** also exhibited moderate to weak cytotoxic activities. Compound **2** had induced moderate activity against the three cancer cell lines KB, Hep, and MCF7, but showed weak activity against cancer cell line Lu. In another case, compound **4** presented IC_{50} values of 122.63-157.93 µg/ml towards three cancer cell lines KB, Hep, and MCF7, but failed to control cancer cell line Lu (IC_{50}>256.0 µg/ml). Compounds **1-2** exhibited cytotoxicity better than compound **3** due to the presence of methoxy groups.

Table 3. Cytotoxic activity of the EtOH extract and isolated compounds 1-4.

No.	IC_{50} values (µg/ml)			
	KB	*Hep*	*Lu*	*MCF7*
1	91.90±3.25	65.90±2.36	134.48±6.64	103.62±4.53
2	36.57±1.78	40.30±2.54	129.68±5.76	59.53±3.78
3	134.92±6.65	98.62±3.85	156.44±7.14	118.86±5.95
4	124.55±6.37	122.63±5.76	>256	157.93±7.26
EtOH	32.96±1.23	22.73±1.08	77.71±2.64	40.20±1.78
Ellipticine	0.36±0.03	0.35±0.03	0.38±0.03	0.30±0.02

Conclusions

Chromatographic separation of the EtOH extract of Vietnamese *C. longa* rhizomes resulted in the isolation and determination of four curcuminoids including curcumin (**1**), demethoxycurcumin (**2**), bisdemethoxycurcumin (**3**), and cyclocurcumin (**4**). Compound **1** was a major compound with 7.215±0.101 mg/g extract, whereas values of 3.927±0.031 and 2.255±0.049 mg/g were found in compounds **2** and **3**, respectively. Curcuminoids **1-4** are now promising antioxidative and α-glucosidase inhibitory agents in *C. longa* extract because their IC_{50} values are comparable to that of resveratrol and acarbose. Both the EtOH extract and compounds **1-4** also exhibited AChE inhibition and cytotoxicity against four cancer cell lines KB, Hep. Lu, and MCF7. Methoxylation has greatly affected biological results...

ACKNOWLEDGEMENTS

This work was financed by the Institute of Chemistry, Vietnam Academy of Science and Technology under grant number VHH.2021.09.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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