



## CHEMICAL CONSTITUENTS FROM THE SEEDS OF *Alpinia blepharocalyx* K. Schum in VIETNAM AND THEIR BIOACTIVES

Ngoc-Tuan Nguyen<sup>1</sup>, Thi-Huong Nguyen<sup>2</sup>, Trung-Hieu Tran<sup>2</sup>, Thanh-Huyen Mai Thi<sup>3</sup>, Nu-Trinh Nguyen Thi<sup>1</sup>, Xuan-Hai Tang<sup>4</sup>, Viet-Hai Ha<sup>5</sup>, Gia-Buu Tran<sup>1</sup>, Dinh-Thang Tran<sup>3,\*</sup>

1. Institute of Biotechnology and Food-technology, Industrial University of Ho Chi Minh City, Vietnam.
2. School of Natural Sciences Education, Vinh University, Vinh City, Vietnam.
3. School of Chemistry, Biology, and Environment, Vinh University, Vinh City 43159, Vietnam.
4. Nghe An Obstetric & Paediatric Hospital, Vinh City, Vietnam.
5. Institute of Natural Product Chemistry, Vietnam Academy of Science and Technology, Hanoi, Vietnam.

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### ABSTRACT.

**Introduction:** Today, *Alpinia* plants are used to treat many diseases such as enterozoa, vomiting, gastralgia, indigestion, etc. in Japan, China, and India. The main second metabolites compounds of *Alpinia* were terpenoids, steroids, alkaloids, diarylheptanoids, flavanones, etc. As a continued program aiming to search for new metabolites from the *Alpinia* plants, *A. blepharocalyx* K. Schum. was collected and studied. **Materials & Methods:** This study aimed to isolate the bioactive compounds from the seeds of *Alpinia blepharocalyx* K. Schum (SAB) and evaluate their anti-cancer and anti-inflammatory effects in Vietnam. The compounds of SAB were determined via MS, IR, and NMR spectrum analysis, and their anticancer activity was tested against cancer cell lines (KB, SK-LU-1, MCF7, and HepG2) via the MTT assay. Moreover, the anti-inflammatory effect of the compounds was measured by evaluating the elastase release inhibition and the assays of the superoxide anion generation. **Results & Discussion:** We identified a sesquiterpene (alpinlepharin A) and eight flavonoids (flavokawain A, 2', 6'-dihydroxy-4'-methoxychalcone, nevaensin, apigenin, apigetrin, cynaroside, rutin, polydatin) from the SAB extract. This compounds showed significant anti-inflammatory (IC<sub>50</sub> values of  $4.3 \pm 0.3$  and  $6.3 \pm 1.7$   $\mu$ M) and cytotoxic activity (IC<sub>50</sub> values of  $15.23 \pm 2.14$  -  $25.81 \pm 2.45$   $\mu$ M/mL) against cancer cell lines (KB, SK-LU-1, MCF7, and HepG2). **Conclusion:** The data suggested *Alpinia blepharocalyx* K. Schum. as a promising functional food for the treatment of inflammatory and cancer-related diseases.

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### Introduction

Medicinal plants have widely been used as since the ancient times to treat various diseases [1-4]. The genus *Alpinia* is an important member of the Zingiberaceae family that includes 230 species [5] and most of them are distributed in Asia, including India, Vietnam, Malaysia, China, and Japan. Today, *Alpinia* plants are used to treat various diseases such as gastralgia, indigestion, vomiting, etc. in Japan, China, and India. Many groups of substances, including terpenoids, steroids, alkaloids, diarylheptanoids, flavanones, etc. have been isolated from this genus [6]. The plant has been traditionally used to treat skin disorders [7]. A number of diarylheptanoids and phenolic compounds have been isolated from the plants. These compounds are known to exhibit in vitro NO inhibitory, antiproliferative, and cytotoxicity activities [8]. In this study, SAB was collected and studied as a continued program aiming to search for new metabolites of the *Alpinia* plants. Herein we wish to publish the characterization of alpinlepharin A (1). Moreover, eight known compounds were isolated from this sample. Moreover, the anticancer, anti-inflammatory effects of the compounds were tested and which provided more information to use this product as a potential functional food in the future.

**Corresponding Author:** Dinh-Thang Tran (Prof.), School of Chemistry, Biology, and Environment, Vinh University, 182 Le Duan Str., Vinh City, Nghe An Province, Vietnam. E-mail – [thangtd@vinhuni.edu.vn](mailto:thangtd@vinhuni.edu.vn)

## Materials and Methods

**Plant Material.** The SAB were collected from the Quephong district of Nghean province, Vietnam in September 2018 and was identified by Dr. Nguyen Quoc Binh, Vietnam National Museum of Nature, Hanoi, Vietnam. A voucher specimen (sample code AB092018VN) was deposited at the School of Chemistry, Biology, and Environment, Vinh University.

**General Methods.** The  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR, HSQC, and HMBC spectra were obtained on the Bruker AV-III 500 NMR spectrometer, with tetramethylsilane (TMS) as the internal standard and chemical shifts were reported in  $\delta$  values (ppm). The high-resolution electrospray ionization (HR-ESI) mass spectra were measured using an Agilent 1200 LC-MSD Trap spectrometer. The preparative HPLC was conducted on the Agilent 218 purification system using a Shim-pack XR-ODS II column ( $2.0 \times 100$  mm, inner diameter  $2.2 \mu\text{m}$ ). Sephadex LH-20 (Sigma Aldrich) and  $40\text{--}60 \mu\text{m}$  silica gel (Merck) were used for gel and column chromatography. The spots of TLC were visualized at 254 and 365nm. TLC was heated silica gel plates sprayed with  $\text{Ce}(\text{SO}_4)_2$  reagent. All solvents used in column chromatography were distilled.

**Anti-inflammatory bioactivity.** The anti-inflammatory bioactivity was determined by elastase release inhibition examinations and the superoxide anion generation [9, 10].

**Cytotoxicity Assay.** The cancer cell lines [HepG2 (ATCC HB8065), MCF7 (ATCC HTB-22), SK-LU-1 (ATCC HTB-57), and KB (ATCC CCL-17)] were maintained in Dulbecco's D-MEM medium [penicillin G (100 UI/mL, L-glutamine (2 mM), 10% fetal calf serum), gentamicin (10  $\mu\text{g/mL}$ ), and streptomycin (100  $\mu\text{g/mL}$ )]. Stock solutions were prepared in DMSO- $\text{H}_2\text{O}$  (1/9 v/v), and cytotoxicity assays were performed in 96-well microtiter plates against cancer cell lines (KB, SK-LU-1, MCF7, and HepG2) ( $3 \times 10^3$  cells/mL) using a modification of the published method [11].

**Extraction and Isolation.** The SAB were dried at  $25^\circ\text{C}$  for 48 h. Then, this material (9.0kg) was extracted with methanol (MeOH) by using the mercerization method ( $3 \times 20$  L) at room temperature. The solvent extract was evaporated under low pressure at  $40\text{--}50^\circ\text{C}$  in order to yield a crude methanol extract (950 g). The crude methanol extract was partitioned by liquid-liquid extraction method with hexane, ethyl acetate, butanol successively. The solvent was recovered under low pressure to afford three crudes including crude hexane (ABH, 20 g), crude ethyl acetate (ABE, 345 g), crude butanol (ABB, 189 g), and crude water (ABW, 126g). The ethyl acetate crude (ABE) was isolated by silica gel column chromatography (CC) with hexane/ethylacetate step gradient system (100/1 – 0/1, v/v) to yield eight fractions (Frs. ABE1-ABE8). Fraction ABE1 (9.8 g) eluted with hexane/acetone (10/1, v/v) was further separated using CC and purified by preparative HPLC to obtain compound **1** (12 mg). Fraction ABE2 (43 g) was subjected to CC eluting with hexane/ethyl acetate solvent mixture (15/1) to give six subfractions (Frs. ABE 2.1-ABE2.6). Purification of fractions ABE2.1 (0.3 g) was performed by using RP-18 eluted with methanol/water (9/1, v/v) to yield compound **2** (35 mg). Purification of fractions ABE2.1 (0.3 g) was subjected to the Sephadex LH-20 eluted with methanol/water (9/1, v:v) to produce compound **4** (22 mg). The fraction ABE5 (29 g) was subjected to CC eluted with chloroform/methanol (20/1, 10/1 v/v) in the order of increasing polarity, to give five subfractions (ABE 5.1-ABE5.5). Purification of fractions ABE5.2 (1.2g) was performed by using RP-18 eluted with methanol/water (3/1, v/v) to produce compound **3** (7 mg). Fraction ABE5.5 (1.5g) was subjected to CC eluting with a solvent mixture of chloroform/methanol (7/1, v/v ) to yield compound **5** (15 mg). The soluble extracts (ABB) were applied to silica gel column chromatography with a chloroform/methanol step gradient system (100/1 – 0/1, v/v) to give seven fractions (Frs. ABB1-ABB7). Fraction ABB1 (0.8g), eluting with chloroform/methanol (9/1, v:v), was purified by RP-18 to give compound **9** (75 mg). Fraction ABB 2 (0.5 g), eluting with methanol/water (1/1, v/v) was purified with Sephadex LH-20 column chromatography to give compounds **8** (15 mg). Fraction ABB4 (1.7 g), eluting with methanol/water (3/1, v/v), was purified with RP-18 CC to yield compounds **6** (28 mg) and **7** (11 mg).

## Statistical analysis

All of the determinations were performed in triplicate. The experimental results were expressed as mean  $\pm$  standard deviation, and the Statgraphics Centurion XVI software was used to conduct statistical analysis. Differences between different treated groups were analyzed using ANOVA and multiple range tests with  $p < 0.05$ .

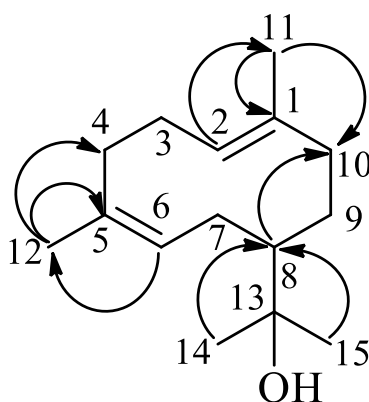
## Results and Discussions

### Chemical constituents of the SAB

To determine the chemical constituents of *A. blepharocalyx*, we studied the characterization and bioactives of nine compounds (**1-9**), which were isolated from SAB. The structures of all compounds were characterized by comparison with the literature. Their structures were identified as alpinepharin A (**1**), flavokawain A (**2**) [12], 2', 6'-dihydroxy-4'-methoxychalcone (**3**) [13], nevadensin (**4**) [14], apigenin (**5**) [15], apigetrin (**6**) [15], cynaroside (**7**) [16], rutin (**8**) [17], and polydatin (**9**) [18].

Compound **1** was obtained as a white powder, and its molecular formula  $\text{C}_{15}\text{H}_{26}\text{O}$ , determined by HR-ESI-MS at  $m/z$  245.1853 [ $\text{M} + \text{Na}$ ] $^+$  (calcd for  $\text{C}_{15}\text{H}_{26}\text{ONa}$ , 245.1881). The  $^1\text{H}$ -NMR spectrum of alpinepharin A (**1**) displayed the

characteristic resonances for four methyl singlets at 1.20 (3H, *s*, H-15), 1.16 (3H, *s*, H-14), and 1.61 (6H, *s*, H-11,12). The multiplet signal at 5.19-5.14 (2H, *m*) was assigned to the two methine protons H-2 and H-6. All this information from  $^{13}\text{C}$  NMR, as well as HMBC, HSQC led to the structure for this compound, which indicated the basic skeleton of **1** was similar to that of helminthogermacrene [19]. The chemical structure of **1** was elucidated as shown in Figure 1 and named trivially alpiblepharin A.



**Fig. 1.** Structure and the important HMBC of alpiblepharin A.

**Table 1.** Phytochemical composition of the seeds of *A. blepharocalyx*

| No | Compounds                           | Bioactivities                                                        |
|----|-------------------------------------|----------------------------------------------------------------------|
| 1  | Alpiblepharin A                     | Anti-inflammatory, anti-oxidative, anti-cancer effects               |
| 2  | Flavokawain A                       | Anti-inflammatory, anti-oxidative, anti-cancer effects               |
| 3  | 2', 6'-dihydroxy-4'-methoxychalcone | Anti-oxidative, anti-cancer effects                                  |
| 4  | Nevadensin                          | Anti-oxidative, anti-cancer effects                                  |
| 5  | Apigenin                            | Anti-oxidative, anti-cancer, anticancer, anti-diabetic effects       |
| 6  | Apigetrin                           | Anti-oxidative, anti-inflammatory, anticancer, anti-diabetic effects |
| 7  | Cynaroside                          | Anti-inflammatory, anti-oxidative effects                            |
| 8  | Rutin                               | Anti-inflammatory, anti-oxidative effects                            |
| 9  | Polydatin                           | Anti-inflammatory, anti-oxidative, anti-cancer effects               |

#### Anti-inflammatory effect of compound 1

In this study, compound **1** displayed the most significant inhibition of superoxide anion generation and elastase release with IC<sub>50</sub> values of  $4.3 \pm 0.3$  and  $6.3 \pm 1.7 \mu\text{M}$ , respectively.

#### Anti-cancer effect of compound 1-9

The purified compounds **1-9** were evaluated for in vitro cytotoxicity against KB, HepG2, SK-LU-1, and MCF7 cancer cell lines. The compounds **2-9** exhibited moderate activity that inhibited cancer cell lines with IC<sub>50</sub> values of  $37.00 \pm 3.66$  to  $55.41 \pm 2.16 \mu\text{M/mL}$ . Compound **1** showed significant cytotoxicity against cancer cell lines with IC<sub>50</sub> values of  $15.23 \pm 2.14$  to  $25.81 \pm 2.45 \mu\text{M/mL}$ . The cytotoxicity may be significant affected by the hydroxyl group. The esterification will decrease the cytotoxic activity of this compound. These data provided a scientific basis for further application of black shallot for the treatment of cancer-related diseases.

#### Conclusion

In this study, the presence of 9 compounds in *A. blepharocalyx* seed extract was identified, including alpiblepharin A, flavokawain A, 2', 6'-dihydroxy-4'-methoxychalcone, nevadensin, apigenin, apigetrin, cynaroside, rutin, polydatin. Moreover, the SAB extract exhibited a stronger anticancer and anti-inflammatory. Alpiblepharin A (**1**) was isolated from natural for the first time. This compound showed significant anti-inflammatory (IC<sub>50</sub> values of  $4.3 \pm 0.3$  and  $6.3 \pm 1.7 \mu\text{M}$ ) and cytotoxic activity against KB, SK-LU-1, MCF7, and HepG2 cancer cell lines (IC<sub>50</sub> of  $15.23 \pm 2.14$  to  $25.81 \pm 2.45 \mu\text{M/mL}$ ). The data indicate *Alpinia blepharocalyx* as a potential food supplements for treating inflammatory and cancer-related diseases.

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# Conflict-of- interest Notification Page:

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

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