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The Chemical Investigation of Four Balanophora Species and Cytotoxicity with Inhibition of NO Production

Nguyen Thanh Tung[1], Nguyen Viet Than[1], Nguyen Quang Hung[2,3], Ain Raal[4*]

[1]Department of Pharmacognosy, Hanoi University of Pharmacy, Le Thanh Tong 15, 11000 Hanoi, Vietnam.
[2]Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST), Hoang Quoc Viet 18, 11300 Ha Noi, Vietnam.
[3]Graduate University of Science and Technology, VAST, Hoang Quoc Viet 18, 11300 Ha Noi, Vietnam.
[4]Institute of Pharmacy, Faculty of Medicine, University of Tartu, Nooruse 1, 50411 Tartu, Estonia.

***Email:** ain.raal@ut.ee*

ABSTRACT

The phytochemistry and biological activity of four rare Balanophora species (B. subcupularis P.C. Tam (BS), B. tobiracola Makino (BT), B. fungosa var. globosa (Jungh.) B.Hansen (BFG), and B. fungosa subsp. indica (Arn.) B.Hansen (BFI)) growing in Vietnam have been studied. The six pentacyclic triterpenoids including β-amyrin, β-amyrone, lupenone, lupeol, β-amyrin acetate, and lupeol acetate could be identified in all GC-MS chromatograms of four species but very different area percentage. The chemical investigation of B. subcupularis and B. tobiracola led to the isolation of coniferyl aldehyde, gallic acid, cinnamic acid, and caffeic acid. Gallic acid and coniferyl aldehyde were isolated from B. subcupularis for the first time, Among isolated compounds, coniferyl aldehyde showed moderate cytotoxicity activity at cancer cell lines MCF-7, A549, Hep3B, and PC3 at the concentration of 100 µg/ml (CS% = 24.05 – 46.61%). Besides, this compound also significantly inhibited NO production (I% = 101.96 and 114.29 at two respectively concentrations of 30 µg/ml and 100 µg/ml).

Key words: *Balanophora subcupularis, Balanophora tobiracola, Isolation, Biological activity, HPTLC, GC-MS*

INTRODUCTION

In Vietnam, Genus *Balanophora* J.R. & G.Forst. consisted of eight species, one subspecies, and one variety with three species including *B. subcupularis*, *B. harlandii*, and *B. tobiracola* were recently recorded to Flora of the country [1-5]. Local people used some species belonging to this genus to treat some different diseases as body aches, abdominal pain or to help strengthen muscles and bones [6]. Chemical investigation of *Balanophora* species led to the isolation of some different classes of compounds such as hydrolyzable tannins, phenylpropanoids, and triterpenes [7]. High-speed counter-current chromatography was used to separate and purify galloyl, caffeoyl, and hexahydroxydiphenoyl esters of glucoses from the aerial parts of the parasitic plant *B. simaoensis* (syn. *B. fungosa* subsp. *indica*) [8]. Recently, two new butenolides were isolated from aerial parts of *B. fungosa* while three new muurolane-type sesquiterpene glycosides were isolated from the whole plant of *B. fungosa* subsp. *indica* [9, 10]. Lately, some lignans and phenolic compounds have been isolated from ethyl acetate fraction of *B. fungosa* var. *globosa* (Jungh.) B.Hansen growing in Vietnam [11]. The comparative HPTLC analysis of five *Balanophora* species including *B. simaoensis*, *B. spicata*, *B. laxiflora*, *B. dioica*, and *B. polyandra* in China led to the identification of some triterpenoids including *β*-amyrin palmitate (balanophorin A), lupeol palmitate (balanophorin B), *β*-amyrin acetate and lupeol acetate in all chromatograms of five species [12].

Our study aimed to investigate the chemical composition and to develop HPTLC and GC-MS to compare the chemical compositions of the aforementioned species, as well as evaluate their biological activity, along with

isolation and identification of some compounds from *B. subcupularis* and *B. tobiracola*. This is the first time the chemical composition of *B. subcupularis* was investigated, as well as some triterpenoids, were identified in four *Balanophora* species using GC-MS, and some biological activity of four *Balanophora* species were evaluated.

MATERIALS AND METHODS

Plant material

Whole plants of *B. fungosa* subsp. *indica* (Arn.) B.Hansen (BFI) (Lao Cai prov., January 2017, 22°19'35" N 103°48'21" E, Voucher specimens (VS) number HNU 024069), *B. fungosa* var. *globosa* (Jungh.) B.Hansen (BFG) (Lam Dong prov., January 2016, 12°05'23"N 108°27'10"E, VS number HNU 024066), and two recently recorded species for Flora of Vietnam including *B. tobiracola* Makino (BT) (Lang Son prov., January 2018, 21°53'33''N 106°22'57''E, VS number HNU 024056) and *B. subcupularis* P.C. Tam (BS) (Dien Bien prov., November 2017, 22°03'56" N 103°06'13" E, VS number HNU 024068) were collected and authenticated by Prof. Phan Ke Loc and MSc. Nguyen Anh Duc, Faculty of Biology, VNU University of Science, Vietnam National University, Hanoi, Vietnam. Voucher specimens of studied species were deposited at the Faculty of Biology, VNU University of Science, Vietnam National University, Hanoi, Vietnam. The sliced plant material was dried in a ventilated oven and stored in sealed PE bags.

Chemicals and reagents

The solvents and chemicals used for extraction, isolation, and TLC analyses are of analytical standard. The reference compounds including caffeic acid, cinnamic acid, coniferyl aldehyde, gallic acid, and lupeol were obtained from Biopurify phytochemicals Ltd. (China).

Extraction and isolation

The dried and powdered whole plants of BFI, BFG, BT, and BS were ultrasonically extracted with methanol (3 times for 30 min). The total extract of each species was concentrated *in vacuo*. Then the crude extract was suspended in water and partitioned between *n*-hexane and ethyl acetate successively. The obtained fractions were evaporated *in vacuo* and used for further analysis.

The ethyl acetate residue of *B. subcupularis* (8 g) was chromatographed on a silica gel column eluted by 1, 10, 20, 40, 80, and 100% of methanol in dichloromethane to give 6 fractions BSE1-6. The fraction BSE1 by a silica gel column eluted by dichloromethane to obtain compound **S1** (3.3 mg) (**1**). The fraction BSE-5 was separated by a silica gel eluted by 0-90% of methanol in dichloromethane to afford **S2** (3.5 mg) (**2**).

The ethyl acetate residue of *B. tobiracola* (10 g) was separated by silica gel eluted by 1, 10, 20, 40, 80, and 100% of methanol in dichloromethane to give 6 fractions BTE1-6. The fraction BTE2 by a silica gel column eluted by 10, 20, 30, and 40 % of methanol in dichloromethane afforded two sub-fractions BTE2.1 and BTE2.2, combined based on TLC analysis. **BT3.1** (4.2 mg) (**3**) was purified from BTE2.2. Fraction BTE5 was chromatographed by Sephadex LH-20 CC with methanol to give 3 sub-fractions BTE5.1-BTE5.3. **BT6.1** (3.1 mg) (**4**) was isolated from BTE5.2 using preparative TLC.

The identification of the structure of compound base on MS and NMR spectral analysis, as well as comparison to commercial standards. ^{1}H NMR spectra were recorded using Bruker Avance III HD spectrometer at 500 MHz. Methanol-d4 or DMSO was used as solvents. Structural assignments were performed by comparing the spectra with literature data.

High-performance thin layer chromatography

High-performance Thin layer chromatography is still a very useful method in the research of medicinal plants because of its simplicity and great flexibility. In our study, the HPTLC of samples was developed using an HPLTC system (CAMAG, Switzerland). The plate was developed in Auto Developing Chamber (ADC-2, CAMAG) using various Eluent Systems with different polarizations. The chromatographic evaluations were done with vision CATs software.

In our previous study, gallic acid, caffeic acid, and methyl caffeate have been identified in the HPTLC chromatogram of four species [11]. In this study, for the identification of cinnamic acid and coniferyl aldehyde, these two compounds were dissolved in methanol to a concentration of 1.0 mg/mL. The crude residues of each species (0.1 g) were dissolved in methanol (10 mL). The same volume (10 µl) of the samples and reference solutions were applied to the same HPTLC plate using Linomat V (CAMAG, Switzerland). After development,

the plate was taken images at a wavelength of 254 nm and 366 nm using TLC-Visualizer (CAMAG). Then the plate was derivatized with anisaldehyde – sulphuric acid (AS) reagent and taken the image in white light.
For the HPTLC analysis of triterpenoids, *n*-hexane residues of each species extract (50 mg) were dissolved in methanol (5 mL). The same volume of each solution (10 µL) was applied on the same HPTLC silica gel 60 F_{254} plate and C_{18} RP-HPTLC plate using Linomat 5 applicator (CAMAG) and respectively developed using two eluent systems *n*-hexane – ethyl acetate (5:1, v/v) and ethyl acetate – acetonitrile (3:2, v/v) using ADC-2. After development, the plates were sprayed with AS reagent, heated at 120°C for 5-10 min, then evaluated in vis. or UV-366 nm.

Gas chromatography-mass spectrometry
Triterpenoids have been investigated by some chromatographic techniques such as gas chromatography (GC) [13, 14], high-performance thin layer chromatography (HPTLC) [15], and mixed-mode liquid chromatography-tandem mass spectrometry [16]. In this study, the GC-MS analysis has been performed to confirm the presence of triterpenoids in *n*-hexane fractions of four *Balanophora* species. The *n*-hexane fractions (10 mg) were dissolved in the mixture of *n*-hexane – chloroform (1:4, v/v) (5 mL) and submitted to filtration over a small silica column gel to obtain the solutions used for GC-MS analysis. The GC-MS studies were performed on a GC system Agilent 7890A coupled with an Agilent 5975C inert MSD and equipped with a DB-5 MS capillary column (30m x 250 µm x 0.25 µm). Column constant flow was set at 1 mL/min. The system was operated under the following conditions: inlet temperature 300 °C; MSD transfer line temperature 300 °C; oven temperature program 200 (3 min) then 2 °C/min to 300 °C, run time 53 min. The mass spectrometer was monitored to scan *m/z* 40-550 with an ionizing voltage at 70 eV. The individual peaks were identified by their fragmentation patterns to those in library NIST 08 thanks to NIST MS search 2.0 software. Some minor peaks in the chromatograms remained unidentified because of the lack of library mass spectra of the corresponding compounds.

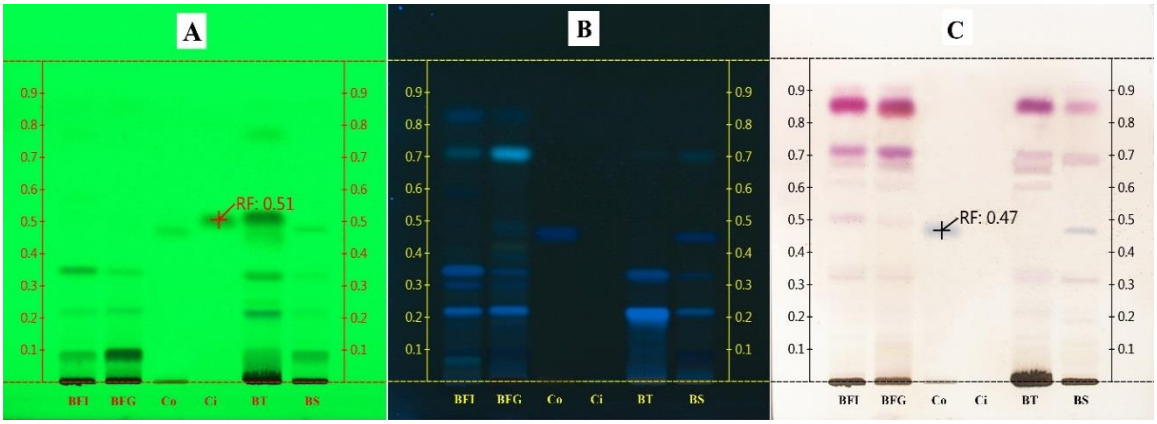
Cytotoxic assay
The cytotoxicity of the total residues of four aforesaid species and some isolated compounds were evaluated by using the previously described method [11], using MTT cell proliferation assay kits. The following four cancer cell lines have been studied: MCF-7 (human breast carcinoma), A549 (human lung carcinoma), Hep3B (human liver cancer), PC3 (human prostate gland carcinoma). The cells were cultured in RPMI-1640 or DMEM at 37ºC with 5% CO_2, supplemented with 10% FBS, penicillin, and streptomycin sulfate. They were grown in a 96-well flat-bottom plate (200 µL, 1×10^5 cells/well, in triplicated) and incubated for 24 h. After that, DMSO solutions of the residues at concentrations of 30 and 100 µg/ml were added to the wells. Then the treated cells were incubated for 48 h at 37ºC, followed by the MTT assay. The absorbance (OD; $\lambda = 570$ nm) was recorded and the cell survival was determined using non-linear regression analysis. Camptothecin (Sigma-Aldrich, St. Louis, Missouri, USA) was used as a positive control.

NO production in LPS-stimulated RAW264.7 cells
Besides, the inhibitory effect of residues on the NO production in LPS-stimulated RAW 264.7 cells was also evaluated using the previously described method [11]. Cells were cultured in DMEM, at 37ºC with 5% CO_2, supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin sulphate (100µg/mL). Then they were seeded in a 96-well plate at 2.5 × 105 cells/well, in triplicate. The cells were treated with two concentrations of samples prepared in DMSO (30 and 100 µg/ml), followed by incubation for 24 h. The Griess method was used to estimate the nitrite concentration in the culture supernatant. Besides, the remaining cell solutions were evaluated by measurement of cell viability with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cardamonin (Sigma-Aldrich, St. Louis, Missouri, USA) was used as a positive control.

RESULTS AND DISCUSSION

HPTLC of balanophora species
For the identification of cinnamic acid and coniferyl aldehyde, the combination of toluene, ethyl acetate, and formic acid (14:10:0.5) (v/v/v) as mobile phase gave well-separated, compact, and symmetrical bands **(Figure 1)**. Some similar profiles of separated bands of *Balanophora* species could be observed in both $\lambda = 254$ nm and $\lambda = 366$ nm before derivatization as well as white light after derivatization. Besides, coniferyl aldehyde (R_f 0.47) could

be detected in the chromatogram of BS as a bright blue band at white light while cinnamic acid (R_f 0.51) could be detected in the chromatogram of BT at UV 254 nm (*B. tobiracola*).

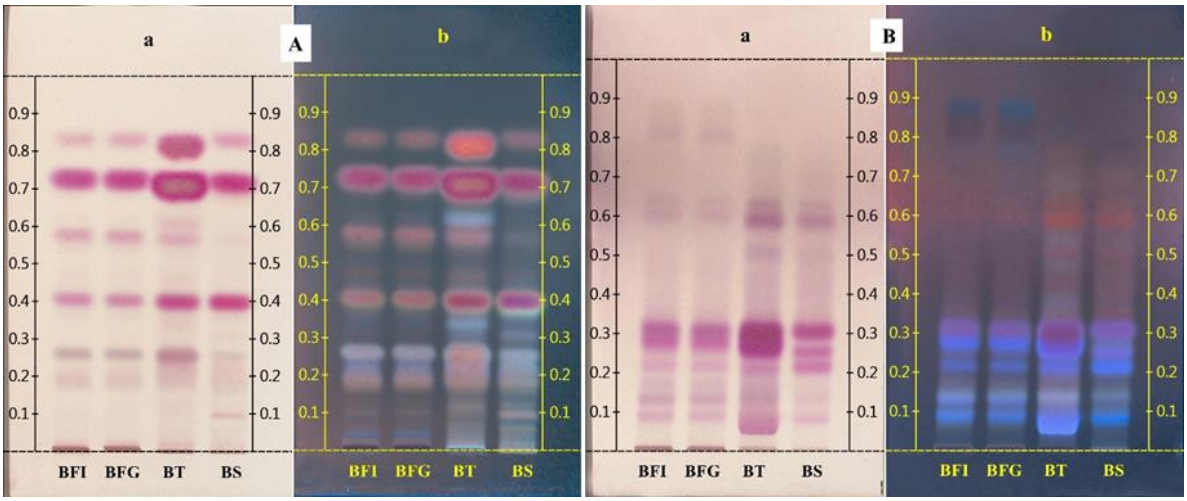


a) **b)** **c)**

Figure 1. HPTLC chromatogram of four species developed with the eluent system toluene – ethyl acetate – formic acid (14:10:0.5) (v/v/v)

Note: a) plate observed at 254 nm, b) Plate observed at 366 nm, c) Plate observed at white light after derivatization. BFG - *Balanophora fungosa* var. *globosa*, BFI - *B. fungosa* subsp. *indica*, BS - *B. subcupularis*, BT - *B. tobiracola*, Co - Coniferyl aldehyde, Ci - Cinnamic acid

The comparative HPTLC chromatograms of *n*-hexane fractions from four species developed on HPTLC silica gel 60 F_{254} plates showed six similar bands **(Figure 2A)** while chromatograms developed on the C_{18} RP-HPTLC plate had five similar bands **(Figure 2B)**. The purple color of the bands indicated that they could be triterpenoids. These results suggested that there were similar components in *n*-hexane fractions of four species.



a) b) a) b)

A) B)

Figure 2. HPTLC chromatogram of *n*-hexane fractions from four species belonging to genus *Balanophora* developed under two different conditions. (A) HPTLC silica gel plate developed with *n*-hexane – ethyl acetate (5:1) at visual illumination (a) and 366 nm (b); (B) C_{18} RP-HPTLC plate developed with ethyl acetate – acetonitrile (3:2) at visual illumination (a) and 366 nm (b)

GC-MS analysis of pentacyclic triterpenes

Total ion chromatograms of *n*-hexane fractions of four *Balanophora* species and chemical structures of some identified compounds are shown in **Figure 3**. Six pentacyclic triterpenes including *β*-amyrone, *β*-amyrin, lupenone, lupeol, *β*-amyrin acetate, and lupeol acetate were identified in all chromatograms of four species but in different area percentage **(Table 1)**.

β-Amyrone (βAm1) was eluted a little bit sooner than *β*-amyrin. The mass spectrum of this metabolite presented fragment ions at *m/z* 218, 203, 189 which are similar to *β*-amyrin. However, there was an additional fragment ion at *m/z* 424 corresponding to molecular formula $C_{30}H_{48}O$ instead of the fragment ion at *m/z* 426 of *β*-amyrin. *B. fungosa* var. *globosa* (12,31 %) và *B. subcupularis* (13,06 %) have the high are the percentage of this component. *β*-Amyrin (βAm2) could be easily identified in chromatograms of four species with the base peak at *m/z* 218 in the mass spectra which is characterized for Δ-12 ursane or olean skeleton, the relative abundance of fragment ion at *m/z* 203 is three times higher than of fragment ion at *m/z* 189; the parent ion peak at *m/z* 426 suggested a molecular formula of $C_{30}H_{50}O$. Among four species, *B. subcupularis* possesses the highest area percentage of this component in the chromatogram (10,41 %).

Lupenone (L1) (1,65 – 11,27 %) and lupeol (L2) (1,20 – 11,25 %) were also identified in all chromatograms of 4 species. Lupenone had a base peak at *m/z* 205 and some other fragment ion at *m/z* 189, *m/z* 409 [$M^+ - CH_3$]; the presence of the parent ion at *m/z* 424 suggested the molecular formula of $C_{30}H_{48}O$. On the other hand, lupeol had a base peak at *m/z* 207 and some other characteristic fragment ion at *m/z* 411 [$M^+ - CH_3$], *m/z* 408 [$M^+ - H_2O$], *m/z* 393 [$M^+ - CH_3 - H_2O$], and the parent ion *m/z* at 426 suggesting the molecular formula of $C_{30}H_{50}O$. The highest is the percentage of lupeol and lupenone in chromatogram belonged to *B. subcupularis*.

β-amyrin acetate (βAm3) was eluted 3 min later than *β*-amyrin. The fragmentations of *β*-amyrin acetate were quite similar to *β*-amyrin but there was an additional present of fragment *m/z* 43 equivalent to acetyl. This component was the main constituent in chromatograms of *B. tobiracola*, *B. fungosa* subsp. *indica*, *B. fungosa* var. *globosa* (31,96 – 39,82 %), however in very low concentration in chromatogram of *B. subcupularis* (0,50 %). Lupeol acetate (L3) is the latest eluted compound among identified pentacyclic triterpenes. This is the main component in all chromatograms of four *Balanophora* species (23,28 – 36,19 %). The mass spectrum of lupeol acetate showed a base peak at *m/z* 189. Some other fragments included *m/z* 468 [M^+], *m/z* 453 [$M^+ - CH_3$], *m/z* 408 [$M^+ - CH_3COO$].

Table 1. Retention indices, formula, and MS data of compounds identified in *n*-hexane fractions of four *Balanophora* species

Compound	RI*	Formula	M^+	Production (relative abundance %)	% Area			
					BFI	BFG	BT	BSP
β-Amyrone (βAm1)	3249	$C_{30}H_{48}O$	424 (10)	218 (100), 203 (62), 44 (26), 219 (19), 189 (16), 207 (14), 83 (14), 95 (14), 41 (13), 205 (13), 409 (6) (M^+-15)	2.8	**12.3**	1.0	**13.1**
β-Amyrin (βAm2)	3278	$C_{30}H_{50}O$	426 (6)	218 (100), 203 (48), 44 (20), 83 (19), 207 (18), 219 (17), 85 (14), 189 (14), 95 (11), 69 (10), 411 (2) (M^+-15)	3.9	1.8	1.5	**10.4**
Lupenone (L1)	3298	$C_{30}H_{48}O$	424 (42)	205 (100), 109 (72), 83 (66), 44 (64), 107 (59), 95 (57), 93 (54), 121 (53), 81 (52), 189 (44), 409 (28) (M^+-15), 381 (6) (M^+- 43)	4.3	4.2	1.7	**11.3**
Lupeol** (L2)	3326	$C_{30}H_{50}O$	426 (40)	207 (100), 83 (70), 189 (69), 95 (67), 44 (65), 135 (62), 109 (61), 107 (60), 121 (58), 93 (58), 411 (18) [M^+-15], 393 (7), 383 (6)	3.0	1.2	1.7	**11.3**
β-Amyrin acetate (βAm3)	3375	$C_{32}H_{52}O_2$	468 (2)	218 (100), 203 (48), 219 (18), 189 (17), 43 (16), 204 (10), 69 (10), 95 (10), 119 (10), 135 (9)	**39.0**	**32.0**	**39.8**	0.5
Lupeol acetate (L3)	3426	$C_{32}H_{52}O_2$	468 (28)	189 (100), 43 (69), 121 (57), 95 (56), 107 (54), 93 (53), 135 (52), 109 (46), 81 (46), 190 (42), 453 (12) [M^+-15], 408 (10)	**23.3**	**30.7**	**30.7**	**36.2**
Total					76.3	82.2	76.4	82.8

*__RI:__ retention indices calculated on a DB-5MS column using a homologous series of straight-chained alkanes (C8-C40) analyzed under the same condition; **Structure of lupeol was confirmed with commercial standard

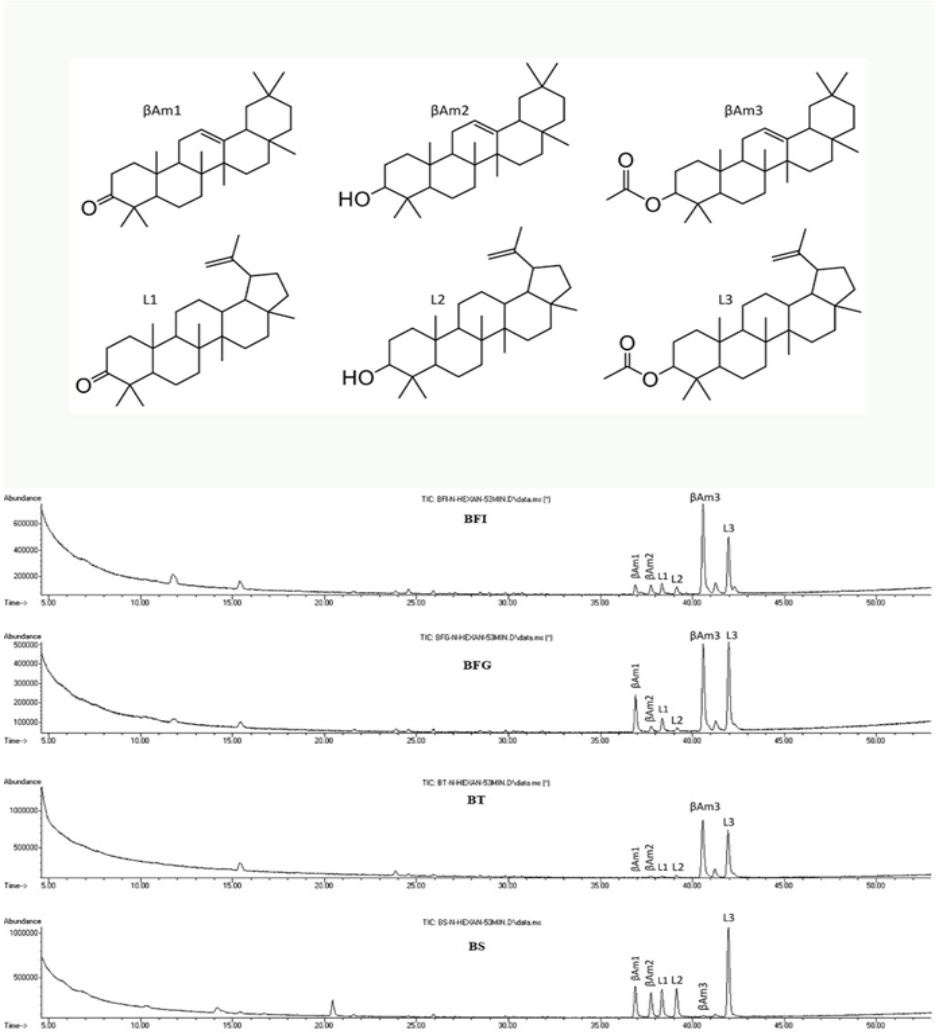


Figure 3. Total ion chromatograms of four *Balanophora* species and chemical structures of identified triterpenes

Note: β-amyrone (βAm1), β-amyrin (βAm2), lupenone (L1), lupeol (L2), β-amyrin acetate (βAm3), lupeol acetate (L3)

Isolated compounds

The ethyl acetate residues of BS and BT were chromatographed via various chromatography columns to obtained four compounds. These isolated compounds were identified as coniferyl aldehyde (**1**), gallic acid (**2**), cinnamic acid (**3**), and caffeic acid (**4**) based on NMR spectral data and MS data, as well as comparison to commercial standards of gallic acid, cinnamic acid, caffeic acid, and coniferyl aldehyde **(Figure 4)**. (see supplementary file)

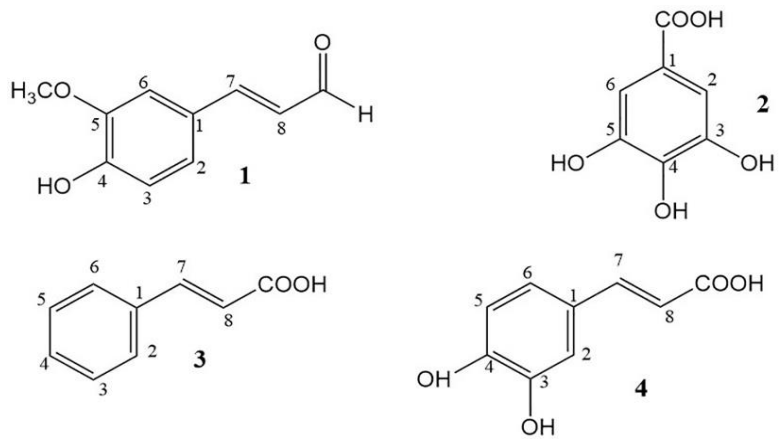


Figure 4. Isolated compounds from ethyl acetate fractions of *B. subcupularis* and *B. tobiracola*

Bioassay

Coniferyl aldehyde showed a significant inhibitory effect on NO production, whereas the inhibitory ability of two taxa of *B. fungosa* (BFI and BFG) at a concentration of 100 µg/mL was 51.26 and 65.01 % **(Table 2)**. On the other hand, *B. subcupularis* and *B. tobiracola* showed very weak activity.

Table 2. Inhibitory effect of the total residues and isolated compounds on NO production

Samples	Concentration (µg/mL)	% Inhibitory	±SD	% Cell survival	±SD
BFI	30	10.08	1.13	99.22	0.84
	100	**51.26**	0.52	93.03	0.44
BFG	30	25.21	1.26	80.77	0.38
	100	**65.01**	0.9	69.62	2.33
BT	30	26.75	2.61	86.47	1.75
	100	47.3	1.36	85.21	2.4
BSP	30	29.19	1.59	92.3	2.08
	100	36.27	2.7	91.27	2.18
Coniferyl aldehdyde	30	**101.96**	0.14	75.25	0.24
	100	**114.29**	0.14	63.65	0.24
Cardamonin*	0.3µM	30.25	0.38	91.45	0.33
	3µM	79.83	1.01	82.96	1.37

*The positive control.

B. subcupularis (BS) is the only species that inhibited weak cytotoxicity against the cancer cell line A549 (human lung carcinoma) at a concentration of 100 µg/mL (CS% = 48.29 %). Total residues of other species demonstrated weak cytotoxicity. Among isolated compounds, coniferyl aldehyde showed cytotoxicity against all four cancer cell lines at a concentration of 100 µg/mL (CS% = 24.05 – 46.61%) **(Table 3)**.

Table 3. Cytotoxicity of the total residues and isolated compounds

Samples	Concentration (µg/mL)	% Cell survival (CS%)							
		MCF-7		A549		Hep3B		PC3	
		CS%	±SD	CS%	±SD	CS%	±SD	CS%	±SD
BFI	30	80.41	1.19	66.48	1.86	67.56	1	84.48	0.14
	100	66.9	1.93	50.32	1.61	45.9	0.84	65.22	0.23
BFG	30	94.35	1.92	68.09	1.72	75.34	1.97	90.51	2.49
	100	78.08	1.72	60.01	2.53	63.73	1.34	68.12	1.9
BT	30	88.36	1.52	64.3	1.97	85.83	1.81	68.39	2.38
	100	65.29	2.05	62.47	1.38	70.68	2.06	59.07	4.25
BS	30	76.47	3.41	62.33	2.06	93.3	1.59	84.67	13.34
	100	72.58	1.95	**48.29**	2.78	88.63	3.11	67.55	3.89
Coniferyl aldehdyde	30	64.22	0.41	64.56	2.95	65.11	0.71	56.9	2
	100	**24.72**	1.2	**46.61**	1.96	**43.07**	3.12	**24.05**	1.13
Camptothecin*	0.1µg/mL	54.46	2.37	50.05	2.1	71.1	1.23	53.81	0.14
	10µg/mL	19.87	1.97	26.52	0.86	26.04	2.19	12.3	0.62

* The positive control. MCF-7 (human breast carcinoma), A549 (human lung carcinoma), Hep3B (human liver cancer), PC3 (human prostate gland carcinoma)

Generally, HPTLC and GC-MS profiles of the *Balanophora* species were analogous with a varied intensity which suggested the presence of similar phytochemicals with different concentrations. Among identified triterpenoids using GC-MS, some compounds possessed important pharmacological activity: lupeol (lupane type) has been shown to possess some significant biological activity such as anti-inflammatory, antiarthritic, antigeogenic, antioxaluric and anticalciuric, and metastatic melanoma inhibitory for human [17], while lupeol acetate dose-dependently inhibited nitric oxide (NO) production, iNOS and COX-2 expression in LPS-stimulated macrophage RAW 264.7 cells and may take part in the antinociceptive and anti-inflammatory effect of *B. spicata* [18].

All six triterpenoids (lupenone, lupeol, lupeol acetate, *β-amyrin, β-amyrin acetate, and lupeol acetate)* were isolated in different species belonging to the genus *Balanophora* J.R.&G. Forst. in previous studies, especially from *B. fungosa* subsp. *indica* (syn. *B. simaoensis*, *B. indica*) with lupenone, lupeol acetate, β-amyrin, β-amyrin acetate, and β-amyron were isolated and identified from this species [7]. Besides, lupeol was isolated from *B. tobiracola* [19]. However, there was no report on isolation or identification of these compounds in *B. fungosa* var. *globosa* and *B. subcupularis*. This is the first time these compounds were identified in these species with the same triterpenoids were identified in GC-MS chromatograms of four species studied by us. Thus, the content of triterpenes is typical to all *Balanophora* species studied by us and other authors, but their different proportions may affect the pharmacological effects of different *Balanophora* species.

Our previous study showed that gallic acid and caffeic acid could be detected in HPTLC chromatograms of the four above-stated species [11]. In this paper, the qualitative HPTLC of coniferyl aldehyde and cinnamic acid showed that there were some differences in their chromatograms with coniferyl aldehyde could be detected in *B. subcupularis* while cinnamic acid could be detected in *B. tobiracola*. The results obtained from GC-MS analyses of four previously mentioned *Balanophora* species are compatible with the HPTLC analysis of *n*-hexane fractions from these species. Thus, the content of triterpenoids is typical to all *Balanophora* species studied by us and other authors [7], but their different proportions may affect the pharmacological effects of different *Balanofora* species.

CONCLUSION

Gallic acid and coniferyl aldehyde were isolated from *B. subcupularis* for the first time, whereas cinnamic acid and caffeic acid were isolated from *B. tobiracola* growing in Vietnam. Besides, six pentacyclic triterpenoids were identified in all chromatograms of four *Balanophora* species but different area percentage. The differences in the proportion of compounds could affect the pharmacological effects of different *Balanophora* species. Among four species, *B. subcupularis* showed weak cytotoxicity against the cancer cell line A549. On the other hand, two taxa of *B. fungosa* (*B. fungosa* subsp. *indica* and *B. fungosa* var. *globosa*) possessed a better inhibitory effect on NO production than two *B. tobiracola* and *B. subcupularis*. Besides, coniferyl aldehyde which is a principal compound of *B. subcupularis* showed cytotoxicity against all four cancer cell lines at a concentration of 100 µg/ml as well as a significant inhibitory effect on NO production.

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