Proceedings of the Estonian Academy of Sciences,
2021, **70**, 1, 40–50
https://doi.org/10.3176/proc.2021.1.01
Available online at www.eap.ee/proceedings

CHEMISTRY

Chemical constituents and biological activities of *Balanophora fungosa* varietas *globosa* growing in Vietnam, as well as comparative chromatography with some species of the genus *Balanophora* J. R. & G. Forst

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Received 6 July 2020, accepted 13 October 2020, available online 18 January 2021

Abstract. The chemical composition and biological activities of *Balanophora fungosa* var. *globosa* (BFG) were studied for the first time. Also, the chemical composition of some other *Balanophora* species was established for comparison. Phenolic compounds isolariciresinol, gallic acid, pinoresinol, methyl caffeate, and epipinoresinol-4-*O*-β-D-glucopyranoside were isolated from *Balanophora fungosa* var. *globosa* collected in Vietnam and identified by the NMR analysis. Some *in vitro* biological activities of the isolated compounds, including the inhibitory effect on NO production and cytotoxic effects, were evaluated. The chromatographic methods were developed to determine the chemical fingerprints of BFG and its very close taxon subsp. *indica* (Arn.) B. Hansen (BFI), also of the two new species recently recorded for the flora of Vietnam *Balanophora tobiracola* Makino (BT) and *Balanophora subcupularis* P.C. Tam (BS). Among the isolated compounds, isolariciresinol showed a moderate inhibitory effect on NO production (I% = 56.02 at concentration of 100 μg/mL), while gallic acid at concentration of 100 μg/mL demonstrated moderate cytotoxicity against cancer cell lines MCF-7 (human breast carcinoma) and PC3 (human prostate gland carcinoma). The HPTLC analysis showed similarities in the chemical compositions of BFG and BFI, as well as the difference between their compositions and these of BT and BS. *O*-caffeoyl-*O*-galloyl-glucoside I, caffeic acid glucoside, *O*-caffeoyl-di-galloyl-β-D-glucoside V, and 1-*O*-caffeoyl-3-*O*-galloyl-4,6-HHDP-β-D-glucoside as principal compounds were identified among 31 phenolic substances of BFI and BFG by using HPLC-MS/MS.

Key words: anti-cancer activity, anti-inflammatory activity, *Balanophora fungosa* subsp. *indica*, *Balanophora subcupularis*, *Balanophora tobiracola*, isolation.

1. INTRODUCTION

Balanophora fungosa var. *globosa* (Jungh.) B. Hansen (BFG) (Fig. 1a), with the Vietnamese name Dó đất sần (Balanophoraceae) is a dioecious parasitic plant, recorded by B. Hansen as an endemic species of Java (Indonesia) and distributed in Java only (Hansen, 1972). Later *B. fungosa* var. *globosa* was recorded in the Checklist of plant species of Vietnam (Ban, 2005) and is one of the two taxa of *B. fungosa* in Vietnam, along with *B. fungosa* subsp. *indica* (BFI) (Fig. 1b). Of these two taxa, BFI has been used in Vietnamese folk medicine to treat some

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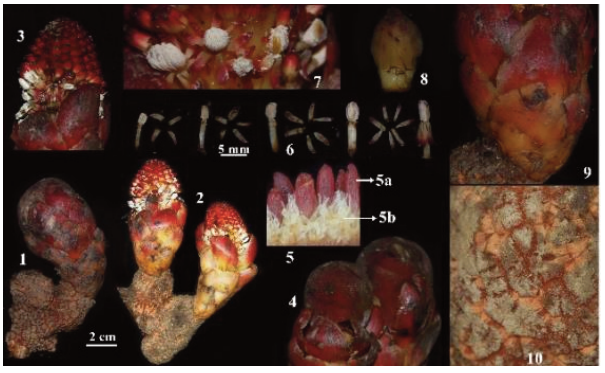
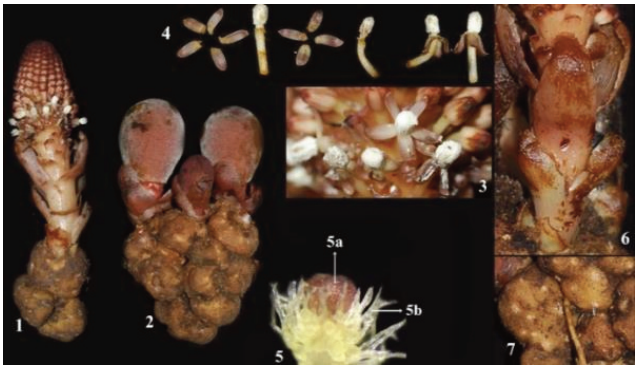
(a)



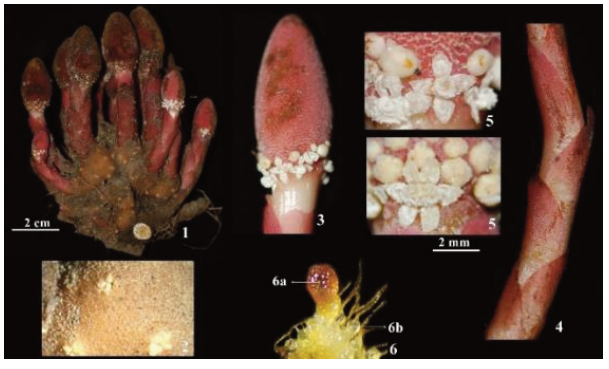
B. fungosa var. *globosa* 1,4. Plant with female inflorescence; 2,3. Plant with male inflorescence; 5. Female flowers (5b) and spadicles (5a); 6,7. Male flowers; 8,9. Leaves; 10. Surface of rhizome.

(b)



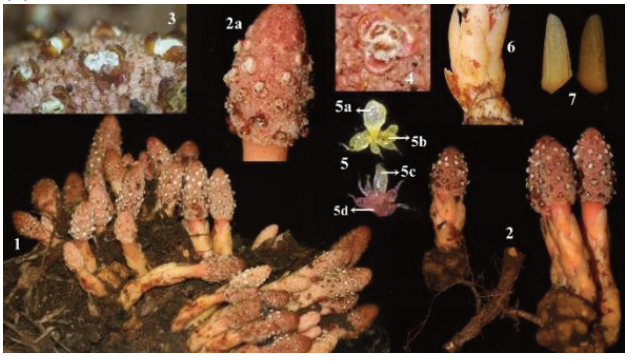
B. fungosa subsp. *indica* 1. Plant with male inflorescence; 2. Plant with female inflorescence; 3,4. Male flowers, 5. Female flowers (5b) and spadicle (5a); 6. Leaves; 7. Surface of rhizome.

(c)



B. subcupularis 1. Plant with androgynous inflorescences; 2. Surface of rhizome; 3. Inflorescence; 4. Scape with leaves; 5. Male flowers; 6. Female flowers (6b) and spadicle (6a).

(d)



B. tobiracola 1,2. Plant with androgynous inflorescence (2a); 3. Surface of inflorescence; 4. Male flower, 5. Female flowers (5b, 5d) and spadicles (5a, 5c); 6. Scape with leaves; 7. Leaves.

Fig. 1. Morphological characteristics of some *Balanophora* species.

diseases, such as abdominal pain and body aches, as well as to be soaked in wine to produce a drink which could help strengthen muscles and bones (Vo, 2012). Recently, *Balanophora subcupularis* P.C. Tam (BS) (Fig. 1c) and *Balanophora tobiracola* Makino (BT) (Fig. 1d) were recorded in the flora of Vietnam.

Phytochemical investigation of species belonging to the genus *Balanophora* resulted in the isolation and identification of various types of compounds, including hydrolysable tannins, phenolic acids, simple phenylpropanoids, lignans, coumarins, and triterpenes (Hou et al., 2009; Wang et al., 2012). There are only a few publications describing chemical constituents of *B. fungosa* (Panthama et al., 2009; Zhou et al., 2019). Some hydrolysable tannins, phenolic acids (gallic acid, ellagic acid), phenylpropanoids (caffeic acid, methyl coniferin, butyl coniferin, brevifolin), pentacyclic triterpenoids (balanophorin A, balanophorin B, monogynol A 3-palmitat, β-amyrin, β-amyrin acetate, β-amyrin stearate and β-amyron) and sterols (daucosterol and β-sitosterol) were isolated from BFI in China (Dai et al., 2005; Fang et al., 2018). Lately, three new muurolene-type sesquiterpene glycosides were isolated from the whole plants of BFI (Bui et al., 2019). The chemical investigation of *B. tobiracola* Makino led to the isolation of six phenylpropanoids (p-methoxycinnamic acid, trans-cinnamic acid, caffeic acid methyl ester, p-coumaric acid, m-coumaric acid, caffeic acid), two triterpenes (β-amyrin acetate, lupeol), two dihydrochalcones (3-hydroxyphloretin, 3-hydroxyphloretin 4'-β-D-glucoside) (Ito et al., 1980), as well as galloyl, caffeoyl and hexahydroxydiphenoyl esters of 3-hydroxyphloretin 4'-β-D-glucoside (Tanaka et al., 2005). The ethyl acetate fractions of BT and BS showed significant xanthine oxidase (XO) inhibitory activity with IC_{50} values of 11.87 and 48.41 μg/mL (Tung et al., 2019). Nevertheless, literature survey indicated lack of published data about the chemical compositions of BFG and BS, although these species have

been used in folk medicine by the local people for a long time.

The aim of this study was the development of HPTLC and HPLC-MS/MS methods to compare the chemical compositions of the aforesaid species, as well as isolation and identification of some compounds from BFG for the first time, along with studying their biological activities.

2. MATERIALS AND METHODS

2.1. Plant material

Whole plants of *Balanophora fungosa* var. *globosa* (Jungh.) B. Hansen (BFG) (12 kg), *B. fungosa* subsp. *indica* (Arn.) B. Hansen (BFI) (10 kg), *B. subcupularis* P.C. Tam (BS) (4 kg), and *B. tobiracola* Makino (BT) (3 kg) were collected (Table 1). The plants were identified by Prof. Phan Ke Loc and MSc Nguyen Anh Duc, Faculty of Biology, VNU University of Science, Vietnam National University, Hanoi, Vietnam (Fig. 1). The voucher specimens (VS) were deposited at the Faculty of Biology, VNU University of Science, Vietnam National University, Hanoi, Vietnam. The plant materials were dried in a ventilated oven and stored in sealed polyethylene bags until analysis.

2.2. Extraction and isolation

The air-dried and powdered whole plant of BFG (3.4 kg) was extracted with methanol (10 litres × 3 times) in a sonic bath for 30 min. The combined extracts were concentrated *in vacuo* to obtain a crude residue (550 g). This crude extract was suspended in water and successively partitioned between *n*-hexane and ethyl acetate to obtain residues from *n*-hexane phase (22.38 g) and ethyl acetate phase (274 g), respectively. The water fraction was evaporated to obtain water-soluble residue.

NMR (^{1}H and ^{13}C) spectra of the isolated compounds were recorded by a Bruker Avance III HD spectrometer. ^{1}H and ^{13}C spectra were measured at 700 MHz and 176 MHz correspondingly. DMSO-d_6, methanol-d_4 and acetone-d_6 were utilized as solvents and their residual peaks (DMSO-d_6: 2.50 ppm for ^{1}H and 39.52 ppm for ^{13}C; methanol-d_4: 3.31 ppm for ^{1}H; acetone-d_6: 2.05 ppm for ^{1}H and 29.84 ppm for ^{13}C) were used for spectrum calibration. Structural assignments were performed by comparing the spectra with literature data.

2.3. Bioassays

2.3.1. NO production in LPS-stimulated RAW264.7 cells

The inhibitory effect of the isolated compounds on the NO production in LPS-stimulated RAW 264.7 cells (Table 1) was evaluated by using the above described method (Dat et al., 2012). RAW 264.7 cells were cultured in DMEM, supplemented with 10% (v/v) FBS, penicillin and streptomycin sulphate with the conditions maintained in a humidified 5% CO_2 and atmosphere at 37 °C. After that, these cells were seeded in a 96-well plate at 2.5×10^5 cells/well. The cells were treated with different concentrations of samples prepared in DMSO, followed by incubation for further 24 h. Nitrite concentration in the culture supernatant was estimated by the Griess method, in which 100 µL of the cultured supernatant was transferred to another 96-well plate before adding 100 µL of Griess reagent. The absorbance of the reaction solution was measured at 570 nm with a microplate reader. In addition, the remaining cell solutions in a 96-well plate were evaluated by measuring 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

Table 1. Information of *Balanophora* species

Id	Scientific names	Collecting time & location	Geographic coordinator	Number of VS
BFG	*Balanophora fungosa* var. *globosa* (Jungh.) B. Hansen	Bidoup-Nuiba National Park, Lam Dong province; January 2016	12°05′23″N 108°27′10″E	HNU 024066
BFI	*B. fungosa* subsp. *Indica* (Arn.) B. Hansen	Sapa city, Lao Cai province; January 2017	22°19′35″N 103°48′21″E	HNU 024069
BS	*B. subcupularis* P.C. Tam	Muong Lay district, Dien Bien province; November 2017	22°03′56″N 103°06′13″E	HNU 024068
BT	*B. tobiracola* Makino	Bac Son district, Lang Son province; January 2018	21°53′33″N 106°22′57″E	HNU 024056

positive control. The inhibitory activity was calculated by the following formula:

$$I\% = \frac{\text{OD (sample)} - \text{OD (blank)}}{\text{OD (LPS)} - \text{OD (blank)}} \times 100.$$

The remaining cell solutions in the cultured 96-well plate were evaluated by measuring cell viability with the MTT assay.

2.3.2. Cytotoxic assays

Cytotoxicity of the isolated compounds (Table 2) against the four cancer cell lines MCF-7, A549, Hep3B, PC3 was estimated by a method described in literature, using MTT cell proliferation assay kits (Scudiere et al., 1988). The cells were cultured in RPMI-1640 or DMEM, supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin sulphate (100 µg/mL) at 37 °C with 5% CO_2. Briefly, cells were seeded (1×10^5 cells/well, in triplicate) in a 96-well flat-bottom plate and incubated overnight. After 24 h, DMSO solutions of the isolated compounds in different concentrations were added to the cells. The treated cells were incubated for 48 h at 37 °C, followed by the MTT assay according to the manufacturer's guidelines. The absorbance (OD; λ = 570 nm) was recorded and a non-linear regression analysis was performed in Excel software to determine the cell survival. In this bioassay, camptothecin (Sigma-Aldrich, St. Louis, Missouri, USA) was used as a positive control:

$$\%\ Cell\ survival\ (CS\%) = \frac{As - Ab}{Ac - Ab} \times 100\,.$$

[*As*: Absorbance of sample; *Ab*: Absorbance of blank; *Ac*: Absorbance of control].

2.4. HPTLC and HPLC-MS/MS analyses

Powdered plant materials (2 g) were extracted with methanol (5 mL × 10 min × 3 times) by sonication. After filtration through a cotton filter, the filtrate was combined and the solvent was evaporated to obtain solid residue, which was kept in refrigerator (2–4 ºC). Before the HPLC analysis, methanol (10 mL) was used to dissolve the residue. After centrifugation at a cooling centrifuge Eppendorf 510R, the supernatants were analysed and stored at –20 ºC.

2.4.1. HPTLC analysis

The HPTLC analyses were performed by means of a HPTLC system including the Applicator CAMAG Linomat 5, the Auto Developing Chamber (ADC-2) and the TLC Visualizer. The same volume (10 µL) of the

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

Samples	Concentration (µg/mL	% Inhibitory	± SD	% Cell survival	± SD
1	30	39.78	0.52	85.02	1.34
	100	**56.02**	0.90	71.19	0.53
2	30	13.73	0.38	75.70	0.04
	100	19.61	0.76	55.83	1.72
3	30	28.29	0.90	74.48	0.42
	100	45.38	0.63	69.61	0.30
4	30	27.45	0.43	70.70	2.07
	100	30.25	0.29	63.42	0.21
5	30	33.61	1.01	79.87	0.87
	100	36.41	0.63	66.40	2.07
Cardamonin*	0.3 µM	30.25	0.38	91.45	0.33
	3 µM	79.83	1.01	82.96	1.37

* Positive control.

methanol extracts of *B. fungosa* var. *globosa*, *B. fungosa* subsp. *indica*, *B. tobiracola* and *B. subcupularis* was applied on the silica gel 60 F_{254} HPTLC plate (10 cm × 10 cm) while the volume of the isolated compound solutions was 5 µL. The HPTLC was developed on the basis of two eluent systems. After development and derivatization with compatible reagents, images of the plates were taken and documented by the TLC Visualizer. The chromatographic evaluations were performed with visionCATs software (Figs 2 and 3).

2.4.2. HPLC-MS/MS analysis

The liquid chromatographic analysis of the extracts was carried out by a 1290 Infinity system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6450 Q-TOF mass spectrometer equipped with a Jetstream ESI source. The separation of compounds was performed by using a reverse-phase HPLC Zorbax 300SB-C_{18} column (2.1 mm × 150 mm; 5 µm; Agilent Technologies) in a stepwise mobile phase gradient of 0.1 % formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 mL/min^{-1} with column temperature of 35 °C. MS/MS detection was performed in negative ion mode in the m/z interval of 50–1000 amu, targeted to 400 amu. The DAD was working at an interval of 200–600 nm and the absorbance of the eluate was continuously monitored at wavelengths of 250, 280, 330, 350, 370, and 590 nm. Phenolic compounds were identified by retention time, mass to charge ratio (*m/z*), UV-vis spectra, and MS fragmentation patterns. Identification was confirmed with commercial standards of quinic acid, caffeic acid, vanillic acid, chlorogenic acid, catechin, kaempferol, myricetin, rutin, quercetin,

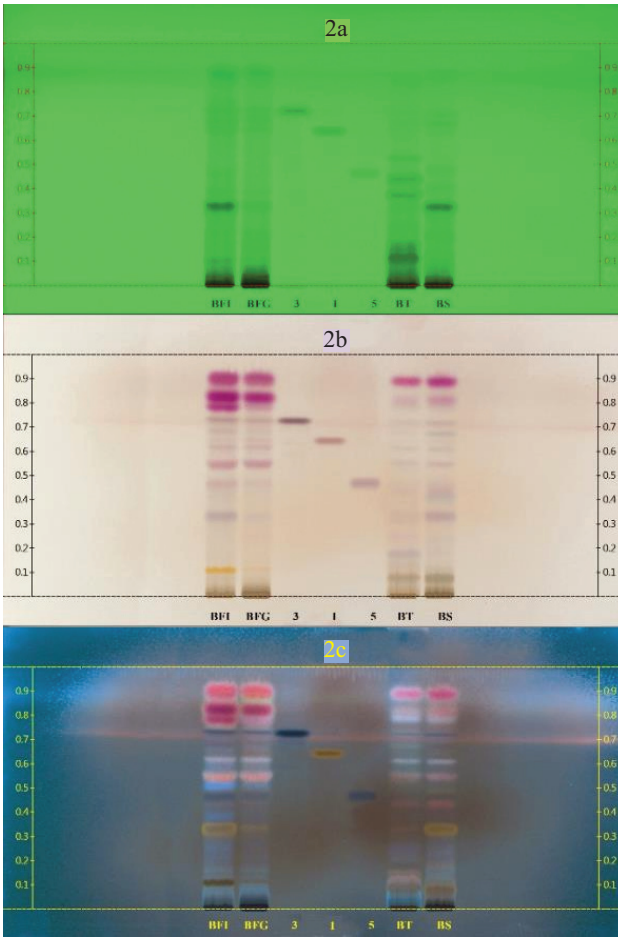


Fig. 2. Thin layer chromatogram developed with the eluent system (1) observed at λ = 254 nm (2a) before derivatization, white light (2b) and λ = 366 nm (2c) after derivatization with anisaldehyde-sulphuric acid reagent (2c).

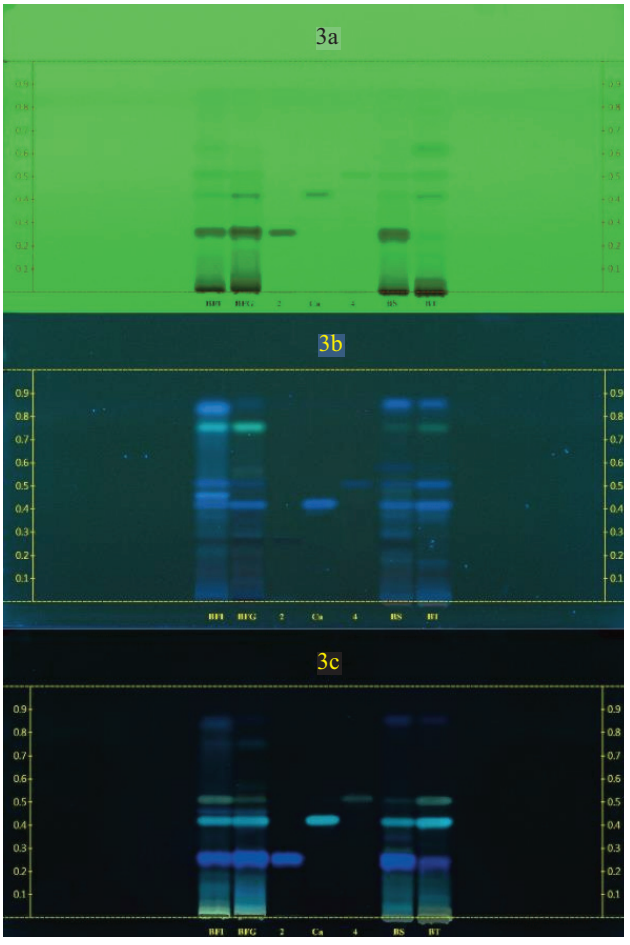


Fig. 3. Thin layer chromatogram developed with the eluent system (2) observed at 254 nm (3a) and ultra violet lamp λ = 366 nm before (3b) and after (3c) derivatization with NP/PEG reagent.

quercetin rhamnoside, quercetin glucoside. External calibration curves were used for quantification of compounds either by UV-absorbance or extracted ion chromatogram (EIC) areas (Table 3). All the details were given in our previous study (Rusalepp et al., 2017).

3. RESULTS AND DISCUSSION

3.1. Isolated compounds

The ethyl acetate residue was purified with column chromatography to obtain five major compounds. Their chemical structures were identified by measuring and analysing NMR spectral data. These compounds were identified as isolariciresinol (**1**) (Eklund et al., 2002), gallic acid (**2**) (Eldahshan, 2011), pinoresinol (**3**) (Deyama et al., 1987), methyl caffeate (**4**) (Xiang et al., 2011) and epipinoresinol-4-*O*-β-D-glucopyranoside (**5**) (El-Domiaty et al., 2002), as illustrated in Fig. 4.

Isolariciresinol: ^{1}H NMR (700 MHz, 25 °C, acetone-d_6): 7.38 (s, 1H), 7.10 (s, 1H), 6.79 – 6.73 (m, 2H), 6.65 (s, 1H), 6.62 (d, J = 7.8 Hz, 1H), 6.19 (s, 1H), 3.99 – 3.94 (m, 1H), 3.86 – 3.83 (m, 1H), 3.82 (d, J = 11.0 Hz, 1H), 3.79 (s, 3H), 3.77 (s, 3H), 3.75 – 3.67 (m, 3H), 3.43 – 3.37 (m, 1H), 2.81 – 2.71 (m, 2H), 2.02 – 1.96 (m, 1H), 1.84 – 1.78 (m, 1H); ^{13}C NMR (176 MHz, 25 °C, acetone-d_6): 148.3, 146.5, 145.8, 145.3, 138.5, 134.0, 128.5, 122.9, 116.9, 115.6, 113.6, 112.0, 65.9, 62.1, 56.3, 56.2, 48.3, 48.1, 40.5, 33.7.

Methyl caffeate: ^{1}H NMR (700 MHz, 25 °C, DMSO-d_6): 9.37 (br s, 2H); 7.48 (d, J = 15.7 Hz, 1H), 7.05 (d, J = 2.1 Hz, 1H), 7.00 (dd, J = 8.3, 2.1 Hz, 1H), 6.76 (d, J = 8.3 Hz, 1H), 6.27 (d, J = 16.2 Hz, 1H), 3.68 (s, 3H); ^{13}C NMR (176 MHz, 25 °C, DMSO-d_6): 167.0, 148.4, 145.6, 145.2, 125.5, 121.4, 115.7, 114.8, 113.7, 51.2.

Pinoresinol: ^{1}H NMR (700 MHz, 25 °C, DMSO-d_6): 8.89 (s, 2H), 6.89 (d, J = 1.5 Hz, 2H), 6.77 – 6.71 (m, 4H), 4.61 (d, J = 4.4 Hz, 2H), 4.14 – 4.10 (m, 2H), 3.76 (s, 6H), 3.72 (dd, J = 9.1, 3.7 Hz, 2H), 3.05 – 3.01 (m, 2H); ^{13}C NMR (176 MHz, 25 °C, DMSO-d_6): 147.5, 145.9, 132.2, 118.6, 115.1, 110.4, 85.1, 70.9, 55.6, 53.6.

Gallic acid: ^{1}H NMR (700 MHz, 25 °C, DMSO-d_6): 12.12 (br s, 1H), 9.14 (br s, 3H), 6.91 (s, 2H); ^{13}C NMR (176 MHz, 25 °C, DMSO-d_6): 167.5, 145.4, 137.9, 120.5, 108.7.

Epipinoresinol-4-O-β-D-glucopyranoside: ^{1}H NMR (700 MHz, 25 °C, MeOD): 7.15 (d, J = 8.2 Hz, 1H), 7.03 (d, J = 1.9 Hz, 1H), 6.98 (d, J = 1.6 Hz, 1H), 6.93 (dd, J = 8.3,

Table 3. Cytotoxicity of the isolated compounds

Samples	Concentration (μg/mL	% Cell survival (CS%)							
		MCF-7		A549		Hep3B		PC3	
		CS%	± SD	CS%	± SD	CS%	± SD	CS%	± SD
1	30	70.26	1.09	74.71	1.33	77.89	1.80	76.35	2.59
	100	59.04	2.20	69.61	2.41	57.97	1.48	63.15	1.28
2	30	71.13	2.09	68.25	3.31	74.46	2.08	66.22	2.54
	100	**35.82**	2.70	50.45	2.14	53.13	2.33	**43.30**	1.42
3	30	69.51	2.43	63.63	2.39	80.25	0.27	66.13	0.08
	100	62.40	1.47	55.96	2.22	57.23	1.65	61.36	0.25
4	30	87.32	1.40	71.53	1.02	73.72	1.40	82.80	2.28
	100	72.55	0.96	64.04	1.21	61.81	0.52	74.78	2.41
5	30	69.91	2.17	60.66	4.25	78.47	1.15	74.41	1.99
	100	63.59	2.13	62.80	8.64	63.73	2.39	66.50	1.37
Camptothecin*	0.1 μg/mL	54.46	2.37	50.05	2.10	71.10	1.23	53.81	0.14
	10 μg/mL	19.87	1.97	26.52	0.86	26.04	2.19	12.30	0.62

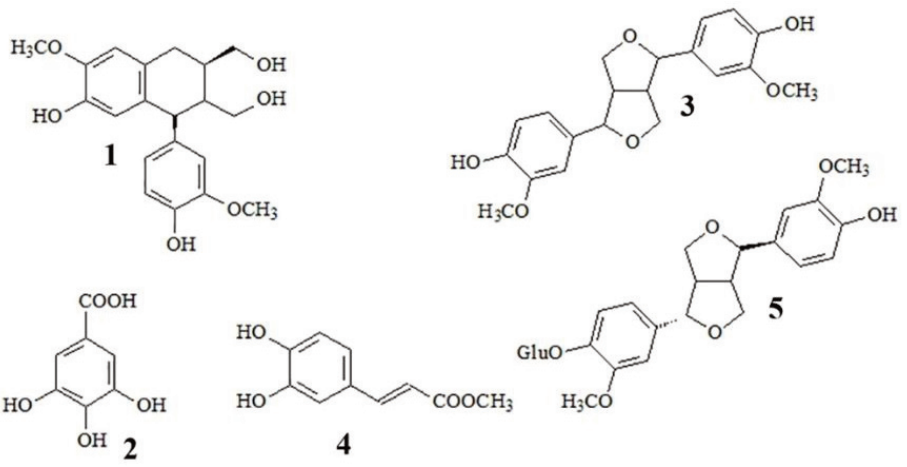


Fig. 4. Isolated compounds from the whole plant of *Balanophora fungosa* var. *globosa*: **1** – isolariciresinol, **2** – gallic acid, **3** – pinoresinol, **4** – methyl caffeate, **5** – epipinoresinol-4-*O*-β-D-glucopyranoside.

1.9 Hz, 1H), 6.82 – 6.77 (m, 2H), 4.89 (d, *J* = 7.5 Hz, 1H), 4.86 (d, *J* = 6.2 Hz, 1H), 4.48 (d, *J* = 6.9 Hz, 1H), 4.14 (d, *J* = 9.2 Hz, 1H), 3.88 – 3.84 (m, 8H), 3.80 (t, *J* = 8.7 Hz, 1H), 3.71 – 3.67 (m, 1H), 3.51 – 3.44 (m, 2H), 3.42 – 3.36 (m, 3H), 2.96 – 2.91 (m, 1H).

3.2. Bioassay

3.2.1. NO production in LPS-stimulated RAW264.7 cells

Isolariciresinol showed moderate inhibitory effect on NO production, whereas the inhibitory ability at concentration of 100 µg/mL was 56.02% (Table 2). Other separated compounds indicated lower activity (13.7–45.4%). Cardamonin used as a positive control showed inhibitory activity of 30.3% and 79.8% at concentrations of 0.3 µM and 3 µM, respectively. Isolariciresinol has been reported to modulate the production of inflammatory mediators through the attenuation of NF-κB transcription signalling (Chiou et al., 2011).

3.2.2. Cytotoxic assays

Gallic acid (GA) indicated moderate cytotoxicity against cancer cell lines MCF-7 (human breast carcinoma) and PC3 (human prostate gland carcinoma) at concentration of 100 µg/mL with the cell survival (CS%) values of 35.8% and 43.3%, respectively (Table 3). Gallic acid showed weak cytotoxicity against two other cancer cell lines A549 and Hep3B. The other studied compounds demonstrated weak cytotoxicity against all the four studied cell lines. Camptothecin used as a positive control showed significant cytotoxicity (12.3–26.5%) at concentration of 10 µg/mL (Table 2). Relevant literature has revealed that GA is able to suppress cell growth and induce apoptosis in PC3 cells in prostate cancer in the range of IC50 concentrations (35 µM) (Saffari-Chaleshtori et al., 2017), while the half-maximal inhibitory concentration value for gallic acid against MCF-7 cells was 18 µg/mL (Rezaei-Seresht et al., 2019).

3.3. HPTLC and HPLC-MS/MS analyses

3.3.1. HPTLC analysis

The chromatogram developed on the basis of the eluent system (1): chloroform–toluene–methanol–25% aqueous ammonia (10:3:6:1) (v/v/v/v) (Fig. 2) showed that all three lignans (**1**, **3**, **5**), isolated from BFG (R_f = 0.64, 0.73 and 0.47, respectively), had equivalent tracks in the chromatogram of BFI. There were nine equivalent tracks in the chromatograms of BFI and BFG, suggesting a similar chemical composition of these two taxa. There were five similar tracks in chromatograms of all the four samples (BFI, BFG, BT, BS) with R_f values of 0.33, 0.54, 0.62, 0.73, 0.82, and 0.90. Among them, the tracks with R_f 0.73 could be attributed to pinoresinol (**3**) (R_f = 0.73). However, isolariciresinol (**1**) (R_f = 0.65) and epipinoresinol-4-*O*-β-D-glucopyranoside (**5**) (R_f = 0.47) could not be detected in the chromatograms of BT and BS (Table 4). Also, the chromatograms of BT and BS possessed some equivalent tracks with R_f values of 0.08 and 0.43, which could not be detected in the chromatograms of BFI and BFG (Table 4). Generally, the chromatograms developed on the basis of the eluent system (1) showed that pinoresinol (**3**) could be detected in the chromatograms of all the four samples while isolariciresinol (**1**) and epipinoresinol-4-*O*-β-D-glucopyranoside (**5**) could be detected only in BT and BS. The chromatographic data also suggested that the two very

Table 4. Evaluation of the HPTLC fingerprinting profile of the methanol extracts of *Balanophora* species, developed with the eluent system chloroform–toluene–methanol–25% aqueous ammonia (10:3:6:1) and observed at white light after derivatization with anisaldehyde-sulphuric acid reagent

R_f values	Area percentage (%)				Substances assigned
	BFI	BFG	BT	BS	
0.08 ± 0.03	–	–	8.24	6.93	
0.11 ± 0.02	**3.48**	**0.80**	–	–	
0.13 ± 0.02	–	–	0.63	–	
0.18 ± 0.03	–	–	–	1.00	
0.33 ± 0.07	**6.79**	**4.01**	**9.85**	**12.14**	
0.43 ± 0.02	–	–	6.49	14.97	
0.46 ± 0.04	**5.12**	**3.28**	–	–	Epipinoresinol-4-*O*-β-D glucopyranoside (5)
0.54 ± 0.04	**6.73**	**8.00**	**4.04**	**3.06**	
0.62 ± 0.04	**3.76**	**4.07**	**2.15**	**2.18**	
0.65 ± 0.01	**1.49**	**2.73**	–	–	Isolariciresinol (1)
0.69 ± 0.03	4.04	–	1.47	4.32	
0.73 ± 0.03	**8.19**	**9.26**	**6.31**	**5.77**	Pinoresinol (3)
0.79 ± 0.03	12.26	–	–	–	
0.82 ± 0.03	23.84	35.73	17.10	16.78	
0.90 ± 0.04	23.52	30.23	37.18	31.56	

close taxa of *B. fungosa* (BFI and BFG) have a similar chemical composition.

Furthermore, the chromatogram developed by using the eluent system (2): toluene–ethyl acetate–formic acid (14:10:1) (v/v/v) (Fig. 3) showed that the tracks equivalent to gallic acid (**2**) (R_f = 0.25), methyl caffeate (**4**) (R_f = 0.42) and caffeic acid (Ca) (R_f = 0.51) could be detected in the chromatograms of all the four samples. However, the difference in the intensities of these tracks in the chromatogram could suggest the difference in the content of each compound in different samples. Also, both the chromatograms of BFG and BFI possessed a track with R_f value of 0.45 and blue inflorescence which could not be detected in the chromatograms of BT and BS.

3.3.2. HPLC-MS/MS analysis

Thirty-one compounds were identified in the methanolic extracts of the both taxa, whereas the major phenolic compounds were *O*-caffeoyl-*O*-galloyl-glucoside isomer I, caffeic acid glucoside, *O*-caffeoyl-di-galloyl-β-D-glucoside isomer V, and 1-*O*-caffeoyl-3-*O*-galloyl-4,6-HHDP-β-D-glucoside (Table 5).

Generally, this is the first time that the chemical composition of *B. fungosa* var. *globosa*, a very rare plant in Vietnam, was investigated. The obtained chromatographic data suggested a similar chemical composition between *B. fungosa* var. *globosa* and its very close taxon *B. fungosa* subsp. *indica,* which in turn is a widely distributed species in Vietnam. What is more, it is the first time the HPTLC analyses of the four species *B. fungosa* subsp. *indica*, *B. fungosa* var. *globosa*, *B. tobiracola* and *B. subcupularis* were developed and evaluated. This could provide useful data for the identification and quality control of these *Balanophora* species. The moderate inhibitory effect of isolariciresinol on NO production may render anti-inflammatory activity to extracts from *Balanophora* species.

Table 5. Characterization of HPLC chromatograms of the methanolic extracts of BFG and BFI

tR, min	Compound	M-H$^-$	Fragment ions
0.6	Gallic acid glucoside	331	313; 271; 211; 193; 169; 125
1.8	Gallic acid	169	125
3.2	Gallic acid glucoside	331	271; 169
5.6–9.9	Coniferin (several isomers)	341	323; 223; 203; 179
7.6	Caffeic acid glucoside	341	323; 233; 203; 179; 135
11.3	Caffeic acid glucoside	341	179; 161; 135
12.6	di-*O*-galloyl-β-D-glucoside isomer I	483	423; 313; 271; 211; 193; 169
13.7	di-*O*-galloyl-β-D-glucoside isomer II	483	313; 271; 211; 193; 169
13.8	p-coumaric acid glucoside	325	307; 281; 195; 187; 163; 145; 119
16.4	Derivative of coniferaldehyde	385	177; 162; 149; 133
18.1	*O*-caffeoyl-*O*-galloyl-glucoside isomer I	493	331; 313; 271; 179; 169
20.1	*O*-caffeoyl-*O*-galloyl-glucoside isomer II	493	475; 433; 313; 271; 241; 169
20.1	*O*-caffeoyl-di-galloyl-β-D-glucoside isomer I	645	627; 585; 493; 423; 331; 271; 193
21.2	*O*-caffeoyl-*O*-galloyl-glucoside isomer III	493	331; 313; 271; 211; 169
21.9	*O*-caffeoyl-di-galloyl-β-D-glucoside isomer II	645	627; 493; 483; 465; 423; 301; 271
21.9	Lariciresinol-glucoside	521	491; 359; 329; 299; 161
23.1	1-*O*-caffeoyl-4,6-HHDP-β-D-glucoside	643	301
23.3	Methyl caffeate	193	178; 161; 134
23.5	Lariciresinol	359	344; 313; 241; 203; 189; 159; 109
24.6	1,3-di-*O*-caffeoyl-β-D-glucoside	503	341; 323; 281; 233; 203; 179; 161
23.8	*O*-caffeoyl-di-galloyl-β-D-glucoside isomer III	645	475; 313
25.0	Epipinoresinol-glucoside	519	357; 311; 151
25.1	*O*-caffeoyl-di-galloyl-β-D-glucoside isomer IV	645	493; 483; 465; 423; 313; 271; 211
25.7	1-*O*-*p*-coumaryl-4,6-HHDP-β-D-glucoside	627	463; 301
26.1	1-*O*-caffeoyl-3-*O*-galloyl-4,6-HHDP-β-D-glucoside	795	751; 589; 493; 419; 301; 275; 249
26.4	*O*-caffeoyl-di-galloyl-β-D-glucoside isomer V	645	493; 483; 465; 423; 313; 271

Continued on the next page

Table 5. *Continued*

tR, min	Compound	M-H$^-$	Fragment ions
27.3	Isolariciresinol	359	329; 299; 256; 189; 159
27.4	2-*O*-caffeoyl-1-*p*-coumaryl-β-D-glucoside	487	469; 341; 323; 233; 203; 179; 161; 135
28.8	6-*O*-caffeoyl-1,3,4-tri-*O*-galloyl-β-D-glucoside	797	645; 627; 475; 295
29.2	1,3-di-*O*-caffeoyl-4,6-HHDP-β-D-glucoside	805	643; 503; 301; 275
31.2	Pinoresinol	357	342; 327; 311; 151; 136

4. CONCLUSIONS

Phytochemical studies of Vietnamese *B. fungosa* var. *globosa* led to the isolation of the five earlier known compounds named isolariciresinol (**1**), gallic acid (**2**), pinoresinol (**3**), methyl caffeate (**4**) and epipinoresinol-4-*O*-β-D-glucopyranoside (**5**). Isolariciresinol indicated moderate inhibitory effect on NO production, while gallic acid at the concentration of 100 μg/mL demonstrated moderate cytotoxicity against two cancer cell lines MCF-7 and PC3. Gallic acid (**2**), pinoresinol (**3**), methyl caffeate (**4**) and caffeic acid could be detected in all the chromatograms of the four samples by using the HPTLC analyses. The chromatographic data of the methanol extracts of BFG and BFI showed high similarity in the chemical composition of these two taxa. The HPLC analyses led to the identification of thirty-one compounds in the methanolic extracts of the both taxa with *O*-caffeoyl-*O*-galloyl-glucoside isomer I, caffeic acid glucoside, *O*-caffeoyl-di-galloyl-β-D-glucoside isomer V, and 1-*O*-caffeoyl-3-*O*-galloyl-4,6-HHDP-β-D-glucoside being the main phenolic compounds.

ACKNOWLEDGEMENTS

This work was supported by Vietnam Academy of Science and Technology (VAST) through the project VAST.CTG.04/16-17. The publication costs of this article were covered by the Estonian Academy of Sciences.

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Vietnamis kasvava *Balanophora fungosa* varietas *globosa* koostisained ja nende bioloogiline aktiivsus ning võrdlev kromatograafiline analüüs mõnede perekonna *Balanophora* J.R. & G. Forst liikidega

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Vietnamis kasvava taime *Balanophora fungosa* varietas *globosa* keemilist koostist ja bioloogilist aktiivsust uuriti selles töös esmakordselt. Samuti võrreldi mõnede *Balanophora* perekonna liikide keemilist koostist. Fenoolsed ühendid isolaritsiresinool (**1**), gallushape (**2**), pinoresinool (**3**), metüülkafeaat (**4**) ja epipinoresinool-4-*O*-β-D-glukopüranosiid (**5**) isoleeriti ning identifitseeriti NMR-analüüsi tulemusel Vietnamist kogutud *B. fungosa* varietas *globosa* droogiproovi põhjal. Isoleeritud koostisainete mõned toimed, nagu NO produktsiooni inhibeeriv ja rakutoksiline efekt, tehti kindlaks *in vitro.* Töötati välja kromatograafilised meetodid *B. fungosa* varietas *globosa* ja sellele väga lähedase taksoni *B. fungosa* subsp. *indica* (Arn.) B. Hansen, aga ka Vietnamist hiljuti avastatud kahe uue liigi *Balanophora tobiracola* Makino ja *Balanophora subcupularis* P.C. Tam (BS) omavaheliseks eristamiseks. Isoleeritud komponentidest näitas isolaritsiresinool keskmise tasemega NO produktsioon I inhibeerivat efekti (I% = 56,02 kontsentratsioonil 100 µg/mL), samas oli gallushappel kontsentratsiooni 100 µg/mL korral keskmine rakutoksiline aktiivsus inimese rinnakartsinoomi rakkudesse (MCF-7) ja eesnäärmekartsinoomi rakkudesse (PC3). HPTLC-analüüs näitas sarnasust *B. fungosa* varietas *globosa* ja *B. fungosa* subsp. *indica* keemilise koostise vahel, mis aga oli erinev *B. tobiracola* ja *B. subcupularis*'e fütokeemilise profiili puhul. Kahes sarnase koostisega taksonis identifitseeriti HPLC-MS/MS-meetodil 31 fenoolset ühendit, millest peamised on *O*-kafeoüül-*O*-galloüülglükosiid I, kohvhappeglükosiid, *O*-kafeoüüldigalloüül-β-D-glükosiid V ja 1-*O*-kafeoüül-3-*O*-galloüül-4,6-HHDP-β-D-glükosiid.