Triterpenoids isolated from the aerial parts of *Buxus latistyla* Gagnep.

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Abstract

Background: *Buxus latistyla* Gagnep. (Buxaceae) has been used as a remedy for treating malaria, haemoptysis, amoebic dysentery etc. However, its chemical constituents are still unknown. Thus, the aim of this study was to identify phytochemicals isolated from this plant. **Materials and method:** Aerial parts of *B. latistyla* were collected from Quang Tri province. Pure compounds were isolated using the combination of chromatographic methods. Structures of isolated compounds were identified by analyzing spectral data of nuclear magnetic resonance as well as by comparing with reported data. **Results & Conclusion:** Five triterpenes were isolated and identified including lupeol (1), lupenone (2), betulin (3), 20(29)-lupene-2α,3α-diol (4), 20(29)-lupene-2α,3α ,28-triol (5). This is the first report about those phytochemicals of *B. latistyla* collected in Vietnam.

Key words: *Buxus latistyla, lupeol, lupenone, betulin, 20(29)-lupene-2α,3α-diol, 20(29)-lupene-2α,3α, 28-triol.*

1. INTRODUCTION

The *Buxus* genus is a part of the Buxaceae family and comprises roughly 70 species of evergreen shrubs. They are mainly found in North America and Eurasia, with their distribution spanning regions such as Pakistan, Turkey, China and Viet Nam [1, 2]. Most *Buxus* species are known to contain a diverse array of chemical compounds, including alkaloids, triterpenoids, steroids, and various others [3 - 5]. This extensive range of compounds has traditionally found application in folk medicine for treating a wide spectrum of ailments such as malaria, tuberculosis, HIV, cancer, skin infections, rheumatism, heart disorders, depression, and fatigue [6, 7].

Within the genus *Buxus*, the species known as *Buxus latistyla* Gagnep. has been identified in various locations across Vietnam, including Nam Dinh, Thanh Hoa and Quang Tri. Traditionally, this plant has been employed by local communities for the treatment of heart ailments, malaria, hemoptysis, amoebic dysentery and edema. However, comprehensive knowledge regarding the chemical composition and biological activity of this species, both within the country and globally, remains incompletes. Consequently, our research aim to elucidate phytochemicals from this plant to contribute to the botanical database of the *Buxus* genus in Vietnam.

2. MATERIALS AND METHODS

2.1. Materials

The research subjects were the aerial parts of the *Buxus latistyla* Gagnep. collected in Quang Tri province in August 2020. The scientific name was determined and confirmed with the support of Dr. Anh Tuan Le (Mien Trung Institute for Scientific Research, Vietnam National Museum of Nature, VAST, Vietnam). Specimen voucher (DHYD-QT-01) is preserved at the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam.

2.2. Methods

Crude extract was carried out through room temperature soaking using methanol. Liquid-liquid extraction method was employed to yield subfractions. The isolation of pure compounds was accomplished using a combination of silica gel column chromatography (Silica gel 60, 0.040-0.063mm, 230-400 mesh ASTM, Merck); reversed-phase silica gel (YMC, 30-50 µm, FuJi Silysia Chemical Ltd.); Diaion HP-20, and Sephadex LH-20 (Merck). The isolation processes were monitored using thin-layer chromatography (TLC) with normal-phase (Merck, Kieselgel 60 F254, 250 µm) and/or reversed-phase (Merck, RP C-18 F254). Compound visualization was performed under ultraviolet (UV) light at wavelengths of 254 nm and 366 nm or by using 10% sulfuric acid solution followed by heating. Structural determination of isolated compounds relied on various spectroscopic techniques, including one-dimensional nuclear magnetic resonance (NMR) spectroscopy (^{1}H NMR, ^{13}C NMR) and two-dimensional NMR experiments (HMBC, HSQC) measured on a Bruker Avance AM500 FT-NMR or Bruker AvanceNeo 600MHz.

3. RESULTS AND DISCUSSION

3.1. Isolation

A total of 3 kg of *Buxus latistyla* plant material was subjected to room temperature maceration using methanol (M) (8 L × 2 times). The combined

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extracts were then concentrated under reduced pressure at a temperature of 50-60°C, yielding a total methanol extract (540 g). This extract was subsequently partitioned with water, followed by liquid-liquid extraction successively with *n*-hexane (H) (1 L × 7 times), dichloromethane (D) (1 L × 7 times), and ethyl acetate (E) (1 L × 7 times). Solvent evaporation under reduced pressure resulted in the corresponding extracts: BLH (80 g), BLD (90 g), BLE (20 g), and the remaining water fraction, BLW (350 g).

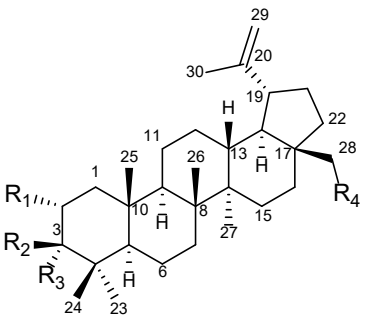
The BLH extract (80 g) was subjected to solid-phase extraction using normal-phase (NP) silica gel, eluted with a gradient solvent system of *n*-hexane:acetone (H:A) (100:0, 60:1, 30:1, 15:1, 7.5:1, 3:1, 1:1, and 0:100, v/v, 1 L each), and finally with methanol (M) to yield nine fractions (BLH1-BLH9). Fraction BLH2 (7.9 g) displayed crystalline characteristics, underwent recrystallization, and provided compound **1** (300 mg). Fraction BLH4 (11 g) was subjected to solid-phase extraction using NP silica gel and was eluted with a gradient solvent system of H:A (100:0, 40:1, 20:1, 10:1, 5:1, 3:1, 1:1, and 0:100, v/v, 500 mL each), yielding eight subfractions from BLH4A to BLH4H. Subfraction BLH4C (2.78 g) was combined with BLH4D and further purified on a silica gel column eluted with H:A (25/1) solvent system, resulting in eight subfractions (BLH4C1–BLH4C8). Subfraction BLH4C1 (0.55 g) was then subjected to another NP silica gel column using H:A (40:1), leading to nine subfractions (BLH4C1A-BLH4C1I). BLH4C1C (186.5 mg) was further purified on a NP silica gel column using H:E:A (40:0.5:0.1), providing eight subfractions (BLH4C1C1-BLH4C1C8). Lastly, BLH4C1C4 underwent reversed-phase chromatography with the solvent system A:M:W (1:1:0.1) on an RP column, yielding compound **2** (23 mg).

The BLD extract (90 g) was subjected to solid-phase extraction using NP silica gel and was eluted with a gradient solvent system of D:M (100:0, 60:1, 30:1, 15:1, 7.5:1, 3:1, 1:1, and 0:100, v/v, 1 L each), yielding eight fractions from BLD1 to BLD8. Fraction BLD1 (16.2 g) was subjected to Sephadex LH-20 column chromatography using D:M (1:1, v/v) to remove chlorophyll. and was further subjected to solid-phase extraction using NP silica gel, eluted with a gradient solvent system of H:A (100:0, 40:1, 20:1, 10:1, 5:1, 3:1, 1:1, and 0:100, v/v, 500 mL each), yielding eight subfractions from BLD1A to BLD1H. From subfraction BLD1E (2.9 g), crystal isolation was performed, resulting in BLD1EX crystals and BLD1E mother liquor. Subfraction BLD1EX was chromatographed on a NP silica gel column with the solvent system D:E (30:1), yielding compound **3** (107 mg). BLD1E and BLD1F were combined as BLD1F. Fraction BLD1F was subjected to Sephadex LH-20 column chromatography, eluted with methanol (M), resulting in three subfractions: BLD1F1, BLD1F2, and BLD1F3. Subfraction BLD1F2 (750 mg) was further purified through NP columns using D:E (25:1, v/v) as an eluent, leading to the isolation of compound **4** (15.8 mg).

The BLE extract (20 g) underwent solid-phase extraction using NP silica gel, following a similar procedure as for the BLD extract, with the solvent system D:M according to a gradient of concentrations (v/v, 1 L each), yielding eight fractions from BLE1 to BLE8. Fraction BLE1 (14.5 g) was dissolved in a minimal amount of solvent and subjected to Sephadex LH-20 column chromatography, eluted with a mixture of D:M (1:1, v/v), which resulted in seven subfractions (BLE1A-BLE1J). BLE1F (2.9 g) was further subjected to Sephadex column chromatography with D:M (1:1, v/v), yielding three subfractions (BLE1F1-BLE1F3). BLE1F2 (750 mg) was subsequently chromatographed on a NP silica gel column using H:E:M (6:1:0.1, v/v/v), providing nine subfractions (BLE1F2A-BLE1F2I). Compound **5** (9 mg) was yielded from the crystallization process of BLE1F2I.

3.2. Structural determination of isolated compounds

Based on experimental data combined with reference to published literature, the chemical structures of the isolated compounds have been determined as (**1**) lupeol, (**2**) lupenone, (**3**) betulin, (**4**) 20(29)-lupene-2α,3α-diol, **(5**) 20(29)-lupene-2α,3α,28-triol.



	R_1	R_2	R_3	R_4
1	-H	-OH	-H	-H
2	-H	=O		-H
3	-H	-OH	-H	-OH
4	-OH	-H	-OH	-H
5	-OH	-H	-OH	-OH

Figure 1. Chemical structures of isolated compounds (**1**-**5**).

Compound **3** was obtained as a white powder. Its nuclear magnetic resonance (NMR) spectroscopy, specifically the 1H NMR spectrum, exhibited characteristic signals of a triterpene compound. The ^{1}H NMR spectrum showed the presence of an oxymethine proton signal at δ_H 3.19 (dd; *J* = 11.4; 4.8 Hz), two oxymethylene protons at δ_H 3.80 (dd; *J* = 10.8; 1.8 Hz) and δ_H 3.34 (d; *J* = 10.8 Hz), and two doublet methylene protons at δ_H 4.68 and 4.58 (d, *J* = 1.8 Hz each). Additionally, six singlet methyl groups were observed at δ_H 1.68, 1.02, 0.98, 0.96, 0.82, and 0.76 ppm. The ^{13}C NMR spectrum displayed signals for thirty carbons, including double bond signals at δ_C 150.5 (C-20) and 109.7 (C-29) ppm corresponding to the isopropenyl group. Oxymethine and oxymethylene carbon signals were observed at δ_C 79.0 (C-3) and 60.6 (C-28) ppm, respectively. The six methyl group signals were observed at δ_C 28.0 (C-23), 19.1 (C-30), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), and 14.8 (C-27) ppm. Proton-carbon connectivity was established through the analysis of 2D NMR spectra, including the heteronuclear single quantum coherence (HSQC) spectrum.

Further analysis using the heteronuclear multiple bond correlation (HMBC) spectrum confirmed the presence of an oxymethylene group attached to C-17, with interactions between H-28 (δ_H 3.80; 3.34) and C-16 (δC 29.2)/ C-17 (δC 47.8)/ C-22 (δC 34.0). The presence of the isopropenyl branch was affirmed through interactions between H-30 (δ_H 1.68) and C-29 (δ_C 109.7)/ C-20 (150.5), as well as between H-29 (δ_H 4.68; 4.58) and C-19 (47.81)/ C-20/ C-30. Additionally, the interaction between H-19 (δ_H 2.38) with C-20/ C-29/ C-30 and with C-13 (δ_C 37.4)/ C-18 (48.8)/ C-21 (29.8) confirmed the attachment position of the isopropenyl group at C-19. Detailed analysis of the HMBC interactions involving the oxymethine proton and the remaining methyl singlet groups further confirmed the presence of an OH group at C-3 and the positions of the methyl groups C-23/C-24. Furthermore, the proton signal H-3 with a large coupling constant (11.4; 4.8 Hz) indicated its axial orientation, demonstrating that the OH group at C-3 had an equatorial orientation, thus corresponding to the 3β-hydroxy configuration. Combining this analysis with the NMR spectroscopic data and comparing them with published literature, compound **3** was identified as **betulin** [8].

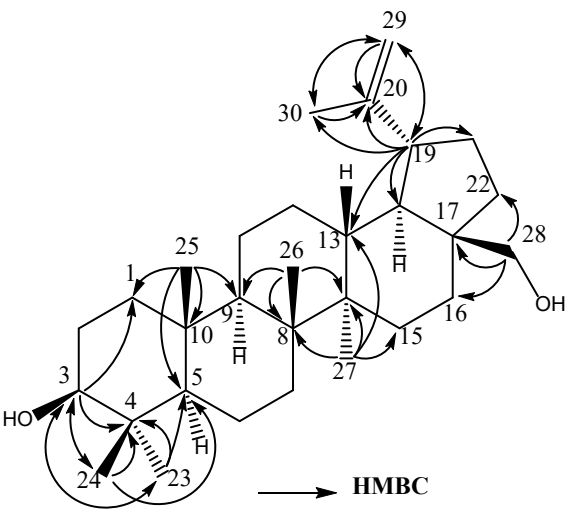


Figure 2. Key HMBC (^{1}H → ^{13}C) of compound **3**

Compound **1** was obtained as white crystals. Its NMR spectra resembled that of compound **3**, except for the presence of a methyl singlet at δ_H 0.79 (s, 3H) corresponding to δ_C 18.0 instead of the oxymethylene group. Comparing these findings with reference literature, this compound was identified as **lupeol** [9, 10].

Compound **2** was obtained as a white powder. Its NMR spectra also exhibited similarities to compound **1**, but the key difference was the presence of a ketone group at δ_C 218.2 ppm instead of the oxymethine group seen in compound **1**. This compound was identified as **lupenone** [11].

Compounds **4** and **5** were also obtained as white powders. The NMR data for compound **4** showed differences from compound **1**, with the presence of two oxymethine groups at δ_H 3.41 (d, *J* = 2.4 Hz, H-3β)/ δC 79.0 (C-3) and δ_H 3.97 (ddd, *J* = 11.4; 3.6; 2.4 Hz, H-2β) corresponding to positions C-2 and C-3. Beside the similarities with compound **4**,

the NMR spectra of compound **5**, displayed signals for an oxymethylene group at δ_H 3.34 (d, *J* = 10.8 Hz, H-28a) and 3.79 (d, *J* = 10.8 Hz, H-28b) ppm corresponding to δ_C 60.6 (C-28) ppm. Detailed analysis of 2D NMR spectra, along with comparisons to those of reference data, confirmed compound **4** as **20(29)-lupene-2α,3α-diol** [12] and compound **5** as **20(29)-lupene-2α,3α,28-triol** [13]. Data of ^{13}C NMR and ^{1}H NMR of isolated compounds were summarized in **Table 1** and **2**.

Lupeol (**1**) has demonstrated effectiveness in reducing the effects of aflatoxin B1, a compound produced by *Aspergillus flavus* fungi known to be a causative agent of liver inflammation and liver cancer [14]. Both lupeol (**1**) and lupenone (**2**) have the ability to selectively and non-competitively inhibit the PTP1B enzyme, showing potential in the treatment of insulin resistance related to diabetes [15]. These two compounds also exhibit anti-inflammatory potential by inhibiting iNOS and COX-2 in RAW 264.7 cells stimulated by LPS [16].

Betulin (**3**) has been shown to have cytotoxic effects on various cancer cell lines, including ovarian cancer, cervical cancer, and glioblastoma, with IC_{50} values ranging from 2.8 to 3.4 µM. It also has lipid-lowering effects in vivo, improving obesity-related conditions caused by dietary habits. Betulin is effective in reducing seizures in mice induced by bicuculline and has anti-inflammatory effects by blocking the tyrosyl phosphorylation process of the 45 kDa protein in human neutrophils. Additionally, betulin can protect the liver from acute damage caused by lipopolysaccharide/D-galactosamine (LPS/D-Gal) and reduce the toxicity of *Streptococcus pneumoniae* in pneumococcal infections [17].

From the studies on these compounds, it is evident that *Buxus latistyla* Gagnep. has the potential to be a valuable plant species for the discovery of bioactive chemical constituents with potential applications in pharmaceuticals or functional foods in the future.

4. CONCLUSION

The current study has identified five known pentacyclic triterpenes including (**1**) lupeol, (**2**) lupenone, (**3**) betulin, (**4**) 20(29)-lupene-2α,3α-diol, **(5**) 20(29)-lupene-2α,3α,28-triol. However, this is the first time these compounds have been isolated from *Buxus latistyla* collected in Vietnam. Among them, three compounds (**1**-**3**) have shown significant biological activities in the literature.

Acknowledgment

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Table 1. ^{13}C NMR data of isolated compounds (**1**-**5**)

Position	δ_C				
	1[b]	**2**[a]	**3**[a]	**4**[a]	**5**[a]
1	38.7	39.6	38.7	42.2	42.2
2	27.5	34.2	27.4	66.7	66.6
3	79.0	218.2	79.0	79.0	79.0
4	38.9	47.4	38.9	38.4	38.3
5	55.3	55.0	55.3	48.3	48.2
6	18.3	19.7	18.3	18.0	18.0
7	34.3	33.6	34.3	34.1	34.0
8	40.9	40.8	41.0	41.1	41.1
9	50.5	49.8	50.4	50.2	50.1
10	37.2	36.9	37.2	38.6	38.6
11	21.0	21.5	20.9	20.9	20.8
12	25.2	25.2	25.3	25.1	25.1
13	38.1	38.2	37.4	38.0	37.2
14	42.9	42.9	42.8	43.0	42.8
15	27.4	27.5	27.1	27.4	27.0
16	35.6	35.6	29.2	35.6	29.2

17	43.0	43.0	47.8	43.0	47.8
18	48.3	48.3	48.8	48.2	48.7
19	48.0	48.0	47.8	48.0	47.8
20	151.0	150.9	150.5	150.9	150.4
21	29.9	29.9	29.8	29.9	29.8
22	40.0	40.0	34.0	40.0	34.0
23	28.0	26.7	28.0	28.5	28.4
24	15.4	21.1	15.4	21.7	21.6
25	16.1	16.0	16.1	17.1	17.1
26	16.0	15.8	16.0	16.0	16.0
27	14.5	14.5	14.8	14.6	14.8
28	18.0	18.0	60.6	18.1	60.6
29	109.3	109.4	109.7	109.4	109.7
30	19.3	19.3	19.1	19.3	19.1

measured in $CDCl_3$; [a]150 MHz; [b]125 MHz

Table 2. ^{1}H NMR data of isolated compounds (**1**-**5**)

No.	**1**[b]		**2**[a]		**3**[a]		**4**[a]		**5**[a]	
	δ_H	**mult. (*J* (Hz))**	**δ_H**	**mult. (*J* (Hz))**	**δ_H**	**mult. (*J* (Hz))**	**δ_H**	**mult. (*J* (Hz))**	**δ_H**	**mult. (*J* (Hz))**
1	1.65*		1.90*		1.65*		1.70*		1.72*	
	0.89	dd (13.0; 4.0)	-		0.89	dd (12.6; 3.6)	1.13*		1.14	dd (12.0; 11.4)
2	1.58*		2.48	ddd (17.4; 10.2; 7.8)	1.58*		3.98	ddd (11.4; 3.6; 2.4)	3.97	ddd (11.4; 3.6; 2.4)
	-		2.41*		-		-		-	
3	3.19	dd (11.5; 5.0)	-		3.19	dd (11.4; 4.8)	3.41	d (2.4)	3.41	d (2.4)
4	-		-		-		-		-	
5	0.68	brd (10.8)	1.30	dd (13.8; 4.2)	0.67	brd (10.2)	1.35*		1.17	m
6	1.54*		1.46*		1.52*		1.40*		1.43*	
	1.39*		-		1.38*		1.35*		1.38*	
7	1.41*		1.44*		1.39*		1.42*		1.42*	
	-		-		-		1.38*		1.38*	
8	-		-		-		-		-	
9	1.28*		1.37*		1.28	dd (12.6; 3.0)	1.41*		1.39*	
10	-		-		-		-		-	
11	1.42*		1.41*		1.41*		1.45*		1.46	m
	1.25	m	1.28	m	1.19*		1.24*		1.25*	
12	1.68*		1.69*		1.63*		1.67*		1.66*	
	1.07*				1.03*		1.07*		1.06*	
13	1.67*		1.69*		1.64*		1.63*		1.64*	
14	-		-		-		-		-	

15	1.71*		1.69*		1.70*		1.65*		1.72*	
	1.01*		1.02*		1.04*		1.00*		1.06*	
16	1.49*		1.49*		1.93*		1.46*		1.93	m
	1.38*		1.38*		1.20*		1.37*		1.25*	
17	-		-		-		-		-	
18	1.38*		1.38*		1.57*		1.16*		1.58	t (11.4)
19	2.38	td (11.0; 5.5)	2.38*	m	2.38	td (11.4; 5.4)	2.37	td (10.8; 6.0)	2.38	m
20	-		-		-		-		-	
21	1.93	m	1.93	m	1.95*		1.90	m	1.97	m
	1.33*		1.32	m	1.40*		1.31*		1.38*	
22	1.42*		1.39*		1.86	ddd (12.6; 8.4; 0.6)	1.37*		1.85	dd (12.0; 8.4)
	1.20	m	1.20	m	1.02*		1.18*		1.03*	
23	0.97	s,3H	1.07	s, 3H	0.96	s, 3H	1.00	s, 3H	1.00	s, 3H
24	0.76	s,3H	1.03	s, 3H	0.76	s, 3H	0.84	s, 3H	0.84	s, 3H
25	0.83	s, 3H	0.93	s, 3H	0.82	s, 3H	0.88	s, 3H	0.87	s, 3H
26	1.03	s, 3H	1.07	s, 3H	1.02	s, 3H	1.02	s, 3H	1.02	s, 3H
27	0.94	s, 3H	0.96	s, 3H	0.98	s, 3H	0.95	s, 3H	0.99	s, 3H
28	0.79	s, 3H	0.81	s, 3H	3.80	dd (10.8; 1.8)	0.79	s, 3H	3.79	d (10.8)
					3.34	d (10.8)			3.34	d (10.8)
29	4.69	d (2.5)	4.69	d (1.8)	4.68	d (1.8)	4.57	dd (2.4; 1.2)	4.68	brs
	4.57	dd (2.5; 1.5)	4.57	d (1.8)	4.58	d (1.8)	4.69	d (2.4)	4.59	brs
30	1.69	s, 3H	1.68	s, 3H	1.68	s, 3H	1.68	s, 3H	1.68	s, 3H

*measured in $CDCl_3$; [a]600 MHz, [b]500 MHz; *mean value, overlapped signal(s)*

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