Isolation and Antibacterial Properties of Phenyl Acrylic Acid Derivatives from *Balanophora elongata* Blume

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Abstract. *Balanophora elongata* (Balanophoraceae) is a tropical parasitic flowering plant 9 cm in height. Four known phenyl acrylic acid derivatives, methyl caffeate (**1**), caffeic acid (**2**), 1,6-di-*O*-caffeoyl-*β*-D-glucopyranose (**3**), and coniferin (**4**), were isolated from this plant. Structural elucidation of the isolated compounds was determined by IR, LC-ESI-MS, 1D and 2D NMR. Extracts and isolated compounds were tested toward some standard human pathogenic bacteria using the agar disk diffusion method. Their inhibition zones were compared to that of chloramphenicol as positive control. Compound **1** showed inhibition toward *Streptococcus mutans*, while compound **3** and **4** inhibited *Staphylococcus aureus*.

Keywords: *antibacterial properties, Balanophora elongata, Balanophoraceae, isolation, parasitic plant, Sumatran Balanophora.*

1 Introduction

Balanophora elongata Blume is a parasitic flowering plant that belongs to the Balanophoraceae family, which consists of 18 genera and is estimated to have more than 50 species distributed around tropical and subtropical forests [1]. Most Balanophora species grow on the terminal roots of *Endospermum malacense*, *Macaranga triloba*, *Villebrunea rubescens*, *Ficus vasculosa*, *Ficus vulva*, *Laportea* sp., and *Ficus* sp. [2].

Phytochemical study of *B. elongata* is still limited, while for *B. laxiflora*, *B. involucrata*, *B. spicata*, *B. papuana*, *B. polyandra*, *B. fungosa*, and *B.*

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tobiracola, the presence of 249 chemical constituents have been reported from 2001 to 2018 [3]. Balanophorin has been recorded as a secondary metabolite originated from *B. elongata* [4]. Bioactivity studies have revealed that isolated compounds of Balanophora species showed antioxidant activity [5]; anti-radical scavenging properties [6][7]; α-glucosidase inhibition [8][9]; cytotoxic toward cancer cell lines, including breast (MCF7), liver (HepG2), lung (SK-LU-1), and epidermal (KB) cancer [5]; xanthine-oxidase inhibition [10]; trypsin and tryptase inhibition [11]; vasodilator [12]; and HIV-1 Env Pseudovirus inhibition [13][14].

Our preliminary study involving antibacterial assay of the methanolic extract of the tuber and flower of *B. elongata* toward some human pathogenic bacteria showed inhibition against some Gram-positive bacteria: *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Micrococcus luteus,* and *Enterococcus faecalis* and Gram-negative bacteria*; Bacillus subtillis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Salmonella typhosa, Salmonella thypi*, and *Vibrio cholerae*. Isolated methyl caffeate at 400 μg/disk from *B. elongata* showed moderate inhibition toward multi drug resistant *P. aeruginosa* (MDR-PA) with an inhibition diameter 19 mm compared to meropenem at 10 μg/disk (41.50 mm) [15].

2 Materials and Method

2.1 Plant materials

The whole plant of *B. elongata* was collected in Malalak, Agam District, West Sumatra in October 2016. Identification of the plant was authenticated by Dr. Nurainas and the voucher specimen (NP001/WS) was deposited in the Herbarium Andalas University (ANDA), Indonesia.

2.2 General Experiment Procedures

Silica gel 60 (70-230 mesh Merck) was used for open column chromatography. UV and IR spectra were recorded on a Shimadzu Pharmaspec 1700 spectrometer and a Perkin Elmer infrared spectrometer. Mass spectra were measured with a Shimadzu LC-PDA-MS/MS. ^{1}H and ^{13}C NMR were obtained with an Avance II 500 MHz Bruker nuclear magnetic spectrometer (^{1}H, 500 MHz and ^{13}C, 125 MHz) and a JEOL nuclear magnetic spectrometer (^{1}H, 400 MHz and ^{13}C, 100 MHz), as described in Syafni *et al.* [16]. Silica gel 60 F_{254} (Merck) plates were used for thin layer chromatography.

2.3 Extraction and Isolation

The fresh plants were divided into flowers (172 g) and tubers (2.4 kg), sliced into small pieces and macerated with MeOH (sample-solvent ratio 1:10) for 3 x 24 h (three times). Each filtrate was combined and concentrated *in vacuo* to get crude extracts of flowers (53 g) and tubers (225 g). The crude extract of flowers (53 g) was dispersed in water and MeOH (2:1) and then partitioned with *n*-hexane (3x100 mL). After fractionation, the water-MeOH fraction was evaporated *in vacuo* to remove the MeOH, extracted with EtOAc (5 x 100 mL) and then with *n*-butanol (3x100 mL) to yield fractions of *n*-hexane (1.1 g), EtOAc (31.0 g) and *n*-butanol (9.1 g). The tuber extract was treated similarly to give a methanolic crude extract (100 g), which was then fractionated in the same way as the methanolic flower extract, *n*-hexane (3x400 mL), EtOAc (4x400 mL), and *n*-butanol (4x400 mL) to give fractions of *n*-hexane (2.1 g), EtOAc (12.0 g), and *n*-butanol (12.0 g).

Flower EtOAc fraction (30 g) was column chromatographed on silica gel and eluted with stepped-up gradient polarity of EtOAc in *n*-hexane and then MeOH through EtOAc to obtain fifteen subfractions. Subfractions 1 and 2 were combined, evaporated, and crystallized from *n*-hexane-EtOAc (1:4) to afford compound **1** (107.5 mg). *n*-Butanolic fraction of flower (9 g) was column chromatographed on silica gel using an increasing polarity of EtOAc by addition of MeOH; thirty subfractions were obtained. Subfractions 21 to 30 were combined, evaporated, and crystallized from MeOH to afford compound **4** (184 mg).

Table 1 Preliminary Study of Antibacterial Screening of Methanolic Extracts from *B. elongata* Towards 12 Bacteria (indicated by zone inhibition, in mm).

Bacteria	Flower		Tuber		Chl.*
	1 mg/disk	0.5 mg/disk	1 mg/disk	0.5 mg/disk	
S. aureus	17.00 ± 0.00	11.00 ± 0.00	9.00 ± 0.00	6.50 ± 0.70	26.00 ± 1.55
Streptococcus mutans	13.00 ± 0.00	9.50 ± 0.70	n.d.	n.d.	23.00 ± 2.65
S. epidermidis	7.50 ± 0.70	6.50 ± 0.70	7.50 ± 0.70	7.00 ± 0.00	18.00 ± 1.20
E. faecalis	18.00 ± 0.00	11.50 ± 0.70	7.50 ± 0.70	6.00 ± 0.00	23.00 ± 2.65
M. luteus	15.50 ± 0.70	11.50 ± 0.70	10.50 ± 0.70	8.00 ± 1.40	23.00 ± 2.65
E. coli	15.50 ± 0.70	9.00 ± 0.00	7.50 ± 0.70	8.00 ± 0.00	18.00 ± 1.20
P. aeruginosa	18.50 ± 0.70	14.50 ± 0.70	11.00 ± 1.40	6.00 ± 0.00	30.00 ± 0.56
V. cholerae	19.50 ± 0.70	14.00 ± 0.00	12.00 ± 1.4	6.50 ± 0.70	25.00 ± 1.64
B. subtilis	17.50 ± 3.50	11.00 ± 1.40	n.d.	n.d.	26.00 ± 1.55
Salmonella thypi	16.50 ± 0.70	13.00 ± 1.40	10.50 ± 0.70	8.00 ± 1.40	25.00 ± 1.64
Salmonella typhosa	17.50 ± 0.70	14.50 ± 0.70	8.50 ± 0.70	7.00 ± 0.00	27.00 ± 1.73
Salmonella typhimurium	18.00 ± 0.00	14.00 ± 1.40	12.00 ± 1.40	7.00 ± 0.00	25.00 ± 1.64

* Chl. = chloramphenicol (30 µg/disk); n.d. = not detected; data significant at $p < 0.05$

Tuber EtOAc fraction (10 g) was column chromatographed as above to give 114 subfractions. Following their TLC profile, subfractions 17 to 25 were combined, evaporated and crystallized from *n*-hexane-EtOAc (1:4) to afford compound **1**

(317.6 mg). Subfractions 26 to 114 were combined and rechromatographed on a silica gel column and eluted with an increasing amount of MeOH in EtOAc to obtain 94 subfractions. Subfractions 1 to 9 were combined, evaporated and crystallized from *n*-hexane-EtOAc (1:2) to obtain compound **2** (42.4 mg). In the same way, subfractions 76 to 81 were directly subjected into a Sephadex LH-20 column chromatographer and eluted with MeOH to afford compound **3** (17.7 mg).

2.4 Antibacterial Assay

Antibacterial screening of the extracts was carried out by disk diffusion, following the modified method of Kirby-Bauer [17][18], and compared to that of chloramphenicol (3 mg/mL) in dimethyl sulfoxide (DMSO) as positive control and DMSO as blank control. The tested bacteria were *V. cholerae* inaba, *Streptococcus mutans* ATCC 25175, *M. luteus* ATCC 10240, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *S. epidermidis* ATCC 12228, *P. aeruginosa* ATCC 27853, *B. subtilis* ATCC 6633, *S. thypi*, *S typhymurium* ATCC 14028, and *S. typhosa* NCTC 786. The bacterial suspensions were prepared by dilution in NaCl 0.9% and 25% of transmittance was measured. The suspension was swabbed onto an agar petri dish. 10μL of positive control and negative control (DMSO) were dropped onto a paper disk (Whattman No. 1, diameter 5 mm) and carefully placed onto a surface cultivation dish. The experiment was done in triplicate for each bacterium. Each dish was then incubated at 37 °C for 16 to 18 hours. The inhibition zones (including the disk diameter) were measured, recorded and expressed as mean ± sd. The result was then statistically analysed using one-way analysis of variance (ANOVA) for each extract and compared with positive control and t-test for each compound with $\alpha = 0.05$. The result suggested that the inhibition zones of all extracts were significantly different at $p < 0.05$ (Table 1) and that the inhibition zones of all compounds were also significantly different at $p < 0.05$ (Table 2).

3 Result and Discussion

The isolated compounds were identified by spectroscopy, particularly by their 1D and 2D NMR (COSY, HSQC and HMBC) data, and then compared to those of data reported in the literature.

Compound **1** was isolated from tubers and flowers as pale-yellow plates (487.6 mg). MP: 160-162 °C. HR-ESI-MS: *m/z* 195.1022 $[M+1]^+$ (calculated for $C_{10}H_{10}O_4$); UV (MeOH) λ_{max} (log ε): 327 (2.67) nm. The IR spectra showed absorption at 3464 cm^{-1} (O-H), 1668 cm^{-1}, 1604 cm^{-1} (C=O), 1531 (C=C) [19]. ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz), see Table 3

and Table 4. Based on these spectroscopic data the compound was identified as methyl caffeate (Figure 1) [20], which is known as an abundant cinnamic acid derivative compound occurring in nature. It has previously been isolated from *Balanophora laxiflora* [5] and from another plant, *Bistorta manshuriensis* [20].

Table 2 Antibacterial Properties of Isolated Compounds Tested at 5 μg/disk.

Bacteria	Inhibition zones (mm)				Chl.*
	1	2	3	4	
S. aureus	n.d.	n.d.	14.00 ± 0,82***	14.00 ± 0.46	29.50 ± 0.66
Streptococcus mutans	18.50 ± 1.80**	6.60 ± 6.60	7.00 ± 0,53	n.d.	27.50 ± 1.32
S. epidermidis	n.d.	6.90 ± 0.50	8.00 ± 0,92	6.50 ± 0.50	23.75 ± 0.95
E. faecalis	n.d.	6.80 ± 0.40	n.d.	6.80 ± 0.26	26.00 ± 1.8
M. luteus	n.d.	n.d.	n.d.	n.d.	27.50
E. coli	11.00 ± 1.80	6.90 ± 0.50	n.d.	7.60 ± 0.35	28.75 ± 1.33
P. aeruginosa	n.d.	7.20 ± 0.44	n.d.	6.80 ± 0.87	31.25 ± 1.28
V. cholerae	n.d.	7.00 ± 0.26	n.d.	6.30 ± 0.26	25.75 ±1.32
B. subtilis	n.d.	6.60 ± 0.30	n.d.	n.d.	28.25 ± 0.51
Salmonella thypi	n.d.	6.90 ± 0.50	n.d.	6.50 ± 0.44	31.50 ± 1.50
Salmonella typhosa	n.d.	8.00 ± 0.80	n.d.	n.d.	29.75 ± 0.97
Salmonella typhimurium	n.d	6.30 ± 0.50	n.d.	6.60 ± 0.52	29.25 ± 0.93

1 = methyl caffeic acid; **2** = caffeic acid; **3** = 1,6-di-*O*-caffeoyl-β-D-glucopyranose; **4** = coniferin; n.d. = not detected; * Chloramphenicol (30 μg/disk); ** 20 μg/disk; *** 40 μg/disk; based on t-test; result significant at $p < 0.05$

Compound **2** was isolated from the tubers as yellowish fine plates. MP: 190.7-192.2 °C; LC-ESI-MS: 181.05 *m/z* $[M+1]^+$ (calculated for $C_9H_8O_4$); UV (MeOH) λ_{max} (log ε): 324 (1.35) nm; the IR spectra showed characteristic absorption bands at 3401 cm^{-1} (O-H); 3225 cm^{-1} (O-H) and 1598 cm^{-1} (C=O) [19]. ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz), see Table 3 and Table 4. The 1D and 2D NMR data showed that the compound was caffeic acid (Figure 1) [21], which has previously been isolated from *B. laxiflora* and *B. involucrata* [6][22] and from another plant, *Erigeron annuus* [21].

Compound **3** was isolated as yellow crystals. MP: 139.5-142.0 °C; LC-ESI-MS: *m/z* 503.25 $[M+H]^-$ (calculated for $C_{24}H_{24}O_{12}$); UV (MeOH) λ_{max} (log ε): 330 nm (4.67); The IR spectra showed characteristic absorption bands at 3265 cm^{-1} (O-H), 1694.5 cm^{-1} (C=O) and 1595 and 1524 cm^{-1} (C=C) [18]. ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz), see Table 3 and Table 4. The ^{1}H and ^{13}C NMR data refer the compound to 1,6-di-*O*-caffeoyl-β-D-glucopyranose (Figure 1) [23], which was isolated from *Balanophora* for the first time. It has also been found in *Coussarea hydrangeifolia* [23].

Compound **4** was isolated as colorless needles. MP: 184-185 °C; LC-ESI-MS: 341.10 *m/z* $[M+1]^+$ (calculated for $C_{16}H_{22}O_8$); UV (MeOH) λ_{max} (log ε): 292 nm (1.69); IR showed characteristic absorption bands 3276 (O-H), 2911 (C=C),

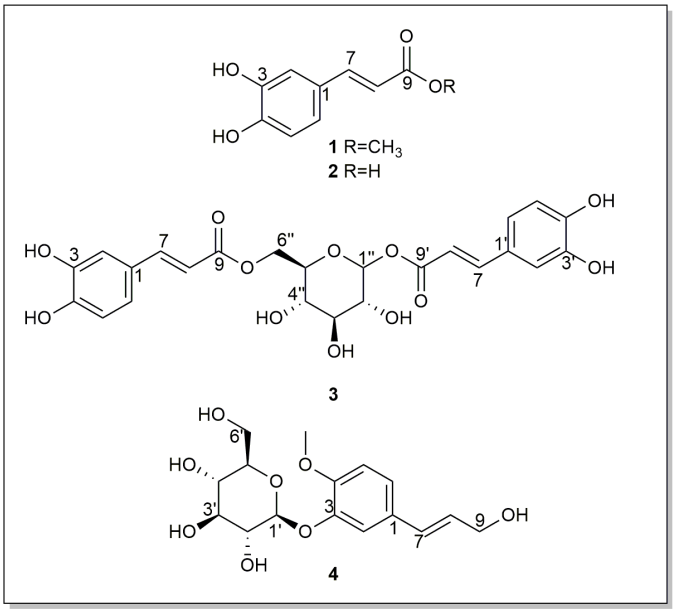

Figure 1 Chemical structures of all isolated compounds (**1**-**4**).

1508 cm^{-1} (C=C). ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz), see Table 3 and Table 4. These data are very similar to those of the spectroscopic data previously reported for coniferin (Figure 1) [24], which has found been in other *Balanophora* species, *B. fungosa* [25], *B. abbreviata* [26], *B. polyandra* [7], *B. involucrata* [6], *B. japonica* [9], and *B. laxiflora* [22].

All isolated compounds were tested against the same human pathogenic bacteria as above. The compounds with glucopyranosyl linked with phenyl acrylic groups (**3** and **4**) showed inhibition towards *S. aureus* at 40 and 5 μg/disk, respectively. Compound **1** had major inhibition towards *S. mutans* at 20 μg/disk and against *E. coli* at 5 μg/disk.

It has been reported that the methyl caffeate (**1**) isolated from *Solanum torvum* showed moderate inhibition toward rat intestinal sucrose and maltose enzyme, with IC_{50} values of 1.5 μM and 2.0 μM respectively; it may be useful as an antidiabetic agent [27]. It also showed moderate antimicrobial activity against *Proteus vulgaris*, *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* with MIC values 50, 25, and 8 μg/ml respectively [28]. It also acted as antioxidant. How it works toward purified ethyl linoleate substrate has been studied previously; surprisingly, it showed strong activity in the initial 2 hours [29]. Methyl caffeate has been successfully synthesized from caffeic acid and also inhibits the growth of *E. coli* and *S. aureus* [30].

Caffeic acid (**2**) has been reported to have antioxidant properties [31] and an *in-vivo* study showed blood pressure lowering properties [32] and hepatoprotective activity [33]. Another research showed antioxidant and hepatoprotective activity and DNA damage reduction [34] as well as anti-hepatocellular carcinoma properties [35]. Caffeic acid has been used as a substrate for the synthesis of caffeic acid derivatives such as methyl-, ethyl-, propyl-, butyl-, pentyl-, and decyl-caffeate; isopropyl-, and isopentyl caffeate; methoxy-ethyl- and 4-methoxybenzyl caffeate; benzyl-, 4-chlorobenzyl-, (di-(4-methoxybenzyl))-, 4-methylbenzyl-, (di-(4-chlorobenzyl))-, and diphenylmethyl caffeate [29]; and also caffeic acid phenethyl ester, which is a potential inhibitor enzyme from honeybee hives [36].

1,6-di-*O*-caffeoyl-*β*-D-glucopyranose (**3**) also exhibited antioxidant activity comparable to that of caffeic acid [23]. On the other hand, it stimulated proliferation of splenocytes cell, peritoneal macrophages, and enhanced the cytotoxicity of natural killer cells. It also showed immunomodulatory activity by regulating expression of Th1 and Th2 related cytokines [37]. No synthesis of this compound exists in the literature. Probably this plant can be useful as a source for immunotherapeutic agents in treating immunity related diseases in the future.

Table 3 ^{1}H NMR Spectral Data of Isolated Compound.

Position	1 (CD_3OD, 400 MHz)	2 (CD_3OD, 500 MHz)	3 (CD_3OD, 500 MHz)	4 (CD_3OD, 400 MHz)
1/1'	-	-	-	-
2	7.0 (1H, *d*)	7.00 (1H, *d*, J=2.1)	7.05 (1 H, *d*, J=1.9)	7.04 (1H, *d*, J= 2.0)
2'	-	-	7.04 (1H, *d*, J=1.9)	-
3/3'	-	-	-	-
4/4'	-	-	-	-
5	6.73 (1H, *d*, J=8.0, 6.8)	6.75 (1H, *d*, J= 8.4)	6.77 (1 H, *d*, J=8.2)	7.08(1H, *d*, J= 8.8)
5'	-	-	6.76 (1H, *d*, J=8.1)	-
6	6.90 (1H, *dd*, J=8.0)	6.90 (1H, *dd*, J=8.3, 2.0)	6.95 (1 H, *dd*, J= 2.4, 8.6)	6.92 (1H, *dd*, J= 8.0, 2.0)
6'	-	-	6.92 (1H, *dd*, J= 1.9, 8.8)	-
7	7.49 (1H, *d*, J=14.0)	7.50 (1H, *d*, J=15.8)	7.65 (1H, *d*, J=15.8)	6.53 (1H, *d*, J= 16.0)
7'	-	-	7.55 (1 H, *d*, J= 15.9)	-
8	6.21 (1H, *d*; J=14.0)	6.19 (1H, *d*, J=15.8)	6.30 (1 H, *d*, J=15.9)	6.27 (1H, *dt*, J= 16.0, 6.0)
8'	-	-	6.27 (1H, *d*, J=15.8)	-
9	-	-	-	4.18 (2H, *dd*, J= 5.2)
1"	-	-	5.60 (1H, *d*, J=7.9)	4.87 (1H, *d*, J= 8.0)
2"	-	-	3.47 (1H, *t*, J=8.2)	
3"	-	-	3.51(1H, *t*, J=8.7)	3.36-3.47 (*m*)
4"	-	-	3.43 (1H, *t*, J=8.9)	
5"	-	-	3.69 (1H, *m*)	
6"	-	-	4.50 (1H, *dd*, J=2.0, 12.1) 4.32 (1H, *dd*, J=5.5, 12.3)	3.68 (*m*)
OCH_3	3.6 (3H, *s*)	-	-	3.84 (3H, *s*)

Coniferin (**4**) has been reported to have antioxidant activity and significant DPPH prooxidant effects [38]. Coniferin has been successfully synthesized from vanillin as starting material [39].

All these compounds are known based on reported references to have various bioactivities, such as antioxidant, antibacterial and antimycobacterial, antidiabetic, anti-hepatocellular carcinoma, hepatoprotective, and immunomodulator activities. One report noted that the whole plant and all parts of *B. elongata* have been used as aphrodisiac [40]. However, more detailed bioactivity study is needed before it can be used as validated traditional medicinal plant.

Table 4 ^{13}C NMR Spectral Data of Isolated Compound.

Position	1	2	3	4
1	126.3	127.9	127.6	132.2
1'	-	-	127.5	-
2	113.7	115.0	114.2	109.9
2'	-	-	114.7	-
3	145.4	146.7	146.7	146.2
3'	-	-	146.6	-
4	148.2	149.6	149.8	149.4
4'	-	-	149.5	-
5	115.1	116.6	116.5	116.4
5'	-	-	116.4	-
6	121.5	123.0	123.3	119.3
6'	-	-	123.0	-
7	145.6	147.3	148.4	129.9
7'	-	-	147.2	-
8	113.4	115.6	115.2	127.4
8'	-	-	115.1	-
9	168.4	171.2	169.1	62.3
9'	-	-	167.6	-
1"	-	-	95.6	101.3
2"	-	-	73.9	73.5
3"	-	-	77.7	76.4
4"	-	-	71.2	69.9
5"	-	-	76.2	76.8
6"	-	-	64.3	61.1
OCH_3	50.6	-	-	55.3

4 Conclusion

Four known phenyl acrylic acid derivatives were isolated and identified from the flower and the tuber of *B. elongata*. Compounds **1** to **4** showed weak inhibition against tested human pathogenic bacteria when compared to positive control (chloramphenicol).

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