ORIGINAL ARTICLE

Antioxidant and antiproliferative activities of n-hexane extract and its fractions from *Blumea balsamifera* L. leaves

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ABSTRACT

Blumea balsamifera is a plant species that has been popularly used to treat a broad spectrum of diseases. In efforts of tackling the increasing threat of cancers, *B. balsamifera* has been studied for its anticancer potentials. Hence, through this research, we aimed to evaluate the antioxidant and antiproliferative activities of n-hexane extract from *B. balsamifera* L. leaves along with its fractionation products. After the n-hexane extract has been obtained, the sample was column chromatographed using gradient elution with n-hexane:ethyl acetate solvent. All the isolation protocols produced 1 n-hexane extract and 10 different fractions (fractions 1–10). Antioxidant and antiproliferative effects of the samples were assessed based on 2,2-diphenyl-1-picrylhydrazyl and brine shrimp lethality test assay, respectively. None of the samples have a strong antioxidant level because all samples yielded IC_{50} of more than 100 ppm – the best of them was fraction 8 with IC_{50} = 113.716 ppm. On contrary, most of the samples were observed to have a potent antiproliferative effect, especially fraction 8 with LC_{50} = 2.00 ppm. Taken altogether, fraction 8 from the n-hexane extract of *B. balsamifera* L. leaves is the most potential candidate for proliferative disease therapy, where further studies confirming the results are required.

Key words: Antioxidant, antiproliferative, brine shrimp lethality test, 2,2-diphenyl-1-picrylhydrazyl, phytomedicine

INTRODUCTION

Ancient traditional medicines, including Chinese traditional medicine, Ayurvedic traditional medicine, African traditional medicine, and so on, have been used as integrative therapies against proliferative diseases.[1] In efforts of finding natural products with anticancer effects, we have conducted research on several plant species including *Myristica fragrans*,[2,3] *Theobroma cacao*,[4-6] and *Annona squamosa*.[7] In addition, in our previous investigation, we found that ethyl acetate extract from *Blumea balsamifera* leaves had a very active antiproliferative activity.[8] *B. balsamifera* is very popular among those who practice ethnomedicines and is believed to possess wound healing, anti-diarrhea, anti-spams, and anti-rheumatism properties.[9] In another study, the *B. balsamifera* extract has been found to reduce the number of rat and human hepatocellular carcinoma cells.[10] Anti-inflammation of

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B. balsamifera essential oils has been evidenced in multiple reports involving the ameliorations of nitric oxygen and pro-inflammatory cytokine regulation.[11,12] Unfortunately, despite its potential, the published research only focused on obtaining the essential oils using hydrodistillation, where extraction using n-hexane solvents is still scarcely reported.

To fill the foregoing research gap, for the first time, this present work reported the bioactivities of n-hexane extract and its fractions from *B. balsamifera* leaves. To assess the antioxidant potential, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay could be a strong modality. A study has used DPPH assay to assess several natural products for their potential as brain-penetrable antioxidants.[13] Moreover, antiglycoxidant effect could also be evaluated based on the scavenging activity against DPPH radicals.[14] As for the antiproliferative activities, they could be screened by employing *Artemia salina* larvae in a brine shrimp lethality test (BSLT) assay. Cytotoxicity in BSLT assay has been found to correlate with antiproliferative effect against certain cancer cells, thus becoming the simple and low-cost method for the initial screening.[15] Therefore, we have employed DPPH and BSLT assays, to investigate the antioxidant and antiproliferative activity of n-hexane extract from *B. balsamifera* leaves along with its derivative fractions.

METHODS

Herbarium specimen and chemicals

Herbarium specimen was collected in October 2020 from the area around Aceh Selatan Regency, Aceh Province, Indonesia (3°21'43.5"N; 97°06'35.6"E). The specimen identification (B/757/UN11.1.8.1/TA.00.01/2019) at the Herbarium Laboratory of Universitas Syiah Kuala confirmed *B. balsamifera* L.

Chemicals used in this study were DPPH, dimethyl sulfoxide (DMSO), silica gel, ethyl acetate, n-hexane, and methanol. Pharmaceutical-grade Vitamin C was purchased from PT. Supra Ferbindo Farma (Jawa Barat, Indonesia). Otherwise stated, all chemicals were analytical grade and purchased from Merck (Selangor, Malaysia).

Extraction and fractionation of *Blumea balsamifera* L. leaves

As much as 3 kg of dried powder of *B. balsamifera* L. leaves was macerated at room temperature using methanol solvent for four cycles (24 h/cycle). The filtrate was separated from the residue using a rotary evaporator (230 mbar; 10°C chiller; 55°C heating bath). The extract was then partitioned using n-hexane solvent to obtain the n-hexane extract sample. In a separating funnel, a mixture of n-hexane solvent and the previously obtained methanol extract was separated. The lipid residue was removed, whereas the soluble layer was collected and reconcentrated using a rotary evaporator with the same operating condition. The sample obtained from this procedure was then labeled as n-hexane extract.

Fractionation was further carried out on the n-hexane extract sample by means of gradient column chromatography (diameter = 5 cm; height = 70 cm). The n-hexane extract (30 g) was impregnated and inserted in a column containing preheated 360 g silica gel (70°Cl 3 min) which was purchased from Merck (Selangor, Malaysia) with a specification of 70–230 mesh american standard testing and material (ASTM). The extract was firstly eluted with 100% n-hexane followed by n-hexane:ethyl acetate with 9:1, 8:2, and 7:3 ratios, sequentially. In total, 215 fractionation tubes were obtained, where each was observed on thin-layer chromatography (TLC). Fractions having the same stain patterns were combined resulting in only 10 fraction samples [Table 1]. The photographs of TLC patterns of each combined fraction obtained from the n-hexane:ethyl acetate solvents of 9:1 and 7:3 are presented in Figure 1.

2,2-diphenyl-1-picrylhydrazyl assay

DPPH assay was performed with two repetitions following the procedure reported previously. Briefly, each of the samples (n-hexane extract and fraction 1–10) was firstly diluted in methanol to obtain 25, 50, and 100 ppm concentrations. The dissolved sample (5 mL) was inserted into a test tube and added with DPPH 0.4 mM (1 mL). The test tube was covered with aluminum foil and homogenized using a vortex mixer and placed in an incubator for 30 min at 37°C. Reduced concentration was analyzed using an ultraviolet-visible mini-1240 spectrophotometer (Kyoto, Japan) at λ =517 nm. The least concentration required to reduce 50% concentration of the reagent (IC_{50}) was calculated based on a linear curve equation. With the same procedure, Vitamin C (3–9 ppm) was determined for its IC_{50} and used as a reference.

Brine shrimp lethality test assay

Before performing the BSLT assay, the *A. salina* eggs were hatched and grew for 48 h. Into each test tube, saline water was added and followed by 10 previously

Table 1: Weights and color appearances of n-hexane extract and its fractions from *Blumea balsamifera* L. leaves

Specimen	Fractionation tube	Weight (g)	Color
n-hexane extract	Not applicable	135.851	Dark green
Fraction 1	1-5	1.16	Dark brown
Fraction 2	6-15	2.10	Dark brown
Fraction 3	16-45	4.19	Green
Fraction 4	46-75	1.6	Green
Fraction 5	76-110	1.81	Green
Fraction 6	111-125	0.84	Green
Fraction 7	126-145	2.67	Green
Fraction 8	146-175	3.39	Green
Fraction 9	176-195	1.7	Green
Fraction 10	196-215	1.5	Green

hatched *A. salina* larvae. n-hexane extract and its fractions (1–10) were dissolved in DMSO so that a variation of concentration of 1, 10, 100, 500, and 1000 ppm was obtained. Each concentration variation was added to the test tube containing *A. salina* larvae and stored under a tubular lamp. A test tube without the n-hexane extract or its fractions was taken as a control. After 24 h, the survived and dead larvae were counted. This procedure was performed in two times repetition, where the number of dead larvae was averaged and presented as a mortality percentage. A linear curve equation was used to calculate the minimum concentration required to cause 50% mortality (LC_{50}).

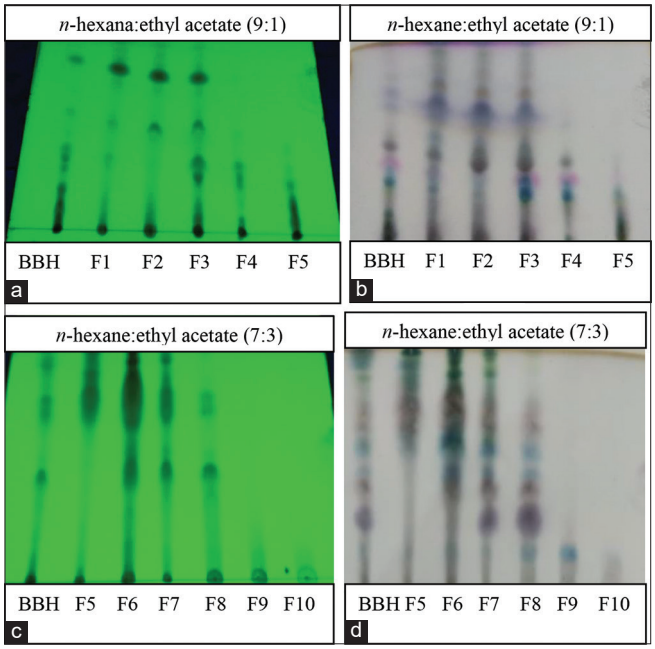

Figure 1: TLC chromatogram of fraction 1–5 eluted with n-hexane: ethyl acetate (9:1) observed under 254-nm UV lamp (a) and with vanillin sulfate reagent (b). TLC chromatogram of fraction 5–10 eluted with n-hexane:ethyl acetate (7:3) observed under 254-nm UV lamp (c) and with vanillin sulfate reagent (d). BBH is the n-hexane extract from *Blumea balsamifera* L. leaves. Fraction samples were indicated by F followed the fraction number, for example, F1 represents fraction 1. TLC: Thin-layer chromatography, UV: Ultraviolet, BBH: n-hexane extract

RESULTS AND DISCUSSION

Yield and color appearance of the n-hexane extract and its fractions

n-hexane extract and its fraction 1–10 from *B. balsamifera* L. leaves appeared in different colors with different weight yields, where the data have been presented [Table 1]. The overall weight of the extract specimen was obtained 135.851 g presented in dark green. The derivative of the extracts (fractions) had green as their basic color but with different intensities.

Antioxidant activity of the extract and its fractions

The antioxidant activities of the samples in this study, based on DPPH scavenging activities, are presented in Table 2. Weak antioxidant was observed in the n-hexane extract with IC_{50} equals to 281.707 ppm. Following the fractionation through column chromatography using gradient elution, the IC_{50} values changed. Poorer antioxidant activity (IC_{50} = 291.357 ppm) was observed in Fraction 1, where the eluent was nonpolar. When the eluent was more polar (fraction 8–10), the IC_{50} values were obtained lower (113.716, 126.423, and 129.201 ppm for fractions 8, 9, and 10, respectively). Among the three, DPPH inhibition values obtained at 100 ppm of fractions 8, 9, and 10 were 44.981%, 42.649%, and 36.633%, respectively. Fractionation was meant to remove the inactive and inert phytocompounds, thus increasing the concentration of active components.[16,17]

Table 2: 2,2-diphenyl-1-picrylhydrazyl radicals scavenging activities of n-hexane extract and its fractions from *Blumea balsamifera* L. leaves

Sample	Concentration (ppm)				
	Inhibition (%)			Linear fitting equation	IC_{50} (ppm)
	25	50	100		
n-hexane extract	1.259	8.511	15.716	y = 0.186x - 2.344	281.707[b]
Fraction 1	5.410	9.094	17.920	y = 0.168x + 0.997	291.357[b]
Fraction 2	3.743	9.607	24.694	y = 0.283x – 3.8	190.413[a]
Fraction 3	11.204	16.264	22.875	y = 0.152x + 7.9	276.49[b]
Fraction 4	5.317	11.974	19.319	y = 0.181x + 1.644	267.145[b]
Fraction 5	10.633	24.915	28.810	y = 0.219x + 8.686	188.766[a]
Fraction 6	20.077	26.093	29.451	y = 0.117x + 18.398	270.737[b]
Fraction 7	21.593	25.708	34.697	y = 0.175x + 17.099	187.534[a]
Fraction 8	23.528	36.936	44.981	y = 0.268x + 19.506	113.716[a]
Fraction 9	22.817	32.051	42.649	y = 0.257x + 17.518	126.423[a]
Fraction 10	0.739	8.056	36.633	y = 0.492x - 13.55	129.201[a]

IC_{50} is defined as the minimum concentration required to yield 50% inhibition. [a]Moderate (101–250 ppm) and [b]weak levels (251–500 ppm) of antioxidant activity. Vitamin C (3, 6, and 9 ppm) was used as the positive control with IC_{50} = 4.326 ppm. IC_{50}: Half-maximal inhibitory concentration

Table 3: Cytotoxic activities against *Artemia salina* larvae of n-hexane extract and its fractions from *B. balsamifera* L. leaves

Sample	Concentration (ppm)						
	Mortality (%)					Linear fitting equation	LC_{50} (ppm)
	1	10	100	500	1000		
n-hexane extract	30	35	40	70	80	y = 0.4367x + 4.2803	44.47
Fraction 1	30	40	45	85	100	y = 0.9671x + 3.9635	11.79
Fraction 2	25	40	90	95	100	y = 1.1627x + 3.9951	7.31
Fraction 3	45	65	75	100	100	y = 1.1333x + 4.4503	3.06
Fraction 4	30	45	60	85	100	y = 0.9687x + 4.0607	9.32
Fraction 5	40	50	65	90	95	y = 0.6229x + 4.5283	5.71
Fraction 6	40	60	70	90	100	y = 0.8955x + 4.4201	4.44
Fraction 7	40	50	55	80	90	y = 0.4695x + 4.5831	7.72
Fraction 8	50	55	75	80	90	y = 0.406x + 4.8776	2.00
Fraction 9	30	50	65	95	100	y = 1.0526x + 4.0888	7.33
Fraction 10	35	50	55	65	75	y = 0.3139x + 4.6138	16.99

LC_{50} is defined as the minimum concentration required to kill half of the population. All brine shrimps observed in control (saline water) survived

Antiproliferative activities of the extract and its fractions

The cytotoxic activities of n-hexane extract and its fractions from *B. balsamifera* L. leaves, based on BSLT assay, are presented in Table 3. The LC_{50} value of n-hexane extract (44.47 ppm) is the poorest among all samples, with a mortality of 40% at 100 ppm concentration. The value dropped rapidly in fraction 1 (LC_{50} = 44.47 ppm), and lower in fraction 2 (11.79 ppm) and fraction 3 (3.06). Nonetheless, fraction 8 had the highest cytotoxicity among all samples with LC_{50} as low as 2.00 ppm. All samples with LC_{50} lower than 30 ppm could be categorized as potent anticancer agents.[18] This value is significantly greater than that obtained from *B. balsamifera* L. leaf extract using ethyl acetate solvent (LC_{50} = 25–100 ppm).[8] Previously, simple methanolic extract from *B. balsamifera* L. leaves was showcased to attenuate human hepatocellular carcinoma cells (*in vitro* and *in vivo*) without affecting hepatocytes in rats.[19] Hence, the fractions from the n-hexane extract of *B. balsamifera* L. leaves could potentially act as an antiproliferative agent and be used in cancer treatment, especially that of fraction 8 with the best LC_{50}. Further studies *in vitro* or *in silico*, including the phytocompound profiling, are warranted to study its anticancer effects as suggested by previous studies.[5,18,20-22]

CONCLUSIONS

Fractionation of n-hexane extract from *B. balsamifera* L. leaves using column chromatography with gradient elution allows to improve the antioxidant and antiproliferative activities. However, none of the samples had an antioxidant level that was <100 ppm, where the antioxidant activity was either moderate or weak. On contrary, antiproliferative activity was found strong (<30 ppm) in all fractions. In future, we will perform the isolation of the pure compound from the most cytotoxic sample (fraction 8) and study its anticancer effect against cancer cell lines.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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