ORIGINAL ARTICLE

Antioxidant and cytotoxicity screenings of ethyl acetate extract from *Annona muricata* leaves and its fractions

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ABSTRACT

Leaves of *Annona muricata* have medicinal potential which has gained attention from researchers around the world. This study has an objective to screen the antioxidant and cytotoxicity of ethyl acetate extract from *A. muricata* leaves and its fraction. The fine powder of *A. muricata* was macerated in methanol and further partitioned using two different solvents, namely n-hexane and ethyl acetate. In this article, we reported the screening results for ethyl acetate extract. Fractionation was then performed on the extract by means of column chromatography by gradient elution resulting in five combined fractions. Brine shrimp lethality test and 1-diphenyl-2-pycrilhidrazil (DPPH) assays were employed to evaluate the cytotoxicity and antioxidant of the extract, respectively. Characterization using gas chromatography-mass spectroscopy (GC-MS) was then conducted. The cytotoxicity of the samples was indicated by median lethal concentration$_{50}$ values ranging from 28.84 to 1023.3 ppm. As for the antioxidant activity, the DPPH median inhibitory concentration$_{50}$ values ranged from 4.12 to 180.66 ppm. GC-MS analysis on the most bioactive fraction revealed the predominating phytochemical contents of neophytadiene, palmitic acid, and phytol. In conclusion, the fraction of ethyl acetate extract from *A. muricata* leaves could potentially act as a strong antioxidant and moderate cytotoxic agent.

Key words: 1-diphenyl-2-pycrilhidrazil, *Annona muricata,* cytotoxicity, ethyl acetate, soursop

INTRODUCTION

The fruit *Anona muricata,* also known as soursop, is a delicacy in several countries and regions. As research on medicinal products receiving worldwide attention,[1-4] *A. muricata* has been suggested to possess a variety of medicinal potentials such as anticancer, antioxidant, anti-inflammatory, antimicrobial, analgesic, antidepressant, and immunostimulant.[5,6] Cytotoxicity of the n-hexane and methanol extracts from this plant has been reported to be lethal, with median lethal concentrations (LC_{50}s) of 84 and 1 ppm, respectively.[7] In the same previous study, 1-diphenyl-2-pycrilhidrazil (DPPH) antioxidant assay revealed median inhibitory concentrations (IC_{50}s) of ranged from 8 to 40 ppm depending on the fractions.[7] Other than being cytotoxic and antiproliferative, as suggested by a review article,[8] leaf extract of *A. muricata* was also recognized for its potential against various cell cancers.[9] With such potential, it is worth to explore *A. muricata* as an alternative

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modalities to fight against the growing cancer prevalence, especially in Indonesia.[10]

The anticancer potential of *A. muricata* was indicated by its phytochemical profile containing alkaloids, essential oils, and acetogenins.[6,11] Acetogenins were found to have specific toxicity against cancer cells without affecting normal cells.[12] The leaf of *A. muricata* contained annonaceous acetogenin – a toxic bioactive substance.[13] There were *in vitro* studies suggesting that the ethyl acetate extracts from *A. muricata* leaves were active against various cancer cell lines including HeLa (IC_{50} = 5.91 ppm),[14] HepG-2 (IC_{50} = 44.55),[15] and MCF-7 (IC_{50} <30 ppm).[16] Taken altogether, exploring potential natural products from *A. muricata*, especially its leaf part, is interesting. Hence, this research sought to screen the bioactivities (antioxidant along with cytotoxicity) of ethyl acetate extract from *A. muricata* leaves and its fractions which were underreported. The phytochemical identification using a semiquantitative method, gas chromatography-mass spectroscopy (GC-MS), was also carried out on a fraction with the highest bioactivities.

METHODS

Leaf specimen

Leaves of *A. muricata* were collected from Montasik, Aceh Besar, Indonesia, and cut into small pieces (1–2 cm). Thereafter, the leaves were cleaned, air-dried (room temperature; no direct sunlight) for 2 weeks, and subsequently crushed into a fine powder (40 mesh). The specimen was collected multiple times with no variation on the quality was observed.

Maceration

The leaf fine powder (538 g) was macerated using methanol (20 L) for 3 h × 24 h in a 30-L sealed glass container. The maceration was carried out at room temperature. Thereafter, the filtrate was filtered, combined, and then concentrated with a rotary evaporator. The resultant extract was partitioned by employing n-hexane (25 L) as well as ethyl acetate (2 L), sequentially, with 2–3 times repetition.

Qualitative phytochemical screening

Screening of phytocompounds contained in the extracts was performed qualitatively by means of observation of bulk changing in the extract following the treatment. The protocol for this screening has been reported previously.[17]

Determination of total phenolic content

First, dissolving the extract (100 mg) was carried out in 10 mL distilled water to retrieve a solution with 10 mg/mL extract concentration. Afterward, 1 mL of the solution was drawn and subsequently added to another 10 mL distilled water for dilution. The diluted extract (0.2 mL) was drawn and added to a test tube containing distilled water and Folin–Ciocalteu reagent with volumes of 15.8 mL and 1 mL, respectively. After the mixture became homogenous, it was left still for 8 min before the addition of Na_2CO_3 (10%; 3 mL). It was left still for another 2 h at room temperature. The measurement of absorbance on a UV-Vis spectrophotometer was carried out at 765 nm.

Determination of total flavonoid content

Dissolving the extract (25 mg) in methanol (25 mL) was carried out first before another dilution with methanol until the concentration was 50 mg/mL. The diluted extract (1 mL) was added with methanol (3 mL), $AlCl_3$ (0.2 mL), potassium acetate 1 M (0.2 mL), along with distilled water (5.6 mL). UV-Vis absorbance was performed on the mixture at 400 nm.

Isolation of the ethyl acetate extract

The isolation was carried out using gravity column chromatography with silica G_{60} (0.2–0.5 mm) as the stationary phase. Gradient elution from nonpolar to polar was used. The eluate was collected using a 50 mL test tube, where thin layer chromatography was performed to guide the combination of the fraction from each test tube.

Brine shrimp lethality test

A. muricata extract solutions were prepared by dissolving the solid extract (50 mg) in 25 mL saline water and 3 drops of DMSO 5% so that the total concentration was 2000 ppm. The extract was dissolved into 50–1000 ppm. Negative control was prepared by mixing DMSO 5% (3 drops) and saline water (5 mL). Thereafter, 48-h newly hatched *Artemia salina* larvae (n = 10) were inserted to each test tube containing extracts or negative control. The incubation at room temperature was carried out under the lamp light (15 Watt) for 24 h. The mortality count was performed in each tube, where the whole assay was carried out in triplicate.

1-Diphenyl-2-pycrilhidrazil assay

Free radical scavenging activity of the *A. muricata* ethyl acetate (AMEA) extract and its fraction was investigated by means of DPPH assay following the guideline published previously.[18,19] DPPH powder (7.9 mg) was firstly dissolved in methanol so that the total volume reached 50 mL. Each of the extract and its fraction was dissolved separately in methanol to retrieve 500 ppm concentration before varied into 6.25, 12.5, 25, 50, and 100 ppm. Thereafter, the two solutions were mixed (4 mL extract: 1 mL DPPH) and incubated (37°C; 30 min). The positive control was ascorbic acid tested under a concentration range of 3–15 ppm. UV-Vis absorbance at 517 nm was measured on a spectrophotometer.

Gas chromatography-mass spectroscopy

A fraction with the most optimal bioactivity was characterized by Thermo Scientific GC-MS. The nonpolar column was used as the stationary phase, while he was

utilized as the mobile phase. The temperature was set at 250°C during the analysis (68.54 min).

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethyl acetate extract

Yields of *A. muricata* crude extracts obtained by using methanol, n-hexane, and ethyl acetate were 20.94, 20.14, and 7.89% w/w, respectively. In this report, we focused on the ethyl acetate extract. The qualitative analysis revealed that the ethyl acetate extract was positive for flavonoids, steroids, terpenoids, phenolics, and saponins. Contrarily, a previous study witnessed the alkaloids (among other phytocompounds) in the ethyl acetate extract.[8] This is indicative that the alkaloids in the extract of the present study were present, but in low concentrations which were not sufficient for qualitative detection. As suggested by a previous study, qualitative screening is useful in the exploration of bioactive extracts.[5,20] For example, tannins might act as antioxidants (both primary and secondary) and antimicrobial agents.[5,20,21] Further, we found that the ethyl acetate extract had total phenolic content (TPC) as well as total flavonoid content (TFC) of 241.3 mg GAE/g dry extract and 51.08 mg QE/g dry extract, respectively. As comparisons, the values indicating the TPC and TFC of the leaf extract of *A. muricata* in this present study are relatively higher, or at least similar, with those reported previously such as ethyl acetate extracts from *Phyllanthus emblica* stem barks, *Limonia acidissima* fruits, as well as *Annona squamosa* stem barks.[17,19,21]

Ethyl acetate isolates

Isolation was carried out on the crude extract by means of column chromatography, where the weight and apparent color of each fraction are presented in Table 1. In total, there were 97 fractions obtained from the isolation, and they were then grouped based on the similarity of stain patterns of the thin layer chromatography. The grouping yielded 5 collective fractions which were labeled as AMEA 1–5, respectively. The range of weight obtained from the isolation was from 0.15 g (50.29%, AMEA 5) to 1.71 (4.41%, AMEA 1). The fractions appeared in dark green (AMEA 1 and 2), brown (AMEA 3 and 5), and yellow colors (AMEA 4).

Brine shrimp lethality test-based cytotoxicity of the ethyl acetate extract and its fractions

The cytotoxicity based on brine shrimp lethality test assay was performed on the ethyl acetate extract of *A. muricata* and its fractions (AMEA 1–5), where the results are presented in Table 2. The crude extract had moderate cytotoxicity with LC_{50} of 28.84 µg/mL. The value was increased following the isolation with a range of 147.91 (AMEA 2)–1023.3 µg/mL (AMEA 1). Other than AMEA 1, all fractions were cytotoxic by using < 1000 µg/mL cut-off value. The elevation of LC_{50} suggests that the fractions have weaker toxicity as compared to their crude extract. A possible explanation for this finding is the intermolecule interactions that could exert synergism and consequently – higher cytotoxicity.[22,23]

Table 1: Results from the qualitative phytochemical screening of fraction groups obtained from the *Annona Muricata* leaf extract

Fraction	Test tube	Weight (g)	Yield (%)	Apparent color
AMEA 1	1–15	1.71	50.29	Dark green
AMEA 2	16–50	0.77	22.65	Dark green
AMEA 3	51–75	0.24	7.06	Brown
AMEA 4	76–95	0.28	8.24	Yellow
AMEA 5	96–97	0.15	4.41	Brown

Total extract weight=3.4 g. AMEA: *A. muricata* ethyl acetate

Table 2: Brine shrimp lethality test cytotoxicity and 1-diphenyl-2-pycrilhidrazil antioxidant of the ethyl acetate extract from *Annona muricata* leaves and its fractions

Sample	BSLT, LC_{50} (µg/mL)	DPPH, IC_{50} (µg/mL)
AMEA	28.84	15.19
AMEA 1	1023.3	180.66
AMEA 2	147.91	11.4
AMEA 3	489.77	42.48
AMEA 4	288.40	59.03
AMEA 5	257.03	4.12
Ascorbic acid	NA	4.12[a]

AMEA: A. *muricata* ethyl acetate of *Annona muricata* leaves, NA: Not applicable, DPPH: 1-diphenyl-2-pycrilhidrazil, BSLT: Brine shrimp lethality test, LC: Lethal concentration, IC: Inhibitory concentration

1-diphenyl-2-pycrilhidrazil-based antioxidant activity of the ethyl acetate extract along with its fractions

The DPPH antioxidant activities of the ethyl acetate extract along with its fraction are presented in Table 3. Compounds with OH moieties or conjugated double bonds in the extract may scavenge the free radical by donating the electron.[24,25] The crude extract has antioxidant potential with IC_{50} = 15.19 µg/mL against DPPH. The activity was further increased following the isolation, observed in AMEA 2 and 5 with IC_{50} of 11.4 and 4.12 µg/mL, respectively. Moreover, AMEA 5 and ascorbic acid (positive control) have similar IC_{50} against DPPH suggesting strong antioxidant potential of this fraction. The fraction had dramatically stronger antioxidant activities when compared to previously reported studies on *A. muricata* leaves (IC_{50} = 140–20 µg/mL).[11,16]

Phytochemical profile of *Annona muricata* ethyl acetate 2

The chromatogram of the GC-MS analysis on fraction AMEA 2 is presented in Figure 1. The chromatogram suggests that many compounds remained in the AMEA 2, though has underwent purification by column chromatography. The identified phytocompounds with a similarity index of over 95% are presented in Table 2. Neophytadiene and palmitic acid were found predominating the fraction with

Table 3: Identified phytocompounds contained in *Annona muricata* ethyl acetate with similarity index>75%

Compounds	Retention (min)	Area (%)	Similarity (%)
Cyclopentane, 1-ethyl-3-methyl-, trans-	4.08	1.40	79.4
4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	28.12	2.74	86.6
Neophytadiene	30.16	12.16	92.2
Phytol	30.67	1.30	83.8
Phytol	31.04	6.09	89.2
Geranyl-a-terpinene	31.79	3.12	78.5
Cyclopropanebutanoic acid, 2-[[2-[[2-[(2pentylcyclopropyl) Methyl] Cyclopropyl] methyl] cyclopropyl] methyl]-, Methyl ester	31.96	2.38	81.3
6,9,12,15-Docosatetraenoic acid, Methyl ester	32.17	1.65	79.3
Palmitic acid	32.59	11.53	88.9
1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-Tetramethyl-, (E, E)-	33.07	1.69	78.5
Phytol	35.48	3.03	78.4
[1,1'-Bicyclopropyl]-2-Octanoic acid, 2'- hexyl, Methyl ester	36.33	1.06	77.6
Ethyl iso-allocholate	48.38	1.20	76.5
Ethyl iso-allocholate	48.51	1.34	76.6

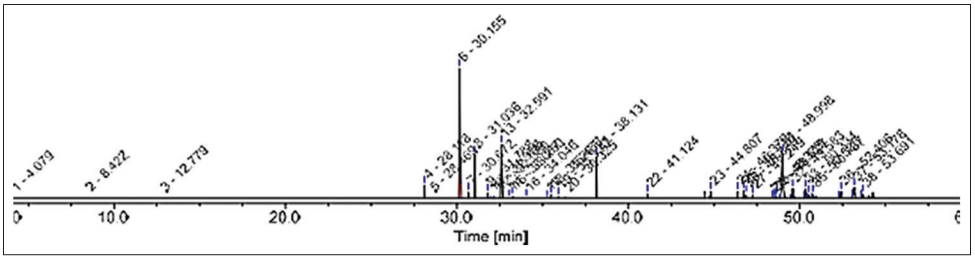

Figure 1: Chromatogram of *Annona muricata* ethyl acetate 2

the relative abundance of 12.16% and 11.53%, respectively. Three peaks were shown close similarity (>75%) with phytol. This profile, where the phytocompounds are both polar and nonpolar, indicates the wide range of compounds present in the extract. Neophytadiene and palmitic acid are known to possess antioxidant activities because of their conjugated double bonds. Anti-inflammatory activities were also witnessed in the two compounds in a previous study. Phytol may act as an antioxidant by donating the electron to free radicals through its OH moieties, while palmitic acid utilizes its conjugated double bond.

CONCLUSIONS

The ethyl acetate extract from *A. muricata* leaves had considerably high antioxidant capacity and activity, along with high cytotoxicity. The fractions had lower cytotoxicity but higher DPPH scavenging activity. Some potential cytotoxic and antioxidant compounds, such as neophytadiene, palmitic acid, and phytol, were found in the fraction with the optimum cytotoxicity and antioxidant activity. We recommend the isolation of pure compounds for future studies.

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Conflicts of interest

There are no conflicts of interest.

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