Antibacterial, cytotoxicity and metabolite profiling of crude methanolic extract from andaliman (Zanthoxylum acanthopodium) fruit

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Abstract. Sibero MT, Siswanto AP, Frederick EH, Wijaya AP, Syafitri E, Farabi K, Murwani R, Saito S, Igaras Y. 2020. Antibacterial, cytotoxicity and metabolite profiling of crude methanolic extract from andaliman (Zanthoxylum acanthopodium) fruit. Biodiversitas 21: 4147-4154. The local community in North Sumatra has utilized andaliman fruit (Zanthoxylum acanthopodium) as spices for traditional cuisines because it has a unique flavor. Information on the antimicrobial activity of Z. acanthopodium fruit against aquaculture pathogens and its bioactivity against leukemia cell lines are limited. The purposes of this study were to evaluate the antimicrobial activity of Z. acanthopodium fruit against Tenacibaculum maritimum, Vibrio alginolyticus, V. anguillarum, V. harveyi that are known as pathogens in aquaculture; to determine cytotoxic property against murine P388 leukemia cells; and to characterize its metabolites profile. The sample was extracted using methanol by the maceration method. Antibacterial assay was conducted by Kirby-Bauer disc diffusion method; while cytotoxicity assay using the XTT method. Proximate analysis showed that Z. acanthopodium fruit contained 63.41% of moisture, 24.73% of crude fiber, 9.81% of crude protein, 6.90% of ash, and 2.55% of crude fat. Several phytochemical components were detected, such as alkaloid, flavonoid, tannin, triterpenoid, and steroid. The GC/MS analysis indicated the presence of various compounds from terpenoid and terpenes derivatives. This study indicated that Z. acanthopodium fruit was not potential as antibacterial agents against the aquaculture pathogens; however, the methanol extract showed cytotoxic potential with IC50 19.14 µg/mL against murine P388 leukemia cells.

Key words: Andaliman, cytotoxicity, leukemia, terpenes, Zanthoxylum

INTRODUCTION

The genus Zanthoxylum is a member of the Rutaceae family, with 225 species found in pantropic countries (Appelhans et al. 2018). In Indonesia, Zanthoxylum acanthopodium, known as andaliman or tuba (in Karonese language), is a species that grows in North Sumatra, Indonesia. This plant was reported to grow in Dairi, North Tapanuli, Sidikalang, Simalungun, Sumbul, and several districts around Lake Toba (Siregar 2003; Sinaga et al. 2015; Asbur and Khairunisayah 2018; Junaeda and Nuralaeni 2019; Saragh and Arsita 2019). Z. acanthopodium fruit is commonly used in traditional cuisine because it gives a unique flavor and taste (Wijaya et al. 2002; Purba et al. 2018; Wijaya et al. 2019). There is still limited information on the use of Z. acanthopodium as a traditional medicine in the community in North Sumatra (Kristanty and Suriawati 2015; Saragh and Arsita 2019).

This plant is well known as a potential producer of essential oils (Wijaya et al. 2002; Asbur and Khairunisayah 2018; Wijaya et al. 2019). The unique flavor of Z. acanthopodium essential oils due to volatile compounds such as β-myrcene, limonene, and citronellal (Wijaya et al. 2019). Previous studies showed the potency of Z. acanthopodium as an antioxidant, antibacterial against human pathogens, anti-inflammatory, and antiacne (Julistiono et al. 2018; Wijaya et al. 2019). The potency of Z. acanthopodium against breast (T47D and 4T1), and cervical cancer cell (HeLa) lines were reported previously (Rosidah et al. 2019; Satria et al. 2019; Syari et al. 2019). However, its potency against leukemia cancer cells has not been determined yet. Leukemia is one of the five types of cancer that causes the highest mortality in Indonesia (WHO 2019). Due to this condition, Z. acanthopodium is expected to have cytotoxic activity against leukemia cancer cells.

Besides its therapeutic benefits, Z. acanthopodium is expected to be used in more widely application such as in the aquaculture industry. One of the common problems in aquaculture is a pathogenic infection caused by Tenacibaculum maritimum, Vibrio alginolyticus, V. anguillarum, V. harveyi (Li et al. 2018; Liu et al. 2016;
Småge et al. 2016). The administration of antibiotics in aquaculture to cure or prevent infection has become a serious problem since it leads to antimicrobial resistance in pathogens (Mo et al. 2015; Santos and Ramos 2018). Moreover, the residue of antibiotic in aquaculture practices also threaten consumer’s health and cause a microbial disturbance in the aquatic environment (Hedberg et al. 2018; Liu et al. 2017). Since Z. acanthopodium has been reported as a potential antimicrobial against human pathogens, this plant is also expected to exhibit the same activity against aquaculture pathogens. Hence, the purposes of this study were to evaluate the antibacterial activity against aquaculture pathogens, evaluate cytotoxic activity against P388 leukemia cancer cells, as well as to characterize metabolites using proximate analysis, phytochemical and chromatography approach.

MATERIALS AND METHODS

Sample preparation
One kg of Z. acanthopodium fruit was purchased from a local farmer in Parsoburan Village, Toba Samosir District, North Sumatra, Indonesia in September 2018. Identification was carried out at the Dharmawangsa University, Medan, Indonesia. The sample was packed in a Styrofoam box to maintain the quality during the shipping process to the Laboratory of Natural Products, Diponegoro University, Semarang, Indonesia. Z. acanthopodium was air-dried under sunshine. Furthermore, the sample was ground using a blender.

Proximate analysis
A total of 200 g of fresh Z. acanthopodium was used for proximate analysis, (moisture, ash, crude fiber, crude protein, and crude lipid) according to the AOAC method (AOAC 2019).

Metabolite extraction
A total of 240 g of the powdered Z. acanthopodium fruit was extracted with methanol with agitation (120 r.p.m., 27 °C) for 24 h. The filtrate was concentrated using a rotary evaporator at 30-35 °C, and the concentrated extract was partitioned with ethyl acetate using a separating funnel. Ethyl acetate extract was concentrated with a rotary evaporator.

Phytochemical analysis
Phytochemical analysis was done to determine the phytochemical compounds of Z. acanthopodium. Qualitative detection of phytochemical compounds was carried out as follow:

Alkaloid
Five grams of Z. acanthopodium fruit powder was immersed in 15 mL of 2N sulfuric acid for 15 minutes and then filtered using filter paper. The filtrate was divided into three and put into three test tubes. Each test tube contained 5 mL of extract. Dragendorff, Mayer, and Wagner reagents were added to the test tube, then the precipitation on the bottom of the test tube was observed. The presence of alkaloids was indicated by the presence of orange to reddish precipitation when added with Dragendorf, white to yellowish precipitation when added with Mayer and brown precipitation when added with Wagner (Harborne 1973; Bhandary et al. 2012).

Flavonoid
The detection of flavonoids was done using the Shinoda method as follows: two grams of Z. acanthopodium fruit powder was extracted with 10 mL of ethanol 96% without evaporation. A test tube containing 5 mL ethanol extract was added with 0.1 mg of Magnesium powder and 1 mL of amyl alcohol, then shaken. Then, 4 mL of ethanol was added to the wall of the test tube carefully. The formation of yellow/orange to reddish color in the amyl alcohol layer indicated the presence of flavonoids (Bhandary et al. 2012; María et al. 2018).

Saponin
One gram for Z. acanthopodium fruit powder was added with 7 mL of water in a test tube and then heated to boiling. Shake test tube vigorously until the foam layer was formed then left for 30 min. The stability of the foam layer was tested by the addition of 1 drop of 2N HCl. A stable foam after the stability test indicated the presence of saponin (Bhandary et al. 2012; María et al. 2018).

Tannin
Ferric chloride test was carried out to detect the presence of tannin in the Z. acanthopodium water extract. Two drops of 1% FeCl3 were added into 2 mL of Z. acanthopodium water extract. A presence of greenish to the black color indicated the presence of tannin (María et al. 2018; Pringgenies et al. 2018).

Steroid/triterpenoid
The Liebermann-Burchard test detected steroid/triterpenoid. Three mL of methanol extract was mixed with several drops of acetic anhydride, then heated using a water bath and cooled down. Several drops of concentrated H2SO4 were added gently through the side of the test tube. The formation of green color in the upper layer and deep red color in the lower layer indicated the presence of steroid/terpenoid (Bhandary et al. 2012; Seow et al. 2013).

Quinone
Borntraeger reaction was performed to detect the presence of free quinones in the Z. acanthopodium methanol extract. Two mL of methanol extract was transferred into the test tube, then 1 mL of 2N NaOH was added. The formation of pink to red/violet color indicated the presence of quinone (Khlif et al. 2015; María et al. 2018).

GC/MS analysis
GC/MS analysis was performed using Shimadzu GCMS-QP2010 SE and Rtx-5MS column package (0.25 mm × 30 m, 0.25 µm). One µL sample (concentration 1 mg/mL) was injected in split mode at a temperature of 300
RESULTS AND DISCUSSION

Proximate analysis was performed to determine primary metabolites of Z. acanthopodium fruit. The result of this analysis is presented in Table 1. The result of proximate analysis (Table 1) showed moisture content of fresh Z. acanthopodium fruit was 61.71%. Z. acanthopodium fruit contained 24.73% of crude fiber, 9.81% of crude protein, 6.90% of ash, 2.55% of crude fat based on a dry weight basis. This result was similar to a previous report by Asbur and Khairunnisyah (2018). Their study stated that the dried Z. acanthopodium fruit content 25.98% of carbohydrate and 8.01% of essential oil. Carbohydrates content in this study was not determined. There is a significant difference between the results of this study with the results of the previous analysis by Asbur and Khairunnisyah (2018) on ash and protein contents was noted between our result and the previous study. Plant maturity, environmental stress and disturbance, fertilizer, geographical distribution and drying method affect an important influence to the proximate content in the plant (Khattak and Rahman 2015; Kovach et al. 1992; Mbah et al. 2012; Oduntan and Olalaye 2012; Vihotogbe et al. 2013) which were not evaluated in this current study. Therefore, the difference of ash and protein contents in our study to the reference was also suggested as the result of those unevaled variables.

In this study, phytochemical screening was carried out to determine secondary metabolites of Z. acanthopodium fruit. The result was presented in Table 2. The phytochemical analysis showed Z. acanthopodium fruit contained alkaloid, flavonoid, saponin, steroid, triterpenoid, and tannin. A review by Singh and Singh (2011) showed that Zanthoxylum extract contained various derivatives compounds from alkaloids, amides, coumarins, flavonoids, lignin, terpenoids, steroids, and steroid. Further analysis using GC/MS showed 20 identified compounds (Figure 1 and Table 3).

The result of the GC/MS analysis successfully identified 20 chemical compounds in the methanol extract of Z. acanthopodium fruit. Sixteen chemical compounds are terpenes and terpenoids derivatives. The other four chemical compounds were structurally identified as aliphatic derived compounds (neoherculin; ethyl linoleate; ethanol, 2-(3,3-dimethylethylidene)-; and 9,12-Octadecadienyl chloride). All of these compounds have been reported in previous studies as secondary metabolites of Zanthoxylum species (Kumar et al. 2016; da Silva et al. 2017; Singh and Singh 2011; Wijaya et al. 2002, 2019) as the component of essential oils from Z. acanthopodium. The results showed that the main compounds of Z. acanthopodium fruit from Parsoburan village, Toba Samosir, North Sumatra were geranyl acetate (26.72%) and neoherculin (20.99%).
Figure 1. Gas chromatogram of *Zanthoxylum acanthopodium* fruit extract

Table 3. Identified chemical content of *Zanthoxylum acanthopodium* fruit

| Library compounds                   | Retention time | Peak area (%) | Structure |
|-------------------------------------|----------------|---------------|-----------|
| Citronellol                         | 6.46           | 1.30          | ![Citronellol Structure](image) |
| Geraniol                            | 6.82           | 7.31          | ![Geraniol Structure](image) |
| Geranyl acetate                     | 8.52           | 26.72         | ![Geranyl Acetate Structure](image) |
| trans-Caryophyllene                 | 9.27           | 1.51          | ![Trans-Caryophyllene Structure](image) |
| 2-hexadecen-1-ol                    | 16.99          | 1.72          | ![2-Hexadecen-1-Ol Structure](image) |
| Ethyl linoleate                     | 17.38          | 1.03          | ![Ethyl Linoleate Structure](image) |
| Neoherculin                         | 17.48          | 20.99         | ![Neoherculin Structure](image) |
| Myrtenol                            | 17.62          | 1.19          | ![Myrtenol Structure](image) |
| 5- (propenyl-2)-1,3,7-nonatriene    | 17.72          | 2.39          | ![5- (Propenyl-2)-1,3,7-Nonatriene Structure](image) |
The result of the antibacterial activity of *Z. acanthopodium* fruit against aquaculture pathogens is presented in Table 4.

**Table 4.** Antibacterial activity of *Zanthoxylum acanthopodium* fruit

| Aquaculture pathogens | Antibacterial activity |
|-----------------------|------------------------|
| *Tenacibaculum maritimum* | NA                     |
| *Vibrio alginoliticus* | NA                     |
| *Vibrio anguillarum* | NA                     |
| *Vibrio harveyi* | NA                     |

Note: NA: not active. Antibacterial assay was done using a concentration of 2 mg/mL.

Several previous studies showed that *Z. acanthopodium* has antibacterial activity against some of the human pathogens bacteria. Julistiono et al. (2018) reported that the hexane extract of *Z. acanthopodium* fruit showed antibacterial activities against *Mycobacterium smegmatis*. A study by Muzafri et al. (2018) showed that the methanol extract had better antibacterial activity than water and hexane extracts against *E. coli*, *Salmonella typhimurium*, and *S. aureus*. However, the results of this study showed that the methanol extract of *Z. acanthopodium* fruit did not exhibit any antibacterial activity against aquaculture pathogens (*T. maritimum*, *Vibrio alginoliticus*, *V. anguillarum*, *V. harveyi*). The absence of antibacterial
property in this study may be due to several factors such as unsuitable organic solvents to extract antibacterial compounds and the inability of the metabolites to inhibit the pathogens (Bacon et al. 2017). Zanthoxylum species are widely reported to have antimicrobial properties (Julistiono et al. 2018; Muzafri et al. 2018), unfortunately, only a few works determine the lead compounds. Tantapakul et al. (2012) successfully isolated 10 compounds from Z. rhetsa, however only one compound, dihydro chelerythrine, performed moderate antibacterial activity against S. aureus and E. coli. There has been no report on the lead compounds of Z. acanthopodium fruit, which have antibacterial property.

In this study, the cytotoxic activity of Z. acanthopodium against murine P388 leukemia cells was carried out. The result is presented in Figure 2 and Figure 3.

Figure 2. Percentage of viable murine P388 leukemia cells treated with Zanthoxylum acanthopodium fruit extract (A) and doxorubicin (B)

Figure 3. Morphological changes of murine P388 leukemia cells treated with: doxorubicin 0.5 µg/mL (C), methanol extract: 0.0025 µg/mL (D), 0.025 µg/mL(E), 0.25 µg/mL (F), 2.5 µg/mL (G), 25 µg/mL (H-I) after 72 h of incubation. Normal cells without treatments (A-B). Red arrows manifest the cell shrinkage; blue arrows manifest the cell blebbing, yellow arrows manifest the cell fragmentation)
The methanol extract of *Z. acanthopodium* fruit had an IC\textsubscript{50} value of 19.14 µg/mL. It means that methanol extract at the concentration of 19.14 µg/mL can inhibit 50% (IC\textsubscript{50}) of the proliferation of murine P388 leukemia cells, while doxorubicin had an IC\textsubscript{50} value of 0.02 µg/mL. (Figure 2). American National Cancer Institute (NCI) considered IC\textsubscript{50} < 30 µg/mL as a potential cytotoxic agent in the preliminary assay (Suffness and Pezzuto 1991), therefore andaliman crude extract was suggested as a potential source of anticancer agent to treat leukemia. Morphological characters of murine P388 leukemia cells were a high density, round shape with green translucent color (Figure 3.a-b). Murine P388 leukemia cells induced by 0.5 µg/mL doxorubicin as a positive control caused cell shrinkage (red arrows) then lead to cell death. It indicated that doxorubicin killed leukemia cells through apoptotic mechanisms because cells were broken down and then bleb (blue arrows) and formed cell fragmentations (yellow arrows) (Elmore 2007; Furusawa et al. 2001). A similar mechanism was indicated by cells treated with methanol extract of *Z. acanthopodium* fruit. The red arrow in Figure 3.i shows that methanol extract induced cell shrinkage and bleb in the murine P388 leukemia cells, but the cell fragmentation was not found in the treatment of methanol extract. Cell shrinkage and cell fragmentation are detected as the early stage of apoptosis, which leads to the formation of apoptotic cells as the late stage in the apoptosis mechanisms (Fink and Cookson 2005; Elmore 2007).

Further study is strongly suggested to determine the mechanism of inhibition of *Z. acanthopodium* fruit extract against cancer cells. This study showed that *Z. acanthopodium* fruit extract has the potential as a natural anticancer against murine P388 leukemia cells. Bioactivity of *Z. acanthopodium* fruit extract may be due to the presence of several bioactive compounds. Several terpenes and terpenoids derivatives have been reported as an anticancer agent such as geranyl acetate, caryophyllene, and β-myrcene (da Silva et al. 2007; Li et al. 2013). Results of GC-MS analysis showed that the methanol extract of *Z. acanthopodium* fruit contained geranyl acetate (26.72%) as the main compound (Table 3).

In conclusion, methanol extract of *Z. acanthopodium* fruit from Parsoburan village, Toba Samosir, North Sumatra, did not present any antibacterial activity against all aquaculture pathogens tested (*Tenacibaculum maritimum*, *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi*). However, methanol extract showed cytotoxic activity against murine P388 leukemia cells with an IC\textsubscript{50} value of 19.14 µg/mL. Phytochemical content of *Z. acanthopodium* fruit was alkaloid, flavonoid, saponin, steroid, triterpenoid, and tannin derivatives. The GC/MS analysis discovered the presence of 20 identified secondary metabolites in methanol extract, and geranyl acetate as its major metabolite.

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