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Chemical Analysis and Biological Activities of *Annona muricata* Leaves Extract from Sarawak

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Abstract: *Annona muricata* (soursop) leaves have been widely known for its ethnomedicinal uses, anti-cancer, anti-microbial and antioxidant activities. Previous studies reported that *A. muricata* have the potential to exhibit anti-cancer, antimicrobial and antioxidant activities. However, there are very few studies of *A. muricata* from Sarawak. These phytochemical and biological studies were aimed to determine the chemical constituents, total flavonoid content, total phenolic content, antioxidant, antimicrobial and anticancer activities. The leaves of *Annona muricata* was extracted using 95% ethanol and yielded 13.03% crude extract. The compound was obtained through isolation and purification by using various chromatographic methods such as radial chromatography and Thin Layer Chromatography (TLC) while the structure was elucidated based on the spectral data such as Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared (FT-IR) and Gas Chromatography-Mass Spectroscopy (GC-MS). The Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) were determined using Folin-Ciocalteu and aluminium chloride methods, respectively. TPC in the ethanolic extract was 195.05 mg GAE/g while the TFC was 64.25 mg QE/g. The antioxidant activity of ethanolic extract which performed using DPPH (2, 2-Diphenyl-1-Pycryl-Hydrazayl) assay has recorded IC_{50} of 21.03 μg/mL. The antiproliferation activity of this extract against three cancer cell lines, namely breast cancer (MCF-7), cervical cancer (HeLa) and liver cancer (HepG2) was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide (MTT) assay. The ethanolic leaf extract was found to be selectively cytotoxic in vitro to HeLa, MCF-7 and HepG2 cell lines with IC_{50} values of 1.4111, 1.7382 and 2.7767 mg/mL, respectively. For the antimicrobial activities, the crude was examined on four bacteria named *Staphylococcus aureus* (SA), *Streptococcus pyogenes* (SP), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA) using MIC and MBC. The extract also showed strong antimicrobial activities with MIC and MBC values of 450 μg/mL. As such *Annona muricata* may have excellent potential for further development for an antioxidant as well as an alternative antibacterial agent.

Key words: *Annona muricate*, total phenolic, total flavonoid, anticancer, antioxidant, antimicrobial

INTRODUCTION

Annona belongs to Annonacae family, is a genus of flowering plants. Annona is the second largest genus after Guatteria where it consists about 160 species of mostly neotropical and afrotropical trees and shrubs. This species is typically grown for its domestic and commercial use and also for the edible and nutritious fruit. In Malaysia, *A. muricata* is easily available throughout the year (Cheong *et al*., 2011).

A.muricata has been widely known and used for its ethnobotanical properties. According to Sathishkumar and Srinivasan (2015) it exhibits pharmacological activities such as cytotoxic, anti-leishmanial, wound healing and anti-microbial. *A. muricata* also contains an active source of substances with anti-cinoceptive and anti-inflammatory activities (De Sousa*et al*., 2010). Most of the parts of *A. muricata* tree such as bark, leaves, fruits as well as its seeds have been used as traditional medicine. For examples, the leaves are used traditionally for insomnia, diabetes, headache, cystitis as well as an anti-inflammatory (Gavamukulya *et al*., 2014) while the decoction of bark, root, seed or leaf are widely used for the preparation of medicine (Coria-Tellez *et al*., 2018).

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There are about 212 bioactive compounds that have been found in *A. muricata* (Coria-Tellez *et al*., 2018). Patel and Patel (2016) reviewed that different parts of *A. muricata* plant contained various chemical compounds such as alkaloids, megastigmanes, flavonol triglycosidesm phenolic, cyclopeptides and essential oils. Besides, Agu and Okolie (2017) study on the phytochemical screening of leaves extracts also reported the presence of tannins, terpenoids, saponins, flavonoids, alkaloids and phenolic. Another study by Gavamukulya *et al*. (2015) supporting this finding where this plant is rich in saponins, alkaloids, terpenoids, flavonoids and other phenolic compounds. Acetogenins is the predominant compound followed by alkaloids, phenols and other compounds (Coria-Tellez *et al*., 2018).

A. muricata exhibits various biological activities such as antioxidant activities, antimicrobial activities and anti-cancer properties. The present *in vitro* study of antimicrobial evaluation of *A. muricata* leaves extract became a major platform for future studies (Solomon-Wisdom *et al*., 2014). The aqueous extract of *A. muricata* leaves has recorded high antioxidants power with an IC_{50} value of 5.8 mg/mL compared to the ethanolic extract with an IC_{50} value of 0.8243 mg/mL (Gavamukulya *et al*., 2014). However, another study showed that the ethanolic leaves extract gave significant antioxidant property (Baskar *et al*., 2007). Besides that, George *et al*. (2015) study revealed that the methanolic leaves extract exhibited higher scavenging potential of free radicals with IC_{50} value of 119 g/mL compared to that of aqueous extract IC_{50} value of 400 g/mL.

Humankind has been used the plant as a medicinal to treat common infectious disease a long time ago before they discovered the existence of microbes. *A. muricata* demonstrated potential against the antimicrobial activity. Vijayameena *et al*. (2013) reported that the ethanolic leaves extract of *A. muricata* gave strong activities against *Pseudomonas* and *Staphylococcus*. Furthermore, the *A. muricata* leaves extract was reported first time by Pinto *et al*. (2017) that it has a broad spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria and as well as the bacterial membranes including both plasma and outer membrane which are the primary targets of the bioactive compounds.

Beside antimicrobial activities properties, *A. muricata* also well known in the community as an alternative option for cancer therapy (Moghadamtousi *et al*., 2015 as cited in Roduan *et al*., 2018). The ethanolic leaves extract showed positive *in vitro* cytotoxic against the same human breast cancer cell lines (Gavamukulya *et al*., 2014). Another finding by Liu *et al*. (2016) showed that the leaves extract of *A. muricata* can cause apoptosis of liver cancer cell (HepG2). Besides, Chen *et al*. (2012) study on *Annona squamosal* found that the seed extract showed the positive result as anti-tumor against human breast carcinoma (MCF-7) and human hepatoma carcinoma (HepG2) with IC_{50} values of 0.25 and 0.36 μg/mL, respectively.

The previous study reported that *A. muricata* have the potential to exhibit anti-cancer, antimicrobial and antioxidant activities. However, there are very few studies of *A. muricata* from Sarawak. Thus, this study aimed to determine the chemical and biological profile on *A. muricata* to reveal basic information or to identify the chemical fingerprint of this *A. muricata* from Sarawak.

MATERIALS AND METHODS

Sample: The leaves of *A. muricata* were collected from the backyard in Kampung Paya Mebi, Kota Padawan, Kuching, Sarawak, Malaysia.

Sample preparation: The fresh leaves were collected and cleaned. The leaves were cut into smaller pieces. The leaves were then dried for about 4 days or until it thoroughly dried in an oven at 40°C. The dried leaves sample was ground until powder form. About 40 g of dried powder sample was soaking in 400 mL ethanol and left overnight for 24 h. The samples were filtered and transferred into Rotary evaporator (Rotavap) to remove the solvent under vacuum condition. The yielded ethanolic extract was kept in a refrigerator (4°C) before being further analyzed.

Isolation and purification

Thin Layer Chromatography (TLC): The sample isolation from each fraction was collected and examined using TLC to identify the purity of a chemical compound. The TLC plate was carried out using the aluminium sheets coated with silica gel 60 F254 (Merck 1.05735) with a thickness of 0.25 mm. The size of the TLC plate of 5×5 and 0.5 cm line was marked at the upper and bottom part. By using a microcapillary tube, the sample was spotted onto the TLC plate after being dissolved in acetone. The plate was placed in a chamber containing mixture and pre-saturated with solvent vapor.

Centrifugal thin layer chromatography (Chromatotron): Apart from TLC plates, HPLC and column chromatography, radial chromatography method (chromatotron) also can be used to separate the chemical compound. Silica gel 60 PF254 containing gypsum (Merck 1.07749.1) was used to prepare the chromatotron plate with a thickness of 0.5 mm. 35 g of the silica was dissolved in 73 mL cold distilled water then stirred and poured onto the plate. The plate was left overnight in the oven at 60°C. The sample was placed through the hole onto the

plate. The partition of the compound on the plate was determined by using UV light. Theeluent was applied to the plate as a mobile phase and each separation was collected by using different vials.

Structure elucidation of chemical compound: The pure chemical components isolated from the ethanolic extract of *A. muricata* leaves was identified by various spectroscopic data such as Nuclear Magnetic Resonance Spectrometry (NMR), Gas Chromatography-Mass Spectrometry (GC-MS), Infrared spectroscopy (IR) and Ultra-Violet Visible spectroscopy (UV-Vis).

Chemical analysis

Total Phenolic Content (TPC): The total phenolic content was performed based on the Folin-Ciolcalteu method (Alizadeh *et al*., 2013). Gallic acid was used for the standard. The standard solutions were preparedat a concentration range of 0.2, 0.4, 0.6, 0.8 and 1.00 mg/mL. For the preparation of the sample, 300 μL of the sample was dissolved in methanol (1 mg/mL) and 750 μL of Folin-Ciocalteure agent (diluted ten times in distilled water) was added in a glass tube. The sample was placed in the incubator for 5 min at room temperature. After that, 3000 μL of 7.5% Na_2CO_3 solution was added and the glass tube was kept in the dark for 90 min. The absorbance was measured at 725 nm by using a UV-visible (LAMBDA 25, Perkin Elmer).

Total Flavonoids Content (TFC): Colorimetric method was used to determine the total flavonoids content of samples (Mohamed *et al*., 2013). Quercetin was used as the standard. The standard solutions were prepared at concentration range from 0-0.14 mg/mL. For the preparation of the sample, 50 μL of the sample (5 mg/mL ethanol) was marked up to 1 mL with ethanol and then added with 4 mL of distilled water followed with 0.3 mL of 5% $NaNO_2$ solution. A 0.3 mL of 10% $AlCl_3$ solution was added and allowed to stand for 5 min. The 2 mL of 1M NaOH solution was added to the mixture after 5 min. of incubation. The solution was topped up to 10 mL with distilled water and the mixture was allowed to stand for 15 min. The absorbance was measured at a wavelength of 510 nm using UV-visible spectrophotometer (LAMBDA 25, Perkin Elmer).

Biological activities

Antioxidant activity: The antioxidant activity of the crude extract was performe dusing DPPH (2,2-Diphenyl-1-Pycryl-Hydrazayl) assay with minor modification as described by Khong *et al*. (2009). The 6 mg of the crude extract was weighed and added with 6 mL of ethanol to prepare the sample stock solution (1 mg/mL). The sample stock solution was further used to prepare a different concentration of 100, 50, 25, 12.5, 6.25 and 3.125 ppm using a series of dilution. The DPPH stock solution was freshly prepared. The 3.94 mg of DPPH was dissolved in 100 mL ethanol to obtained 0.04 mg/mL concentration solution. In each sample, 1 mL of DPPH solution was added into each sample. The mixture was then incubated in the dark room at room temperature for 30 min. The absorbance was measured at 517 nm. The percent inhibition of free radical was calculated with the equation as shown below:

$$\text{Inhibition (\%)} = \frac{\text{A blank} - \text{A sample}}{\text{A blank}} \times 100\%$$

Antimicrobial activity

Preparation of Nutrient Agar (NA): The 28 g/L of NA powder was dissolved in distilled water and stirred well for the process of preparing the agar. The mixture was then autoclaved at 121°C for 15 min. The mixture was poured aseptically into petri dishes after the solution becomes warm enough.

Preparation of Nutrient Broth (NB): Agar was prepared by dissolving 13 g/L of NB powder in distilled water and mixed well. The mixture was then autoclaved at 121°C for about 15 min.

Bacterial strain: Four bacteria were used to test the sample, namely two Gram-positive bacteria which are *Streptococcus pyogenes* (SP) and *Staphylococcus aureus* (SA) and two Gram-negative bacteria which are *Escherichia coli* (EC) and *Pseudomonas aeruginosa* (PA). All the bacterial strains were cultured overnight at 37°C in nutrient agar.

Minimum Inhibitory Concentration (MIC) assay: The nutrient broth and nutrient agar solutions were prepared and sterilized along with pipette, pipette tips and beakers using an autoclave. A of sample stock solution with a concentration of 1800 μg/mL was prepared by dissolving 3.6 mg of sample in 2.0 mL of Dimethyl Sulfoxide (DMSO). The preparation of agar onto the petri dish was conducted in laminar flow. The solidified agar was kept in the refrigerator. The bacteria were cultured by adding the bacteria in warm NB into the bottles. The bottleswere then incubated for 24 h at 30°C.

A 100 μL of sample stock solution was added into each column of row A and B. After that, 100 μL of NB was added and diluted in the concentration ranged between 1800-14.07 g/mL. Twelve columns were prepared. Column 9 and 10 were added with 100 μL streptomycin sulphate as the positive control while column 11 and 12 were filled with 100 μL DMSO as the negative control. In each row and column (A-H) (Table 1), 100 μL of four types of bacteria were added onto the 96-well plates and it was then covered, sealed and incubated overnight at 37°C.

Table 1: The 96-well plates arrangement of MIC assay of different bacteria

	SA		SP		EC		PA		Positive control			Negative control
Variables (µg/mL)	1	2	3	4	5	6	7	8	9	10	11	12
A (1800)												
B (900)												
C (450)												
D (225)												
E (112.5)												
F (56.25)												
G (28.13)												
H (14.07)												

EC: *Escherichia coli*; PA: *Pseudomonas aeruginosa*; SA: *Staphylococcus aureus*; SP: *Streptococcus pyogenes*; Positive control: atreptomycin; negative control: DMSO

The arrangement of MIC assay on 96 well plates is shown in Table 1. The presence of turbidity and a pellet on the well bottom was observed and recorded.

Minimum Bactericidal Concentration (MBC) assay: MBC was performed to determine the number of organisms survived by observing the activity of the bacterial growth which evaluation was extended from the result of MIC. From the MIC test, the solution from the last clear well was taken and then pipetted onto the surface of the agar in a petri dish. The solution was spread gently using a sterilized glass rod. The petri dish was left overnight at a temperature of 37°C. The amount of concentration at 99% of the killed bacteria was recorded as the MBC values.

Anticancer activity: The sample was tested for its antiproliferative activity on three cancer cell lines which are breast cancer (MCF-7), cervical cancer (HeLa) and liver cancer (HepG2). All the cancer cell lines were obtained from the Thai Traditional Medicine College, Rajamangala University of Technology, 12130 Thanyaburi Pathumthani, Thailand. All cell lines were cultured and kept in an incubator at 37°C in 5% CO_2 atmosphere. The antiproliferation activity for this sample was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay as described by Korawinwich *et al.* (2016).

RESULTS AND DISCUSSION

Characterization of DB1: DB1 compound has been isolated as white transparent crystal. The mass spectrum revealed the molecular ion peak at m/z 207.1318 where it indicated the molecular formula of this compound was $C1_1H1_3NO_3$. Besides, the base peak for this compound was at m/z = 105 giving of 100% natural abundance while the other peaks observed were m/z 149 and 77. The mass fragmentation pattern of DB1 is shown as in Fig. 1-4.

Determination of Total Flavonoid Content (TFC): Flavonoid can be found ubiquitously in plants. Colorimetric method was used to determine the Total Flavonoids Content (TFC) in *A. muricata* ethanolic leaves extract. The TFC of *A. muricata* was spectrophotometrically analyzed and was expressed in term of quercetin based on the standard curve equation with $R^2 = 0.9882$.

In an ethanolic extract of *A. muricata*, the TFC was 64.25 mg QE/g of dried sample. This finding showed slightly higher compared to the previous study where the TFC reported was 9.96 mg QE/g. Furthermore, a study by Bryan-Thomas (2016) also r evealed that the leaves extract of *A. muricata* exhibited lower TFC value of 0.8 mg QE/g (Fig. 5).

Determination of Total Phenolic Content (TPC): The total phenolic content of *A. muricata*ethanolic extract was determined by using Folin-Ciocalteu reagent. The calibration curve of gallic acid was plotted ($R^2 = 0.9996$) as shown in Fig. 6 which was used to determine the Total Phenolic Content (TPC) of the sample. The TPC for the ethanolic extract of *A. muricata* was 195.05 mg GAE/g.

The TPC was higher (195.05 mg GAE/g) compared to the previous study reported by Gavamukulya *et al.* (2014) where the value was 0.37293±0.15 mg/mL GAE/g. However, the TPC in hot pressurized ethanol extract was 93.2±2.0 mg GAE/g (Moraes *et al.*, 2016) and slightly lower compared to the finding in this study. Phenolic compounds are one of the major contributions to antioxidant activity.

Antioxidant activity using DPPH assay: The 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging assay was used to determine the free radical capability of leaves extract of *A. muricata*. The absorbance was determined at 517 nm for its reduction of the photochemical present. The antioxidant activity was evaluated in term of inhibition concentration (IC_{50}).

The IC_{50} for ethanolic extract of *A. muricata* was 21.03 µg/mL. The IC_{50} value obtained from this finding was higher compared to the previous study by Gavamukulya *et al.* (2014) which was 0.8243 mg/mL. This finding is accepted as TPC of the ethanolic extract was higher. Besides, Moein and Moein (2012) study revealed the antioxidant activity has a positive correlation with the

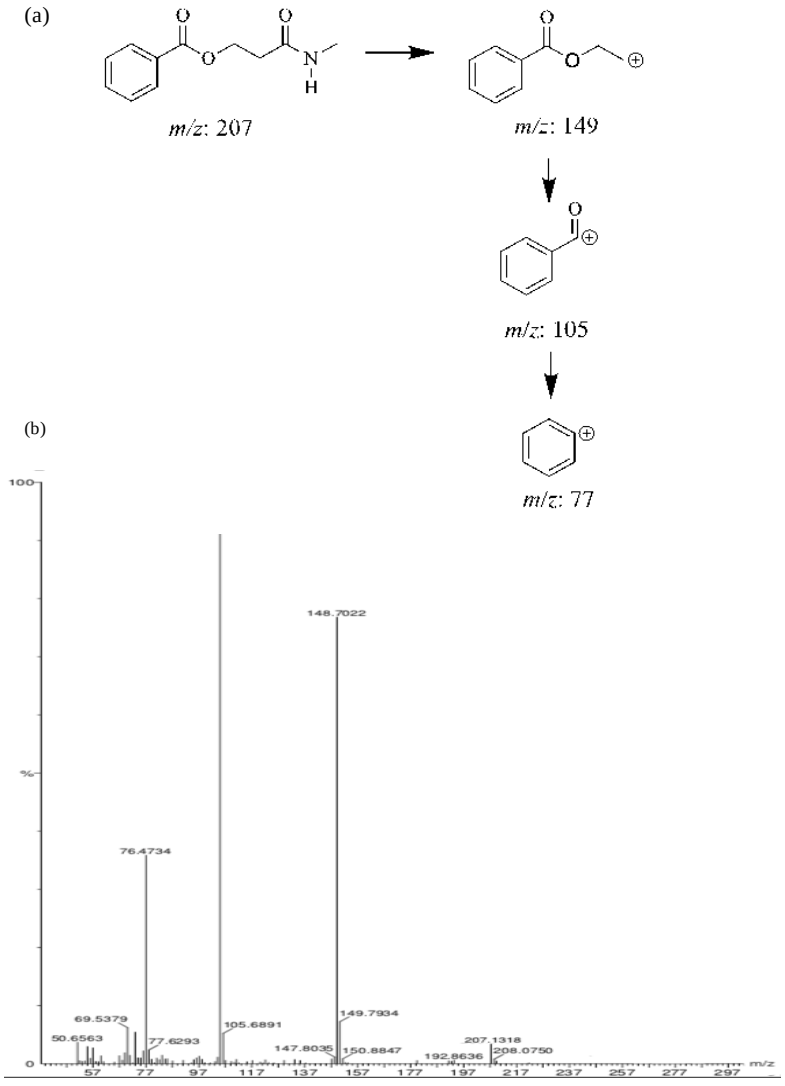

Fig. 1: a, b) Mass spectrum of DB1

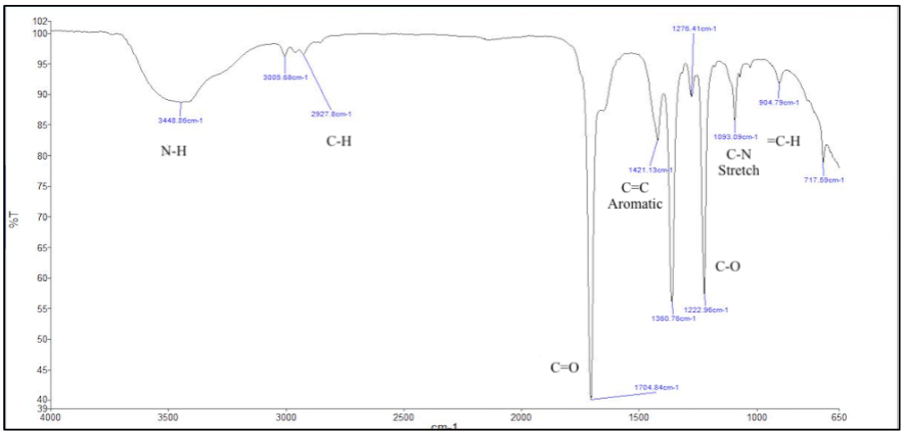

Fig. 2: IR spectrum of DB1

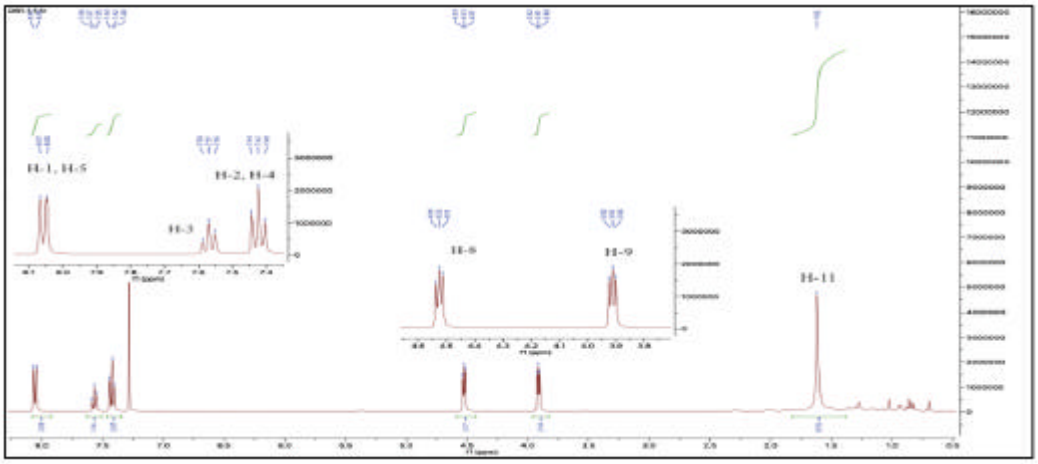

Fig. 3: ^{1}HNMR spectrum of DB1 ($CDCl_3$, 400 MHz)

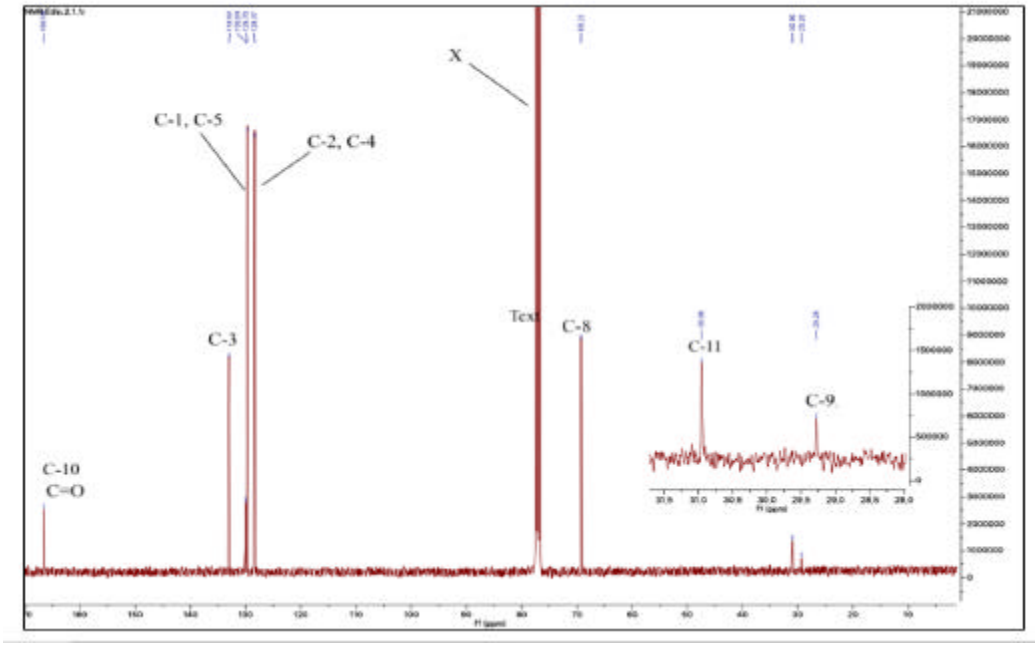

Fig. 4: ^{13}C NMR spectrum of DB1 ($CDCl_3$, 400 MHz)

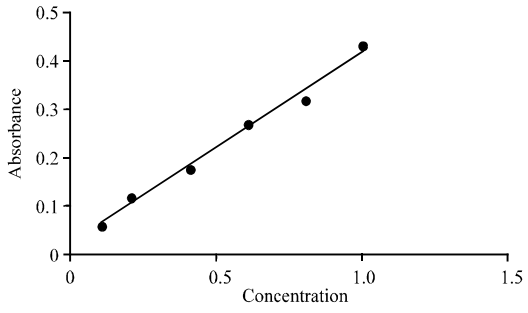

Fig. 5: Calibration curve of quercetin

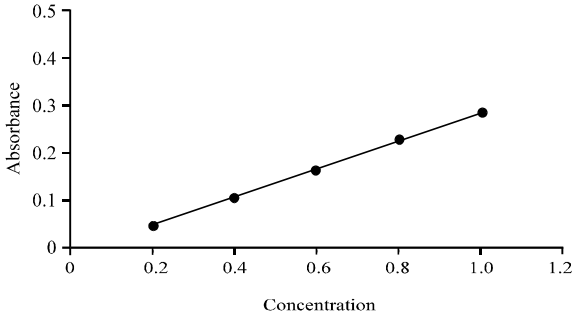

Fig. 6: Calibration curve of gallic acid

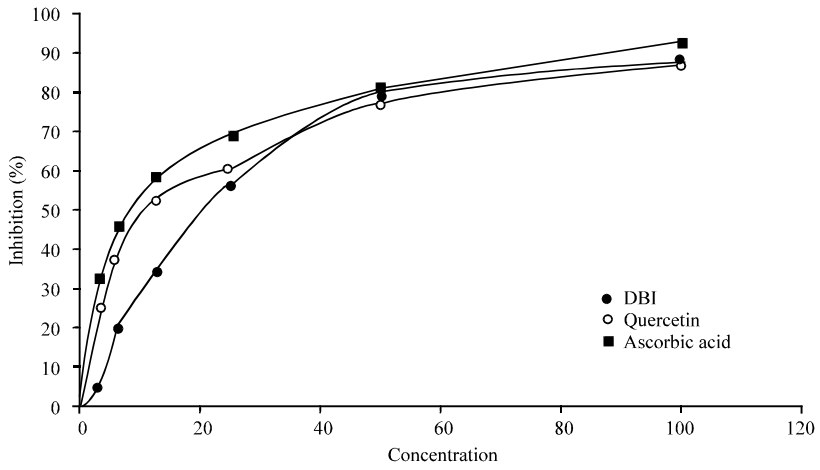

Fig. 7: Antioxidant activity of *A. muricata*

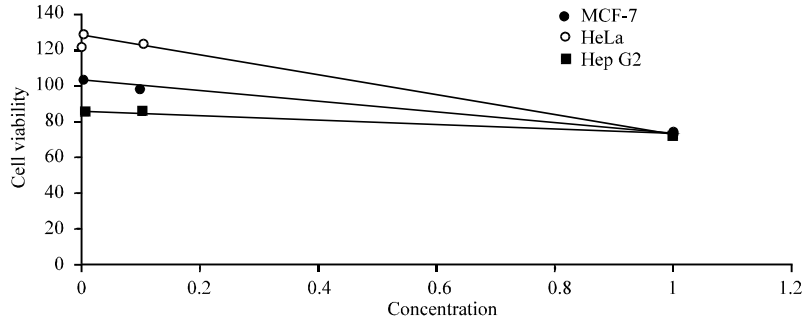

Fig. 8: Cell viability of ethanolic extract of *A. muricata* leaves extract

content phenolic compounds. Furthermore, this positive relationship also supported by Luzia and Jorge (2012) which stated that the phenolic compounds were one of the significant contributions to the antioxidant activity (Fig. 7).

Anticancer activities using MTT assay: Microculture assay based on the metabolic reduction of MTT was used to determine the cytotoxicity against three cancer cell lines, human breast adenocarcinoma cell line (MCF-7), human cervix cell line (HeLa) and human liver cell line (HepG2). In this study, the ethanolic extract of *A. muricata* leaves was evaluated for its cytotoxicity against MCF-7, HeLa and HepG2 cell lines (Table 2). Besides, three types of drugs named cisplatin, doxorubicin and fluorouracil were used as positive control. All of these drugs were commonly used in the chemo therapy to treat those cancer cells.The cell viability of ethanolic extract of *A. muricata* on MCF-7, HeLa and the HepG2 cell lines is shown in Fig. 8.

Table 2: Anticancer activity of *A. muricata* ethanolic leaves extract

	IC_{50} (mg/mL) of the extracts on cancer cell lines		
Samples	MCF-7	HeLa	HepG2
DB	1.7382	1.4278	2.7767
Cisplatin	0.0409	0.0398	0.0397
Doxorubicin	0.1291	0.1130	0.0519
Fluorouracil	3.4277	8.3664	3.7676

DB: *A. muricata* ethanolic leaves extract; Drugs: Cisplatin, doxorubicin, fluourocil

The ethanolic leaves extract was found to be selectively cytotoxic in vitro to HeLa, MCF-7 and HepG2 cell lines with IC_{50} values of 1.4111, 1.7382 and 2.7767 mg/mL, respectively (Table 2).

The *A. muricata*ethanolic extract showed weak inhibition against the three cancer cell lines when compared to the cisplatin and doxorubicin. Nevertheless, *A. muricata* is a better anticancer agent compared to fluorouracil. It can be said that the *A. muricata* from Sarawak showed positive activity towards breast cancer cell lines. Moreover, Najamuddin *et al.* (2016) revealed in his study that only one out of 19 samples of *A. muricata*

Table 3: MIC and MBC activities of *A. muricata*

	Bacteria concentration (µg/mL)							
	SA		SP		EC		PA	
Samples	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Streptomycin sulfate	14.07	14.07	14.07	14.07	14.07	14.07	14.07	14.07
DB	450	450	450	450	450	450	450	450

DB: *A.muricata* ethanolic leave extract; SA: *Staphylococcus aureus*, SP: *Streptococcus pyogenes*, EC: *Escherichia coli*, PA: *Pseudomonas aeruginosa*; <500 µg/mL: strong, 501-1500 µg/mL: moderate >1500 µg/mL: weak

samples showed anticancer activities toward MCF-7 which obtained from different state namely Johor, Melaka, Negeri Sembilan, Perlis, Selangor and Perak.

Antimicrobial activities: The findings revealed that the crude extracts inhibited all the growth for both Gram-positive and Gram-negative bacteria at Minimum Inhibitory Concentration (MIC) values of 450 µg/mL while Minimum Bactericidal Concentration (MBC) was the same values of 450 µg/mL. The results were summarized in Table 3. The *A. muricata* extract possessed strong inhibition against all of the bacteria.

The findings revealed that *A. muricata* ethanolic extract is a potent antimicrobial agent against all of the bacteria comparable with the standard antibiotic streptomycin. This finding is supported by Vijayameena *et al.* (2013) study which the ethanolic leaves extract of *A. muricata* demonstrated strong antimicrobial activities against *Pseudomonas* and *Staphylococcus*. Furthermore, Rajeswari *et al.* also confirmed that the leaves extract of *A. muricata* was used for the treatment of diarrhea, pneumonia, urinary tract infection and some skin disease. As such the *A. muricata* is a potential antimicrobial agent, especially, to inhibit *Escherichia coli* bacteria that caused diarrhoea. According to Gavamukulya *et al.* (2017) the bioactivity efficiency of the *A. muricata* leaves extract is depends on the kind of solvent used in the extraction. The ethanolic extract of *A. muricata* showed significant antibacterial activity against *Staphylococcus aureus* (Gavamukulya *et al.*, 2017).

Total flavonoid content and total phenolic content of the ethanolic extract of *A. muricata* were 64.25 mg QE/g and 195.05 mg GAE/g, respectively. The antioxidant assay based on DPPH free radical scavenging activity revealed that the ethanolic leaves extract of *A. muricata* has strong scavenging activity with an IC_{50} value of 21.03 µg/mL. Both flavonoid and phenolic content contributed to the high antioxidant activity because the flavonoid content and phenolic content showed a positive relationship with antioxidant activity.

For the anticancer activity, the ethanolic leaves extract was found to be selectively cytotoxic *in vitro* to HeLa, MCF-7 and HepG2 cell lines with IC_{50} values of 1.4111, 1.7382 and 2.7767 mg/mL, respectively. However, the ethanolic leaves extract of *A. muricata* showed stronger activity when compared to fluorouracil, chemotherapy drugs.

Besides, the crude extract demonstrated strong inhibition for both Gram-positive and Gram-negative bacteria namely *Staphylococcus aureus* (SA), *Streptococcus pyogenes* (SP), *Escherichia coli* (EC) and *Pseudomonas aeruginosa* (PA) in the MIC and MBC assays. This finding revealed that the *A. muricata* is a potential antimicrobial agent.

CONCLUSION

Further study on *A. muricata* leaves can be carried out with similar chemical analysis study and biological activities on different extraction methods and solvents extraction. Findings from this study are promising for further development of the pharmaceutical products. Furthermore, the medicinal properties of this plant can be further evaluated and developed for innovative products which could contribute to the higher income nation. As such, this finding provided useful information and contributed to the importance of the chemotaxonomic study of this genus from the Sarawak Region.

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