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Alkaloids from the root of Indonesian *Annona muricata* L

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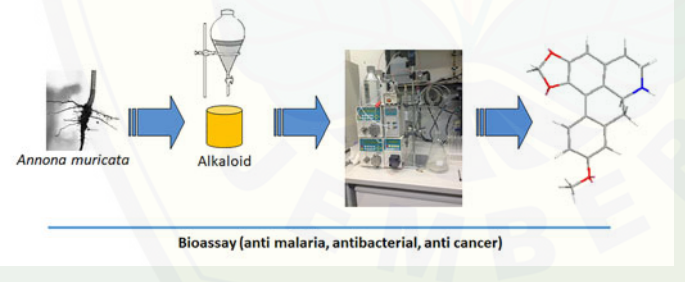
ABSTRACT

Annona muricata L. has been used traditionally in Indonesia to treat disease. Phytochemical studies on the alkaloid fractions from the root of *Annona muricata* L. from Malang-Indonesia resulted in the isolation of an unreported benzylisoquinoline alkaloid (+)-xylopine **5** as well as four known alkaloids (**1**–**4**). The crude methanol extract and alkaloid fractions were tested against *Plasmodium falciparum* K1 and against bacteria (*Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter buamanii*, *Pseudomonas aeruginosa*, Methicillin-resistant *Staphylococcus aureus*) with insignificant activities (MIC > 32 μg/mL). Individual alkaloids were tested against a human suspension cancer cell line (HL-60 leukemia cells) and two human fibroblastic cancer cell lines (A549 lung cancer cells and HepG2 liver cancer cells) in which compound **5** was the most toxic alkaloid with IC_{50} values ranging from 20 to 80 μM.



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Indonesian medicinal plant; *Annona muricata*; alkaloid; anti-cancer; anti-malarial; anti-bacteria

1. Introduction

The Indonesian archipelago is acknowledged as a source of around 6,000 recorded medicinal plants, but only a few selected species have been investigated for the source of their potency (Nugraha and Keller 2011). The indigenous population of

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Figure 1. Alkaloids obtained from the root bark of *Annona muricata* L.

Indonesia has relied on medicinal plants for traditional therapy against diseases, including the use of *Annona muricata* L. to treat malaria and bacterial infections.

Annona muricata L. originated from tropical America and spread throughout Southern Eastern China, South East Asia to Northern Australia. It is part of the Annonaceae family which contains more than 2,000 species within 130 genera. The genus Annona itself consists of 119 species including at least four, commonly used both as an edible fruit and as a medicine (*Annona cherimola* Mill., *Annona muricata* L., *Annona squamosal* L., *Annona reticulata* L.) with other species commonly used only in traditional medication including *Annona ambotay* Aubl., *Annona senegalensis* Pers.

To the indigenous Indonesian population, the leaves have been prepared to treat boils, spasm and as aphrodisiac agents (Syamsuhidayat and Hutapea 1991). In Indonesia, the fruit and leave are currently used to treat breast cancer while the stem and root have been used to treat malarial fever.

In the early 20th century, elemental examination of the *Annona* plants began on the leaves, fruits and seeds. Study on volatile compounds of Vietnamese *Annona glabra* L., *Annona squamosal* L., *Annona muricata* L. and *Annona reticulata* L. comprised of β-caryophyllene as the major constituent (Thang et al. 2013). Since 1980s with the development of chemical analysis instrumentation, a series of acetogenin were isolated from the *Annona* genus provided promising anti-cancer activity (Nugraha 2015). Phytochemical studies of *A. muricata* L. produced around 127 compounds of which almost 90% were acetogenins (Nugraha 2015). Notable acetogenins isolation work included the isolation of dieporeticanin-1, dieporeticanin-2, dieporeticenin, trieporeticanin, diepomuricanin, reticulatacin and solamin from seeds of Vietnamese *Annona reticulata* L. (Tam et al. 1994) and the isolation of robustocin from the seeds of Brazilian *Annona muricata* L. (Gelye et al. 2000). Annonacin, goniothalamicin and isoannonacin were moluscicidal potent acetogenins isolated from the leaves of Brazilian *Anonna muricata* L. (Luna et al. 2006). Here, we reported the isolation of the previously unreported alkaloid constituents of the root of *Annona muricata* L. and their bioactivities.

2. Result and discussion

The root bark of *A. muricata* was collected from Malang-Indonesia and was initially powdered to ease extraction. The initial extract was obtained by stirring plant samples with methanol, then acidification with HCl solution followed by back extraction

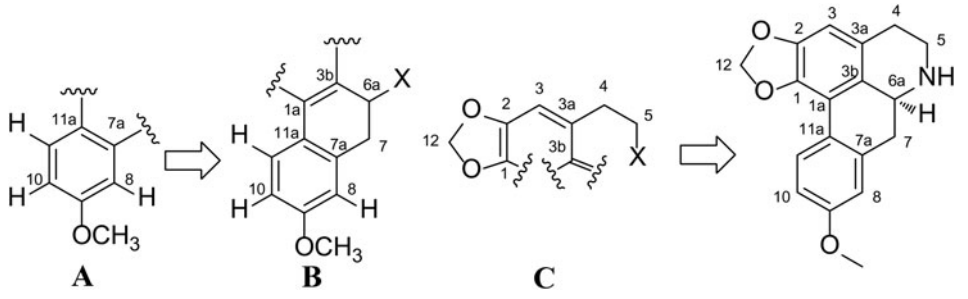

Figure 2. Progressive molecular structure build of **5** based on NMR spectral analysis.

(CH_2Cl_2). Basification of the aqueous layer with ammonia solution followed by back extraction (CH_2Cl_2) produced an alkaloids fraction. Semi-preparative normal-phase HPLC was able to isolate the alkaloids (+)-coclaurine **1** (Kashiwada et al. 2005), (+)-reticuline **2** (Oliveira da Cruz et al. 2011), argentinine **3** (Lopez-Martin et al. 2002), atherosperminine **4** (Lu et al. 1985) and the previously unreported (+)-xylopine **5** (Figure 1), with argentinine **3** reported here for the first time from *A. muricata*. The only previously reported examples of this alkaloid came from the related species *A. montana*, and therefore, might be useful in future chemotaxonomical studies.

Compound **5** was isolated as brown solid. ^{1}H-NMR and ^{13}C-NMR spectral analysis are illustrated in the experimental section. The ^{13}C-NMR spectrum revealed eighteen carbon resonances and APT spectral analysis confirmed the presence of eight quaternary (C), five methane (CH), four methylene (CH_2), and one methyl (CH_3) carbon atoms. Analysis of the ^{1}H-NMR spectra suggested the presence of one methoxy (-OCH_3) and one methylenedioxy (-O-CH_2-O) moiety while the IR spectral analysis indicated no N-H bending at 1600 cm^{-1} and one medium N-H stretching signal at 3300 cm^{-1}, suggesting the existence of a secondary amine (R-NH-R). Therefore, the existence of oxygen (x3), hydrogen (x1) and nitrogen (x1) atoms in the molecular structure is suggested. The molecular formula of $C_{18}H_{18}NO_3$ was confirmed by HRESI-MS analysis with a peak at *m/z* 296.1295.

The ^{1}H-NMR spectrum of **5** showed a set of resonances at δ_H 8.00 (1H, d, $^3J = 7.5$ Hz), 6.83 (1H, d, $^3J = 7.5$ Hz) and 6.81 (1H, s), assigned to H11, H10, H8, respectively (Figure 2A). This 1,3,4 tri-substituted aromatic ring contained quaternary carbons C7a, C9 and C11a which were assigned using gHMBC spectral analysis to resonances at δ_C 137.7, 160.6 and 125.0 ppm, respectively. In the ^{1}H-NMR spectrum, the singlet at δ_H 3.81 (3H, s) was distinctively assigned to –OCH_3 which the gHMBC spectral analysis indicated a proton-carbon correlation with C9. In total, this suggested a 1,3,4-trisubstituted aromatic ring unit of compound **5** (Figure 2A).

Further analysis of the ^{1}H-NMR spectrum showed resonances at δ_H 2.87 (1H, m) and 2.70 (1H, m), assigned to the methylene H7a and H7b, respectively. Through gCOSY spectral analysis, these protons showed *ortho* proton-proton correlations with the proton resonance at δ_H 3.90 assigned to H6a (1H, m). Further, gHMBC spectral analysis indicated a three bond proton-carbon correlation between H11 and carbon resonance at δ_C 117.3 assigned to C1a while H8 also indicated a three bond correlation with the carbon resonance at δ_C 37.4 assigned as the methylene C7. In the gHMBC spectral analysis, proton H7a, H7b and H6a showed proton-carbon correlation with the carbon

Table 1. Anti-plasmodium and antibacterial activities of crude methanol extract and alkaloid fraction from the root of *Annona muricata* L.

Entry	Anti-plasmodium (IC_{50}, μg/mL)	Antibacterial (MIC, μg/mL)				
	PF	EC	KP	AB	PA	MRSA
Crude methanol extract	i	>32	>32	>32	>32	>32
Alkaloid fraction	i	>32	>32	>32	>32	>32
Coistin	na	0.06	0.03	0.03	≤0.025	na
Daptomycin	na	na	na	na	na	1
Mafloquine	0.0290*	na	na	na	na	na

Note: * in μM; i: inactive; na: not available; PF: Plasmodium falciparum; EC: Escherichia coli; KP: Klebsiella pneumonia; AB: Acinetobacter baumannii; PA: Pseudomonas aeruginosa; SA: Methicillin-resistant Staphylococcus aureus.

resonance at δ_C 127.2, assigned to C3b. Together with the previous analysis, the molecular structure building block B was established (Figure 2B).

Further, the ^{1}H-NMR spectrum showed two proton systems at δ_H 6.03 (1H, s) and 5.90 (1H, s), assigned to a distinct acetal methylene group $H12_A$ and $H12_B$, respectively (Figure 2C). The gHMBC spectral analysis showed a proton-carbon correlation between the acetal methylene protons and two quaternary carbons at δ_C 143.3 and 148.6, assigned to C1 and C2, respectively. Further analysis of ^{1}H-NMR spectrum showed a singlet proton at δ_H 6.51, assigned to H3 which gHMBC spectral analysis showed the proton to correlate with C2 and carbon resonance at δ_C 127.2, assigned to C3b. Moreover, gHMBC spectral analysis showed H3 to correlate with methylene carbon resonance at δ_C 29.8 assigned as C4. The ^{1}H-NMR and gCOSY spectra showed proton resonances at δ_H 2.99 (m, 1H) and 2.67 (m, 1H), assigned to $H4_A$ and $H4_B$, respectively whereas proton resonances at δ_H 3.33 (m, 1H) and 2.97 (m, 1H) were assigned to $H5_A$ and $H5_B$, respectively. The chemical shift of methylene carbon C5 at 44.0 clearly suggested an electronegative group was adjacent, presumably the nitrogen functional group. This was confirmed by IR experiment which showed a distinct secondary amine stretch at 3310 cm^{-1}. Therefore, the molecular structure of fragment C was established (Figure 2C). By combining this evidence above, the molecular structure of xylopine was established (Figure 2D) with selected proton-carbon correlations summarised in the Figure S5 (see supplementary information).

This alkaloid has only previously been reported in its sinister (*R*) optically active form with previously rotations of $[\propto]_D^{25}$ of −21.5° (Bhaumik et al. 1979), −23.4° (Roblot et al. 1983), −38° (Lu et al. 1985) in methanol. Here, we report for the first time the isolation of the opposite enantiomer, *dextro* xylopine **5** with an optical rotation of $[\propto]_D^{25}$ +81° in methanol and the (*S*) stereogenic carbon. The previously isolated (-) isomer of xylopine was present in *A. reticulate*, *A. squamosal*, *A. Montana*, but our studies show the existence of (+)-xylopine alkaloid for the first time.

Biological testing of the crude methanol extract and alkaloid fractions revealed insignificant anti-bacterial activities against *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter buamanii*, *Pseudomonas aeruginosa* and Methicillin-resistant *Staphylococcus aureus* (Table 1). Testing against *Plasmodium falciparum* also indicated no anti-malarial potency. This result is contrary to traditional claims and uses in malarial and fever therapy. Further bioactivity testing revealed significant cytotoxicity against several cancer cell lines (Figure S10).

In addition, the methanol extract of the leaf was previously reported to possess low antimicrobial activity against *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* with MIC values of 1024, 256, 1024 μg/mL, respectively (Dzotam et al. 2016). Insignificant antimicrobial activity was also reported on the hydroethanolic extract of the leaves against *Staphylococcus aureus* (MIC $\geq$ 1024 μg/mL) (Bento et al. 2013). *Bacillus subtilis* and *Staphylococcus aureus* were reported to be sensitive against benzene extract of wood of Brazilian *Annona ambotay* with inhibition diameter of 10 and 9 mm, respectively. The same result was produced by ethanolic extract of Brazilian *Annona cherimolia* with inhibition diameter value of 14 and 11 mm, respectively (Takahashi et al. 2006). Crude ethanolic extract of leaves, stem and root of Cameroonian *Annona muricata* possessed anti-fungal activity against *Cadida albicans*, *Candida glabrata*, *Candida cruzei*, *Candida lusitaniae*, *Cryptococcus neoformans*, *Candida paapsilosis* and *Candida tropicalis* with MIC values vary from 1.9 to 15 mg/mL (Simo et al. 2018). An additional study reported the crude methanol extract of leaves and stem of *A. muricata* were able to inhibit *Plasmodium falciparum* W2 growth by 36.8 and 26.3% at concentration of 10 μg/mL, respectively (Osorio et al. 2007). The crude leave extract species derived from various habitats in Malaysia revealed anti-breast cancer (MCF7) with IC_{50} values ranging from 221.67 to 799.67 μg/mL with no environmental effect discussed (Najmuddin et al. 2016).

Previously pharmacological reports on the five alkaloids in this study revealed reticuline to have gram-positive selective antimicrobial against *Staphylococcus aureus* with MIC value of 500 μg/mL (Costa et al. 2013). (+)-Coclaurine dimerization was reported to increase its antiplasmodial activity (Kashiwada et al. 2005). Interestingly, typical dimerization of benzylisoquinolines such as dehatrine, also possessed potent anti-malarial activity against a chloroquine-resistant *Plasmodium falciparum* K1 with an IC_{50} value of 0.17 μM (Kitagawa et al. 1993). (+) Reticuline and atherosperminine were reported to inhibit *Plasmodium falciparum* with IC_{50} values of 10.9 and 5.80 μM, respectively (Böhlke et al. 1996; Nasrullah et al. 2013).

Five compounds were tested across three cell lines which included a human suspension cancer cell line (HL-60 leukemia cells) and two fibroblastic cell lines (A549 lung cancer cells and HepG2 liver cancer cells). Figure S9A–C shows the typical concentration-response curves obtained from the MTT assay of each compound with the three cell lines. The IC_{50} values are summarized in Figure S10. Compounds **1** and **2** were relatively nontoxic across all three cell lines. Their IC_{50} values were greater than 300 μM and could not be determined due to solubility limitations. The most toxic compound across the three cell lines ($P < 0.05$–0.0001) was compound **5**, exhibiting the lowest IC_{50} values (ranging from approximately 20–80 μM)).

Previous cytotoxicity studies revealed that Madin–Darby bovine kidney cells were sensitive to (+) reticuline with a maximum non-toxic concentration of 101.5 mM (Orhan et al. 2007). The natural product, coclaurine, isolated from Annona squamosa, was previously reported to exhibit cytotoxicity against colon cancer cells (HCT116), human breast cancer cells (MCF-7) and human liver cancer cells (HepG2) with IC_{50} values of 28.9, 53.8 and 5.9 mM, respectively (Al-Ghazzawi 2019). While the reported IC_{50} value for coclaurine in HepG2 cells is lower than that reported in this study, earlier reports correspond to a 72-h (versus 24-h) treatment. The alkaloid, argentinine,

isolated from Annona montana possessed significant cytotoxicity against the human colon cancer cell line HT-29, the human lung carcinoma cell line, A549, the murine leukaemia P388 and human KB cells with ED_{50} values of 9.9, 33.5, 15.0 and 15.5 mM respectively (Wu et al. 1993); however, the treatment period was not reported. Atherosperminine did not show significant cytotoxicity against colon tumour HT-29, murine leukaemia P-388 and human KB cells (Jow et al. 2004; Wu 2006). Xylopine from Xylopia laevigata (Mart.) R.E. Fr. was reported to cause cells apoptosis on human colon carcinoma HCT116 through p53-independent pathway (Santos et al. 2017).

3. Experimental

3.1. General experimental procedures

Silica gel (Merck Silica Gel 60, 0.063–0.200 mm). Analytical HPLC was performed in a Waters 1525 binary HPLC pump coupled with Waters 2487 dual λ absorbance detector. A symmetry ® C_{18} column was used (4.9 × 150 mm, 5 μm). Semi-preparative HPLC was performed in a Waters LC system coupled with Waters 2489 UV/Visible detector. An OBD Sunfire™ C_{18} semi-preparative HPLC column (19 × 150 mm, 5 μm) was used. ESI-MS spectra were collected from Waters platform LCZ mass spectrometer (low resolution) and Waters QTof Ultima mass spectrometer (high resolution). MS/MS experiment were carried out on Waters (micromass) Quattro micro™. NMR spectra were obtained from Varian Unity Inova-500 MHz NMR spectrometer. Biological assays for anti-malarial activities were performed at the National Centre for Genetic Engineering and Biotechnology, Thailand. The antimicrobial screening was performed by CO-ADD (The Community for Antimicrobial Drug Discovery), funded by the Welcome Trust (UK) and The University of Queensland (Australia).

3.2. Plant material

Annona muricata L. (Annonaceae) root were collected from Malang, Indonesia and identified at the School of Pharmacy, University of Jember, Indonesia, where sample vouchers are kept under accession AMR. The root was cleaned, washed, sliced, sun-dried and then powdered.

3.3. Extraction

A suspension of the powdered root (200 g) in methanol (2.5 L) was stirred for 24 hr, and then filtered and the filtrate concentrated producing a brown sticky semi-solid (28.1 g). A portion of extract (10.0 g) was acidified with 5% HCl and extracted with CH_2Cl_2 (3 × 200 mL), the water layer was then basified with NH_4OH solution until pH 12. This solution was then extracted with CH_2Cl_2 (4 × 200 mL) and the organic layer was vacuum dried to produce alkaloid extract (1.35 g).

3.4. Isolation

The alkaloid extract (500 mg) was re-dissolved in MeOH (10 mL) and filtered through HPLC filter (0.45 μm). The sample was subjected to semi-preparative normal HPLC with gradient eluent from 90% to 55% solvent A within 50 minutes (solvent A: 0.1% TFA in H_2O; solvent B: 0.1% TFA in acetonitrile) to produce (+)-coclaurine **1**, (+)-reticuline **2**, argentinine **3**, atherosperminine **4** and previously unreported (+)-xylopine **5**.

(+)-xylopine (**5**)

Brown solid (0.32 mg/g dried plant sample); $[\propto]_D^{25}$ of +81° (methanol); IR [cm^{-1}] 3300 (m), 2919 (m), 2850 (m), 1650 (m), 1606 (s), 1500 (s), 1225 (s), 1051 (s), 447 (s); ^{13}H-NMR (500 Hz, CD_3OD): δ 8.00 (d, $^3J = 7.5$, 1H, H11), 6.83 (d, $^3J = 7.5$, 1H, H10), 6.81 (s, 1H, H8), 6.51 (s, 1H, H3), 6.03 (s, 1H, H12a), 5.90 (s, 1H, H12b), 3.90 (m, 1H, H6a), 3.81 (s, 3H, C9-OCH_3), 3.33 (m, 1H, H5a), 2.99 (m, 1H, H4a), 2.97 (m, 1H, H5b), 2.87 (m, 1H, H7a), 2.70 (m, 1H, H7b), 2.67 (m, 1H, H4b); ^{13}C-NMR (125 Hz, CD_3OD): δ 160.6 (C9), 148.6 (C2), 143.3 (C1), 137.7 (C7a), 129.6 (C11), 127.5 (C3b), 127.2 (C3a), 125.0 (C11a), 117.3 (C1a), 114.5 (C8), 113.4 (C10), 107.9 (C3), 102.0 (C12), 55.7 (C9-OCH_3), 54.6 (C6a), 44.0 (C5), 37.4 (C7), 29.8 (C4); ESMS: 296 $(M+H)^+$ HRESIMS: calculated 296.1287, found 296.1295 ($C_{18}H_{18}NO_3$)

3.5. Bioactivity testing

Anti-malarial activity was determined against *Plasmodium falciparum* K1 based on the microculture radioisotope technique (Desjardins et al. 1979). Cytotoxicity was tested against human suspension cancer cell line (HL-60 leukemia cells) and the human fibroblastic cancer cell lines (A549 lung cancer cells and HepG2 liver cancer cells) (see supplementary information). Anti-viral activity was tested against herpes simplex virus type 1 using a green fluorescent protein assay (Haritakun et al. 2010). Antimicrobial activities were tested against *Escherichia coli* ATCC 25922 (GN_001), *Klebsiella pneumoniae* ATCC 700603 (GN_003), *Acinetobacter baumannii* ATCC 19606 (GN_034), *Pseudomonas aeruginosa* ATCC 27853 (GN_042) and *Staphylococcus aureus* ATCC 43300 (MRSA) (GP_020).

4. Conclusion

Our investigation into the phytochemistry of the root of *Annona muricata* L. has resulted in the isolation of five alkaloids including the new compound, (+)-xylopine **5**. We reported the isolation of argentinine for the first time from the *Annona muricata* L. with the only previous reports coming from the two closest species *Annona muricata* and *Annona Montana*. Therefore, this alkaloid might be useful for chemotaxonomical studies. Bioactivity testing revealed compound **5** as the most toxic constituent among the alkaloids of the root of *Annona muricata* L.

Acknowledgment

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Disclosure statement

No potential conflict of interest was reported by the authors.

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