A phenolic ester from Aglaia loheri leaves reveals cytotoxicity towards sensitive and multidrug-resistant cancer cells

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Abstract

Background: Bioactivity-guided fractionation of extracts of Aglaia loheri Blanco (Meliaceae) yielded a cytotoxic isolate, termed Maldi 531.2[M + H]+. This phenolic ester was further investigated for its in vitro cytotoxicity toward human CCRF-CEM leukemia cells and their multi-drug resistant (MDR) subline, CEM/ADR5000. The intrinsic mitochondrial membrane potential (ΔΨm) and induction of apoptosis by this isolate were evaluated.

Methods: Chromatography techniques, mass spectrometry and proton NMR were employed to isolate Maldi 531.2[M + H]+. XTT cell proliferation and viability assay was used for cytotoxic test, and JC-1[5',5',6,6'-tetrachloro-1',3',3'-tetracyanomethoxybenzimidazol] carbocyanine iodide was used to assess ΔΨm and initiation of apoptosis; Annexin V/FITC-PI staining was employed to analyse apoptosis.

Results: Maldi 531.2[M + H]+ was cytotoxic towards both CCRF-CEM and CEM/ADR5000 cells with IC50 values of 0.02 and 0.03 μM, respectively. The mitochondrial membrane potential (ΔΨm) of MDR cells was significantly reduced in a dose-dependent manner leading to apoptosis as detected by flow cytometric Annexin V-FITC/PI staining.

Conclusion: Maldi 531.2[M + H]+ may be a potential anti-cancer drug candidate whose mode of action include reduction of the mitochondrial membrane potential and induction of apoptosis.

Keywords: Aglaia loheri, Cytotoxicity, Multi-drug resistance, Apoptosis, JC-1 mitochondrial membrane potential, Annexin V-FITC.

Background

Hematologic malignancies account for a considerable percentage of cancers worldwide [1]. A common type of this malignancy is acute lymphoblastic leukemia (ALL). ALL involves rapid transformation of a single normal progenitor cell [2] triggered by several factors associated with exposure to radiation and chemicals. These factors cause genetic damage, resulting in complete destruction of cellular regulatory controls on proliferation, differentiation and apoptosis [3-6]. Since leukemia is a systemic disease, its cure depends on chemotherapy [7] rather than surgery. A new approach to increasing effectiveness to chemotherapy includes the use of various molecularly targeted drugs derived from natural plant products [8].

The research on Aglaia loheri was part of a larger project to explore the potential medicinal value of plants endemic to the mountain area of Kanawan, Morong (Bataan, Philippines) which is part of the ancestral domain of indigenous people called Aetas. From personal communications with the indigenous people, it was learned that some of the plants were used by the Aetas for medicinal or nutritional purposes. A. loheri is actually part of their diet. However, due to accounts from published literature on other Aglaia species, we hypothesized that A. loheri may be cytotoxic towards cancer cells.

The genus Aglaia (family Meliaceae) is an important source of unique bioactive natural products, which contain a cyclopenta[b]tetrahydrobenzofuran skeleton and include more than 50 naturally occurring derivatives collectively
called rocaglamides [9]. This group of compounds was found to be effective against thymidine kinase-deficient \textit{Herpes simplex} virus type 1 (HSV-1) and phosphonoacetate-resistant HSV-1 strains [9], including human amoebiasis caused by \textit{Entamoeba histolytica} [10]. These compounds were shown to inhibit necrosis factor (NF)-B induced gene activation in human T cells [11], HIV-1 infection [12], and initiation and promotion of chemically-induced cancer on mouse skin [13]. Amide esters are structurally related to bisamides and flavaglines from \textit{Aglaia sp.} [14] and exhibited anti-fungal [15], insecticidal [16], anti-tuberculosis and anti-viral activities [17]. Remarkably, this class of compounds also induces apoptosis in resistant human cancer cells [18–20]. For example, silvestrol, a novel compound from \textit{Aglaia} was active against cancer cells \textit{in vitro} and \textit{in vivo} [20,21]. It induced apoptosis in prostate carcinoma cells through the mitochondrial/apoptosome pathway without activation of caspase-3 or −7 [19] and in human B-leukemia cells, by reducing Mcl-1 expression due to inhibition of translation with subsequent mitochondrial damage [21].

In the present study, crude extracts from leaves of \textit{A. loheri} Blanco were subjected to bioassay-guided isolation by means of various chromatography techniques. The resulting active principle was further analyzed and characterized by mass spectroscopy and nuclear magnetic resonance (NMR). The isolated active principle was investigated for its cytotoxicity towards cancer cells. The mitochondrial membrane potential (ΔΨ\textsubscript{m}) was analyzed as a key indicator of cell viability [22,23] and induction of apoptosis as an important parameter of cell integrity.

Methods

Kits and reagents

Analytical grade ethyl acetate and hexane were used for extraction. Analytical grade chloroform and methanol were used for gravity column chromatography. Silica gel 60 G 0.063-0.200 mm (Merck; Germany) was used for gravity column chromatography. Pre-coated gel 60 G F\textsubscript{254} plates 0.25 mm thick (Merck, Darmstadt, Germany) was used for thin layer chromatography (TLC). Iodine crystals and UV were used to visualize separation monitored by analytical TLC. Doxorubicin was purchased from Sigma Chemical Company, USA. Dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) was used to dissolve the test samples. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Promega, USA. XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide), XTT labelling reagent (sodium 3′-[1-phenylaminocarbonyl]-3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) and electron-coupling reagent (N-methyl dibenzoylazine methyl sulphate [0.383 mg/mL (1.25 mM)]) in sterile phosphate buffered saline (PBS) were purchased from Roche (Mannheim, Germany). JC-1 and annexin V-FITC detection kit was obtained from eBioscience (Frankfurt, Germany); propidium iodide and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) from Sigma-Aldrich (Taufkirchen, Germany).

Cell culture and supplements

Human colon cancer cell line (HCT116) was obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were grown in McCoy’s 5a modified medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% inactivated fetal bovine serum (Invi- trogen, USA) and 1% penicillin-streptomycin (100 U/mL) (Invitrogen, USA). The cell line was maintained in a humidified incubator containing 5% CO\textsubscript{2} at 37°C.

Human leukemic cells, CCRF-CEM and their multi-drug-resistant subline, CEM/ADR5000 [24], were obtained from Dr. Axel Sauerbrey (Department of Pediatrics, University of Jena, Germany). The cells were maintained in a humidified environment at 37°C and 5% CO\textsubscript{2}. The cells were grown in RPMI 1640 [–] L-glutamine containing 1% penicillin (10 U/mL), streptomycin 10 µg/mL, and 10% heat-inactivated fetal bovine serum (FBS) (all obtained from Gibco Invitrogen, Germany). Drug resistance of CEM/ADR5000 cells was maintained by weekly treatment with 5000 ng/mL. P-glycoprotein expression without over-expression of other ATP-binding cassette transporters in CEM/ADR5000 cells has been reported [25,26].

Peripheral blood mononuclear cells (PBMC) were isolated from freshly collected whole blood sample using Ficoll-paque solution (Histopaque-1077) (Sigma) by cen-trifugation [27]. The cells were washed twice with RPMI-1640 and were re-suspended in same culture medium (RPMI-1640). Cells (0.5 mL of cell suspension) were seeded in a 24-well sterile plate at a density of 10^6/mL prior to activation with 0.5 mL of 10 µg/mL of phytohemagglutinin (PHA). Addition of 0.5 mL of PHA into each well (0.5 mL RPMI-1640 to one control group) adjusted the number of cells to a final density of 5 × 10^7/mL per well. The cells were incubated at 37°C and 5% CO\textsubscript{2} for 3 days prior to XTT assay.

Collection of plant material

Fresh and mature leaves of \textit{A. loheri} Blanco were collected in the morning from its natural habitat in Morong (Bataan, Philippines). Voucher specimen of the plant under Accession No. 124162 was deposited and authenticated by the Jose Vera Santos Herbarium of the Institute of Biology, University of the Philippines (Diliman, Quezon City, Philippines). The leaf specimens were collected in the morning from its natural habitat in Morong (Bataan, Philippines). Voucher specimen of the plant under Accession No. 124162 was deposited and authenticated by the Jose Vera Santos Herbarium of the Institute of Biology, University of the Philippines (Diliman, Quezon City, Philippines). After drying, the leaf samples were homogenized using a blender.
Concentration and solvent fractionation
Homogenized leaf samples were soaked in ethanol for 48 h. The soluble ethanolic extract was filtered and the filtrate was concentrated in vacuo at 40°C using a rotary evaporator (Heidolph, Schwabach, Germany) yielding a concentrated ethanolic crude extract (ECE). ECE was assayed for cytotoxicity by using MTT assay [28] against HCT116 cells. The concentrated ECE was diluted in approximately 200 mL ethanol and was partitioned exhaustively with hexane and ethyl acetate successively. Both hexane soluble extract and ethyl acetate soluble extract were concentrated in vacuo at 40°C separately which yielded concentrated hexane extract (HE) and concentrated ethyl acetate extract (EAE). Finally, the two concentrated crude fractions were tested for their cytotoxicity by the MTT assay.

Determination of band profiles by thin layer chromatography (TLC)
A small amount of the EAE was spotted 5 cm above the bottom edge of the 5 × 7.5 cm TLC plate using a very thin glass tube. The extract was air-dried on the TLC plate which was then placed in eluent in the developing chamber so the bottom of the plate is in the liquid. Several eluents with increasing/decreasing concentration ratio (ranging from 1:9 to 9:1) of chloroform to methanol were prepared before subjecting the cells to MTT assay. Each MDP contained A. loheri extracts/isolate in varying concentrations ranging from 125–1000 μg × mL⁻¹ for crude extracts and 7.8–62.5 μg × mL⁻¹ for pure isolates. Cells were harvested and seeded in a sterile, flat-bottom 96-well microtiter plates at a density of 3.5 × 10⁵ cells per mL, then were incubated for 24 h at 37°C in a humidified incubator with 5% CO₂. After 24 h incubation, 10 μL from MDP preparation were added to the cells in each well to make final concentrations of 50, 25, 12.5 and 6.25 μg × mL⁻¹ for crude extracts and 7.8, 15.6, 31.25, 62.5 μg × mL⁻¹ for the pure isolate. The cells were then incubated for 72 h. After incubation, the media was discarded, and 20 μL of MTT were added to each well. The MTT solution was made by dissolving 5 mg MTT (Sigma-Aldrich) per mL phosphate buffer solution. The cells were then incubated for 4 h before adding 150 μL of DMSO (Sigma, USA) to dissolve the formazan crystals. At least two independent tests with three replicates each were performed in this experiment. The absorbance of the formazan crystals was then taken with dual wave-measurement at 570 nm and 620 nm using a Bio-Rad microplate reader (Munich, Germany).

Furthermore, a colorimetric XTT-based assay was applied according to the manufacturer’s protocol. Two independent tests were conducted. Cells grown in cell culture flasks were harvested. A 100 μL aliquot of 2.0 × 10³ cells/mL were seeded into each well of a 96-well microtiter cell culture plate, and were immediately treated with various concentrations (0.001 to 30 μM) of Maldi 531.2[M + H]⁺, the isolate, with six replicates per dose level. Two sets of control groups were employed: one control group was suspended only in nutrient media while another was suspended in nutrient media containing 0.02% dimethyl sulfoxide (DMSO), the solvent to dissolve Maldi 531.2[M + H]⁺. After incubating for 72 h at 37°C and 5% CO₂, 50 μL of a mixture of XTT labeling reagent and electron-coupling reagent in sterile phosphate buffered saline (PBS) were added and incubated at 37°C for 4 h. The absorbance of the water-soluble
formazan formed was measured at 490 nm and 655 nm using a Tecan microplate reader (Sunrise-Basic Tecan, Crailsheim, Germany) and is directly proportional to the number of living cells in the culture. The viability of the treated cells was compared to the viability of the DMSO-treated cells. Percent growth inhibition (Ip) was calculated using the formula: Ip = 100 \[ \frac{(A_t - A_c)}{A_t} \] where \( A_t \) is the absorbance of the Maldi 531.2[M + H]+-treated cells and \( A_c \) is the absorbance of the DMSO-treated cells. The IC50 concentration was estimated by logarithmic regression [29] where IC50 is the inhibitory concentration of Maldi 531.2 [M + H]+ required to inhibit growth of 50% of the incubated cell population.

Mitochondrial membrane potential (ΔΨm) analysis
JC-1 is a membrane permeable dye that selectively accumulates in the mitochondria, where it reversibly changes color as membrane potential (ΔΨm) increases over values of about 80–100 mV. JC-1 is widely used for quantifying mitochondrial membrane potential by measuring the fluorescence intensities and is therefore an indicator of initiation of cell apoptosis [30]. To assess the effect of Maldi 531.2[M + H]+ on ΔΨm, 10^6 cells were incubated for 24 and 48 h (37°C, 5% CO2) with three doses (0.05, 0.5 and 1.0 μM). Two aliquots of cells were treated with the control drugs: 10 μg/mL doxorubicin and 0.05 μM CCCP (Sigma-Aldrich) and incubated at 37°C and 5% CO2. An incubation time of 10 min was sufficient, because of the rapid stimulation of membrane polarization by CCCP. An incubation of more than 10 min with CCCP greatly reduces the fluorescence intensity in the FL2 channel (564–606 nm) converting a red fluorescence signal to a green signal. Afterwards, the cells were stained with JC-1 for 30 min at 37°C and 5% CO2. The membrane sensitive JC-1 dye was prepared according to the manufacturer’s recommended concentration of 2.5 μg/million cells/mL from a stock solution of 1 mg/mL prior to use (eBioscience, Germany). The dye was pre-warmed in a 37°C water bath prior to addition to treated and untreated cells. Stained cells were then analyzed by flow cytometry (FACS®Calibur (Becton Dickinson, Heidelberg, Germany) and CellQuest Pro (Becton Dickinson) analysis software. Excitation and emission settings were 488 nm, 515–545 nm (FL1 channel) for annexin-V-FITC and 564–606 nm (FL2 channel) for PI.

Statistical analysis
Data were expressed as mean ± SEM of two independent tests. JC-1 and annexin V-FITC fluorescence intensity data were subjected to one-way analysis of variance followed by Tukey’s multiple comparison test. A probability of less than or equal to 0.05 was considered statistically significant to reject the null hypothesis.

Results
Bioactivity-guided isolation of an active fraction from A. loheri
Approximately 15.23 g (1.96%) of ethyl acetate crude extract were obtained from 777 g of dried ground leaf samples of A. loheri after solvent partitioning. Cytotoxicity of the extracts was tested towards HCT116 by the MTT assay. The ethyl acetate fraction was cytotoxic exhibiting an IC50 concentration of 5.47 μg/mL. Ethyl acetate extract was subjected to gravity column chromatography utilizing silica gel-60 as stationary phase packed in 3.5 cm diameter, 37 cm long glass column, and 9:1 chloroform-methanol as mobile phase. This chromatographic separation yielded 10 column fractions, where CF1 revealed the highest cytotoxicity (IC50 of 3.82 ± 0.02 μg/mL). For comparison, doxorubicin was used as control drug and revealed an IC50 of 3.74 ± 0.14 (Table 1). Further gradient fractionation of CF1 yielded 11 sub-fractions (SF) whose IC50 values are shown in Table 2. SF9 was the most cytotoxic one. Therefore, this fraction was subjected to HPLC to further purify the active compound. Four distinct peaks were generated. The

Annexin V/FITC binding assay
Annexin V/FITC staining was performed to evaluate apoptosis induced by Maldi 531.2[M + H]+. Annexins are calcium-dependent, phospholipid-binding proteins that specifically bind to phosphatidylserine (PS) in the plasma membrane [31]. Under normal physiological conditions, PS is predominantly located in the cytosolic layer of the plasma membrane. Upon initiation of apoptosis, PS is translocated from the cytosolic layer of the plasma membrane to the extracellular layer for recognition and uptake by phagocytes, where it can be detected by fluorescently-labeled Annexin V (eBioScience). Cells were exposed to the active isolate (0.05, 0.5, 1.0 μM) and incubated for 24 h at 37°C and 5% CO2. For positive controls, cells were treated with 10 μg/mL doxorubicin (Sigma-Aldrich) and 10 μM camptothecin separately and were also incubated for 24 h at 37°C and 5% CO2. The annexin-V assay was performed according to the protocol provided in the annexin V-FITC detection kit (eBioScience). Briefly, 5.0 × 10^5 treated cells were resuspended in 50 μL of 1× annexin-staining buffer containing 10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2 (pH 7.4) and incubated in the dark with 2.5 μL of annexin V conjugate for 15 min at 25°C. After incubation, the final volume of each sample was adjusted to 500 μL followed by the addition of 1.25 μL of 1 mg × mL^-1 propidium iodide (PI). Apoptotic cells were analyzed using a FACS®Calibur (Becton Dickinson, Heidelberg, Germany) and CellQuest Pro (Becton Dickinson) analysis software. Excitation and emission settings were 488 nm, 515–545 nm (FL1 channel) for annexin-V-FITC and 564–606 nm (FL2 channel) for PI.

Statistical analysis
Data were expressed as mean ± SEM of two independent tests. JC-1 and annexin V-FITC fluorescence intensity
eluates collected from peaks 1, 2 and 3 were colorless while peak 4 was greenish in color. Cell viability test results after cytotoxicity assay are shown in Table 3.

Chemical characterization of the bioactive A. loheri fraction
Bioactivity-guided fractionation of a crude extract of A. loheri leaves led to the isolation of a bioactive component (peak 3), an amorphous white powder of approximately 2.0 mg (total yield = 1.3%) which exhibited a molecular ion peak at m/z 531.2[M + H]+ (Figure 1), which was termed Maldi 531.2[M + H]+.

The chemical shift values and their assignment to Maldi 531.2[M + H]+ was based on its 1H NMR spectrum (Table 4). The 1H NMR spectrum indicated that Maldi 531.2[M + H]+ contains aromatic rings (δ 6.7-7.9 ppm), a phenolic group (δ 4.54 ppm), methoxy groups (δ 3.61-3.66 ppm), and several aliphatic chain structures (δ 1.87-3.61 ppm). As can be deduced from the one-dimensional NMR analysis, the probable structure of Maldi 531.2[M + H]+ may be an aromatic (phenolic) ester.

Cytotoxicity of Maldi 531.2[M + H]+ towards sensitive and multidrug-resistant tumor cells
Figure 2 shows the effects of Maldi 531.2[M + H]+ on the viability of CCRF-CEM cells and their multidrug-resistant sub-line, CEM/ADR5000. The active principle reduced mean cell viability of the two human leukemic cell types in a dose-dependent manner. A significant decrease in cell viability was observed at 0.03 μM and this further decreased with increasing concentration. Few cells survived at a dose range of 0.3 to 30 μM. The IC50 of Maldi 531.2[M + H]+ on CCRF-CEM cells was 0.02 μM and on CEM/ADR5000 cells 0.03 μM, indicating that CEM/ADR5000 cells were only 1.5-fold cross-resistant to Maldi 531.2[M + H]+. For comparison, CEM/ADR5000 cells were more than 1000-fold resistant towards doxorubicin compared to parental CCRF-CEM.

Table 1 IC50 values of gravity column chromatographic fractions of Aglaia loheri extracts run in silica gel-60 and 9:1 chloroform: methanol tested for toxicity against HCT 116

| Column fraction (CF) | IC50 (μg/mL) |
|---------------------|-------------|
| 1                   | 3.82 ± 0.02 |
| 2                   | 3.84 ± 0.07 |
| 3                   | 3.88 ± 0.10 |
| 4                   | 3.86 ± 0.12 |
| 5                   | 3.98 ± 0.08 |
| 6                   | 3.84 ± 0.09 |
| 7                   | 5.07 ± 0.16 |
| 8                   | 26.60 ± 0.60|
| 9                   | 13.50 ± 0.54|
| 10                  | NLI         |
| Doxorubicin         | 3.74 ± 0.14 |

Table 2 Gravity column chromatographic sub-fractions (SF) of A. loheri CF1 run in silica gel-60 and various gradients of increasing amount of ethyl acetate

| Sub-fractions (SF) | Gradient (Percent ratio of eluent: hexane to ethyl acetate) | IC50 (μg/mL) |
|--------------------|-------------------------------------------------------------|-------------|
| 1                  | 85:15                                                       | NLI         |
| 2                  | 83:17                                                       | 42.06 ± 0.67|
| 3                  | 81:19                                                       | 35.17 ± 4.63|
| 4                  | 78:22                                                       | 31.61 ± 6.52|
| 5                  | 75:25                                                       | 40.55 ± 7.24|
| 6                  | 71:29                                                       | NLI         |
| 7                  | 67:33                                                       | NLI         |
| 8                  | 63:37                                                       | NLI         |
| 9                  | 59:41                                                       | 3.96 ± 0.07 |
| 10                 | 55:45                                                       | 4.98 ± 0.50 |
| 11                 | 50:50                                                       | 11.92 ± 1.29|
| Doxorubicin        |                                                             | 4.31 ± 0.06 |

Table 3 IC50 values of eluates collected from individual HPLC peaks

| Peaks | Amount collected (mg) | IC50 (μg/mL) |
|-------|-----------------------|-------------|
| 1     | 0.2                   | 5.25 ± 0.54 |
| 2     | 0.3                   | 21.35 ± 0.67|
| 3     | 2.0                   | 4.52 ± 0.71 |
| 4     | 0.4                   | 5.57 ± 0.36 |
| Doxorubicin |                      | 4.10 ± 0.28 |

The chemical shift values and their assignment to Maldi 531.2[M + H]+ was based on its 1H NMR spectrum (Table 4). The 1H NMR spectrum indicated that Maldi 531.2[M + H]+ contains aromatic rings (δ 6.7-7.9 ppm), a phenolic group (δ 4.54 ppm), methoxy groups (δ 3.61-3.66 ppm), and several aliphatic chain structures (δ 1.87-3.61 ppm). As can be deduced from the one-dimensional NMR analysis, the probable structure of Maldi 531.2[M + H]+ may be an aromatic (phenolic) ester.

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cells [32]. Maldi 531.2[M + H] + was also tested against HCT 116 cells yielding a mean IC₅₀ of 3.9 μg/mL (data not shown). However, an MDR suspension cell line and its corresponding sensitive line, were used for the later experiment due to the ease of maintaining and handling suspension cell line over that of adherent cell line which need constant trypsinization. Although it is important that the cytotoxic agent be determined to be specific only to cancer cells, it is an established fact that many of the presently used cancer chemotherapeutic agents are not specific against tumor/malignant cells only. They also affect fast dividing normal cells such as those in the blood, in the digestive tract and other organs leading to a number of side effects like vomiting, hair loss, and the like. Hence, a number of studies that seek to mitigate these effects on normal cells such as on drug delivery specifically targeting cancer cells are being actively explored. We also suggest this as an offshoot of this study as part of the recommendation or future directions or follow up studies.

In connection with this, a cell viability test was performed separately using PHA-activated peripheral blood mononuclear cells (PBMC). After exposing the cells with various concentrations of Maldi 531.2[M + H] +, it was found to be cytotoxic (IC₅₀: 5.06 μM) as seen in Figure 3. However, despite its cytotoxic effects on PBMC, the level of toxicity is much less than its effects toward CCRF-CEM and ADR5000/CEM cells.

Quantitation of intrinsic mitochondrial membrane potential by flow cytometry
Figure 4 shows the mitochondrial membrane potential of CEM/ADR5000 cells treated for 24 and 48 h with various doses of Maldi 531.2[M + H] +. A significant ΔΨₘ reduction was observed in cells treated with the active principle in a dose-dependent manner 24 h after treatment compared with untreated cells. A further significant reduction of ΔΨₘ with Maldi 531.2[M + H] +-treated cells was observed after 48 h. However, reduction in Maldi 531.2[M + H] +-treated cells was not significantly different from the positive controls doxorubicin and CCCP after 24 h, but significantly decreased in those cells treated with 0.5 and 1.0 μM Maldi 531.2[M + H] + including those of doxorubicin-treated cells after incubation for 48 h.

| PPM   | Multiplicity | J coupling | TYPE                     |
|-------|--------------|------------|--------------------------|
| 1.87  | m            |            | NHCO-phenyl              |
| 2.70  | s            |            | -OCO-alkyl or phenyl     |
| 3.19  | m            |            | N-propyl CH₂OH           |
| 3.42  | m            |            | Methyl CH₃OH             |
| 3.61  | m            |            | CH₂-OH                   |
| 3.62  | s            |            | -OCH₃                    |
| 3.66  | s            |            | -OCH₃                    |
| 3.72  | m            |            | -OCO-CH₃                 |
| 3.82  | d            | 6.72       | -OCO- phenyl or methyl   |
| 3.97  | dd           | 7.92; 6.24 | Isopropyl CH-OH          |
| 4.23  | d            | 14.22      | -OCO-phenyl or n-propyl  |
| 4.58  | bs           |            | Phenolic –OH?            |
| 5.49  |              |            | -OCO-phenyl              |
| 6.17  | d            | 1.92       | Aromatic H               |
| 6.28  | d            | 1.80       | Aromatic H               |
| 6.61  | dd           | 8.24; 3.00 | Aromatic H               |
| 6.89  | d            | 6.90       | Aromatic H               |
| 6.99  | m            |            | Aromatic H               |
| 7.03  | m            |            | Aromatic H               |
| 7.11  | dd           | 8.94; 3.00 | Aromatic H               |
| 7.90  | s            |            | Aromatic H               |

s: singlet; bs: broad singlet; d: doublet; dd: doublet of doublet; m: multiplet.
In the present study, we report the isolation of a cytotoxic combolites with varying bioactivities [33]. Plant extracts usually contain many secondary metabolites with varying bioactivities [33]. To date, Aglaia still represents a promising and rich source for novel compounds. The isolation and purification of Maldi 531.2[M + H]⁺ from A. loheri proved to be difficult, because the isolated active component, was only present in very low amounts. Despite this limitation, we were able to investigate the bioactivity of Maldi 531.2[M + H]⁺ towards cancer cells. Based on mass spectrometry analyses, this compound does not reveal any similarity with other compounds isolated from other Aglaia species [19,20]. As shown in Tables 5 and 6, the molecular ion peaks of other Aglaia compounds are distinct from that of our A. loheri isolate with m/z 531.2[M + H]⁺, indicating that this represents a still unknown compound at least for the genus Aglaia.

The isolate could possibly be related to bisamides and flavaglines isolated by Kim et al. [19] and Salim et al. [20] based on their cytotoxic and anticancer activities.

**Annexin-FITC binding assay**

Apoptosis was induced by Maldi 531.2[M + H]⁺ in a dose-dependent manner (Figure 5). Compared with untreated cells, early apoptosis did not significantly differ in all treatments, but significantly increased in late apoptosis in a dose-dependent manner. Compared with camptothecin and doxorubicin-treated cells Maldi 531.2 [M + H]⁺ reduced the occurrence of early apoptotic, but not late apoptotic and dead cells over time.

**Discussion**

Plant extracts usually contain many secondary metabolites with varying bioactivities [33]. In the present investigation, we report the isolation of a cytotoxic component of A. loheri, termed Maldi 531.2[M + H]⁺. The cytotoxic properties of genus Aglaia was previously recognized by several groups [34]. Therefore, chemical studies of the constituents of Aglaia focused primarily on its toxic components resulting in the structure elucidation of numerous phytochemical compounds [34]. To date, Aglaia still represents a promising and rich source for novel compounds. The isolation and purification of Maldi 531.2[M + H]⁺ from A. loheri proved to be difficult, because the isolated active component, was only present in very low amounts. Despite this limitation, we were able to investigate the bioactivity of Maldi 531.2[M + H]⁺ towards cancer cells. Based on mass spectrometry analyses, this compound does not reveal any similarity with other compounds isolated from other Aglaia species [19,20]. As shown in Tables 5 and 6, the molecular ion peaks of other Aglaia compounds are distinct from that of our A. loheri isolate with m/z 531.2[M + H]⁺, indicating that this represents a still unknown compound at least for the genus Aglaia.

The isolate could possibly be related to bisamides and flavaglines isolated by Kim et al. [19] and Salim et al. [20] based on their cytotoxic and anticancer activities.

Cytotoxic activities were also observed in other isolated compounds from A. tenuicaulis and A. forbesii such as amide esters [14,17], which also demonstrated bioactivity, e.g. anti-inflammatory and antimicrobial [35], antiviral [17], and anticancer activities [36].

Phenolic esters inhibit carcinogenesis and reduce progression of cancer. Understanding the molecular basis of their anticancer properties based on structure and activity is important for the rational design of novel chemotherapeutic agent [37]. Maldi 531.2[M + H]⁺ is a phenolic derivative. Structure, lipophilicity and antioxidant properties of the compounds are major factors affecting the cytotoxicity of the compound due to the presence of a number of hydroxyl (–OH) ring components and by the length of the alkyl esters within the structure [37]. Lipophilicity goes
with the number of alkyl esters, i.e. the longer the alkyl esters in the molecule, the greater its anticancer effect [37]. For example, propyl esters are more effective against tumor cells than methyl esters. In addition, –OH groups are responsible for the antioxidant property in phenolic compounds and their methylation diminishes their cytotoxicity [38]. However, lipophilicity and antitumor activity were difficult to correlate, because other structural characteristics may also account for the cytotoxicity of Maldi 531.2[M + H]+. This includes the position of the phenolic substituents such as aromatic rings, carbons, carboxyl group (C = O), and the alkyl chains.

Apoptosis is a fundamental, energy-dependent biological mechanism [31,39], which can be triggered by a diverse array of stimuli, including phytochemicals [40,41]. The reduction of ΔΨm by Maldi 531.2[M + H]+ in the present study strongly indicates initiation of apoptotic events and inhibition of cell proliferation by dissipation of the mitochondrial membrane potential. Mitochondria are dynamic organelles, as they constantly produce ATP [42]. A decrease of the mitochondrial membrane potential results in loss of mitochondrial function as it is an important component of the electro-chemical gradient that is generated across the inner mitochondrial membrane during electron transport and oxidative phosphorylation [43]. Thus, ΔΨm is used as an indicator of cell integrity [23], which is critical for normal function of mammalian cells, maintaining the function of mitochondrial proteins in the production of ATP and reactive oxygen species (ROS). Alterations of the mitochondrial membrane potential are indicative of changes in mitochondrial metabolic activity (Acton 2004) leading to cell cycle arrest and apoptosis [44].

Two biochemical assays were used to establish apoptosis: annexin V and JC-1 mitochondrial membrane potential assay. The results of these two assays are sufficient to establish the occurrence of apoptosis. JC-1 assay evaluates extent of apoptosis through an end point which is the loss of the mitochondrial membrane potential reflected by the entry and aggregation of the dye inside the mitochondrial matrix. This event presupposes that cytochrome C has been released, an event associated with the loss of the mitochondrial membrane potential. These two events however may not always go together since it has been shown that loss of mitochondrial membrane potential is not necessarily required for cytochrome c release [45].

In the case of the cytotoxicity effect of Maldi 531.2 [M + H]+ as shown in cell viability tests and annexin V-FITC binding assay, the difference in IC50 values with XTT at 0.02-0.03 uM and the non-significant difference of cytotoxic effect at 0.05 uM with annexin V FITC binding assay for apoptosis, relative to untreated cells, is the time of observation. The cytotoxicity assay required 3 days (72 hours) of incubation with varied concentrations of the drug/ natural product to determine the growth inhibitory concentration which is the IC50 where 50% of the population of cells have died already at a range of concentration of 0.02 uM-0.03 uM for CCRF-CEM and CEM/ADR5000 respectively. In the case of apoptosis, observation time was only 24 hours after treatment purposely to check only the ability of Maldi 531.2[M + H]+ to “initiate” apoptosis. The optimum number of hours for Maldi 531.2[M + H]+ must not exceed 24 hours. If cells were incubated longer than 24 hours with Maldi 531.2[M + H]+, i.e. after 32 or 48 hours, all cells were dead which may significantly affect flow cytometric reading for apoptosis. Annexin V-FITC binding assay was used

### Table 5 List of unknown compounds previously isolated from Aglaia edulis tested and found active against several human cancer cell lines (Kim et al., 2006) [19]

| Compound number | m/z  | Elemental formula | Assigned name of the compound |
|-----------------|------|-------------------|-------------------------------|
| 1               | ^    | ^                 | Aglaroxin A                   |
| 2               | 584.1875 | C18H31NO9Na* | Aglaroxin A-1-O-Acetate |
| 3               | 614.1999 | C18H31NO9Na* | 3’-methoxyaglaroxin A-1-O-Acetate |
| 4               | 693.2421 | C18H31NO9Na | 19,20-dehydrodulirone A |
| 5               | *    | *                 | Edulirin A                     |
| 6               | **   | **                | Edulirin A-10-O-Acetate        |
| 7               | 665.2469 | C36H38N2O8Na | 9,20-dehydroedulirin A         |
| 8               | 667.2608 | C36H38N2O8Na | Isoedulirin A                  |
| 9               | ***  | ***               | Isoedulirin B                  |
| 10              | 316.1225 | C13H20N2O5Na | Aglamide A                    |
| 11              | 335.1079 | C17H20N2O2SNa | Aglamide B                    |
| 12              | 323.1732 | C13H20N2O2Na | Aglamide C                    |
| 13              | 254.1151 | C13H20N2O2Na | Aglamide D                    |

^ - known compound; ^ ^ - no assigned compound name; * - similar compounds.

### Table 6 List of compounds previously isolated from Aglaia foveolata tested and found active against cancer (Salim et al., 2007) [20]

| Compound number | m/z  | Elemental formula | Assigned name of the compound |
|-----------------|------|-------------------|-------------------------------|
| 1               | ^    | ^                 | Silvestrol                    |
| 2               | 675.2682 | C36H38N2O8Na* | ^^                            |
| 3               | *    | *                 | ^^                            |
| 4               | *    | *                 | ^^                            |
| 5               | 673.2528 | C36H38N2O8Na | ^^                            |
| 6               | 661.2529 | C2H17NO2Na | ^^                            |
| 7               | 559.3606 | C2H17NO2Na | ^^                            |

^ - known compound; ^^ - no assigned compound name; * - similar compounds.
to detect PS exposure on the membranes of apoptotic cells with high affinity. Annexin V binding assay detects apoptotic cells significantly even earlier than detections based on alteration in gross DNA structures [31]. Although huge efforts have been undertaken to identify P-glycoprotein inhibitors to overcome multidrug resistance [46,47], clinical trials failed as yet [47,48]. Another concept is to use non-cross-resistant cytotoxic compounds to kill multidrug-resistant tumors. Remarkably, CEM/ADR5000 cells did not reveal considerable cross-resistance to Maldi 531.2[M + H]+ (1.5-fold), although CEM/ADR5000 cells were highly resistant to anthracyclines, Vinca alkaloids, taxanes and other established drugs as previously reported [32]. This is a remarkable result, since it implies that Maldi 531.2[M + H]+ might be suitable to kill refractory tumors, which are resistant to standard chemotherapy.

Conclusion

In conclusion, Maldi 531.2[M + H]+ showed considerable activity against sensitive and multidrug-resistant tumor cells by dissipation of the mitochondrial membrane potential and induction of apoptosis. A. loheri and Maldi 531.2[M + H]+ may be promising for further drug development. Further studies are required to unravel the full potential of Maldi 531.2[M + H]+ as novel drug candidates for cancer therapy.

Abbreviations

ALL: Acute lymphoblastic leukemia; CCCP: Carbonyl cyanide 3-chlorophenylhydrazone; CCRF-CEM: Cytosine arabinoside human leukemia cells; CEM/ADR5002: Multidrug resistant leukemia cells; CF: Column fraction(s); EAE: Ethyl acetate extract; ECE: Ethanolic concentrated extract; HCT116: Human Colon cancer cell line; HE: Hexane extract; HPLC: High liquid performace chromatography; MDP: Master dilution plate; MTT: 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide); NMR: Nuclear magnetic resonance; PRBC: Peripheral blood mononuclear cells; PHA: Phytohemagglutinin; PI: Propidium iodide; PS: Phosphatidyserine; SF: Sub-fractions of CF; TLC: Thin layer chromatography; XTT: 2,3-bis(2-methoxy-4-nitro-S)-sulfophenyl-2H-tetrazolium-5-carboxanilide).

Competing interests

The authors declare that there are no competing interests involved in this research study.

Authors' contributions

SJ and TE planned and supervised the research work (SJ in the Philippines; TE in Germany). SJ helped in collection of plant materials, funded (through her research grant) the isolation of Maldi 531.2[M + H]+; TE funded expenses for all assays in the laboratory (Germany); ED performed the isolation of Maldi 531.2[M + H]+ (in the Philippines) and all assays in Germany. All authors read and approved the final manuscript.

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References

1. Lucas DM, Still PC, Perez LB, Grever MR, Kinghorn AD: Potential of plant-derived natural products in the treatment of leukemia and lymphoma. Curr Drug Targets. 2010, 11:812–822.
2. Reaman GH: Pediatric oncology: current views and outcomes. Pediatr Clin North Am. 2002, 49:1305–1318.
3. Pui CH, Relling MV, Pharm D, Downing JR: Mechanisms of disease: acute lymphoblastic leukemia. New Engl J Med 2004, 350:1553–1548.
4. Kawamata N, Ogawa S, Zimmermann M, Kato M, Sanada M, Hernminkki K, Yarnatomo G, Nannya Y, Koehler R, Flohr T, Miller CW, Harbott J, Ludwig WD, Stanulla M, Schrappe M, Bartram CR, Koeffler HP: Molecular allelokatotyping of pediatric acute lymphoblastic leukemias by high-resolution single nucleotide polymorphism oligonucleotide genomic microarray. Blood 2008, 111:776–784.
5. Bungaro S, Dell'Orto MC, Zangrandario A, Basso D, Gorletta T, Lo Nigro L, Lenz A, Young BD, Basso G, Bacciato S, Biondi A, Tekonnie G, Cazzagniga G: Integration of genomic and gene expression data of childhood ALL without known aberrations identifies subgroups with specific genetic hallmarks. Genes Chromosomes Cancer 2009, 48:223–235.
6. Parker H, Wright SL, Stewart AR, Bailey S, Bown NP, Hall AG, Harrison CJ: A comprehensive analysis of the CDKN2A gene in childhood acute lymphoblastic leukemia reveals genomic deletion, copy number neutral loss of heterozygosity, and association with specific cytogenetic subgroups. Blood 2009, 113:100–107.
7. Shacter E, Williams JA, Hinson RM, Sentürker S, Lee Y: Oxidative stress interferes with cancer chemotherapy: inhibition of lymphoma cell apoptosis and phagocytosis. Blood 2000, 96:307–313.
8. Effert T: Personalized Cancer Medicine: From molecular diagnostics to targeted therapy with natural products. Planta Med 2010, 76:1143–1154.
9. Lipipun V, Kurokawa M, Sutsiris R, Taweechotipatr P, Poomthyn P, Hattori M, Shiraki K: Efficacy of Thai medicinal plant extracts against herpes simplex virus type 1 infection in vitro and in vivo. Antiviral Res 2003, 60:175–180.
10. Tasanor O, Brem B, Leitsch D, Binder M, Duchene M, Greger H, Wernsdorfer W: Development of a pharmacodynamic screening model with Entamoeba histolytica. Wien Klin Wochenschr 2007, 119:58–59.
11. Proksch P, Edrada R, Ebel R, Bohnenstengel FI, Nugroho BW: Structure activity relationships of antiproliferative rocaglamide class of compounds structurally related to bisamides and flavaglines. Phytochemistry 2007, 68:928–938.
12. Efferth T: Personalized Cancer Medicine: From molecular diagnostics to targeted therapy with natural products. Planta Med 2010, 76:1143–1154.
13. Proksch P, Edrada R, Ebel R, Bohnenstengel FI, Nugroho BW: Structure activity relationships of antiproliferative rocaglamide class of compounds structurally related to bisamides and flavaglines. Phytochemistry 2007, 68:928–938.
14. Inad A: Cancer chemopreventive activity of odorine and odorinol from Aglaia odorata. Biol Pharm Bull 2001, 24:1282–1285.
15. Proksch P, Edrada R, Ebel R, Bohnenstengel FI, Nugroho BW: Structure activity relationships of antiproliferative rocaglamide class of compounds structurally related to bisamides and flavaglines. Phytochemistry 2007, 68:928–938.
16. Knewkomo N, Greger H, Sangchote S: Antifungal activity of flavaglines from Aglaia species. Agric Sci J 2006, 37:5 (Suppl.):66–71.
17. Proksch P, Edrada R, Ebel R, Bohnenstengel FI, Nugroho BW: Structure activity relationships of antiproliferative rocaglamide class of compounds structurally related to bisamides and flavaglines. Phytochemistry 2007, 68:928–938.
18. Proksch P: Structure activity relationships of antiproliferative rocaglamide derivatives from Aglaia species (Meliaceae). Z Naturforsch C 1999, 54:55–60.
19. Kim S, Chin YW, Su BN, Riswan S, Kardonno LBS, Afnastiin JJ, Chai H, Farnsworth NR, Cordell GA, Swanson SM, Kinghorn AD: Cytotoxic flavaglines and bisamides from Aglaia edulis. J Nat Prod 2006, 69:1769–1775.

20. Salim AA, Chai H, Rachman I, Riswan S, Kardonno LBS, Farnsworth N, Carcache-BLANCO E, Kinghorn D: Constituents of leaves and stem bark of Aglaia foveolata. Tetrahedron 2007, 63:7926–7934.

21. Lucas DM, Edwards RB, Lozanski G, West DA, Shin JD, Vargo MA, Davis ME, Rozewski DM, Johnson AJ, Su BN, Goettl VM, Heerema NA, Lin TS, Lehman A, Zhang X, Jarjura D, Newman DJ, Byrd JC, Kinghorn AD, Grever MR: The novel plant-derived agent silvestrol has B-cell selective activity in chronic lymphocytic leukemia and acute lymphoblastic leukemia in vitro and in vivo. Blood 2009, 113:4656–4666.

22. Diaz G, Setzu MD, Zucca A, Isola R, Diana A, Murre R, Sogos V, Gremo F: Subcellular heterogeneity of mitochondrial membrane potential: relationship with organelle distribution and intercellular contacts in normal hypoxic and apoptotic cells. J Cell Sci 1999, 112:1077–1084.

23. Acton B, Junisaca A, Junisca L, Casper R: Alterations in mitochondrial membrane potential during pre-implantation stages of mouse and human embryos. Mol Hum Reprod 2004, 10:33–32.

24. Kimmig A, Gielen F, Neumann M, Frese G, Handgretinger R, Kardos G, Diddens H, Niethammer D: Susceptibility of multidrug-resistant human leukemia cell lines to human interleukin 2-activated killer cells. Cancer Res 1990, 50:753–769.

25. Efferth T, Sauerbrey A, Olbrich A, Gebhart E, Rauch P, Weber HO, Hengstler JG, Halatsch ME, Volm M, Tew KD, Ross DD, Funk JO: Molecular modes of action of artesunate in tumor cell lines. Cytotechnology 1993, 12:171–212.

26. Gillet JP, Efferth T, Steinbach D, Hamel J, de Longueville F, Bertholet V, Remacle J: Microarray-based detection of multidrug resistance in human tumor cells by expression profiling of ATP-binding cassette transporter genes. Cancer Res 2004, 64:8987–8993.

27. Katal P, Sachandani D, Pinney C, Lieberman MW: Cytokine production in cell culture by peripheral blood mononuclear cells from immunocompetent hosts. Clin Diag Lab Immunol 1998, 5:1(1).

28. Mossmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 1983, 65:53–63.

29. Joshi SC, Verma AR, Mathela CS: Antioxidant and antibacterial activities of the leaf essential oils of Himalayan Lauraceae species. Food Chem Toxicol 2010, 48:38–40.

30. Pett PX, Leceur H, Zorn E, Dauguet C, Mignotte B, Gougeon ML: Alterations in mitochondrial structure and function are early event of desamethasone-induced thymocyte apoptosis. J Cell Biol 1995, 130:157–167.

31. Zhang G, Gurtu V, Kain SR, Yan G: Early detection of apoptosis using a fluorescent conjugate of annexin V. Biotechniques 1997, 23:525–531.

32. Efferth T, Konkimalla VB, Wang YF, Sauerbrey A, Fuchtbauer S, Zindl F, Matern J, Volm M: Prediction of broad spectrum resistance of tumors towards anticancer drugs. Clin Cancer Res 2008, 14:2405–2412.

33. Vu H, Pham NB, Quinn RJ: Direct screening of natural product extracts using mass spectrometry. J Biomol Screen 2008, 13(4):265–275.

34. Mueller AN, Samuel R, Chase MW, Pannell CM, Greger H, Aglaia (Meliaceae): an evaluation of taxonomic concepts based on DNA data and secondary metabolites. Am J Botany 2005, 92:534–543.

35. Crotoruo R, Van den Broek LA, Frissen AE, Davidecu CM, Peter P, Bueriu CG: Lipase catalyzed synthesis aromatase esters of sugar alcohols. World Acad Sci Eng Technol 2011, 76:484–489.

36. Lee YF, Don MI, Hung PS, Shen YC, Lo YS, Chang CF, Hu JK: Cytotoxicity of phenolic acid phenethyl esters on oral cancer cells. Cancer Lett 2005, 223:19–25.

37. Machado NFL, Calheiros R, Gaspar A, Garrido J, Borges F, Marques MP: Antioxidant phenolic esters with potential anticancer activity: solution equilibria studied by raman spectroscopy. J Raman Spectrosc 2008. doi:10.1002/jrs.2083.

38. Fiuza SM, Gomes C, Teixeira L, da Cruz MT G, Cordeiro MNDS, Milhazes N, Borges F, Marques MP: Phenolic acid derivatives with potential anticancer properties – a structure-activity relationship study. Part 1: methyl, propyl and octyl esters of caffeic and gallic acids. Bioorg Med Chem 2004, 12:3581–3589.

39. Eimore S: Apoptosis: a review of programmed cell death. Tactiol Pathol 2007, 35:495–516.

40. Fulda S: Modulation of apoptosis. Planta Med 2010, 76:1075–1079.

41. Liberles SD, Schreiber SL: Apoptosis-inducing natural products found in utero during murine pregnancy. Chem Biol 2000, 7:365–372.

42. Tait SWG, Green DR: Mitochondria and cell death: outer membrane permeabilization and beyond. Mol Cell Biol 2010, 11:521–632.

43. Heerdt BG, Houston MA, Augenlicht LH: The intrinsic mitochondrial membrane potential of colonic carcinoma cells is linked to the probability of tumor progression. Cancer Res 2005, 65:9861–9867.

44. Heerdt BG, Houston MA, Anthony GM, Augenlicht LH: Initiation of growth arrest and apoptosis of MCF-7 mammary carcinoma cells by tributyrin, a triglyceride analogue of the short-chain fatty acid butyrate, is associated with mitochondrial activity. Cancer Res 1999, 59:1584–1591.

45. Ly JD, Grubb DR, Lawen A: The mitochondrial membrane potential (∆Ψm) in apoptosis; an update. Apoptosis 2003, 8:115–128.

46. Ford JM, Hatt WN: Pharmacologic circumvention of multidrug resistance. Cytotechnol 1993, 12:171–212.

47. Eichhorn T, Efferth T: β-glycoprotein and its inhibition in tumors by phytochemicals derived from Chinese herbs. J Ethnopharmacol 2012, 141:557–570.

48. Tiwari AK, Sodani K, Dai CL, Ashby CR Jr, Chen ZS: Revisiting the ABCs of multidrug resistance in cancer chemotherapy. Curr Pharm Biotechnol 2011, 12:570–594.

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