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SHORT REPORTS

Neuroblastoma SH-SY5Y cytotoxicity, anti-amyloidogenic activity and cyclooxygenase inhibition of *Lasianthus trichophlebus* (Rubiaceae)

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Abstract

The anti-amyloidogenic potential and cyclooxygenase anti-inflammatory activity of *Lasianthus trichophlebus* extracts were evaluated. The MeOH extract (LTM) and chloroform extract (LTC) exhibited significant cytotoxic inhibition against the neuroblastoma SH-SY5Y cell with an IC_{50} of 17.52 µg/mL and 12.28 µg/mL, respectively. Thioflavin T assay indicated the LTC extract inhibition (70.56% at 50 µg/mL) to be statistically comparable ($p < 0.05$) to the positive control. Cyclooxygenase inhibition against COX-1 and COX-2 enzymes gave IC_{50} values for the LTM extract to be 18.20 and 29.60 µg/mL, respectively; while, the LTC extract showed 4.11 and 2.78 µg/mL, respectively. LC–MS of the LTM extract identified 22 putative compounds, which may prove to be pharmacologically relevant. This study has provided potential insights into the utilization of *L. trichophlebus* to develop safer plant-based agents for anti-inflammatory or neurodegenerative diseases.

Keywords *Lasianthus* · Cytotoxicity · Neuroblastoma · Cyclooxygenase

Introduction

Genus *Lasianthus* Jack consists of 225 species, with the highest diversity in tropical and subtropical Asia (Arshed and Alejandro 2016). These plants are used in several types of traditional folk medicine, including treatments for fever and blood loss with *Lasianthus lucidus* (Choudhury et al. 2014) and as an unspecified antidote using the fruit of *Lasianthus andamanicus* (Sharief 2007). The root decoction of *Lasianthus oblongus* was used by post-partum mothers to accelerate the contraction of expanded organs (Ong et al. 2012). Recent pharmacological studies revealed the antioxidant potential of *Lasianthus hartii* (Yang et al. 2017), anti-inflammatory effect of *L. oblongus* (Saha et al. 2004), and cytotoxicity against human ovarian 2780 cells by *Lasianthus acuminatissimus* (Li et al. 2006). Secondary metabolites from *Lasianthus* species were isolated using several chromatographic methods and elucidated with spectroscopic techniques. These included the presence of anthraquinones and their glycosides and sesquiterpenes from *L. acuminatissimus* (Li et al. 2006), iridoid glucosides from *Lasianthus wallichii* (Takeda et al. 2002), *Lasianthus hartii* (Yang et al. 2017), and *Lasianthus verticillatus* (Al-Hamoud et al. 2019), megastigmane glucosides from *Lasianthus fordii* (Takeda et al. 2004), and triterpenoids and steroids from *Lasianthus gardneri* (Dallavalle et al. 2004).

As part of our research in studying the endemic and indigenous Rubiaceae plants of medicinal importance from the Philippines (Castro et al. 2016; Olivar et al. 2018; Tan et al. 2012, 2014), we investigated the *Lasianthus trichophlebus* Hemsl. ex. F.B. Forbes & Hemsl. This species is found in South China, Thailand, Vietnam, Malay Peninsula, Philippines, and Indonesia and grows from a short shrub to reach 1–2-m tall (Zhu 2002). No study on the phytochemicals or bioactivity of *L. trichophlebus* has been previously reported.

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Hence, the present study of *L. trichophlebus* describes its cytotoxicity against a neuroblastoma cell line (SH-SY5Y), amyloid-beta aggregation potential, inhibitory effects against COX-1 and COX-2, and the metabolite profile of its extracts.

Materials and methods

Plant materials

Fresh leaves of *L. trichophlebus* were collected from the province of Antique, Philippines (11° 24′ 33.59″ N, 122° 04′ 2.40″ E) in April 2016. The plant morphology was identified by Grecebio Jonathan Alejandro, a Philippine Rubiaceae specialist. After a year, this species was molecularly authenticated using five DNA barcoding loci (Arshed et al. 2017). A voucher specimen was kept at the UST-Herbarium (USTH 012462).

Extraction and fractionation of extracts

Air-dried, ground leaves of *L. trichophlebus* (2.7 kg) were subjected to MeOH extraction overnight. The filtrate was collected and concentrated under reduced pressure using a rotary evaporator set at 45 °C. The process was repeated thrice using a total of 22.0 L MeOH. The extraction yielded 247.34 g of the MeOH extract (LTM). A portion (190 g) of the marc was suspended in dist. H_2O and partitioned successively with hexane and $CHCl_3$. The hexane and $CHCl_3$ layers were, respectively, dried with anhydrous Na_2SO_4 and concentrated in vacuo to obtain the hexane (LTH, 27.7 g) and $CHCl_3$ extracts (LTC, 8.0 g). The remaining aqueous layer was freeze-dried using a lyophilizer to obtain the aqueous extract (LTA, 9.4 g).

ATP cell cytotoxicity assay

Fibroblast N9 and neuroblastoma SH-SY5Y cells were maintained in DMEM supplemented with 10% FBS, 1% kanamycin, and 1% penicillin. Cell cultures were maintained at 37 °C in 5% CO_2 and passaged once per week. The cells were sub-cultured into a 96-well plate at 1×10^3 cells/well and incubated for 24 h. The cells were treated with plant extracts and incubated for 48 h for fibroblast N9 cells and 72 h for neuroblastoma SH-SY5Y cells. The media was removed, and the wells were washed with PBS. Fresh media (100 µL) were added and incubated for another 30 min. CellTiter-Glo® luminescent reagent (100 µL) was added and the luminescence was measured using a PerkinElmer Victor-3® multi-plate reader (PerkinElmer, Waltham, MA, USA).

Thioflavin T (ThT) fluorescence assay

The amyloid-β_{1-42} (10 µM) dissolved in PBS (pH 7.4) was incubated at 37 °C for 24 h in the presence or absence of the plant extracts. Then, ThT solution (20 µL, 50 µM) in glycine–NaOH buffer (pH 9) was added. The fluorescence signal was measured (excitation wavelength, 450 nm; emission wavelength, 510 nm) using a PerkinElmer Victor-3® multi-plate reader. The percentage of aggregation inhibition was calculated using the following equation: $[(1 - I_{Fi}/I_{Fc}) \times 100\%]$, where I_{Fi} and I_{Fc} are the fluorescence absorbance with and without the inhibitors, respectively, after subtracting the background fluorescence of the ThT solution (Xia et al. 2019).

Cyclooxygenase assay

To a solution of 150 µL of 100-mM Tris, 10 µL of plant extracts dissolved in DMSO, 10 µL of 1000 µM Hemin, and 10 µL of 250 U/mL COX-2 or COX-1 enzyme were added. Indomethacin was used as the positive control, and DMSO served as the negative control. The mixture was incubated at 25 °C for 15 min. After incubation, 10 µL of 200-µM amplex red was added to the mixture. Then, 10 µL of 2000-µM arachidonic acid was added, and the reaction fluorescence absorbance was monitored for 2 min using Varioskan Flash at 535 nm (excitation) and 590 nm (emission).

The percent inhibition of the samples and the positive control was determined based on the averaged slope of each replicate using the following formula:

$$\% \text{ Inhibition} = \left[(\text{slope uninhibited} - \text{slope inhibited})/\text{slope uninhibited}\right] \times 100.$$

"Slope uninhibited" is the slope of the line from the fluorescence intensity vs. time plot of the negative control group, and "slope inhibited" is the slope of the line from the fluorescence intensity vs. time plot of the samples/positive control.

LC–MS metabolite profiling

LC–MS/MS analysis was performed using a Xevo G2-S Qtof (Waters Corp., Singapore). The separation was achieved using a BEH HSS T3 column (50 × 2.1 mm internal diameter). The system delivered a constant flow of 0.4 mL/min, and the mobile phase consisted of 5% CH_3CN in MeOH and 0.1% HCOOH in H_2O. The injection volume was 1 µL. For operation in MS/MS mode, a mass spectrometer with an electrospray interface (ESI) was used, and the parameters were set as follows: capillary voltage, 3.0 kV for negative mode; source temperature, 120 °C; desolvation temperature,

400 °C; cone gas flow, 100 L/h; and desolvation gas flow, 1000 L/h. Low collision energy at 6 V, high collision energy at 20–50 V, and lock mass solution at 1 ng/µL were used to calibrate mass accuracy. All LC–MS/MS data were processed by the MassLynx version 3.5 NT Quattro data acquisition software. For putative compound identification, accurate mass screening was carried out using the UNIFI data analysis software. The acquired MS spectra were subjected to library matching using the Traditional Chinese Medicine (TCM) library that is integrated within the UNIFI analysis software. Annotation of the candidate masses was based on the accurate mass match, isotopic ratio match, and precursor ion intensity counts. The criteria for a component ID to be considered a good match are as follows: a mass accuracy error ≤ 5 mDa or ≥ -5 mDa, and a response precursor for precursor ion ≥ 2000.

Statistical analysis

All values were reported as means ± SD. Statistical significance was analyzed using one-way ANOVA and Levene's test followed by Tukey's HSD test. $p < 0.05$ was considered statistically significant.

Results and discussion

In vitro cell toxicity using fibroblast N9

Methanolic extraction and solvent partitioning of *L. trichophlebus* leaves resulted in obtaining the different extracts. All extracts were dried free of solvent prior to their use in the different assays. Initially, the toxicity of the *L. trichophlebus* crude methanolic extract (LTM) was determined in vitro using the fibloblast N9 cell line (Fig. 1). The LTM extract did not show any significant cytotoxicity after 48-h incubation even at the highest concentration of 50 µg/mL. Maximum cell growth inhibition of 15% was noted at 50 µg/mL. The percentage cell viability of the LTM extract was comparable to the negative control (0 µg/mL) at $p < 0.05$. The in vivo acute oral toxicity of the LTM extract is reported in the Online Resource material.

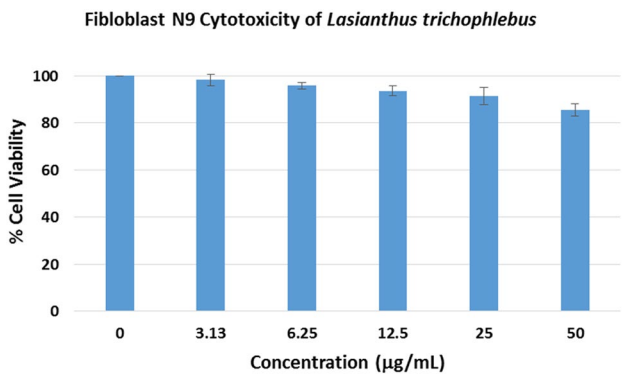

Fig. 1 Cell viability of the *Lasianthus trichophlebus* crude methanolic extract (LTM) against fibroblast N9 cell line. The viability was determined by ATP assay and is expressed as % of the control (0 µg/mL). Results are expressed as means ± SD of three trial experiment

Cyclooxygenase-1 and -2 inhibition

The cyclooxygenase pathways have been widely accepted as major enzymatic routes dealing with inflammatory progression in mammalian cells. Hence, their clinical relevance has also been noted in several review articles (González-Périz and Clària 2007). Medicinal drugs exhibiting anti-inflammatory activity were also prioritized in pharmacological treatment dealing with degenerative inflammatory diseases (Malik et al. 2017). COX-1 inhibition in gastric mucosal cells led to decreased prostaglandin synthesis (Saha et al. 2004), which would be responsible for the significant gastric toxicities imposed by nonsteroidal anti-inflammatory drugs (NSAIDs) due to the loss of local protection. COX-2 inhibition was more desirable for delivering therapeutic outcomes for inflammation, pain, cancer, and neurological diseases (Blobaum and Marnett 2007).

The results of COX-1 and COX-2 screening assays of the *L. trichophlebus* extracts at 10 µg/mL are presented in Fig. 2. Among the four tested extracts, LTC showed the highest inhibition towards both COX-1 (61.63%) and COX-2 (57.75%). The LTM extract also showed enzyme inhibitions greater than 50%. The LTM extract gave 54.01% against COX-1 and 57.07% inhibition using COX-2. The

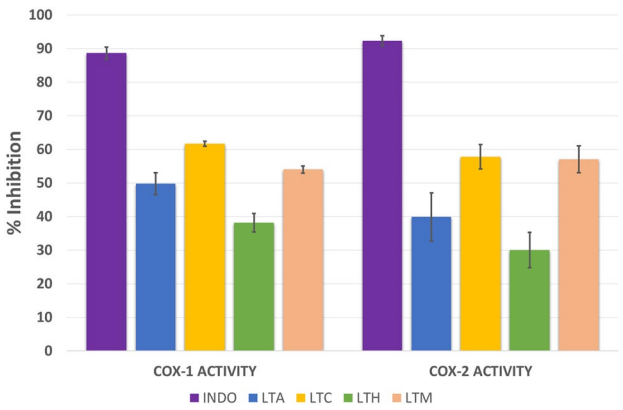

Fig. 2 In vitro cyclooxygenase of *Lasianthus trichophlebus* extracts at 10 µg/mL. Indomethacin (INDO, 4.0 mM) was used as the positive control. The % inhibition was expressed as means ± SD of three trial experiment. Only LTM and LTC extracts exhibited > 50% inhibition to the COX-1 and COX-2 enzymes. At $p < 0.05$, there is a significant difference on the % inhibition of INDO to the plant extracts. *LTA* aqueous extract, *LTC* chloroform extract, *LTH* hexane extract, *LTM* crude methanol extract

other extracts, hexane (LTH) and aqueous (LTA), gave enzyme inhibitions of less than 50%. All extracts exhibited significantly lower inhibition of COX-1 and COX-2 compared to the standard drug, indomethacin ($p < 0.05$), which gave 88.61% inhibition for COX-1 and 92.28% inhibition for COX-2.

As only LTM and LTC extracts gave greater than 50% inhibition to the cyclooxygenase enzymes, we determined their 50% maximal inhibitory concentration (IC_{50}) using 0.5, 1, 5, 10, 40, 70, and 100 µg/mL concentrations. The LTM extract exhibited IC_{50} values of 18.20 and 29.60 µg/mL for COX-1 and COX-2, respectively. The IC_{50} values for LTC were 4.11 and 2.78 µg/mL for COX-1 and COX-2, respectively. Based on these values, the LTC revealed a more potent IC_{50} compared to LTM. Extensive literature search also indicated limited studies on the anti-inflammatory effect of *Lasianthus* extracts. The *L. oblungus* crude extract gave 85.97% inhibition (at 250 µg/mL) on nitric oxide production from macrophages (RAW 264.7 cells) (Saha et al. 2004).

Neuroblastoma SH-SY5Y cell viability

Different methods of in vitro cell viability and cytotoxicity assays were established (Ishiyama et al. 1996) and applied for drug screening and development as they are rapid, inexpensive, and allow low-cost animal study. Adenosine triphosphate (ATP) is the most important chemical energy source for all biological processes in cells. As such, measuring cellular ATP is the most sensitive method for probing cellular stages and determining their viability. Cells tended to limit their ability to produce ATP when they were stressed, mutilated, or subjected to limited nutrient availability. The ATP assay utilized the conversion of luciferin to oxyluciferin catalyzed by luciferase in the presence of Mg^{2+} ions and ATP. Hence, the luminescent signals correlated linearly with the ATP concentrations or cell numbers (Garcia and Massieu 2003; Mueller et al. 2004).

The cellular viability of neuroblastoma SH-SY5Y cells was explored in the presence of LTM and LTC extracts via the ATP assay. As depicted in Fig. 3, both extracts at various concentrations inhibited SH-SY5Y cell growth. There is no significant difference ($p < 0.05$) in the percentage cell viability of both extracts at the 0.39 µg/mL lowest concentration when compared to the negative control (0 µg/mL). However, significant difference ($p < 0.05$) was observed for the other percentage inhibitions for both extracts. The IC_{50} of the LTM extract was determined as 17.52 µg/mL, while the LTC extract gave an IC50 of 12.28 µg/mL. Since the neuroblastoma SH-SY5Y cells are used in neurological studies

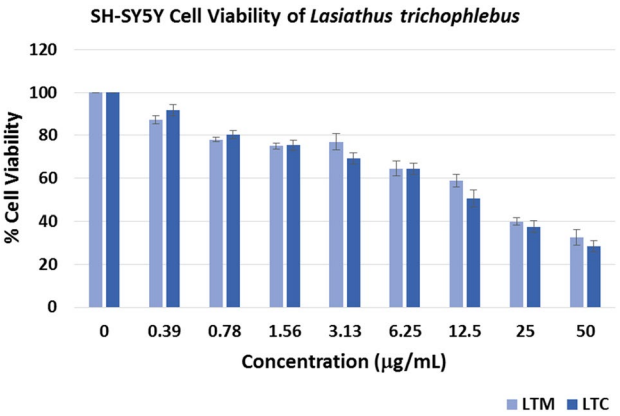

Fig. 3 Cell viability of the *Lasianthus trichophlebus* crude methanolic extract (LTM) and chloroform extract (LTC) against the neuroblastoma SH-SY5Y. The viability was determined by ATP assay and is expressed as % of the control (0 µg/mL). Results are expressed as means ± SD of three trial experiment

Table 1 ThT assay results of *Lasianthus trichophlebus* extracts

Sample	$A\beta_{1-42}$ aggregation inhibition (%)[a]
Phenol red (18 µg/mL)[b]	68.03 ± 2.33
LTM extract (50 µg/mL)	44.02 ± 1.26
LTM extract (5 µg/mL)	28.49 ± 1.68
LTC extract (50 µg/mL)	70.56 ± 2.46*
LTC extract (5 µg/mL)	31.67 ± 3.98

*Statistically comparable to the positve control at $p < 0.05$

[a]The values are expressed as mean ± SD of three trial experiments

[b]The positive control

including Parkinson's disease (PD), Alzheimer's disease (AD), and traumatic brain injury (TBI) (Wu et al. 2011), the results of the cytotoxicity assay may offer promising leads to the discovery of pharmacologically active compounds in neurological diseases.

Thioflavin T assay

Alzheimer's disease (AD) is a fatal neurodegenerative disorder associated with dementia, deterioration of cognitive functions, and memory loss. Several factors have been identified to enhance AD progression, including abnormal β-amyloid (Aβ) deposition, tau protein aggregation, decreased acetylcholine, oxidative stress, and neuroinflammation of the nervous system (Xia et al. 2019). Currently, only five compounds (donezipil, tacrine, rivastigmine,

galantamine, and memantine) are available and approved in the market to reduce the symptoms associated with AD (Alghazwi et al. 2019). The control of Aβ deposition is an important approach for AD treatment as it damages neuronal and mitochondrial function, leading to oxidative stress and neuroinflammation.

As part of our on-going program on research involving AD (Giau et al. 2019, Giau and An 2019), we have tested the potential of the LTM and LTC extracts (Table 1) to inhibit the aggregation of $A\beta_{1-42}$ deposition using the thioflavin-T (ThT) fluorescence assay utilizing phenol red as the positive control. Moderate percentage inhibition was noted for the LTM extract at 50 µg/mL (44.02%) and 5 µg/mL (28.49%), and the LTC extract at 5 µg/mL (31.67%). A strong inhibition of 70.56% was observed for the LTC extract at 50 µg/mL. This value is also statistically comparable to the inhibition of the positive control at $p < 0.05$.

Metabolite profiling

LC–MS metabolite profiling of the LTM extract has putatively identified 22 secondary metabolites (Figs. 4, 5, Table 2). Based on the literature, these compounds could be categorized as "good match" standards in the TCM library (Chen 2011; He et al. 2019), as listed in Table 2. The following compounds were previously isolated and identified from different *Lasianthus* species: benzyl 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (**2**), asperuloside (**6**), citroside A (**10**), and paedroside (**16**) from *L. wallichii* (Takeda et al. 2002); lasianthionoside A (**4**), B (**3**), and C (**9**), asperuloside (**6**), citroside A (**10**), deacetylasperuloside (**13**), and methyl deacetylasperuloside (**17**) from *L. fordii* (Takeda et al. 2004); damnacanthol 11-methyl ether (**1**), asperulosidic acid (**5**), asperuloside (**6**), tachioside (**7**), isotachioside (**8**), lasianthuoside A (**19**), B (**14**), and C (**20**), damnacanthol (**15**), and deacetyl asperulosidic acid (**21**) from *L. acuminatissimus* (Li et al. 2006); lupeol (**12**), β-sitosterol (**18**), and lupenone (**22**) from *L. gardneri* (Dallavalle et al. 2004); and leonuriside A (**11**), asperuloside (**6**), and asperulosidic acid (**5**) from *L. hartii* (Yang et al. 2017). Interestingly, several compounds were found to be bioactive (Table 2). These bioactive compounds might be responsible for the observed biological activity of the *L. trichophlebus* extracts. Although these compounds were putatively identified using untargeted LC–MS, all compounds were present in other *Lasianthus* species, thus supporting their common biogenetic pathway in the genus *Lasianthus*. The antioxidant activity of several compounds and the phenolic functionality have been reported to have an effect on the inhibition of amyloid-*beta* aggregation. In particular, the presence of the aromatic moiety forms a non-covalent interaction with the amino acid residues, while the hydroxyl groups are capable of hydrogen bonding in amyloid-*beta* (Dhouafli et al. 2019).

Conclusions

This is the first report on the pharmacological activities associated with cyclooxygenase inhibition and amyloid-*beta* aggregation potential of *Lasianthus trichophlebus*. The crude methanolic and semi-polar chloroform extracts exhibited significant inhibition against neuroblastoma SH-SY5Y cells and cyclooxygenase-1 and -2 enzymes; while, the chloroform extract exhibited strong inhibition using the ThT assay. The present results demonstrated that *L. trichophlebus* could be a source of pharmacologically relevant compounds with potentially new biologically active materials obtained from its extracts. Studies of *L. trichophlebus* also provided potential insights into the utilization of plant materials to develop safer plant-based agents for anti-inflammatory or neurodegenerative diseases.

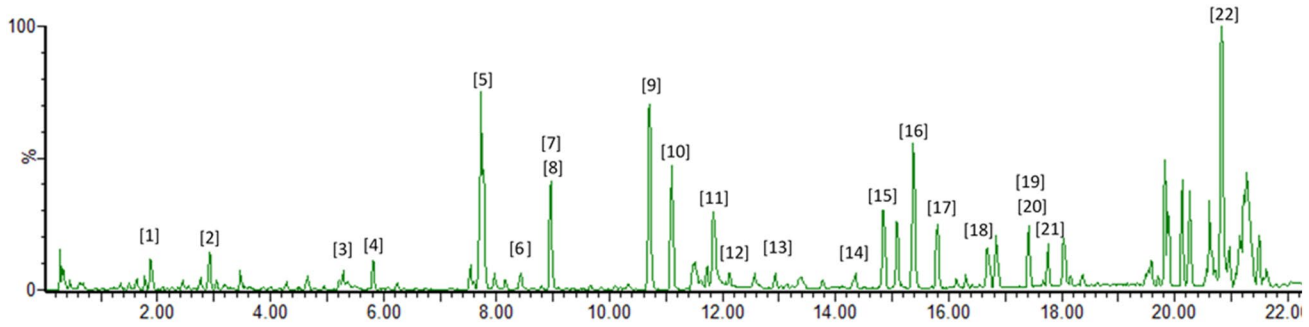

Fig. 4 Chromatogram of *Lasianthus trichophlebus* MeOH extract (LTM)

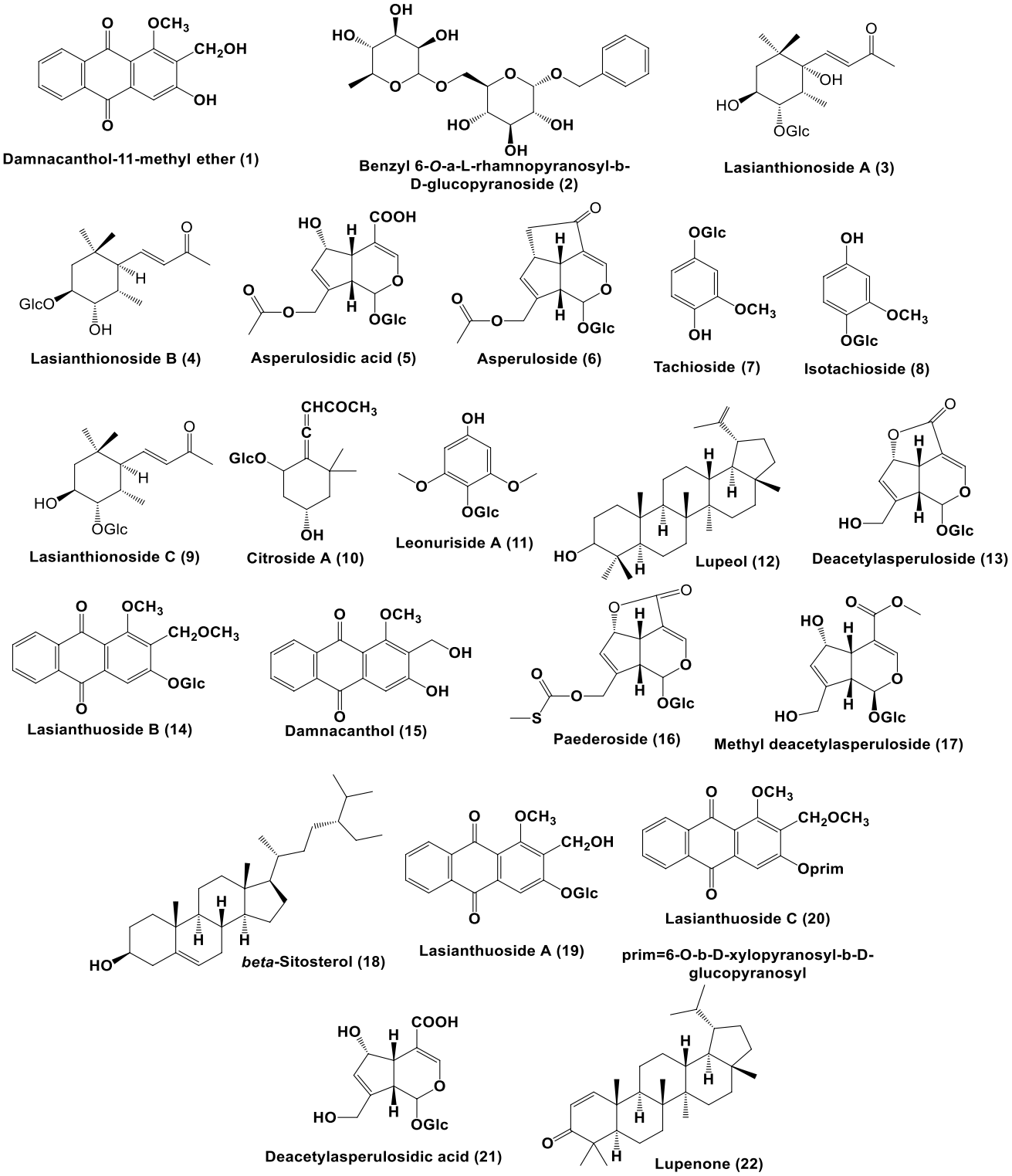

Fig. 5 Structures of putative compounds of *Lasianthus trichophlebus* MeOH extract (LTM)

Table 2 Secondary metabolites from *Lasianthus trichophlebus* MeOH extract (LTM)

RT	Exact mass		Elemental composition	Error (ppm)	Putative identity	Associated biological activity
	Calculated	Observed				
1.88	298.08412	298.08540	$C_{17}H_{14}O_5$	4.35	Damnacanthol-11-methyl ether (**1**)	
2.92	326.12131	326.12120	$C_{12}H_{22}O_{10}$	−0.31	Benzyl 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (**2**)	
5.29	388.20310	388.20340	$C_{19}H_{31}O_8$	−1.03	Lasianthionoside B (**3**)	
5.81	404.19560	404.19680	$C_{19}H_{31}O_9$	−2.96	Lasianthionoside A (**4**)	
7.73	432.12677	432.12820	$C_{18}H_{24}O_{12}$	3.29	Asperulosidic acid (**5**)	
8.43	414.11621	414.11570	$C_{18}H_{22}O_{11}$	−1.2	Asperuloside (**6**)	Treatment of rheumatoid arthritis (Li et al. 2006) Antiangiogenic (Camero et al. 2018)
8.97	302.10016	302.10050	$C_{13}H_{18}O_8$	1.13	Tachioside (**7**)	Antioxidant (DPPH) (Simlai and Roy 2013)
8.97	302.10016	302.10050	$C_{13}H_{18}O_8$	1.13	Isotachioside (**8**)	Antioxidant (DPPH) (Simlai and Roy 2013)
10.71	388.19980	388.19940	$C_{19}H_{31}O_8$	0.77	Lasianthionoside C (**9**)	
11.09	386.19406	386.19370	$C_{19}H_{30}O_8$	−1.03	Citroside A (**10**)	
11.84	332.11073	332.11180	$C_{14}H_{20}O_9$	3.2	Leonuriside-A (**11**)	Anti-oxidant (FTC) (Sugaya et al. 1998)
12.11	390.18311	390.18190	$C_{25}H_{26}O_4$	−3.07	Lupeol (**12**)	Antiinflammatory (Geetha and Varalakshimi. 2001) Antiangiogenic (You et al. 2003)
12.93	372.10565	372.10540	$C_{16}H_{20}O_{10}$	−0.8	Deacetylasperuloside (**13**)	
14.35	460.13700	460.13840	$C_{23}H_{24}O_{10}$	3.04	Lasianthuoside B (**14**)	Antitumor (Li et al. 2006)
14.84	284.06848	284.06700	$C_{16}H_{12}O_5$	−5.26	Damnacanthol (**15**)	Antibacterial (Comini et al. 2011)
15.37	446.08829	446.08620	$C_{18}H_{22}O_{11}S$	−4.7	Paedroside (**16**)	
15.80	404.13187	404.13100	$C_{17}H_{24}O_{11}$	−2.22	Methyl deacetylasperuloside (**17**)	
16.84	414.38617	414.38650	$C_{29}H_{50}O$	0.72	β-Sitosterol (18)	Antiinflammatory, Antipyretic (Gupta et al. 1980) Anthelminthic, antimutagenic, analgesic (Villasenor et al. 2002)
17.41	446.12100	446.12080	$C_{22}H_{22}O_{10}$	−0.45	Lasianthuoside A (**19**)	Antitumor (Li et al. 2006)
17.41	592.17900	592.17760	$C_{28}H_{32}O_{14}$	−2.36	Lasianthuoside C (**20**)	Antitumor (Li et al. 2006)
17.67	390.11621	390.11720	$C_{16}H_{22}O_{11}$	2.56	Deacetylasperulosidic acid (**21**)	In vivo antioxidant (Ma et al. 2013)
20.83	424.37051	424.36950	$C_{30}H_{48}O$	−2.35	Lupenone (**22**)	Antidiabetic (Xu et al. 2014)

RT retention time in minutes

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Author contribution MAT and SSAN conceptualized the study; MWDL performed the experiments; GJDA collected and identified the plant materials; MAT, SSAN, GJDA wrote and revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval The in vivo acute oral toxicity in the Online Resource material was approved by the Institutional Animal Care and Use Committee (IACUC) at the Research Center for Natural and Applied Sciences, University of Santo Tomas, and the Philippine Bureau of Animal Industry and Animal Research Permit.

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