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Research article

Isolation of indole alkaloids from *Kopsia larutensis* King & Gamble and their effects on histamine and β-hexosaminidase inhibitory in RBL-2H3 cell line

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Key words: *Kopsia larutensis*; Indole alkaloids; Histamine; β-hexosaminidase; Anti-allergic activity; RBL-2H3 cells.

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Abstract

This present study was aimed to isolate the indole alkaloids from various parts of *K. larutensis* King & Gamble. The indole alkaloids were isolate and identify by various chromatography and spectroscopic techniques. The major isolated compounds were tested for anti-allergic activity by evaluation of histamine and β-hexosaminidase release inhibitory activities in RBL-2H3 cells. The extracts of *K. larutensis* stem bark and leaves gave a new compound identified as kopsilarutensinine (**1**), two known compounds namely (-)-eburnamine (**2**) and kopsinine (**3**), and tetrahydroalstonine (**4**) which first time being reported in this plant. Crude alkaloids of stem bark and leaves showed highest inhibitory activities as compared to other parts. Meanwhile, compound **4** exhibited the highest inhibitory significantly among the isolates. These results were supported by docking simulation study which showed binding of compound **4** with responsible key residues. In conclusions, indole alkaloids from *K. larutensis* have potential as alternative anti-allergic agent.

Introduction

Kopsia genus (family: Apocynaceae) is widely distributed around South-eastern Asia especially in Peninsular Malaysia and Sarawak. There are about 18 species of *Kopsia* which occur in Malaysia including *Kopsia larutensis* King & Gamble. *Kopsia* is well-known as a source of many novel indole alkaloid compounds that gives some useful bioactivities such as antimicrobial, anticancer, anti-inflammatory and antitussive effects [1-7]. However, only few study reported on biological activities of *K. larutensis* indole alkaloid such as cerebrovascular protection and antitussive effects [7-8]. Many available anti-allergic agents have shown various side effects which the usage of herbal medicine and isolated active moieties has become a trend to provide an alternative way for treatment of allergies. Therefore, a lot of anti-allergic studies have been conducted on various compounds isolated from numerous plant [9-11]. However, there is no anti-allergic study ever conducted on alkaloids from *Kopsia* species.

It is well known that allergic reaction has been associated with the degranulation of mast cells. In response to type 1 immediate allergic reaction, stimulated mast cell release histamine which is the most potent mediators of allergic reaction together with β-hexosaminidase [12]. β-Hexosaminidase, an essential enzyme responsible for glycoprotein metabolism in cell homeostasis is stored in secretory granule of mast cells and always been used as biomarker to determine mast cells degranulation process [9, 13-14].Therefore, this study was undertaken to determine the anti-allergic potential from one of *Kopsia* species which is extract of *K. larutensis* and its indole alkaloids by evaluating histamine and β-hexosaminidase release inhibitory activities in RBL-2H3.

Experimental

Materials and Methods

Materials

Minimum Essential Eagle Medium (MEM) and penicillin/ streptomycin were obtained from Gibco (Thermo Fisher Scientific Inc. USA). Anti-DNP-IgE (Monoclonal anti-DNP), fetal bovine serum (FBS) and dinitrophenylated bovine serum albumin (DNP-BSA) were purchased from Sigma Aldrich, USA. *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide was obtained from Thermo Fisher, UK.

Plant materials

K. larutensis were collected from FRIM Malaysia. The samples were identified by the botanist at University Kebangsaan Malaysia and specimens were deposited at UKM Herbarium with voucher number UKMB5971.

Extraction & isolation of indole alkaloid

The dried samples (stem-bark, 2 kg; leaves, 2 kg; roots, 2 kg) of *K. larutensis* were extracted by simple maceration method. The grounded sample of plant was soaked with absolute methanol at room temperature for 72 hours, three times to yield the methanol extract. The crude alkaloid was

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obtained by acid-base extraction method. Briefly, the methanol extract was acidified by 5% aqueous H_2SO_4 (100 mL). Acidified extract was basified with 10% Na_2CO_3, followed by partitioned with $CHCl_3$. The alkaloid layer was then collected by using separating funnel. Na_2SO_4 was added to remove water content. The alkaloid layer was concentrated by using rotary evaporator.

Crude alkaloid of stem-bark (8.61 g) was subjected to CC over silica gel (SiO_2, 230-400 mesh, 2 x 70 cm, 500 g) (repeated 4x) eluting with increasing polarity solvent system DCM (100%), DCM: MeOH (9.5:0.5, 9.2:0.8, 9:1, 8:2, 5:5) and MeOH (100%) to obtain 34 fraction (F1-F34). Fraction F11 (70 mg) was further separated by silica gel (50 g, 2 x 30 cm) with solvent system hexane: EA (5:5, 3:7), EA (100%) and MeOH (100%) to obtain 70 fraction (CF1-70). Compound **1** (6.1 mg) was precipitated during collection of fraction CF11 which were eluted with hexane: EA (3:7). Fraction F12 (61.5 mg) was purified using $CHCl_3$: MeOH (9.5: 0.5) to give compound **2** (6.0 mg). The combination of all other fraction was further separated with Sephadex LH-20 to form fraction i5 (22.5 mg). This fraction was purified using $CHCl_3$: MeOH (9: 1) to give compound **3** (2.1 mg). Meanwhile, crude alkaloid of leaves (3.35 g) was also subjected to CC over silica gel (SiO_2, 230-400 mesh, 2 x 70 cm, 100 g) eluting with increasing polarity solvent system $CHCl_3$ (100%), $CHCl_3$: MeOH (9.5:0.5, 9.2:0.8, 9:1, 8:2, 5:5) and MeOH (100%) to obtain 51 fraction (L1-L51). Further isolation of combined fraction L20-L23 (457.2 mg) with solvent system $CHCl_3$: MeOH (9.5:0.5) to obtain fraction D1-D73. Combined fraction 63-73 (17.8 mg) was purified using $CHCl_3$: MeOH (9:1) to give compound **4**. All compound structure was identified by using various spectroscopy technique such as mass spectrometry (HR-ESI-MS) (Hewlett Packard GC 5890 series II), nuclear magnetic resonance spectroscopy (NMR) (FT-NMR 600 MHz and 150 MHz; AVANCE-III model, Bruker, Madison, USA), infra-red spectroscopy (FT-IR) (100 FT-IR; Perkin Elmer Model, Massachusetts, USA) and UV spectroscopy (UV1601, Shimadzu, Kyoto, Japan).

Kopsilarutensinine (**1**): White crystal (6.1 mg). TLC: R_f 0.49 (hexane: ethyl acetate (3:7) as TLC solvent system). UV λ_{max} (log ε) 230 (1.57); FT-IR (ATR) V_{max} cm^{-1}: 3747, 3616, 3445, 2920, 2842, 2804, 1670, 1642, 1601, 1462, 1457, 1429; ^{1}H-NMR and ^{13}C-NMR: Table 1; ESI-MS *m/z* calcd. for $C_{21}H_{24}N_2O_3$ 352.1787; found 351.1746 $[M-H]^-$.

(-)-Eburnamine (**2**): Yellowish oil (6 mg). TLC: R_f 0.63 ($CHCl_3$: MeOH (9:1) as TLC solvent system). FT-IR (ATR) V_{max} cm^{-1}: 3730, 3429, 3377, 3286, 3179, 1643, 1671, 1457, 1128, 1062, 1025; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 0.88 (1H, *br td*, *J*= 3.6, 4.2 Hz, H-15), 0.94 (3H, *t*, *J*= 0.6 Hz, H-18), 1.29 (1H, *br d*, *J*= 13.2 Hz, H-14), 1.40 (1H, *d*, *J*= 13.2 Hz, H-15), 1.58 (4H, *m*, H-17, H-19), 1.72 (2H, *br q*, H-14), 2.08 (1H, *m*, H-19), 2.36 (3H, *m*, H-17), 2.39 (3H, *m*, H-3), 2.54 (2H, *m*, H-3, H-6), 2.96 (1H, *m*, H-6), 3.24 (1H, *ddd*, *J*= 5.4, 6.0 Hz, H-5), 3.31 (1H, *dd*, *J*= 6.0, 6.6 Hz, H-5), 3.90 (1H, *s*, H-21), 5.60 (1H, *br s*, OH), 5.63 (1H, *q*, H-16), 7.18 (2H, *m*, H-10, H-11), 7.49 (1H, *dd*, *J*= 7.2 Hz, H-9), 7.74 (1H, *d*, *J*= 7.8 Hz, H-12). ^{13}C-NMR (150 MHz, $CDCl_3$): δ 7.5 (C-18), 16.8 (C-6), 25.2 (C-15), 28.7 (C-19), 36.9 (C-20), 43.8 (C-17), 44.4 (C-3), 50.9 (C-5), 58.8 (C-21), 76.8 (C-16), 105.8 (C-7), 112.1 (C-12), 118.1 (C-9), 120.0 (C-10), 121.4 (C-11), 128.7 (C-8), 132.4 (C-2), 136.6 (C-13); ESI-MS *m/z:* $[M+H]^+$ 297 [15].

Kopsinine (**3**): Yellowish oil (2.1 mg). TLC: R_f 0.48 (dichloromethane: MeOH (9.5:0.5) as TLC solvent system). FT-IR (ATR) V_{max} cm^{-1}: 3594, 3511, 3351, 2921, 2859, 2351, 2252, 2202, 2147, 1965, 1734, 1460, 1366, 1239, 742; ^{1}H-NMR (600 MHz, $CDCl_3$): δ1.27 (6H, *m*, H-14, H-15, H-17, H-19), 1.42 (4H, *m*, H-18, H-19), 1.55 (1H, *d*, *J*= 12.6 Hz, H-6), 1.93 (2H, *m*, H-14, H-18), 2.66 (1H, *m*, H-6), 2.79 (1H, *m*, H-16, H-17), 2.90 (1H, *t*, *J*= 17.4 Hz, H-5), 2.99 (1H, *s*, H-21), 3.03 (2H, *d*, *J*= 8.4 Hz, H-3), 3.14 (1H, *m*, H-3), 3.37 (1H, *q*, H-5), 3.77 (4H, *s*, OMe), 6.67 (1H, *d*, *J*= 11.4 Hz, H-12), 6.76 (1H, *ddd*, *J*= 1.6, 9.6, 11.1 Hz, H-10), 7.00 (1H, *ddd*, *J*= 1.8, 9.6, 11.4 Hz, H-11), 7.21 (1H, *d*, *J*= 9.6 Hz, H-9). ^{13}C-NMR (150 MHz, $CDCl_3$): δ 17.0 (C-14), 31.7 (C-20), 32.1(C-17), 33.9 (C-18, C-19), 34.6 (C-6), 36.3 (C-15), 43.8 (C-16), 47.6 (C-3), 50.8 (C-5), 52.1 (OMe), 58.0 (C-7), 66.6 (C-2), 68.3 (C-21), 110.9 (C-12), 120.0 (C-10), 121.9 (C-9), 126.8 (C-11), 149.0 (C-13), 174.9 (CO); ESI-MS *m/z*: $[M+H]^+$ 339 [16].

Tetrahydroalstonine (**4**): White crystal (3.5 mg). TLC: R_f 0.60 ($CHCl_3$: MeOH (9:1) as TLC solvent system). FT-IR (ATR) V_{max} cm^{-1}: 3471, 1680, 1615, 1436, 1375, 1297, 1190 cm^{-1}; ^{1}H-NMR (600 MHz, $CDCl_3$): 1.25 (2H, *s*, H-18), 1.98 (2H, *m*, H-6, H-14), 2.25 (2H, *t*, *J*= 12.0 Hz, H-20), 2.62 (1H, *td*, *J*= 6.0 Hz, H-5), 2.70 (1H, *d*, *J*= 6.0 Hz, H-15), 2.73 (1H, *d*, *J*= 6.0 Hz, H-21), 2.94 (1H, *d*, *J*= 6.0 Hz, H-3), 2.96 (1H, *d*, *J*= 6.0 Hz, H-5), 2.99 (1H, *m*, H-21), 3.10 (1H, *m*, H-7), 3.36 (1H, *d*, *J*= 12.0 Hz, H-2), 3.81 (3H, *s*, OMe), 4.20 (1H, *m*, H-19), 7.08 (1H, *t*, *J*= 6.0, 12.0 Hz, H-11), 7.13 (1H, *t*, *J*= 6.0, 12.0 Hz, H-10), 7.29 (1H, *d*, *J*= 12.0 Hz, H-9), 7.46 (1H, *d*, *J*= 6.0 Hz, H-12), 7.81 (1H, *br s*, NH). ^{13}C-NMR (150 MHz, $CDCl_3$): δ21.7 (C-18), 23.3 (C-6), 31.4 (C-15), 34.3 (C-14), 36.7 (C-20), 52.0 (OMe), 52.9 (C-5), 59.9 (C-21), 52.3 (C-3), 130.0 (C-2), 66.9 (C-19), 108.3 (C-16), 110.7 (C-12), 118.1 (C-10), 119.4 (C-9), 121.4 (C-11), 127.3 (C-8), 130.0 (C-13), 135.9 (C-17), 176.0 (CO); ESI-MS *m/z*: $[M+H]^+$ 353 [17-18].

Preparation & Cell viability of RBL-2H3 cells

RBL-2H3 cells which were provided by American Type of Cell Culture (ATCC), were cultured in Minimum Essential Medium Eagle (MEM) containing 10% of fetal bovine serum and 1% of antibiotic (penicillin/ streptomycin) in a culture flask. The cells were maintained at 37°C with 5% of CO_2. The cytotoxic effect of samples on RBL-2H3 cells was

determined by using standard MTT assay method. The percentage of viability was calculated and only cell viability percentage more than 90% was used for further assay to ensure maximum mast cell degranulation can be achieved [19].

Histamine release assay

RBL-2H3 cells ($2x10^5$ cells/mL) were cultured in 24-well plate. Monoclonal mouse anti-DNP IgE (0.45 µg/mL) was added and incubated for 24 hr. On completion of incubation period, monoclonal mouse anti-DNP IgE-treated cells were washed twice with Siraganian buffer (NaCl 119 mM, KCl 5 mM, $MgCl_2$ 0.4 mM, PIPES 25 mM, NaOH 40 mM with pH 7.2) followed by addition of 160 µl of Siraganian buffer incubated for 10 min at 37°C. After incubation, the IgE-sensitized cells were treated with IC_{10} value of samples for 10 min followed by addition of 20 µl of dinitrophenyl-labeled bovine serum albumin (DNP-BSA) (10 µg/mL) and further incubated for 20 min. 50 µl of supernatant was withdraw from each well and added in 96-well plate. Histamine concentration was quantified by ELISA technique (ELab Science, China). ELISA was performed in triplicate by following the manufacturer's instructions. The percentage inhibition of histamine release was calculated as follows [10, 20]: Inhibition of histamine release (%) = [1 - (sample- negative control)/ (positive control - negative control)] x 100.

β-hexosaminidase release assay

Inhibitory effects on release of β-hexosaminidase from RBL-2H3 cell line were evaluated by following method of Tewtrakul & Subhadirasakul (2007) with minor modification by using 0.1 M Glycine buffer, pH 10.0 as stop solution [20, 21]. Meanwhile, the percentage inhibition of β-hexosaminidase release were calculated as follows: Inhibition of β-hexosaminidase release (%) = [1 - (test - negative control)/ (positive control - negative control)] x 100.

β-hexosaminidase activity assay

Inhibitory effects on activity of β-hexosaminidase from RBL-2H3 cell line were also evaluated by using the method of Tewtrakul & Subhadirasakul (2007) with minor modification which 0.1 M Glycine buffer, pH 10.0 was used as stop solution [20, 21]. The percentage inhibition of β-hexosaminidase activity were calculated as follows: Inhibition of β-hexosaminidase release (%) = [1 - (test - negative control)/ (positive control - negative control)] x 100.

Statistical analysis

The results were expressed as a mean ± S.E.M of three determinations. The IC_{10} values were calculated using the Microsoft Excel program. Statistical significance was tested by using One-way ANOVA, followed by Dunnett's test.

Docking Simulation Study

This study was conducted by using Intel Core 2 Quad Q6600 (2.4 Hz) Linux PC with SYBYL 7.3 (Tripos). The radius of docking is 6.5 Å and the residue of docking been chosen. The crystalography of β-hexosaminidase is provided by Protein Data Bank with code 2GK1.pdb. It consists of homodimeric subunit which is the alfa-subunit been chosen for docking process while, *N*-acetyl-*D*-glucosamine-thyazoline as substrate. Flexible docking process with 10 conformation is conducted between apo-protein complex and tested compound. The best score and docking position been chosen before the interaction of tested compound and β-hexosaminidase been identified.

Results and Discussion

Isolation and structural elucidation

Extraction of the alkaloid was done in the usual manner followed by extensive chromatography of the crude alkaloid yielding several indole alkaloids among which one new compound was identified as kopsilarutensinine (**1**) (Figure 1), while another known compound, tetrahydroalstonine (**4**) is being reported for the first time in *K. larutensis*. In addition, two known compounds, (-)-eburnamine (**2**) and kopsinine (**3**) were also identified. Compound **2-4** were found to be identical in all of their characteristics including NMR data with (-)-eburnamine [15], kopsinine [16] and tetrahydroalstonine [17, 18].

Compound **1** was isolated as a white crystal with melting point range of 180-183°C. Its molecular formula $C_{21}H_{24}N_2O_3$ (*m/z* at 351.1746 $[M-H]^+$, calc. 352.1787) was deduced from HRESIMS. Meanwhile, the IR spectrum showed a band of hydroxyl (-OH) and amine (-NH) groups at range of 3200-3650 cm^{-1}.

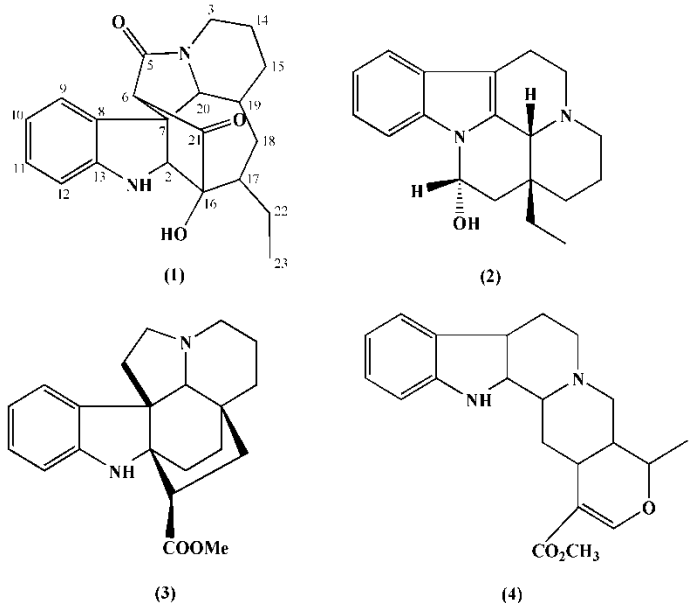


Figure 1. Chemical structures of kopsilarutensinine (1) (-)-eburnamine (2), kopsinine (3) and tetrahydroalstonine (4).

The ^{1}H-NMR spectrum accounted for total of 24 protons. The broad singlet peak at δ_H 5.65 and δ_H 8.12 referred to proton of hydroxyl group (OH) at C-16 and N-H group of indole nucleus respectively. Besides, there is a presence of triplet peak at shielded region (δ_H 0.91) for H-23 because of methyl groups at C-23 neighboring with methylene (CH_2) at H-22 (δ_H 1.82). The aromatic proton peaks of indole nucleus showed at range of 7.23-7.54 ppm. Proton H-12 showed doublet peak which overlapped with solvent peak $CDCl_3$ at δ_H 7.27. The doublet peak of H-12 formed because of ortho coupling with H-11 ($J_{12, 11}$= 9 Hz). The position of H-12 also proved by HMBC analysis that shows ^{2}J correlation for C-11 and C-13 with H-12, while C-8 have ^{3}J correlation with H-12 (Table 1, Figure 2).

Meanwhile, ^{13}C-NMR spectrum showed the presence of 21 carbon peaks in this structure including one methyl carbon (CH_3), five methylene carbon (CH_2), nine methine carbon (CH) and six quaternary carbon (C). There is also presence of 6 aromatic carbon peaks at C-8 (δ_C 138.7), C-9 (δ_C 124.3), C-10 (δ_C 128.5), C-11 (δ_C 126.1), C-12 (δ_C 131.9), C-13 (δ_C 140.1) and 2 peaks at aliphatic region of indole nucleus which is C-2 (δ_C 55.1) and C-7 (δ_C 37.9). The spectrum also showed carbonyl carbon at deshielded region which is C-5 (δ_C 172.7) and C-21 (δ_C 203.8). The spectrum data of ^{1}H-NMR and ^{13}C-NMR was supported by 2D-NMR data by correlation analysis of HMBC and HSQC (Table 1 and Figure 2). Therefore, compound (**1**) was identified as kopsilarutensinine or IUPAC name's was 5-ethyl-6-hydroxy-2,3,3a,3a1,5,6,6a,7-octahydro-1H,4H-6,12-methanoindolizino[1',8':2,3,4]cyclohepta[1,2-b]indole-13,15(12H)-dione.

Anti-allergic activity of crude extract

Inhibitory effects of crude alkaloid samples (Table 2) on degranulation activity of RBL-2H3 cells were determined by measuring the release of β-hexosaminidase which is the granule marker. All the crude alkaloids demonstrated inhibition of β-hexosaminidase release. Interestingly inhibitory activity of all sample from *K. larutensis* was higher than ketotifen fumarate (positive control) (IC_{10}= 1.37μg/mL, 41.8 ± 1.0 %). Whereas *K. larutensis* (leaves) (IC_{10}= 3.01 μg/mL) showed the most potent inhibitory activity (52.0 ± 3.0 %). To identify the mechanism responsible for the inhibitory activity of samples on β-hexosaminidase release, the effect of *K. larutensis* on the β-hexosaminidase activity was evaluated and it was observed that *K. larutensis* (leaves) gives approximately same percentage inhibition with positive control (13.2± 0.4%). It proved that the *K. larutensis* (leaves) gives effects towards antigen-induced degranulation process of mast cells and not prone to inhibition of β-hexosaminidase activity.

Table 1. The data comparison of ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) for kopsilarutensinine (1)

Position	Kopsilarutensinine (1)					
	δ_H	*J* (Hz)	δ_C	HSQC	HMBC	
					$^{2}J_{CH}$	$^{3}J_{CH}$
2	2.18, *s*	-	55.1	55.1	-	-
3	2.64, *t*	12.3	42.8	42.8	-	C-20
	3.44, *q*	7.4	-	-	-	C-20
5	-	-	172.7	-	-	-
6	2.75, *s*	-	75.5	-	-	C-2,20
7	-	-	37.9	-	-	-
8	-	-	138.7	-	-	-
9	7.54, *ddd*	7.5, 6.0, 1.6	124.3	124.3	C-8,10	C-11,13
10	7.44, *dd*	7.5, 6.0	128.5	128.5	C-9,11	C-8,12
11	7.32, *ddd*	7.2, 6.6, 0.8	126.1	126.1	C-10, 12	C-9,13
12	7.26, *d*	9.0	131.9	131.9	C-11,13	C-8
13	-	-	140.1	-	-	-
14	1.72, *dt*	13.2, 3.0	32.9	32.9	-	C-19
	2.00, *dt*	12.0, 3.8	-	-	-	-
15	2.05, *m*	-	40.2	40.2	-	C-18
	3.02, *m*	-			C-14	C-3
16	-	-	84.0	-	-	-
17	1.26, *ddd*	13.5, 9.0, 4.6	32.9	32.9	C-18,22	C-2,19
18	1.43, *m*	-	35.8	35.8	C-17	C-15
19	2.28, *dd*	15.0 ,6.0	40.2	40.2	C-18	C-14,17
20	3.10, *d*	1.2	54.6	54.6	-	-
21	-	-	203.8	-	-	-
22	1.82, *m*	-	22.5	22.5	-	C-18
	2.45, *ddd*	12.3, 9.9, 2.0	-	-	C-17	-
23	0.91, *t*	7.5	7.4	7.4	-	-
NH	8.12, *br s*	-	-	-	-	-
OH	5.65, *br s*	-	-	-	-	-

Table 2. The inhibition percentage of *K. larutensis* crude alkaloids on histamine and β-hexosaminidase in RBL-2H3 cell

Samples *K. larutensis* Crude Alkaloid	Histamin Release Inhibition (%)	β-hexosaminidase Release Inhibition (%)	β-hexosaminidase Activity Inhibition (%)	IC_{10} (µg/mL)
Bark	48.12 ± 8.2 ***	51.47 ± 5.8	1.80 ± 0.33	2.17
Leaves	42.98 ± 8.5 ***	51.99 ± 3.0	12.22 ± 1.53	3.01
Roots	36.70 ± 5.2 ***	50.94 ± 2.7	9.84 ± 0.77	1.61
Ketotifen fumarate (positive control)	45.22 ± 10.0	41.80 ± 1.0	13.16 ± 0.35	1.37

Each value represents the mean ± SEM (n = 3). Significantly different from the positive control, ***p ≤ 0.001, **p≤ 0.005, * p ≤ 0.01.

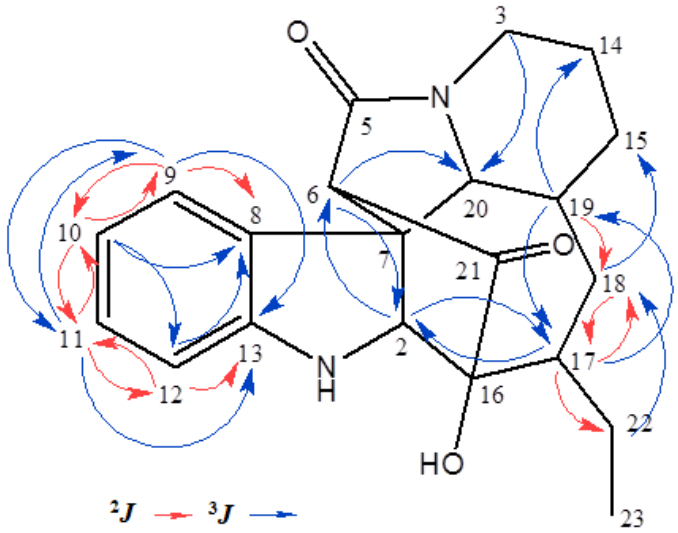


Figure 2. HMBC correlations of kopsilarutensinine (1).

Besides that, crude alkaloids from different part of *K. larutensis* were also screened for their inhibitory effect on histamine release in RBL-2H3 cells. *K. larutensis* crude alkaloids extract of stem-bark (48.1 ± 8.2 %) showed optimal inhibition effect on histamine release as compared to others significantly.

Anti-allergic activity of pure compounds

All four indole alkaloids isolated from leaves and stem-bark of *K. larutensis* have been used after the anti-allergic screening of crude alkaloids in RBL-2H3 cells. Based on Table 3, tetrahydroalstonine (**4**) (IC_{10}= 11.78 µg/mL) showed significantly high percentage of inhibition of β-hexosaminidase (60.6 ± 2.7%) as compared to other compounds. Besides that, the other three indole alkaloids still show significantly higher inhibition percentage than positive control. These results proved that among tested indole alkaloid compounds, tetrahydroalstonine (**4**) possesses high potential to inhibit the degranulation process of mast cells.

β-hexosaminidase activity assay was carried out to confirm that the results obtained are because of degranulation process inhibition in mast cells and not due to the inhibition of enzyme activity [14]. The results on Table 3 also show that all four compounds tested gave low inhibition percentage significantly (<15%) which proved that the high β-hexosaminidase release inhibition results are because of the effects of compounds towards degranulation process of mast cells. However, based on study conducted by Larson & Mitre (2012) which found that the inhibition percentage of histamine release is giving more sensitive results to detect the mast cells degranulation as compared to β-hexosaminidase release [22]. Therefore, the histamine release assay has been conducted to identify the inhibition effects of indole alkaloid compounds isolated from *K. larutensis* towards degranulation process of mast cells.

Histamine is one of important mediators that involves in degranulation process that play role in the early phase reaction of type I hypersensitivity [23-25]. Based on the results, all four indole alkaloid compounds tested showed lower histamine release inhibition percentage as compared to positive control significantly. However, the crude alkaloid of *K. larutensis* especially the stem-bark part have shown potential to inhibit the histamine release (48.1 ± 8.2%). Therefore, it is suggested that the positive results obtained from crude alkaloids of *K. larutensis* are because of the synergistic effects of compounds present in the crude alkaloids tested.

Docking simulation study of tetrahydroalstonine (4)

Based on the results of β-hexosaminidase release assay shown in Table 3, indole alkaloid of tetrahydroalstonine (**4**) (IC_{10}= 11.78 µg/mL) exhibited highest inhibition percentage as compared to other compounds. Therefore, *in silico* docking analysis was carried out to support the provided data. This analysis was also used to identify the interaction between tetrahydroalstonine (**4**) and β-hexosaminidase. β-Hexosaminidase A (HexA) was used in this analysis because only HexA has a potential to degrade GM2 into GM3 ganglioside by hydrolysis of *N*-acetyl-*D*-glucosamine (NAG) terminal which is an important event in degranulation process of mast cells [26]. Besides, *N*-acetyl-*D*-glucosaminthiazoline (NGT) was used as an inhibitor on binding site of α-subunit HexA. There are two important amino acids that become a residue in this reaction which are αAsn423 and αArg424. These residues are only present in α subunit and critically used on enzyme activity with negative charge substrate [27]. The α subunit for HexA consists of 529 amino acids, but only 492 residue has been used in this analysis which the HexA crystal provided by Protein Data Bank with code 2GK1.pdb.

Table 3. The inhibition percentage of indole alkaloids compounds *K. larutensis* on histamine and β-hexosaminidase in RBL-2H3 cell

Samples	Histamin Release Inhibition (%)	β-hexosaminidase Release Inhibition (%)	β-hexosaminidase Activity Inhibition (%)	IC_{10} (µg/mL)
Kopsilarutensinine (**1**)	9.27 ± 0.01***	56.78 ± 5.67	4.27 ± 0.56***	3.73
(-)-Eburnamine (**2**)	14.35 ±0.01***	59.58 ± 4.11*	13.75 ± 0.76	5.51
Kopsinine (**3**)	22.63 ± 0.05**	60.48 ± 6.62*	7.66 ± 0.45***	7.06
Tetrahydroalstonine (**4**)	23.92 ± 0.23**	60.59 ± 2.68*	14.03 ± 0.32	11.78
Ketotifen fumarate (positive control)	45.22 ± 10.0	41.80 ± 1.0	13.16 ± 0.35	1.37

*Each value represents the mean ± SEM (n = 3). Significantly different from the positive control, ***$p \leq 0.001$, **$p \leq 0.005$, * $p \leq 0.01$.*

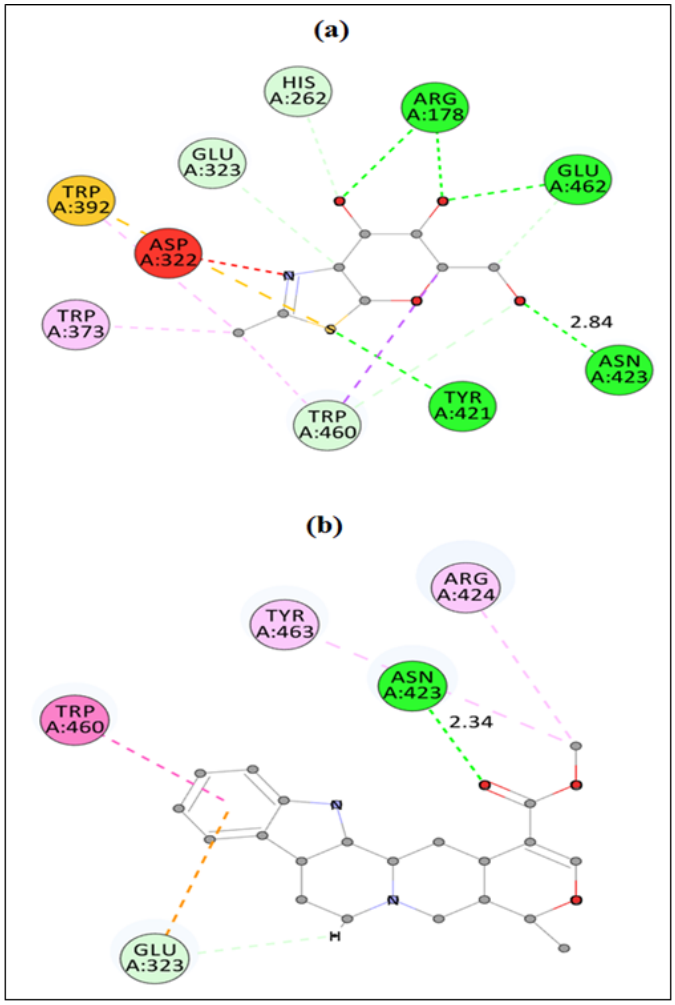


Figure 3. Interaction binding between residue and (a) 2GK1 crystal; (b) tetrahydroalstonine (4).

Ten position of flexible docking method was carried out and the results show that tetrahydroalstonine (**4**) fit in α subunit structure of β-hexosaminidase. Tetrahydroalstonine (**4**) also showed hydrogen bond formation at αAsn423 with strength 2.34 as compared to ligand that shows same hydrogen bond formation at αAsn423 with strength 2.84. Meanwhile, αArg424 residue gives pi (π) bonding with tetrahydroalstonine (**4**) as compared to ligand which form hydrogen bonding. Besides, few weak bonding were also detected such as αTRP460, αTYR463 and αGLU323 (Figure 3). The results obtained supported the previous assay data that tetrahydroalstonine (**4**) is able to inhibit β-hexosaminidase release and has high potential to inhibit the type I hypersensitive allergic activities of mast cells.

Conclusion

In conclusion, the findings indicated that tetrahydroalstonine (**4**) isolated from *K. larutensis* showed potential to be an anti-allergic agent via β-hexosaminidase inhibition. However, further studies should be carried out to discover the mechanism of action, responsible for anti-allergic effect of tetrahydroalstonine (**4**).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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