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Histamine and Beta-Hexosaminidase Inhibitory Effects of Crude Alkaloid from *Kopsia arborea* Blume in RBL-2H3 Cell Lines

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Abstract

***Kopsia arborea* Blume is one of the plants in the Apocynaceae family, found in southeastern Asia and Malaysia. It has been used widely in traditional medicines. Extracts and pure isolates of *Kopsia* species have been reported to exhibit various biological activities. In this study, crude alkaloid derived from different parts of *K. arborea* was evaluated for their inhibitory activity on histamine and beta-hexosaminidase release in rat basophilic leukemic (RBL-2H3) cells. The effect of *K. arborea* on histamine, beta-hexosaminidase release and beta-hexosaminidase activity in RBL-2H3 cell was determined by using sandwich enzyme-linked immunosorbent assay (ELISA). On the other hand, cytotoxic effect of crude alkaloid in RBL-2H3 cell was evaluated by standard 3-(4, 5-dimetiltiazol-2-Yl)-2, 5-difeniltetrazolium (MTT) assay. The results showed that all samples significantly inhibit ($p \leq 0.001$) the histamine release in RBL-2H3 cell except *K. arborea* (roots). However, % inhibition of histamine release demonstrated by crude alkaloid extracted from *K. arborea* leaves was higher as compared to ketotifen fumarate that acts as positive control. Meanwhile, crude alkaloid extracted from *K. arborea* (roots) exhibited the highest inhibition of beta-hexosaminidase release and activity than ketotifen fumarate. These results indicated that crude alkaloid from this plant consist of bioactive compounds which have the ability to inhibit mast cell degranulation via inhibition of histamine and beta-hexosaminidase release and have potential to provide as alternative anti-allergic agent.**

Keywords*: *RBL-2H3 cells; Histamine; Anti-allergic activity; Alkaloid; Kopsia arborea

Introduction

It is well known that allergic reaction has been associated with the degranulation of mast cells. A number of mediators that play a key role in propagation of allergic reaction are released by mast cells [1]. In response to type 1 immediate allergic reaction, stimulated mast cell release histamine which is the most potent mediators of allergic reaction [2,3]. Histamine along

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with other mediators promotes smooth muscle contraction, vasodilation and increase vascular permeability [4]. Beta-hexosaminidase, an essential enzyme responsible for glycoprotein metabolism in cell homeostasis, is stored in secretory granule of mast cells [5]. Activated mast cells release beta-hexosaminidase together with histamine. Inhibition of beta-hexosaminidase release is a key strategy to restrain the degranulation process of mast cells [6-8]. Therefore, various anti-allergic studies involved the use of beta-hexosaminidase as a marker for mast cell degranulation in rat basophilic leukemia (RBL-2H3) cell line.

Many anti-allergic agents available have shown various side effects [6]. For centuries mankind has been using the medicinal plants for ailment of various diseases. Therefore, the usage of herbal medicine and isolated active moieties can provide an alternative way for treatment of allergies. Previous studies have reported that alkaloid compound isolated from numerous plant species such as *N*-methylbuxifoliadine-E, *N*-methylatalaphylline, citrusinine-I [8], ganocalicine A [9], curine [10], dichotomines [11], and warifteine [12] possessed anti-allergic activities. However, there is no anti-allergic study ever conducted on alkaloids from *Kopsia* species.

Kopsia genus which is a member of Apocynaceae family is a well-known source of indole alkaloid. There are about 18 species of *Kopsia* which occur in Malaysia including *K. arborea* which is widely distributed around southeastern Asia especially in Peninsular Malaysia and Sarawak [13-15]. In Malaysia, many *Kopsia* species are used in traditional medicine for the treatment of ulcerated noses in tertiary syphilis, skin infections and wounds [16]. Various extracts and active compounds isolated from *Kopsia* species also have been reported to exhibit numerous biological activities such as antimicrobial, anti-cancer, anti-inflammation, antipyretic and antitussive [17-19]. In the current study, one of *Kopsia* species which is *K. arborea* (synonym *Kopsia scortechinii* King and Gamble) have been investigated and the inhibitory effects of *K. arborea* crude alkaloid on histamine and beta-hexosaminidase release in RBL-2H3 cells were evaluated.

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 and ketotifen fumarate which act as positive control were purchased from Abcam Biochemicals, Cambridge, USA.

Plant materials

K. arborea were collected from the forest of Kelantan, Malaysia. The samples were identified by the botanist at Universiti Kebangsaan Malaysia and the specimens were deposited at UKM Herbarium with voucher number UKMB40000.

Extraction of crude alkaloids

The dried sample of *K. arborea* was 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 alkaloids were

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. Percentage yield of each part of plant sample are listed in TABLE 1.

TABLE 1. **Extraction yields of each part of plant samples**

Sample		Weight (g)			Yield (%)
		Dried powder	Methanol extract	Crude alkaloid	
K. arborea	Bark	500	81.82	4.81	5.88
	Leaves	500	94.91	49.06	51.69
	Roots	500	107.45	29.19	27.17

Preparation and cell viability of RBL-2H3 cells

RBL-2H3 cells is a widely used in allergy studies because of their mucosal mast cell characteristic such as abundant of IgE receptor on membrane and able to release mediators after IgE-sensitization [20]. Although, Passante and Frankish report that RBL-2H3 cells is no more suitable to represent mast cells in allergy studies but as our study act as a preliminary study on identifying the potential of crude alkaloids of *K. arborea* on histamine and beta-hexosaminidase release inhibition [21]. Thus, RBL-2H3 cells is still considered relevant to be chosen as a model for mast cell IgE-mediated degranulation in this study.

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 [22].

Histamine release assay

RBL-2H3 cells (2×10^5 cells/ml) were cultured in 24-well plate. Monoclonal mouse anti-DNP IgE (0.45 μg/ml) was added and incubated for 24 hrs. 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: [10,23].

Inhibition of histamine release (%)=[1 - (Sample- negative control)/(Positive control - Negative control)] × 100.

Beta-hexosaminidase release assay

Inhibitory effects on release of beta-hexosaminidase from RBL-2H3 cell line were evaluated by following method of Tewtrakul and Subhadirasakul with minor modification by using 0.1 M Glycine buffer, pH 10.0 as stop solution [23,24]. Meanwhile, the percentage inhibition of beta-hexosaminidase release were calculated as follows:

Inhibition of beta-hexosaminidase release (%)=[1-(test - Negative control)/(Positive control - Negative control)] × 100.

Beta-hexosaminidase activity assay

Inhibitory effects on activity of beta-hexosaminidase from RBL-2H3 cell line were also evaluated by using the method of Tewtrakul and Subhadirasakul with minor modification which 0.1 M Glycine buffer, pH 10.0 was used as stop solution [23,24]. The percentage inhibition of beta-hexosaminidase activity were calculated as follows:

Inhibition of beta-hexosaminidase release (%)=[1-(test - Negative control)/(Positive control - Negative control)] × 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 the Dunnett's test.

Results and Discussion

Effects of the crude alkaloid on beta-hexosaminidase release and activity

Beta-hexosaminidase is an enzyme that is stored inside the mast cell granule which is released along with histamine when degranulation process occurs [25]. Inhibitory effects of crude alkaloid samples on degranulation activity of RBL-2H3 cells were determined by measuring the release of beta-hexosaminidase which is the granule marker [6]. Productions of beta-hexosaminidase from RBL-2H3 cells were measured by using ELISA and results are demonstrated as percentage inhibition. As shown in TABLE 2, the results demonstrated that all tested crude alkaloid extract from *K. arborea* inhibited the release of beta-hexosaminidase. Inhibitory activity of *K. arborea* (leaves) and *K. arborea* (roots) was higher as compared to ketotifen fumarate which is the positive control (IC_{10}=1.37 µg/ml, 41.80 ± 1.0%).

Beta-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 [6]. The results on TABLE 2, show that, all sample tested except *K. arborea* (roots) gave low inhibition percentage significantly (<15%) which proved that the high beta-hexosaminidase release inhibition results are because of the effects of crude alkaloid samples towards degranulation process of mast cells. However, based on study conducted by Larson and Mitre which found that the inhibition percentage of histamine release is giving more sensitive results to detect the mast cells degranulation as compared to beta-hexosaminidase release [26]. Therefore, the histamine release assay has been conducted to identify the inhibition effects of crude alkaloid extract of *K. arborea* towards degranulation process of mast cells.

TABLE 2. **Inhibitory effects of *K. arborea* crude alkaloids on the release of histamine and β-hexosaminidase in RBL-2H3 cells**.

Sample		Histamine Inhibition (%)	B-hexosaminidase Release Inhibition (%)	B-hexosaminidase Activity Inhibition (%)	IC_{10} (µg/ml)
K. arborea	Bark	37.55 ± 5.8***	41.95 ± 4.8	14.45 ± 2.0	3.82
	Leaves	50.62 ± 3.8***	43.14 ± 2.5	5.73 ± 1.54	2.58
	Roots	44.69 ± 7.0	54.84 ± 1.5*	17.51 ± 1.61	4.32
Ketotifen fumarate		45.22 ± 10.0	41.80 ± 1.0	13.17 ± 0.35	1.37

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

Effects of the crude alkaloid on histamine release

Histamine is one of important mediators that involves in degranulation process that play role in the early phase reaction of type I hypersensitivity [4,7,27]. Three crude alkaloids extract from different parts of *K. arborea* were also screened for their inhibitory effect on histamine release in RBL-2H3 cells by using ELISA. The results are shown in TABLE 2 and it was observed that the entire crude alkaloids sample exhibited the histamine release from IgE-sensitized RBL-2H3 cells. Among all the tested sample, crude alkaloids extract derived from leaves (IC_{10}=2.58 µg/ml) had shown the maximum inhibition of histamine release significantly (50.62 ± 3.8%). Moreover the inhibitory effect of sample derived from leaves of *K. arborea* was higher than positive control (45.22 ± 10.0%).

These finding suggest that crude alkaloid of *K. arborea* (leaves) possessed high potential as an alternative anti-histamine agent. However, further exploration of this plant is needed to determine the bioactive compound responsible for activity. The further mechanism behind inhibition of histamine release by all sample of crude alkaloid extract still need to be investigated. The isolation and purification of potent crude alkaloid extract are being carried out to determine the inhibitory effects of isolated compounds on histamine and beta-hexosaminidase release in RBL-2H3 cell lines.

Conclusion

As conclusion, the results described that among all the tested crude alkaloid extract, *K. arborea* (leaves) shows the strongest inhibition of mast cell degranulation. This findings indicated that crude alkaloid extract from this plant have potential bioactive compounds which able to inhibit mast cell degranulation via inhibition of histamine and beta-hexosaminidase release and provides an insight for discovery of new alternative anti-histamine agent for allergy treatment.

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