Inhibition of the histamine and leukotriene B_4 release from rat peritoneal exudate cells by six Bangladeshi plants

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Running Title: **Anti-allergy of Bangladeshi plants**

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Summary

Alcohol extracts of six Bangladeshi medicinal plants were investigated to search out new sources of supplements or drugs beneficial to human health. All the extracts had polyphenols and anti-oxidative activity, and inhibited the release of both histamine and leukotriene B_4 (LTB_4) induced by the calcium ionophore A23187 from rat peritoneal exudate cells. The extract of mangrove apple (*S. caseolaris*) had strong anti-oxidative activity and reducing power. The extracts of white mangrove (*A. officinalis*), pan lota (*D. uliginosa*), beach hibiscus (*H. tiliaceous*), sapodilla (*M. zapota*) and mangrove apple (*S. caseolaris*) inhibited both histamine and LTB_4 releases significantly, suggesting them to be useful for the development of an anti-allergic supplement and drugs.

Keywords: Antioxidant, Anti-allergy, Anti-histamine release, Anti-leukotriene release, Mangrove tree

Introduction

Anti-oxidative compounds are reported to suppress the reactive oxygen species (ROS). The pathogenesis of diseases like cardiovascular disorders, cancer, aging, inflammation, and brain dysfunction is accompanied by the production of free radicals leading to oxidative stress. Anti-oxidants are used in food industry and pharmaceuticals as additives. Widely used synthetic anti-oxidants are now under question due to their side effects like carcinogenicity [1]. Therefore in response to the growing consumer concern, search for anti-oxidants and/or anti-oxidant principles from natural sources have gained interest and many plants reportedly having potential anti-oxidant activity [2, 3]. Phenolic compounds, which are secondary metabolites in plant materials, are known to be responsible for anti-oxidant effect. Fruits and vegetables are the main sources of phenolic compounds of human diet. Other sources, such as grain, herbs and spices, also have received particular attention as important sources of anti-oxidants [4, 5].

In early spring, many people suffer allergic reactions to cedar pollen in Japan. Allergies are a damaging immune response and classified into four types. Type 1 plays an important role in reactions to food and environmental allergens [6, 7]. Mast cells play a crucial role in many physiological changes during an immediate allergic response through the production and release of chemical mediators such as histamine and eicosanoid [8, 9, 10]. So, compounds which inhibit the release of histamine and eicosanoid from mast cells will reduce allergic symptoms. Reportedly, various polyphenols in foods and beverages have anti-allergic activities [11, 12, 13, 10].

Bangladesh has the largest mangrove forests in the world which has been a vast source of bioactive compounds. Mangrove plants have been used in traditional folk medicines and extracts from mangrove species are widely used throughout the world. They provide a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins [14]. In a previous paper [15], Bangladeshi fruits were screened for anti-oxidative, anti-amylase, anti-glucosidase and anti-histamine release activities. In this current study, six Bangladeshi plants traditionally used as folk medicines have been screened for both anti-histamine and anti-leukotriene B_4 (LTB_4) release activity and anti-oxidative activity to find medicinal plants which can be used in the food, cosmetic and pharmaceutical industries to prepare functional foods, cosmetics, and drugs with anti-allergic or anti-oxidative activity.

Materials and Methods

Chemicals

Folin-Ciocalteu's phenol reagent, fish gelatin, and histamine dihydrochloride were purchased from Sigma-Aldrich Co (St. Louis, MO). A-23187, bovine serum albumin (BSA), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Wako Pure Chemical Industry, Ltd., Osaka, Japan. Gallic acid was purchased from Nacalai Tesque, Kyoto, Japan. All of these chemicals and other reagents were of analytical grade.

Plant materials

Leaves of *Avicennia officinalis* Linn. (Avicenniaceae; white mangrove), *Deris uliginosa* (Leguminosae; pan lota), and *Sonneratia caseolaris* Linn. (Sonneratiaceae; mangrove apple), leaves and stems of *Hibiscus tiliaceous* Linn. (Malvaeceae, beach hibiscus) were collected from the Sundarbans' mangrove forest, Bangladesh. Bark of *Manilkara zapota* (L.) Royen. (Sapotaceae; sapodilla), and seeds of *Switenia mahagoni* (Meliaceae; mahogany) were collected from the plants in and around the Khulna University Campus, Bangladesh. They were taxonomically identified by experts at the Bangladesh National Herbarium, or authenticated at Forest and Wood Technology Discipline, Khulna University, Bangladesh. These plant materials were cut into small pieces and sun-dried. The dried materials were ground into a powder with a grinder. The powders were stored separately in an air tight container and kept in a cool, dark, and dry place.

Preparation of extracts of the plants

About 400 g of powdered material was taken in a clean, flat bottomed glass container (4 L) and soaked in 1.3 L of 80% methanol for *A. officinalis*, *H. tiliaceous*, *M. zapota*, and *S. mahagoni* or 80% ethanol for *D. uliginosa*, and *S. caseolaris*. The container with its contents was sealed and kept for a period of 7 days with occasional shaking and stirring. The whole mixture then underwent coarse filtration through a piece of clean, white cotton material followed by filtration through Whatmann filter paper and the filtrate was concentrated by using a rotary evaporator (Bibby RE200, Sterlin Ltd., UK) to obtain the crude extract. The yields of the samples were 12-15% after these procedures. The crude extracts (20 mg) were dissolved in ethanol (1 mL) for experiments.

For fractionation, one gram (1 g) of the extract was taken in an air tight bottle. Hereafter, n-hexane was added to prepare hexane fraction of the sample. After vigorous shaking and filtration through filter paper n-hexane was evaporated and the dried solid was taken as a hexane fraction. Precipitate on the filter paper was taken in a separate air tight bottle and chloroform was added. Chloroform fraction was prepared by evaporation of chloroform after filtration through a filter paper. Subsequently following the same procedure as above, ethyl acetate, then ethanol, and at last distilled water was used to obtain ethyl acetate, ethanol, and water fractions. Twenty milligram (20 mg) of the fraction was dissolved in 1 ml ethanol or distilled water for conducting experiments.

Determination of total phenolic content (TPH)

The total concentration of phenolics (TPH) in the extracts was determined according to the Folin-Ciocalteu method [16] with gallic acid (GA) as the standard and expressed (μM) as gallic acid equivalents (GAE)/mM of the extract [17].

DPPH radical scavenging activity

The reaction mixture (total volume, 3 mL), consisting of 0.5 mL of a 0.5 M acetic acid buffer solution at pH 5.5, 1 mL of 0.2 mM DPPH in ethanol, and 1.5 mL of a 50% (v/v) ethanol aqueous solution with the extracts, was shaken vigorously [18, 19]. After incubation at room temperature for 30 min, the amount of DPPH remaining was determined by measuring absorbance at 517 nm.

Reducing power

The reducing power of the extracts was determined according to the method of [20]. Phosphate buffer (2.5 mL, 0.2 M, pH 6.6) containing 0.4 mg/mL of extract was prepared. Then it was added to 2.5 mL of 1% (w/w) potassium ferricyanide, and mixed. After incubation at 50 ^{o}C for 20 minutes, the mixture was mixed with 2.5 mL of 10% (w/w) trichloroacetic acid and centrifuged at 650g for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. Then the absorbance of this solution was measured at 700 nm. One mM potassium ferrocyanide in the buffer solution, which is produced from potassium ferricyanide by reduction, produced absorbance of OD 0.985 at 700 nm in a cell with a 1-cm long light path. Ascorbic acid (40 µg/mL phosphate buffer) served as a positive control.

Preparation of rat peritoneal exudate cells (PECs)

Male SD rats (8 weeks old) were purchased from Kyudo Co., Ltd., Tosu, Japan. Twenty milliliters of Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$ and 5.6 mM glucose, p^H 7.4) containing 0.1% (w/w) BSA was injected into the peritoneal cavity. After the abdomen was gently massaged for 2 min, the cavity was opened, and the fluid containing the peritoneal exudate cells (PECs) was collected with a Pasteur pipette. The cells were gently washed with Tyrode buffer and then centrifuged at 200 x g for 10 min at 4 ^{o}C. To remove contaminating erythrocytes by hypotonic lysis, the cell pellets were re-suspended in a modified ammonium chloride buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 10 mM EDTA.2Na, p^H 7.4) and incubated for 5 min at 4 ^{o}C. The cell suspension was then centrifuged at 200 x g for 5 min at 4 ^{o}C and the cells were re-suspended in the Tyrode buffer at 2 x 10^6 cells/mL. Cell viability was measured by trypan blue staining and mast cells were identified by toluidine blue staining [13]. The cell viability of this preparation was more than 95% and the proportion of mast cells was 5-10% of all the cells [9].

Measurement of the inhibition of histamine release

Rat peritoneal exudate cells (PECs; 500 µL, 2 x 10^6) were suspended in 48 µL of 25 mM $CaCl_2$, 12 µL of various concentrations of samples and/or 120 µL of 5 µM A23187 solution, and then the volume was adjusted to 1.2 mL with tyrode buffer, and incubated for 20 min at 37 ^{o}C. The reaction was terminated by incubating for 5 min at 4 ^{o}C. The cell suspension was then centrifuged at 300 x g for 10 min, and the amount of histamine in the supernatant was measured [13].

The histamine content was measured by fluorometric assay [21]. The percent inhibition of histamine release was calculated with the following formula: inhibition of histamine release (%) = (histamine release without extract – histamine release with extract) x 100/histamine release without extract. The negative control was the histamine content without stimulation. The positive control was that after stimulation by A23187. All results were expressed as the mean ± SD of at least four determinations (n = 4).

Measurement of the inhibition of leukotriene B_4 (LTB_4) release

The LTB_4 release was measured similarly as described in Measurement of the inhibition of histamine release [10].

The reaction was terminated by adding 50 µL of the mixture of acetonitrile : methanol (6 : 5 v/v) and kept at -20 ℃ for 30 min. To measure LTB_4, the internal standard, 250 ng prostaglandin B_2 (PGB_2) was added to the mixture. Then, the samples were centrifuged at 10000xg for 10 min.

The LTB_4 and PGB_2 in the supernatant was measured by reverse-phase high performance liquid chromatography (LC-10AD, Shimadzu Co.) with ODS-A column (150 x 6.0 mm, 5 µm particle size). A mixture of acetonitrile : methanol : 5 mM ammonium acetate aqueous solution (6 : 5 : 9, v/v/v) was used as a mobile phase with a flow rate of 1.1 mL/min. LTB_4 and the internal standard PGB_2 were detected by absorbance at 280 nm (SPD-10AVP, Shimadzu, Co.). Quantification of LTB_4 was done by comparing their peak areas with those of known amounts of authentic standards and correction for recovery.

Results

Total phenolic (TPH) content, DPPH radical scavenging activity and reducing power

The total phenolic (TPH) content, DPPH radical scavenging activity, and reducing power of the extracts of six Bangladeshi plants were measured and shown in Table 1. The extract of *S. caseolaris* has much TPH and DPPH radical scavenging activity (IC_{50} = 18 µg/mL). Reportedly, the activity of anti-oxidants is concomitant with the development of reducing power [22]. As expected, this extract's reducing power is also large (O.D. 2.11). Generally, extracts with a higher phenolic content show more DPPH radical scavenging activity as well as reductive activity. Here we found a strong correlation (r = 0.96) between total phenolic content and DPPH radical scavenging activity, & r = 0.95 between TPH content and the reducing power of all extracts.

Table 1. Total polyphenolic content, DPPH radical scavenging activity at 50 µg/mL, and reducing power at 0.4 mg/mL of six Bangladeshi plants.

Name of plant	Total polyphenol content (µM GAE)	DPPH radical scavenging activity (%)	Reducing power (O.D.)
A. officinalis	329 ± 14	18.3 ± 1.3	0.49 ± 0.03
D.uliginosa	556 ± 25	3.7 ± 0.8	0.43 ± 0.02
H. tiliacious	312 ± 7	10.0 ± 1.3	0.63 ± 0.06
M. zapota	222 ± 4	3.8 ± 3.8	0.42 ± 0.02
S. caseolaris	2039 ± 66	87.7 ± 0.2	2.11 ± 0.30
S. mahagoni	577 ± 11	7.2 ± 1.5	1.06 ± 0.04

One mM potassium ferrocyanide in the buffer solution, which is produced from potassium ferricyanide by reduction, produced absorbance of O.D. 0.985 at 700 nm in a cell with a 1-cm-long lightpath.

Inhibition of histamine release

We examined the effect of the extracts on the release of histamine from rat PECs. The PECs (1 x 10^6/mL) were stimulated with 5 μM A23187 for 20 min in the presence of the extracts. As shown in Figure 1, all examined extracts except that of *S. mehagoni* inhibited significantly the release of histamine from the cells at the same concentration of polyphenols (2 μM GAE). The dose-dependence inhibitory effect of the extract of *A. officinalis* was examined and is shown in Figure 2.

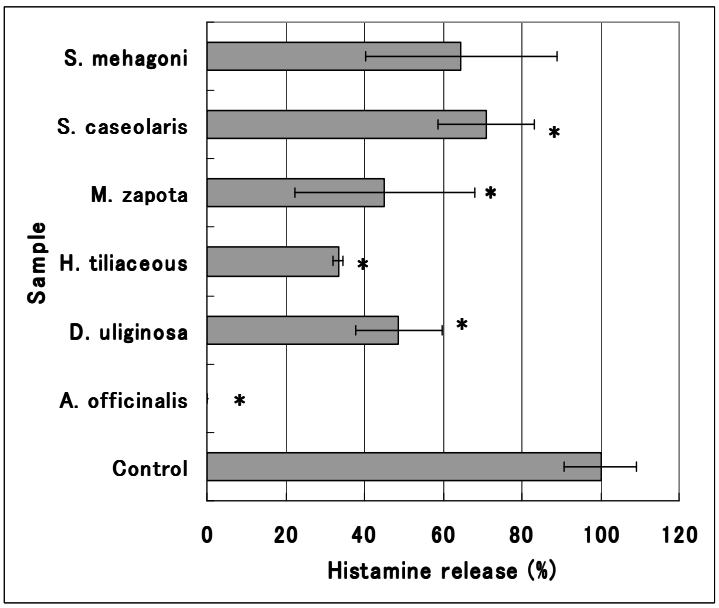

Figure 1. Inhibition of histamine release activity by the extracts of six Bangladeshi plants. The release of histamine from rat PECs caused by the Ca^{2+} ionophore A23187 was measured with the same concentration of polyphenol, 2 μM GAE of the extracts. The data are shown as means ± SD (bars) for four experiments. *P < 0.05 by Student's t test for the comparison between the control and extracts.

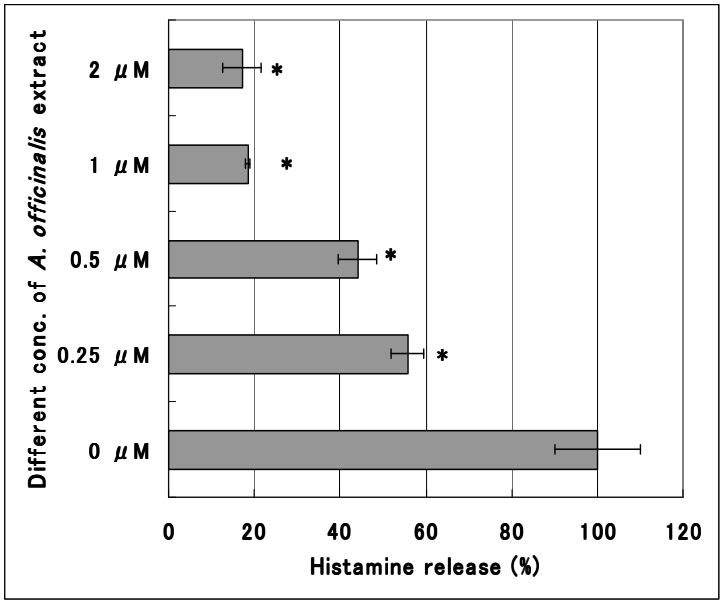

Figure 2. Dose-dependent inhibition of histamine release by the extract of *A. officinalis*. The release of histamine from rat PECs caused by the Ca^{2+} ionophore A23187 was measured in the presence of various concentrations of the extract. The data are shown as means ± SD (bars) for four experiments. *P < 0.05 by Student's t test for the comparison between the control and extract.

As a preliminary experiment, the extract (*A. officinalis*) was further extracted by various solvents to study the components which inhibit the release. The solvent of the fractions was evaporated by an evaporator, and the solid was dissolved in ethanol or distilled water (DW). Then, inhibition of the release of histamine by the fractions was measured as shown in Figure 3. Most activity showed from the chloroform fraction, but activity was also observed in the hexane and ethyl acetate fractions.

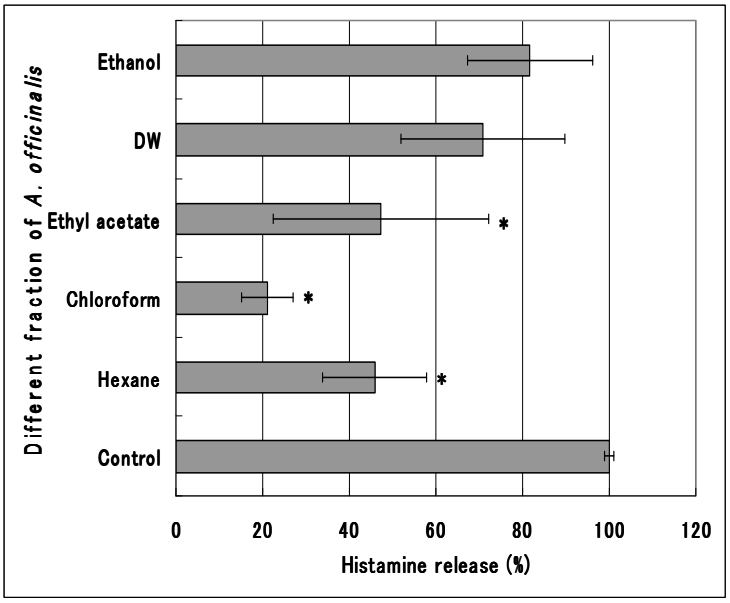

Figure 3. Inhibition of histamine release activity by different fractions of *A. officinalis*. The release of histamine from rat PECs caused by the Ca^{2+} ionophore A23187 was measured in the presence of samples (80 μg/mL). The data are shown as means ± SD (bars) for four experiments. *$P < 0.05$ by Student's *t* test for the comparison between the control and extracts.

Inhibition of LBT_4 release

We examined the effect of the extracts on the release of leukotriene (LTB_4) from rat PECs. The PECs (1 x 10^6/mL) were stimulated with 5 μM A23187 for 20 min in the presence of the extracts. As shown in Figure 4, all extracts examined inhibited significantly the release of LTB_4 from the cells at the same concentration of polyphenols (2 μM GAE) of each extract.

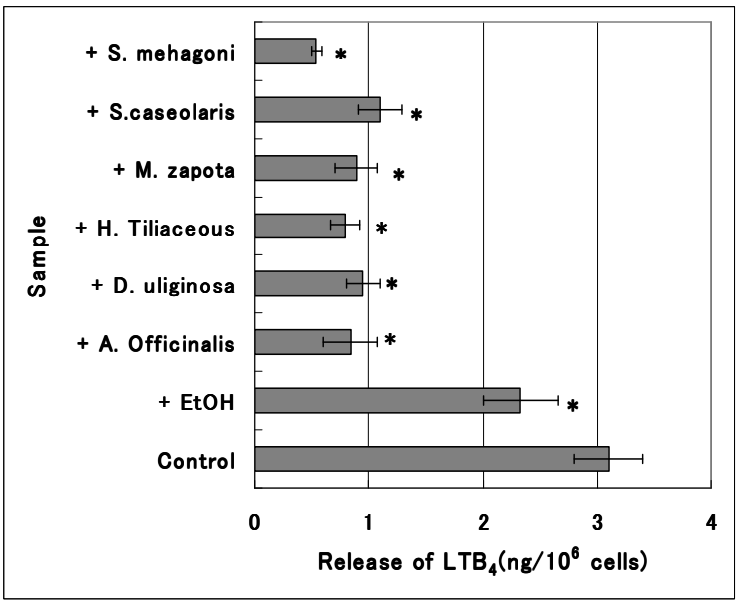

Figure 4. Inhibition of LTB_4 release activity by the extracts. The release of LTB_4 from rat PECs caused by the Ca^{2+} ionophore A23187 was measured with the same concentration of polyphenol of each extract, 2 μM GAE. The data are shown as means ± SD (bars) for four experiments. *$P < 0.05$ by Student's *t* test for the comparison between the control and extracts.

Discussion

Many of the health-promoting activities of fruit, such as anti-cancer, anti-diabetic, anti-mutagenic, anti-microbial, and anti-allergic effects, may be related to anti-oxidative activity. A relationship between TPH content and anti-oxidative and anti-allergic activities has already been reported [23]. In a previous paper [15], we reported that extracts of some Bangladeshi fruits have beneficial properties such as anti-oxidative, anti-amylase, anti-glucosidase and anti-histamine-release activity. The Bangladeshi mangrove plants examined in this study also have anti-oxidative and anti-histamine-release activity. Here a strong correlation between TPH content and scavenging DPPH radical suggested that the level of scavenging activity of the extracts was closely related to their phenolic groups. A very high correlation between TPH content and the reducing power of these extracts also suggested that the anti-oxidative activity increase with increases in polyphenol content [22]. Notably, the extract of *S. caseolaris* has high TPH and

strong DPPH scavenging activity (IC_{50} = 18 μg/mL). Its reducing power is also large. *S. caseolaris* is a small tree distributed in tidal creeks and mangrove swamps of Bangladesh. The fruit is used as a poultice, on sprains and swellings. The fermented juice of the fruit is useful in arresting hemorrhage and stop-bleeding treatment of piles [28]. Reportedly, anti-oxidants such as polyphenols are very important for the prevention of cardiovascular disorders, cancer, aging, inflammation and brain dysfunction [24, 25, 26, 27]. So the extracts of *S. caseolaris* may be useful to develop supplements and cosmetics with anti-oxidative activity.

A type I allergy is an immediate hypersensitive reaction to, for example, food or environmental allergens [6, 7]. Mast cells play a crucial role in the pathogenesis of this type of allergy through the production and release of chemical mediators such as histamine and eicosanoid [9, 10], which trigger various pathophysiological events in the acute phase of the reaction, including an increase in vascular permeability, the contraction of bronchial smooth muscle or production of mucus, and neutrophil chemotaxis [8, 29]. Therefore, it is important to inhibit the release of mediators for the prevention and/or alleviation of allergic symptoms. All extracts examined inhibited the release of both histamine and LTB_4 from PECs **(**Figure 1 and Figure 4). Reportedly, polyphenols play an important role in the suppression of histamine and LTB_4 release [11, 12, 13, 10] and gallic acid, which is very ubiquitous in mangrove trees, inhibits histamine release from mast cells, mediated by the modulation of cAMP and intracellular calcium [30]. In a previous paper, we have reported anti-histamine-release activity of Excoecaria agallocha, a mangrove well grown in the Sundarbans' [31]. From the dose-inhibition relationship of histamine release, the IC_{50} value of *A. officinalis* was estimated to be about 0.5 μM GAE (Figure 2). The mangrove tree, *A. officinalis*, commonly known as Baen or Kala baen in Bangladesh, is a tall (25 m) tree widely distributed in coastal forests in Southeast Asia. The seeds, barks, leaves and fruits of this tree are used as food or folk medicine. The barks and leaves are used in the treatment of asthma, diabetes, and rheumatism [32]. The extract of this tree may be of use for the development of supplements, cosmetics and drugs with anti-allergic activity.

Since extracts of other mangroves also inhibited the release of both histamine and LTB_4, further experiments are necessary to clarify the component(s) of the extracts responsible for these activities. It is also necessary to examine whether they reduce the type I allergy when administered to humans. It is worth searching for other beneficial effects of these medicinal plants in future.

Conclusion

The extracts of six Bangladeshi plants were screened for activities beneficial to health. The extract of *S. caseolaris* had strong anti-oxidative activity. The extracts examined inhibited the release of both histamine and LTB_4 induced by A23187 from rat PECs, suggesting the presence of type I anit-allergy activity. The extracts of these trees should be of use in the development of supplements or drugs with anti-oxidative and anti-allergic activity.

Acknowledgment

This work was supported by grant-in-aid no. 18 · 06189 from the Japan Society for the Promotion of Science (JSPS), Ministry of Education, Science, and Culture of Japan. The authors thank Prof. Matsudomi, N., Faculty of Agriculture, Yamaguchi University for useing the fluorescence spectroscope.

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