Anti-allergic Activity of *Boesenbergia thorelii* Rhizome Extract

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บทคัดย่อ

สารสกัดชั้นเอทานอล และชั้นน้ำจากเหง้าของพืชในวงศ์ขิง ทั้ง 8 ชนิดได้แก่ *Boesenbergia thorelii* (Gagnep.) Loes. (กระชายป่า), *Boesenbergia longiflora* (Wall.) Kuntze. (ว่านเปรี้ยว), *Boesenbergia* sp.1 (ไก่แดง), *Boesenbergia* sp.2 (ไก่ดำ), *Kaempferia angustifolia* Roscoe. (เฒ่าหนังแห้ง), *Kaempferia marginata* Carey. (กระแจะจันทน์), *Kaempferia rotunda* L. (ทิพยเนตร) และ *Kaempferia* sp. (เปราะป่า) ได้ถูกนำมาศึกษาฤทธิ์ต้านภูมิแพ้โดยใช้ RBL-2H3 cell line พบว่าสารสกัดชั้นเอทานอลของเหง้ากระชายป่า มีฤทธิ์ต้านภูมิแพ้ดีที่สุด โดยให้ค่า IC_{50} เท่ากับ 23.0 μg/mL รองลงมาได้แก่สารสกัดชั้นน้ำของกระชายป่า (IC_{50} = 26.3 μg/mL), ไก่แดง (ชั้นเอทานอล, IC_{50} = 38.3 μg/mL) และ กระแจะจันทน์ (ชั้นน้ำ, IC_{50} = 38.4 μg/mL) ในขณะที่สารสกัดอื่นมีฤทธิ์ปานกลางถึงน้อยมาก (IC_{50} = 49.4- > 100 μg/mL) การทดสอบพิษเฉียบพลันในหนูถีบจักร พบว่าขนาดของสารที่ทำให้หนูถีบจักรตาย 50 % เมื่อป้อนสารสกัดเอทานอลจากเหง้ากระชายป่าในตัวผู้และตัวเมียมีค่าเท่ากับ 471 mg/kg และ 636 mg/kg ตามลำดับ อาการผิดปกติที่พบในหนูถีบจักรได้แก่ การสูญเสียของกิริยาตอบสนอง, นอนหลับ และ ตาย จากการประเมินฤทธิ์ทางชีวภาพเบื้องต้นของสารสกัดในชั้นต่างๆ (bioassay-guided) พบว่าสารสกัดชั้นคลอโรฟอร์มมีฤทธิ์ต้านภูมิแพ้ได้ดีที่สุด โดยมีค่า IC_{50} เท่ากับ 22.9 μg/mL ตามด้วยสารสกัดชั้นน้ำ เอทานอล และเฮกเซน ซึ่งมีค่า IC_{50} เท่ากับ 49.6, 54.6, 59.1 μg/mL ตามลำดับ ในขณะที่ชั้นเอทิลอะซีเตทไม่มีฤทธิ์ (IC_{50}= >100 μg/mL) สารสกัดชั้นคลอโรฟอร์มจากเหง้ากระชายป่า สามารถแยกสารบริสุทธิ์ได้ 4 ชนิดคือ asaronaldehyde, β-sitosterol-D-glucoside, protocatechuic acid methyl ester และ 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols โดยสาร 4 ชนิดนี้เป็นสารที่แยกได้ครั้งแรกจากพืชนี้ ผลการทดสอบฤทธิ์ต้านภูมิแพ้พบว่า asaronaldehyde มีฤทธิ์ต้านภูมิแพ้สูงสุด โดยมีค่า IC_{50} เท่ากับ 24.3 μM รองลงมาคือ β-sitosterol-D-glucoside (IC_{50} = 63.3 μM) และ 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (IC_{50} = 72.6 μM) ตามลำดับ ในขณะที่ protocatechuic acid methyl ester ไม่มีฤทธิ์ (IC_{50} > 100 μM) ซึ่งชี้ให้เห็นว่าผลการทดสอบฤทธิ์ต้านภูมิแพ้ของ asaronaldehyde (24.3 μM) มีค่าสูงกว่าสารมาตรฐาน, ketotifen fumarate (IC_{50} = 41.1 μM) จากการศึกษาดังกล่าว

สรุปได้ว่า กระชายป่าและสารบริสุทธิ์ที่แยกได้มีฤทธิ์ต้านภูมิแพ้ และสนับสนุนการใช้เหง้ากระชายป่าในการรักษาโรคภูมิแพ้

Thesis Title Anti-allergic Activity of *Boesenbergia thorelii* Rhizome Extract

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Abstract

Ethanolic and water extracts from the rhizomes of eight selected Zingiberaceous plants, including *Boesenbergia thorelii* (Gagnep.) Loes. (Kra Chai Pa), *Boesenbergia longiflora* (Wall.) Kuntze. (Wan Priao), *Boesenbergia* sp.1 (Kai Dang), *Boesenbergia* sp.2 (Kai Dam), *Kaempferia angustifolia* Roscoe. (Thao Nhang Haeng), *Kaempferia marginata* Carey. (Kra Jae Jhun), *Kaempferia rotunda* L. (Thip-pa-ya-Nate) and *Kaempferia* sp. (Prauh Pa) were tested for their anti-allergic activities using a RBL-2H3 cell line. The ethanolic (EtOH) extract of *B. thorelii* exhibited the most potent anti-allergic effect with an IC_{50} value of 23.0 µg/mL, followed by *Boesenbergia thorelii* (water, IC_{50} = 26.3 µg/mL), *Boesenbergia* sp.1 (EtOH, IC_{50} = 38.3 µg/mL) and *Kaempferia marginata* (water, IC_{50} = 38.4 µg/mL), whereas other plants possessed moderate and mild activities. (IC_{50} = 49.4- > 100 µg/mL). In the acute toxicity test, the LD_{50} values for oral administration of the ethanol extract of *B. thorelii* in male and female mice were 471 mg/kg and 636 mg/kg, respectively. The signs of toxicity included loss of righting reflex, sleep and death were observed. From bioassay guided fractionation, the result showed that the chloroform fraction exhibited the most potent anti-allergic effect against antigen-induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells with an IC_{50} value of 22.9 µg/mL, followed by the water fraction, EtOH extract and hexane fraction with IC_{50} of 49.6, 54.6 and 59.1 µg/mL, respectively whereas the ethyl acetate fraction was apparently inactive (IC_{50} >100 µg/mL). Four compounds were isolated from the chloroform fraction of *B. thorelii* : asaronaldehyde, β-sitosterol-D-glucoside, protocatechuic acid methyl ester and 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols, Moreover, these four compounds were isolated for the first time from this plant. The result indicated that asaronaldehyde was the most active inhibitor of the allergic reaction with an IC_{50} value of 24.3 µM, followed by β-sitosterol-D-glucoside (IC_{50} = 63.3 µM) and 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (IC_{50} = 72.6 µM), respectively whereas protocatechuic acid methyl ester was inactive (IC_{50} > 100 µM). It was indicated that anti-allergic

effect of asaronaldehyde (24.3 μM) was higher than that of ketotifen fumarate (IC_{50}= 41.1 μM), the positive control. These results suggest that *B*. *thorelii* and its compounds exert anti-allergic activity and support the use of *B. thorelii* for treatment of allergy and allergic-related diseases.

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CONTENTS

		Page
CONTENTS		x
LIST OF TABLES		xiii
LIST OF FIGURES		xiv
LIST OF SCHEMES		xv
LIST OF ABBREVIATIONS AND SYMBOLS		xvi
CHAPTER 1 INTRODUCTION		
1.1 General introduction		
1.1.1	Immediate hypersensitivity (type I): allergy	1
1.1.2	Antigen	3
1.1.3	Cells	3
1.1.4	Antibody	6
1.1.5	Degranulation	8
1.1.6	Mediators of early phase	9
1.1.7	Mediators of late phase	10
1.2 Literature review		
1.2.1	Thai medicinal plants used for anti-allergic activity	14
1.2.2	Review of active compounds containing in Thai medicinal plants used for anti- allergic activity	16
1.2.3	Plant	23
1.3 Background and objectives		24
CHAPTER 2 EXPERIMENTAL		
2.1 General		25
2.2 Chemicals and reagents used for cell culture		25
2.3 Plant material		26

CONTENTS (continued)

	Page
2.4 Screening for anti-allergic activity of some selected plants in the genus *Boesenbergia* and *Kaempferia*	30
2.5 Isolation and structure elucidation	30
2.6 Structure elucidation	32
2.7 Animals	32
2.8 Acute toxicity test of *Boesenbergia thorelii* extract in mice	33
2.9 Anti –allergic activity assay	
2.9.1 Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells	33
2.10 Statistical analysis	35
CHAPTER 3 RESULTS AND DISCUSSION	
3.1 Effect of some selected plants in the genus *Boesenbergia* and *Kaempferia* on the release of β-hexosaminidase from RBL-2H3 cells	36
3.2 Isolation and structure elucidation	
3.2.1 Isolation	40
3.2.2 Structure elucidation	
3.2.2.1 Asaronaldehyde (compound **1**)	42
3.2.2.2 β-sitosterol-D-glucoside (compound **2**)	44
3.2.2.3 Protocatechuic acid methyl ester (compound **3**)	47
3.2.2.4 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (compound **4**)	49
3.3 Acute toxicity effect of *B. thorelii* extract in mice	51
3.4 Anti –allergic activity assay	
3.4.1 Effect of fractions from *B. thorelii* rhizomes and isolated compounds on the release of β-hexosaminidase from RBL-2H3 cells	51

CONTENTS (continued)

	Page
CHAPTER 4 CONCLUSION	56
REFFERENCES	57
APPENDIX	64
VITAE	85

LIST OF TABLES

Table		Page
1	Allergic diseases	2
2	Active constituents isolated from Thai medicinal plants used for allergy treatment	16
3	Anti-allergic activity [a] of some selected plants in the Zingiberaceae family	37
4	^{1}H and ^{13}C NMR spectral data of compound **1** ($CDCl_3$; 500 MHz for ^{1}H) and reference	43
5	^{1}H NMR spectral data of compound **2** (DMSO-d_6; 500 MHz for ^{1}H) and reference	45
6	^{1}H NMR spectral data of compound **3** ($CDCl_3$; 500 MHz for ^{1}H) and reference	48
7	^{1}H NMR spectral data of compound **4** ($CDCl_3$; 500 MHz for ^{1}H) and reference	50
8	Anti-allergic activity [a] of fractions from *B. thorelii* rhizomes	54
9	Anti-allergic activity [a] of compounds from *B. thorelii* rhizomes	55

LIST OF FIGURES

Figure		Page
1	Positive feedback sustains the T cell response as long as antigen (allergen) is available and more IL-4 and more IgE are produced. The IL-4 produced may favor IL-4 production by T cells responding to other antigents	8
2	Mediators of the early phase of allergy have different consequences depending on the target tissue	10
3	Following exposure to cat fur, this patient has severe asthma symptoms that return after an interval of several hours	11
4	Chronic allergic inflammation: compare this with the changes of acute asthma in the bronchus in Figure 2	12
5	Drug treatment of allergy	13
6	Chemical structures of constituents isolated from Thai medicinal plants used for allergy treatment	18
7	The pictures of some Zingiberaceous plants	26
8	The procedure of *B. thorelii* rhizomes extraction	31
9	The anti-allergic activity test	35
10	Dose-response curves of water (**A**) and EtOH (B) extracts of some selected plants in the Zingiberaceae family against allergic reaction	39
11	The chemical structures of compounds isolated from the *B. thorelii* rhizomes	41
12	Dose-response curves of various compounds from the chloroform fraction of *B. thorelii* extract against allergic reaction	53

LIST OF SCHEMES

Scheme		Page
1	Hydrolysis reaction of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (PNAG)	35

LIST OF ABBREVIATIONS AND SYMBOLS

br	broad (for NMR signals)
d	doublet (for NMR signals)
dd	doublet of doublet (for NMR signals)
EIMS	electron-impact mass spectroscopy
et al.	et alibi
g	gram
h	hour
IC_{50}	inhibitory concentration at 50% of tested subject
J	nuclear spin-spin coupling constant (in Hz)
kg	kilogram
L	liter
M	multiplet (for NMR signals)
mg	milligram
MHz	megahertz
min	minute
ml	milliliter
MS	mass spectroscopy
MW	molecular weight
m/z	mass-over-charge ratio
NMR	nuclear magnetic resonance
P	*P*-value
pH	potential of hydrogen
ppm	part per million
s	singlet (for NMR signals)
S.E.M	standard error mean
UV-VIS	ultraviolet-visble
δ	Chemical shift in ppm

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

^{o}C	degree of Celsius
/	per
μg	microgram
μM	micromolar
%	percentage
®	trade name
pH	The negative logarithm of the hydrogen ion concentration
S.E.M.	Standard error of mean

CHAPTER 1

INTRODUCTION

1.1 General introduction

1.1.1 Immediate hypersensitivity (type I): allergy

The immunological definition of atopy is an immediate hypersensitivity reaction to environmental antigens, mediated by IgE. Such reactions tend to run in families and these families are said to have inherited the atopy trait. Although the term allergy was originally defined as altered reactivity to exogenous antigens, it is now often used synonymously with atopy. Allergic diseases include anaphylaxis, rhinitis, asthma, some types of dermatitis or eczema and food allergies. The distinction between true allergy and other reactions is important because some of the treatments for allergy would be inappropriate for other types of reaction (Nairn and Helbert, 2002).

Usually, allergies are very rapid reactions mediated by IgE. However, some allergic reactions continue for a long time (for example, when the environmental antigen cannot be easily avoided) and they develop into a late phase reaction characterized by T cell infiltrates. This is called the late phase response. The types of allergy are shown in Table 1 (Nairn and Helbert, 2002).

Table 1 Allergic diseases (Nairn and Helbert, 2002).

Clinical disease	Symptoms	Characteristics
Asthma	Reversible airways obstruction in the bronchi	Infection, exercise and changes in temperature can also cause changes in air flow
Rhinitis	Discharge, sneezing and nasal obstruction	
Allergic conjunctivitis	Redness of conjunctiva and itchy eyelids	Often co-exists with rhinitis
Urticaria	Usually acute; itchy edema of the cutaneous tissues; the lesion is identical to that induced by skin prick testing	Allergy accounts for about two thirds of cases of urticaria
Angioedema	Usually acute; non-itchy edema of the subcutaneous tissues	Some forms, for example lip swelling, may be manifestations of food allergy
Atopic eczema	Usually chronic; itchy inflammation	A minority of cases are caused by food allergy
Anaphylaxis	Low blood pressure, angioedema and airways obstruction	

1.1.2 Antigen

Antigens that trigger allergic reactions are referred to as allergens. Allergens are present in the environment as small particles or low-molecular-weight substances that penetrate the body after being inhaled, eaten or administered as drugs. Inhaled antigens include pollens, fungal spores and the faces of the house dust mite. Animal allergens include salivary proteins; these become airborne after the animal has licked itself and the saliva dries on the fur. Some insect venoms are allergens and these are injected directly into the skin (Nairn and Helbert, 2002).

For food allergens to cause symptoms they must either be absorbed through the mucosa of the mouth and lips (in which case they cause symptoms very rapidly) or be resistant to digestive enzymes and low pH.

An important part of the treatment of allergy is identification and avoidance of allergens. Careful history taking facilitates identification of allergens. For example, a patient with a runny nose (rhinitis) is likely to be sensitive to aeroallergens. If symptoms occur predominantly in summer, grass pollens are the likely culprit. If symptoms occur all year round and mainly indoors, sensitivity to house dust mite feces is likely. House dust mite allergy occurs in areas where a cold climate dictates the need for central heating, heavy bedding and thick carpets- the habitat of this mite (Nairn and Helbert, 2002).

1.1.3 Cells

Basophils and mast cells are two functionally similar but distinct cell types that play pivotal roles in the initiation and development of type I hypersensitivity reactions. Granulocyte basophils are circulating cells representing less than 1% of the white cell population, while mature mast cells can be found exclusively in tissues and in particular in regions located at interfaces with the external environment, such as lungs, skin and mucosal surfaces. Despite their similarity, for many years it was postulated that these two cell types did not derive from common

progenitors but were descended from different lineages subsequently, various objections to this hypothesis have been put forward suggesting a possible common origin (Passante and Frankish, 2009).

After crosslinking of their IgE-bound Fc receptors (FcεRI) by allergens, basophils and mast cells release a range of preformed and newly synthesised mediators that evoke a potent immune allergic response. While preformed mediators such as histamine, heparin, chemotactic factors, tryptases and chymases are stored in granules and released upon exocytosis, newly synthesised mediators such as prostaglandin D2 (PGD2), the leukotrienes C4 and D4 (LTC4, LTD4) and platelet activating factor (PAF) are produced and secreted after cell stimulation (Passante and Frankish, 2009).

It is also well established that both mast cells and basophils take part in the adaptative immune response to pathogens. Mast cells, due to their "sentinel locations" at the interfaces of the body with its external environment, such as the lungs and intestine, are one of the first activators of the inflammatory response, not only releasing cytokines and chemoattractants for other immune cells such as neutrophils and macrophages but also phagocytosing invading pathogens and presenting the antigen to T cells. The role of basophils during host defence is less well known, but they are thought to sustain the inflammatory process with the release of immunoregulatory cytokines such as interleukin 4 (IL-4), interleukin 13 (IL-13) and tumour necrosis factor α (TNF-α) (Passante and Frankish, 2009).

Granule contents

Mast cell enzymes. Mast cell granules contain a number of proteolytic enzymes including tryptase and chymotrypsin. These enzymes increase mucus secretion and smooth muscle contraction, for example, bronchi. In addition, they cleave and activate components of the complement and kinin pathways, which promote inflammation (Nairn and Helbert, 2002).

Histamine: Histamine causes smooth muscle contraction in the gut and lungs in an attempt to expel worms. Histamine increases vascular permeability and provides a chemotactic signal to attract more white cells to the site of worm infestation. Histamine cause marked itching in the skin, possibly to draw the attention of an infested host to the presence of skin parasites (Nairn and Helbert, 2002).

Cytokines: Like activated macrophages, mast cells produce a range of cytokines to promote and extend the inflammatory response. Tumor necrosis factor (TNF) is preformed and present in granules and will activate local endothelium to enhance diapedesis of more inflammatory cells. Mast cells also produce other cytokines after stimulation, and, unlike those produced by macrophages, these stimulate T helper 2 (T_H2) responses. interleukin (IL) 4 activated T_H2 cells and IL-3 and IL-5 stimulate eosinophil production and activation. IL-4 and IL-5 also skew the adaptive immune response away from a T_H1 response (Nairn and Helbert, 2002).

Tryptase: Tryptase is a major protease released by mast cells; its exact role is uncertain, but it can cleave C3 and C3a. Tryptase is found in all human mast cells but in few other cells and thus is a good marker of mast cell activation (Anand and Routes, 2009).

Chemotactic factors: An eosinophilic chemotactic factor of anaphylaxis causes eosinophil chemotaxis; an inflammatory factor of anaphylaxis results in neutrophil chemotaxis. Eosinophils release major basic protein and, together with the activity of neutrophils, can cause significant tissue damage in the later phases of allergic reactions (Anand and Routes, 2009).

Newly formed mediators

Prostaglandins D2: Produced mainly by mast cells; bronchoconstrictor, peripheral vasodilator, coronary and pulmonary artery vasoconstrictor, platelet aggregation inhibitor, neutrophil chemoattractant, and enhancer of histamine release from basophils (Anand and Routes, 2009).

Leukotrienes C4 and D4: Potent bronchoconstrictors, increase vascular permeability, and cause arteriolar constriction (Anand and Routes, 2009).

Platelet-activating factor (PAF): PAF is synthesized from membrane phospholipids via a different pathway from arachidonic acid. It aggregates platelets but is also a very potent mediator in allergic reactions. It increases vascular permeability, causes bronchoconstriction and causes chemotaxis and degranulation of eosinophils and neutrophils (Anand and Routes, 2009).

β -Hexosaminidase

This granule-associated enzyme is an exoglycosidase that has been extensively used to monitor mast cell degranulation as it is released in parallel with histamine. Its role during the inflammation process is not known, but it has been hypothesised that it could act in concert with tryptases and chymases for the degradation of glycoproteins and proteoglycans of the extracellular matrix (an important event during the remodelling of the inflamed tissue). The enzyme β-hexosaminidase shows optimal activity at low pH (pH = 4.5), a typical condition during inflammatory processes. β-Hexosamidinase is commonly used as a marker for degranulation in both Rat basophilic leukemia (RBL-2H3) cells and rat mast cell protease (RPMC) since it is released in parallel with histamine (Church *et al*., 2003).

1.1.4 Antibody

IgE is required for type I hypersensitivity reactions. B cells class switch to IgE production when they are co-stimulated by interleukin (IL) 4, secreted by T helper 2 (T_H2) cells. Once IgE is produced**,** it binds to the high affinity receptor FcεRI, expressed on resting mast cells resident in tissues and eosinophils that have been activated and migrated into tissues. If antigen

crosslinks the IgE bound to the FcεRI, these cells release the mediators of type I hypersensitivity. On stimulation, mast cells also release more IL-4. This provides a positive feedback system for the production of more IgE and T_H2 cells. Thus, once a T cell response to an antigen has deviated towards production of T_H2 cytokines, positive feedback sustains and enhances the response (Figure 1). IgE production is inhibited by interferon γ (IFN-γ) secreted by T_H1 type T cells (Nairn and Helbert, 2002).

Very high levels of IgE are seen in patients infected with parasites, for example schistosomiasis. Levels of IgE may also be high in people who have inherited the atopy trait, although they can be normal. The presence of IgE against specific allergens is required for allergic reactions to that allergen, but this dose not always predict clinical symptoms. Specific IgE can be measure using enzyme-linked immunosorbent assays (ELISA), although in diagnosing allergy, it is more often useful to do skin prick testing (Nairn and Helbert, 2002).

Skin prick testing is preferred method for allergy testing. When the antigen crosslinks IgE on mast cell FcεRI, there is a rapid release of histamine, causing a local flare and wheal reaction. Skin prick tests give immediate results, which the patient can see, they are cheap and they may be more reliable than specific IgE testing. Skin prick testing is generally safe. Skin prick testing is not possible when patients have taken antihistamines or have extensive atopic dermatitis (Nairn and Helbert, 2002).

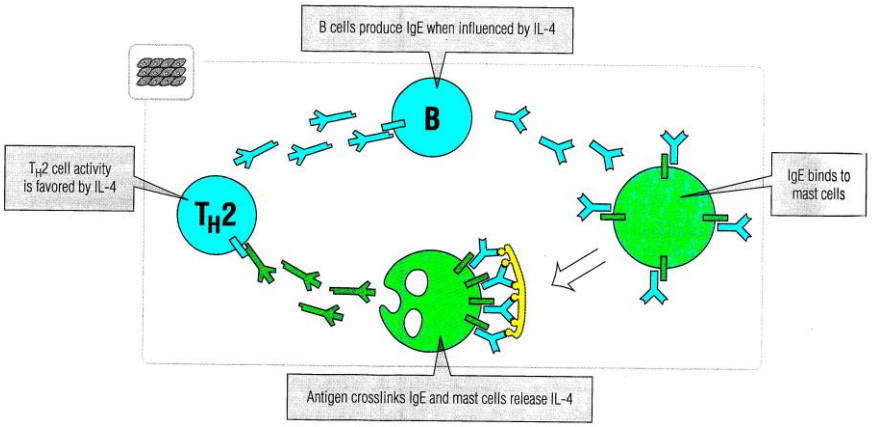


Figure 1 Positive feedback sustains the T cell response as long as antigen (allergen) is available and more IL-4 and more IgE are produced. The IL-4 produced may favor IL-4 production by T cells responding to other antigents (Nairn and Helbert, 2002).

Rat basophilic leukemia (RBL-2H3) cells display properties of mucosal-type mast cells. The RBL-2H3 cells contain several hundred thousand IgE receptors on the membrane surface, and after sensitization with mouse monoclonal IgE, the cells respond to antigen and release histamine. Therefore, RBL-2H3 cells are used as a model cell line for histamine release. (Nakatani *et al*., 2002).

1.1.5 Degranulation

RBL-2H3 cells, like mast cells and basophils, respond with degranulation following crosslinking of their IgE-bound FcƐRI by multivalent allergens, with the release of a range of preformed and newly synthesised mediators that evoke a potent immune allergic response. Degranulation of RBL- 2H3 cells, as a consequence of FcƐR-stimulation, reflects the behaviour of both mucosal mast cells (MMC) and basophils in respect of their response to immunological stimuli and has been extensively reviewed (Gilfillan and Tkaczyk, 2006). In fact,

degranulation of basophils was largely modelled on studies using RBL-2H3 cells (Kepley et al 1998). RBL-2H3 cells may also degranulate due to a variety of non-immunological stimuli, and differences exist between MMC, RBL-2H3 and connective tissue mast cells (CTMC) (Passante and Frankish, 2009).

1.1.6 Mediators of early phase

The early phase of allergy is caused by mediators released by mast cells when IgE bound to FcεRI is crosslinked by allergen. Anaphylaxis is the most serious type of allergy and can occur when allergen enters the body from any route. Mast cells release preformed histamine and rapidly synthesize prostaglandins through the cyclooxygenase pathway. These mediators cause vasodilatation and an increase in vascular permeability. Fluid shifts from the vascular to the extravascular space and there is a fall in vascular tone. The result of widespread mast cell activation is a dramatic fall in blood pressure, which is characteristic of anaphylactic shock (Nairn and Helbert, 2002).

In other forms of allergy, there are more localized changes in blood vessels, restricted to the site of allergen entry. For example, in allergic rhinitis, inhaled allergens stimulate mast cells in the nasal mucosa. There is then vasodilatation and edema in the nose, causing nasal stuffiness and sneezing. Leukotrienes, products of the lipooxygenase pathway, increase mucus secretion, which cause the discharge characteristic of allergic rhinitis (Nairn and Helbert, 2002).

Increase mucus secretion in the bronchi also occurs in asthma and contributes to the airflow obstruction. However, in the lungs leukotrienes cause smooth muscle contraction and this has the most dramatic effects on airflow reduction (Figure 2).

All of these effects can take place within minutes of exposure to allergen. Symptoms persist whilst exposure to allergen continues. Even if the patient is able to avoid the allergen, late-phase response may then occur (Nairn and Helbert, 2002).

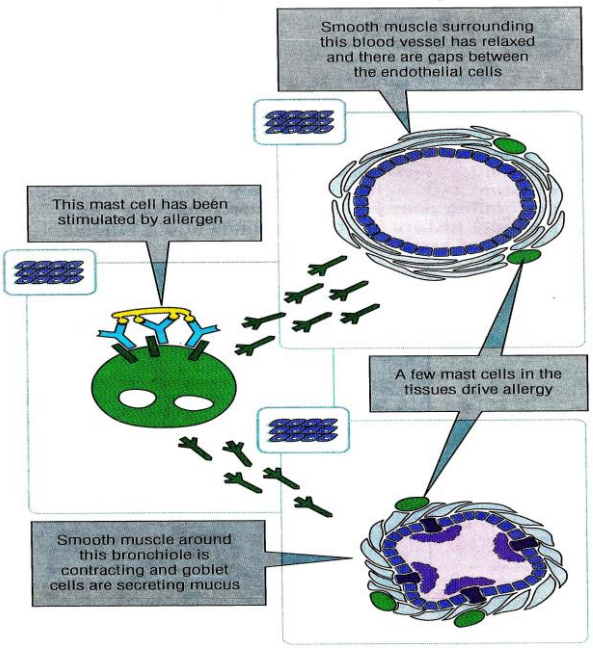


Figure 2 Mediators of the early phase of allergy have different consequences depending on the target tissue (Nairn and Helbert, 2002).

1.1.7 Mediators of late phase

Type I hypersensitivity reactions are generally characterized by immediate symptoms after exposure to allergens. For example, an asthmatic patient allergic to cats will develop airways obstruction, characterized by wheeze, seconds after exposure to cat fur. The symptoms improve after an hour or so as the immediate response dies down (Figure 3).

Several hours after the acute episode, the airflow in the bronchi may deteriorate again, reflecting the migration of leukocytes, particularly eosinophils, into the bronchi in response to chemokines. The late phase may last several hours (Figure 3).

In some individuals, this process become self perpetuating as T_H2 cells in the bronchial wall secrete cytokines such as IL-4 and more attractant chemokines (Figure 4). The result is chronic allergic inflammation in the airways. Mediators release by eosinophils include peroxidase, eosinophil major basic protein and cationic protein, which all cause direct damage to

bronchial tissue. As a result of the chronic allergic inflammation, the bronchial smooth muscle is hypertrophic and mucus secretion is increased; airflow becomes persistently, rather than intermittently reduced (Nairn and Helbert, 2002).

Infections exacerbate the inflammation and symptoms by attracting more inflammatory cells to the airway. Once the lining of the airway has become inflamed, it is susceptible to any irritant, for example cigarette smoke, and changes in endogenous steroid secretion (Nairn and Helbert, 2002).

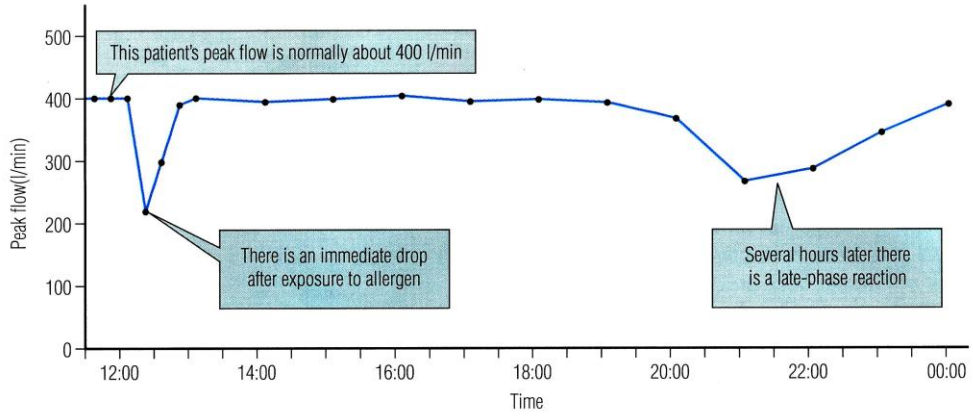


Figure 3 Following exposure to cat fur, this patient has severe asthma symptoms that return after an interval of several hours (Nairn and Helbert, 2002).

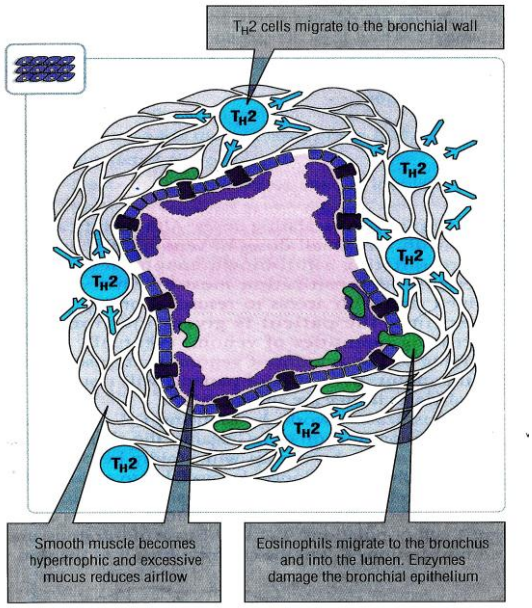


Figure 4 Chronic allergic inflammation: compare this with the changes of acute asthma in the bronchus in Figure 2 (Nairn and Helbert, 2002).

Treatment

General measures in the treatment of allergy include identifying and avoiding possible allergens. This is not always possible when the allergen is widespread in the environment, for example grass pollen. Other treatments involve the used of drugs or desensitization (Nairn and Helbert, 2002).

Drug treatments

Drug can be useful in both preventing and treating allergic reactions. Some drugs block the end effect of mediator release; for example β_2-adrenergic agonists, such as salbutamol, mimic the effects of the sympathetic nervous system and work mainly by preventing smooth bronchial muscle contraction in asthma (Figure 5). Epinephrine (adrenaline) is an

important drug used in the treatment of anaphylaxis. In anaphylaxis, the blood pressure falls dramatically because fluid shifts out of blood vessels and into the tissues when vessel permeability increases. Epinephrine (adrenaline) can increase the blood pressure and reverse airways obstruction and be life saving in anaphylaxis.

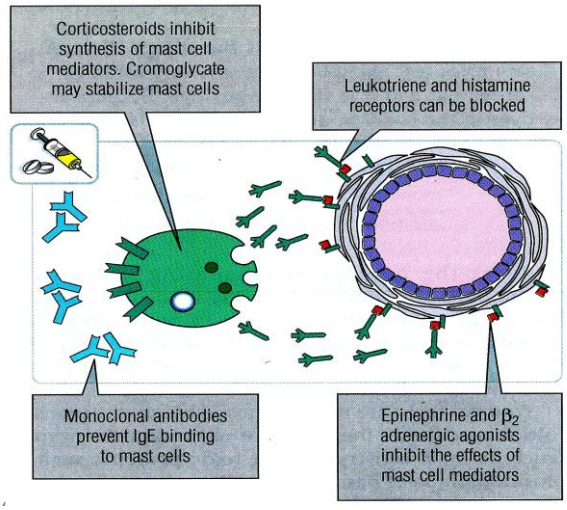


Figure 5 Drug treatment of allergy (Nairn and Helbert, 2002).

Corticosteroids are widely used in the prevention of symptoms in patients with allergy. It can prevent the immediate hypersensitivity reaction, the late phase and chronic allergic inflammation. To avoid side effects, corticosteroids are often given topically in allergies, for example inhaled steroids are used in asthma.

Sodium cromoglicate has some effects in preventing allergy attacks. It is thought to work by stabilizing mast cells and reducing degranulation (Nairn and Helbert, 2002).

Specific receptor antagonists block the effects of leukotrienes. Montelukast, for example, reduces the amount of airways inflammation in asthma. Antihistamine block specific histamine receptors. Antihistamines are not very useful in asthma, because this mediator is not released by lung mast cells, but they have an important role in allergies affecting the skin and nose.

Newer drugs that block the effects of the T_H2 type cytokines, IL-3, IL-4 and IL-5, have not been shown to improve symptoms in allergy.

Omalizumab is a monoclonal antibody that binds to IgE and prevents it binding to the FcƐR. Omalizumab reduces the number of circulating and airway eosinophils and prevents both the immediate and late phase of asthma after exposure to house dust mite. Omalizumab is a humanized chimeric antibody and it is, therefore, thus less likely to provoke anti-mouse antibodies.

To date, there are no drugs available to clinicians to block specifically the effects of chemokines and enzymes such as mast cell tryptase or toxic proteins such as eosinophil major basic protein (Nairn and Helbert, 2002).

1.2 Literature review

1.2.1 Thai medicinal plants used for anti-allergic activity

There have been many reports on the anti-allergic effects of some Thai medicinal plants such as *Curcuma longa* Linn. (Yano *et al*., 2000), *Garcinia mangostana* Linn. (Nakatani *et al*., 2002), *Curcuma zedoaria* Roscoe. (Matsuda *et al*., 2004), *Alpinia galangal* L. (Yoshikawa *et al*., 2004), *Dioscorea membranacea* Pierre. (Tewtrakul *et al*., 2006), *Zingiber cassumunar* Roxb. & *Curcuma mangga* Valeton & Zijp. (Tewtrakul *et al*., 2006), *Suregada multiflora* (A. Juss) Baill. (Cheenpracha *et al*., 2006), *Kaempferia parviflora* Wall. ex Baker. (Tewtrakul *et al*., 2008), *Atalantia monophylla* Correa. (Chukaew *et al*., 2008), *Mangifera indica*

Linn., *Musa sapientum* Linn., *Hibiscus esculentus* Linn., *Tamarindus indica* Linn. and *Artocarpus integer* Merr. (Tewtrakul *et al.*, 2008), *Centella asiatica* (Linn.) Urban. (George *et al*., 2009), *Rhinacanthus nasutus* Kurz. (Tewtrakul *et al.*, 2009), and Zingiber z*erumbet* L. (Yob *et al*., 2011).

The fruit hull of *Garcinia mangostana* Linn. is used as a traditional medicine in Southeast Asia for anti-inflammatory, anti-diarrhoea, antiulcer and antiseptic purposes. *Alpinia galangal* L. has been used to treat stomachache and as a carminative, antiflatulant, antifungal and anti-itching agent. *Dioscorea membranacea* Pierre. has long been used as common ingredients in many preparations, including those used in treatments of dermopathy, lymphopathy, inflammation, cancers, venereal diseases, and leprosy. *Suregada multiflora* (A. Juss) Baill. has been reported to contain anti-human immunodeficiency virustype 1 (HIV-1) protein, GAP31, and also exhibit the inhibitory effect on the infection and replication of herpes simplex virus (HSV) (Bourinbaiar *et al*., 1996). *Kaempferia parviflora* Wall. ex Baker. has been used for treating allergies, gastrointestinal disorders, fungal infections and impotence. Various parts of *Atalantia monophylla* Correa. have been used for folk medicine for several purposes such as the treatment of chronic rheumatism, paralysis (Basa, 1975), antispasmodic, stimulant and hemiplegia (Panda, 2004). The essential oil from the leaves showed antimicrobial and strong inhibitory activities against some pathogenic fungi (Prasad, 1988), whereas a decoction of the leaves is often applied for itching and other skin complaints (Panda, 2004). Some stilbenes isolated from the aerial parts of *Artocarpus integer* Merr. have been reported to have anti-malarial activity against *Plasmodium falciparum* (Boonlaksiri *et al.*, 2000). *Centella asiatica* (Linn.) Urban. is used as anti-bacterial, anti-microbial, wound healing , anti-oxidant and analgesic drug (Cheng *et al*., 2000). *Zingiber cassumunar* L. has been used for treatment of inflammation and skin diseases (Jeenapongsa *et al.*, 2003) and *Zingiber zerumbet* L. as an antiflatulant and anti-inflammatory agent (Wutthithamavet, 1997). The rhizome of *Curcuma longa* Linn. has long been used in Thai traditional medicine for treatment of itching and other skin diseases, whereas *Curcuma zedoaria* Roscoe. has been used as a substitute for *Curcuma longa* Linn. and has recently been reported to show anti-allergic activity. Seed of *Mangifera indica* Linn. and *Tamarindus indica* Linn. have been used in Thai traditional medicine for wound healing, whereas *Musa sapientum* Linn. and

Hibiscus esculentus Linn. have been used as tonic for the treatment of ulcer (Pengcharoen, 2002). The root and whole plant of *Rhinacanthus nasutus* Kurz. have been used in Thai traditional medicine by topical application (alcoholic extract) for treatment of Tinea versicolor, ringworm, itching and skin diseases (Wutthithamavet, 1997; Farnsworth and Bunyapraphatsara 1992). Different parts of this plant have been also used for the treatment in a range of other diseases such as diabetes, hypertension, eczema, pulmonary tuberculosis, herpes, hepatitis, and various skin diseases, and the active components of this plant have been widely investigated. (Gotoh *et al*., 2010).

1.2.2 Review of active compounds containing in Thai medicinal plants used for anti-allergic activity

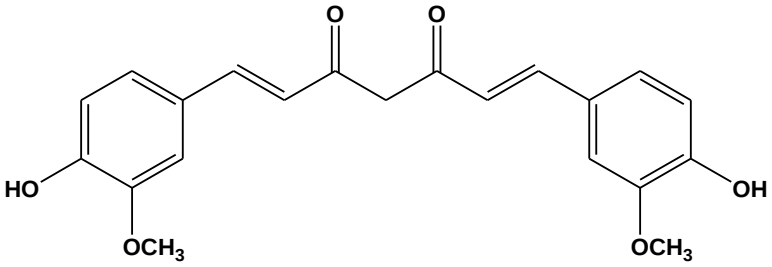
There have been many reports of active constituents isolated from Thai medicinal plants. The identified active compounds are presented in Table 2 and Figure 6.

Table 2 Active constituents isolated from Thai medicinal plants used for allergy treatment

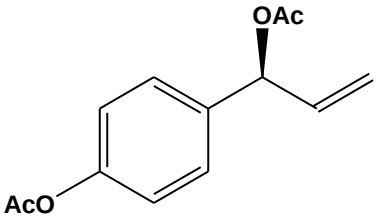
Active constituents	Plant name	Part -used	References
Curcumin	*Curcuma longa*	Rhizome	Yano *et al*.,2000
1'-Acetoxychavicol acetate	*Curcuma zedoaria*	Rhizome	Matsuda *et al*., 2004
Dioscorealide A Dioscorealide B	*Dioscorea membranacea*	Rhizome	Tewtrakul *et al*., 2006
Dioscoreanone Diosgenin	*Dioscorea membranacea*	Rhizome	Tewtrakul *et al*., 2006
ent-16-Kaurene-3β, 15β, 18-triol (1) *ent*-3-Oxo-16-kaurene -15β, 18-diol (2) *ent*-16-Kaurene-3β, 15β -diol (3)	*Suregada multiflora*	Stem bark	Cheenpracha *et al*., 2006

Table2 (Continued)

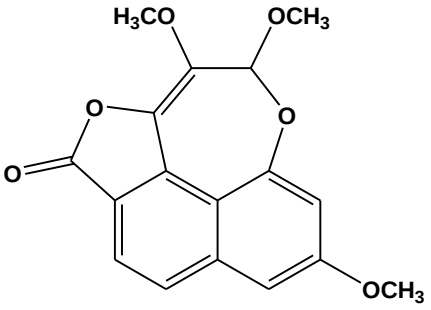
Active constituents	Plant name	Part -used	References
Abbeokutone (4) Helioscopinolide A (5) Helioscopinolide C (6) Helioscopinolide I (7)	*Suregada multiflora*	Stem bark	Cheenpracha *et al*., 2006
5-Hydroxy-3, 7, 3', 4'-tetramethoxyflavone 5-Hydroxy-7-methoxyflavone 5-Hydroxy-7, 4'-dimethoxyflavone	*Kaempferia parviflora*	Rhizome	Tewtrakul *et al*., 2008
N-methylcyclo-atalaphylline-A Buxifoliadine-E Citrusinine-I	*Atalantia monophylla*	Root	Chukaew *et al*., 2008
Rhinacanthin-C (1) Rhinacanthin-D (2) Rhinacanthin-N (3)	*Rhinacanthus nasutus*	Leaf	Tewtrakul *et al*., 2009



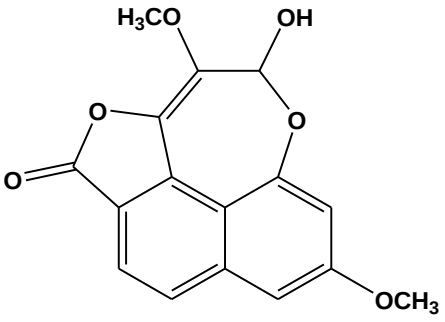
Curcumin



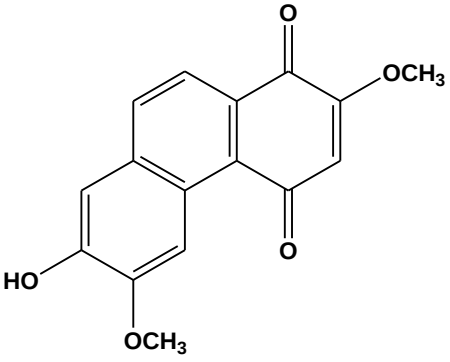
1'-Acetoxychavicol acetate



Dioscorealide A

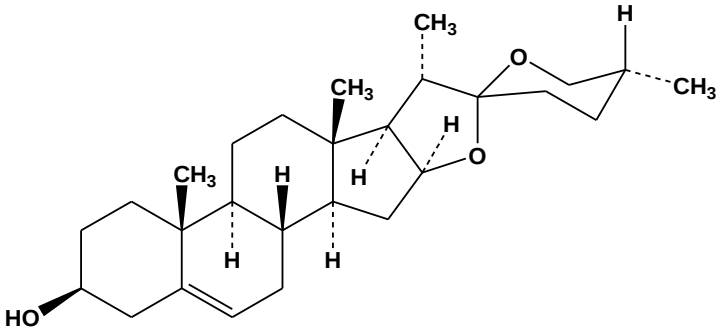


Dioscorealide B

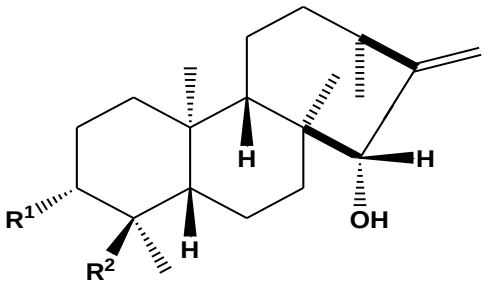


Dioscoreanone

Figure 6 Chemical structures of constituents isolated from Thai medicinal plants used for allergy treatment



Diosgenin



1) R^1= OH, R^2= CH_2OH

2) R^1= =O, R^2= CH_2OH

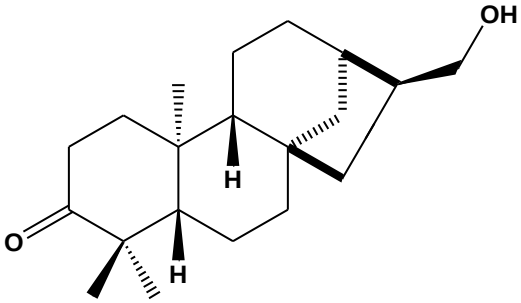
3) R^1= OH, R^2= CH_3

ent-16-Kaurene-3β, 15β, 18-triol (1)

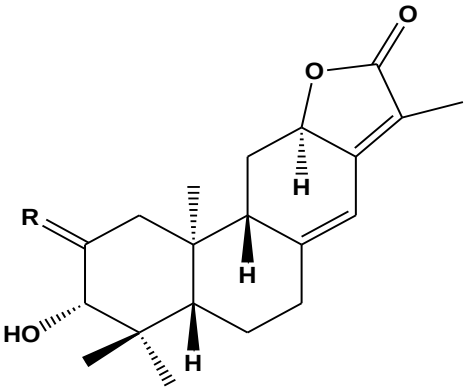
ent-3-Oxo-16-kaurene-15β, 18-diol (2)

ent-16-Kaurene-3β, 15β-diol (3)

Figure 6 Chemical structures of constituents isolated from Thai medicinal plants used for allergy treatment



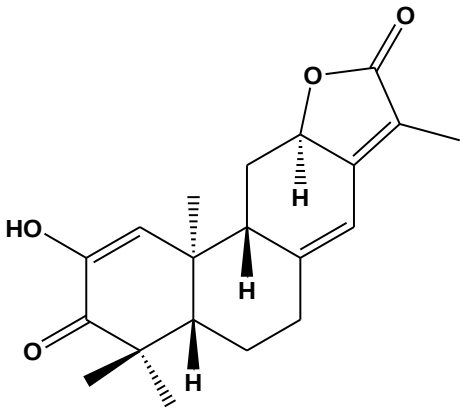
Abbeokutone (4)



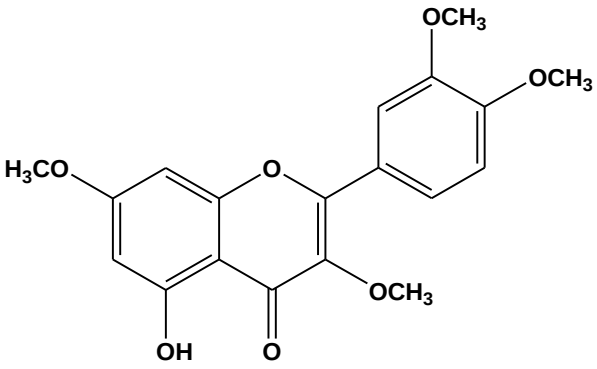
5) R= H_2

6) R= O

Helioscopinolide A (5), Helioscopinolide C (6)

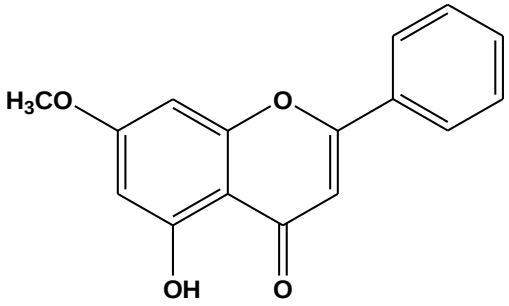


Helioscopinolide I (7)

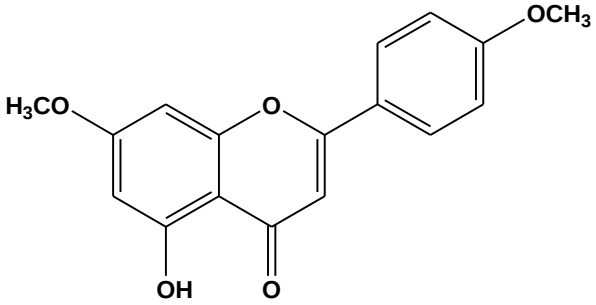


5-Hydroxy-3, 7, 3', 4'-tetramethoxyflavone

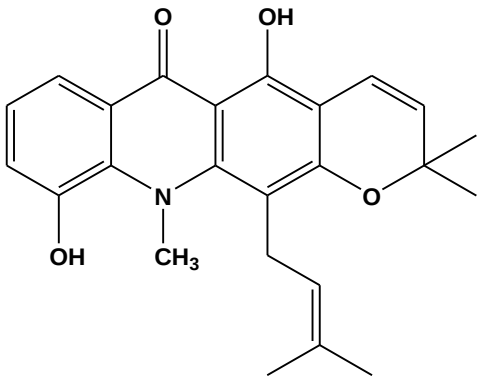
Figure 6 Chemical structures of constituents isolated from Thai medicinal plants used for allergy treatment



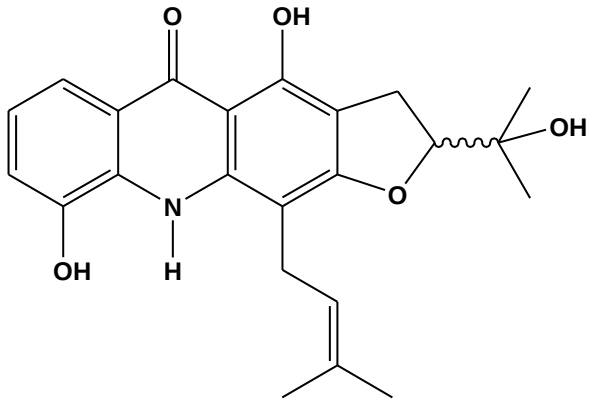
5-Hydroxy-7-methoxyflavone



5-Hydroxy-7, 4'-dimethoxyflavone

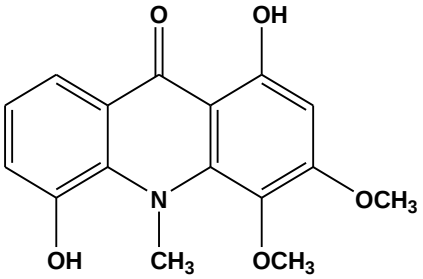


N-methylcyclo-atalaphylline-A

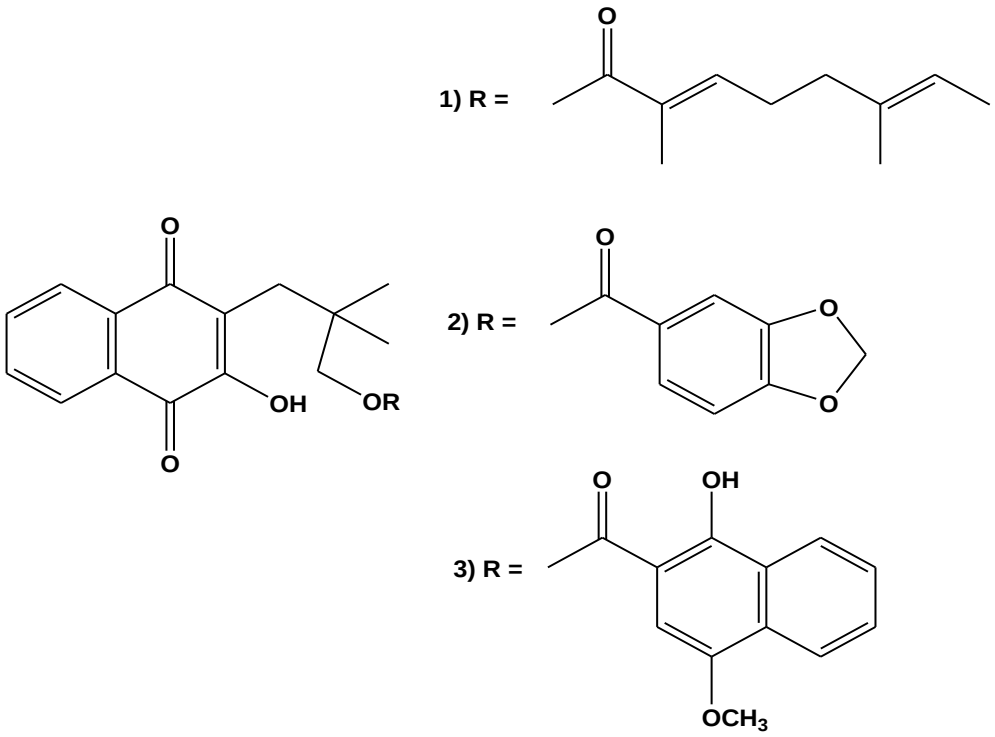


Buxifoliadine-E

Figure 6 Chemical structures of constituents isolated from Thai medicinal plants used for allergy treatment



Citrusinine-I



(1) Rhinacanthin-C (2) Rhinacanthin-D (3) Rhinacanthin-N

Figure 6 Chemical structures of constituents isolated from Thai medicinal plants used for allergy treatment

1.2.3 Plant

The Zingiberaceae is among the plant families which are widely distributed throughout the tropics particularly in Southeast Asia. Zingiberaceae is one of the largest plant family from the order Zingiberales, with approximately 50 genera and over 1,000 species (Holttum, 1950). Zingiberaceae species grow naturally in damp, shaded parts of the low-land or on hill slopes, as scattered plants or thickets. Most members of the family are easily recognized by the characteristic aromatic leaves and fleshy rhizome when both of them are crushed and also by the elliptic to elliptic-oblong leaves arranged in two ranks on the leaf-shoot. Various ginger rhizobia provide health-promoting effects and have been utilized to treat certain illnesses such as nausea, motion sickness, stomachache, asthma, diarrhea, digestive disorder, vomiting, rheumatism, swelling, common cold, cough and other disorders (Li *et al*., 2007). Several studies have revealed that the members of the Zingiberaceae family consist of a wide variety of active phytochemicals and possess antioxidative, anti-inflammatory, anticancer and anti-tumour promoting activity (Ling *et al*., 2005).

Boesenbergia, a member of Zingiberaceae, are composed of approximately 80 species worldwide and 19 of which are indigenous to Thailand (Saensouk and Larsen, 2001). Only *B. rotunda* is cultivated commercially and its rhizomes have been used for medicinal (i.e. treatment of colic disorder and as an aphrodisiac in folk medicine; (Trakoontivakorn *et al*., 2001) and culinary purposes. The rhizomes of this species contain active constituents against HIV-1 protease (Tewtrakul *et al*., 2003) and those exhibiting anti-tumor, anti-mutagenic and anti-inflammatory activities (Murakami *et al*., 1994; Trakoontivakorn *et al*., 2001; Tuchinda *et al*., 2002), whereas *B. pandurata* is commonly used in Southeast Asia as a food ingredient, a folk medicine for the treatment of several diseases such as aphthous ulcer, dry mouth, stomach discomfort, leukorrhea, and dysentery (Cheenpracha *et al*., 2006). Moreover, it has also been used as self-medication by AIDS patients in Thailand. As regards its biological activities, *B. pandurata* exhibits antibacterial (Ungsurungsie *et al*., 1982), antifungal (Achararit *et al*., 1983), anti-inflammatory, analgesic (Panthong *et al*., 1989) and insecticidal activities (Areekul *et al*.,

1987). Due to these properties, *Boesenbergia* species has gained attention as important sources of active constituents for medicinal treatment.

Boesenbergia thorelii, locally known in Thai as Kra-Chai-Pa belongs to the Zingiberaceae family and is widely cultivated in Thailand, Malaysia and Laos. Sometimes referred to as Thai Waan Petch Krub, Kra Chai Khao. This plant is a perennial ground herb that grows to 30 cm height with brown rhizomes. It has not been previously reported for its pharmacological activities and bioactive constituents. However, there was a report on the evaluation of genetic variation and evolutionary relationships of *Boesenbergia* in Thailand using multilocus DNA fingerprints generates by AFLR analysis (Techaprasan *et al*., 2008).

1.3 Background and objectives

Since the rhizomes of *B.thorelii* have not been previously reported for its pharmacological activities and bioactive constituents.

The objectives of this present investigation are therefore as the following;

1. To study on anti-allergic effect of *B. thorelii* crude extract, using an enzyme β-hexosaminidase as a biomarker for antigen-induced degranulation in RBL-2H3 cell line
2. To isolate and elucidate the structures of pure compounds from *B.thorelii* extract
3. To study on anti-allergic effect of pure compounds from *B. thorelii* extract
4. To study acute toxicity of ethanol extract from *B. thorelii*

CHAPTER 2

EXPERIMENTAL

2.1 General

All solvents for general purposes and chromatography were analytical grade. Thin layer chromatography (TLC) was performed on Merck® pre-coated silica gel 60 F_{254} plates (0.20 mm thickness). Chromatographic separations performed over the columns of C18 (10 mm, 250 nm) (Alltech Associates, Inc). Visualization was done with observation under UV light (254 nm). The optical density was measured with a microplate reader (Bio-Tek instruments, Inc). IR spectrophotometer from JASCO IR-810, Japan Spectroscopic, Japan and UV-VIS spectrophotometer (Genesis-6, Bio-Thermo scientific, USA) were used.

2.2 Chemicals and reagents used for cell culture

Minimum Essential Medium Eagle (MEM), anti-DNP-IgE (Monoclonal anti-DNP), dinitrophenylated bovine serum albumin, Tween 80 and phosphate buffer saline (PBS) were purchased from Sigma Aldrich (Sigma Aldrich, Missouri, USA). Fetal calf serum (FCS) was purchased from Gibco (Invitrogen, California, USA). Penicillin-streptomycin was purchased from Invitrogen (Invitrogen, California, USA). 24-well and 96-well microplates were purchased from Nunc (Nunc, Birkrød, Denmark). Other chemicals were obtained from Sigma Aldrich (Sigma Aldrich, Missouri, USA).

2.3 Plant material

The rhizomes of some Zingiberaceous plants including *Boesenbergia thorelii* (Gagnep.) Loes. (Kra Chai Pa), *Boesenbergia longiflora* (Wall.) Kuntze. (Wan Priao), *Boesenbergia* sp.1 (Kai Dang), *Boesenbergia* sp.2 (Kai Dam), *Kaempferia angustifolia* Roscoe. (Thao Nhang Haeng), *Kaempferia marginata* Carey. (Kra Jae Jhun), *Kaempferia rotunda* L. (Thip-pa-ya-Nate) and *Kaempferia* sp. (Prauh Pa) were all bought from the JATUJAK market in Bangkok, Thailand. The voucher specimens are Songklanakarind Pharmacy (SKP) 206022001, SKP 2060200-101, SKP 2060200-201, SKP 2060200-301, SKP 206110101, SKP 206111301, SKP 206111801 and SKP 2061100-101, respectively. The plant materials were identified by Dr. Jarun Maknoi and kept in the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand (Figure 7).

Boesenbergia thorelii (Gagnep.) Loes.

Figure 7 The pictures of some Zingiberaceous plants

Boesenbergia longiflora (Wall.) Kuntze.

Kaempferia marginata Carey.

Figure 7 Continued


Kaempferia rotunda L.


Kaempferia sp. (Prauh Pa)

Figure 7 Continued

Kaempferia angustifolia Roscoe.

Boesenbergia sp.1 (Kai Dang)

Boesenbergia sp.2 (Kai Dam)

Figure 7 Continued

2.4 Screening for anti-allergic activity of some selected plants in the genus *Boesenbergia* and *Kaempferia*

Ten grams of each dried plant were powdered and extracted successively by reflux for 3 h with 200 ml of ethanol (EtOH) and water, separately. The solvents were removed under reduced pressure to give ethanolic and water extracts, respectively. Stock solution (10 mg /mL) of the extracts were prepared in dimethylsulfoxide (DMSO) and stored at 4^{o}C until use.

2.5 Isolation and structure elucidation

Four kilograms (dried weight) of *Boesenbergia thorelii* (Gagnep.) Loes. were ground and macerated with ethanol four times (24 L×4) at room temperature. The EtOH extract (231.25 g) was then concentrated and partitioned between 90% MeOH and hexane, removed of MeOH, added of water and partitioned with chloroform. After that, the water layer was partitioned with ethyl acetate (EtOAc). Each partition was evaporated to dryness *in vacuo* to give residues of hexane (93.65 g), chloroform (107.31 g), EtOAc (1.28 g) and water fractions (15.4 g) (Figure 8), respectively.

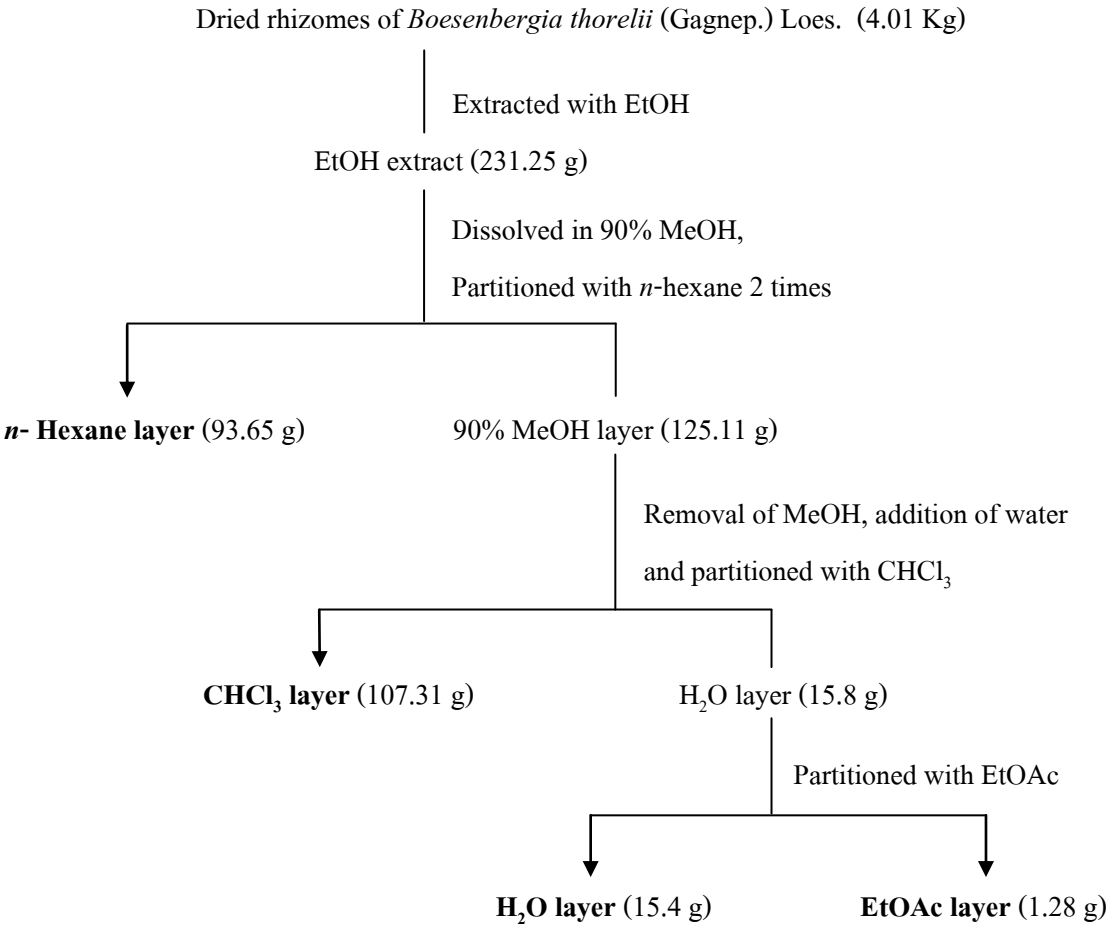


Figure 8 The procedure of *B. thorelii* rhizomes extraction

The chloroform fraction (30.0 g) which possessed marked anti-allergic activity on the release of β-hexosaminidase was chromatographed on silica gel (800 g), using hexane/EtOAc (100:0 to EtOAc 100%) and EtOAc/methanol (100:0 to methanol 20%), to afford fifteen fractions (F1-F15). Fraction F5 (2.76 g) was subjected to column chromatography on 120 g of silica gel eluted with hexane/EtOAc (70:30 to EtOAc 100%) which finally afforded asaronaldehyde (**1**) (white needles, 20 mg).

Fraction F12 (1.75 g) was subjected to column chromatography on 100 g of silica gel eluted with $CHCl_3$/EtOAc (100: 0 to EtOAc 100%), EtOAc/ methanol (100:0 to

methanol 20%) to give twelve subfractions (F1a-F12a). Subfraction F12a gave β-sitosterol-D-glucoside (**2**) (white powder, 15 mg).

Fractions (F6-F7) (1.28 g) were purified by column chromatography on 100 g of silica gel using hexane/EtOAc (50:50 to EtOAc 100%) to give eight subfractions (F1b-F8b). Further column chromatography of the subfraction F6b (35 mg) was on 50 g of silica gel using hexane/EtOAc (70: 30 to EtOAc100%) to obtain protocatechuic acid methyl ester (**3**) (pale yellow solid, 10 mg). Subfraction F7b was purified by preparative TLC with EtOAc 100% (eluted 2 times) to afford 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (**4**) (white solid, 16 mg). Moreover, these four compounds were isolated for the first time from *B. thorelii*.

2.6 Structure elucidation

Structure elucidation of compounds were interpreted using spectroscopic techniques including ultraviolet visible spectroscopy (UV-Vis spectroscopy), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS).

2.7 Animals

Male and female Swiss albino mice with the weight ranging from 30-40 g were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat-Yai, Songkhla,Thailand. They were fed with standard rodent diet and water *ad libitum*. Animal study protocol was approved by The Animal Ethic Committee, Prince of Songkla University (MOE0521.11/303).

2.8 Acute toxicity test of *Boesenbergia thorelii* extract in mice

The 50% lethal dose (LD_{50}) of the ethanol extract of *B. thorelii* rhizome was estimated by the up-and down method in mice (Bruce, 1985). The animals were fasted for 6 h prior to dosing. Doses were adjusted by a constant multiplicative factor; viz. 1.5, for this experiment. The dose for each successive animal was adjusted up or down depending on the previous outcome. The extract was homogenized in Tween-80 (1%) and dissolved in distilled water and orally administered in a single dose by gavage using a stomach tube to both groups of male and female mice. Animal behaviors were observed individually at least once during the first 30 minutes after administration, periodically during the first 8 hours and daily thereafter, for a total of 7 days. The signs of toxicity were observed including tremor, convulsion, diarrhea, hyperactivity, sedation, grooming, loss of righting reflex, increased or decreased respiration, coma and death.

2.9 Anti –allergic activity assay

2.9.1 Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells

Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells were evaluated by the following modified method (Matsuda et al, 2004). Briefly, RBL-2H3 cells were dispensed in 24-well plates at a concentration of 2×10^5 cells/well using minimum essential medium eagle (MEM) containing 10% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 U/mL), and anti-dinitrophenyl immunoglobulin E (anti-DNP IgE) (0.45 μg/mL), then incubated overnight at 37°C in 5% CO_2 for sensitization of the cells. The cells were washed twice with 500 μL of Siraganian buffer (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM piperazine-*N-N'-bis* (2-ethanesulfonic acid) (PIPES), 0.1%

bovine serum albumin (BSA) and 40 mM NaOH, pH 7.2) and then incubated in 160 μL of Siraganian buffer for an additional 10 min at 37 ^{o}C. After that, 20 μL of test sample solution was added to each well and incubated for 10 min, followed by addition of 20 μL of antigen (DNP-BSA, final concentration is 10 μg/mL) at 37^{o} C for 20 min to stimulate the cells to degranulate. The supernatant was transferred into a into 96-well plate and incubated with 50 μL of substrate (1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) (PNAG, scheme 1) in 0.1 M citrate buffer (pH 4.5) at 37 ^{o}C for 1 h. The reaction was stopped by adding 200 μL of stop solution (0.1M Na_2CO_3 / $NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm (Figure 9). The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to Siraganian buffer (final DMSO concentration was 0.1%). The inhibition (%) of the release of β-hexosaminidase by the test samples was calculated by the following equation, and IC_{50} values were determined graphically:

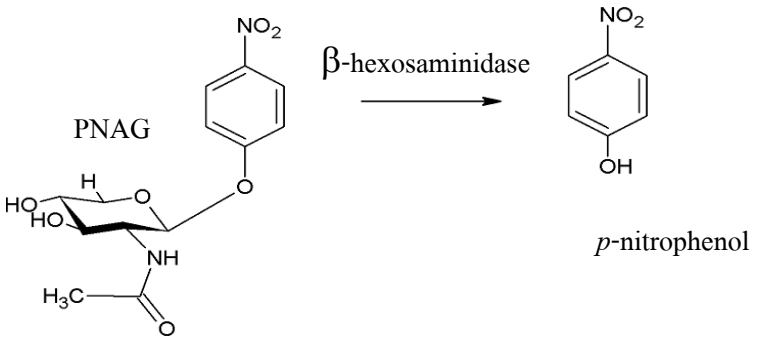
$$\% \text{ Inhibition} = \left[1 - \frac{T - B - N}{C - N}\right] \times 100$$

where; Normal (*N*) = DNP-BSA (-), test sample (-)

Control (*C*) = DNP-BSA (+), test sample (-)

Test (*T*) = DNP-BSA (+), test sample (+)

Blank (*B*) = DNP-BSA (-), test sample (+)



Scheme 1 Hydrolysis reaction of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (PNAG)

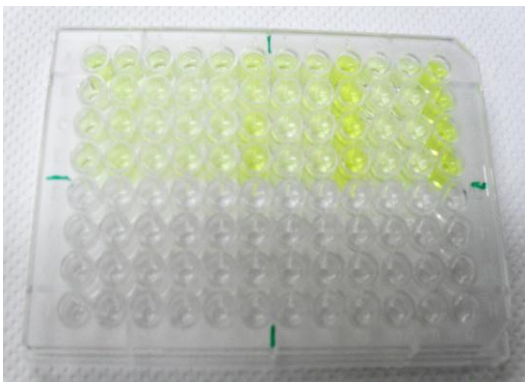


Figure 9 The anti-allergic activity test

2.10 Statistical analysis

The results were expressed as mean ± S.E.M. of four determinations at each concentration for each sample. The IC_{50} values were calculated using the Microsoft Excel program. Statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Effect of some selected plants in the genus *Boesenbergia* and *Kaempferia* on the release of β-hexosaminidase from RBL-2H3 cells

The EtOH and water extracts from the rhizomes of eight selected Zingiberaceous plants, including *Boesenbergia thorelii* (Gagnep.) Loes., *Boesenbergia longiflora* (Wall.) Kuntze., *Boesenbergia* sp.1, *Boesenbergia* sp.2, *Kaempferia angustifolia* Roscoe., *Kaempferia marginata* Carey., *Kaempferia rotunda* L. and *Kaempferia* sp. were tested for their anti-allergic activities using the rat basophilic leukemia strain 2H3 (RBL-2H3) cell line. The EtOH extract of *Boesenbergia thorelii* (Gagnep.) Loes., locally known in Thai as Kra Chai Pa, was the most active inhibitor of the allergic reaction with an IC_{50} value of 23.0 μg/mL, followed by *Boesenbergia thorelii* (Gagnep.) Loes. (water, IC_{50} = 26.3 μg/mL), *Boesenbergia* sp.1 (EtOH, IC_{50} = 38.3 μg/mL), *Kaempferia marginata* Carey. (water, IC_{50} = 38.4 μg/mL) *Boesenbergia* sp.2 (EtOH, IC_{50} = 49.4 μg/mL) and *Kaempferia angustifolia* Roscoe. (water, IC_{50} = 55.6 μg/mL), whereas other *Boesenbergia* and *Kaempferia* spp. had much lower activities (IC_{50} = 70.1- > 100 μg/mL). The result showed that the IC_{50} values of EtOH and water extracts of *Boesenbergia thorelii* (Gagnep.) Loes. (IC_{50} = 23.0 and 26.3 μg/mL, respectively) were comparable to that of the positive control, ketotifen fumarate (IC_{50} = 20.2 μg/mL) as shown in Table 3 and Figure 10.

Table 3 Anti-allergic activity [a] of some selected plants in the Zingiberaceae family

Plants	% Inhibition at various concentrations (μg/mL)				IC_{50} (μg/mL)
	0	10	30	100	
Boesenbergia thorelii (Gagnep.) Loes. (Water)	0.0±2.1	17.0±8.9	54.0±6.6**	96.1±3.0**	26.3
B. thorelii (EtOH)	0.0±9.8	23.5±3.5	64.4±1.2**	86.6±1.0**	23.0
Boesenbergia longiflora (Wall.) Kuntze. (Water)	0.0±3.9	-	-	48.9±0.8**	>100
B. longiflora (EtOH)	0.0±3.9	-	-	-21.6±2.2	>100
Boesenbergia sp.1 (Water)	0.0±2.1	-	-	47.2±2.1**	>100
Boesenbergia sp.1 (EtOH)	0.0±5.6	35.1±4.5**	47.5±7.8**	79.4±2.5**	38.3
Boesenbergia sp.2 (Water)	0.0±3.9	-	-	23.1±0.7	>100
Boesenbergia sp.2 (EtOH)	0.0±8.7	24.9±8.6*	32.7±9.7*	86.6±1.1**	49.4
Kaempferia marginata Carey. (Water)	0.0±2.1	27.4±6.7*	36.7±5.5**	109.4±7.0**	38.4
K. marginata (EtOH)	0.0±3.9	-	-	-51.3±0.9**	>100

Table 3 (Continued)

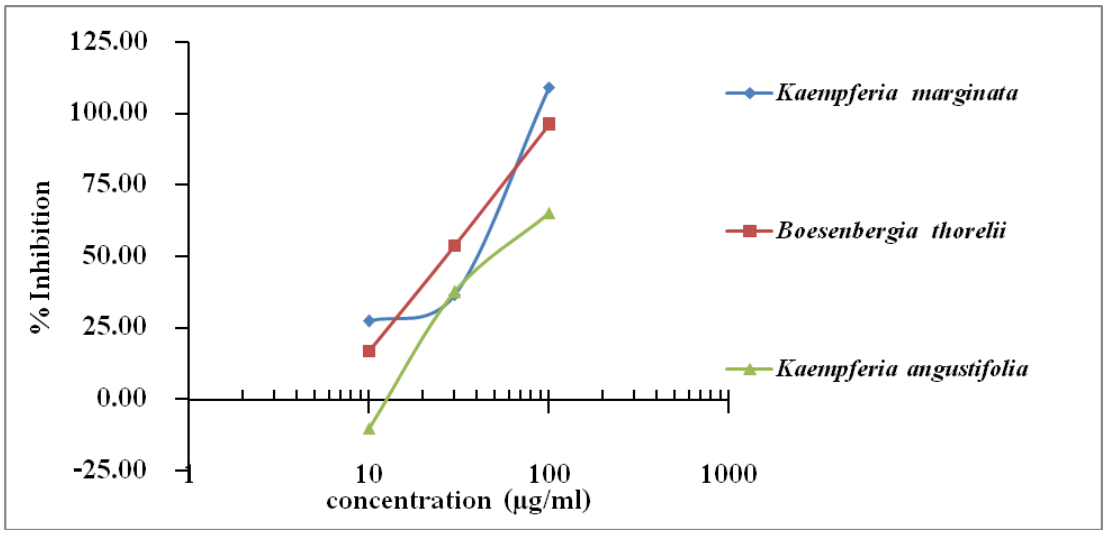
Plants	% Inhibition at various concentrations (μg/mL)				IC_{50} (μg/mL)
	0	10	30	100	
Kaempferia sp. (Water)	0.0±3.9	-	-	48.7±5.1**	>100
Kaempferia sp. (EtOH)	0.0±3.9	-	-	-36.9±4.7**	>100
Kaempferia angustifolia Roscoe. (Water)	0.0±3.9	-10.1±2.9	37.7±4.8**	65.4±7.4**	55.6
K. angustifolia (EtOH)	0.0±4.3	-	-	10.1±4.2	>100
Kaempferia rotunda L. (Water)	0.0±8.7	-	-	39.8±5.0**	>100
K. rotunda L. (EtOH)	0.0±12.5	4.5±12.9	18.5±5.9*	73.1±0.2**	70.1
Ketotifen fumarate (positive control)	0.0±6.9	12.8±0.5	38.3±3.2*	68.2±1.5**	47.5 μM (20.2)[b]

"-" means "not done"; Statistical significance, *$p < 0.05$; **$p < 0.01$

[a] Each value represents mean ± S.E.M. of four determinications.

[b] Value in parenthesis is an IC_{50} in μg/mL.

A



B

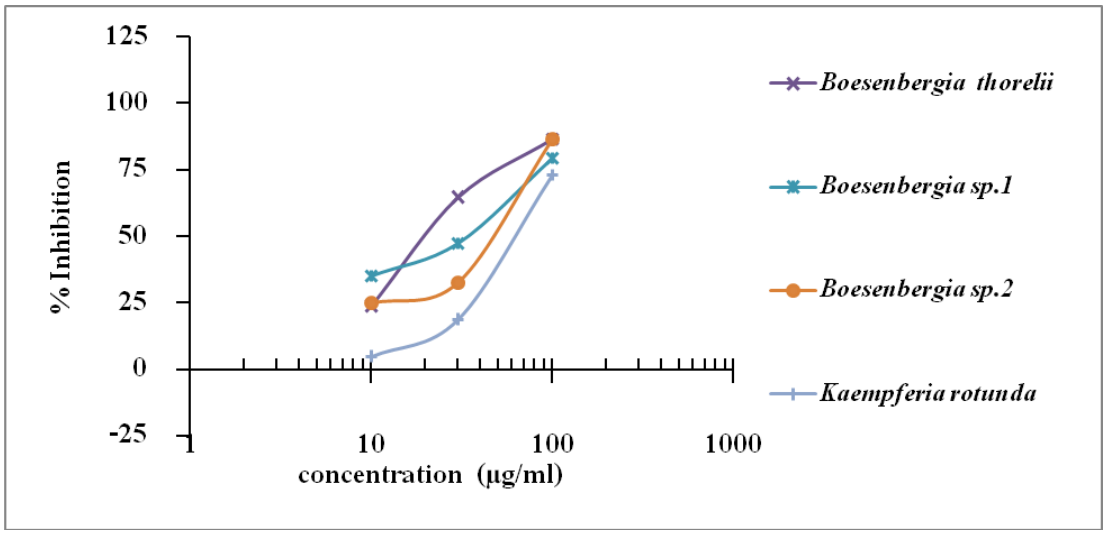


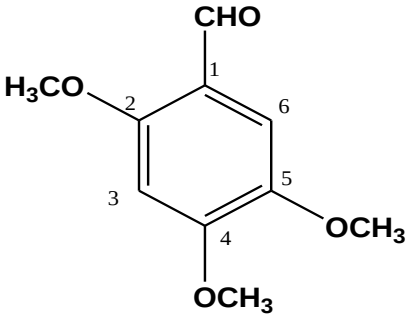
Figure 10 Dose-response curves of water (**A**) and EtOH (B) extracts of some selected plants in the Zingiberaceae family against allergic reaction

3.2 Isolation and structure elucidation

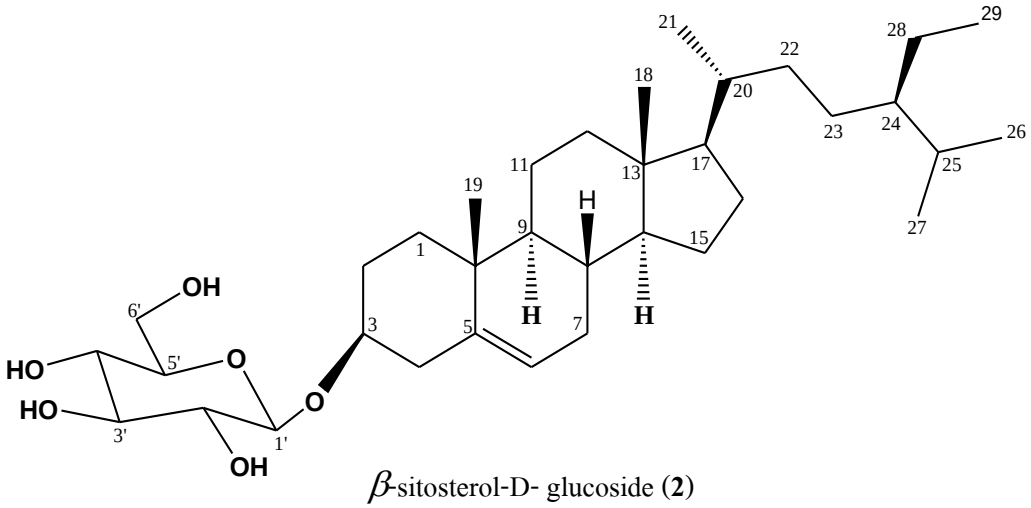
3.2.1 Isolation

The fresh rhizomes *Boesenbergia thorelii* (Gagnep.) Loes. were bought from a JATUJAK market in Bangkok, Thailand. Four kilograms (dried weight) of *B. thorelii* were ground and macerated with ethanol four times (24 L×4) at room temperature. The EtOH extract (231.25 g) was then concentrated and partitioned between 90% MeOH and hexane, removed of MeOH, added of water and partitioned with chloroform. After that, the water layer was partitioned with ethyl acetate (EtOAc). Each partition was evaporated to dryness *in vacuo* to give residues of hexane (93.65 g), chloroform (107.31 g), EtOAc (1.28 g) and water fractions (15.4 g), respectively.

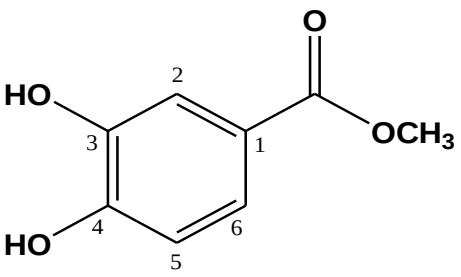
The present study on the chloroform fraction of *Boesenbergia thorelii* (Gagnep.) Loes. (IC_{50}= 22.9 μg/ml) resulted in the isolation and characterization of compounds 1-4 (Figure 11). These four compounds were isolated for the first time from this plant which were identified as asaronaldehyde (**1**), β-sitosterol-D-glucoside (**2**), protocatechuic acid methyl ester (**3**) and 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (**4**). The compounds were identified on the basis of spectroscopic methods and comparison with the data reported in the literature.



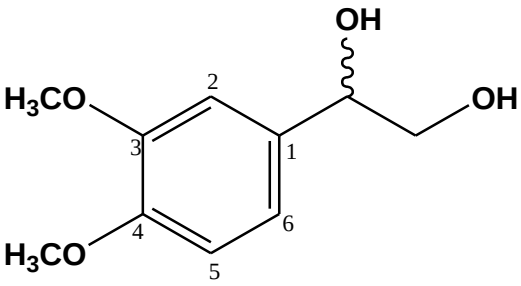
asaronaldehyde (**1**)



β-sitosterol-D- glucoside (**2**)



protocatechuic acid methyl ester (**3**)

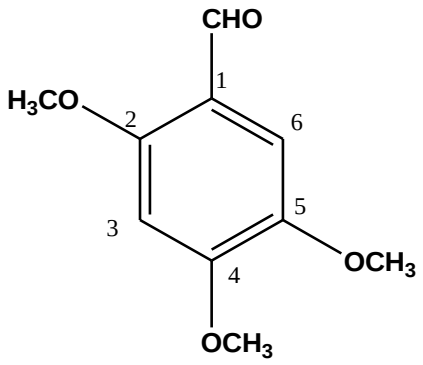


2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (**4**)

Figure 11 The chemical structures of compounds isolated from the *B. thorelii* rhizomes

3.2.2 Structure elucidation

3.2.2.1 Asaronaldehyde (2, 4, 5-trimethoxybenzaldehyde, compound 1)

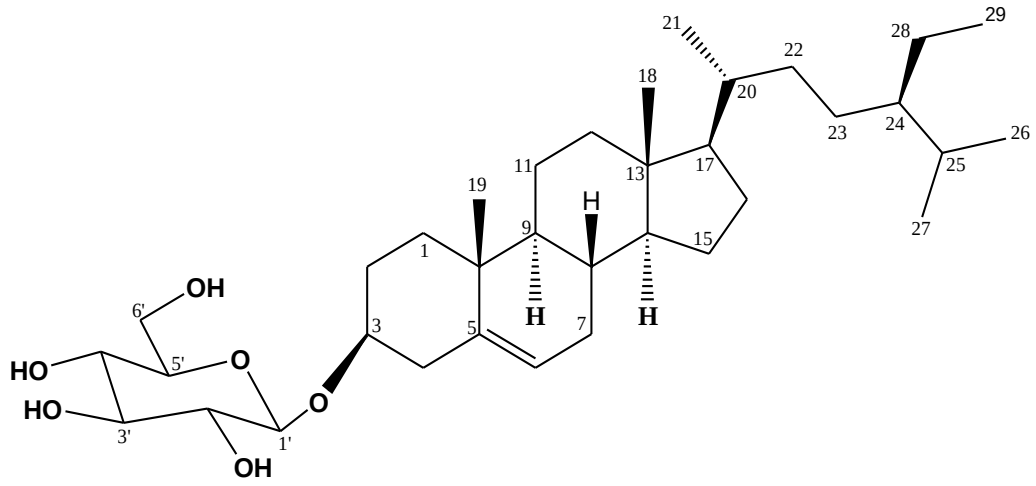


The compound **1** was obtained as a feathery white needles (20 mg): mp 112-114^{o}C from chloroform fraction by column chromatography hexane/EtOAc (70:30 to EtOAc 100%) and analyzed as $C_{10}H_{12}O_4$, MW 196. The UV spectrum showed absorption bands at 241, 275 and 340 nm. The IR spectrum showed absorption band for carbonyl group (1662 cm^{-1}). It displayed 500 MHz ppm signals ($CDCl_3$). In the ^{1}H NMR spectrum of compound **1** (Table 4) showed one aldehyde group signal at δ 10.30 (1H, *s*, CHO) and three methoxyl protons signal at δ 3.95 (3H, *s*, 2-OCH_3), 3.90 (3H, *s*, 4-OCH_3), 3.85 (3H, *s*, 5-OCH_3) and two substituted benzene protons at δ 7.31(1H, *s*, H-6), 6.47 (1H, *s*, H-3). Furthermore, the ^{13}C NMR spectrum (Table 4) indicated the presence of 10 carbons including those of one aldehyde group signal at δ 188.02 (CHO), methoxyl carbons signal at δ 56.18 (2-OCH_3), 56.27 (4-OCH_3) and 56.21 (5-OCH_3), six benzene carbons at δ 117.32 (C-1), 158.62 (C-2), 95.91 (C-3), 155.76 (C-4), 143.55 (C-5) and 108.98 (C-6). These data indicated that **1** was asaronaldehyde. The data was confirmed by comparison with spectral analysis data reported in the literatures (Patra and Mitra, 1981 and Sinha *et al*., 2003).

Table 4 ^{1}H and ^{13}C NMR spectral data of asaronaldehyde (**1**) ($CDCl_3$; 500 MHz for ^{1}H) and reference

Positions	^{1}H (mult.; *J* in Hz), compound **1**	^{13}C (mult.), compound **1**	^{1}H (mult.; *J* in Hz, Sinha *et al*., 2003), reference
1	-	117.32 (C-1)	-
2	-	158.62 (C-2)	-
3	6.47 (1H, *s*, H-3)	95.91 (C-3)	6.50 (1H, *s*, H-3)
4	-	155.76 (C-4)	-
5	-	143.55 (C-5)	-
6	7.31 (1H, *s*, H-6)	108.98 (C-6)	7.33 (1H, *s*, H-6)
7	10.30 (1H, *s*, CHO)	188.02 (CHO)	10.32 (1H, *s*, CHO)
8	3.95 (3H, *s*, 2-OCH_3)	56.18 (2-OCH_3)	3.98 (3H, *s*, 2-OCH_3
9	3.90 (3H, *s*, 4-OCH_3)	56.27 (4-OCH_3)	3.93 (3H, *s*, 4-OCH_3)
10	3.85 (3H, *s*, 5-OCH_3)	56.21 (5-OCH_3)	3.88 (3H, *s*, 5-OCH_3)

3.2.2.2 β- sitosterol-D-glucoside (compound 2)



The compound **2** was subjected to column chromatography and eluted with $CHCl_3$ / EtOAc (100: 0 to EtOAc 100%), 10% methanol in ethyl acetate and gave white powder (15 mg): mp 275-277°C. It has a molecular formula of $C_{35}H_{60}O_6$, MW 576.85. Strong absorption due to many hydroxyl groups (3400 cm^{-1}) in the IR spectrum. It displayed 500 MHz ppm signals (DMSO-d_6). The ^{1}H-NMR was shown as one olefinic proton signal at δ 5.32 (H-6), two angular methyl groups at δ 0.64 (*s*, H-18), δ 0.94 (*s*, H-19), an isopropyl (δ 0.89 (H-26), 0.92 (H-27), 1.62 (H-25) and ethyl (δ 0.85 (H-29), 1.26 (H-28) groups, one oxymethine proton at δ 3.63 (*m*, H-3) and the signal of strong absorption due to many hydroxyl groups at δ 3.63 (H-5'), 4.02 (H-2'), 4.22 (H-3', 4'), 4.40 (H-6'a), 4.82 (H-6'b), 5.06 (H-1') (Table 5). Spectra suggested that the compound was a steroidal glycoside. These data indicated that **2** was β-sitosterol-D-glucoside. The compound was identified as β-sitosterol-D-glucoside by comparing the ^{1}H NMR data with reported value (Kadowaki *et al*., 2003).

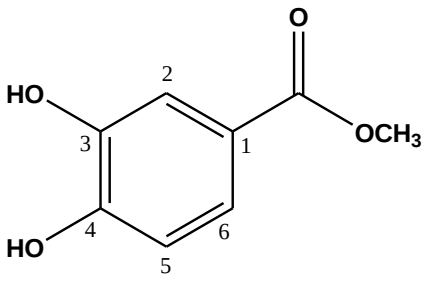
Table 5 ^{1}H NMR spectral data of β-sitosterol-D-glucoside (**2**) (DMSO-d_6; 500 MHz for ^{1}H) and reference

Positions	^{1}H (mult.; *J* in Hz), compound **2**	^{1}H (mult.; *J* in Hz, Kadowaki *et al.*, 2003), reference
1	1.79 (5H, *m*, H-1)	1.71 (5H, *m*, H-1)
2	2.11 (1H, *m*, H-2)	2.13 (1H, m, H-2)
3	3.63 (2H, *m*, H-3, 5')	3.97 (2H, *m*, H-3, 5')
4	2.37 (1H, *m*, *J* = 11.0 Hz, H-4)	2.47 (1H, *m*, *J* = 12.0 Hz, H-4)
5	-	-
6	5.32 (1H, *t*, *J* = 3.0 Hz, H-6)	5.35 (1H, *t*, *J* = 3.0 Hz, H-6)
7	1.93 (2H, *m*, H-7)	1.87 (2H, *m*, H-7)
8	1.50 (1H, *m*, H-8)	1.50 (1H, *m*, H-8)
9, 14	0.93 (2H, *m*, H-9, 14)	0.92 (2H, *m*, H-9, 14)
10	-	-
11, 20, 22	1.31(5H, *m*, H-11, 20, 22)	1.38(5H, *m*, H-11, 20, 22)
12, 17	1.11(2H, *m*, H-12, 17)	1.10 (2H, *m*, H-12, 17)
13	-	-
15	1.56 (2H, *m*, H-15)	1.54 (2H, *m*, H-15)
16, 28	1.26 (4H, *m*, H-16, 28)	1.26 (4H, *m*, H-16, 28)
18	0.64 (3H, *s*, H-18)	0.66 (3H, *s*, H-18)
19	0.94 (3H, *s*, H-19)	0.93 (3H, *s*, H-19)
21, 24	1.0 (5H, *m*, *J*=7.0 Hz, H-21)	0.98 (5H, *m*, H-21)
23	1.22 (2H, *m*, H-23)	1.23 (2H, *m*, H-23)
25	1.62 (1H, *m*, H-25)	1.68 (1H, *m*, H-25)
26	0.89 (3H, *d*, *J*=6.5 Hz, H-26)	0.86 (3H, d, *J* = 6.0 Hz, H-26)

Table 5 (Continued)

Positions	^{1}H (mult.; *J* in Hz), compound **2**	^{1}H (mult.; *J* in Hz, Kadowaki *et al*., 2003), reference
27	0.92 (3H, *d*, *J*=6.5 Hz, H-27)	0.87 (3H, *d*, *J* = 6.0 Hz, H-27)
29	0.85 (3H, *t*, *J*=7.5 Hz, H-29)	0.89 (3H, *t*, H-29)
1'	5.06 (1H, *d*, *J* = 7.6 Hz, H-1')	5.06 (1H, *d*, *J* = 7.6 Hz, H-1')
2'	4.02 (1H, *dd*, *J* = 7.0 Hz, H-2')	4.07 (1H, *dd*, *J* = 7.6 Hz, H-2')
3', 4'	4.22 (1H, *m*, *J* = 8.0 Hz, H-3', 4')	4.30 (2H, *m*, H-3', 4')
5'	-	-
6'a	4.40 (1H, *dd*, *J* = 11.5, 5.5 Hz, H-6'a)	4.43 (1H, *dd*, *J* = 11.6, 5.1 Hz, H-6'a)
6'b	4.82 (1H, *dd*, *J* = 14.0, 5.0 Hz, H-6'b)	4.58 (1H, *dd*, *J* = 11.0, 6.0 Hz, H-6'b)

3.2.2.3 Protocatechuic acid methyl ester (Methyl 3, 4- dihydroxybenzoate, compound 3)



The compound **3** was isolated as a pale yellow solid (10 mg): mp 106-107^{o}C from chloroform fraction using column chromatography hexane/EtOAc (70: 30 to EtOAc100%). The molecular formula of **3** was proposed to be $C_8H_8O_4$ as observable in the EI mass spectrum, which showed a molecular peak at *m/z* 168.9. The UV spectrum showed maximum absorption bands at 215, 259 and 290 nm which revealed the presence of conjugated system. The IR spectrum showed absorption bands at 3447 cm^{-1} and 1629 cm^{-1} for a hydroxyl group and a carbonyl group, respectively. It displayed 500 MHz ppm signals ($CDCl_3$). In the ^{1}H NMR spectrum of compound **3** (Table 6) showed two hydroxyl groups at δ 6.04 (2H, *brs*, OH), tri-substituted benzene protons at δ 7.56 (1H, *d*, $J_{2,6}$ = 2.0 Hz, H-2), 6.94 (1H, *d*, $J_{5,6}$ = 8.5 Hz, H-5) and 7.67 (1H, *dd*, $J_{6,5}$ = 8.5 Hz, $J_{6,2}$ = 2.0 Hz, H-6). The presence of a methyl carbonate group was derived from the resonance of - OCH_3 signal at δ 3.94.

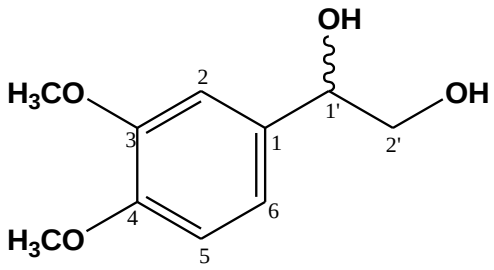
Compound **3** then was assigned to be tri-substituted benzene derivative with two hydroxyl groups and a methyl carbonate group. These data indicated that **3** was protocatechuic acid methyl ester according to the basis of its spectroscopic data and comparison with the previously reported data (Chin *et al.*, 2008a).

Table 6 ^{1}H NMR spectral data of protocatechuic acid methyl ester (**3**) ($CDCl_3$; 500 MHz for ^{1}H) and reference

Positions	^{1}H (mult.; *J* in Hz), compound **3**	^{1}H (mult.; *J* in Hz, Chin *et al*., 2008a)* , reference
1	-	-
2	7.56 (1H, *d*, $J_{2,6}$ = 2.0 Hz, H-2)	7.39 (1H, *d*, $J_{2,6}$ = 2.0 Hz, H-2)
3, 4	6.04 (1H, *br*, OH)	-
5	6.94 (1H, *d*, $J_{5,6}$ = 8.5 Hz, H-5)	6.80 (1H, *d*, $J_{5,6}$ = 8.3 Hz, H-5)
6	7.67 (1H, *dd*, $J_{6,5}$ = 8.5 Hz, $J_{6,2}$ = 2.0 Hz, H-6)	7.34 (1H, *dd*, $J_{6,5}$ = 8.3 Hz, $J_{6,2}$ = 2.0 Hz, H-6)
7	-	-
8	3.94 (3H, *S*, OCH_3)	3.80 (3H, *S*, OCH_3)

* ^{1}H NMR in reference was run in Acetone-d_6

3.2.2.4 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (compound 4)



The compound **4** was purified by preparative TLC with EtOAc 100% (eluted 2 times) and gave white solid (16 mg): mp 89-90°C. The molecular formula was determined to be $C_{10}H_{14}O_4$ which supported by the existence of fragment ion peaks at *m/z* 198 in the EI mass spectrum. The UV spectrum gave absorption maximum at λ 203, 242 and 278 nm. The IR spectrum of compound **4** suggested the presence of hydroxyl group 3401cm^{-1} and phenyl ring (s) (1593, 1516, 877 cm^{-1}). It displayed 500 MHz ppm signals ($CDCl_3$). In the ^{1}H NMR spectrum of compound **4** (Table 7) showed the aromatic proton signals at δ 6.83 (1H, *d*, $J_{5,6}$ = 8.0 Hz), 6.88 (1H, *d*, $J_{2,6}$ = 2.0 Hz) and 6.91 (1H, *dd*, $J_{6,5}$ = 8.0 Hz, $J_{6,2}$ = 2.0 Hz). The ^{1}H-NMR spectrum also showed a carbinol proton signal at δ 4.77 (1H, *dd*, *J* = 8.0, 3.5 Hz) and carbinol proton signals at δ 3.73 (1H, *dd*, *J* = 11.0, 3.5 Hz) and 3.65 (1H, *dd*, *J* = 11.0, 8.0 Hz) and two methoxyl protons signal at δ 3.88 (3H, *s*, 3-OCH_3) and 3.86 (3H, *s*, 4-OCH_3). Thus, the structure of **4** was determined to be 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols. The data was confirmed by comparison with spectral analysis data reported in the literature (Takenaka *et al*., 2000).

Table 7 ^{1}H NMR spectral data of 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (**4**) ($CDCl_3$; 500 MHz for ^{1}H) and reference

Positions	^{1}H (mult.; *J* in Hz), compound **4**	^{1}H (mult.; *J* in Hz, Takenaka *et al*., 2000), reference
1	-	-
2	6.88 (1H, *d*, *J* = 2.0 Hz)	6.93 (1H, *d*, *J* = 2.0 Hz)
3	-	-
4	-	-
5	6.83 (1H, *d*, *J* = 8.0 Hz)	6.85 (1H, *d*, *J* = 8.0 Hz)
6	6.91(1H, *dd*, *J* = 8.0, 2.0 Hz)	6.91 (1H, *dd*, *J* = 8.0, 2.0 Hz)
7	3.88 (3H, *s*, 3-OCH_3)	3.88 (3H, *s*, 3-OCH_3)
8	3.86 (3H, *s*, 4-OCH_3)	3.90 (3H, *s*, 4-OCH_3)
1′	4.77 (1H, *dd*, *J* = 8.0, 3.5 Hz)	4.78 (1H, *dd*, *J* = 8.0, 3.5 Hz)
2′	3.73 (1H, *dd*, *J* = 11.0, 3.5 Hz,	3.75 (1H, *dd*, *J* = 11.0, 3.5 Hz,
	3.65 (1H, *dd*, *J* = 11.0, 8.0 Hz)	3.67 (1H, *dd*, *J* = 11.0, 8.0 Hz)

3.3 Acute toxicity effect of *B. thorelii* extract in mice

In the acute toxicity test, the LD_{50} values for oral administration of the ethanol extract of *B. thorelii* in male and female mice were 471 mg/kg and 636 mg/kg, respectively. The signs of toxicity included loss of righting reflex, sleep and death were observed.

3.4 Anti –allergic activity assay

3.4.1 Effect of fractions from *B. thorelii* rhizomes and isolated compounds on the release of β-hexosaminidase from RBL-2H3 cells

This examination bases on the colorimetric assay of the mediators released from the degranulated mast cells and basophils after binding with antigen-specific IgE antibodies to receptors on mast cells or basophils. The colorimetric assay is to determine *p*-nitrophenol derived from *p*-nitrophenyl-N-acetyl-β-D-glucosaminide catalyzed by β-hexosaminidase enzyme (Ozaka *et al*., 1993; Lee *et al*., 2004). The results are expressed as the percentage of intracellular β-hexosaminidase release into the medium.

EtOH extract, hexane-, chloroform-, ethyl acetate- and water fractions from *Boesenbergia thorelii* (Gagnep.) Loes. rhizomes were then carried out to investigate for their anti-allergic activities. As shown in Table 8, the chloroform fraction of *B.thorelii* extract exhibited the most potent anti-allergic effect against antigen-induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells with an IC_{50} value of 22.9 μg/mL and higher effect than that of the positive control, ketotifen fumarate (IC_{50}= 27.4 μg/mL), followed by the water fraction, EtOH extract and hexane fraction with IC_{50} of 49.6, 54.6 and 59.1 μg/mL, respectively whereas the ethyl acetate fraction was apparently inactive (IC_{50}>100 μg/mL).

The chloroform fraction of *B. thorelii* extract was subjected to chromatographic methods led to the isolation of four compounds: asaronaldehyde (**1**), β-sitosterol-D-glucoside (**2**), protocatechuic acid methyl ester (**3**) and 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (**4**) which were also tested for their anti-allergic activities using RBL-2H3 cells. The result indicated that asaronaldehyde (**1**) was the most active inhibitor of the allergic reaction with an IC_{50} value of 24.3 μM, followed by **2** (IC_{50} = 63.3 μM) and **4** (IC_{50} = 72.6 μM), respectively (Table 9). Whereas compound **3** was inactive (IC_{50} > 100 μM). It was shown that anti-allergic effect of **1** (24.3 μM) was two fold higher than that of ketotifen fumarate (IC_{50}= 41.1 μM), the positive control.

Moreover, from the present study, these four compounds were isolated for the first time from *B. thorelii*. It was reported that asaronaldehyde was also found in *Acorus calamus* (Patra and Mitra, 1981), *Piper sarmentosum* (Likhitwitayawuid *et al*., 1988), *Daucus carota* L. and *Carrot puree* (Czepa and Hofmann, 2003). β-sitosterol-D-glucoside from *Tribulus terrestris* (Deepak *et al*., 2002), *Hydrangea chinensis* (Khalil *et al*., 2003 and Kadowaki *et al*., 2003) and *Ficus septic*a (Lansky *et al*., 2008). Protocatechuic acid methyl ester from *Elaeagnus pungens* (Zhao *et al*., 2006), *Schisandra verruculosa* (Wilaira *et al*., 2006), *Paris verticillata* (Lee *et al*., 2008), *Polygonum orientale* (Li *et al*., 2009) and *Vitex agnus-castus* (Azizuddin and Choudhary, 2010) and 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols from *Fraxinus Americana* (Takenaka, *et al*., 2000).

Regarding biological activities of the isolated compounds. The fungicidal activity of asaronaldehyde (**1**) treated with various concentrations against six phytopathogenic fungi, were determined *in vivo*. Compound **1** showed strong and moderate fungicidal activity against *Phytopthora infestans* and *Rhizoctonia solani*, at 100% and 68% of the control values, respectively, at a concentration of 1,000 mg/L, but not against cucumber gray mold caused by *Botrytis cinerea*, barley powdery mildew caused by *Erysiphe graminis*, rice blast caused by *Pyricularia grisea* and wheat leaf rust caused by *Puccinia recondite* (Lee, 2007). β-Sitosterol-D-glucoside (**2**) has been reported for antibacterial activity (Bayor *et al*., 2009), uv-radiation protection, anti-oxidant, moisture holding (Fan, 2010), antimicrobial (Chung *et al*., 2005), antiatherogenic (Zhao *et al*., 1990) and gastroprotactive activities (Navarrete *et al*, 2002).

Protocatechuic acid methyl ester (**3**) has been reported for antioxidant activity against the DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical (Azizuddin and Choudhary, 2010). Compound **3** exhibited moderate inhibitory activity on the three tumor cell lines and on human lymphocyte proliferation. It also showed a strong scavenging activity for DPPH free radical, only slightly lower than ascorbic acid (Wilairat *et al*., 2006). This is the first report on biological study and chemical constituents of *Boesenbergia thorelii* (Gagnep.) Loes.

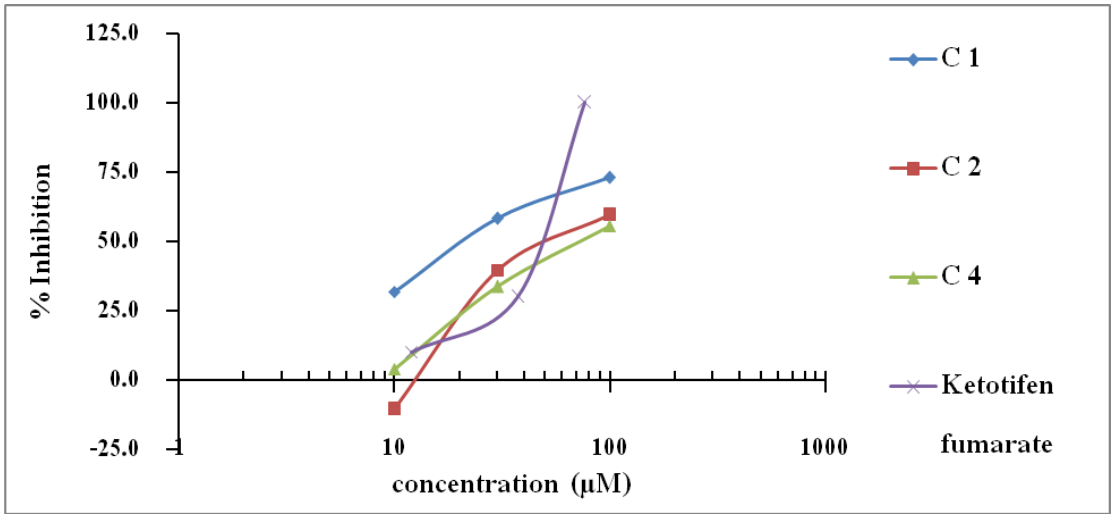


Figure 12 Dose-response curves of various compounds from the chloroform fraction of *B. thorelii* extract against allergic reaction comparing with ketotifen fumarate (positive control)

Table 8 Anti-allergic activity [a] of fractions from *B. thorelii* rhizomes

Sample	% Inhibition at various concentrations (μg/mL)					IC_{50} (μg/mL)
	0	3	10	30	100	
EtOH extract	0.0±1.8	-	12.1±5.1*	36.0±3.1**	64.0±1.0**	54.6
Hexane fraction	0.0±5.0	-	3.4±2.0	25.0±6.3**	87.4±4.5**	59.1
Chloroform fraction	0.0±3.0	-	44.5±5.9**	51.6±3.5**	95.7±3.3**	22.9
EtOAc fraction	0.0±3.4	-	11.2±0.8*	35.2±7.7**	43.4±9.6**	>100
Water fraction	0.0±3.0	-	16.2±6.2*	34.5±0.2**	67.7±10.0**	49.6
Ketotifen fumarate (positive control)	0.0±1.5	-	-12.1±3.8	28.8±1.6**	62.6±5.4**	64.4 μM (27.4 μg/mL)

"-" means "not done"; Statistical significance, *$p < 0.05$; **$p < 0.01$

[a] Each value represents mean ± S.E.M. of four determinications.

Table 9 Anti-allergic activity [a] of compounds from *B. thorelii* rhizomes

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
Asaronaldehyde (**1**)	0.0±8.1	-	31.8±4.0**	58.3±2.2**	73.0±3.2**	24.3
β-sitosterol-D-glucoside (**2**)	0.0±3.6	-	-10.7±9.0	39.6±1.7**	59.5±8.0**	63.3
Protocatechuic acid methyl ester (**3**)	0.0±1.4	-	6.5±1.9	35.0±1.2**	48.7±5.3*	>100
2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (**4**)	0.0±1.3	-	3.9±6.7	33.6±4.2**	55.5±1.0**	72.6
Ketotifen fumarate (positive control)	0.0±4.0	-	12.0±2.1*	37.3±3.5**	77.0±2.1**	41.1

"-" means "not done"; Statistical significance, *$p < 0.05$; **$p < 0.01$

[a] Each value represents mean ± S.E.M. of four determinications.

CHAPTER 4

CONCLUSION

Investigation of the chemical constituents from chloroform fraction of *Boesenbergia thorelii* rhizomes extract led to the isolation of four compounds: asaronaldehyde (**1**), β-sitosterol-D- glucoside (**2**), protocatechuic acid methyl ester (**3**) and 2-hydroxy-1-(3, 4-dimethoxyphenyl) ethanols (**4**). The structure elucidations were confirmed by spectroscopic techniques, physical properties and comparing with the reported literatures. These four compounds were isolated for the first time from this plant. Therefore, this is the first report on the anti-allergic effect using an enzyme β-hexosaminidase as a biomarker for antigen-induced degranulation in RBL-2H3 cell line and acute toxicity of ethanol extract and compounds from *B. thorelii* rhizomes.

In the acute toxicity test, the signs of toxicity included loss of righting reflex, sleep and death were observed. The LD_{50} value for oral administration of the ethanol extract of *B. thorelii* in male and female mice were 471 mg/kg and 636 mg/kg, respectively.

The crude extracts and the pure compounds **1** - **4** were examined for their anti-allergic activities against antigen-induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells. The chloroform fraction and asaronaldehyde (IC_{50} = 24.3 μM) showed higher anti-allergic activities than that of ketotifen fumarate (IC_{50} = 41.1 μM), the positive control. Whereas other compounds had much lower activities (63.3- > 100 μM).

This work has demonstrated that *B. thorelii* are among the potential sources for anti-allergic agents and has potential to be developed as a pharmaceutical preparation for treatment of allergy and allergic-ralated diseases.

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APPENDIX

APPENDIX-A

PREPARATION OF REAGENTS

1. Minimum Essential Medium Eagle (MEM) of RBL-2H3 cells

1.1 Incomplete media

Ingredients	1L	Storage
MEM	1 pack	2-8^{o}C
Sodium hydrogen carbonate	0.1 g	RT
1 N NaOH or 1N HCl	Adjusted to pH 7.0	RT
Sterile water	Adjusted to 1 L	

1.2 Complete media

Ingredients	
Incomplete media	900 mL
10% Fetal calf serum (FCS)	100 mL
1% Penicillin-streptomycin	10 mL
Sterile by filtration (0.22 µm) and stored at 2-8^{o}C	

2. Trypsin-EDTA solution

0.25 trypsin	0.5 g
0.02% EDTA-2Na	0.04 g
PBS	200 mL

3. Phosphate buffer saline (PBS)

Ingredients	
NaCl	80 g
$Na_2HPO_4.12H_2O$ (Na_2HPO_4 11.5 g, NaH_2PO_4 9.7 g, Add water 1,000 mL)	29 g
KCl	2 g
KH_2PO_4 (KH_2PO_4 2.55 g)	2 g
Ultra pure water	900 mL
Autoclave 121^{o}C, 30 min	

4. Reagent for determination β-hexosaminidase

4.1 Siraganian buffer (Buffer A)

Ingredients		**g/L**
NaCl	119 mM	6.594
KCl	5 mM	0.373
Glucose	5.6 mM	1.009
$MgCl_2.6H_2O$	0.4 mM	0.081
$CaCl_2.2H_2O$	1 mM	0.147

4.1 (Continued)

Ingredients		**g/L**
PIPES	25 mM	7.559
BSA	0.1%	1.000

4.2 0.1 M Citric buffer (pH 4.5)

Ingredients		**g/500 mL**
Citric acid monohydrate	0.1 M	10.51
Trisodium citrate dehydrate	0.1M	14.71

4.3 0.1 M Na_2CO_3 buffer solution (pH 10.0)

Ingredients		**g/500 mL**
Na_2CO_3	0.1 M	5.3 g
$NaHCO_3$	0.1M	4.2 g

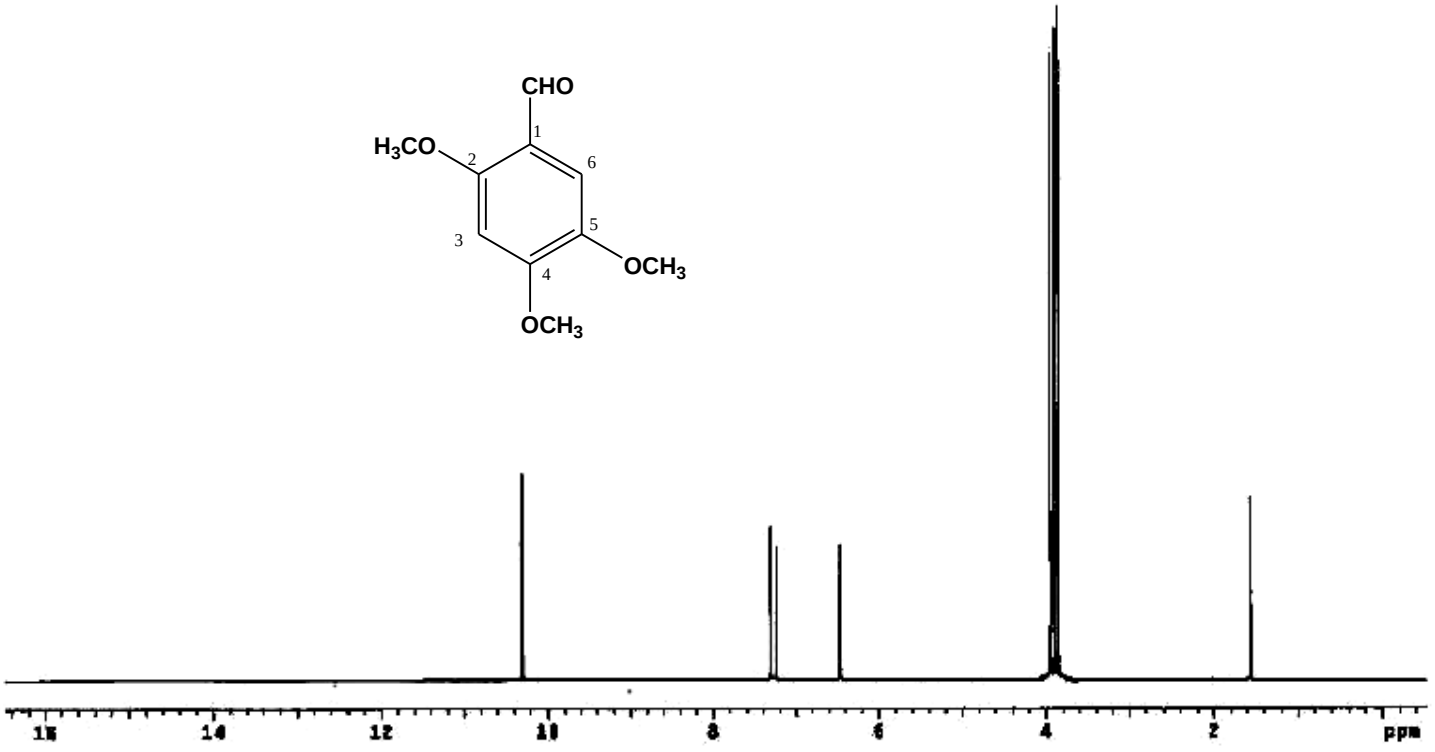
4.4 DNP-IgE solution 50 µg/mL

Ingredients	
DNP-IgE	0.5 mL
PBS	9.5 mL

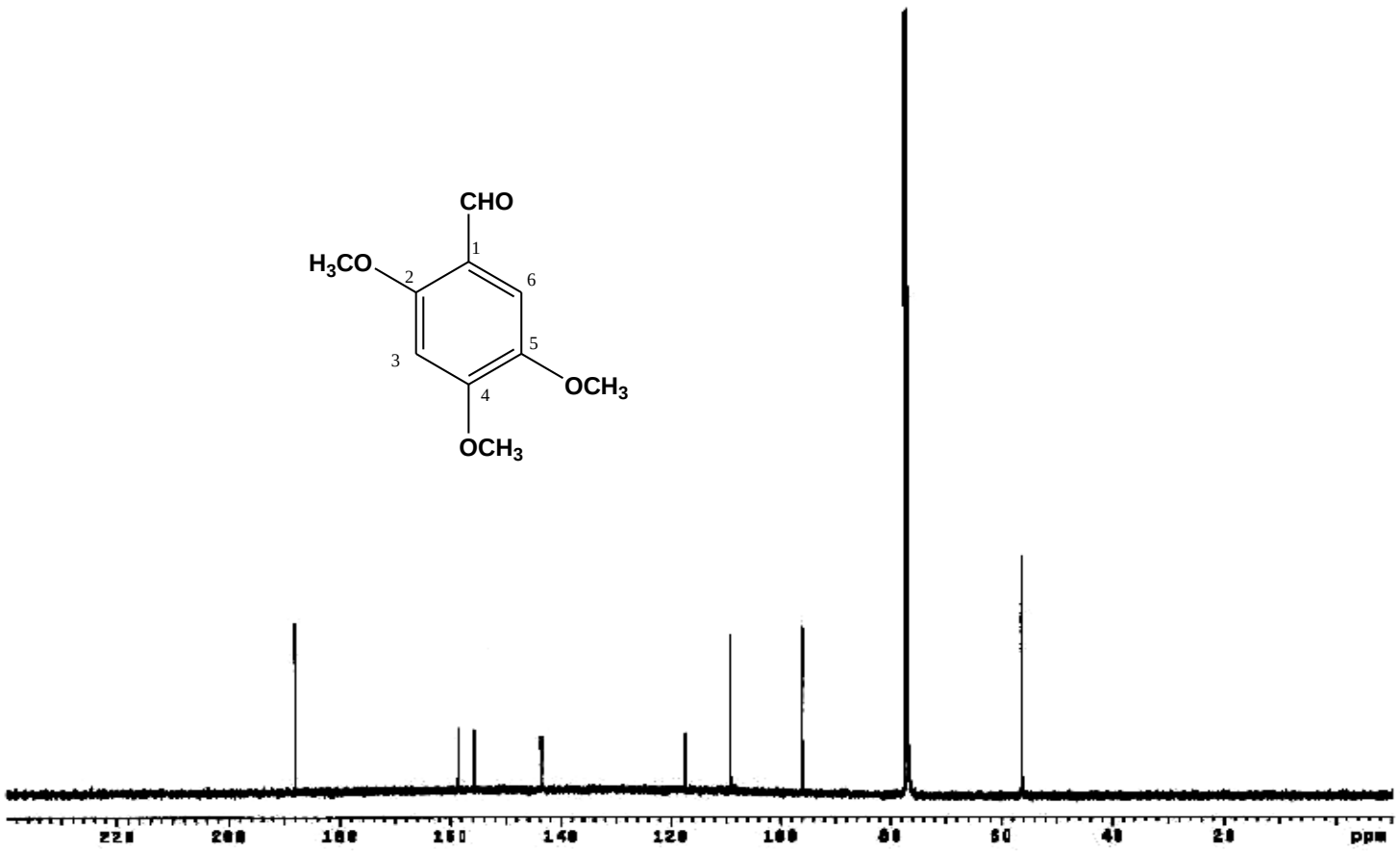
4.5 DNP-BSA solution

Ingredients	
DNP-BSA	4 g
Buffer A	40 mL

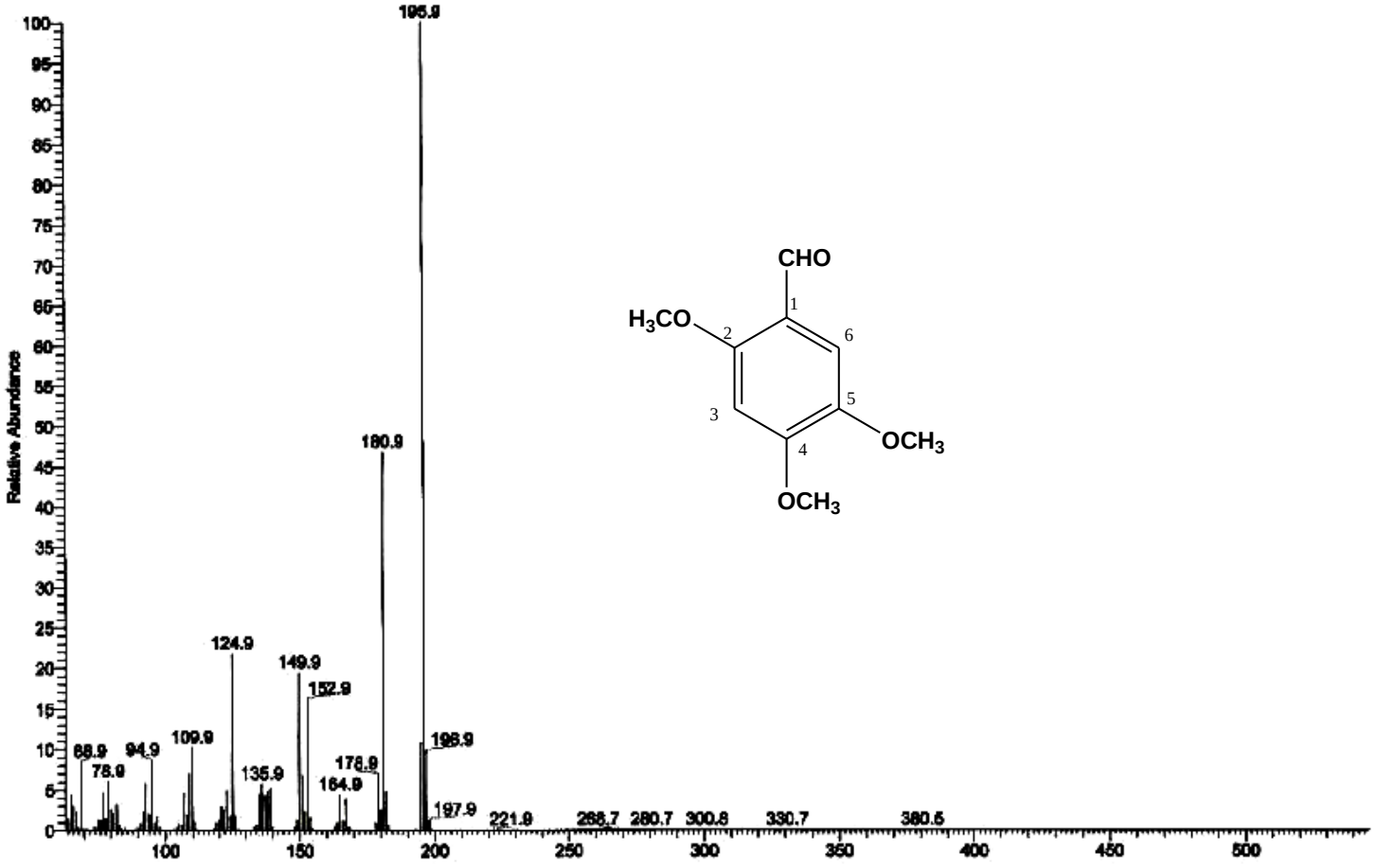
APPENDIX-B



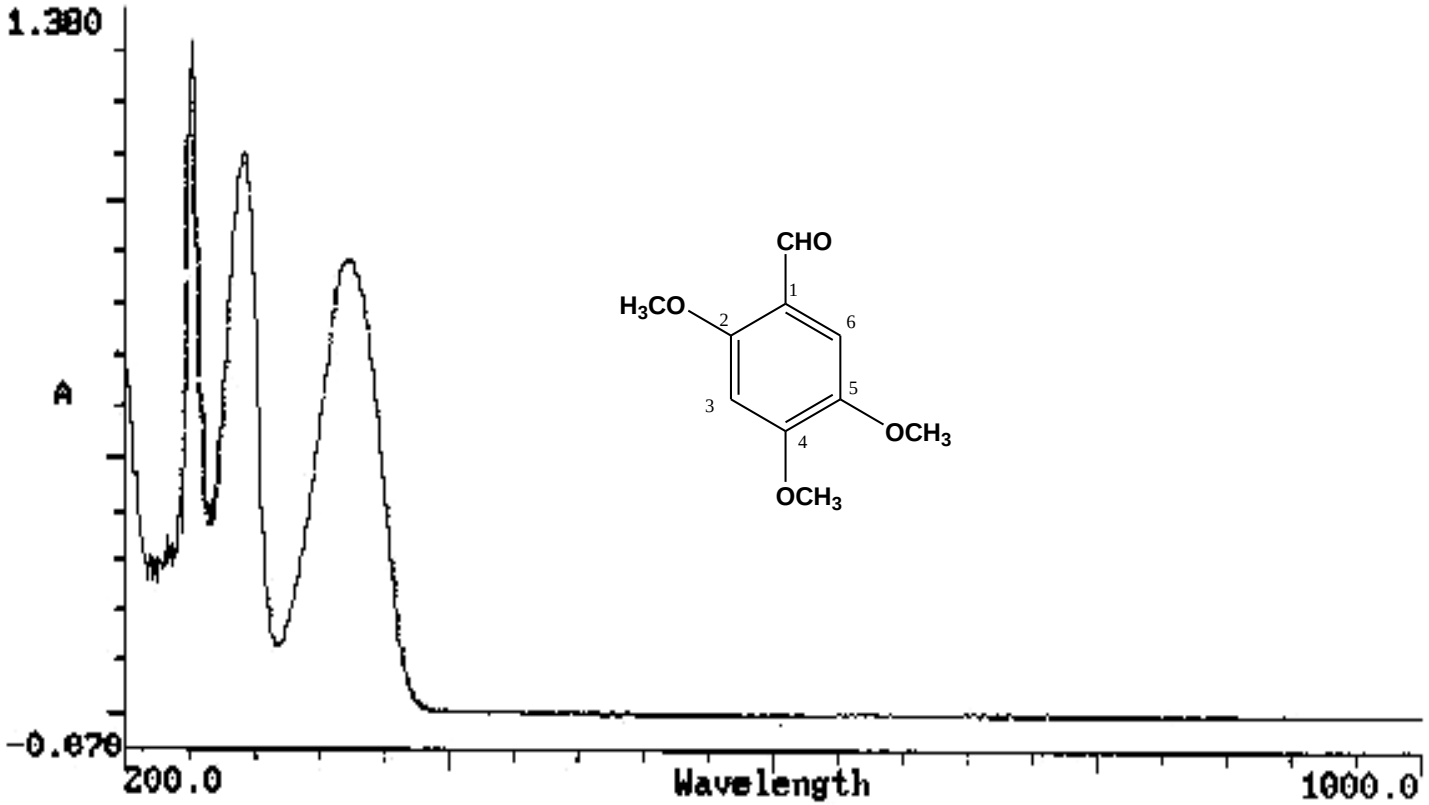
^{1}H NMR spectrum of compound **1** ($CDCl_3$; 500 MHz)



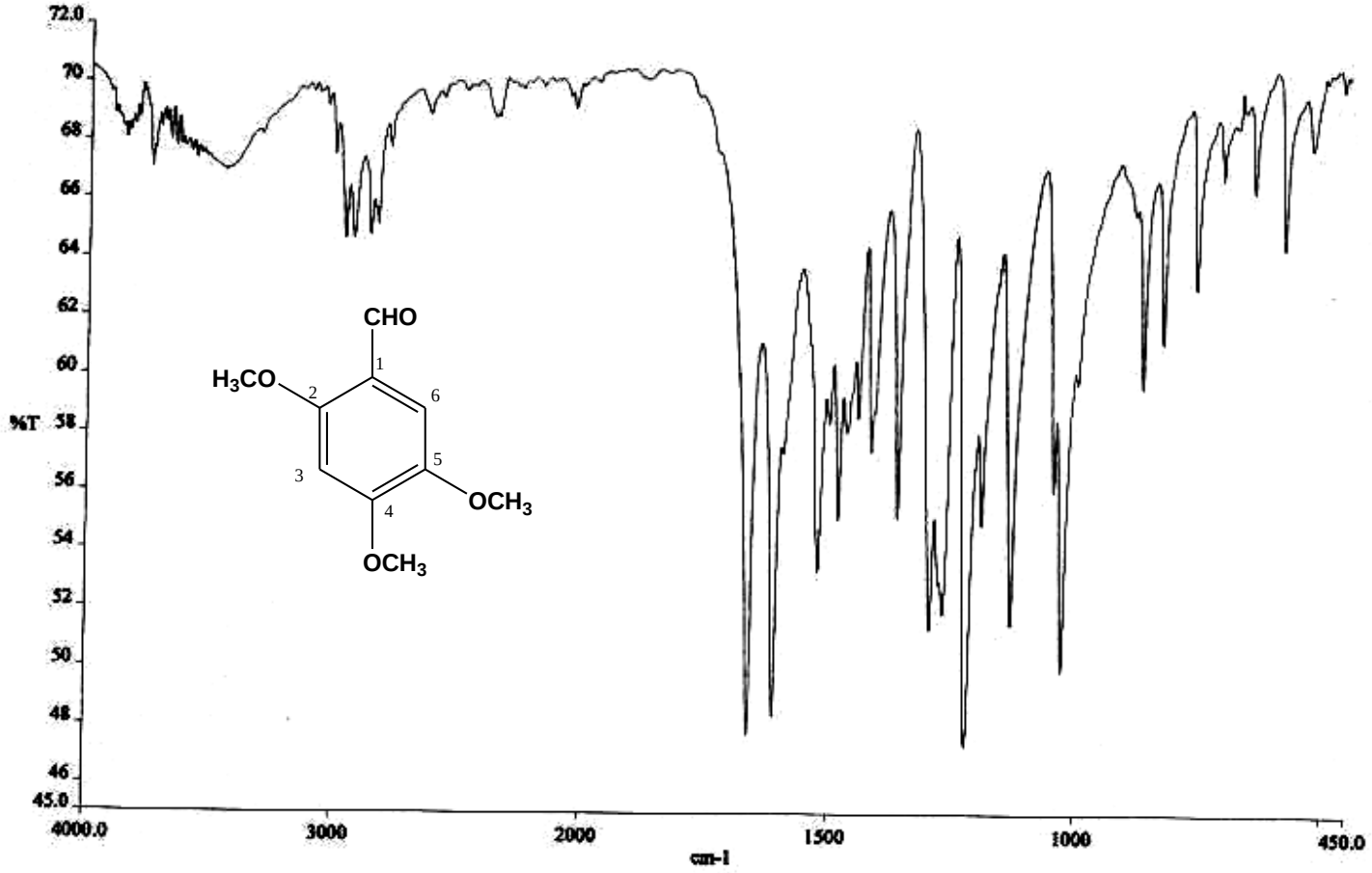
^{13}C NMR spectrum of compound **1** ($CDCl_3$; 125 MHz)



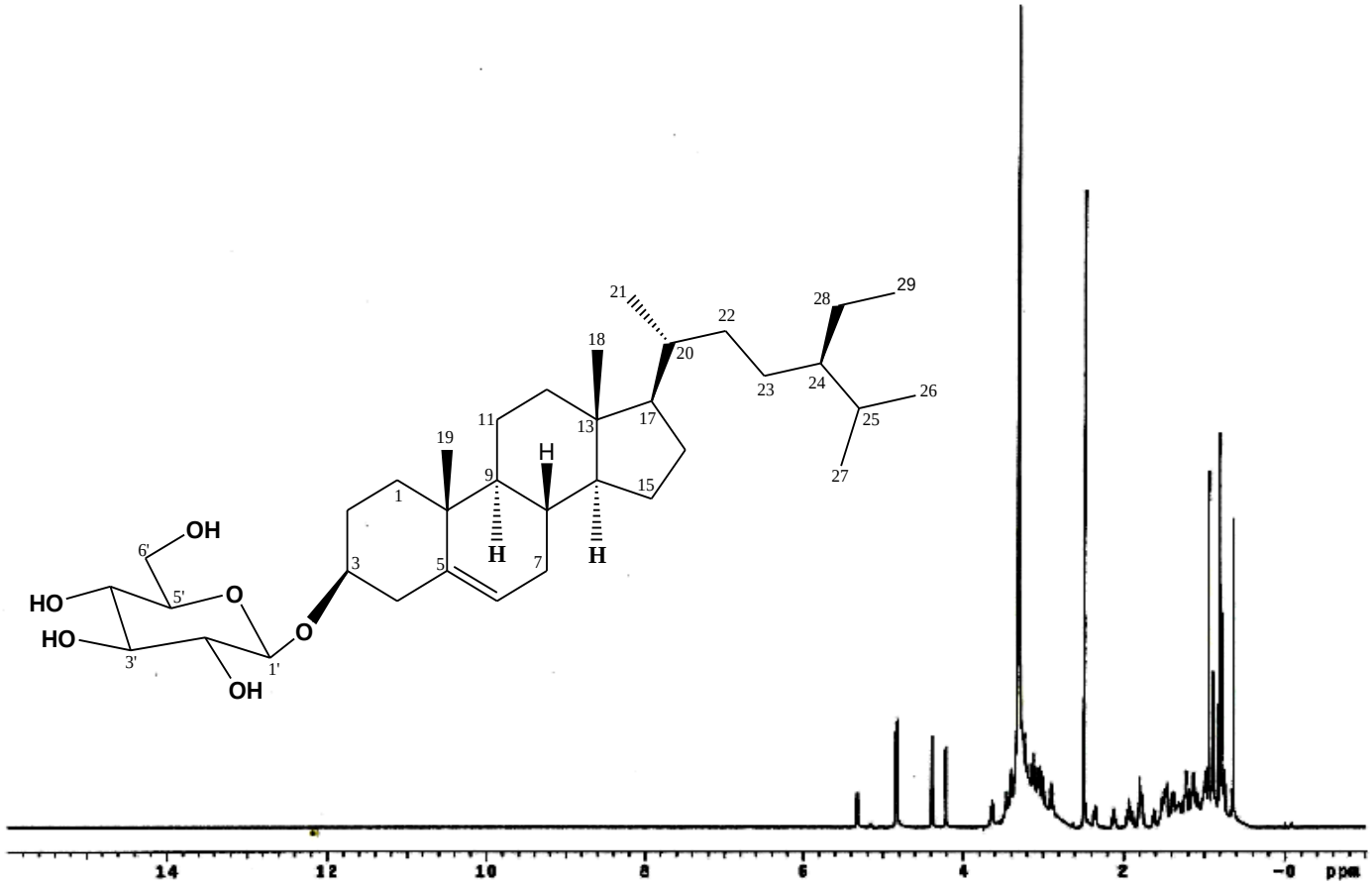
EI mass spectrum of compound **1**



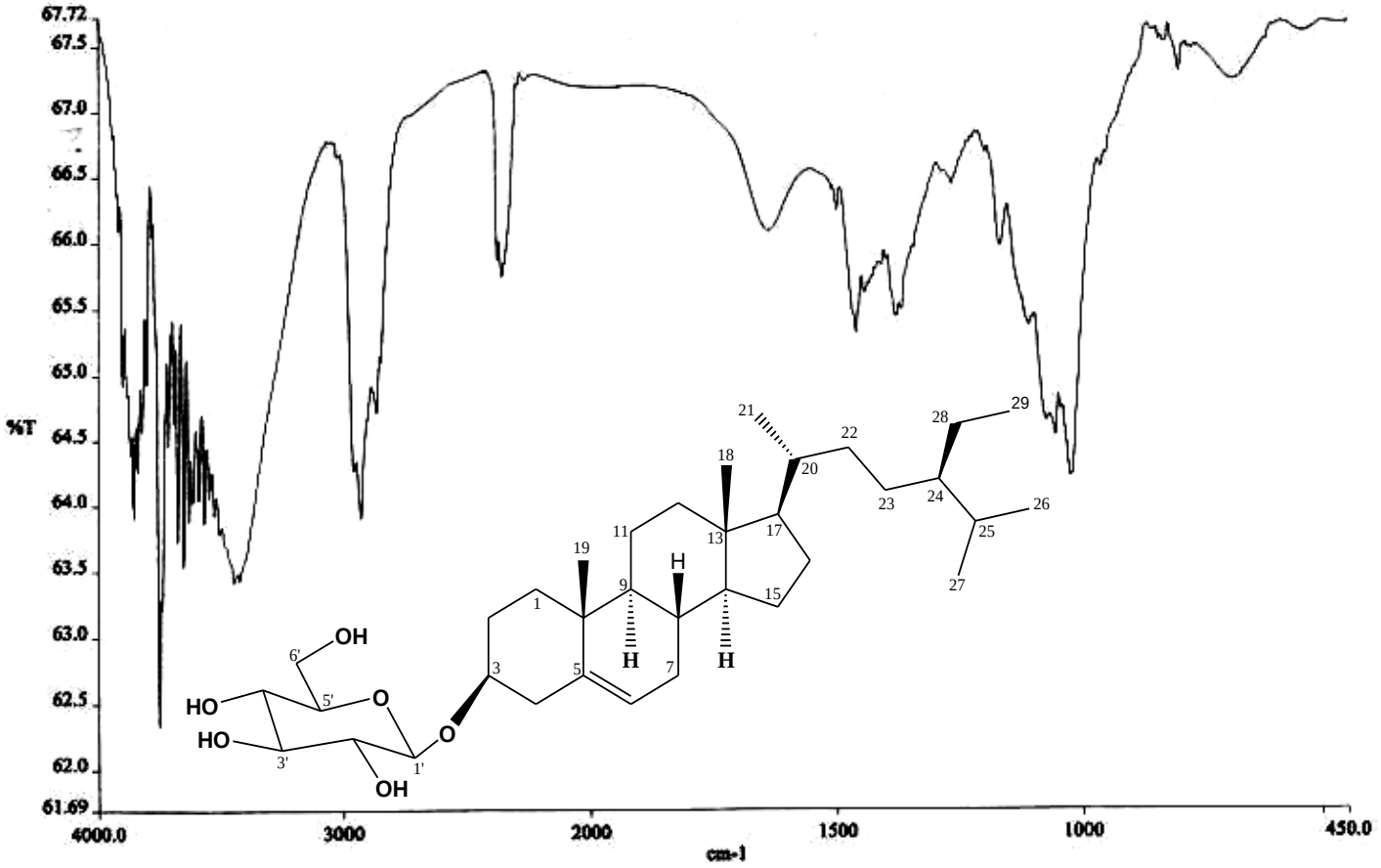
UV spectrum of compound **1** ($CHCl_3$)



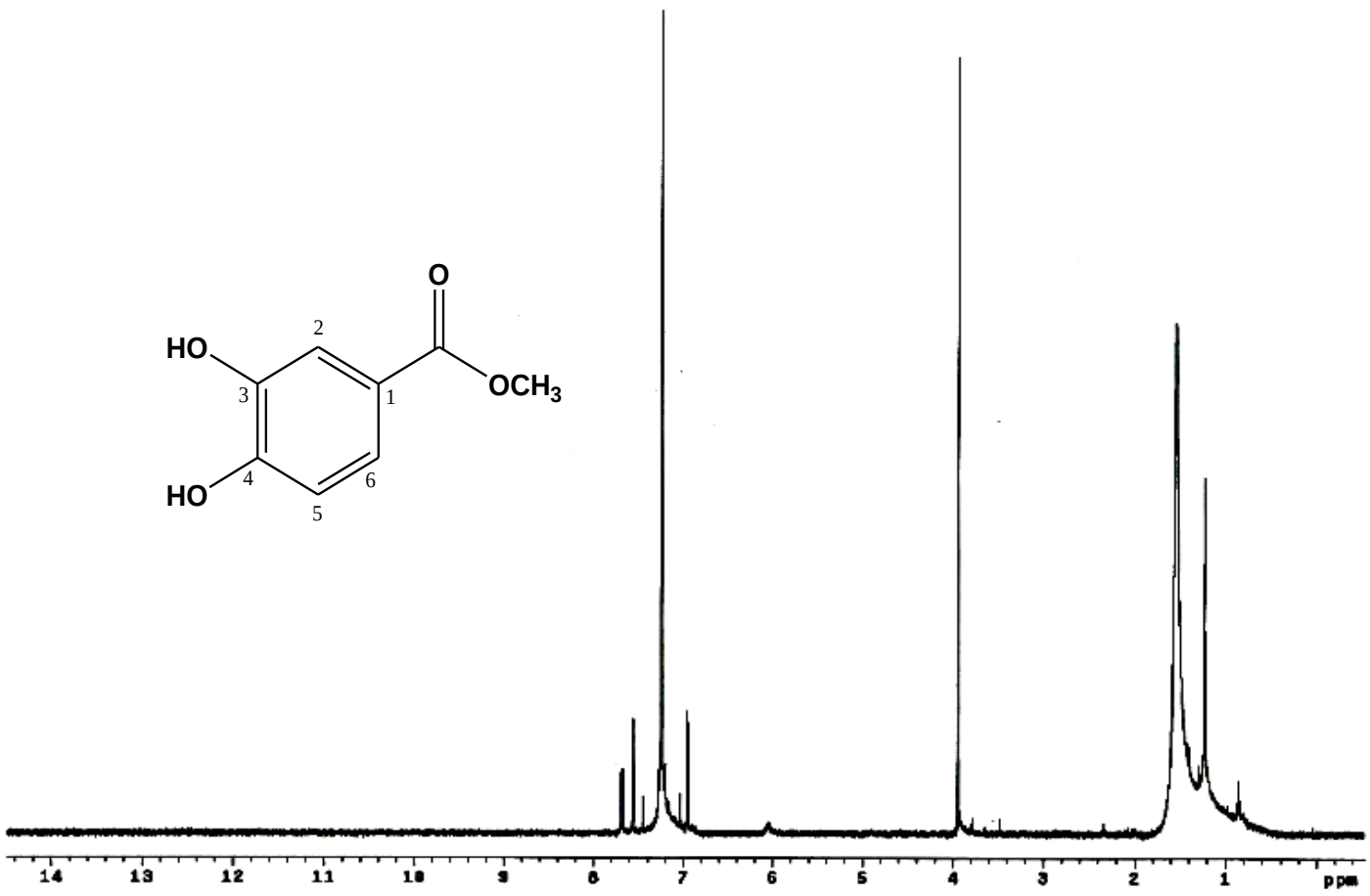
IR spectrum of compound **1** (KBr)



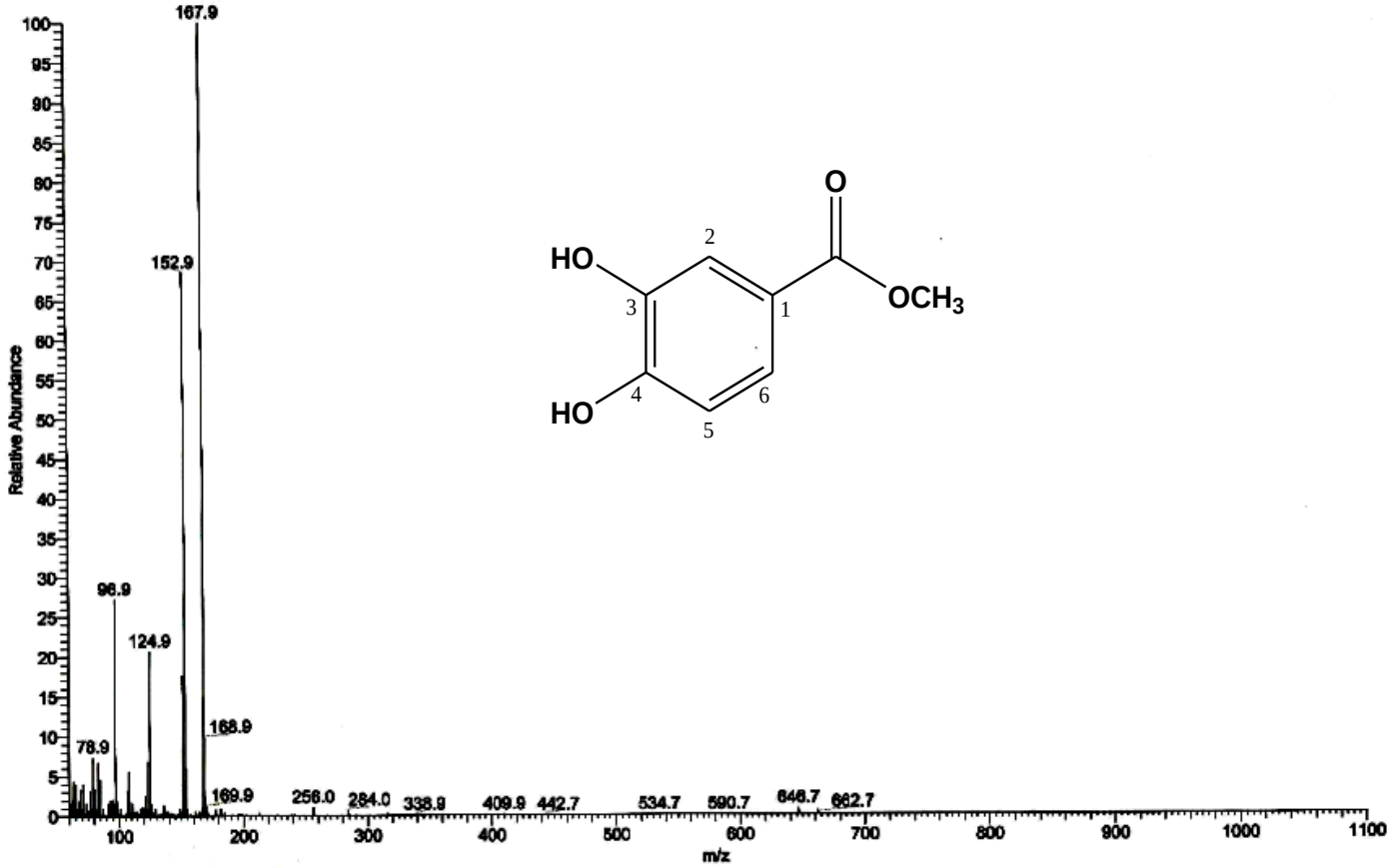
^{1}H NMR spectrum of compound **2** (DMSO-d_6; 500 MHz)



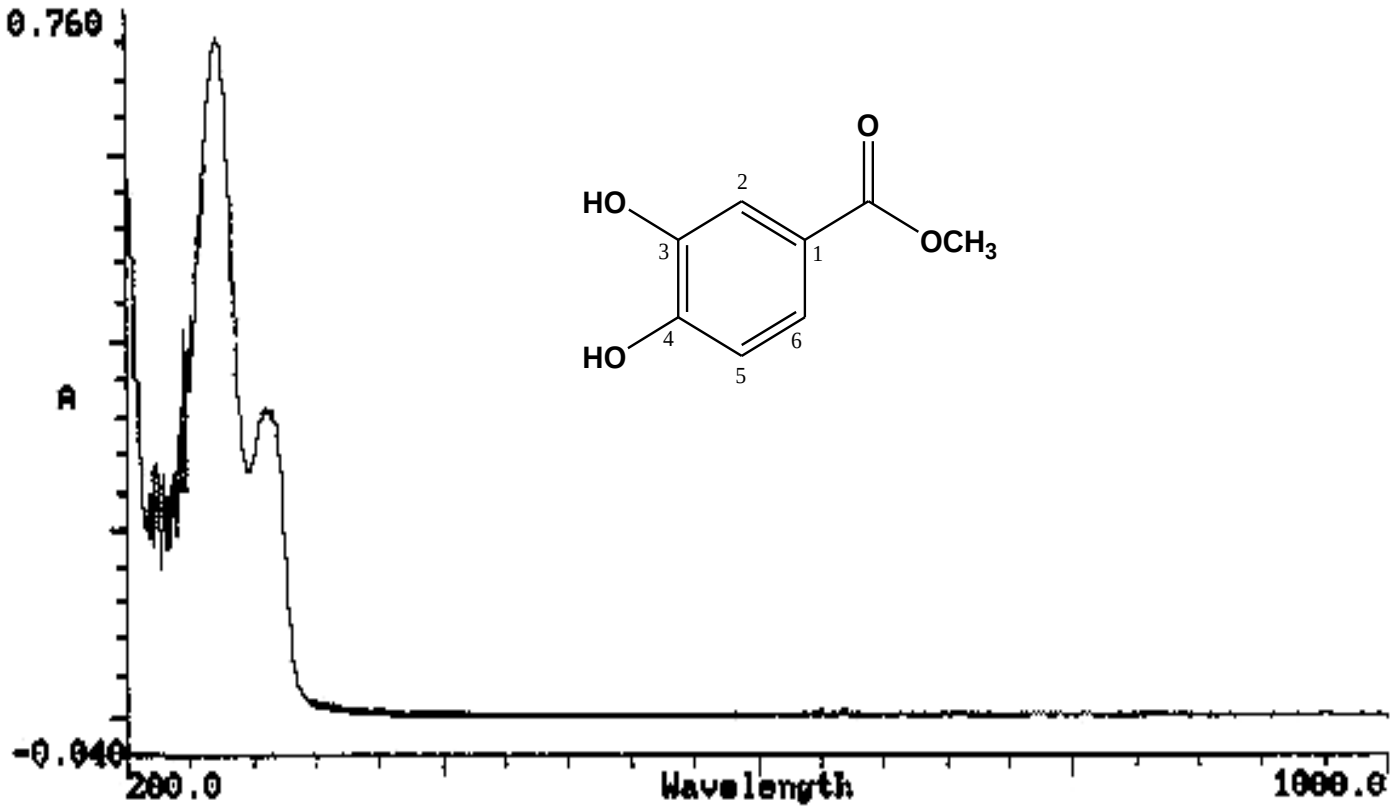
IR spectrum of compound **2** (KBr)



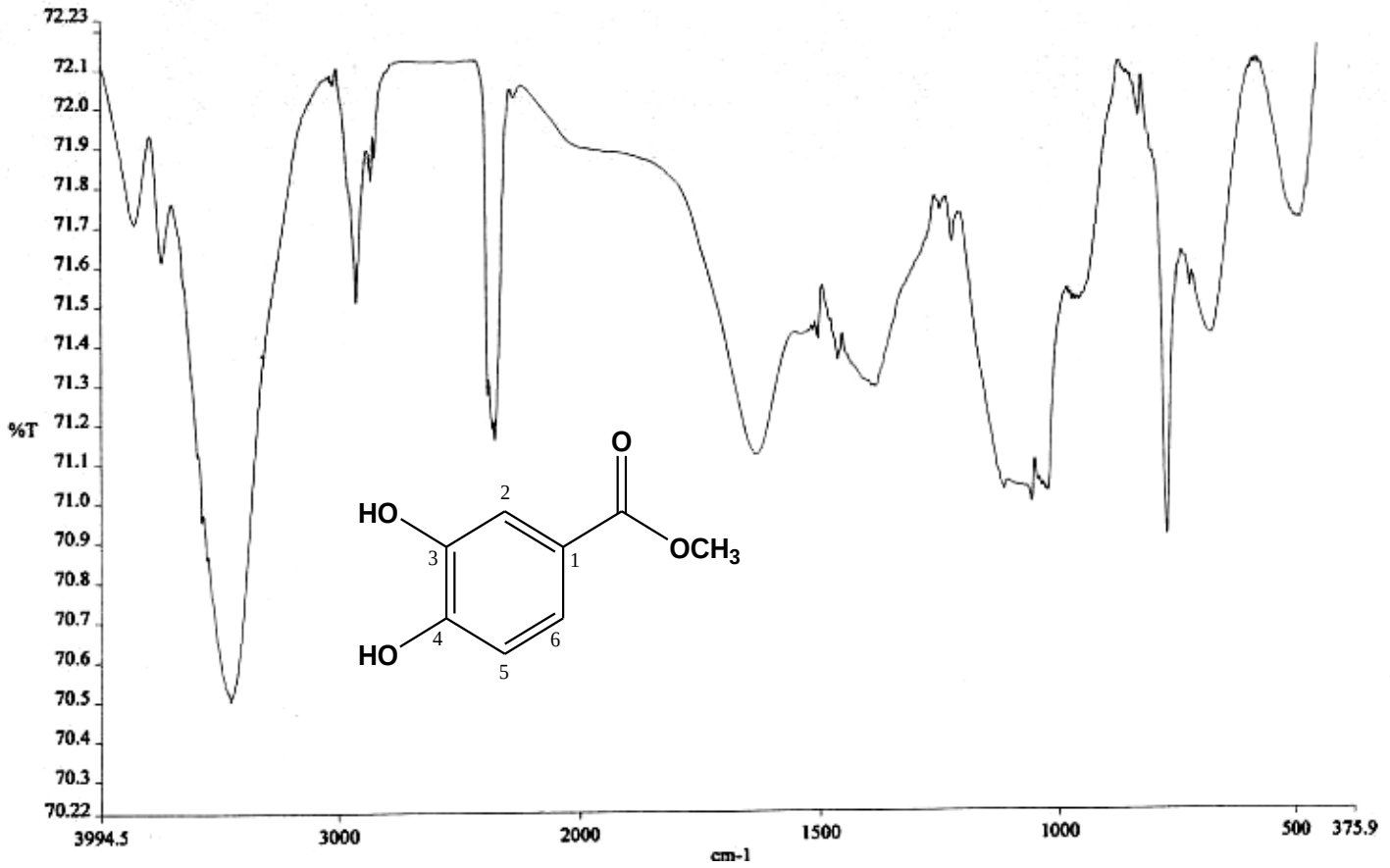
^{1}H NMR spectrum of compound **3** ($CDCl_3$; 500 MHz)



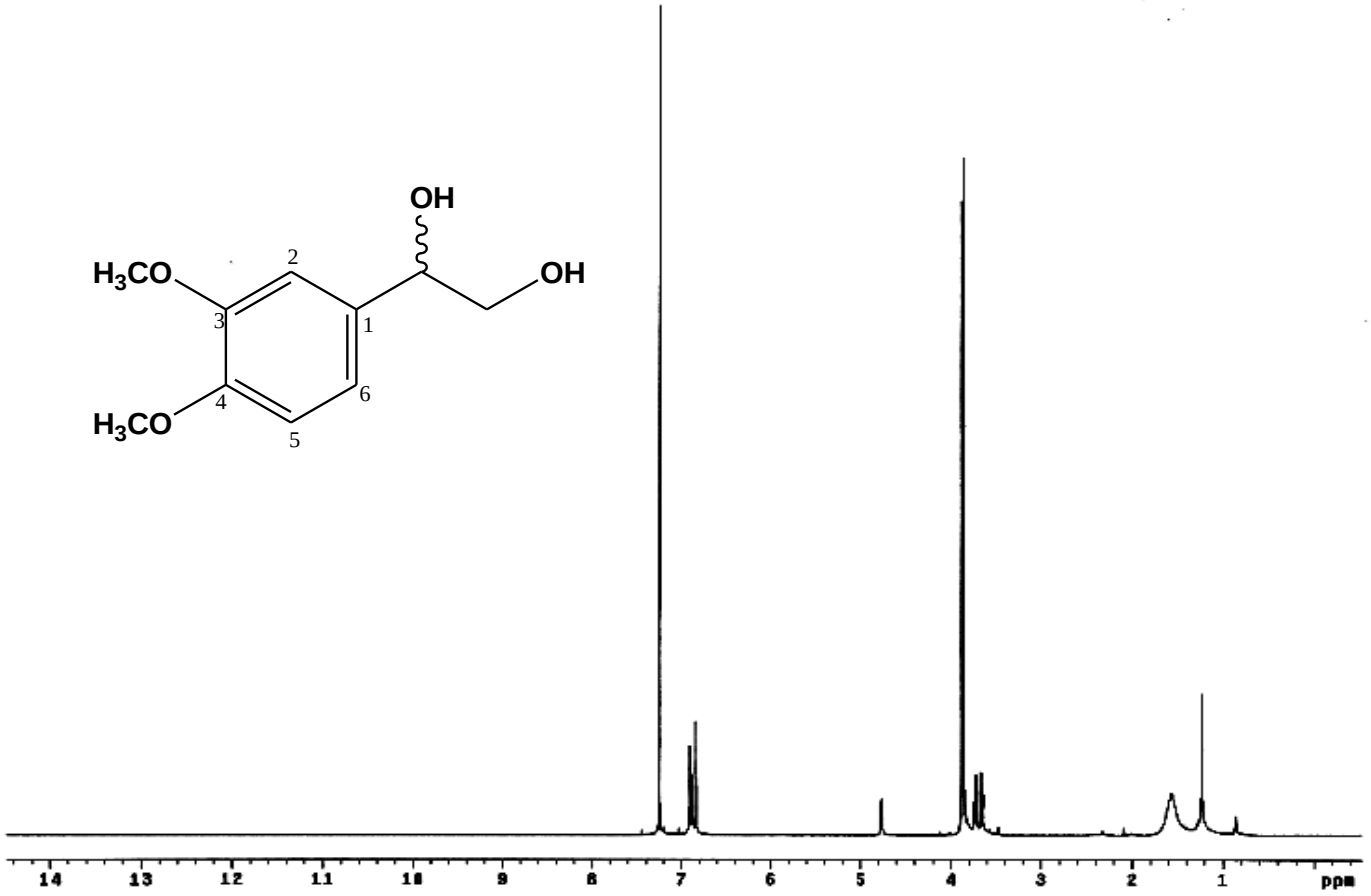
EI mass spectrum of compound **3**



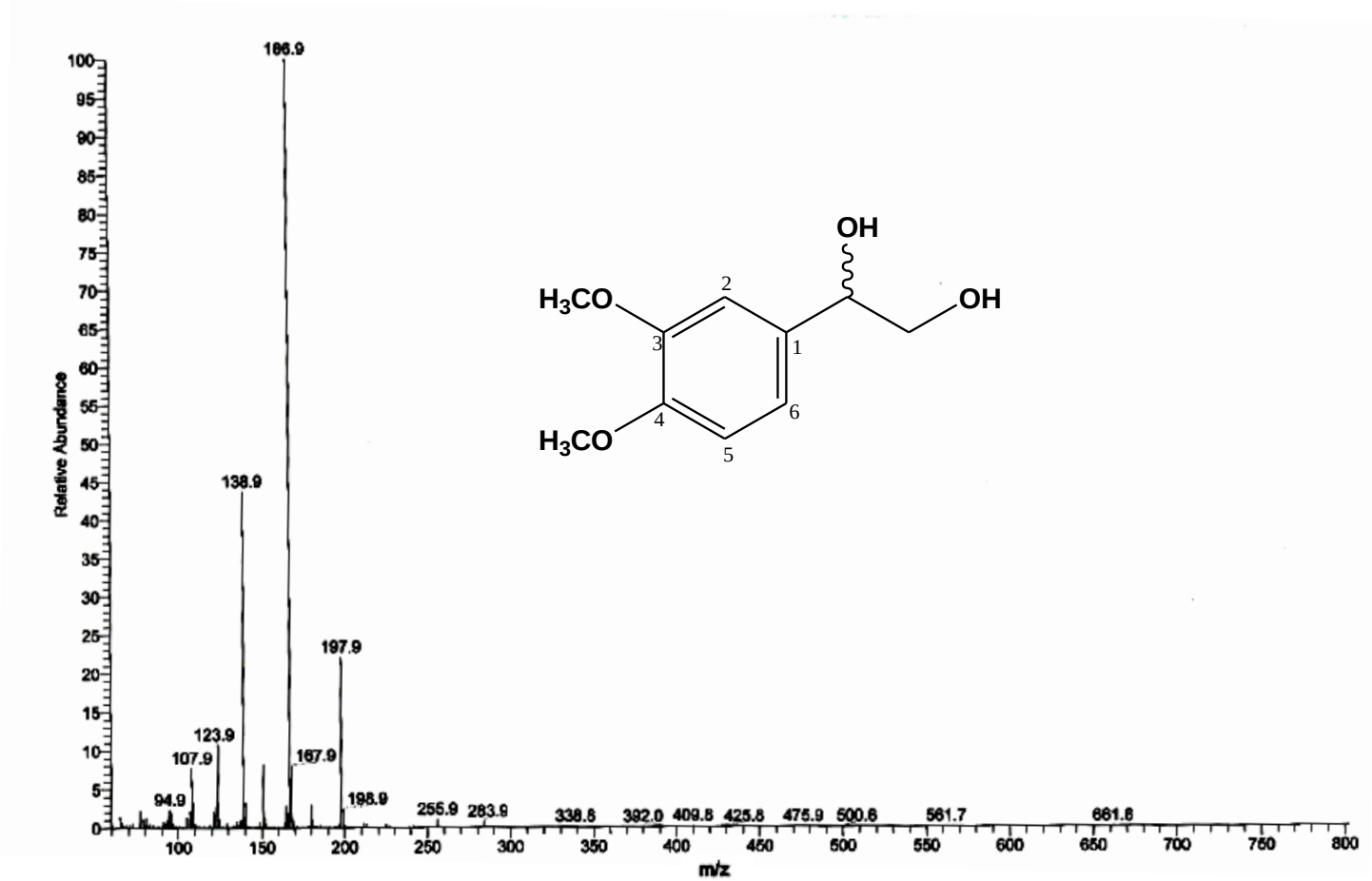
UV spectrum of compound **3** ($CHCl_3$)



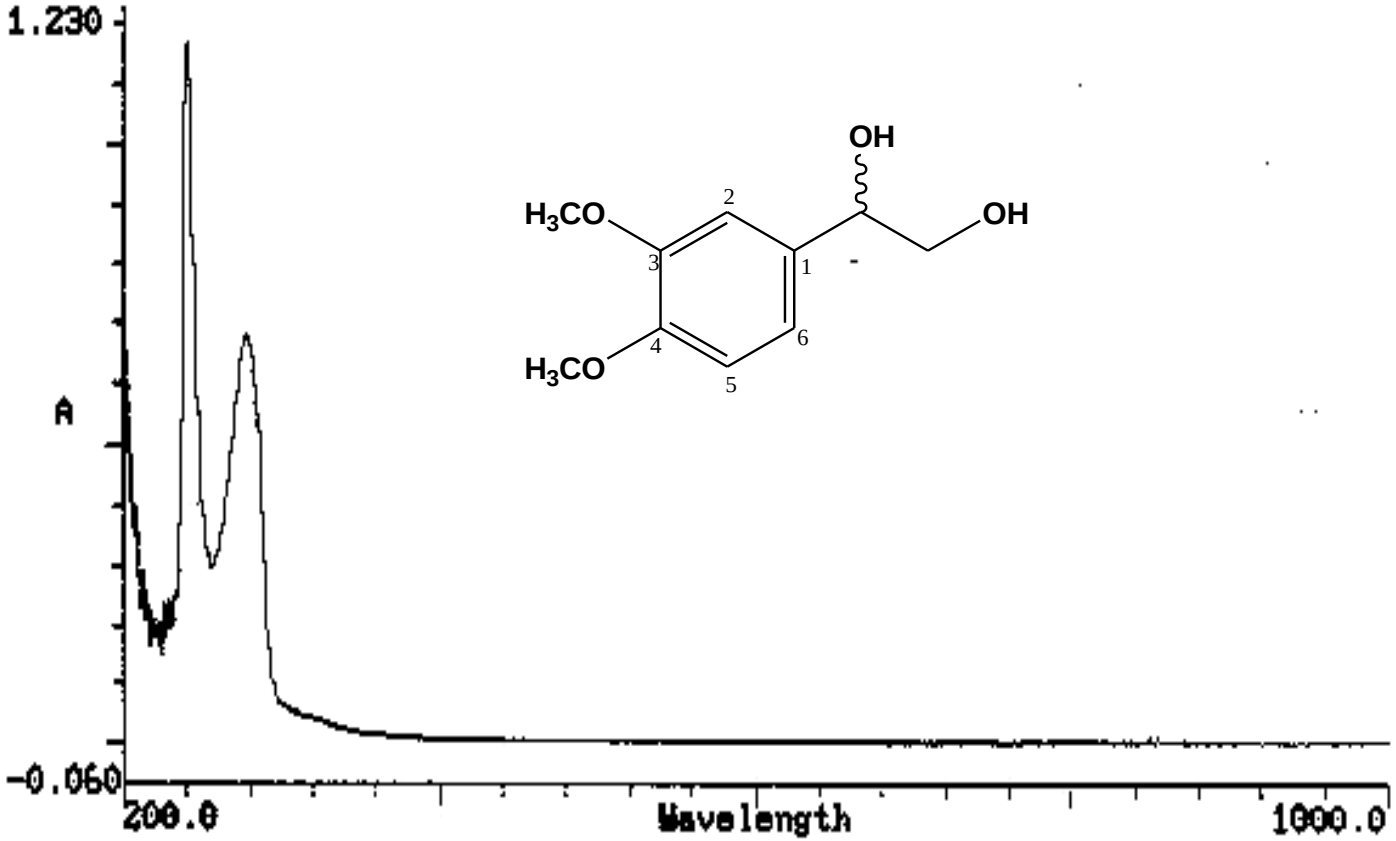
IR spectrum of compound **3** (KBr)



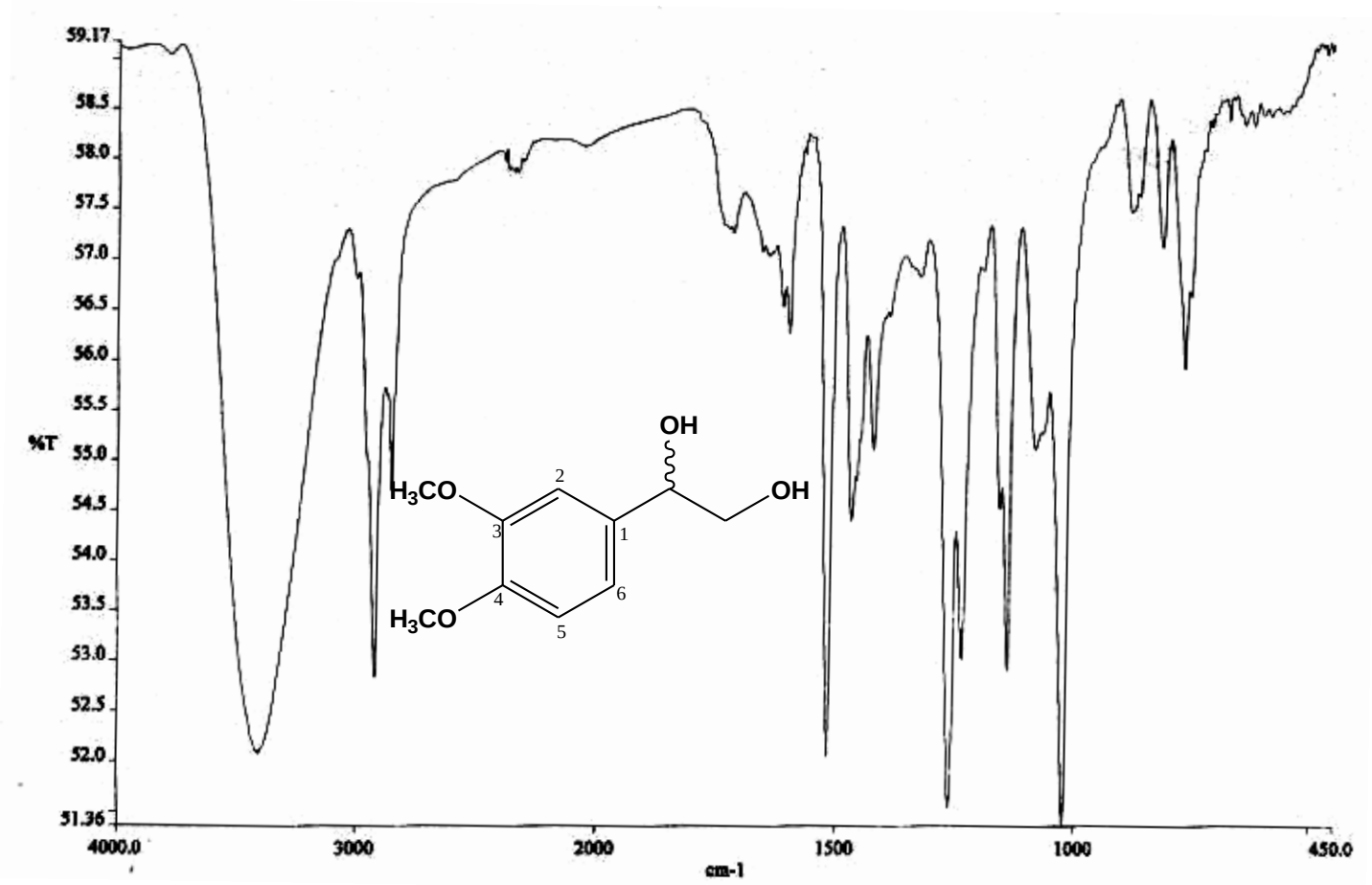
^{1}H NMR spectrum of compound **4** ($CDCl_3$; 500 MHz)



EI mass spectrum of compound **4**



UV spectrum of compound **4** ($CHCl_3$)



IR spectrum of compound **4** (KBr)

VITAE

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Bachelor of Science (Chemistry)	Prince of Songkla University	2008

List of Publication and Proceedings

Madaka, F. and Tewtrakul, S. 2011. Anti-allergic activity of some selected plants in the genus *Boesenbergia* and *Kaempferia*. Songklanakarin Journal of Science and Technology. 33: 301-304.

Madaka, F. and Tewtrakul, S. 2011. Anti-allergic activity of some selected plants in the genus *Boesenbergia* and *Kaempferia*. PERCH-CIC CONGRESS VII: Theme: Chemistry, Environment and Society. Proceedings, 4-7 May, 2011. Jomtien Palm Beach Hotel & Resort Pattaya, Chonburi, Thailand.