ELASTASE AND INFLAMMATION INHIBITORY FROM *LABISIA PUMILA*

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To my beloved son Aqil Miqail, husband Alif Firdaus,
my mother Madzni Yacob,
my family and my family in laws.

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ABSTRACT

Labisia pumila (*L. pumila*) is widely reported to exhibit antioxidant, anti-microbial and anti-cancer activities. The goal of this study was to identify and quantify triterpenoid compounds from *L. pumila* leaves which are responsible for anti-inflammatory and anti-elastase activities by using bioassay-guided fractionation. The leaves of *L. pumila* were sequentially extracted with n-hexane, ethyl acetate, methanol, and water using serial exhaustive extraction procedure. Qualitative phytochemical analysis revealed the presence of phenolics, flavonoids, terpenoids, cardiac glycosides and saponins in the extracts. Each extract was assessed for total triterpenoids and polyphenols content by using vanillin-perchloric acid assay and Folin-Ciocalteu's reagent, respectively. The ethyl acetate extract has the highest total triterpenoids content (78.94 mg oleanolic acid equivalent (OAE)/g dried extract) and appreciable amount of total polyphenols (38.93 mg gallic acid equivalent/g dried extract). The extracts were then tested for their anti-inflammatory and anti-elastase activities. The ethyl acetate extract showed the highest anti-inflammatory activity via the inhibition of cyclooxygenase-2 (half-maximal inhibitory concentration (IC_{50}) of 34.32 µg/ml) and elastase (IC_{50} of 17.14 µg/ml). The correlation analysis of anti-inflammatory and anti-elastase ability of *L. pumila* with its total triterpenoids and total polyphenols content showed positive relationship. Further fractionation of the ethyl acetate extract by solid phase extraction afforded sample fraction EAF-B with high total triterpenoids content (91.50 mg OAE/g dried fraction) and anti-elastase activity (IC_{50} of 13.27 µg/ml). Rapid liquid chromatography-electrospray ionization-tandem mass spectrometry method was developed and validated for the identification and quantification of pentacyclic triterpenoids with elastase inhibition property from the sample fraction EAF-B. Three pentacyclic triterpenoids, namely betulinic acid, oleanolic acid and lupeol were identified. The content of betulinic acid, oleanolic acid and lupeol in the fraction were 2.67, 5.04 and 1.47 mg/g dried fraction, respectively. The limits of detection and quantification ranged from 0.04 to 0.12 µg/ml and 0.11 to 0.65 µg/ml, respectively. The response surface methodology analysis showed that solvent-solid ratio ($p<0.01$), extraction time ($p<0.05$) and temperature ($p<0.01$) were statistically significant factors affecting the total triterpenoids extraction. The optimum extraction conditions obtained were: solvent-solid ratio of 37.76, extraction time of 4.24 hours and extraction temperature of 55.27 °C. The results suggested that *L. pumila* has promising anti-inflammatory and anti-elastase activity that could serve as potential source of natural elastase inhibitor in treating inflammatory skin disorders and photoaging.

ABSTRAK

Labisia pumila (*L. pumila*) telah dilaporkan menunjukkan aktiviti anti-pengoksidaan, anti-mikrob dan anti-kanser. Tujuan kajian ini adalah untuk mengenalpasti dan mengetahui jumlah sebatian triterpenoid di dalam *L. pumila* yang bertanggungjawab untuk aktiviti-aktiviti anti-keradangan dan anti-elastase melalui pemeringkatan berpandukan bioesei. Daun *L. pumila* diekstrak dengan n-heksana, etil asetat, metanol dan air menggunakan kaedah pengekstrakan lengkap bersiri. Analisis kualitatif fitokimia mendedahkan kehadiran fenolik, flavonoid, terpenoid, kardiak glikosida dan saponin di dalam ekstrak. Setiap ekstrak diuji untuk mengetahui jumlah kandungan triterpenoid dan polifenolik masing-masing menggunakan kaedah assai vanillin-asid perklorik dan reagen Folin-Ciocalteu. Ekstrak etil asetat mengandungi jumlah kandungan triterpenoid tertinggi (78.94 mg setara asid oleanolik (OAE))/g ekstrak kering) dan jumlah polifenolik yang ketara (38.93 mg setara asid galik/g ekstrak kering). Ekstrak kemudiannya diuji untuk aktiviti anti-keradangan dan anti-elastase. Ekstrak etil asetat menunjukkan aktiviti anti-keradangan tertinggi melalui perencatan aktiviti siklooksigenase-2 (kepekatan perencatan setengah maksimal, (IC_{50}) 34.32 µg/ml) dan elastase (IC_{50} 17.14 µg/ml). Analisis korelasi antara anti-keradangan dan anti-elastase dengan jumlah kandungan triterpenoid dan polifenolik menunjukkan hubungan yang positif. Penyaringan ekstrak etil asetat melalui kaedah pengekstrakan fasa pepejal menghasilkan sampel pecahan EAF-B dengan jumlah triterpenoid (91.50 mg OAE/g pecahan kering) dan aktiviti anti-elastase (IC_{50} 13.27 µg/ml) yang tinggi. Kaedah kromatografi cecair-pengionan elektrosembur-tandem spektrometri jisim telah dibangunkan untuk mengenalpasti dan pengkuantitian triterpenoid yang mempunyai sifat perencatan elastase dari sampel pecahan EAF-B. Tiga triterpenoid iaitu asid betulinik, asid oleanolik dan lupeol telah dikenalpasti. Kandungan asid betulinik, asid oleanolik dan lupeol di dalam pecahan EAF-B telah dikenalpasti masing-masing sebagai 2.67, 5.04 dan 1.47 mg/g pecahan kering. Analisis kaedah permukaan gerak balas menunjukkan nisbah pelarut-pepejal ($p<0.01$), masa pengekstrakan ($p<0.05$) dan suhu pengekstrakan ($p<0.01$) merupakan faktor statistik bermakna untuk pengekstrakan triterpenoid. Keadaan pengekstrakan optimum didapati ialah: nisbah pelarut-pepejal pada 37.76, masa pengekstrakan pada 4.24 jam dan suhu pada 55.27 °C. Keputusan ini mencadangkan bahawa *L.pumila* mempunyai aktiviti anti-keradangan dan anti-elastase yang berpotensi untuk dijadikan sumber perencat elastase semulajadi untuk mengubati penyakit keradangan kulit dan fotopenuaan.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AP-1	-	Activator protein 1
BBD	-	Box-Behnken Design
BBIC	-	Bowman-Birk inhibitor concentrate
COX	-	Cyclooxygenase
DMBA	-	7,12-dimethylbenz(a)anthracene
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
ECM	-	Extracellular matrix
ELSD	-	Evaporative light scattering detector
ERT	-	Estrogen replacement therapy
ESI	-	Electrospray ionization
ESI-MS	-	Electrospray ionization tandem mass spectrometry
GAE	-	Gallic acid equivalent
HPLC	-	High-performance liquid chromatography
IC_{50}	-	Half-maximal inhibitory concentration
LOD	-	Limit of detection
LOQ	-	Limit of quantification
LOX	-	Lipooxygenase
MMPs	-	Matrix metalloproteinases
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NOAEL	-	Non-observable adverse effect level
OAE	-	Oleanolic acid equivalent
PCOS	-	Polycyctic ovary syndrome
RP-HPLC	-	Reverse phase-high-performance liquid chromatography
RSM	-	Response surface methodology

SELDI-MS	-	Surface-enhanced laser desorption/ionization mass
SPE	-	Solid phase extraction
UV	-	Ultraviolet

LIST OF APPENDICES

CHAPTER I

INTRODUCTION

1.1 Background of Study

Ultraviolet (UV) radiation is known to have deleterious effects on human skin, including photoaging, skin cancer, and inflammatory skin disorders. UV radiation is regarded as the most significant external cause of photoaging and inflammatory skin disorders (Baumann, 2007). Inflammatory skin disorders, particularly sunburn occur acutely in response to excessive exposure to the sun, while photoaging and skin cancer resulted from an accumulated damage caused by repeated exposures to UV radiation. Skin produces free radicals or reactive oxygen species under repeated UV radiation, which then leads to oxidative stresses and inflammatory responses in the dermal or epidermal layer resulting in damage to cell membranes, lipids, proteins, and DNA. Cyclooxygenase-2 (COX-2) is one of the major pro-inflammatory enzymes implicated in the modulation of inflammation and can be upregulated in macrophages, monocytes, and fibroblasts by various stimuli during the inflammatory process. Therefore, inhibition of COX-2 is considered as one of the therapeutic targets for treatment of inflammatory diseases. During inflammation, the first immune cells that respond are mostly neutrophils, which are quick-response immune cells that recognize and destroy bacteria (Brinkmann *et al.*, 2004).

Neutrophils are typical inflammatory cells that secrete neutrophil elastase in the acute inflammation phase resulting from UV radiation damage. Normally, the activity of neutrophil elastase released by polymorphonuclear leukocytes is strictly regulated by its endogenous inhibitors (Korkmaz, Moreau and Gauthier, 2008). However, in certain inflammatory conditions, for example upon excessive exposure of UV radiation to human skin, a large number of neutrophils are infiltrated and migrated to the site of inflammation. The abnormal exocytosis of this neutrophil elastase results in an imbalance between elastase and its inhibitor which consequently causes severe tissue damages. Therefore, it can be said that neutrophils were also among the major mediators of inflammation parallel to other pro-inflammatory mediators. Thus, neutrophil elastase is an important regulator of the inflammatory process that could be used as a therapeutic approach in skin inflammatory disorders such as psoriasis and atopic dermatitis.

Besides that, neutrophils also play a pivotal role in the process of photoaging of human skin. Upon repeated exposure of UV radiation to the human skin, neutrophil infiltrates the skin and released neutrophil elastase. This enzyme has the capability of degrading elastin along with other extracellular matrix (ECM) components (Siedle, Hrenn and Merfort, 2007). Even though elastin is known to be quite resistant to proteolytic degradation due to its insolubility and its nature of being hard to be hydrolysed (Schmelzer *et al.*, 2012), continuous exposure of elastin to elastase activity subsequently leads to serious and irreversible damage to elastin fibres. Polymerized connective tissue fibres causes the decreases of skin elasticity, hence leading to skin sagging. Several studies have demonstrated that skin photoaging and the wrinkling effect of the skin is significantly correlated with increased enzymatic activity of neutrophil elastase along with other enzymes such as collagenase and matrix metalloproteinases (MMPs) (Kim *et al.*, 2010; Chattuwatthana and Okello, 2015).

Since neutrophil elastase plays a critical role in both inflammatory process and massive degradation of important ECM proteins, the inhibition of neutrophil elastase activity is now considered as another approach to suppress inflammatory skin disorders and photoaging (Wiedow *et al.,* 1990; Kim *et al.,* 2010). In the last decade, recognition of neutrophil elastase as a promising therapeutic target in inflammatory disorders has led to an increased to the scientific interest in the discovery of new inhibitors of this enzymatic activity. Various synthetic and natural elastase inhibitors are currently under development to combat inflammatory disorders. Inhibition of the elastase activity could also be used as a valuable method to protect the skin against skin photoaging and maybe useful to prevent skin wrinkles. Since the past decade, natural compounds and plant extracts have been the targets of research as the potential source to explore for efficient elastase inhibitors to overcome neutrophil elastase deleterious effects. Moreover, natural ingredients are easier to be absorbed by the outer layer of the skin, hence lowering the possibility of skin allergy problems.

1.2 Statement of Problems

Plants have been one of the sources of medicine to treat various illnesses since ancient time. Natural elastase inhibitors derived from plant extracts have been discovered by many researchers. Previously, *in vitro* study on the inhibition of human neutrophil elastase activity by traditional Yemeni medicinal plants was conducted by Alasbashi and Melzig (2008). Several studies showed that all of the plants tested were used to treat inflammation related diseases (Sukumar *et al*., 1995; Mandal *et al*., 2003; Lukhobe *et al*., 2006). Inhibition effect of elastase activity by traditional Chinese medicine *Diospyros kaki* was studied by An *et al.* (2005). This plant has been used in Chinese medicine to treat various skin problems including pimples and eczema. The effect of 22 Korean traditional herbal medicines upon the elastase inhibitory was conducted by Kim *et al*. (2014) However, to date, natural elastase

inhibitors from *Labisia pumila*, a traditional Malaysian medicinal plant, has not been reported before.

L. pumila, a member of the Primulaceae family is commonly known in Malaysia as Kacip Fatimah. Traditionally, this plant has been used by the indigenous women of the Malay Archipelago to facilitate childbirth in inducing and hastening delivery and also as a post-partum medication to help contract the birth channel, to delay conception and to regain body strength (Burkill, 1935). Recently, the demand for *L. pumila* based product has increased drastically in the herbal and commercial industry (Ibrahim and Jaafar, 2011). The increased demand of this herb as a result of the extensive studies done by various researchers who have documented the biological activities and phytochemical constituents of this plant. Phytoestrogenic effects of this herb has been proved by many researchers through *in vitro* and *in vivo* studies (Jamal *et al*., 2003; Ayida *et al*., 2007; Al-Wahaibi *et al*., 2008; Fazliana *et al*., 2009, Manneras *et al*., 2010; Shuid *et al*., 2011). In addition, this plant is also famously known to exhibits strong antioxidative activities (Norhaiza, Maziah and Hakiman, 2009; Choi *et al*., 2010; Chua *et al*., 2011; Karimi and Jaafar, 2011).

The aging process is highly related to the antioxidant defence of the skin; as a result, the antioxidative activity of *L. pumila* is expected to exhibit anti-aging potential. *L. pumila* was also reported to inhibit TNF-α production and MMPs expression (Choi *et al*., 2010). It also has been discovered that the water extract of *L. pumila* restored the synthesis of type 1 pro-collagen which was reduced in the presence of ultraviolet-B (Choi *et al*., 2010). This discovery suggests that *L. pumila* has tremendous potential to be used in anti-aging cosmetic ingredients. On the other hand, the anti-inflammatory activities of *L. pumila* have been studied previously via the inhibition of the expression of TNF-α, COX-2 and nitric oxide (NO) (Choi *et al*, 2010; Karimi, Jaafar and Ahmad, 2013). However, to date, there is no scientific evidence available on the anti-elastase activity of *L. pumila*. Other beneficial biological activities of this plant include antimicrobial, anti-stress, aphrodisiac and anticarcinogenic effect (Ali and Khan, 2011; Kour *et al*., 2010; Asiah, Nurhanan and Ilham, 2007; Pihie *et al*., 2011).

In recent years, secondary metabolites extracted from medicinal plants have been extensively investigated as a source of medicinal agent due to its health-promoting effects. Various researches have been conducted on hundreds of medicinal plants in a quest to find the secondary metabolites that exhibits elastase inhibitory activity. Through all of the studies, the elastase inhibitory activity from medicinal plant extracts were belief to be contributed by the anti-inflammatory compounds namely triterpenoids, phenolics and flavonoids (Lee *et al*., 2001; Sultana and Lee, 2007; Kim *et al*., 2009a; Onar *et al*., 2012; Kacem, 2013).

Triterpenoids are the most important groups of natural anti-inflammatory compounds (Safayhi and Sailer, 1997). Pentacyclic triterpenoids such as ursolic acid, oleanolic acid, betulinic acid, boswellic acid, and lupeol are used in nutraceuticals for treating a wide spectrum of diseases ranging from skin inflammation and cancer to diabetic and rheumatism and cardiovascular related diseases (Alqahtani *et al*., 2013; Siddique and Saleem, 2011). The inhibitory activities of betulinic acid, oleanolic acid, and lupeol against human neutrophil elastase have been described as similar as of other pentacyclic triterpenoids (ursolic acid, glycyrrhetinic acid and glycyrrhizin) (Feng *et al*., 2013). A number of acidic triterpenoid compounds have anti-inflammatory properties and this has been related to the inhibition of leukocyte elastase by these compounds as reported by Ying *et al*. (1991).

Several studies reported that the biological activities of *L. pumila* were the result of the combination of individual phytochemical compounds present in the plant which are phenolics, flavonoids, carotenoids, ascorbic acids, triterpenoid saponins, alkenyl compounds and benzoquinone derivatives. Total phenolics and flavonoids in *L. pumila* was studied by many researchers (Norhaiza, Maziah and Hakiman, 2009; Chua *et al*., 2011; Karimi, Jaafar and Ahmad, 2011). However, no data was reported in literature regarding the total triterpenoids content of *L. pumila*.

Recently, the growing interests in the natural phytogenic drugs have led to an increasing need for an efficient extraction methods that can isolate the phytogenic compounds without damaging the chemical structures or its biological activities. Among these methods, serial exhaustive extraction is the most popular since it is considered as a non-aggressive method that does not harsh the active ingredients present in the plant extract. In order to exhaustively extract all of the active ingredients (hydrophilic and hydrophobic constituents) available in the interested medicinal plant, polar and non-polar solvents have been used without heating or with low heat. Previously, as reviewed by Abdullah *et al*. (2013), most of the extraction techniques used in the extraction process of *L. pumila* is conventional techniques whether by soaking or maceration. Up till now, serial exhaustive extraction method for the extraction of valuable compounds in *L. pumila* plants has not been reported before.

High performance liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) has been found to be the most useful method for the identification, separation, and quantification of triterpenoids. However, there is no LC-ESI-MS/MS method reported for the simultaneous quantification of betulinic acid, oleanolic acid, and lupeol from the leaves of *L. pumila*. The extraction conditions can alter the extract biochemical profiles which subsequently influence its bioactive potential. The maximum extraction efficiency of targeted components can be obtained by optimising the extraction conditions. Response surface methodology (RSM) is a well-accepted approach for optimising the extraction conditions and this method requires fewer experimental trials to evaluate the influence of multiple parameters such as extraction time, extraction temperature and solvent-solid ratio and their interactions (Zhen-ming *et al*., 2011; Li *et al.*, 2011) However, the effects of extraction conditions on extraction efficiency of elastase inhibitors from *L. pumila* leaves has not been reported till now.

1.3 Objective of Study

The objective of this study was to establish the fingerprinting of *L. pumila* leaf extract for the identification of triterpenoid compounds which are responsible for the inhibition of human elastase activity.

1.4 Scope of Study

The scopes of this study are:

1. To determine total polyphenols and triterpenoids content in *L. pumila* leaves extract and in the fractions of ethyl acetate extract of *L. pumila* leaves.
2. To examine the anti-inflammatory and anti-elastase potential of *L. pumila* leaves extract.
3. To evaluate the relationship between anti-inflammatory and anti-elastase activity of *L. pumila* leaves with the phytochemicals content of the extracts.
4. To establish an analytical method by LC-ESI-MS/MS in order to identify and quantify the triterpenoid compounds which were responsible for the elastase inhibition activity of *L. pumila* leaves.
5. To optimize the extraction condition of triterpenoid compounds that exert the elastase inhibitory activity from *L. pumila* leaves by employing Response Surface Methodology (RSM).
6. To provide a preparative study for the extraction and fractionation of ethyl acetate extract of *L. pumila* leaves for optimum recovery of triterpenoid compounds.

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