

**FRACTIONATION OF *FICUS DELTOIDEA* LEAVES EXTRACT USING SOLID
PHASE EXTRACTION ON ANTI-AGEING ACTIVITY IN VITRO**

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ABSTRACT

Ficus deltoidea (*F. deltoidea*) belongs to moraceae. Vitexin and isovitexin that have high antioxidant property are considered as the chemical markers of *F. deltoidea* leaves. The objective of this research was to investigate the fractionation of *F. deltoidea* leaves using solid phase extraction on anti-ageing activity *in vitro*. The leaves of *F. deltoidea* were extracted by methanol by using ultrasonic extraction method and further fractionated using different concentration of methanol. Antioxidant activity of *F. deltoidea* leaves fractions and extract was analyzed through 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability test and total flavanoid content (TFC) activity. The result shows that 80% methanol 20% water fraction (F4) have higher percentage of scavenging activity and flavonoid content with 87.16% and 237.57 mg, respectively. The quantification of biomarkers (vitexin and isovitexin) was performed using high performance liquid chromatography (HPLC) and the result showed that F4 fraction have higher percentage of vitexin (11.02%) and isovitexin (0.49%) compared to other fractions and extract. Moreover, cytotoxicity study on human skin fibroblasts cell (HSF 1184) also demonstrated that F4 has higher percentage of cell viability with value 175.29% at 100 µg/mL. The anti-ageing activity of *F. deltoidea* leaves fractions and extract were further evaluated using sircol collagen assay, inhibition of elastase assay, hyaluronidase assay and lipoxygenase assay, where F4 also showed the strongest activities compared to other fractions and extract for all assays with the value of 8.9 µg collagen concentration, 83%, 97.82%, and 88.56%, respectively. Taken together, as F4 contains high amount of vitexin and isovitexin, therefore these compounds have the potential to be further developed as anti-ageing agent.

ABSTRAK

Ficus deltoidea (*F. deltoidea*) tergolong dalam moraceae. Viteksin dan isoviteksin yang mempunyai antioksidan yang tinggi merupakan penanda kimia yang terdapat dalam daun *F. deltoidea*. Objektif kajian ini dilakukan untuk pemeringkatan daun *F. deltoidea* menggunakan pengekstrakan fasa pepejal secara *in vitro* untuk aktiviti anti-penuaan. Daun *F. deltoidea* diekstrak menggunakan metanol dan pemeringkatan dijalankan menggunakan kepekatan metanol dengan kaedah ultrasonik yang berbeza. Aktiviti antioksidan pecahan dan daun mentah *F. deltoidea* dianalisis melalui ujian aktiviti pemerangkapan 2,2-diphenil-1-picrilhrazil dan jumlah kandungan flavanoid. Keputusan yang diperolehi menunjukkan campuran 80% metanol : 20% air (F4) mempunyai peratusan yang tertinggi bagi aktiviti memerangkap dan kandungan flavanoid masing-masing dengan nilai 87.16% dan 237.57 mg. Pengukuran biomarker (viteksin dan isoviteksin) ditentukan menggunakan kromatografi cecair prestasi tinggi dan keputusan menunjukkan pecahan F4 mempunyai peratusan viteksin (11.02%) dan isoviteksin (0.49%) yang tinggi berbanding ekstrak dan pecahan yang lain. Tambahan pula, kajian sitotoksik terhadap kulit manusia dijalankan menggunakan sel fibroblast (HSF 1184) menunjukkan F4 juga mempunyai peratusan daya maju sel tertinggi iaitu 175.29% pada kepekatan 100 µg/mL. Kajian aktiviti anti-penuaan terhadap pecahan dan ekstrak daun *F. deltoidea* seterusnya dikaji melalui kaedah kolagen sirkol, perencatan elastase, hialuronidase dan liposiginase mendapati F4 juga menunjukkan aktiviti yang terkuat berbanding pecahan dan ekstrak keatas kesemua aktiviti masing-masing dengan nilai 8.9 µg , 97.82%, 97.82% dan 88.56%. Secara keseluruhannya, F4 mempunyai kandungan viteksin dan isoviteksin yang tinggi di mana sebatian ini berpotensi dibangunkan sebagai agen anti-penuaan.

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TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	ACKNOWLEDGEMENT	
	ABSTRACT	v
	ABSTRAK	vi
	TABLE OF CONTENT	vii
	LIST OF TABLES	viii
	LIST OF FIGURES	ix
	LIST OF ABBREVIATIONS	x
1	INTRODUCTION	1
	1.1 Research Background	1
	1.2 Problem Statement	4
	1.3 Objective	5
	1.4 Scope of Research	6
	1.5 Significant of study	6
2	LITERATURE REVIEW	7
	2.1 Skin	7
	2.1.1 Elements in the skin	8
	2.1.1.1 Skin Cells	8
	2.1.1.2 Skin Fibers	9
	2.2 Ageing	10

2.3	Skin Ageing	10
2.3.1	UV radiation and its effects on the human skin	11
2.3.2	Mechanisms of skin ageing	13
2.3.3	Hyaluronic acid and skin ageing	14
2.4	<i>Ficus deltoidea</i>	16
2.4.1	Phytochemical properties of <i>Ficus deltoidea</i>	17
2.4.1.1	Vitexin	18
2.4.1.2	Isovitexin	19
2.4.1.3	Chemical features of vitexin and isovitexin	19
2.4.2	Biological activity of <i>Ficus deltoidea</i>	21
2.4.2.1	Anti-inflammatory activity	21
2.4.2.2	Antioxidant activity	22
2.5	Ultrasonic Assisted Extraction	23
2.6	Solid Phase Extraction	25
2.6.1	The Effect of Fractionation of Phytochemical on Health Benefits	28
3	METHODOLOGY	30
3.1	Introduction	30
3.2	Chemicals	32
3.3	Preparation of Raw Materials and Extraction Process	33
3.4	Fractionation of <i>Ficus deltoidea</i> extract using Solid Phase Extraction	33
3.5	Quantification of vitexin and isovitexin in <i>Ficus deltoidea</i> leaves fractions	35
3.5.1	High Performance Liquid Chromatography (HPLC)	35
3.6	Analysis on antioxidant activity	36
3.6.1	DPPH radical scavenging activity	36
3.6.2	Total Flavanoid Content (TFC)	36
3.7	Effect of <i>Ficus deltoidea</i> leaves extract and fractions on anti-ageing activity	37
3.7.1	Cell Line	37
3.7.2	Cell Seeding	37

3.7.3	Medium Renewal	37
3.7.4	Cell counting and evaluation on viable cells	38
3.7.5	Cell viability assay	38
3.7.5.1	UV irradiation and treatment	38
3.7.5.2	MTT assay	39
3.8	Measurement of Collagen content by Sircol Collagen Assay (SCA)	39
3.8.1	Sample Preparation	40
3.8.2	Determination of Collagen Assay	40
3.9	In vitro elastase inhibition assay	41
3.9.1	Preparation of Tris-HCL buffer	41
3.9.2	Preparation of enzyme solution	41
3.9.3	Preparation of substrates	41
3.9.4	Serial dilution of Ascorbic Acid	41
3.9.5	Serial dilution of crude extract and fractions	42
3.9.6	Determination of elastase inhibition activity	42
3.10	Anti-inflammatory activity of <i>Ficus deltoidea</i> leaves fraction	43
3.10.1	Hyaluronidase inhibitory assay	43
3.10.2	Lipoxygenase inhibitory assay	44
3.11	Statistical analysis	45
4	RESULTS AND DISCUSSION	46
4.1	Detection and Quantification of vitexin and isovitexin in <i>Ficus deltoidea</i> leaves fraction	46
4.2	Antioxidant activity of <i>Ficus deltoidea</i> leaves fractions	48
4.3	Anti-ageing Activity of <i>Ficus deltoidea</i>	52
4.3.1	Cell Cytotoxicity Study	52
4.3.2	Collagen synthesis study	55
4.3.3	Elastase Assay	57
4.3.4	Anti-inflammatory activity of <i>F. deltoidea</i> leaves fractions	59
5	CONCLUSION	62
	REFERENCES	63

LIST OF TABLE

TABLE NO.	TITLE	PAGE
2.1	The description of skin cells	9
2.2	Description of skin fibers	9
2.3	The structure of compound isolated from <i>F. deltoidea</i>	18
2.4	Type of extraction method	23
3.1	List of chemical and solvent used	32
3.2	Sample ritual of for each well in 96-well plate for elastase inhibition assay of <i>F. deltoidea</i> leaf fraction and crude extract.	43
4.1	Quantitative analysis of vitexin and isovitexin in <i>Ficus deltoidea</i> leaves fractions	47
4.2	IC50 of Inhibition of elastase assay by <i>F. deltoidea</i> leaves fractions.	58

LIST OF FIGURE

FIGURE NO	TITLE	PAGE
2.1	Structure of human skin	7
2.2	UVA and UVB penetration into human skin	11
2.3	Mechanisms of ageing	13
2.4	Photo of <i>Ficus deltoidea</i>	16
2.5	Structure of vitexin and isovitexin	20
2.6	Schematic diagram of ultrasound-assisted extraction	24
2.7	SPE method	27
3.1	Project outline	30
3.2	An illustration of SPE fractionation process	33
3.3	Block of diagram HPLC system	34
3.4	Sircol collagen assay flowchart	39
4.1	The percentage of free radical scavenging activity observed among fractions.	48
4.2	Percentage of total flavanoid content in <i>F. deltoidea</i>	50
4.3	Cell viability of HSF cells cultured with increasing concentration of <i>F. deltoidea</i> leaves fractions and extract using MTT assay	53
4.4	Collagen contains in <i>F. deltoidea</i> leaves fractions	55
4.5	<i>F. deltoidea</i> leaves fractions on inhibition of elastase assay	56
4.6	Inhibition of Hyaluronidase assay activity of <i>F.s deltoidea</i> leaves fractions and extract	59
4.7	Inhibition of Lipoxygenase assay activity of <i>F. deltoidea</i> leaves fractions and extract	60

LIST OF ABBREVIATION

UV	Ultra violet
ROS	Reactive Oxygen Species
AP-1	Activator protein-1
MMP	Matrix metalloproteinase
ECM	Extracellular matrix
SPE	Solid phase extraction
MMP-1	Matrix metalloproteinase-1
MMP-9	Matrix metalloproteinase-9
MMP-3	Matrix metalloproteinase-3
HA	Hyaluronic acid
GAG	Glycosaminoglycan
HYAL	Hyaluronoglucosaminidase
DFT	Density Functional theory
iNOS	inducible nitric oxide synthase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
UAE	Ultrasound-assisted extraction
TFC	Total flavonoid content
MTT	3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
LOX	Lipoxygenase
HPLC	High performance liquid chromatography
UPM	Universiti Putra Malaysia
MeOH	Methanol
F1	Fraction 1
F2	Fraction 2
F3	Fraction 3

F3	Fraction3
F4	Fraction4
F5	Fraction5
TFA	Trifloroaceticacid
DMEM	Dulbecco's modified essential medium
FBS	Fetal Bovine Serum
CO2	Carbon dioxide
HSF	Human skin fibroblast
DMSO	Dimethyl sulfoxide
SCA	Sircol collagen assay
HCL	Hydrochloric acid
SANA	5 mM N-Succinyl-Ala-Ala-Ala-nitroanilide
HNE	human neutrophil elastase
Na₂H₂PO₄	Sodium phosphate
NDGA	Nordihydroguaiaretic acid
SPSS	Statistical Package Social Science
ANOVA	Analysis of variance
UAE	Ultrasonic assisted extraction

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Ageing is a natural phenomenon, a physiological change that is certain to be experienced by each living organism. It is a complex metabolic process that occurs over a period of time through growth, development and maturity. The ageing process is said to happen when the energy to maintain the structural and functional molecules that are synthesized in our life is no longer accessible (Hayflick, 2004). As the bodies reach maturity, the skin appearance and characteristics change. In general, ageing is more prominently seen on skin where thin, dry, unblemished, elasticity-depleted texture and fine wrinkles are the common indicators (Rogers *et al.*, 2008).

Skin is the largest organ of the body with multiple cell types and structures that exhibits multiple functions, among them is a protective barrier between internal organs and outer environment (Fearnley, 2009). The outer part of skin is composed of fibro, protein, collagen and elastin. Collagen is one of the major building block of skin which responsible for elasticity and strength of the skin.

Skin ageing can be divided into intrinsic and extrinsic ageing (Bennett & Cooper, 2009). Intrinsic ageing is determined primarily by oxidative metabolism,

genetic and hormonal factors while extrinsic ageing emphasize on the exposure to the solar ultraviolet (UV) radiation. This UV radiation will induce free radical damage and increase Reactive Oxygen Species (ROS), which responsible for oxidative stresses and inflammatory responses in the dermal or epidermal layer by destructing the connective tissues fibers. Free radical damage will bring about DNA damage, protein and gene modifications. High level of ROS will induce the transcription of Activator protein 1 (AP-1). AP-1 is responsible in regulation of cell growth and differentiation. AP-1 strongly regulated the transcription of Matrix metalloproteinase (MMP), which causes further degradation of mature fibrillar collagen in the skin which contributes to skin ageing (Yin, 2014). Bissett *et al* (1987) reported that the decrease of skin elasticity with premature ageing is significantly correlated with increased elastase and hyaluronidase activity. Therefore, the inhibition of these enzymes may be the most effective therapy to improve the structure of collagen in the extracellular matrix (ECM) and control its metabolism (Mukherjee *et al.*, 2011).

The widespread awareness on depletion of ozone layer and the danger of UV radiation reaching directly to the surface of the Earth has made the society at large become more alert regarding the effect of this harmful ray on their skin. Nevertheless, skin care is not just a matter of health but an affair of beauty as well, which lead to the ever growing skin-based research conducted by various interested parties. In the turn of the decade, high accessibility of information regarding skin care products by consumer demands a greater need for anti-ageing products that are scientifically-proven in its efficiency (Rogers *et al.*, 2008).

Consuming proper food choice might be the larger part of what makes skin age gracefully while remaining healthy, strong and disease-free. Powerful anti-ageing benefits can be obtained from wide range of natural food, spices and herbs that carry high incidence of antioxidant properties. Traditional herbs have been proved to be safe and effective for ageing-related problem and very much often attracted the growing industry of skin-care product with niche in herb-based medicine (Hoffmann, 2013). Treatment of the skin with products containing plant-

derived antioxidant ingredients has been proven useful for the prevention of UV-mediated cutaneous damage. Various parts of the plant are available to be processed and consumed in the form of powder, tablets and extract.

This study attempts to focus on *Ficus deltoidea*, (*F. deltoidea*) a traditional herb from the genus *Ficus* of Moraceae family. *F. deltoidea*, locally known as 'Mas Cotek' is widely distributed in Peninsular Malaysia and a popular medicinal herb among Malay. This miraculous herb has been used traditionally to treat wounds and sore, used as an antidiabetic treatment and an after-birth tonic to contract the uterus and vaginal muscle (Bunawan *et al.*, 2014). *F. deltoidea* also reported to possess powerful antioxidant activity which is good for anti-ageing (Mohd *et al.*, 2015).

Bunawan *et al.* (2014) reported the biological properties of *F. deltoidea* which are antioxidant, antidiabetic, anti-inflammatory, anti-ulcerogenic, wound healing activity, anti-bacterial activity and anticancer activity. Wide ranges of chemical compound have been identified from leaves of *F. deltoidea*. Some of the compound that is stated to be present in the *F. deltoidea* is flavonoids such as isovitexin, vitexin, proanthocyanidins, flavan-3-ol monomers and flavones glycosides (Misbah *et al.*, 2013). The volatile compound identified is mainly product of shikimic acid pathway, terpenoids and aliphatic groups. Vitexin and isovitexin has been reported can be effective for the prevention of free radical scavenging (Kim *et al.*, 2005). Isolation and purification process are important to identify the bioactive compound in *F. deltoidea* leaves.

Fractionation is a separation process in which a certain quantity of a mixture is divided into smaller quantities in which of the composition vary according to the gradient (Baynes, 2017). The use of fractionation could isolate the structural and morphology of identifiable entity for subsequent analysis, the emphasis being on purity at the expense of yield. During Solid phase extraction (SPE), the target analyte and structurally related compounds are adsorbed onto a solid, stationary phase. The solid phase is then washed with selective eluents in order to eliminate any interfering

substances and to reduce the complexity of the matrix. Finally, the bound target analyte is recovered by an elution step (Sigma, 1998). The solvent should enable rapid elution of the analyte from the solid phase. The recovery of organic compounds by SPE is highly dependent on the polarity of the eluents. A study conducted by Barbosa *et al* (2013) proved that purification obtain from the fractionation process yield higher antioxidant activity than the crude extract. Therefore, by applying this approach, reliable information about the potential of chemical compound in the *F. deltoidea* extracts on anti-ageing activity could be well understood and ultimately, a product which can slow the ageing process and make for a younger-looking skin could be produced.

1.2 Problem Statement

Exposure of the skin to UV occurs from both natural and artificial sources. The sun is the main source of UV radiation on human while the depletion of the ozone layer would intensifies the harmful exposure to humankind and the environment. As stated by Sudcl *et al.* (2005), people without natural protection are estimated to account up to 90% visible skin ageing due to the effect of sunlight on the skin. As people age, their concern towards outward appearance increases profoundly and the concept of delaying the process of ageing seems appealing to most person. Today's anti-ageing market is expanding to incorporate diverse consumer concerns.

Recently, many herbs and natural products has been receiving public interest as complement and alternative medicine. Herbs which contain antioxidant and anti-inflammatory are used in cosmetic and dermatological products to improve signs of extrinsic ageing (Ho *et al.*, 2010). *F. deltoidea* extracts have been found to have photo-protective effects on epidermal cell line. A study reported by Mohd *et al.* (2015) stated that *F. deltoidea* has very strong antioxidant properties. Antioxidant can neutralize and stimulate the production of collagen and restore skin elasticity, thus can slow down the ageing process (Watson, 2013). Moreover, Zino *et al.* (1997)

reported the ability of *F. deltoidea* antioxidant that can delay some effects of ageing. A study conducted by Hasham *et al.* (2013) reported that skin ageing was strongly related to the inflammatory process. Leaves of *F. deltoidea* also have been confirmed devoid any toxic elements as reported by Shafei *et al.* (2011) and Farsi *et al.* (2013) also showed that *F. deltoidea* do not have any potential to induce mutation.

Flavanoids are a class of secondary plant phenolics with significant antioxidant and chelating properties (Seawan & Jimtaisong, 2013). Furthermore, flavonoids protect plants from solar UV radiation and scavenge UV generated ROS (Shirley, 1996). Therefore, flavonoids have three different photoprotection effects including UV absorption, direct and indirect antioxidant properties, and modulating several signaling pathways. Vitexin and isovitexin are flavones, which is one kind of flavanoid. These compound have been drawing more attention antioxidant activity and anti-inflammatory activity that can help in slowing the ageing process (He *et al.*, 2016). In order to isolate the bioactive compound in *F. deltoidea* leaves, fractionation method by using Solid Phase Extraction (SPE) was used in this study. Therefore, the proposed project is expected to yield novel insight on *F. deltoidea* leaves fractions on anti-ageing effects and lead to better understanding of anti-ageing properties of *F. deltoidea* leaves which is important and can give benefits to pharmaceutical and cosmeceutical industry.

1.3 Objective

The objective of this research was to investigate the fractionation of *Ficus deltoidea* leaves using solid phase extraction on anti-ageing activity *in vitro*.

1.4 Scope of Research

The scopes of research were:

1. Fractionation of *F. deltoidea* leaves extract by using solid phase extraction (SPE) method.
2. Determination of antioxidant properties of *F. deltoidea* leaves fractions using DPPH free radicals scavenging assay and total flavanoid content.
3. Evaluation of vitexin and isovitexin in *F. deltoidea* leaves fractions using HPLC.
4. Observation and investigation of anti-ageing effects caused by UVB irradiation of *F. deltoidea* leaves fractions on fibroblast cell line.
5. Evaluation of anti-ageing properties of *F. deltoidea* leaves fractions by using sircol collagen assay, MTT assay, elastase assay, hyaluronidase assay, and lipoxygenase assay.

1.5 Significant of Study

The finding of this study will rebound the benefit to pharmaceutical and cosmeceutical industry. From the result, fractionation of bioactive compound by using SPE method can isolate vitexin and isovitexin at mixture of 80% methanol : 20% water. This fraction shows the highest ability to slow down the ageing problem by ability to proliferate high percentage of fibroblast cells, produce more collagen content, inhibit elastase activity lipoxygenase and hyaluronidase assay.

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