EXTRACTION OF VITEXIN AND ISOVITEXIN FROM *FICUS DELTOIDEA* FOR SKIN BARRIER ENHANCEMENT

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ABSTRACT

The understanding of plant extracts' effect on the skin permeability barrier characteristic is important for the development of natural ingredient based cosmetic product. However, the study on the strategy of skin barrier treatment using local plants for topical use is limited. Ficus deltoidea (FD) was chosen in this study as it possesses strong antioxidant, anti–melanogenic and photo–protective activities. The objectives of this study were to optimize the extraction conditions of vitexin and isovitexin compounds from FD leaves, and to evaluate their effects on the skin hydration and skin barrier function properties in vitro and in vivo. FD extract was successfully obtained through ultrasonic–assisted extraction and optimally designed using Box–Behnken design by response surface methodology. Methanol concentration, sonication time and solvent to sample ratio were the independent variables, while yields of vitexin and isovitexin were the dependent variables. The optimal yield of FD extracts was 32.29 ± 0.08 mg/g and 35.87 ± 0.09 mg/g, for vitexin and isovitexin respectively. The optimum extraction conditions were 77.66 % methanol concentration, 20.03 minutes sonication time, and 19.88 mL/g solvent to sample ratio. The optimum extraction conditions required less solvent concentration up to 77.66 %, which is 3 % lower as compared to the preliminary experiment. The cell viability assay showed that the cell growth of human skin fibroblasts treated with FD extract was effectively increased in concentration–dependent manner, compared to untreated control and ascorbic acid. In vitro study showed that FD extract induced keratinocyte differentiation by enhancing the expression of differentiation marker genes of transglutaminase 1, caspase 14, ceramide synthase 3, involucrin, and filaggrin using real–time polymerase chain reaction. The in vivo efficacy study was conducted on 20 female subjects using tape stripping method and the study evaluated the trans–epidermal water loss, hydration, melanin, erythema and elasticity. In addition, the skin lipid analysis using high performance thin layer chromatography showed that the presence of FD extracts increased the ceramide content, and ceramide/cholesterol ratio, compared to other samples. This condition proved the ability of FD extract to enhance the skin barrier function through improvement of skin hydration and epidermal lipid integrity. Overall, the in vitro and in vivo studies have successfully demonstrated the efficacy of FD extracts in the formation of an effective moisture barrier which includes: corneocyte strengthening, lipid processing and natural moisturizing factor generation.

ABSTRAK

Pemahaman mengenai kesan ekstrak tumbuhan terhadap fungsi pertahanan kulit adalah penting untuk pembangunan produk kosmetik yang berasaskan bahan semulajadi. Walau bagaimanapun, kajian mengenai strategi rawatan pertahanan kulit menggunakan tumbuhan tempatan secara topikal adalah sangat terhad. Pokok Mas Cotek (FD) telah dipilih di dalam kajian ini kerana memiliki sifat antioksida yang tinggi serta aktiviti pencerahan, dan fotolindung. Objektif kajian ini adalah untuk mengoptimumkan keadaan pengekstrakan vitexin dan isovitexin daripada daun FD, dan mengkaji kesan vitexin dan isovitexin terhadap hidrasi kulit dan pertahanan kulit secara *in vitro* dan *in vivo*. Ekstrak FD berjaya diperoleh melalui kaedah pengekstrakan berbantu ultrasonik secara optimum dengan menggunakan rekabentuk Box–Behnken melalui kaedah tindakbalas permukaan. Kepekatan metanol, masa sonikasi dan nisbah pelarut kepada sampel adalah pemboleh ubah tidak bersandar, sementara itu hasil vitexin dan isovitexin adalah pemboleh ubah bersandar. Hasil optimum ekstrak FD adalah 32.29 ± 0.08 % dan 35.87 ± 0.09 % masing-masing untuk vitexin dan isovitexin. Keadaan pengekstrakan yang optimum ialah pada kepekatan metanol 77.66 %, masa sonikasi 20.03 minit, dan nisbah pelarut kepada sampel 19.88 mL/g. Proses optimum memerlukan kepekatan pelarut yang kurang sehingga 77.66%, iaitu 3 % lebih rendah berbanding eksperimen awal. Ujian keupayaan sel menunjukkan bahawa ekstrak FD secara efektif meningkatkan pertumbuhan sel–sel fibroblas kulit manusia dan pertumbuhan ini bergantung pada kepekatan ekstrak, berbanding dengan sel yang tidak dirawat dan asid askorbik. Kajian *in vitro* menunjukkan bahawa ekstrak FD dapat mendorong pembezaan keratinosit dengan meningkatkan ekspresi gen penanda pembezaan transglutaminase 1, caspase 14, ceramide synthase 3, involucrin, dan filaggrin menggunakan tindak balas berantai polimeras masa sebenar. Kajian keberkesanan *in vivo* telah dilakukan ke atas 20 subjek wanita dengan menggunakan kaedah pelucutan pelekat dengan menentukan kehilangan air daripada trans–epidermis, penghidratan, melanin, eritema dan keanjalan. Selain itu, analisa komposisi lipid kulit menggunakan kromatografi lapisan nipis berprestasi tinggi menunjukkan bahawa ekstrak FD secara signifikan meningkatkan kandungan ceramide, dan nisbah ceramide/kolesterol berbanding dengan sampel lain. Ini menunjukkan bahawa ekstrak FD dapat meningkatkan fungsi pertahanan kulit melalui peningkatan penghidratan kulit dan integriti lipid epidermis. Secara keseluruhan, kajian *in vitro* dan *in vivo* ini berjaya menunjukkan keberkesanan ekstrak FD dalam pembentukan pertahanan kelembapan berkesan yang juga merangkumi: pengukuhan sel korneosit, pemprosesan lipid, dan penghasilan faktor pelembap semula jadi.

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LIST OF ABBREVIATIONS

AD	–	Atopic Dermatitis
AE	–	Atopic Eczema
ANOVA	–	Analysis of variance
BBD	–	Box–Behnken Design
C	–	Carbon
Ca^{2+}	–	Calcium Ions
CAMP	–	Cathelicidin Antimicrobial Peptide
CCD	–	Central Composite Designs
cDNA	–	Complementary Deoxyribonucleic Acid
CE	–	Corneocyte Envelope
CERs	–	Ceramide
CERS 3	–	Ceramide Synthase 3
CHOL	–	Cholestrol
CO_2	–	Carbon Dioxide
CoA	–	Coenzyme
COX–2	–	$Cyclooxygenase_2$
CREB	–	CAMP Response Element Binding Protein
CSO_4	–	Cholesterol Sulphate
CV	–	Coefficient of Variation
DMEM	–	Dulbecco's Modified Essential Medium
DMSO	–	Dimethyl Sulfoxide
DNA	–	Deoxyribonucleic Acid
DPPH	–	Dihydrosphingosine

EEMCO	–	European Group for Efficacy Measurement of Cosmetics and other Products
EGF	–	Epidermal Growth Factor
FBS	–	Fetal Bovine Serum
FD	–	*Ficus deltoidea*
FFAs	–	Epidermal Growth Factor
FLG	–	Filaggrin
FRAP	–	Ferric–Reducing Antioxidant Power
FTC	–	Ferric thiocyanate
GAPDH	–	Glyceraldehyde 3–phosphate dehydrogenase
GNI	–	Gross National Income
GST	–	Glutathione S–transferase
H_2O	–	Water
HA	–	Hyaluronic Acid
HaCaT	–	Human Keratinocytes Cell
HEKn	–	Human Epidermal Keratinocytes
HKGS	–	Human Keratinocyte *Growth Supplement*
HMG–CoA	–	Hydroxy–3–methylglutaryl–coenzyme
HPTLC	–	High Performance Thin Layer Chromatography
HSF	–	Human Skin Fibroblast
HSV	–	Herpes Simplex Virus
IL	–	Interleukin
iNOS	–	Inducible Nitric Oxide Synthase
INV	–	Involucrin
LB	–	Lamellar Bodies

MAE	–	Microwave–Assisted Extraction
MAPK	–	Mitogen–Activated Protein Kinase
MARDI	–	Malaysia Agriculture Research and Development Institute
mBD3	–	Mouse β–defensin 3
MITF	–	Microphthalmia–associated Transcription Factor
min	–	Minutes
MMP–1	–	Metalloproteinase–1
MPA	–	Multi Probe Adapter
mRNA	–	Messenger Ribonucleic Acid
MTT	–	3–(4,5–Dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide
NF–κB	–	Nuclear Factor–kappab
NKEA	–	National Key Economic Area
NMF	–	Natural Moisturizing Factor
NMRR	–	National Medical Research Register
NO	–	Nitric Oxide
NSAIDs	–	Nonsteroidal Anti–Inflammatory Drugs
OFAT	–	One Factor at a time
PAK 1	–	p21–Activated kinase 1
PCA	–	Pyrrolidone Carboxylic Acid
PGE_2	–	Prostaglandin E2
PMNs	–	Polymorphonuclear Leukocytes
RECUKM	–	Research Ethics Committee Universiti Kebangsaan Malaysia
RM	–	Ringgit Malaysia
RNA	–	Ribonucleic Acid

RNS	–	Reactive Nitrogen Species
ROS	–	Reactive Oxygen Species
RSM	–	Response Surface Methodology
RT–PCR	–	Real–Time Polymerase Chain Reaction
SC	–	Stratum Corneum
SD	–	Standard Deviation
SEM	–	Standard Error Mean
SG	–	Stratum Granulosum
SPSS	–	Statistical Package for the social sciences
TBA	–	Thiobarbituric Acid
TEWL	–	Trans–epidermal Water Loss
TG	–	Triglyceride
TGase–1	–	Transglutaminase 1
TLC	–	Thin Layer Chromatography
TNF– α	–	Tumor Necrosis Factor–Alpha
UAE	–	Ultrasonic–assisted Extraction
UGT	–	*UDP–glucuronosyltransferase*
UKMMC	–	Universiti Kebangsaan Malaysia Medical Centre
UPM	–	Universiti Putra Malaysia
US	–	United States
UVB	–	Ultraviolet B
α –MSH	–	α–Melanocyte–stimulating hormone

LIST OF SYMBOLS

℃	–	Degree Celsius
R^2	–	R–squared
g	–	Gram
mg	–	Miligram
mm	–	Milimeter
mM	–	Milimolar
ppm	–	Parts per million
w/w	–	Weight per weight
v/v/v	–	Volume per volume per volume
σ	–	Standard deviation
µm	–	Micrometer
nm	–	Nanometer
ml	–	Milliliter
mbar	–	Millibar
µg	–	Microgram
µl	–	Microlitre
ms	–	Micro–siemens
cm	–	Centimeter
hz	–	Hertz
khz	–	Kilohertz
N	–	Sample
W	–	Watt

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Skin is the largest organ of the human body. The primary function of skin is to protect the body from unwanted influences in the environment and prevent water loss. The main barrier of the skin is in the outermost layer of the skin, stratum corneum (SC). The SC consists of corneocytes surrounded by the lipid regions, organized into parallel stacks of lamellar bilayers. These two structural compartments are known as 'bricks and mortar model'. The water–repellent lamellar layers consist of three main SC lipids: ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA) in an approximately 1:1:1 molar ratio (Feingold, 2007; Wertz, 2006). An equimolar mixture of SC lipids is important to maintain the permeability barrier homeostasis and excellence epidermal barrier function. The barrier act as critical defensive functions not only towards the outward water and electrolytes but deflecting the ultraviolet (UV) light before a deeper penetration occurred into the skin (Berkers et al., 2019).

There are several factors that can affect the role of the skin barrier. Recently, the increased levels of trans–epidermal water loss (TEWL) after mechanical disruption, epithelial differentiation defects such as lack of filaggrin (FLG) and altered SC intercellular lipid composition were proven as the triggered factors (Czarnowicki et al., 2017; Smith et al., 2017). Skin barrier defect is associated with the increased proliferation of keratinocytes which is known as cornification or keratinization, as well as increased production of inflammatory cytokine in the local

tissue. Alterations in the epidermal cornification process would also affect the formation of the skin barrier (Hanel et al., 2013). Dysregulated of these processes are closely connected with several skin diseases such as psoriasis and atopic dermatitis (AD).

The uprising of consumer concern towards natural ingredients consumption increased the market demands for natural cosmetic products. It is also supported by the presence of highly potential natural ingredients in the product. The alternative treatments using certain types of plants and their isolated compounds were traditionally used for medical purposes. The high popularity of herbs and plants in cosmetic are due to many advantages such as an environmentally friendly and have fewer side effects as compared to the synthetic based cosmetics. Natural cosmetics are believed to be safer for people and friendlier to the nature (Elsner and Maibach, 2005; Chen, 2009). Furthermore, synthetic based cosmetic contains occlusive ingredients (petroleum based, silicone based, zinc oxide, mineral oil, mineral wax) that may lead to the skin reaction, allergies and irritation, especially those with sensitive skin (Purnamawati et al., 2017). In addition, cosmetics products with hazardous chemicals could give harmful effects prior to the long–term usage. Owing to the side effects of the synthetic ingredients, it is crucial to choose a lower risk natural treatment that could also effectively react. Thus, natural cosmetic especially from plants may provide a beneficial skin health effect.

Ficus deltoidea (FD) or widely known as Mas Cotek in Malaysia, is a plant originated from Moraceae family. It has been traditionally used as medicine for several diseases such as diabetes and relieving pain. The presence of antioxidant compounds in FD such as flavonoids, tannins, polyphenols, saponins, triterphenoids, and proanthocyanins was previously reported by Malaysia Agriculture Research and Development Institute (MARDI). Several researches were reported on the application of FD in biological activities such as anti–melanogenic, anti–inflammatory, antinociceptive, antioxidant and anti–photoaging effects (Oh et al., 2011; Zakaria et al., 2012; Hasham et al., 2013; Misbah et al., 2013). The isolated

compounds of vitexin and isovitexin were mostly detected as the biomarker of FD for many assays (Abdullah et al., 2009; Choo et al., 2012; Farsi et al., 2014). Furthermore, Malaysian Herbal Monograph (MHM) also declared that vitexin and isovitexin are the biomarkers for FD plant.

An efficient extraction procedure that able to limit the decomposition of the extracted compound during the process is extremely important. This method is crucial to optimize the chemical constituents in plant material. Insertion of ultrasound in the extraction part of the process is by far the most convenient. It is commonly known as ultrasonically assisted extraction (UAE). Pan et al. (2003) reported on the efficient application of ultrasonic method in flavonoid extraction. The mechanism of UAE is attributed to the mechanical and cavitation efficacies, which could possibly result in the disruption of the cell wall, particle size reduction, and enhanced mass transfer across the cell membrane (Hossain et al., 2012). Furthermore, the processing parameters such as solvent concentration, sonication time and solvent to sample ratio are also important to achieve the optimum concentration of bioactive compounds. Recently, the Response Surface Methodology (RSM) optimization analysis using a statistical software tool is highly recommended as it enables the measurement of the effects of multiple factors and their interactions on one or more response variables with fewer runs and faster performance (Bezerra et al., 2008). In this study, the production of FD extract was mainly carried out through UAE and optimized using Box Behnken design by Design–Expert software.

Different model studies such as cell–based assay and human skin biophysical and molecular changes are developed to investigate the potential use of natural plant products or plant extracts for the prevention or treatment in skin barrier diseases. The formation of normal skin barrier components (corneocytes and intercellular lipids) requires two different events to be completed. The first event is the maturation of cornified envelope (CE) that replaces cell membrane of viable keratinocytes with the aggregates of proteins such as involucrin (INV) and loricrin tightly cross–linked each other by transglutaminase–1 (TGase–1). The second event is the anucleated corneocyted, flattened by the compressive action of FLG on keratin intermediated filaments (KIF). This part should be filled with natural moisturizing factor (NMF)

components such as 2–pyrrolidone–5–carboxylic acid (PCA), urocanic acid (UCA) and other amino acids derived from degradation of FLG through hydrolysing processes which involves caspase 14 (Joo et al., 2015). Therefore, an *in vitro* study of gene expression on INV, ceramide synthase 3 (CERS 3), TGase–1, caspase 14 and FLG can be determined using quantitative Real–time Polymerase Chain Reaction (Real–time PCR) to evaluate the effect of plant extract on skin hydration and barrier function towards human epidermal cell. Real–time PCR was used to investigate the transcriptome in skin barrier, resulting in the identification of various pathomechanisms, such as abnormalities in epidermal differentiation (Lu et al., 2009; Winge et al., 2011), inflammatory pathways (Guttman–Yassky et al., 2007; Saaf et al., 2008), and lipid homeostasis (Mathay et al., 2011).

Meanwhile, an *in vivo* tape stripping model has been widely accepted as a dermal sampling technique for the human study, or specifically in dermatology (Dreher et al., 2005, Svoboda et al., 2017; Choi et al., 2018). This method was conducted to gradually remove the corneocytes layer using an adhesive film (Weerheim and Ponec, 2001). Epidermal components such as proteins and lipids which were collected in the adhesive film are further extracted and analysed using various chromatography techniques. Jamin et al. (2019) developed a quick and easy method for the quantification of ceramides, cholesterol, and free fatty acids using High–Performance Thin Layer Chromatography (HPTLC) with UV detection. Similarly, Kilic et al. (2019) used HPTLC to measure different cholesterol/fatty acid/ceramide of lipid lamellae ratios in the different age of subjects. These evaluation techniques could establish the differences in the skin structure and function after the treatment of plant extract *in vitro* and *in vivo*.

1.2 Problem Statements

The rise of natural cosmetic products on the market is triggered by the current demand for going green. Consumer concern and the immense potential of natural ingredients lead to widespread usage in different industries (e.g. pharmaceutical, nutraceutical and cosmeceutical). Certain herbs are currently popular because their ingredients may not only be rich in antioxidants, but also have protective effects on the skin. FD is a popular medicinal herb in Malaysia and well–known for its beneficial effects towards women's health (Oh et al., 2011; Zakaria et al., 2012; Choi et al., 2018). However, research on epidermal protecting activity *in vitro* and on human study is still limited. FD contains various bioactive compounds such as flavonoids, vitexin, isovitexin, saponins, tannins, polyphenols, triterpenoids, and proanthocyanins. The active biomarker compounds of vitexin and isovitexin have biological activities such as anti–hypertensive, anti–inflammatory, antispasmodic, antimicrobial, anti–melanogenic, and high antioxidant (Abdullah et al., 2009; Hasham et al., 2014). Yet, their effects on epidermal cells remain to be elucidated.

The growing demand of plant extract was not only on the quantity basis, but also on the quality basis, including for the utilization of green extraction. However, non–standardized procedures of extraction may lead to the degradation of bioactive compounds in the plant extract. The FD leaves were extracted using UAE and optimized using Box–Behnken Design (BBD) in Design Expert software, according to biomarker compounds of vitexin and isovitexin. Unlike the conventional extraction methods, the UAE method for the extraction of active compounds from plants is a suitable, cost effective, faster kinetic, efficient in producing higher yield and low effect on plants physiologically active compounds (Fomo et al., 2020). Alim et al. (2016) and Ong et al. (2016) also reported UAE as an efficient in flavonoid extraction of FD plants compared to conventional extraction.

In vitro models based on human cells are among the reliable methods to study the epidermal barrier protecting activities of FD extract. It is widely used since the authorities of the European Union have prohibited the testing of final cosmetic products or part of their ingredients on laboratory animals. They also banned the marketing of cosmetic products tested on animals outside the EU (Regulation (EC) No 1223/2009) and legal restrictions have further been argued for the development and assessment of *in vitro* models that involve cutaneous tissues (Savic and Paunovic, 2018). The *in vitro* models were used in this study to determine the eventual toxic properties of FD on skin and their effects on skin hydration and barrier function. To the best of knowledge, this is the first study to identify the skin moisturizing effect of FD extract by modulating expression of FLG and caspase 14, which involved in NMF production in corneocytes. FD extract was also investigated on the potential of enhancing the synthesis of CER that provide the mechanism of anti–aging properties which supported the effect on skin barrier function.

On top of that, the human study was also determined based on the TEWL factor. TEWL is a marker of damaged epidermal barrier function, and increased levels of TEWL after mechanical disruption, such as tape stripping, are correlated to the lower hydration values (Choi et al., 2018). In addition, the tape stripping method is also a non–invasive method that allows the accessibility of the skin tissue, the process of measuring skin lipids both quantitatively and qualitatively (Jacobi et al., 2005; Svoboda et al., 2017). An improved HPTLC method was conducted for quantitative analysis of SC lipids. The developing solvent was optimized and the calibration curved was developed using the Michaelis Menten equation.

The understanding on the FD extract effects and mechanisms on the skin barrier activity towards the response of topical application is important for the skin care products development, as well as for the prevention and treatment of skin diseases. The correlation between skin and usage of cosmetic is always a concern in the cosmetic industry. However, the study about FD topical application on skin barrier function was poorly reported. Thus, this study was aimed to obtain a better insight of the skin structure, function, and physiology after the treatment with FD extract.

1.3 Objective

The objectives of this study are as follows:

a) To optimize the extraction conditions for vitexin and isovitexin compounds from *Ficus deltoidea* leaves by ultrasonic assisted extraction using Box–Behnken Design by Response Surface Methodology with three parameters of methanol concentration (%), sonication time (min) and solvent to sample ratio (mL/g).
b) To evaluate the effect of *Ficus deltoidea* extract on the skin hydration and barrier function in human skin cells.
c) To investigate the effect of *Ficus deltoidea* extract based formulation on the skin biophysical properties and lipid composition during skin barrier recovery.

1.4 Scopes of the Research

The scopes of the research were identified to achieve the objectives. The scopes are listed as follows:

a) Identification of processing parameters (methanol concentration (%), sonication time (min) and solvent to sample ratio (mL/g)) that effect the biomarkers content of *Ficus deltoidea* extract using one factor at a time (OFAT) method.
b) Optimization of *Ficus deltoidea* extract processing conditions on the extraction of the biomarkers content using Response Surface Methodology (RSM) with Box–Behnken Design (BBD). Methanol concentration (%), sonication time (min) and solvent to sample ratio (mL/g) were the independent variables, while yield of vitexin and isovitexin were the dependent variables.

c) Characterizing of cell viability on *Ficus deltoidea* extract using MTT assay on human skin fibroblast (HSF) cell lines.
d) Investigation of *Ficus deltoidea* extract on gene expressions of involucrin (INV), ceramide synthase 3 (CERS 3), transglutaminase 1 (TGase–1), caspase 14 and filaggrin (FLG) using Real–time Polymerase Chain Reaction (Real–time PCR) in determining skin hydration and barrier function of human skin cells.
e) Evaluation of skin barrier recovery on *Ficus deltoidea* extract based formulation using a non–invasive tape stripping technique.
f) Evaluation of skin biophysical changes on 20 female subjects of 20–35 years old after application of *Ficus deltoidea* extract based formulation. Skin TEWL, hydration, melanin, erythema index and elasticity were evaluated by using multiprobe adapter systems (MPA) for 14 days.
g) Investigation of lipid composition on the application of *Ficus deltoidea* extract based formulation using High Performance Thin Layer Chromatography (HPTLC).

1.5 Significance and Original Contributions of the Research

FD has a wide spectrum of traditional uses, as well as in terms of the biological and pharmacological properties. The scientific findings on the effect of FD extract may provide useful insight into the understanding of FD mechanism of action *in vitro* and *in vivo* study. New emerging lipid composition data suggested that FD extract had demonstrated the potential skin barrier activities. The results obtained through this study would beneficially provide a critical observation on the correlation of epidermal lipids variation and skin barrier function. The ordered alignment of lipid formed a closed system to prevent TEWL and provided the SC more impermeable in maintaining the skin barrier function. Thus, it is also a preliminary step towards the real application of FD topical uses for skin treatments. Additionally, FD extract was proven as the potential treatments for the skin ailments based on the previous studies.

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