# ANTI INFLAMMATORY AND ANTI OXIDANT PROPERTIES OF PURIFIED STILBENE AND CRUDE EXTRACTS OF PERLIS *Vitis vinifera* ON CULTURED HUVECs

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*In the memories of my late grandmother,*

*Tok Baayah binti Che Mat*

*May ALLAH rest her soul in eternal peace (1927- April 2013)*

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# ABSTRACT

Air-dried and particle form of stems and leaves of *Vitis vinifera* cultivated in Perlis, Malaysia were extracted using cold, back, methanolic and ethanolic extraction methods. The ethyl acetate stems extract by back extraction (STBE EA) possessed the highest total phenolic content (916.27 ± 0.01) by Folin-Ciocalteu assay and radical scavenging activity at $IC_{50}$ = 18 μg/mL by DPPH assay. The extracts was further investigated by thin layer chromatography resulted in detection of resveratrol at $R_f$ = 0.425 in STBE EA using petroleum ether:ethyl acetate at ratio 2:3. Advanced screening method of phenolic profiling by LC-MS/MS has detected seven phenolic compounds which were identified through LC-MS/MS library and Mass Bank Software. The compounds are *trans*-resveratrol, *trans*-cinnamic acid, ε-viniferin, salicylic and caffiec acid, catechin, and epigallocatechin. Purification of the STBE EA has resulted in the isolation of *trans*-stilbene which was identified as 3, 4', 5-trihydroxystilbene (*trans*-resveratrol) by Nuclear Magnetic Resonance. The STBE EA and *trans*-resveratrol exhibited protective effects on HUVECs against oxidative cell damage by hydrogen peroxide ($IC_{50}$ = 205 μM) with survival rate 17.69% ($IC_{50}$ = 1 μg/mL) and 40.80% ($IC_{50}$ = 0.01 μM) and induced nitric oxide 3.41 ($IC_{50}$ = 0.001 μg/mL) and 3.96 mm ($IC_{50}$ = 0.001 μM) respectively. STBE EA and *trans*-resveratrol possessed dual inhibition of COX-2 and 15-LOX 85.07 ± 1.20 (COX-2) and 60% with $IC_{50}$ at 7.5 μM (15-LOX) respectively. Furthermore purified *trans*-resveratrol exhibited better inhibition at 96.87 ± 0.10% for COX-2 and 79.57% for 15-LOX. This study demonstrated that STBE EA extracts and *trans*-resveratrol isolated from *Vitis vinifera* stems posses potent anti-oxidant and anti-inflammatory properties.

# ABSTRAK

Pengekstrakan batang dan daun *Vitis vinifera* yang ditanam di Perlis, Malaysia diekstrak menggunakan teknik rendaman sejuk, bersiri, metanol dan etanol. Keseluruhan 16 ekstrak menjalani penyaringan terhadap ciri-ciri anti-oksida. Ekstrak etil asetat batang (STBE EA) melalui kaedah pengekstrakan bersiri memiliki kandungan jumlah fenol tertinggi (916.27 ± 0.01) melalui kaedah Folin-Ciocalteu dan pemerangkapan radikal bebas pada $IC_{50}$ 18 μg/mL melalui kaedah DPPH. Komposisi fenolik di dalam ekstrak terus dikaji dan menunjukkan kehadiran resveratrol di $R_f$ = 0.425 pada ekstrak STBE EA menggunakan petroleum eter:etil asetat pada nisbah 2:3. Kaedah saringan profil fenolik oleh LC-MS/MS berjaya mengesan tujuh sebatian fenolik yang dikenal pasti melalui pengumpulan data LC-MS/MS dan perisian Mass Bank. Kompaun-kompaun dikenali sebagai *trans*-resveratrol, asid *trans*-cinnamic, ε-viniferin, asid salisilik, asid caffiec, catechin, dan epigallocatechin. Penulenan STBE EA telah menemui *trans*-stilbene yang telah dikenal pasti sebagai 3, 4 ', 5-trihydroxystilbene (*trans*-resveratrol) berdasarkan kaedah spektroskopi oleh Resonans Magnetik Nuklear. STBE EA dan *trans*-resveratrol masing-masing menunjukkan kesan perlindungan pada HUVECs terhadap kerosakan oksidatif sel oleh hidrogen peroksida ($IC_{50}$ = 205 μM) dengan peningkatan sel hidup 17.69% ($IC_{50}$ = 1 μg/mL) dan 40.80% ($IC_{50}$ = 0.01 μM) dan nitrik oksida teraruh pada kadar 3.41 ($IC_{50}$ = 0.001 μg/mL) dan 3.96 mm ($IC_{50}$ = 0.001 μM). STBE EA dan *trans*-resveratrol memiliki dwi-perencatan COX-2 dan 15-LOX pada 85.07 ± 1.20 dan 60% dengan $IC_{50}$ pada 7.5 μM masing-masing. *Trans*-resveratrol tulen menunjukkan perencatan tertinggi pada 96.87 ± 0.10% untuk COX-2 dan 79.57% untuk 15-LOX. Kajian ini menunjukkan bahawa ekstrak STBE EA dan *trans*-resveratrol yang diasingkan daripada batang *Vitis vinifera* memiliki sifat-sifat anti-oksida dan anti-radang.

# TABLE OF CONTENTS

# LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| $^{\circ}C$ | Degree celcius |
| µg | Microgram |
| µM | Micromolar |
| $^{13}C$ | 13-Carbon |
| 15-LOX | 15-Lipooxygenase |
| $^{1}H$ | 1-Hydrogen |
| 5-LOX | 5-Lipooxygenase |
| ANOVA | Analysis of variance |
| C | Carbon |
| CC | Column chromatography |
| $CO_2$ | Carbon dioxide |
| COX-1 | Cyclooxygenase-1 |
| COX-2 | Cyclooxygenase-2 |
| DMSO | Dimethyl sulfoxide |
| DPPH assay | 2,2-diphenyl-1-picrylhydrazyl |
| EGM-2 | Endothelial growth media-2 |
| EIA | Enzyme immunoassay |
| ESI | Electrospray ionization |
| *et al.*, | And others |
| *etc.* | *Et cetera* |
| EtOH | Ethanol |
| g | Gram |

| | |
|---|---|
| GAE | Gallic acid equivalent |
| GC | Gas chromatography |
| $H_2O_2$ | Hydrogen peroxide |
| $H_2O_2$ | Hydrogen peroxide |
| He | Helium |
| HUVECs | Human umbilical vein endothelial cells |
| Hz | Hertz |
| $IC_{50}$ | Inhibitory concentration at 50 % |
| L/min | Litre/minutes |
| LC | Liquid chromatography |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| LDL | Low density lipoprotein |
| LTs | Leucotrienes |
| LV80 ET | Leaves crude extracts of *Vitis vinifera* derives from 80% ethanol extraction techniques using ethanol to water |
| LV80 ME | Leaves crude extracts of *Vitis vinifera* derives from 80% methanol extraction techniques using methanol to water |
| LVBE EA | Leaves crude extracts of *Vitis vinifera* derives from back extraction techniques using methanol |
| LVBE ME | Leaves crude extracts of *Vitis vinifera* derives from back extraction techniques using methanol |
| LVBE PE | Leaves crude extracts of *Vitis vinifera* derives from back extraction techniques using methanol |
| LVCE EA | Leaves crude extracts of *Vitis vinifera* derives from cold extraction techniques using ethyl acetate |
| LVCE ME | Leaves crude extracts of *Vitis vinifera* derives from cold extraction techniques using methanol |
| LVCE PE | Leaves crude extracts of *Vitis vinifera* derives from cold extraction techniques using petroleum ether |
| LXs | Lipoxins |

| | |
|---|---|
| *m/z* | Mass to charge ratio |
| MeOH | Methanol |
| mg | Milligram |
| MHz | Mega hertz |
| min | Minute |
| mL | Milliliter |
| mM | Millimole |
| $MS^2$ | Mass spectrometry/mass spectrometry |
| MTT assay | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| $N_2$ | Nitrogen |
| NaOH | Sodium Hydroxide |
| nm | Nanometer |
| NMR | Nucleus magnetic resonance |
| NO | Nitric oxide |
| OH | Hydroxyl group |
| ROS | Reactive oxygen species |
| SFC | Supercriticial fluid chromatography |
| $SiO_2$ | Silica gel |
| SPSS | Statistical analysis system |
| ST80 ET | Stem crude extracts of *Vitis vinifera* derives from 80% ethanol extraction techniques using ethanol to water |
| ST80 ME | Stem crude extracts of *Vitis vinifera* derives from 80% methanol extraction techniques using methanol to water |
| STBE EA | Stem crude extracts of *Vitis vinifera* derives from back extraction techniques using ethyl acetate |
| STBE ME | Stem crude extracts of *Vitis vinifera* derives from back extraction techniques using methanol |
| STBE PE | Stem crude extracts of *Vitis vinifera* derives from back extraction techniques using petroleum ether |
| STCE EA | Stem crude extracts of *Vitis vinifera* derives from cold extraction techniques using ethyl acetate |
| STCE ME | Stem crude extracts of *Vitis vinifera* derives from cold |

| | |
|---|---|
| | extraction techniques using methanol |
| STCE PE | Stem crude extracts of *Vitis vinifera* derives from cold extraction techniques using petroleum ether |
| TPC | Total phenolic content |
| USDA | United states department of agriculture |
| UV | Ultra Violet light |
| v/v | Volume over volume |
| VLC | Vacuum liquid chromatography |
| w/w | Weight over weight |
| WHO | World Health Organisation |

# LIST OF APPENDICES

# CHAPTER 1

# INTRODUCTION

## 1.1. Background of Research

Consumption of plant derived medicine has experienced rapid growing interest and is increasing extensively in both traditional and modern medicinal purpose. Since Sumerian civilisation dated 400 years before the Common Era, the therapeutic use of plants has been discovered. In western medicine, it has been reported that Dioscorides has successfully produced *De Materia Medica,* a compilation of information on more than 600 species of plants with medicinal value (Borzelleca *et al*., 2008) while Hippocrates has been recorded using approximately 4000 different plant species for medical purpose. Natural products play a major role in the ancient traditional medicine system.

In decades, the uses of natural derived medicine were still in practice by Chinese, Ayurveda, and Egyptian (Sarker *et al*., 2006). According to the statistic data by World Health Organization (WHO), herbal plant have been used for healthcare requirements by more than 80% of the world population, generally in developing countries (Farnsworth *et al*., 1985; Canter *et al*., 2005). After extensive research over 50 years, the National Institutes of Health have recently suggested that a diet rich in fresh foods derives from plant may reduces the risk of diseases including inflammation and cardiovascular diseases. Indeed, the right foods may act as medicines which increase the strength of immune system.

Cragg *et al*. (2002) has reported that, several numbers of modern drugs have been developed from medicinal plants due to their rich sources of bioactive compounds over the last century. Natural compounds such as phenolic acids, stilbenes, flavonoids, isoflavonoids, and lignans present diversely in plants may be responsible for their health benefits.

Based on previous studies, Vitaceae family is one of dicotyledonous flowering plants that produce many bioactive compounds in their biological activity. *V.vinifera* or commonly known as grapevine which belong to Vitaceae family are native to Mediterranean region, and Central Europe. It is liana flaky bark, with alternate leaves, palmately lobed and broad. The fruit is a berry known as grape. Grape was originally used in winemaking and the leaves were widely used in Indian Ayurvedic medicinal purposed. Since 1980 from its first trial to be grown in Malaysia, grape has experienced rapid growing interest in Malaysia's agricultural sector due to its high demand of local grown grape fruits. The sector itself has grown up to thousand hectares in 2010. After pruning season, large quantity of stems and leaves would remain on the field producing between 0.62 to 2.03 kg per plant. It is estimated that average pruning waste yielded approximately 5 tonnes per hectare of plant per year.

Ignat *et al*. (2011) proposed that phenolic compounds including flavonoids and stilbenes were found abundantly in agro-industrial residues compare to fruits and fresh vegetables itself. Phenolic compound comprise a large group of secondary metabolites obtain from phenylalanine and widely distributed throughout the plant. Phenolic compounds plays a pivotal role as imparting colours in plants' parts (leaves and fruits), appealing or repelling insects, and plant self-protection against harmful attack or injuries and ultraviolet radiation.

Chemically, phenolic compounds comprising an aromatic ring bearing one or more hydroxyl groups, including their derivatives. More than 8000 phenolic compounds have been identified in plants which predominantly substituted derivatives of hydrobenzoic and hydrocinnamic acids (Robbins, 2003; Wrolstad, 2005). These derivatives differ in patterns of hydroxylation process of their aromatic rings (Harborne, 1994). The phenolic composition and its biosynthesis in plant are highly influence by viticulture and various biotic factors such as temperature, sunlight, rainfall, pathogenesis and soil nutrient content.

Downey *et al*., (2006) observing the reduction in grape berry colour in hot climate. An additional climate impact on phenolic compounds is the positive relationship between sunlight exposure and increased flavonol accumulation. Mori *et al*., (2005) reported that anthocyanin levels in Cabernet Sauvignon grapes are higher when day temperatures are constant at 20˚C compared to 30˚C. Therefore, increasing in anthocyanin content is highly related with grapes grown at higher altitudes. However, this association is complicated by the effect of diurnal differences in temperature with lower night temperature result in greater accumulation of anthocyanins.

Increasing in epidemiological studies and data collections suggests that high intake of fresh plant-based food provides an extensive number of health benefits against degenerative disease. Due to diverse phenolic composition in the plant, it possessed antioxidant activity which contributes to their protective effects against cardiovascular disease and cancer (Howitz *et al*., 2003; Cho *et al*., 2006; Valenzano *et al*., 2006). Several specific plant phenolic compounds derives from plant extracts such as resveratrol and quercetin have been reported to exhibit anti-oxidant and anti-inflammatory via *in vitro* or *in vivo* (Pearson, 2008).

Therefore, in assessing the potential value of *V.vinifera* residues, it is pertinent to determine the phenolic compounds composition of its crude extracts and its anti-oxidant and anti-inflammatory properties. It has been shown that anti-oxidant and anti-inflammatory property varies with types of phenolic compounds present in the plant. Furthermore, the combination between phenolic compounds or together with other phytochemicals in extracts may react by different mechanisms, often interact synergistically or inhibitorily (Fuhrman *et al*., 2000; Graversen *et al*., 2008).

Inflammation is a basic fundamental pathological mechanism which underlies an array of disease (Tedgui, 2001). The inflammatory reaction may occurs by interactions between inflammatory cells such as neutrophils, lymphocytes, and monocytes, and vascular cells - endothelial cells and smooth muscle cells. The nature of inflammatory reaction can potentially be stimulated by multiple cytokines and growth factors which are present at sites of inflammation. Endothelial cells and smooth muscle cells must integrate the signals produced by these multiple factors to effectively regulate the immunoinflammatory response during the expression of adhesion molecules, cytokines, chemokines, matrix metalloproteinases, and growth factors.

Research in vascular biology has progressed astonishingly in last decade, resulting in a better understanding of the vascular cell responses to inflammatory stimuli and identification of major intracellular inflammatory signalling pathways, particularly nuclear factor- κB (NF-κB) system. NF-κB may be found in all animal cell types and entailed in cellular response to stimuli stress, cytokines, free radicals, and oxidized Low Density Lipoprotein (LDL).

This study is designed to screened and build phenolic compound profiling, isolating targeted stilbene and investigate the anti-oxidant and anti-inflammatory effect of this tropical isolated stilbene on Human Umbilical Vein Endothelial Cell (HUVEC) by COX-2, 15-LOX and Nitric Oxide (NO) assay.

## 1.2. Problem statement

Phenolic compounds and its biosynthesis were present in plant tissue as phytoalexins which is induced by biotic and abiotic stresses. Hence, the amount of phenolic compounds in grape is based on the variety of grapevine and influenced by viticultural (greenhouse, *etc*.) and environmental factors such as light, environment temperature, altitude, soil nutritional status, rain, pathogenesis, and other various development process (Downey *et al*., 2006). Thus, the attention of this research is focused on the stilbenes content from grapes of tropical countries such as Malaysia which has a hot, humid tropical climate throughout the year.

## 1.3. Objectives of study

The objectives of this research are:

1. To evaluate total phenolic content and anti-oxidant activities of plant crude extracts by Folin-Ciocalteu and DPPH assay.
2. To develop LC-MS/MS library and rapid screening of phenolic compounds in selected crude extract of *V.vinifera*.
3. To isolate, purify, identify, and quantify targeted compound from selected active crude extract by Vacuum Liquid and Column Chromatography, and NMR Spectro-analysis.
4. To evaluate oxidative damage by hydrogen peroxide and protective effect of purified stilbene and selected crude extract on HUVECs
5. To evaluate the anti-inflamatory effect of purified stilbene and selected crude extract by production of Nitric Oxide and dual inhibition of COX-2 and 15-LOX.

## 1.4. Scope of study

In this research, stems and leaves of *V.vinifera* were extracted using four different extraction methods. The total phenolic content and anti-oxidant activities were evaluated by Folin-Ciocalteu and DPPH assay. Rapid screening in phenolic compounds profiling were analysed by simple thin layer chromatography technique and LC-MS/MS spectra. The selected crude extract by screening of its potential antioxidant properties and phenolic compounds profile was further undergo partitioning by using Vacuum Liquid Chromatography technique, purifies using column chromatography and was assessed by using $^{1}H$ and $^{13}C$ of NMR spectral data. NMR was used to evaluate the purities of compound isolated and so as to identify the structure of isolated compound. The anti-inflammatory effect of purified compound and selected crude were evaluated on Human Umbilical Vein Endothelial Cell (HUVEC) via COX-2 inhibition assay, 15-LOX colometric inhibition assay and nitric oxide production by Griess assay.

## 1.5. Significant of Study

Nature has been a fundamental source of therapeutic agents for centuries which led to the developments of modern drugs typically based on their used in ancient traditional medicine. However, these modern derives drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) have its negative side-effects on human. Thus, the results obtain from this study will be used as further guidance in research work in findings new bioactive compounds led to more effective natural anti-inflammatory medicine with less side effects on human consumption. This study will contribute mainly in the science and clinical chemistry.

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