



UNIVERSITI PUTRA MALAYSIA

ANTIOXIDATIVE AND ANTI-INFLAMMATORY ACTIVITIES OF *Hibiscus cannabinus* L. LEAF AQUEOUS EXTRACT AND ITS POTENTIAL BENEFIT IN EXPERIMENTAL ATHEROSCLEROSIS IN VITRO

DARYL B. JESUS ARAPOC

FPSK(M) 2014 12



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By

DARYL B. JESUS ARAPOC

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

February 2014

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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February 2014

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Kenaf or scientifically known as *Hibiscus cannabinus* has been reported to be widely cultivated in West Africa, Bangladesh, India and Southern China. The objective of this study is to evaluate the nutritional composition of *H. cannabinus* leaf as well as its *in vitro* antioxidant activities and anti-inflammatory properties towards human umbilical vein endothelial cell (HUVEC). Fresh *H. cannabinus* leaves were collected from Taman Pertanian Universiti, Universiti Putra Malaysia (UPM). The mixture of the dried powder leaf and water were incubated in the water bath at 40°C for 12 hour in order to produce the aqueous extract. Proximate, elemental and vitamins analysis were done on fresh *H. cannabinus* leaves to determine the nutritional composition. Then, 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and Ferric Reducing/Antioxidant Power (FRAP) assay was done on *H. cannabinus* leaves aqueous extract to determine antioxidant power whereas the phenolic and flavonoid content of *H. cannabinus* leaves aqueous extract were assessed by total phenolic content (TPC) and total flavonoid content (TFC) assays. Cytotoxic assessment of *H. cannabinus* leaves aqueous extract was performed by exposing the HUVECs to the extract at concentrations ranging from 50 to 1000 µg/ml for 24 hr with complete medium. The inhibitory concentration (IC₅₀) of hydrogen peroxide (H₂O₂) and the effective concentration (EC₅₀) of *H. cannabinus* leaves aqueous extract in preventing H₂O₂-induced cell injury were assessed using the MTT assay. The antioxidative and anti-inflammatory effects of *H. cannabinus* leaves aqueous extract on H₂O₂-induced cell injury were carried out by seeding and divided HUVECs into three groups; the positive control (PC) group, HUVECs were exposed to either 250 µM H₂O₂ or 10 ng/ml TNF-α alone; the treated groups HUVECs were incubated with various concentrations of extracts (50, 100, 200 and 400 µg/ml) for 30 minutes prior exposed to H₂O₂ (250 µM) or TNF-α (10 ng/ml); the

negative control (NC) groups, HUVECs were incubated with culture medium only. The cells were incubated for 24 hours at 37 °C with 5% CO₂ supply for analysis of antioxidant enzymes activities (SOD, GPx and Catalase) and lipid peroxidation level (MDA) as well as anti-inflammatory effects (NO, VCAM-1, ICAM-1, MCP-1 and M-CSF). All results of this study were then statistically validated using one-way ANOVA, SPSS version 16.

The result showed that *H. cannabinus* leaf contained carbohydrate, protein and fat with very high level of moisture with 79.2%. On the other hand, the leaf contained considerable values of Vitamin A, Vitamin C, Vitamin B1, Vitamin B2 and Vitamin E. For the mineral analysis, it was found that *H. cannabinus* leaf contained high level of potassium and calcium and other mineral traces were included magnesium, ferum, selenium, and zinc. The leaf phenolic content was $3.21 \pm 0.10\text{mg (GAE)/0.1gml}^{-1}$ extract and the flavonoid content was $2.17 \pm 0.54\text{mg (QE)/0.1gml}^{-1}$ extract. TPC and TFC results correlated positively with DPPH and FRAP with 92.4% and $3.8\text{ }\mu\text{mol Fe (II) sulphate/g}$ respectively. *H. cannabinus* leaves aqueous extract was found to be non-toxic to the cells as no inhibitory concentration (IC₅₀) and significantly attenuate the cytotoxicity effect of H₂O₂ at 50µg/ml. *H. cannabinus* leaves aqueous extract doses within concentration range of 50-400 µg/ml protected the cells against cellular damage and caused significant reduction in the anti-oxidative enzyme (SOD, GPx and Catalase) activities ($p < 0.05/p < 0.01$) with reduction of NO production in comparison with the PC. Besides that, the expressions of VCAM-1, ICAM-1, MCP-1 and M-CSF in the *H. cannabinus* leaves aqueous extract-treated groups were lowered ($p < 0.05/p < 0.01$) than PC. These findings suggest that *H. cannabinus* leaf possesses antioxidative (polyphenols and flavonoid) and anti-inflammatory properties and that it attenuates the initial stage of atherogenesis *in vitro*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**AKTIVITI ANTIOKSIDA DAN ANTI-INFLAMASI EKSTRAK DAUN *Hibiscus
cannabinus* L. DAN POTENSI KEBAIKAN KE ATAS EKSPERIMENTAL
ATEROSKLEROSIS *IN VITRO***

Oleh

DARYL B. JESUS ARAPOC

Februari 2014

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Kenaf atau nama saintifiknya *Hibiscus cannabinus* dilaporkan ditanam secara meluas di Afrika Barat, Bangladesh, India dan China Selatan. Objektif kajian ini adalah untuk mengkaji komposisi nutrien, aktiviti antioksidan (*in vitro*) dan keupayaan anti-inflamasi ke atas sel endotelial vena uri manusia (HUVEC). Daun segar *H. cannabinus* dikutip di Taman Pertanian Universiti Putra Malaysia (UPM). Daun-daun tersebut kemudian dibersihkan, dikeringkan dan dikisar menjadi serbuk. Pengekstrakan akuas dijalankan melalui inkubasi campuran serbuk daun dan air pada suhu 40 °C selama 12 jam. Analisis proksimat dijalankan ke atas daun segar bagi mengkaji komposisi nutrien. Ujian 5-1-diphenyl-2-picrylhydrazyl (DPPH) dan ujian *Ferric Reducing/Antioxidant Power* (FRAP) dijalankan ke atas ekstrak akuas daun *H. cannabinus* bagi menguji keupayaan antioksidan manakala kandungan fenolik dan flavonoid ekstrak akuas daun *H. cannabinus* diuji melalui ujian jumlah kandungan fenolik (TPC) dan ujian jumlah kandungan flavonoid (TFC).

Ujian sitotoksik dijalankan dengan mendedahkan HUVEC kepada akuas ekstrak *H. cannabinus* pada julat kepekatan 50 hingga 1000 µg/ml selama 24 jam dengan medium kultur lengkap. Hasil ujian mendapati ekstrak tidak memberi kesan toksik kepada HUVEC kerana tiada kepekatan merencat (IC₅₀) diperolehi. IC₅₀ bagi hidrogen peroksida (H₂O₂) dan kepekatan efektif (EC₅₀) bagi ekstrak dalam melindungi HUVEC daripada kecederaan disebabkan oleh H₂O₂ diuji dengan menggunakan ujian sitotoksik iaitu dengan menganalisa bilangan sel hidup. Hasil kajian mendapati 250 µM H₂O₂ telah mengurangkan bilangan sel hidup sebanyak 50% (IC₅₀). Kesan antioksidan dan anti-inflamasi ekstrak ke atas kerosakan HUVEC kesan aruhan H₂O₂ diteruskan dengan mengkultur HUVEC dan dibahagikan kepada tiga kumpulan iaitu kumpulan positif, kumpulan negatif dan kumpulan-kumpulan rawatan. Kumpulan kawalan positif (PC) diaruhkan dengan 250 uM H₂O₂ atau 10 mg/ml TNF-α sahaja. Manakala kumpulan

rawatan ditambah ekstrak dengan pelbagai kepekatan (50, 100, 200 dan 400 mg/ml) iaitu 30 minit sebelum HUVEC diaruhkan dengan 250 μM H_2O_2 atau 10 mg/ml TNF- α sahaja. Kumpulan kawalan negatif (NC) hanya dikultur dengan medium kultur lengkap. Sel-sel dimasukkan ke dalam inkubator selama 24 jam pada suhu 37°C dengan bekalan gas karbon dioksida (CO_2) sebanyak 5% bagi analisis aktiviti enzim antioksidan (SOD, GPx, Katalase), paras peroksidasi lipid (MDA) dan analisis anti-inflamasi (NO, VCAM-1, ICAM-1, MCP-1 dan M-CSF). Semua keputusan dalam kajian ini dianalisis secara statistik menggunakan ANOVA satu hala, SPSS versi 16.

Keputusan menunjukkan daun *H. cannabinus* mengandungi karbohidrat, protein dan lemak serta kandungan kelembapan yang tinggi iaitu 79.2%. Selain itu, daun *Hibiscus cannabinus* mengandungi Vitamin A, Vitamin C, Vitamin B1, Vitamin B2 dan Vitamin E. Analisis mineral pula mendapati daun *H. cannabinus* mengandungi mineral kalium dan kalsium yang tinggi selain mineral lain seperti magnesium, besi, selenium dan zink. Kandungan fenolik (TPC) bagi ekstrak adalah 3.21 ± 0.10 mg (GAE/0.1gml⁻¹ ekstrak dan kandungan flavonoid (TFC) adalah 2.17 ± 0.54 mg (QE)/ 0.01 gml ekstrak. Keputusan TPC dan TFC berhubung kait secara positif dengan keputusan DPPH dan FRAP iaitu 92.4% dan 3.8 μmol Fe (II) sulfat/g ekstrak. Julat kepekatan ekstrak (50-400 $\mu\text{g/ml}$) didapati dapat melindungi HUVEC daripada kerosakan dan menurunkan aktiviti enzim antioksidan (SOD, GPx dan Katalase) secara signifikan ($p < 0.05$) dan menurunkan pengeluaran NO berbanding PC. Selain itu, ekspresi VCAM-1, ICAM-1, MCP-1 dan M-CSF bagi kumpulan rawatan adalah rendah ($p < 0.05$). Hasil kajian secara keseluruhan mendapati daun *H. cannabinus* mempunyai keupayaan bertindak sebagai antioksidan (kandungan polifenol dan flavonoid) dan anti-inflamasi di mana ianya berpotensi untuk mengurangkan risiko aterosclerosis secara *in vitro*.

ACKNOWLEDGEMENTS

Thanks God whom gives me willing and strength to finish all the works until the last word of this thesis. Here, I would like to express my gratitude and appreciation to those that have stay around me along the journey of my study.

First and foremost, I wish to show my gratitude to my current supervisor, Associate Professor Dr Norhaizan Mohd Esa. Thank you for your guidance, encouragement, and support. I could not finish this project work without your great and brilliant suggestion. I would also like to thank my extraordinary co-supervisor, Dr. Che Norma Mat Taib for her help and caring in helping me to do this thesis.

Not forgetting admirable Professor Dr Zulkhairi Amom who gave me lots of inspirations and guidance from the beginning of this project. Your deeds would be forever remembered.

My next appreciation goes to all the people in Human Anatomy Lab. To all Staff, Thank you for all the help and understandings. Special thanks and gratitude to all the members of Professor Dr Zulkhairi's group; especially Nor Amalina, Kamal, Ihsan and Hasnah.

Last but not least, a million thanks to my beloved parents (Jesus and Jenny) and my siblings whom always been my backbone and main motivator. God Bless You All.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

%	:	percent
<	:	less than
=	:	equal to
>	:	more than
±	:	approximately or about
$\cdot\text{OH}$	:	hydroxyl radical
$^1\text{O}_2$	:	singlet oxygen
ANOVA	:	one-way variance analysis
ATCC	:	American Type Cell Collection
ATP	:	Adenosine triphosphate
BHA	:	Butyl hydroxyanisole
BHT	:	butylated hydroxytoluene
BSA	:	Bovine Serum Albumin
Ca^{2+}	:	calcium ion
CAM	:	Cell adhesion molecule
CAT	:	Catalase
cDNA	:	complimentary DNA
Cl^-	:	chloride ion
CO_2	:	carbon dioxide
CSF	:	Colony stimulating factor
Cu	:	Cuprum
CVD	:	Cardiovascular disease
DMEM	:	Dulbeco's Modified Eagle's Medium
DMSO	:	dimethyl sulphoxide
DNA	:	deoxyribonucleic acid
DPPH	:	1,1-diphenyl-2-picrylhydrazyl
EC	:	Endothelium Cell
EDTA	:	Ethylenediaminetetraacetic acid
EGF	:	Epidermal Growth Factor
ELISA	:	enzyme-linked immunosorbent assay
EMSA	:	electrophoretic mobility shift assay
eNOS	:	Endothelial nitric oxide
ET-1	:	Endothelin-1

FCS	:	Fetal Calf serum
Fe ²⁺	:	Ferrous ion
Fe ³⁺	:	Ferric ion
FGF	:	Fibroblast growth factor
g	:	gram
GM-CSF	:	granulocyte macrophage -CSF
GPx	:	Glutathione peroxidase
GSH	:	Glutathione
GSSG	:	glutathione reductase
GTP	:	guanosine triphosphate
H ₂ O	:	water
H ₂ O ₂	:	hydrogen peroxide
HDL	:	High Density Lipoprotein
HPLC	:	High Performance Liquid Chromatography
HUVEC	:	Human Umbilical Vein Endothelium Cell
IC ₅₀	:	Inhibition concentration 50 %
ICAM-1	:	Intercellular CAM-1
IL	:	Interleukine
L	:	liter
LDL	:	Low Density Lipoprotein
LOO•	:	lipid peroxide radical
LOOH	:	Lipid hidroperoxide
LPS	:	Lipopolysaccharide
LSGS	:	low serum growth supplement
M	:	molar
MCP-1	:	Monocyte Chemotaxis Protein-1
M-CSF	:	Macrophage Colony Stimulating Factor
MDA	:	malondialdehyde
Mg	:	miligram
Min	:	minute
ml	:	militer
mM	:	milimolar
Mn	:	Manganese
mRNA	:	messenger RNA

MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	:	nicotinamide adenine dinucleotide
NADP	:	nicotinamide adenine dinucleotide phosphate
NBT	:	nitroblue tetrazolium
NH ₃	:	Ammonia
nm	:	nanometer
nmol	:	nanomol
NO	:	nitric acid
NO ₂	:	nitric oxide
O ₂	:	Oxygen
O ₂ [•]	:	superoxide anion
O ₂ ^{2•-}	:	superoxide anion radical
°C	:	degree of celcius
OD	:	Optical Density
OD	:	optical density
OH [•]	:	hydroxyl radical
ONOO	:	peroxynitrite
Ox-LDL	:	oxidized LDL
p	:	probability
PBS	:	phosphate buffer saline
PECAM-1	:	platelet endothelium- CAM-1
PUFA	:	Polyunsaturated Fatty acid
r.p.m	:	rotation per minutes
RNA	:	Ribonucleic acid
RNS	:	Reactive Nitrogen Species
ROS	:	reactive oxygen species
RT-PCR	:	Real Time- Polymerase Chain Reaction
SD	:	standard deviation
SOD	:	superoxide dismutase
TBA	:	thiobarbituric acid
TCA	:	trichloroacetic acid
TNF-α	:	Tumor Necrosis Factor Alpha
UV	:	Ultraviolet

VCAM-1	:	Vascular CAM-1
VEGF	:	Vascular Endothelial Growth Factor
W	:	Watt
WHO	:	World Health Organisation
Zn	:	Zinc
α	:	Alpha
β	:	Beta
γ	:	Gamma
$\mu\text{g/ml}$	:	microgram per milliliter
μl	:	microliter
μM	:	micromolar

CHAPTER I

INTRODUCTION

1.1 Background

Atherosclerosis is one of the major etiologies that contributed to cardiovascular disease (CVD) problems (Yang *et al.*, 2005; James, 2004). It is a condition characterized by the accumulation of lipid in the artery wall with chronic inflammatory (Ross, 1993). Clinical investigations indicated that inflammation is an important risk factor for the development of atherosclerosis and it interacts with established risk factors such as hypercholesterolemia, hypertension and cigarette smoking (James, 2004; Binder *et al.*, 2002 Galina and Belmont, 2001). Aside from common risk factors, the risk of atherosclerosis increases with excessive rise in the concentration of low density lipoprotein (LDL) with involvement of free radical which contributed to the formation of oxidized LDL (oxLDL) (Lowenstein and Matsushita, 2004; Franco and Cinzia, 2003; Ross, 1993). This event played and contributed central roles in development of atherosclerosis (Dandona *et al.*, 2007; Ridker *et al.*, 2005; Pradhan and Ridker 2002).

Up regulation of adhesion molecules such as vascular adhesion molecule (VCAM) and intercellular cell adhesion molecule (ICAM) as well as chemokine such as monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF) describe as the earliest event involved in atherosclerosis. The up regulation may due to immune response caused by pro-inflammatory cytokines such as Tumor necrosis factor-alpha (TNF- α) (Sica *et al.*, 1990). During the event of inflammatory, VCAM-1 and ICAM-1 responsible in the attraction, adherence and migration of monocytes into sub endothelial cells (Zhang *et al.*, 2002). Both VCAM-1 and ICAM-1 which are soluble adhesion molecules were also postulated to contribute and extent the severity of atherosclerosis which both significantly correlated with the thickening of carotid intima-media, an index of early atherosclerosis (Rohde *et al.*, 1998). MCP-1 is a protein that stimulates the recruitment of monocytes toward sites of injury and infection. Expression of MCP-1 was found to be increased in arteries of non-human primates with hypercholesterolemia in which the medial smooth muscle cells responsible for most of the expression (Yu *et al.*, 1992). Macrophage colony stimulating (M-CSF) factor play roles as regulator for the proliferation, differentiation and survival of hemopoietic progenitor cells into mature macrophages (Morstyn *et. al.*, 1988). It was suggested that M-CSF play an

important role in the atherosclerotic lesions as the production of M-CSF and its gene expression had been detected locally in the lesion.

Free radicals are molecules or atom that usually unstable and reactive due to absent of one or more electron in the atom or molecules. Roles of free radicals such as reactive oxygen species (ROS) which included superoxide radical and hydroxyl has been emphasized involve in phagocytosis, inflammation, apoptosis and redox signaling (Bahorum *et al.*, 2006; Chandra *et al.*, 1994). However, excessive production of free radicals due to oxidative stress may cause damage towards cell structures and functions that leads toward inflammations (Kaygusuz *et al.*, 2003; Lefer and Grandner, 2000). This condition leads toward endothelial dysfunction (Cai and Harrison, 2000) where lipid peroxidation resulted when the polyunsaturated fatty acids in the cell membranes being the main targets of ROS (Kaygusuz *et al.*, 2003). In the event of hypercholesterolemia, LDL in sub-endothelial space can be oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) and results in the formation of oxidized LDL (oxLDL) (Lamon and Hajjar, 2008; Cai, 2005). Inflammatory process initiates by oxLDL which resulted in the recruitment of monocytes (Gleissner, 2007).

Since ancient times, animals, mineral and mostly plants have been use for medicinal purposes. Herbalism is still being popularly practiced in modern medicine worldwide (Mahady, 2001). Decoction and infusion of herbs' stems, leaves and root has been popularly used among Malaysian as source to prevent or treat various diseases. Raw young leaves of the herbs are also being included in their daily meal (Ong, 2006; Goh, 2004). Despite of it's claimed for possessing various medicinal properties, unfortunately there are likely less to none being supported by scientific evidence.

Hibiscus cannabinus also known as kenaf or hemp. *H. cannabinus* is one of the most important species of *Hibiscus* in Nigeria (Falusi, 2004). Instead of Malaysia, it has been widely cultivated in Bangladesh, West Africa, India and Southern China (Falusi 2004) for its commercial stem's used. Upon its high potential commercial value, government of Malaysia has provided RM 12 million under 9th Malaysian Plan 2006-2010 for research and development of *H. cannabinus* based industries which emphasizing more on diversifying and commercializing the downstream *H. cannabinus* L-based industries such as pulp and paper industries (Mohd Edeerozey *et al.*, 2007). Cultivation of *H. cannabinus* worldwide mostly is focusing on the utilization of its fiber. In harvesting process of kenaf stalk, the leaf of Kenaf usually will be left dried and regarded as agri-wastes. However in Cameroon, some parts of *H. cannabinus* are being eaten. For example, their people consumed *H. cannabinus*' leaves as vegetables and its seed being roasted and prepared as beverage just like coffee

(Katende *et al.*, 1999). *H. cannabinus* L. also being used in African folk medicine to treat anemia, to relief toothache, sore gum, to release dysentery as well as to cure pellagra (Akinpelu, 2001; Mote, Thomas and Barbosa Filho, 1985). Some studies by Agbor *et al.*, (2002, 2001) found that *H. cannabinus* possessed hematinic, hepatoprotective and antioxidative properties. However, there are still less scientific evidence found yet to support its claimed to have various medicinal properties. Thus, there was a need to define its nutritional and medicinal benefits through scientific research.

1.2 Problem Statement

Coronary heart disease (CHD) also known as coronary artery disease and ischemic heart disease which has been seen to be increasing and very common in most industrialized society. It can be prevented by focusing on the risk factor contributing to CHD. Ministry of Health Malaysia (2012) has reported that the death caused by circulatory failure such as heart disease or diseases of pulmonary circulation in 2011 was the highest (25.64%) among other ten principal causes of death in Ministry of Health hospitals. It's a disease that's traditionally confined to the middle and older age population, but an increasing affliction of the younger age group is a disturbing trend world over. In overcoming this disease, antioxidant had been seen as a good alternative ways in preventing it. It is believed that antioxidants help to reduce the incidence of degenerative diseases such as arthritis, arteriosclerosis, cancer, heart disease, and inflammation and brain dysfunction. In addition, antioxidants were reported to retard ageing (Feskanich *et al.*, 2000; Gordon, 1996; Halliwell, 1996) besides preventing or delaying oxidative damage of lipids, proteins and nucleic acids caused by reactive oxygen species. Despite being agri-waste, *Hibiscus cannabinus* leaf might possess antioxidant properties. Hence, this study focused on *H.cannabinus* leaf as potential antioxidant source and its benefits towards attenuating atherosclerosis.

1.3 Significant of the Study

Nowadays antioxidant has been proven in various scientific researches done by scientists that play important role in reducing risk of certain disease such as cancer, cardio and cerebrovascular diseases (Chung *et al.*, 1999). Compounds such as flavonoids, polyphenols, carotenoids and tocopherol are among the compounds that attributed to the antioxidant properties. Polyphenols are known to be the most abundant and ubiquitously distributed group of plant secondary metabolites (Bravo, 1998). It has been documented to possess high antioxidant properties in several studies (Katalinic *et al.*, 2004; Rice-Evans *et al.*, 1997).

There has been a growing interest in natural products and most people has become aware in replacing the therapeutic drugs and synthetic supplements with the natural herb as medical drug as well as for the maintenance and improvement of health and wellness due to its lower side effect and more economic factor. Medicinal plants have been used for a disease prevention and cure for years. Most of the medicinal plants were used in various traditional ways such as eaten raw and infusion of the plant's part (Ong, 2006; Goh, 2004). However, there are abundant of medical plants with very minimum scientific information to support its traditional benefit claims due to lack of research done. Since *Hibiscus cannabinus* is planted commercially in Malaysia, it is an opportunity to scientifically study the benefits of *Hibiscus cannabinus* leaves instead of being given to animal as feed and being thrown out as agri-waste. Thus this study will further offer a basic knowledge on the benefits of *Hibiscus cannabinus* leaves in improving health especially as a potential anti-atherogenic therapeutic plant.

1.4 Objectives of the Study

1.4.1 General Objective

The objective of this study was to evaluate *H. cannabinus* leaves' potential in attenuating early stage of atherosclerosis *in vitro* by using human vein endothelial cells (HUVECs).

1.4.2 Specific Objectives

The specific objectives of this study were:

1. To determine the nutritional properties of *Hibiscus cannabinus* leaves via proximate and mineral analysis.
2. To determine total phenolic content (TPC), total flavanoid content (TFC) and antioxidant activities of *H. cannabinus* leaves aqueous extract via 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) and Ferric Reducing/Antioxidant Power (FRAP) assays.
3. To determine the toxicity of *H. cannabinus* leaves aqueous extract on HUVECs and its ability to reduce the oxidation activity in the present of H_2O_2 in HUVECs via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

4. To determine the effect of *H. cannabinus* leaves aqueous extract to reduce Lipid Peroxidation (MDA) index in HUVECs exposed to H_2O_2 .
5. To determine the effect *H. cannabinus* leaves aqueous extract on the activity of antioxidative enzyme, Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and Catalase (CAT) in HUVECs exposed to H_2O_2 .
6. To determine the effect of *H. cannabinus* leaves aqueous extract on the expression of pre-inflammatory biomarker, nitric oxide (NO), adhesion molecules (VCAM and ICAM) and cytokines (MCP-1 and M-CSF) on HUVECs induced by TNF- α .

1.5 Hypotheses

1. *H. cannabinus* leaves aqueous extract possess antioxidant properties. No IC_{50} is observed in HUVECs exposed to *H. cannabinus* leaves aqueous extract.
2. Hydrogen peroxide (H_2O_2) shows toxic effect on HUVECs as IC_{50} is achieved in HUVECs upon exposure towards H_2O_2 .
3. *H. cannabinus* leaves aqueous extract shows antioxidative effect on HUVECs as EC_{50} is demonstrated in *H. cannabinus* leaves aqueous extract pre-treated HUVECs exposed to H_2O_2 .
4. The lipid peroxidation index biomarker (MDA) of H_2O_2 -induced HUVECs treated with *H. cannabinus* leaves aqueous extract decreases in the present of *H. cannabinus* leaves aqueous extract.
5. Nitric oxide (NO) concentration in TNF- α induced HUVECs decrease in *H. cannabinus* leaves aqueous extract pre-treated groups.
6. Concentrations of adhesion molecules and cytokine in TNF- α induced HUVECs decrease in *H. cannabinus* leaves aqueous extract pre-treated groups.
7. Increase of antioxidant enzymes concentrations (SOD, GPx and CAT) in H_2O_2 -induced HUVECs treated with *H. cannabinus* leaves aqueous extract.

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