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POTENTIAL HYPOCHOLESTEROLEMIC EFFECT OF MORINGA OLEIFERA LEAVES' EXTRACT

by
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ABSTRACT
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Cholesterol is a lipid component of membrane structure and a precursor of many biomolecules. Disturbance in serum cholesterol homeostasis leads to an increase in cholesterol level, that underlie cardiovascular diseases and atherosclerosis. While cholesterol biosynthesis occurs in all nucleated cells, the liver plays a key role in distributing and eliminating cholesterol. Biosynthesis of Cholesterol occurs via a complex pathway known as mevalonate pathway with the rate determining step catalyzed by HMG-CoA reductase, an important target for drug development.

Statins are competitive inhibitors of HMG-CoA reductase enzyme. Their therapeutic value is not limited to their effect on cholesterol pathway, but to the increase in plasma membrane LDL receptors, consequently hypocholesterolemia. However, the chronic administration of statins, have been reported to cause side effects including myopathies, and liver dysfunction, which necessitated the need to find safer and more affordable medications. Interest in alternative medicine, such as herbal medicine, has increased in recent years, providing a non-costly source for treatment of ailments. Moringa Oleifera (MO) has been recognized for its nutritional, environmental, and medicinal value. All parts of the tree are edible and have been used in curing diseases. The MO leaves are commonly consumed as hot tea beverage. Many beneficial effects have been attributed to the tea drink, including hypoglycemia, antioxidant, and hypocholesterolemic effects.

In vitro and in vivo studies, using organic solvents extracts, of MO leaves demonstrated its effect on regulating cholesterol level, by controlling contributors to cholesterol pool. We hereby hypothesize that chronic administration of MO leaves water extract (MOE) will maintain cholesterol homeostasis. We investigate, in this study, the effect of chronic treatment of MOE on key enzymes controlling cholesterol level specifically: HMG-CoA reductase and LDL receptors.

The viability of HepG2 cells treated (24hrs) with varying concentration (0.01% -0.1%) of MOE, decreased in a concentration dependent manner; with EC50 ranging between 0.02-0.03%. In all subsequent experiments, we opted to treat cells, for extended periods (24, 48, 72hrs), with MOE, at sub EC50. The metabolic activity was confirmed by assaying: viability (MTT and trypan blue assays); ROS level (NBT assay), and ATP (luciferase assay) level. In addition, HMG-CoA reductase and LDL receptors, transcription (RT-PCR) and translational levels (western blotting, immunostaining) were assessed in MOE treated cells. The effect on intracellular cholesterol level, as well as the direct effect of MOE on pure HMGCOA reductase were also determined.

We hereby report in MOE-treated HepG2 cells a concentration dependent, but time independent: a) decrease in the viability; b) increase in ROS and c) decrease in ATP level. Importantly, these findings suggest a strict mechanism controlling ROS and ATP levels maintaining thus metabolic activity of HepG2 cells.

Moreover, MOE treated cells caused a significant decrease in HMG-CoA reductase with a concomitant increase in LDL receptors, gene expression levels. No change in the house keeping gene (GAPDH) level was detected. Unexpectedly however, the translated protein level of GAPDH, decreased in MOE treated cells, hence actin was used instead to assess the relative variation in the tested proteins. While a non-significant decrease in the translated HMG-CoA reductase protein was obtained, both pre-mature and mature LDL receptors levels increased significantly. The increase in the level of the LDL receptors was also confirmed using immunostaining. Extracted intracellular cholesterol level showed a small, but not significant, decreased 48-hours following MOE treatment.

To sum up, our results demonstrate a hypocholesterolemic potential of MOE. It increases the LDL receptors favoring cholesterol uptake into the cells and decreases HMG-CoA reductase gene expression impacting on cholesterol biosynthetic pathway. In addition, we identified another target of MOE, namely GAPDH enzyme that links glycolysis to electron transport chain. The decrease in the activity of this enzyme will favor flux of glucose into HMP stimulating production of NADPH controlling ROS level in MOE treated cells favoring proliferation and viability.

Finally, our findings provide a link between dietary practices and disease prevention. A recommended daily intake of MOE (common tea drink) may have a beneficial effect regulating cholesterol level, hence protecting subjects with low risk from developing heart disease.

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ABBREVIATIONS

MO	Moringa Oleifera
MOE	MO leaves' extract
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SOD	Superoxide dismutase
TCM	Traditional Chinese medicine
CAT	Catalase
HepG2	Liver hepatocellular cells
Caco-2	Cancer coli-2 cells
MCF-7	Human breast cancer cells
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA reductase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamineteraacetic acid
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
A547	Adenocarcinomic human alveolar basal epithelial cells
ATP	Adenosine triphosphate
Cyt C	Cytochrome complex
Smac/Diablo	Second mitochondria-derived activator of caspases
AIF	Apoptotic inducer factors
PBMCs	Peripheral blood mononuclear cells
Hek293	Human embryonic kidney 293 cells

HCT116	Human colon cancer cells
HT29	Human colon cancer cells
LDL-R	Low density lipoprotein receptor
NADPH	Nicotinamide-adenine dinucleotide phosphate

CHAPTER I

INTRODUCTION

A. Natural Products

Natural products possess a variety of structural and chemical diversity that cannot be matched by any chemically synthesized compounds. Over the centuries, the use of natural products and herbal extract in treating ailments was common practice shared between civilizations. They were based on experiences and beliefs that were passed down through generation. Written documents trace the use of plants in medicine to great civilizations such as Chinese, Indians, and North Africans (Alostad et al., 2020; F.-S. Li & Weng, 2017; Williamson, 2001).

The first written report on medicinal plants for the preparation of herbal drugs was found on a 5000-years-old Sumerian clay slab from Nagpur. Over 250 referenced plants were described being used in almost 12 drug mixtures. In addition, Hippocrates, the founder of Greek medicine, as well as his pupil, Aristotle, extensively elaborated on the medicinal properties of some plants. Moreover, it was around 350 BC and c. 287 BC that Theophrastus, the father of botany, wrote the book "*Historia Plantarum*" in which he describes and classifies plants based on their structure, growth, and medical usage (Bernardini et al., 2018; Vuolo et al., 2019).

Other important historical evidence of herbal medicine was authored by Pedanius Dioscorides, a Greek physician and surgeon who wrote "*De Materia Medica*". The manuscript is a pharmacopoeia that describes 600 therapeutic medicinal plants as a series of

scientific studies. From the sixteenth century on, this pharmacopeia had been the principal reference work on pharmacology used across Europe and the Middle East (Bernardini et al., 2018; Phillipson, 2001; Vuolo et al., 2019; J. Wang et al., 2019).

The beginning of the 19th century witnessed an increased interest in isolating bioactive compounds from medicinal plants. Following the discovery of quinine, the use of phytochemicals from plant extracts in clinical and scientific research gained more recognition and popularity for their possible pharmaceutical application. This shaped today current drug development processes that consider plants as a source for new drugs and therapeutic agents (Bernardini et al., 2018; Phillipson, 2001; Vuolo et al., 2019; J. Wang et al., 2019).

1. Traditional medicine

The World Health Organization (WHO), defined traditional medicine as "the sum total of the knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness" (Williamson, 2001).

Traditional medicine consists of a complex mix of practices and beliefs that values the body's equilibrium and state of health, being a direct function of the body, mind and soul state (figure 1) (Wagner & Elmadfa, 2003; H.-X. Yang et al., 2020; Zhang et al., 2018).

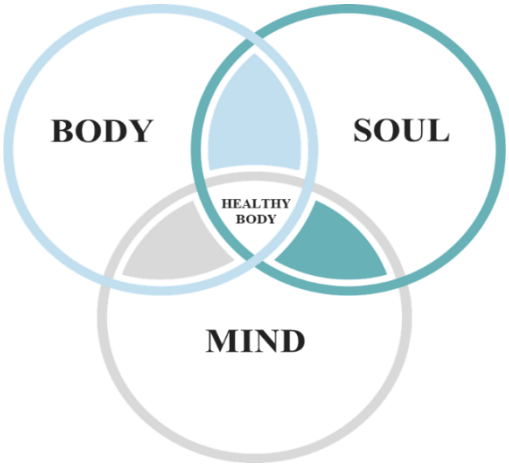


Figure 1: Traditional medicine principal

Therefore, treatments used in traditional medicine can be divided into three categories (Qiu, 2007):

- Medication therapies using herbal medicines and/or medicines derived from animal parts and/or minerals
- Non-medication therapies, such as, massage, physical therapy, meditation, hypnosis, and yoga
- Mixed therapies combining medication and non-medication therapies.

It is worth noticing that although traditional medicine practices may vary with regional cultures and beliefs, all share the same principle caring for the individual's overall health rather than treating a specific disease. For this reason, traditional medicine has

become tremendously popular and a reliable therapeutic alternative. According to the WHO, 80% of the world's population rely on traditional medicine (Alostad et al., 2020; F.-S. Li & Weng, 2017; Wachtel-Galor & Benzie, 2011).

In fact, compared to western medicine, traditional medicine applications were more successful, such as the use of traditional Chinese medicine (TCM) in the treatment of severe acute respiratory syndrome reported by WHO in 2003. Moreover, in Africa, traditional herbal medicine has been used for decades to treat HIV (Tilburt & Kaptchuk, 2008).

Encompassed within traditional medicine is the common use of herbal extract in the treatment of diseases. Herbs were widely used in crude herbal teas drinks, syrups, infusions, ointments, liniments, and powders (figure 2) (Wachtel-Galor & Benzie, 2011).

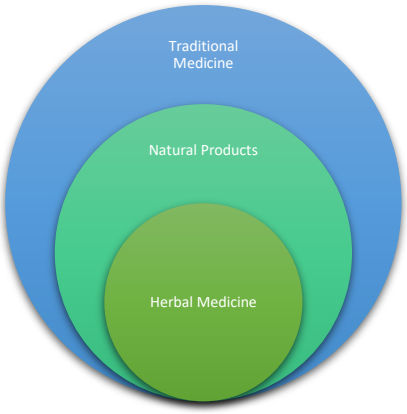


Figure 2: The branches of traditional medicine and its correlation

Plant extracts encompass compounds classified as primary and secondary metabolites (figure 3). Primary metabolites consist of small molecules such as sugars, amino acids, tricarboxylic acids, or Krebs cycle intermediates, proteins, nucleic acids and polysaccharides which play a role of primary plant metabolites in basic life functions such

as cell division and growth, respiration, storage and reproduction (A. Hussein & A. El-Anssary, 2019).

On the other hand, secondary metabolites are the end products of the primary metabolisms. Biotransformation occurring in plants leading to wide variety of biomolecules. Different from the primary metabolites, secondary metabolites are not required for the growth and maintenance of cellular function in the plants, but they are important in their survival and increasing lifespan. They function by exerting defense mechanisms that support survival and favor adaption to environmental changes and threats (Ibc Cib Cib МКБ et al.; Mendoza & Silva, 2018; Mera et al., 2019; Sinha et al., 2019; Thirumurugan et al., 2018) (figure 3).

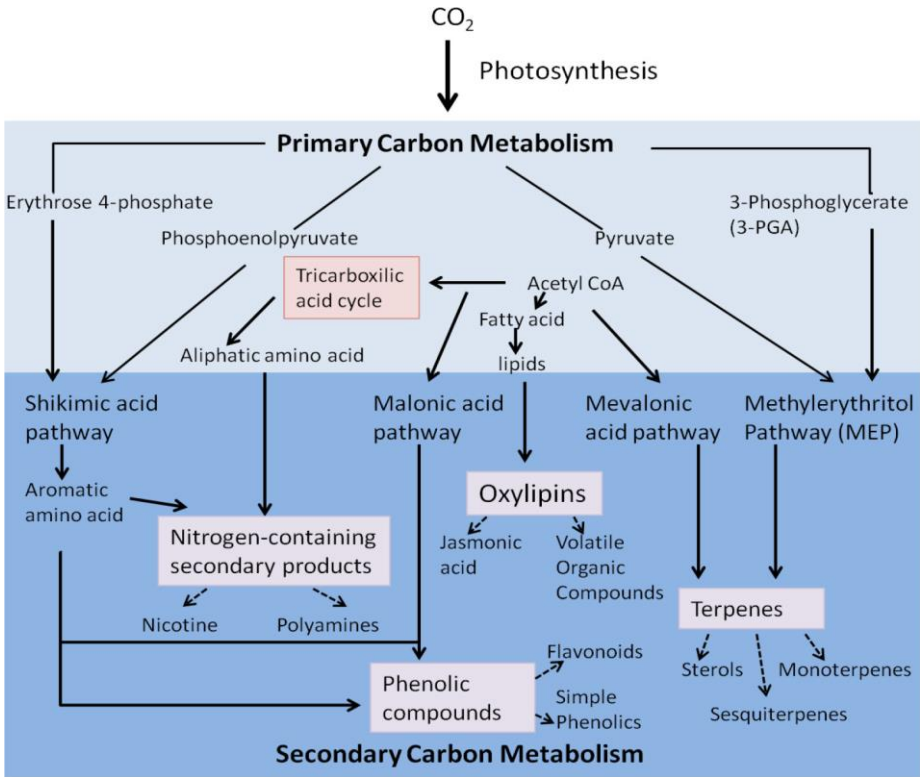


Figure 3: Correlation between Primary metabolic products and Secondary metabolites (Sinha et al., 2019)

Secondary metabolites are responsible for plants' medicinal properties attributed to their ability to serve as: substrates for biochemical reactions; cofactors for enzymatic reactions; inhibitors enzymatic reactions; for sequestrants of undesirable constituents; agonists/antagonists to cell surface and/or intracellular receptors; scavengers of reactive or toxic chemicals; enhancers of absorption and/or stability of essential nutrients; source growth factors (substrate) to beneficial gastrointestinal bacteria; or as possible selective inhibitors of deleterious intestinal bacteria (Sinha et al., 2019).

The efficacy, however, of an herbal medicine is not correlated with the activity of a single specific bioactive compound but to the synergistic action of a mix of ingredients, leading to its the therapeutic result (Kaurinovic & Vastag, 2019; Y. Yang et al., 2013;

Zhang et al., 2018). Therefore, by isolating its individual constituents and recombining them it is often difficult to reproduce its therapeutic effect, which is multitarget.

Hence, the synergetic effect of a herbal extract is complex and difficult to predict, as it involves the multiple action of different compounds on the same or different targets, involved in various pathways (Y. Yang et al., 2013).

B. Moringa oleifera

Horseradish tree, Drumstick tree, Ben oil tree, Miracle tree, and “Mother’s Best Friend", are all names given to the members of the Moringaceae family of perennial angiosperm plants, Moringa oleifera (MO). The tree was cultivated by natives of sub-Himalayan regions and throughout India for ages. It was first described 5000 years ago as a medicinal plant and quickly spread all over the ancient Greek, Roman, and Egyptian world (Fahey, 2005; Ganatra et al., 2012; Niju et al., 2019).

MO tree is drought-tolerant, fast-growing, multi-purpose, easily accessible plant, that has spread through tropical and subtropical areas (Fahey, 2005; Ganatra et al., 2012; Niju et al., 2019). It is valued worldwide for its economic, environmental, nutraceuticals, food supplementation and multiple medicinal properties (Fahey, 2005; Ganatra et al., 2012).

1. MO in Environmental

MO has been used throughout the world and is recognized for its applicability in water purification and conservation, as wood fuel, as dye and in soil conservation (Fahey, 2005). Recent studies have investigated the potential use of methyl-esters of MO seeds oil

as a biodiesel resource, which showed the high cetane number reported for a biodiesel fuel so far (Niju et al., 2019; Rashid et al., 2008).

2. MO in Nutrition

MO has been used as a dietary supplement for its incredible macro- and micro-nutrients content. Cooked or mixed with food, all parts of the tree can be used for its nutritional value as a food additive (Lindgren et al., 1985; Mahmood et al., 2010; Saini et al., 2016). For example, in India the pod has been used in the preparation of traditional dishes such as sambhar, corma, dal, cutlet, and curry recipients (Ganatra et al., 2012; Mahmood et al., 2010).

The tree is rich in minerals, essential amino acids, vitamins (A, B3, B4, B6, B9, C, E, β-carotene), and a wide range of phytochemicals including: glucosinolates, isothiocyanates, sitosterols, quercetin, kaempferol phytosterol, pterygospermin, zeatin, caffeoylquinic acid (Dhakad et al., 2019; Ma et al., 2019; Saini et al., 2016).

MO leaves are richer in iron, Vitamin A, Vitamin C, calcium and potassium when compared to spinach, carrots, oranges, milk and bananas, and contain protein quality competitive to that of milk and eggs (Fahey, 2005) (figure 6).Therefore, WHO (WHO Readers Forum, 1999) and several non-governmental organizations (NGO) around the world[1] have advocated MO as a potential alternative food source for alleviating

[1] Trees for Life, ECHO, Church World Service, GIANT, Helen Keller International and Santé et Nature

malnutrition in countries like Africa and some countries in Asia and Latin America (Kumar et al., 2019).

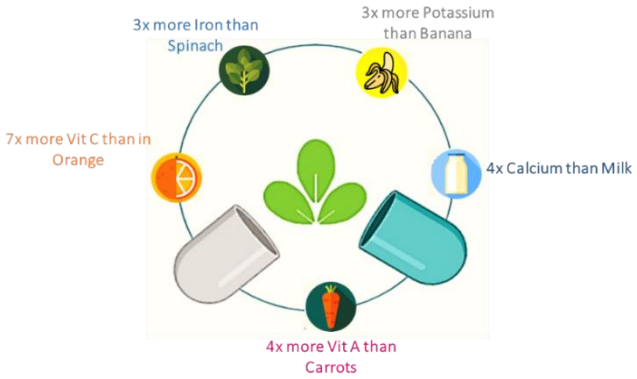


Figure 4: Important nutritional factors present in MO leaves

3. MO in Traditional medicine

All parts of the MO tree, including the stems, leaves, flowers, pods, and seeds, have been widely used in the treatment of various ailments. Reports in the oldest Ayurvedic manuscripts on medicine (Charak and Sushruta Samhita) described the usage of MO against asthma, anemia, intestinal worms, cardio-vascular disorder, headache, skin infections and others (Ganatra et al., 2012; Zhao, 2018).

Studies have attributed the wide medical application of MO tree to a unique combination of bioactive compounds including polyphenol, phenolic acids, flavonoids, alkaloids, glucosinolates, isothiocyanates, tannins and saponins (Dhakad et al., 2019; Ma et al., 2019; Saini et al., 2016). MO bioactivity has been associated with its antioxidant, anticancer, antidiabetic, anti-inflammatory and antihyperlipidemic potential.

a. Antioxidant Effect of MO

Excess production of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) can lead to DNA damage, and lipid and protein oxidation in cells. Antioxidants provide a defense system of the body against those free radicals. Although our body has its sources of antioxidants such as glutathione peroxidase, catalase, and superoxide dismutase (SOD); exogenous antioxidants provide additional protection against free radicals. Natural antioxidants, provided exogenously by food and medicinal plants, help in balancing ROS and RNS levels, while, exhibiting a range of protective biological effects against inflammation, cancer and aging (Oyenihi et al., 2014).

The potent antioxidant activity of MO has been largely described using extracts of different parts of the plant (Aju et al., 2019; Gupta et al., 2012; Kumbhare et al., 2012; B.N. Singh et al., 2009). It is well established that leaves of MO have the highest source of antioxidant compared to its seeds, and roots (Xu et al., 2019). Moreover, MO antioxidant potential has been highly associated with the presence of different phytochemicals.

Phenolic enriched ethanol extract of MO leaves was reported to reverse the toxic effect of CCl4 in treated rats by stimulating the expression and activity of antioxidant enzymes such as SOD and catalase (CAT) (Verma et al., 2009).

In addition, the antioxidant potential of MO leaves is also linked to the free radical scavenging activity due to the presence of phenolic and flavonoid compounds in the extract (D. Singh et al., 2014).

b. Anticancer Effect of MO

Cancer is the result of genetic mutations that lead to the dysregulation of cellular biological processes including enhanced cellular proliferation and diminished programmed

cell death (PCD), including apoptosis, autophagy, and programmed necrosis (Labi & Erlacher, 2015; Ouyang et al., 2012).

Many landmarks studies show that medicinal plants exert strong therapeutic against cancer (Cragg & Pezzuto, 2016; Mo & Elson, 2004). Paclitaxel, a drug obtained from the bark of Taxus brevifolia presents one of the most successful stories in plant-based research, highly used in the treatment of breast cancer (Amaral et al., 2019). Many herbal derived drugs have shown promising results against cancer, activating pro-death signals leading to apoptosis (Cragg & Pezzuto, 2016).

Several studies on MO extracts have reported its effect on signaling pathways that impact cell cycle arrest, cell proliferation, and apoptosis (Berkovich et al., 2013; Fahey et al., 2018; Madi et al., 2016; Reda et al., 2017; Thurber & Fahey, 2009). Ethanolic extract of MO leaves and bark caused a decrease in cell motility, cell apoptosis, and colony formation in colorectal and breast cancer cell lines, as well as apoptosis (Al-Asmari et al., 2015). Similarly, MO was demonstrated to exert apoptosis on different human cancer cell lines (HepG2, Caco-2, and MCF-7) (Charoesin et al., 2014). Furthermore, Bharali et al. (2003) proved that the topical application of MO seed and pod extract could help reduce skin papillomas in mice (Bharali et al., 2003).

In our laboratory, we have reported the effect of aqueous MO leaves' extract (MOE) on A547 cells (Madi et al., 2016). MOE reduced cell survival, inducing apoptosis. The effect was mediated by targeting the mitochondrial function by depolarizing membrane potential, depleting ATP, increasing ROS generation, while inducing the overexpression of pro-apoptotic proteins such as Cyt C, Smac/Diablo and AIF (cytochrome complex, second mitochondria-derived activator of caspases and apoptotic inducer factors) and subsequent

activation of nuclear PARP (Poly (ADP-ribose) polymerase) cleavage and/or caspases activation. Other studies using MO leaves extract reported similarly increase in DNA damage, lipid peroxidation and activation caspases (Caspase 9, Caspase 3 and Caspase 7) in HepG2, PBMCs, and Hek293 cell lines (Tiloke et al., 2019).

We have recently reported the effect of MOE on 3 colon cancer cell lines: HCT116, Caco2, and HT29. MO treatment showed increase in cell death mediated by increase in ROS, disturbances in membrane integrity and cell cycle arrest (Reda et al., 2017).

Many reports have attributed MO anticancer potential to its unique variety of glucosinolates compounds, that are present in all MO species (Berberich & Hegele, 2019; Fahey et al., 2018; Jain et al., 2010; Thurber & Fahey, 2009).

c. Antidiabetic Effect of MO

Diabetes mellitus refers to a group of metabolic alterations characterized by hyperglycemia resulting from defects in insulin secretion, action, or both (Yeh et al., 2003). Medicinal herbs have long been reported to be a potential therapy against diabetes. The mechanism so far has been linked to the presence of phytochemicals that may induce increases in insulin secretion, increasing glucose transport into muscle and fat tissues, reducing intestinal glucose absorption, and preventing glucose production in hepatocytes (Kooti et al., 2016; Oyenihi et al., 2014; Yeh et al., 2003).

The antidiabetic potential of different MO extraction has been demonstrated in many animal models (Adisakwattana & Chanathong, 2011; Edoga et al., 2013; Gupta et al., 2012; Khan et al., 2017). In streptozotocin-induced diabetic albino rats, treatment for 21 days with methanol extract of MO pods caused a significant decrease in serum glucose and

an increase in serum insulin after 21 days of treatment (Gupta et al., 2012). Similar observations were reported using MO aqueous leaf extract (Edoga et al., 2013).

In vivo studies performed on MO- treated type 1 and 2 diabetes induced rats provided evidence of improved carbohydrate digestion and significant restoration of hepatic and pancreatic histoarchitecture of the cells (Khan et al., 2017).

d. Anti-Inflammatory and Immunomodulatory Effect of MO

Inflammation is a natural physiological response of the body against hazardous stimuli such as pathogen infections, foreign substances, and pro-inflammatory molecules. Several plants (cinnamon, turmeric, ginger, willow bark and others) were reported to regulate inflammatory response through the activation or inhibition of proinflammatory cytokines (Miller et al., 1999). For example, the willow bark extract has been used for its analgesic effect in treating patients with osteoarthritis (Schmid et al., 2001). What is more, the anti-inflammatory potential of turmeric treated rats mediated by the upregulation of peroxisome proliferator-activated receptor-γ (PPAR-γ) was demonstrated (Siddiqui et al., 2006).

Similarly, the anti-inflammatory potential of MO has been attributed to its immunosuppressive and immunostimulatory effect (figure 5). Treatment of RAW 264.7 cells with MO root, leaf, fruit and flower extract, significantly suppressed the secretion and expression of nitric oxide (NO), prostaglandin E2 (PGE2), interleukin- (IL-) 6, IL-1β, tumor necrosis factor-alpha (TNF-α), nuclear factor-kappa B (NF-κB), inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2) (Lee et al., 2013; Tan et al., 2015) .

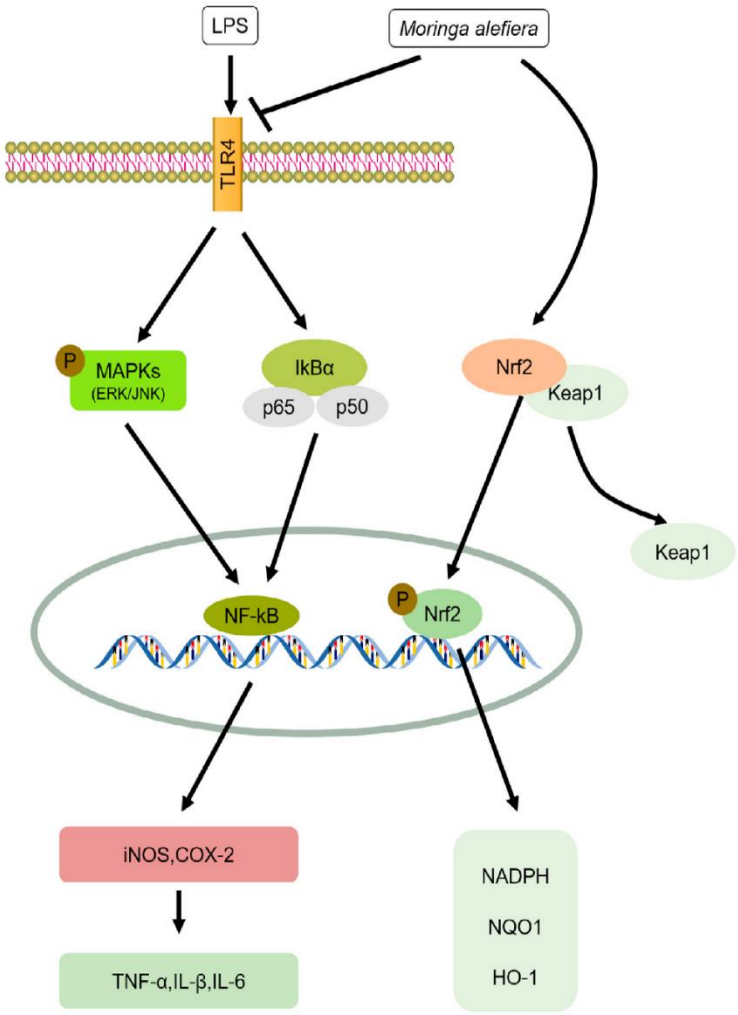


Figure 5: The anti-inflammatory mechanisms of M. oleifera (Kou, Li, Olayanju, Drake, & Chen, 2018)

e. Hypolipidemic Effect of MO

Elevated levels of cholesterol, triglyceride, and low-density lipoprotein cholesterol, and/or decrease in circulating high-density lipoprotein cholesterol (HDL) are the main risk factors contributing to the prevalence and severity of atherosclerotic cardiovascular disease (El-Tantawy & Temraz, 2019). The use of natural products to reduce serum lipid levels and to improve health and wellness has grown in the past years.

In-vivo studies using MO leaves decreased total cholesterol (TC), LDL and triglycerides (TG) levels in different animal models such as rabbits and rats (Chumark et al., 2008; Ghasi et al., 2000). In addition, MO leaves impacted the molecular mechanism that regulated cholesterol levels (Adisakwattana & Chanathong, 2011; Almatrafi et al., 2017; Waterman et al., 2015). MO enriched phenolic leaf extract increased LDL receptor gene expression and decreased HMG-CoA reductase activity in HepG2 cells (Tabboon et al., 2016).

Similarly, in vivo studies on high-cholesterol diet fed rats treated with polyphenol-enriched extract of MO leaves, showed a significant decrease in HMG-CoA reductase activity and cholesterol absorption accompanied by an increases in fecal excreted bile acids resulting in a decrease in serum total cholesterol levels. (Reddy et al., 2017).

Table 1: List of studies using MO leaves in the treatment of dyslipidemia

Reference	**Type of MO Extraction**	**Type of study**	**Results**
Chumark et al. (2008)	Aqueous extract	ex-vivo: High cholesterol fed rabbits	↓TC ↓LDL ↓HDL ↓TG ↓carotid plaque formation
Nambiar et al. (2010)	Tablet of MO leaves	Human: 35 hyperlipidemic subjects (17 treated and 18 control)	↓TC ↑HDL-C ↓non-HDL-C
Almatrafi et al. (2017)	Moringa leaves supplemented in the diet	in-vivo/ ex-vivo: High cholesterol fed guinea pigs	↓TC ↓LDL ↓HDL ↓TG ↓mRNA LDLR/ CD68/ SRBP1c/ DGAT2/ PPARγ
Jain et al. (2010)	Methanolic extract	in-vivo: High fat diet fed rats	↓TC ↓LDL ↑HDL ↓VLDL ↓TG ↑fecal cholesterol ↓atherogenic index
Reddy et al. (2017)	Polyphenols-enriched extract	in- vivo: High cholesterol diet fed rats	↓TC ↓LDL ↓TG ↓HMG-CoA activity
Tabboon et al. (2016)	Phenolics-enriched extract	in-vitro: HepG2 cells	↓ICC ↓ HMG-CoA activity ↑LDLR activity ↑mRNA LDLR/ HMG CoA

C. Cholesterol

Cholesterol was first isolated from gallstones by Doctor François Poulletier de la Salle in 1758, who described it as being a crystalline fatty wax that years later was referred to as cholesterine by Doctor Michel Chevreul (1815) (Craig & Malik, 2020; Rudel & Edwards, 2019; Scharnagl et al., 2001). The term comes from the Greek, meaning "solid bile" (kholē= 'bile' and stereos= 'stiff') since it was found in human and animal bile.

Cholesterol serves as a precursor for biomolecules such as vitamin D, steroids hormones and bile acid (Craig & Malik, 2020; Sudhakaran & Surolia, 2012). Structurally it is composed of four fused rings and a flexible hydrocarbon tail, conferring a hydrophobicity to the molecule (Chaudhuri & Anand, 2017; Liscum, 2008; Lusis & Pajukanta, 2008)(figure 6).

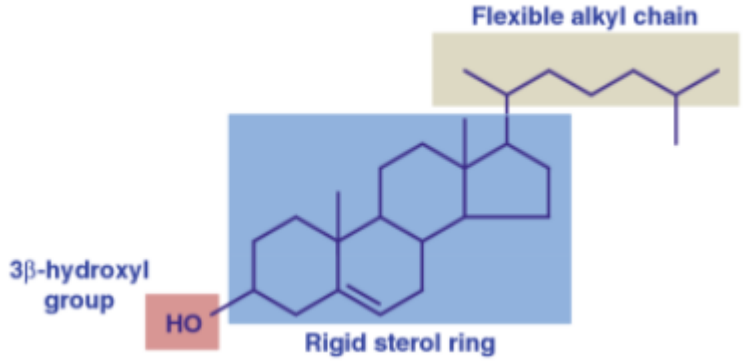


Figure 6: Chemical structure of cholesterol (Sudhakaran & Surolia, 2012)

Cholesterol homeostasis is tightly regulated to prevent its accumulation and abnormal deposition which has been linked to cardiovascular disease, neurodegenerative diseases and cancers (Adorni et al., 2019; Huang et al., 2016; Riobo, 2012; Shen et al., 2016). Maintaining, therefore cellular cholesterol level, strict regulatory mechanism exist

that controls its de novo cholesterol biosynthesis, storage, uptake, and excretion of cholesterol (Luo et al., 2020).

1. Cholesterol biosynthesis

Cholesterol is actively synthesized in different tissues such as the liver, intestines, adrenal glands, and reproductive organs (Espenshade, 2013).

The liver remains the main organ responsible for the distribution of exogenous and endogenous cholesterol pool, as well as excretion (J.Z. Li et al., 2010; Russell, 1992; P. Singh et al., 2013). Figure 7 shows a simplified schematic diagram of the complex biosynthesis pathway of cholesterol that involves the following steps:

- HMG-CoA synthesis: The conversion of 3 acetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by the action of HMG-CoA synthase.
- Mevalonate formation: HMG-CoA reductase catalyze the rate determining biotransformation of HMG-CoA into mevalonate, that is further converted into isopentyl pyrophosphate (IPP).
- Formation of Squalene: Briefly, the isomerization of IPP into dimethylallyl diphosphate (DMAPP) is followed with a series of head-to-tail condensations generating geranyl pyrophosphate (GPP), farnesyl diphosphate (FPP) and ultimately squalene.
- Cholesterol synthesis: Squalene undergoes epoxidation followed by intramolecular rearrangement producing lanosterol. Finally, lanosterol is converted into cholesterol in a multistep process.

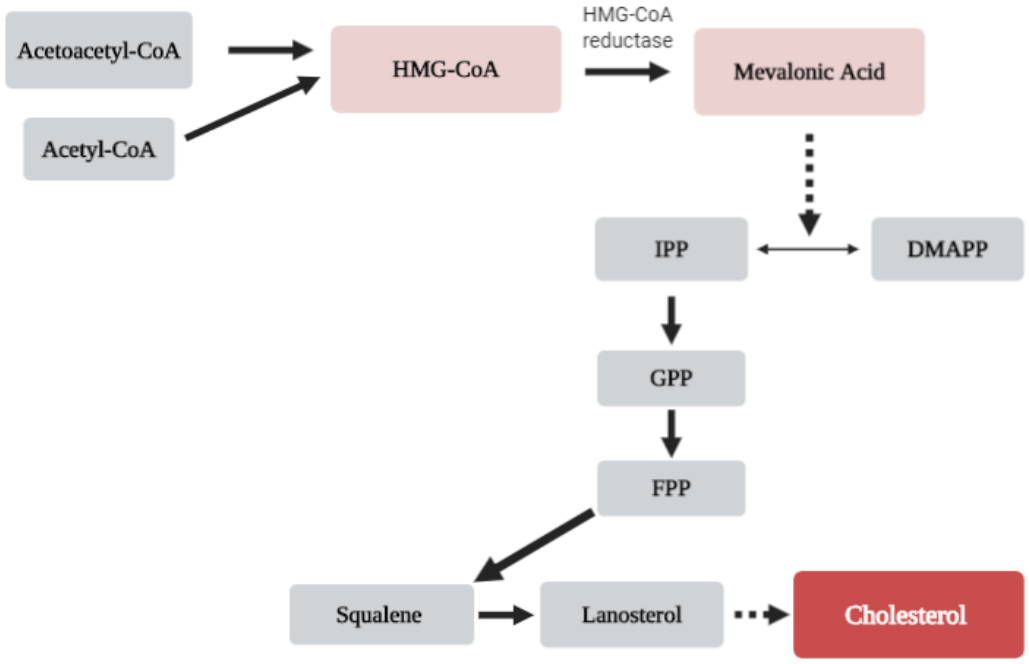


Figure 7: Cholesterol biosynthesis pathway

2. Cholesterol Homeostasis

Intracellular cholesterol levels are maintained by the dynamic balance between dietary cholesterol (exogenous); *de novo* biosynthesized cholesterol (endogenous); cholesterol esterification (store); bile acid (excretion), as shown in figure 8 (Afonso et al., 2018; Berg et al., 2002).

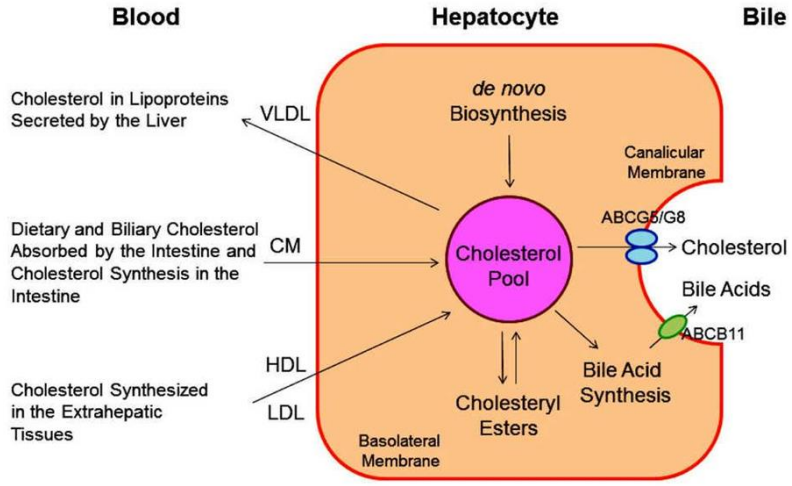


Figure 8: Hepatic cholesterol pool (H. H. Wang, Garruti, Liu, Portincasa, & Wang, 2017)

Being highly hydrophobic, the transportation and distribution of free and esterified cholesterol, along with triglyceride (TG) is facilitated by lipoprotein (Afonso et al., 2018; Berg et al., 2002; Cerqueira et al., 2016; Enns, 2010; H.H. Wang et al., 2017) (figure 9). Lipoproteins are classified into chylomicrons (CM), very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Hu et al., 2010; Movsesian, 2016; H.H. Wang et al., 2017). The different types of transporters are distinguished by size, density, electrophoretic mobility, composition, apoprotein associated, and function (Hu et al., 2010; Movsesian, 2016; H.H. Wang et al., 2017).

The exogenous lipoprotein pathway starts with the incorporation of dietary lipids into CM. Digested dietary lipids are absorbed as fatty acid, monoglycerides and cholesterol which are then packed into molecules known as CM and released into the lymphatic system. CMs are responsible for distributing dietary TG to various tissues for energy or storage purposes (Daniels et al., 2009; Owen & McIntyre, 1982).

The presence of apoprotein C2 (ApoC2) on CM, the activator of lipoprotein lipase (LPL) present in capillary endothelial cells favors the release of hydrolyzed TG into muscles and adipose tissue. The remain CM, rich in exogenous cholesterol, is taken up by the liver by LDL receptors and LDL receptors related protein (LRP) through binding to the CM surface molecule protein known as apoprotein E (ApoE) (Daniels et al., 2009; Owen & McIntyre, 1982).

In the liver, the endogenous lipoprotein pathway starts with the formation of VLDL, responsible for the transportation of TG and cholesterol to tissues. In the circulation, as VLDL delivers TG to extra hepatic tissue, such as muscle and adipocyte, it is transformed into IDL, that can be either returned to the liver, where it is taken up and metabolized or it can remain in the circulation where it will be converted into LDL (Daniels et al., 2009; Owen & McIntyre, 1982; H.H. Wang et al., 2017).

The remained IDL known as LDL, will be then taken up by the liver where it can be recycled in the Golgi apparatus to make more lipoproteins (Daniels et al., 2009; Heuman et al., 1989; Owen & McIntyre, 1982; H.H. Wang et al., 2017; Wilson & Rudel, 1994).

Cholesterol present in peripheral cells is transported from the plasma membranes of peripheral cells to the liver and intestine by a process known as reverse cholesterol transport (RCT) that is associated with the activity of HDL. HDL is responsible for providing a reservoir of apolipoproteins C, which is required for the metabolism of chylomicrons and very low-density lipoproteins (VLDL), and for the direct and indirect routes of RCT (Brufau et al., 2011; Schober et al., 2020).

In the direct pathway, cholesterol from peripheral tissues is transported to the liver by the taking up of HDL cholesterol via HDL hepatocellular surface receptors, such as

specific scavenger receptors known as scavenger receptor B1 (SR-B1) (Brufau et al., 2011; Schober et al., 2020).

In the indirect route, HDL cholesterol ester is first transferred to apolipoprotein (Apo) B, such as LDL, VLDL, IDL and CM, in exchange for triglycerides (Hu et al., 2010; Movsesian, 2016; H.H. Wang et al., 2017). Subsequently, Apo B containing lipoproteins are taken up via receptor-mediated endocytosis (Brufau et al., 2011; Schober et al., 2020).

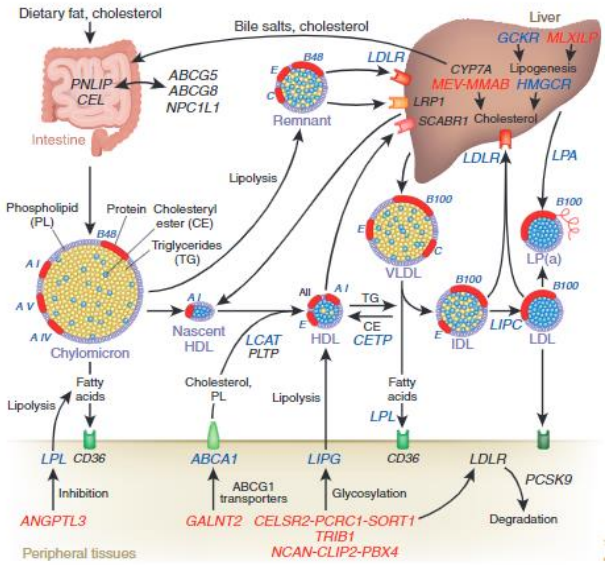


Figure 9: Lipoprotein pathways in the body (Lusis & Pajukanta, 2008)

3. Regulation of Intracellular Cholesterol Concentrations

Levels of cholesterol is maintained via many processes involving a feedback mechanism that controls the transcription and translation of LDL-R and HMG-CoA reductase (Go & Mani, 2012; Nair, 2013).

The crosstalk behind the absorbed cholesterol and the de novo synthesized cholesterol was first recognized by doctors Michael Brown and Joseph Goldstein in 1974,

who showed the role of LDL, and its receptor (LDL-R) in the regulation levels of cholesterol and hence cholesterol biosynthesis via regulation of HMG-CoA reductase (Go & Mani, 2012; Nair, 2013).

This study has shown that, in patients with familial hypercholesterolemia, the absence or deficiency of functional LDL-R impaired the uptake of LDL by the liver and other tissue; consequently, there is an increase in LDL plasma levels and then cholesterol levels. In addition, the study demonstrated that internalization of LDL suppressed cellular cholesterol synthesis by inhibiting the rate limiting enzyme HMG-CoA reductase (J. L. Goldstein & Brown, 1984; Joseph L. Goldstein & Brown, 1990) (figure 10).

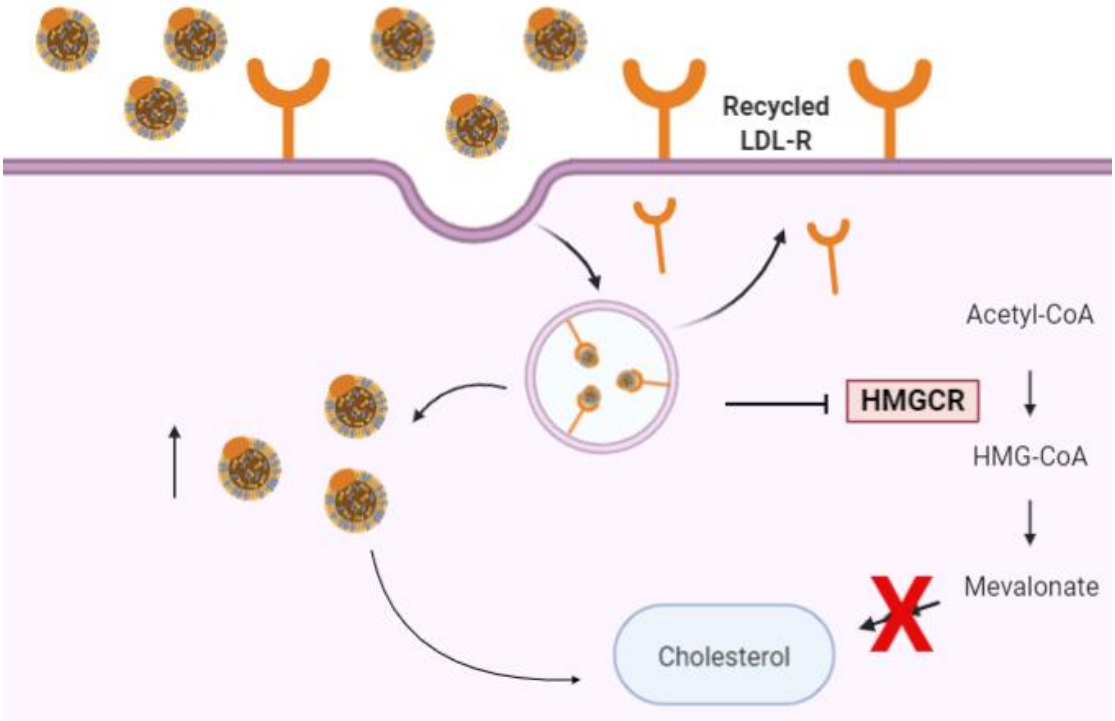


Figure 10: Feedback mechanism on intracellular cholesterol level

a. HMG-CoA reductase

HMG-CoA reductase catalyzes the rate limiting step of cholesterol biosynthesis. The human HMG-CoA reductase gene is 24 kbp, located on the chromosome 5, is composed of 20 exon and 19 introns (Friesen & Rodwell, 2004) (figure 11).

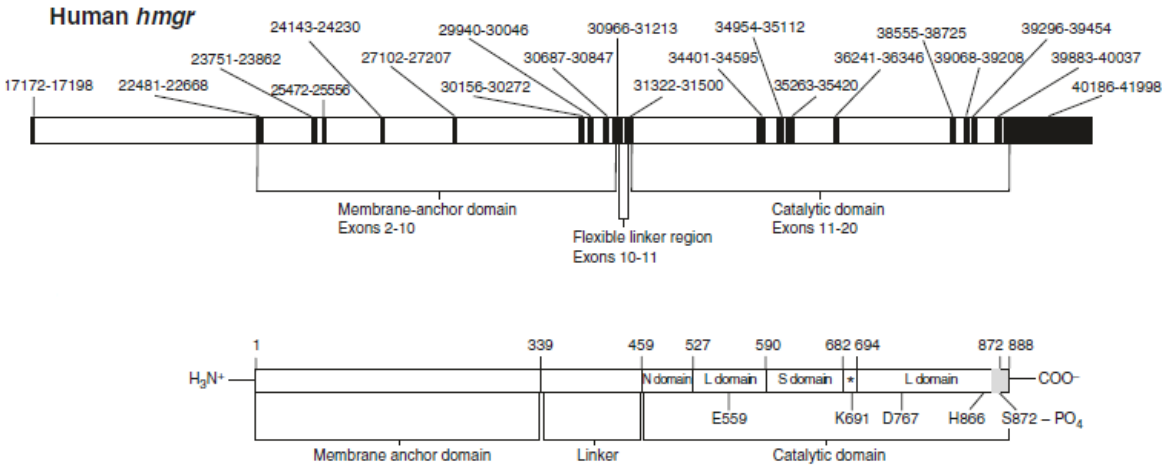


Figure 11: Schematic representation of the human hmgr gene and the human HMG-CoA reductase (Friesen, J. et. al.,2004)

Its mRNA encodes two isoforms that are produced by alternative splicing of the exon 13 (Medina & Krauss, 2009). Studies have reported differences between enzymatic activity as well as its responses to statin inhibition. The alternatively spliced transcription, absent exon 13, has shown less activity, as it encodes in part of the enzyme catalytic domain and statins biding site (Medina & Krauss, 2009; Stormo et al., 2012) (figure 12).

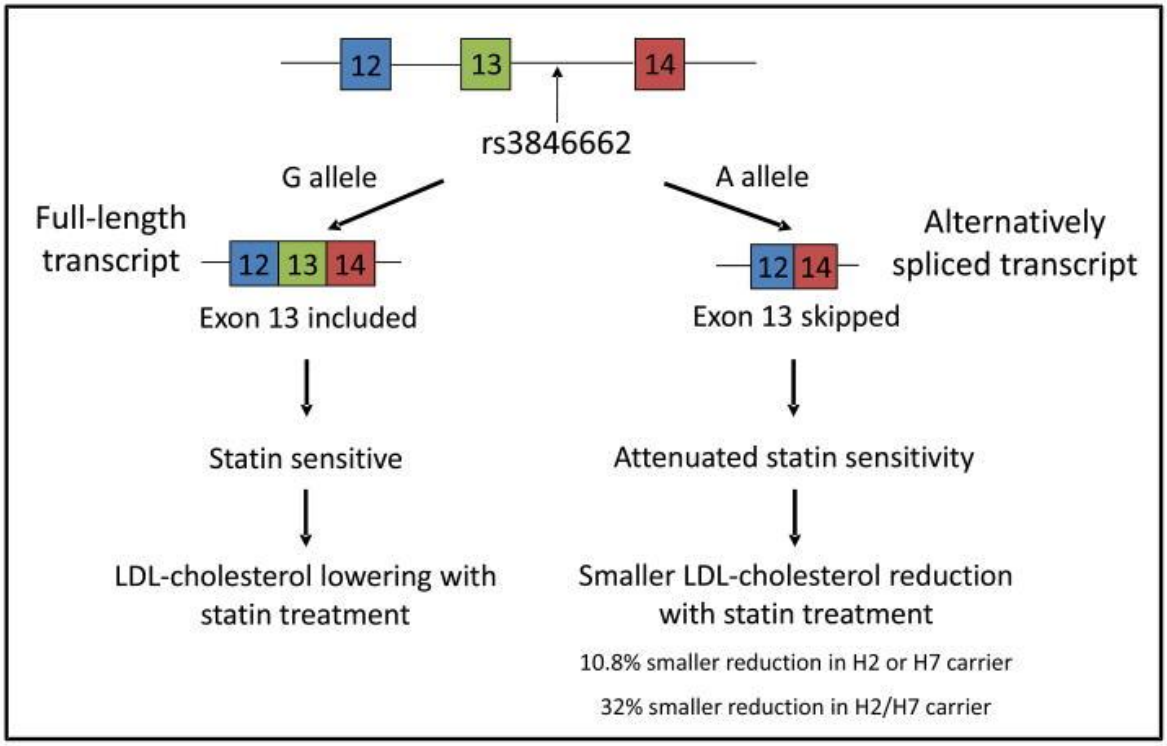


Figure 12: Scheme of the alternative splicing of HMG-CoA reductase (Medina, M. W. et al., 2009)

Structurally, the HMG-CoA reductase consists of 8 transmembrane glycoproteins located in the endoplasmic reticulum (ER). This enzyme is made of two major domains: the NH2-terminal domain anchoring the enzyme to the ER, and the hydrophilic COOH-terminal found in the cytoplasm consisting of the catalytic domain (Friesen & Rodwell, 2004) (figure 13).

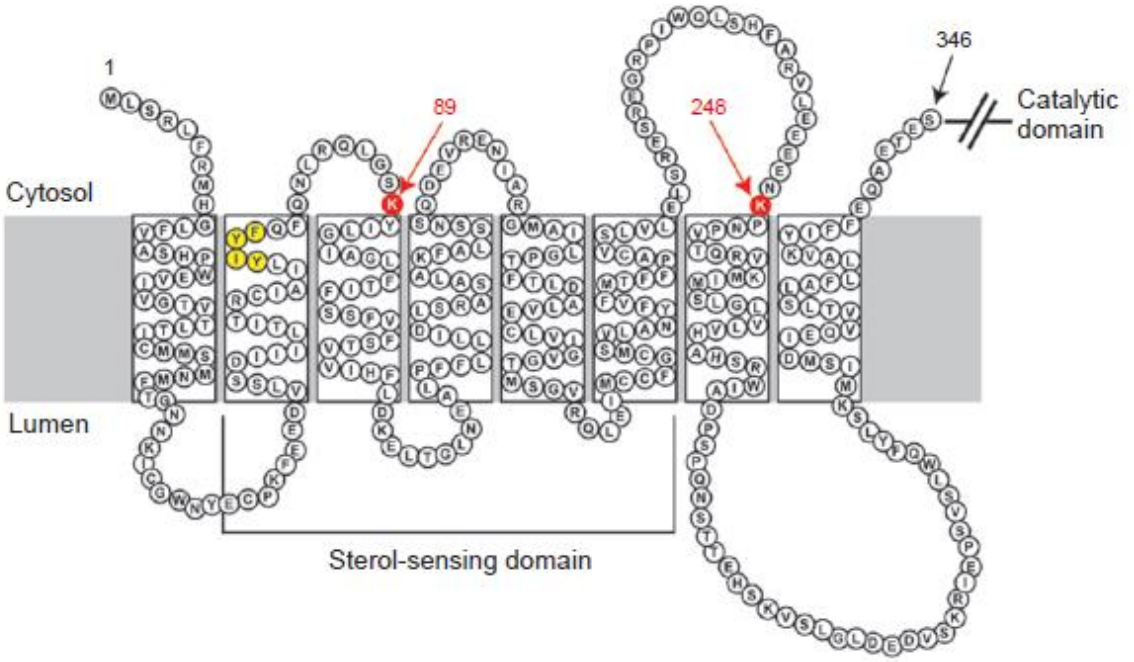


Figure 13: HMG-CoA reductase structure (DeBose-Boyd, R., 2008)

The enzyme is extensively regulated at the transcriptional, translational, and post-translational levels.

i. Transcription Regulation of HMG-CoA reductase

Transcription of HMG-CoA reductase is regulated by sterol regulatory element-binding proteins (SREBPs), which also regulates the transcription of LDL-R (Brown & Goldstein, 1997; Radhakrishnan et al., 2010).

SREBPs constitute a part of membrane-anchored transcription factors family, which coordinates and regulates cellular uptake, transport, and utilization of cholesterol. Three members of the SREBP family (SREBP-2/ 1-a/ 1-c) were identified, which share three conservative domains: NH2-terminal transcription factor domain; middle hydrophobic

region; and COOH-terminal regulatory domain located in the cytoplasm (Brown & Goldstein, 1997; Burg & Espenshade, 2011; Nair, 2013; Ness & Chambers, 2000).

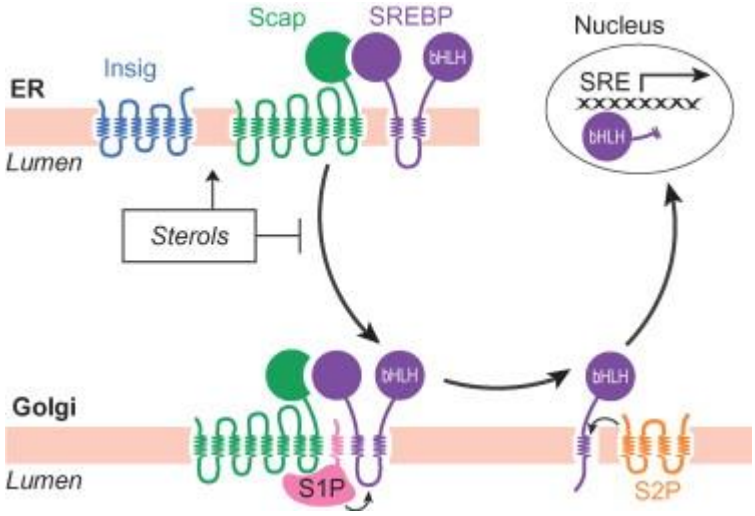


Figure 14: SREBP domains and activation (Radhakrishnan et al., 2010)

Translocation of SREBP to the nucleus, hence activation of HMG-CoA reductase and LDL-R transcription, is regulated by: SREBP-cleavage activating protein (SCAP), site 1 protease (S1P), site 2 protease (S2P) and insulin-induced gene (Insig) (Burg & Espenshade, 2011; Cerqueira et al., 2016; Friesen & Rodwell, 2004).

SCAP behaves as a cholesterol sensor and an escort protein for SREBP to the Golgi complex. When intracellular cholesterol levels are low, Scap/SREBP forms a heterodimer complex that is transported to the Golgi complex, where SREBP gets activated by two consecutively cleavages catalyzed by S1P and S2P (Brown & Goldstein, 1997; Nair, 2013; Ness & Chambers, 2000) (figure 14).

However, when intracellular cholesterol is high, the translocation of Scap/SREBP complex is blocked, which is triggered by the binding of cholesterol to Scap, and which favors the interaction of Scap/SREBP complex with the protein Insig. Consequently,

transcription of HMG-CoA reductase gene is inhibited (Brown & Goldstein, 1997; Nair, 2013; Ness & Chambers, 2000) (figure 15).

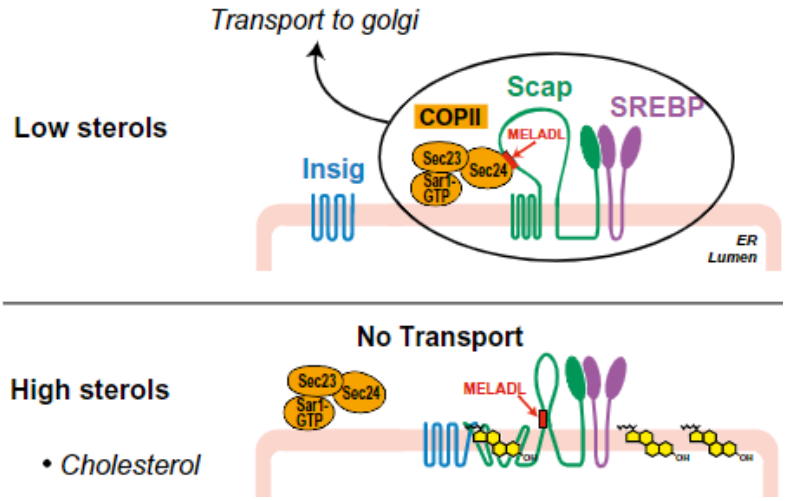


Figure 15: Inhibition of SREBP transportation by Insig activity (Radhakrishnan, Sun, Espenshade, Goldstein, & Brown, 2010)

ii. Circadian regulation

Circadian rhythms refer to physiological processes that repeat over a period of approximately 24 hours and ensure that internal physiology is synchronized with the external environment (Hussain & Pan, 2009; Schroor et al., 2019; Y. Yang et al., 2013).

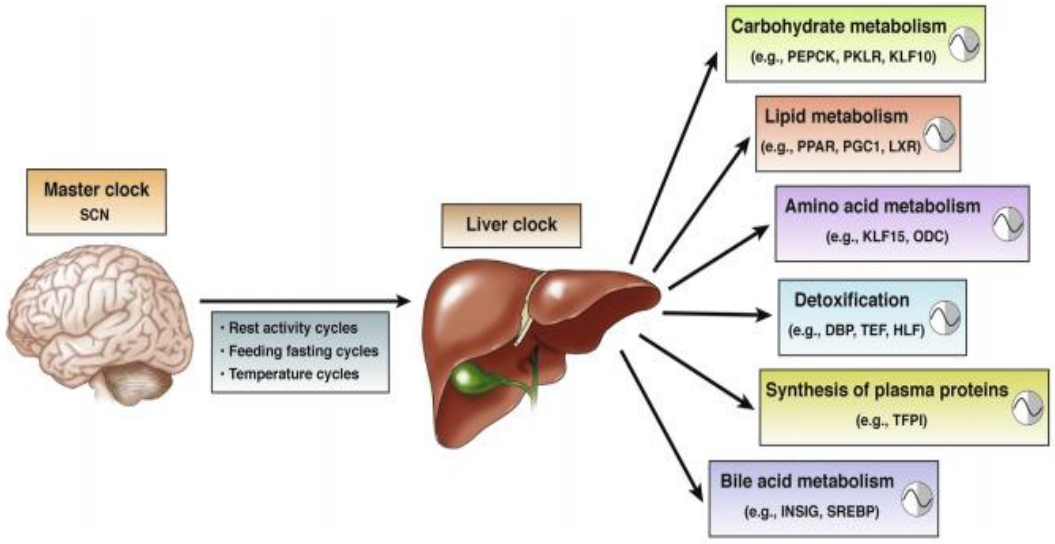


Figure 16: SCN synchronize metabolic function throughout the body (Reinke & Asher, 2016)

Accumulating evidence showed the impact of the circadian clock as well as dietary cholesterol in activity of HMG-CoA reductase (Hussain & Pan, 2009; Schroor et al., 2019; Y. Yang et al., 2013) (figure 16). According to Shapiro and Rodwell (1969) HMG-CoA reductase exhibits striking diurnal variations with peak activity at night. Mutation of clock gene showed a strongly suppressed gene expression of HMG-CoA reductase in mice (Kudo et al., 2008).

iii. Post-transcriptional Regulation

Another vital mechanism of feedback regulation of hepatic HMG-CoA reductase relies on the translation, stability, and catalytic activity of the enzyme (Jiang et al., 2018; Myant, 1981; Radhakrishnan et al., 2010).

Brown et al. (1900), reported that translation of HMG-CoA reductase is influenced by farnesylated protein which can affects stability of mRNA causing degradation (Joseph L. Goldstein & Brown, 1990). Moreover, Gayer et al. (1995) showed that lovastatin treated C100 cell lines result in a decrease in HMG-CoA reductase mRNA and consequently decrease in its translation levels (Gayen & Peffley, 1995b).

At the protein level of regulation, the process of regulation can be divided into two classes: short-term regulation (Insig-independent) and long-term regulation (Insig-dependent) (Guixa-Gonzalez et al., 2017; Luo et al., 2020).

Furthermore, the translated protein is regulated covalently and reversibly by phosphorylation and dephosphorylation that is triggered by changes in the glucagon/insulin ratio or AMP/ATP ratio.

The energy states dictate the activity of HMG-CoA reductase. During low energy state (AMP/ATP is high) cell economizes on energy expenditure by inhibiting protein synthesis, lipogenesis, including cholesterol biosynthesis. They are mediated by AMP-activated protein kinase (AMPK) activity that is allosterically activated by AMP (Burg & Espenshade, 2011; Guixa-Gonzalez et al., 2017; Luo et al., 2020) (Figure 17).

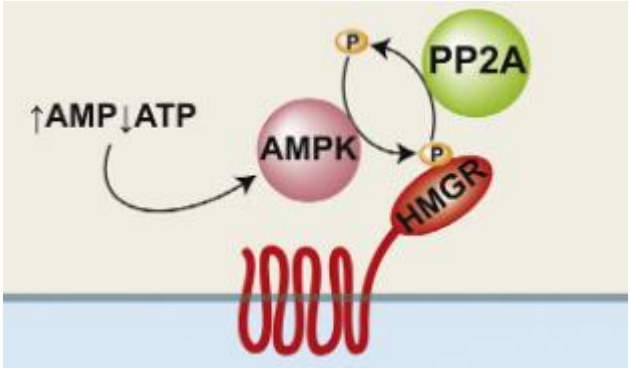


Figure 17: Phosphorylation of HMG-CoA reductase (Burg & Espenshade, 2011)

On the other hand, cyclic AMP (cAMP), a second messenger, the level of which rises with the increase in the glucagon/insulin ratio. This has dual effect phosphorylating HMG-CoA reductase and phosphorylating a phosphoprotein phosphatase inhibitor (PPI) making it active, hence inhibiting the dephosphorylation of the HMG-CoA reductase (Beg et al., 1985, 1987) (figure 18). When the glucagon/insulin ratio is low, phosphatases are activated which will dephosphorylate HMG-CoA reductase are activated, leading to its activation and the dephosphorylation of PPI making it inactive (Rao & Ramakrishnan, 1975).

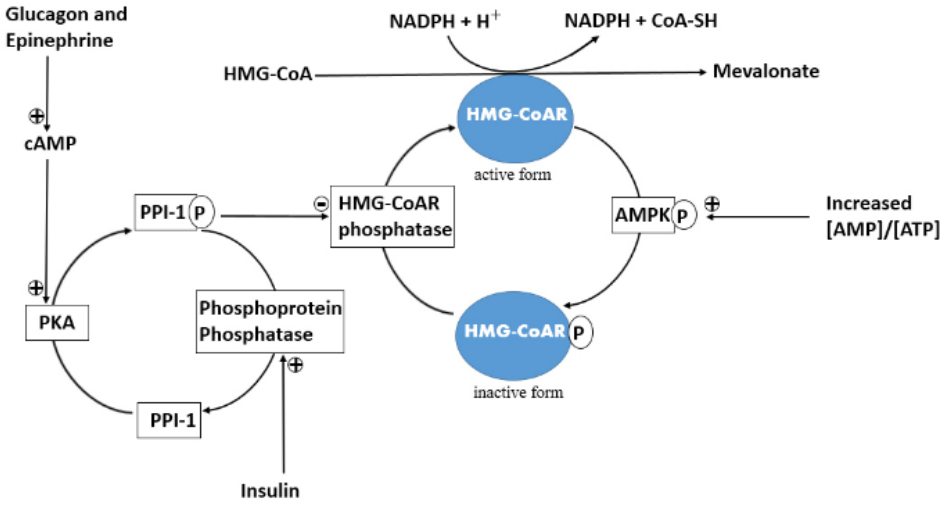


Figure 18: HMG-CoA reductase and PPI regulation (Bathaei et al., 2016)

Furthermore, stability of the protein, hence the half-life of the HMG-CoA reductase depends on intracellular cholesterol/sterols levels (Burg & Espenshade, 2011; DeBose-Boyd, 2008; Ness & Chambers, 2000). The increased levels of cholesterol trigger the biding of Insig to HMG-CoA reductase NH2-terminal region favoring the ubiquitination of the enzyme and further degradation (Beg et al., 1985, 1987; Burg & Espenshade, 2011) (figure 19). Studies have reported that upon a decrease in intracellular cholesterol levels HMG-CoA reductase half-life reaches around 10 h. However, increased levels of cholesterol may decrease the protein half-life around 10-folds (~ 45 min) (Espenshade, 2013).

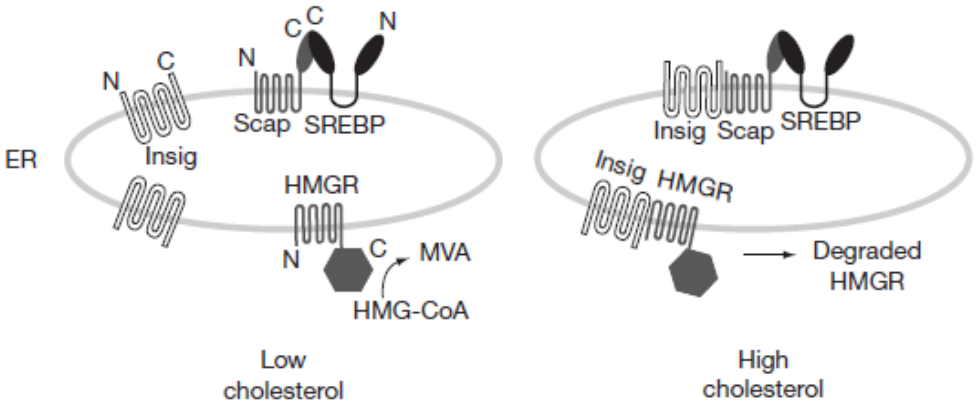


Figure 19: Mechanisms involved in the stability of HMG-CoA reductase upon cellular cholesterol concentration (Espenshade, P, 2013)

b. <u>LDL receptor</u>

The LDL receptor plays a critical role in cholesterol homeostasis. The LDL receptor is responsible for transporting cholesterol carried by LDL into the cells by receptor-mediated endocytosis. LDL-R protein is encoded by a family of gene located on the short arm of chromosome 19 composed of 18 exons and 17 introns. Different isoforms have been reported to exist, produced by alternative splicing (Tveten et al., 2006; Yuan et al., 2018; Zhu et al., 2007) (figure 20). According to the UCSC Genome Browser database, six different isoforms were identified for the LDL-R gene (Human, 2013). Tveten et al. (2006)

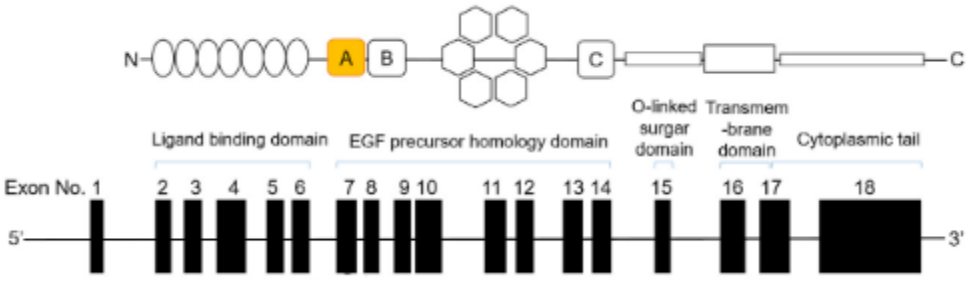


Figure 20: LDL receptor transcript (Yuan et al., 2018)

reported four isoforms involving the splicing of exon 4 and 12, with splicing of exon 4 being the most abundant.

The receptor includes an extracellular domain, a transmembrane domain, and a cytoplasmic domain (figure 21).

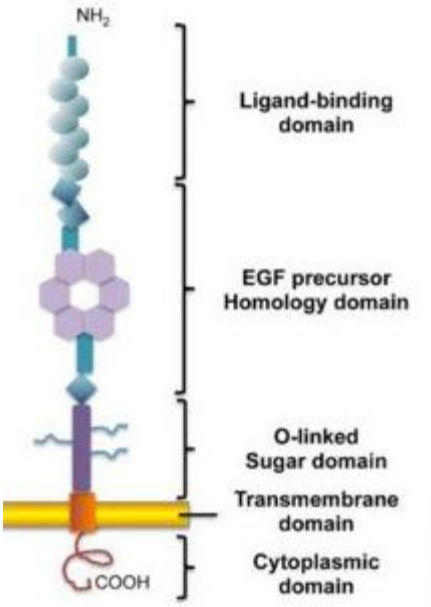


Figure 21: LDL receptor structure (Benito-Vicente et al., 2018)

The life cycle of the receptor is controlled by the rate of its biosynthesis, surface localization, internalization, recycling, and degradation (Huff et al., 2014; Nair, 2013; H.-X. Yang et al., 2020).

Cholesterol regulates levels of LDL-R: an increase in intracellular cholesterol results in the decrease of LDL-R expression, which is influenced by the transcription factor SREBP and its levels in the cellular membrane (Huff et al., 2014; Nair, 2013; H.-X. Yang et al., 2020).

The fate of LDL-R following its internalization, LDL-R is degraded by proprotein convertase subtilisin/kexin type 9 (PCSK9); or becomes a target for degradation in the plasma membrane by inducible degrader of LDLR- IDOL. Those two proteins play an

important role in the levels of LDL-R present in the cell surface and the degradation ratio of the receptor (H.-X. Yang et al., 2020).

Just like the LDL receptor and HMG-CoA reductase, PCSK9 gene expression is regulated by the activation of the SREBP. PCSK9 interacts with the extracellular portion of LDL receptor sequestering it and reroutes it from the recycling pathway toward degradation (Go & Mani, 2012; Huff et al., 2014; Nair, 2013; H.-X. Yang et al., 2020) (figure 22).

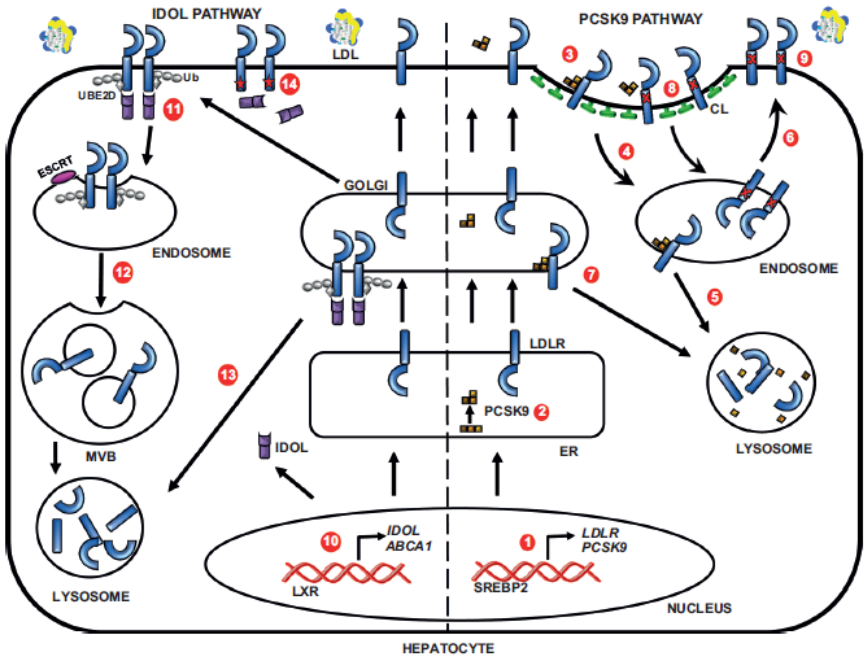


Figure 22: Degradation mechanism of LDL receptor (Huff, Assini, & Hegele, 2014)

4. Cholesterol deregulation in diseases

As mentioned before, cholesterol is an important component of lipid rafts and has been recognized for: its ability to regulate cell membrane proteins, receptor trafficking, signal transduction, as well as influencing cell membrane fluidity (Lingwood & Simons, 2010; Simons & Ikonen, 1997; S.-T. Yang et al., 2016). However, its accumulation in the body has been recently associated with the increased risk of several diseases, one of which is cancer (Daniels et al., 2009; J.Z. Li et al., 2010; Lindgren et al., 1985).

Therefore, some studies suggested that cholesterol present in the cell membrane may activate some oncogenic pathway, thus inducing cell differentiation, cell proliferation and tumor formation (Huang et al., 2016; Shen et al., 2016). Likewise, being an important component of neuronal and glial membranes and a key constituent of myelin, cholesterol deregulation has been linked to neurodegenerative disorders, such as Alzheimer's disease, Huntington's disease, Parkinson's disease, Niemann-Pick type C disease and Smith-Lemli Opitz syndrome (Adorni et al., 2019; Jeong et al., 2018; Riobo, 2012). The underlying mechanism has not been clearly identified but most hypotheses have associated it with lipid raft integrity and the activation of important cell signaling pathway (Adorni et al., 2019; Jeong et al., 2018; Riobo, 2012).

Finally, the causal relation between cholesterol levels and cardiovascular disease has been comprehensively studied and proved. In the last few decades, many therapeutic approaches to lower plasma cholesterol levels have been investigated (Adorni et al., 2019; Jeong et al., 2018; Riobo, 2012).

a. Cholesterol treatment

Since 1950 several epidemiologic studies have been conducted to reveal the correlation between cholesterol and coronary heart disease. The linear relation between the blood level of cholesterol and the incidence of heart attacks have been well confirmed; hence the interest in cholesterol lowering therapy including dietary interventions or changes in life style. (Nair, 2013; Trapani, 2012).

Several drugs have been introduced over the years that interfere with cholesterol pathways at different sites, some of which were withdrawn from the market because of the side effects, such as Triparanol and Clofibrate (Endo, 2010; Nair, 2013; Trapani, 2012)..

In 1976, Compactin was identified and introduced as the first competitive inhibiter of HMG-CoA reductase. Currently it is recognized as the precursor of the statins drug family'. Dr. Akira Endo had isolated an active principle from a fermentation broth of Penicillium citrinum, which can inhibit the enzyme activity (Endo, 2010; Nair, 2013; Trapani, 2012).

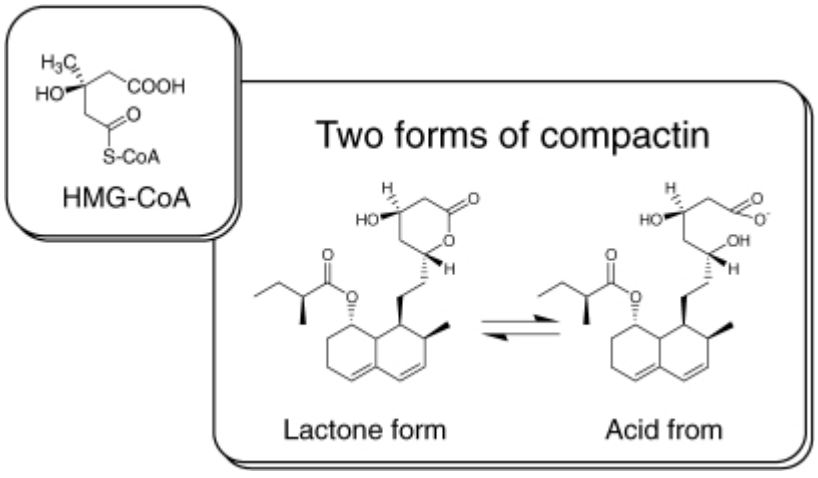


Figure 23: Compactin and HMG-CoA structures (Endo, 2010)

The mechanism by which Compactin inhibits the HMG-CoA reductase enzyme relies on the similarity between the drug and substrate structure (figure 23). Due to the inhibition of the enzyme HMG-CoA reductase, a significant increase in the number of LDL receptors on the cell surfaces can be observed; thus, leading to a decrease in the blood LDL cholesterol (Dreyer et al., 2018; Endo, 2010). It was structurally modified, developed and introduced in the market with names' such as Lovastatin, Simvastatin, Pravastatin, Fluvastatin, Atorvastatin, Cerivastatin, and the two most recent drugs Rosuvastatin and Pitavastatin (Dreyer et al., 2018; Endo, 2010) (figure 24).

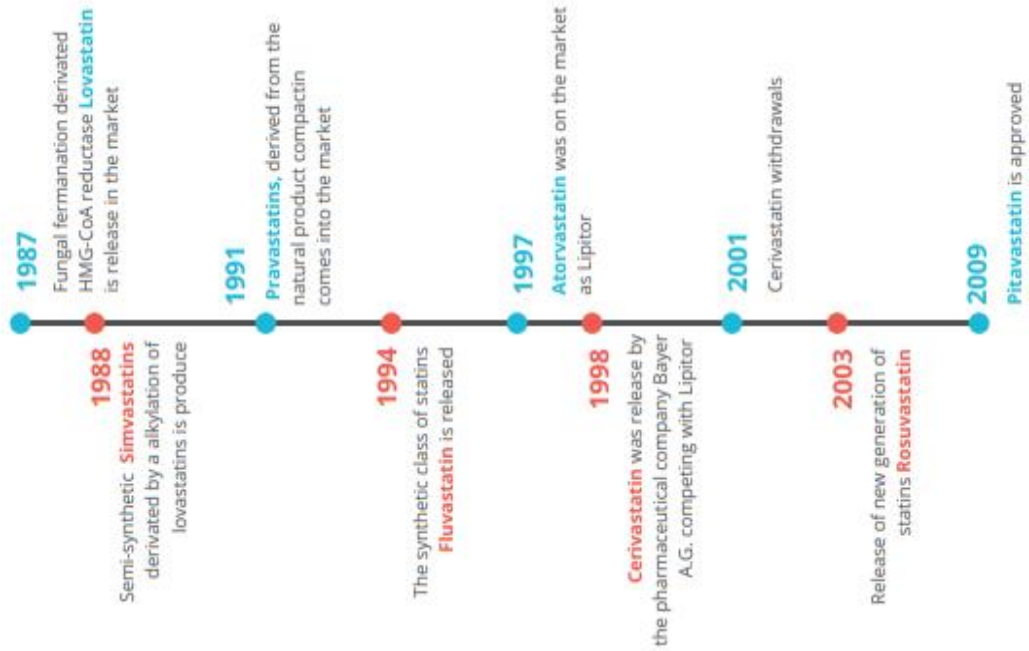


Figure 24: Statins timeline

This large family of drugs, known as statins, share a common mechanism of action, although differing in terms of their chemical structures, pharmacokinetic profiles, and lipid-modifying efficacy (Dreyer et al., 2018) (figure 25).

Characteristic	Atorvastatin	Cerivastatin	Fluvastatin	Lovastatin	Pravastatin	Simvastatin
Prodrug absorption	No	No	No	Yes	No	Yes
(%)	Rapid	No data	98	30	35	60–85
Bioavailability (F)						
(%)	12	60	24	<5	17	<5
Excretion (%)						
Urine	<2	24	5	10	20	13
Feces	>98	70	90	83	70	60
Half-life (hours)	14 (parent)	2–3	<1	3–4	1.8	3
Protein binding (%)	≥90	>99	98	>95	50	95
CYP substrate	CYP3A4	CYP3A4 CYP2C8	CYP2C9	CYP3A4	Sulfation	CYP3A4
Major metabolites contributing to lipid-lowering effects	Yes	Yes	No	Yes	No	Yes
Lipophilicity	Lipophilic	Lipophilic	Hydrophilic	Lipophilic	Hydrophilic	Lipophilic

Figure 25: Differences between statins according to it pharmacodynamic (Dreyer et al., 2018)

Overall, their structures can be divided into three parts: an analog of the target enzyme-substrate, HMG-CoA; a complex hydrophobic ring structure that is covalently linked to the substrate analog and which is involved in binding of the statin to the reductase enzyme; and side groups on the rings that define the solubility properties of the drugs and their pharmacokinetic properties (Chong et al., 2001; Dreyer et al., 2018).

They can be classified into three different groups, based on their potential to decrease the LDL cholesterol concentration in the blood. The first class includes Pravastatin and Fluvastatin, which are considered the least potent statins. The second class encompasses Atorvastatin and Simvastatin which are until today, considered the best-selling statins. Finally, the third generation, represented by Rosuvastatin and Pitavastatin, are the most potent and efficient of all statins (Chong et al., 2001; Dreyer et al., 2018).

To this day, statins remain the most common treatment used in the management of hypercholesterolemia. Although widely prescribed, statins are not universally well tolerated (Dreyer et al., 2018; Endo, 2010). A growing number of patients have reported symptoms of statin intolerance even at low doses. One of the most common side effects is muscle-related side effects (MRSE) which affects 5-10% of statin-treated patients and has become

a major obstacle for some patients to continue their lipid-lowering therapy (Chong et al., 2001; Endo, 2010; McFarland et al., 2014). Alternative therapies are thus needed to reduce the risk of cardiovascular disease and reinstate lipid balance in hypercholesterolemic patients.

D. Objectives of the Study

Increase in blood is considered the most important risk factors underlying cardiovascular disease (CVDs). Statins have been the recommended drug of choice to control cholesterol level. However, several collateral effects were reported in statin treated patients including myopathy and liver dysfunction. Hence the need for an alternative preventive or curative therapy is necessary.

Traditional medicine has used plants and herbal extracts in the treatment of many diseases. The interest in natural extracts is increasing for their efficacy and low cost. Moringa Oleifera (MO), a valuable crop rich in nutrients, has been widely investigated demonstrating great benefits to human health. Being highly consumed as raw, cooked, or boiled as tea drink, people have attributed many beneficial effects in the treatment of many ailments: cancer, lipo-proteinemia, diabetes, hypertension, inflammation, and others.

Moringa leaves is widely consumed as hot drink in many countries. Studies have shown a change in the lipoprotein profile in serum of animals consuming MO extracts (organic or aqua-alcoholic). In this study we plan to investigate the effect of MO leaves extracts, mimicking people consumption. More specifically, we postulate that chronic ingestion of MO leaves water extract (MOE) will impact cholesterol level, hence may have a preventive effect against hypercholesterolemia. To substantiate the MOE

hypocholesterolemic effect, we plan to study the biochemical effect of long exposure of liver cells to MOE on cholesterol homeostasis.

Liver is the main site for cholesterol synthesis, distribution, and excretion, we aim to study the effect of in vitro MOE on HMG-CoA reductase and LDL receptors using human hepatic carcinoma cells (HepG2). Towards this goal, we will:

1. Determine whether long exposure (24, 48, 72 hours) of HepG2 cells to MOE is not cytotoxic, hence is metabolically active.
    a) Verify viability using MTT and trypan blue exclusion assays
    b) Determine ROS and ATP levels
2. Examine the effect of long exposure of HepG2 to MOE on the gene expression of HMG-CoA reductase and LDL receptor using RT-PCR
3. Examine the effect of long exposure of HepG2 cells to MOE on protein expression of both LDL receptors and HMG-COA reductase using Western blotting Effect of MOE on expression of LDL receptors using immunostaining
4. Determine the effect of long exposure of HepG2 cells to MOE on plasma membrane expression of LDL receptors using fluorescent immunostaining
5. Assess the direct effect of MOE on purified HMG-CoA reductase enzyme activity
6. Determine intracellular cholesterol level in MOE treated HepG2 cells

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Moringa leaves

Pulverized leaves of Moringa were provided by Mr. Salah Abdoun, an agricultural engineer from Sudan, Khartoum. Leaves were washed with distilled water and allowed to dry at room temperature in a dark and aerated place. Dried leaves were grounded and stored in the dark, in a glass jar at room temperature.

2. Cancer cell line

Human liver cancer cells (HepG2, cat# HB-8065) were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA.

3. Disposable lab ware

- Cell culture plastic wares were purchased from Corning.
- Microscope cover glasses for immunofluorescent assays (cat # G15973C) were purchased from GAIGGER.
- Microscope glass slides for immunofluorescent assays (cat # B4 0401) were purchased from Knittel Glass.

4. Cell Culture Reagents and In Vitro Kits

Sigma-Aldrich

- Dublecco's Modified Eagle's Medium- High glucose (DMEM) cat# D5796
- Fetal Bovine Serum (FBS) Heat Inactivated cat# F9665
- Trypsin-EDTA solution 1X cat# T3924
- Phosphate buffered saline (PBS) without calcium and magnesium cat# D8537
- HMG-CoA Reductase Assay Kit (cat# CS1090)
- Cholesterol Quantitation Kit (cat# MAK043
- ATP Kit (cat# MAK190)

Biowest

- Penicillin-Streptomycin Solution 100x (PEN-Strep) cat# L0022-100

GE health care

- RNA Kit (cat#25050072)

Qiagen

- cDNA Kit (cat#205311)

3. Chemicals and reagents

Sigma-Aldrich

- Dimethyl sulfoxide (DMSO; 41640); Bovine Serum Albumin (BSA; A2153); Thiazolyl Blue Tetrazolium (MTT; M5655); IGEPAL CA-630 (I8896)

ACROS Organics

- Nitro Blue Tetrazolium Chloride (NBT; A0317685)

MERCK

- Potassium Hydroxide pellets (KOH; C140532); Pure Acetone

VWR-Chemicals

- Methanol; Ethanol Absolute

Invitrogen

- ProLong Gold antifade reagent with DAPI (Mounting Medium; P36931)

Bio-Rad

- 0.2 µm pore–size nitrocellulose membrane

4. Antibodies:

ABCAM

- LDL-R, rabbit recombinant monoclonal (ab52818)
- HMG-CoA reductase, rabbit polyclonal (ab214018)
- Anti-beta Actin, rabbit polyclonal (ab8227)

Santa Cruz Biotechnology

- GAPDH, mouse monoclonal (H2114)

5. Buffer Preparations

- PBS 1X was prepared by diluting 100 ml of PBS 10X up to 1 liter of ddH2O
- 10% BSA-PBS was prepared by dissolving 1 g of BSA in 10 ml of PBS 1X
- Sucrose Hepes Tris–Base buffer (SHT, pH 7.4): 250mM sucrose-10mM Hepes-50mM Tris-Base buffer was prepared by dissolving 85.6g of sucrose, 2.83g of Hepes and 60g of Tris-Base dissolved in 1 Liter ddH2O and adjusting its pH.

- Lysis buffer: 1µl of triton (1%) added to 1mL of SHT.
- LaemmLi (4X): 8mL of 10% SDS, 1mL of 100% glycerol, 2mL of 5% ß-Mercaptoethanol and 100µl of 0.01% bromophenol blue.
- Running Buffer (5X): 15.1 g of Tris-HCl, 72 g of Glycine and 5 g of SDS were mixed with ddH_2O up to 1000mL
- Transfer Buffer (1X): 3.03 g of Tris-Base, 11.4 g of Glycine and 1 mL of 10% SDS were dissolved in methanol (200mL) and diluted up to 1000 mL with ddH_2O.
- Tris-Buffered Saline (TBS 10X): 30 g Tris-HCl and 80 g of Sodium Chloride were mixed with ddH2O up to 1000mL
- Washing buffer (1X): 100 ml of TBS and 900 ml of H_2O and 200 ml of Absolute methanol were mixed, and 1 ml of 10% SDS were added
- Blocking buffer (5%): 2.5 g of fat free milk were mixed in 50 ml of washing buffer
- Acrylamide (30%): 30g Acrylamide and 0.8g N, N-methylene-bisacrylamide were mix with ddH_2O up to 100 mL
- Resolving gel (10%): for 10 mL gel, 4 mL H_2O, 3.3 mL of 30% acrylamide, 2.5 mL Tris-HCl (1.5M, pH 8.8), 50 µl 10% SDS, 100 µl Ammonium Persulfate (APS, 10%) and 5 µl N, N, N', N'-Tetramethyl ethylenediamine (TEMED) were mixed.
- Stacking gel (5%): for 5 mL of stacking gel was prepared by mixing 3.4 mL ddH_2O, 830µl (30%) acrylamide, 630µl Tris-HCL (0.5M, pH6.8), 50µl SDS (10%), 50µl APS (10%) and 5 µl TEMED.

6. Equipment

The following equipment were used in the study:

- Centrifuge 5416 and centrifuge 5810 (eppendorf)
- Hematocytometer (Fisher scientific) (cat # 0267110)
- Inverted microscope (Axiovert 25)
- Mini spin centrifuge (Thermo)
- Centrivap Console (Labconco)
- AES-2010 Speed Vac System (Thermo Savant)
- UV-VIS scanning Spectrophotometer (UV-2101 PC) (SHIMADZU)
- Fluoroskan™ FL Microplate Fluorometer and Luminometer (Thermo)
- LSM710 confocal microscope (Zeiss)
- Scientific™ Multiskan™ GO UV/V (Thermo).

B. Methods

1. Preparation of MO Extract

In order to mimic people's daily consumption of *Moringa* as an herbal tea drink, MO water extract (MOE) was prepared by soaking the pulverized leaves' in hot ddH_2O while stirring for 30 minutes. The extract was centrifuged, the aqueous portion (infusion) was filtered, aliquoted (1ml) and stored at -20°C for later use. Different stocks with concentrations ranging between 0.05%-1% were prepared.

2. Cell Culture

HepG2 cells were incubated in a humidified 5% CO_2 incubator at 37ºC and cultured in DMEM (500 mL) supplemented with 10% FBS and 0.5% Pen-Strep. Cells were treated using a freshly prepared master solution of DMEM-MOE (10:1v/v) for each stock concentration.

1. Viability assay

The cytotoxic effect of varying MOE concentration on HepG2 cells was determined using the colorimetric MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and trypan blue exclusion assay.

a. MTT Assay

MTT assay is a colorimetric assay for measuring cell metabolic activity. It is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidoreductase enzyme to metabolize MTT into purple-colored formazan. Briefly, HepG2 cells were seeded in 96-well plates (1×10^4, 100 µL DMEM), incubated at 37 °C for 24 hours, followed by treatment with different final concentrations of MOE ranging between (0.01%-0.1%). 10 µL of MTT (5 mg/mL in PBS) and 100 µL of media were added to each well and incubated for 4 hours at 37 °C. The media was discarded, and the formed formazan crystals were dissolved with 50 µL DMSO and incubated for 20 minutes at 37ºC.

Color intensity was measured using the ELISA reader at 595 nm. Viability was calculated as follows:

$$\%\,Viabilitty = 100 - \%Death = \frac{Absorption\ \ Treated\ cells}{Absorption\ Control\ cells} \times 100\%$$

b. Trypan Blue

Trypan Blue is a stain used to quantify live cells by labeling dead cells exclusively. Loss of membrane integrity of non-viable cells allows trypan blue to permeate cells, dyeing them dark blue. On the other hand, live cells with intact plasma membrane will exclude the dye remaining colorless. Briefly, HepG2 cells were plated in 12 well plates (1×10^5 cells,1 mL/well) at 37 °C for 24 hours and treated with different concentrations of MOE (0.005,0.01,0.015 and 0.02%) for 24, 48 and 72 hours. Following trypsinization, control and treated cells were collected by centrifugation and re-suspended in media. An equal volume of the cell suspension and trypan blue (1v/1v) were mixed and loaded into a hemocytometer where both viable and dead cells were counted. The % cell viability was calculated as follows:

$$\%\, Viability = 100 - \%Death = \frac{\sum Alive\ Cells}{\sum(Alive\ Cells\ + Dead\ Cells)} \times 100\%$$

3. Determination of Intracellular ROS level

ROS levels were assessed using the nitro-blue tetrazolium (NBT) assay. This assay based on the ability of superoxide anion radical to reduce NBT into blue formazan. In brief, HepG2 cells were seeded in 96-well plates (1×10^4 cells, 100 µl/well) and treated for 24, 48 and 72 hours with different concentrations of MOE (0, 0.005, 0.01 and 0.015 %). Media was aspirated, NBT (1 mg/mL in ddH_2O, 100 µL) was added, and plates were incubated (1 hour) in a humidified 5% CO_2 incubator at 37ºC. Cells were then washed with methanol (100 µl/well) and left to dry at room temperature. Blue formazan crystals were consecutively dissolved with KOH (2M, 120 µl) followed by DMSO (140 µl). The

absorption of the developed color was measured using a microplate reader at 630 nm. ROS levels were calculated relative to a control as follows:

$$\%\ NBT\ Reduction = \frac{Absorption\ \ Treated\ cells}{Absorption\ Control\ cells} \times 100\%$$

$$\%\ ROS\ Production = 100 - \%\ NBT\ Reduction$$

4. HMG-CoA Reductase Activity Assay

The effect of MOE on the activity of pure HMG-CoA reductase was determined using HMG-CoA reductase activity assay kit (Sigma-Aldrich) following manufacturer's instruction manual. The reaction assay (96 well plate) of final volume 200 µL per well contained: 10 µL of MOE (final concentration: 0.01, 0.015 and 0.02), 4 µL NADPH (400 µM), 12 µL HMG-CoA substrate (0.3 mg/ml) and 171 µL of 1 X assay buffer. Reaction was initiated by addition 2 µL of the enzyme HMGR (0.50–0.70 mg/ml) to each well.

$$HMG - CoA + 2\ NADPH + H^{+} \xrightarrow{HMGR} mevalonate + 2\ NADP^{+} + CoASH$$

Oxidation of NADPH was kinetically monitored, at 37°C, by measuring absorbance (340 nm) every 10 seconds over 20 minutes, using a microplate spectrophotometer reader. Results were expressed as % of the control specific activity of the enzyme in the absence of MOE. Pravastatin (1 µL, 100 µM) was used as a positive inhibitory control of HMGR activity.

5. Cholesterol levels determination

Cholesterol levels in control and MOE-treated (0.015%) HepG2 cells were determined using Cholesterol Quantitation kit (MAK043) from Sigma-Aldrich, following manufacturer's instructions. Cells were seeded in petri dishes ($1x10^6$ cells/ 10ml) and treated with MOE (0.015%) for 24, 48 and 72 hours. Cells were scraped, collected by centrifugation (900 rpm, 10min), washed, and resuspended with 1 ml of ice-cold PBS. An aliquot of 200 µL was removed for protein quantification. Cells from the remaining 800 µL suspension were pelleted down by centrifugation (1500 rpm, 5 min), and were homogenized in lipid extracting solution (200µL) composed of: chloroform: isopropanol: IGEPAL (7 ml:11 ml: 0.1 ml). The lipid extract was centrifuged (13,000g ,10 minutes) while the pellet was discarded, the organic phase was transferred to a new tube, and consecutively heated using a heating block (50°C for 30 minutes), followed by evaporation under vacuum using a centrifugal concentrator unit (30 minutes).

Extracted lipids were dissolved in the kit cholesterol Assay Buffer (200µL) then a sample of 50 µL was transferred into 96 well plates onto introduced 50 µL of kit provided reaction mixture and incubated for 60 minutes at 37 °C. Absorbance (570 nm) was measured and level of cholesterol was determined relative to a standard curve, normalized to protein level determined by Bradford assay, using BSA standard.

6. Expression of HMG CoA reductase and LDL receptors genes by q-PCR

The gene expression of two important proteins involved in cholesterol homeostasis, HMG-CoA reductase, and LDL receptor, were examined comparing MOE-treated with control cells. This involved:

a. RNA isolation and quantification

RNA samples were extracted from HepG2 cells line using total RNA kit according to manufacturer's instruction manual. Briefly, cells were lysed by vortexing in a mix of beta mercapto-ethanol: RA1 buffer provided by the kit (3.5 µL: 50 µL). The lysate was then transferred to the violet color RNA spin mini filter unit and centrifuged at 11000xg (1 minute). The eluted filtrate was mixed with ethanol (350ul of 70%), transferred into the light blue RNA spin mini column, centrifuged (8000g, 30seconds) followed by washing with a desalting buffer solution (MDB kit buffer), centrifuged (11000xg, 1 minute) followed by DNase digestion adding DNase I solution (kit) to remove DNA contamination. The filter was washed twice with each of RA2 and RA3 consecutively. Finally, the filter was eluted in RNase-free water (100µL), centrifuged (11000xg,1 minute) and the purity and quantity of eluted RNA was determined using a Nano Drop.

b. cDNA synthesis

Reverse transcription was performed using QuantiTect Reverse Transcription Kit. Two steps were involved: elimination of genomic DNA followed by reverse transcription.

To eliminate genomic DNA, the eluted RNA sample (1 µg) was incubated at 42°C for 2 minutes, with gDNA wipeout buffer (2 µl) and RNase-free water in a final volume of 14 µl. This comprises a DNA free RNA sample that will be used for reverse transcription.

Reverse transcription was then carried out in a final volume of 20ul composed of DNA-free- RNA sample (14ul), 1 µL of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript RT Buffer, and 1 µl of RT Primer Mix. The reaction was incubated for 15 min (42°C) followed by inactivation step of the enzyme by heating for 3 minutes at 95°C The reactions were stored at –20°C for later use.

c. q-RT-PCR

Real-time PCR using QuantiFast® SYBR® Green was used to quantify gene expression. The reaction assay of final volume of 10 µL, was composed of: 2µl of cDNA (1:10 dilution), 5 µl of QuantiFast SYBR Green PCR Master Mix, 0.5 µl of each of the forward and reverse primer each and 2 µl of RNase DNase free water.

The PCR amplification was carried out on CFX 384 and CFX 96 PCR Bio-Rad machines following a similar program for both HMG-CoA reductase enzyme and LDL receptor as follows: initial denaturation at 95 º C for 5 minutes; 40 cycles of denaturation at 95 ºC for 30 seconds; annealing at 60 ºC for 30 seconds; extension at 72 ºC for 45 seconds; and a final melting curve at 60 ºC for 5 seconds and 95 °C for 50 seconds.

Non-template controls (NTC) containing RNase-free water were used for each primer as a negative control. The recorded cycle threshold (Ct) values of the targeted genes were used to calculate the relative quantitation (RQ) using the delta-delta Ct (ΔΔCt) method, which were calculated as follows:

$$\Delta\Delta Ct = (Average\ Ct\ treated - Average\ Ct\ GAPDH) - (Average\ Ct\ control - Average\ Ct\ GAPDH)$$

$$RQ = 2^{-\Delta\Delta Ct}$$

Table 2: The sequence of primer used in RT-qPCR

Gene	Primer (Forward)	Primer (Reverse)
HMGCR	5-ACAATAAGATCTGTGGTTGGAATTATGA-3'	5-CCTAAAATTGCCATTCCACGAGCAATAT-3'
LDLR	5-CTGAAATCGCCGTGTTACTG-3'	5-GCCAATCCCTTGTGACATCT-3'

7. Indirect immunofluorescence assay

To determine the protein expression levels of LDLR, HepG2 cells were seeded onto glass cover slips placed in 12 well plate and incubated overnight (1x 10^5 cells/well). Cells were then treated with MOE (0.015%) at 37°C in 5% CO_2 for 24, 48 and 72 hours. All the subsequent steps were performed at room temperature. The cover slips were washed, fixed with 100% methanol (-20 °C for 10 minutes) followed by a permeabilization step with 100% acetone (-20 °C for 1 minute). Cells were blocked with 2% BSA-PBS (1 hour, room temperature) and incubated with primary anti-LDLR antibody (1:250ul) overnight at 4 °C, followed by 3 washes with 1X PBS (5 minutes each) and incubated with goat anti-Rabbit antibody (1/1000 diluted in 1% BSA-PBS) for 1 hour at room temperature, washed with PBS and finally treated with mounting solution with DAPI. Confocal images were captured with a confocal microscope.

8. ATP level determination

ATP level in MOE- treated HepG2cells was determined using Luminescence ATP detection Assay Kit (Sigma) following manufacturer's instruction. HepG2 cells were seeded for 24 hrs in 12-well plates (1x 10^5 cells/well), then treated with MOE (0.015%) at 37°C in

5% CO2 for 24, 48 and 72 hours. Cells were then collected by trypsinization, centrifuged, washed (1ml PBS, 1X), and lysed for 5 min, using lysis reagent provided by the kit. Luciferase enzyme (50 ul) was then added to a sample of the cell lysate (50 µl) and introduced into a black MTP plate. Luminescence was instantaneously measured using a microplate fluorometer/ luminometer (cat #: 5210470, Thermo Scientific). ATP level I treated HepG2 cells was compared with a control of untreated cells expressed as follows:

$$\%\ ATP\ levels = \frac{Bioluminescence\ Treated\ Cells}{Bioluminescence\ Control\ Cells}\ X\ 100\%$$

9. Western Blot

a. Sample preparation

Control and treated cells were scraped, and collected by centrifugation (900 rpm, 10 min). The pellet was washed twice with ice-cold PBS and resuspended in 200 µL of lysis buffer. Protein Level was determined using Bradford assay. Protein samples 20 µl, corresponding to 40mg of protein, were mixed with 5 µL Laemmeli (4x) and denatured by heating each sample for 10 minutes at 95°C.

b. Western blot analysis

Samples were loaded (20 µl/well) on 10% SDS-PAGE and allowed to migrate in 1X running buffer, for over 3 hours at 80 volts. Transfer of proteins from gel to nitrocellulose membrane was done using BIO-RAD electro-transfer setup for 2 hours at 80 volts in a cold room.

Next, membranes were blocked in 5% milk for 1 hour, followed by overnight incubation with the primary antibody in a cold room. The membranes were washed with TBS-tween (3X, 10 minutes each), incubated for 1 hour at room temperature with the appropriate secondary antibody diluted (1:5000- 1: 10,000) and finally washed (3X, 15 minutes each) with TBS-tween buffer. Protein bands were visualized using enhanced chemiluminescence (ECL kit), and their expression level were quantified using ImageJ software, normalized to house-keeping gene (β-actin band), with fold expression determined relative to the control.

10. Bradford Assay

Protein levels were measured using Bradford protein assay. This assay based on the binding of protein molecules to the dye, Coomassie, under acidic conditions results in a color change from brown to blue. Briefly, in a 96-well plate 5 µl of each of sample is added to 200 µl of diluted Bradford reagent in ddH2O (1:4 v/v). Following 10 minutes incubation at room temperature, the absorption of the mixture was read at 595 nm using ELISA plate reader. Protein levels were determined using stander curve of known concentration of bovine serum albumin (BSA).

11. Statistical analysis

Microsoft Excel software was used to analyze data and determine the statistical significance of the results. p-value < 0.05 is considered significant. For each parameter tested, a set of at least three biological replicates was considered. What is more, for each

parameter, both, inter-categorical statistical significance, and significance relative to the control were analyzed. All graphs were generated using GraphPad Prism.

CHAPTER III

RESULTS

A. Cytotoxicity of MOE on HepG2 cells

1. MOE decreases HepG2 cell viability

The cytotoxic effect of MOE on HepG2 cells was initially screened, using MTT assay, to estimate the EC50 value.

Findings indicate that treating HepG2 cells at 24 hours with MOE, at a final concentration varying between 0.01-0.1%, caused a significant ($p=0.0006$) dose dependent decrease in cell viability. A maximal cell death of 90% ($p<0.0001$) occurred at 0.1% MOE as showed in Figure 26. The estimated EC50 ranged between 0.02- 0.03 %.

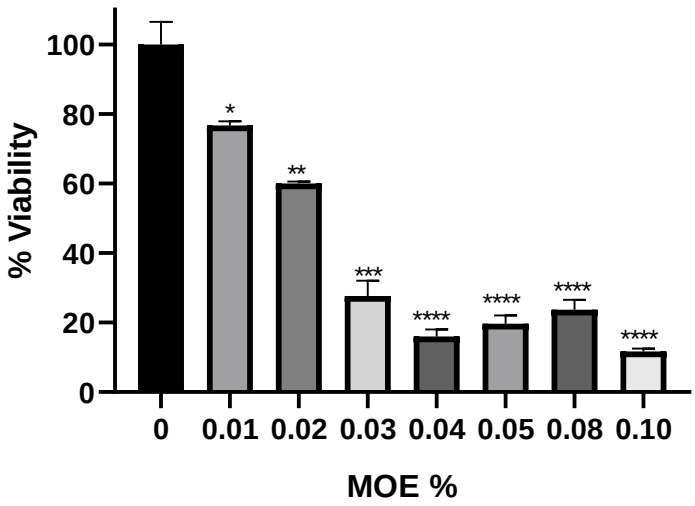


Figure 26: Effect of MOE on viability of HepG2 cells. Cells were treated for 24 hours with MOE following which viability was assessed using MTT assay. Results represent the average ± SEM of 9

determinations from 3 different experiments. Asterisks on bars represent the statistical significance compared to the control. (***), and (****) correspond to P < 0.0005 and 0.0001, respectively.

In order to evaluate the hypocholesterolemic effect of MOE, we have opted in all subsequent experiments to treat cells at MOE concentrations <EC50 which maintains the viability and metabolic activity.

2. Viability of MOE (sub EC50) treated HepG2 cells is time independent

HepG2 cells were treated for an extended period of time with MOE at sub EC50 concentration. Viability of cells treated for 24, 48, 72 hours with MOE concentrations were assessed using both MTT and trypan blue exclusion assays. Results from both assays show a similar profile (Figure 27. a-b) of a dose-dependent and time-independent response. Results presented in Figure 27, shows no significant change in the viability of HepG2 cells treated at a specific concentration for different times (24,48, 72 hours), indicating the ability of cells to multiply and proliferate. For instance, at 0.015% MOE (subEC50), viability of HepG2 cells (60-80%) showed no significant difference (p> 0.9999) treated for 24, 48 and 72 hours.

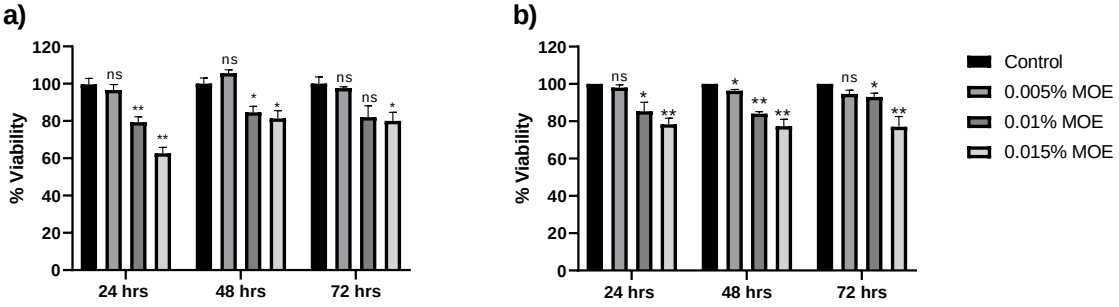


Figure 27: Viability of HepG2 cells treated for 24-, 48- and 72-hours with MOE concentration (0.005-0.015%). The viability was assessed using a) MTT assay and b) Trypan blue exclusion assay. Results represent the average ± SEM of 9 determinations from 3 different experiments. Asterisks on bars represent

the statistical significance compared to the control. (*) and (**) correspond to P < 0.05 and 0.01, respectively.

3. ROS production in MOE treated cells is time independent

Antioxidants (flavonoids, vitamin c) are among the many components present in the *moringa* extract. Therefore, we examined if treating cells with MOE (sub EC_{50}) has any bearing on ROS level. Using NBT assay, MOE-treated cells show a reduction in NBT in a dose-dependent manner, indicating a significant increase in ROS levels (Figure 28). However, the subsequent elevation in ROS levels was time independent. For instance, compared to control cells, 60% of the NBT were reduced by HepG2 cells treated with 0.015% MOE for 24 or 48 or 72 hours (Figure 28) Indicating no significant increase in generated ROS (40 %) occurred over time.

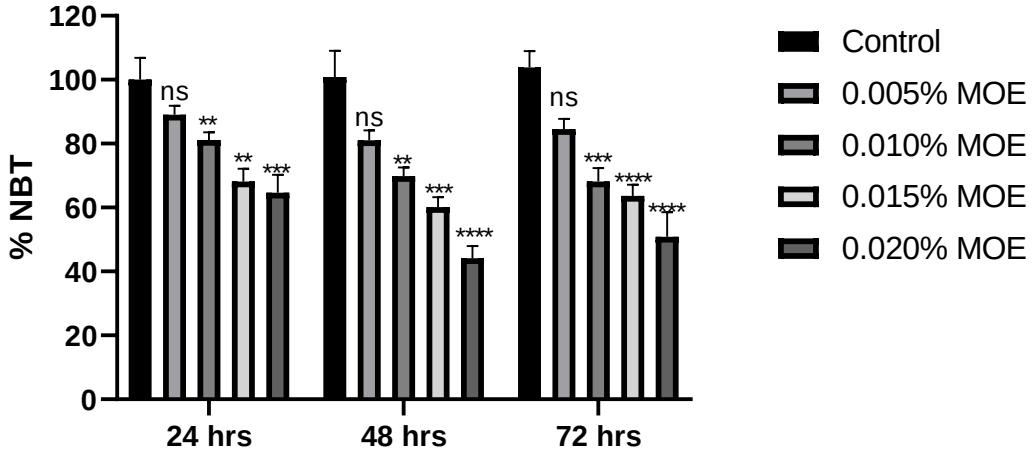


Figure 28: Level of ROS in MOE-treated HepG2 cells. Cells were treated with MOE concentration (0.005-0.02%) for 24, 48 & 72 hours following which NBT reduction was determined. Loss of cellular ability to reduce NBT is indicative of ROS generation. Results represent the average ± SEM of 9 determinations from 3 different experiments. Asterisks on bars represent the statistical significance compared to the control. (*), (**), and (****) correspond to P < 0.05, 0.01, and 0.001, respectively.

4. Decrease in ATP levels in MOE treated HepG2 is time independent

HepG2 cells treated for 24 hours with MOE caused a significant decrease of 44% in ATP level respectively to the control with no further significant decrease ($p>0.9999$) following 48- and 72-hours treatment (48%, 62%) as per Figure 29. This indicates that MOE treatment exerts a strict metabolic control on energy production in addition to maintaining cell viability.

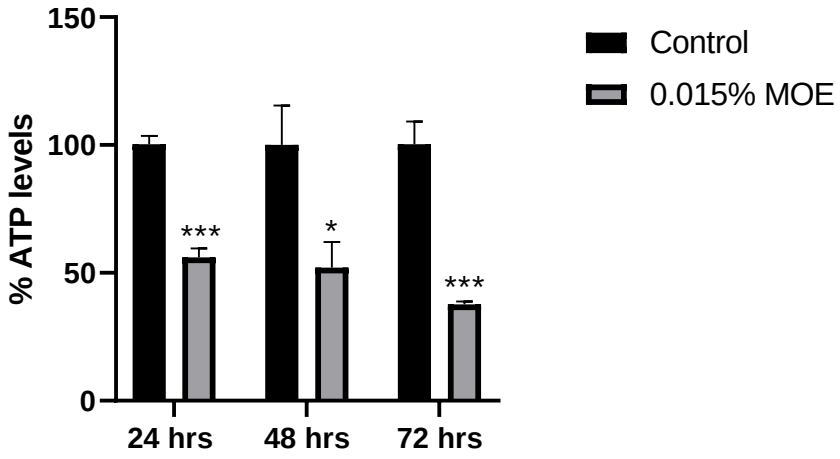


Figure 29: ATP levels in MOE-treated HepG2 cells with time 24-, 48- and 72-hours treatment. Results represent the average ± SEM of 6 determinations from 2 different experiments. Asterisks on bars represent the statistical significance compared to the control. (*), and (***) correspond to P < 0.05, and 0.0001, respectively.

B. MOE influences the transcription of key proteins regulating cholesterol homeostasis

Intracellular cholesterol level affects plasma cholesterol level by controlling the expression and activity of HMG-CoA reductase and LDL-R. The effect of MOE on the gene expression levels of both proteins was investigated.

1. MOE decreases HMG-CoA reductase gene expression

qRT-PCR studies indicate that MO-treated HepG2 cells show a down-regulation in HMG-CoA reductase gene expression levels at 24 hours ($p < 0.0053$) and 48 hours ($p < 0.0017$) (Figure 31). Following 24 hours of treatment with MOE, a decrease in HMG-CoA gene expression was apparent, with a small change after 48 hours. qRT-PCR analysis was normalized against the housekeeping gene GAPDH.

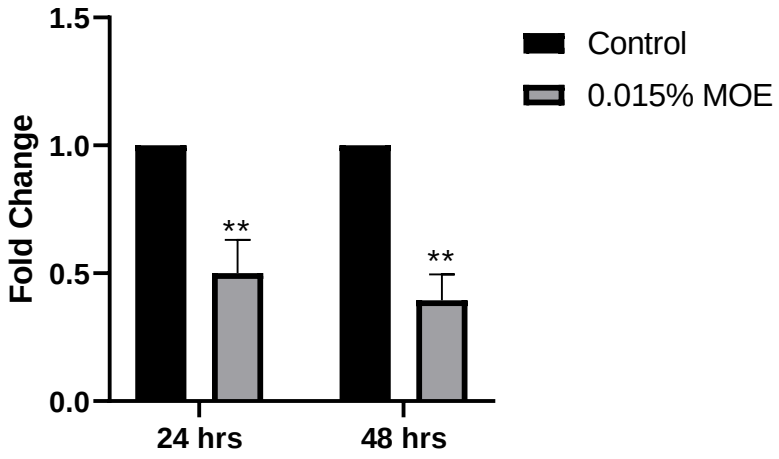


Figure 30: Effect of MOE (0.015%) HMG-CoA reductase gene expression. Results represent the average ± SEM of 3 determinations from 3 different experiments. Asterisks on bars represent the statistical significance compared to the control. (**) correspond to P < 0.01.

2. MOE effect on LDL receptor transcription

MOE treatment induced a significant increase in LDL-R gene expression levels at 24 hours timepoint ($p = 0.0176$) with a similar increase after 48 hours ($p = 0.0434$) (Figure 31). Results were normalized to the expression of the housekeeping gene GAPDH which remained constant after treatment.

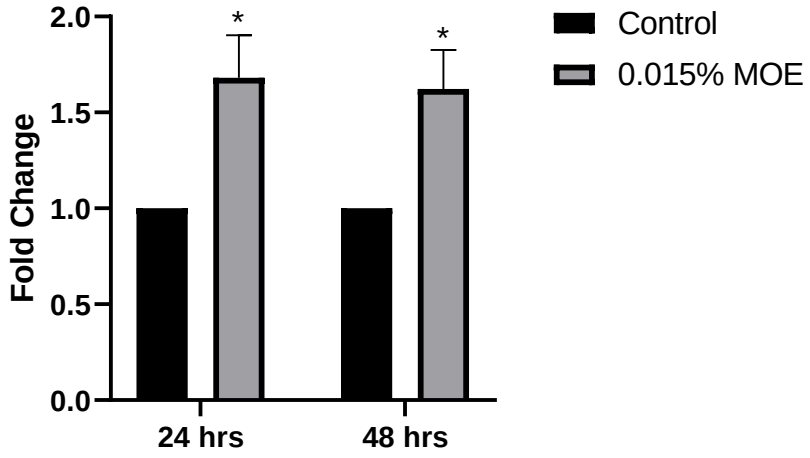


Figure 31: Effect of MOE (0.015%) on LDL-R gene expression. Results represent the average ± SEM of 3 determinations from 3 different experiments. Asterisks on bars represent the statistical significance compared to the control. (*) correspond to P < 0.05.

C. Impact of MOE treatment on protein expression and activity of LDL-R, HMG-CoA reductase and GAPDH

MOE evoked an altered gene expression profile through acting on central players involved in cholesterol homeostasis. E investigated the effect of MOE in the translation of LDL-R and HMG-CoA reductase protein using western blot analysis. Expression levels were compared to GAPDH and β-actin.

1. LDL-R activity and protein levels after 24- and 48 hours MO treatment

The impact of MOE treatment on the protein levels and activity of LDL receptor were performed using two methods, the western blot and immunohistochemistry staining:

a. MOE increased LDL-R protein levels

LDL-R exists in cell in two forms: pre-mature (100kDa) and mature glycosylated (150kDa). The expression on both pre-mature and mature showed a significant increase at 24- and 48-hours post-treatment with MOE.

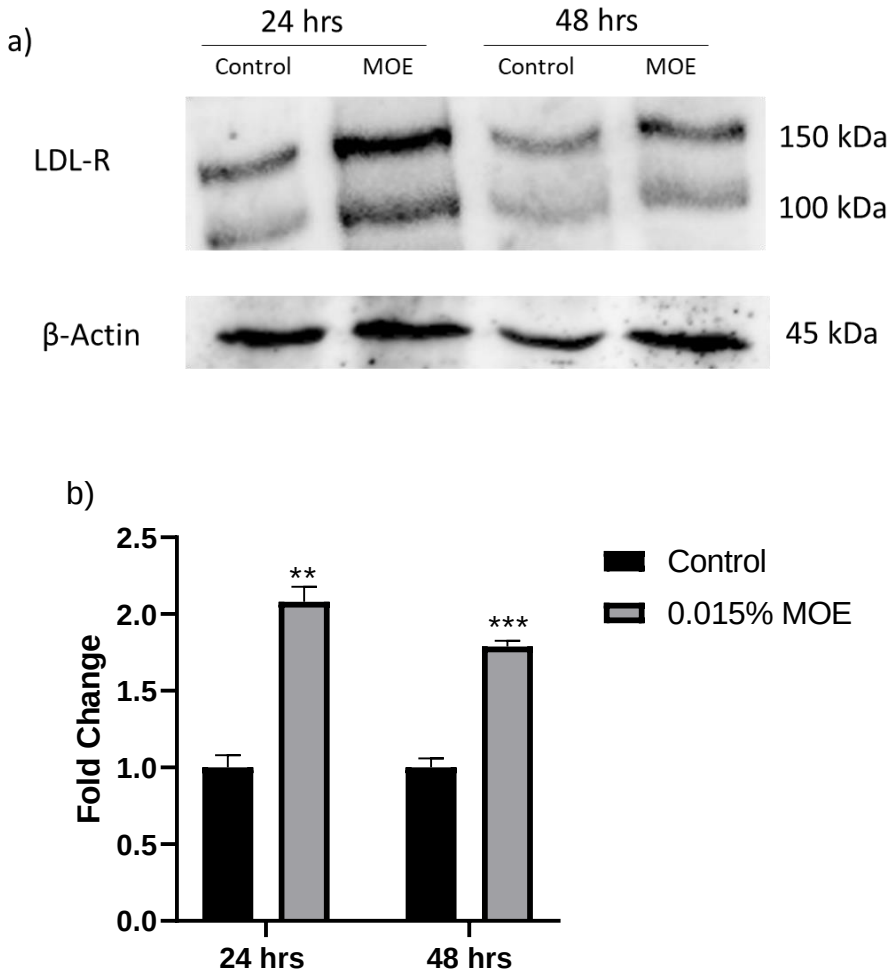
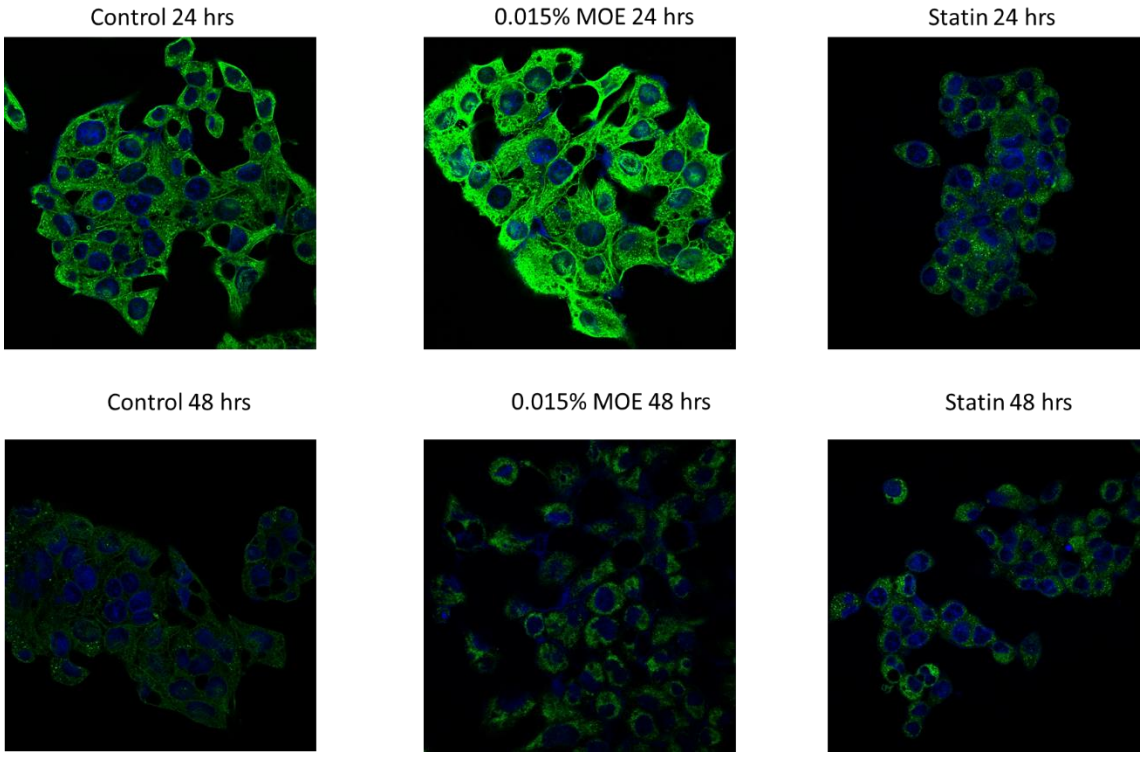


Figure 32: Western blot of LDL-R protein in HepG2 cells a) Western blot of LDL-R protein levels b) Quantification of LDL-R protein levels MO treated HepG2 cells. Images were quantified using ImageJ program. Results represent the average ± SEM of determinations from 3 different experiments. Asterisks on bars represent the statistical significance compared to the control. (**) and (***) correspond to P< 0.001, and <0.0001, respectively.

b. MOE effect on LDL-R cellular activity (Immunofluorescence)

The hypocholesterolemic potential of MOE was correlated with the protein levels of LDL-R on cells. Treatment with 0.015% of MOE increased (25%) LDL-Rs on HepG2 cell surface significantly (p=0.00072) 24 hours post-treatment. Moreover, following 48 hours of treatment with MOE, a no significant, increase in the LDL-R was noticed (10%, p=0.082) (Figure 33). Mevastatin (50mM) were used as a positive control.



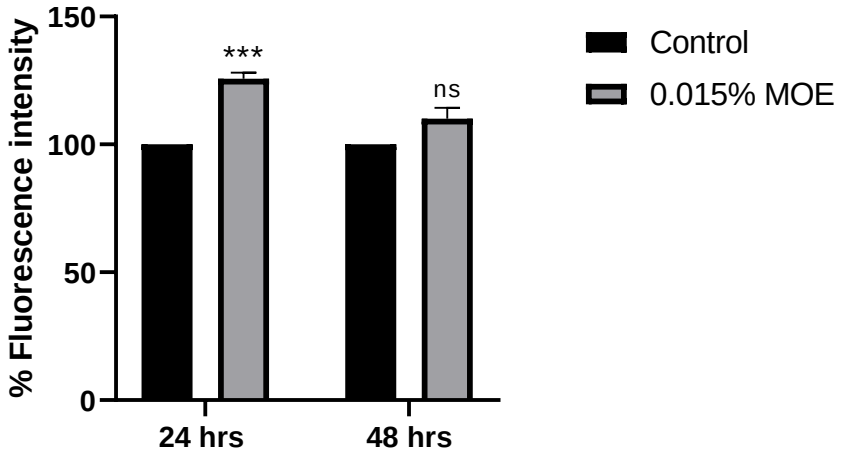


Figure 33: Immunofluorescent detection of LDL-R expression on HepG2 cell surface after 24 and 48 hours of MOE treatment. Cells were treated with 0.015% MO extract for 24 hr. and 48 hours. DAPI dye was used to stain nuclei in blue. Images were visualized using confocal fluorescent microscopy. Results represent the average ± SEM of 3 determinations from 3 different experiments. Asterisks on bars represent the statistical significance compared to the control. (*) correspond to P < 0.001.

2. GAPDH and HMG-CoA reductase protein levels in MO treated cells

MOE did not cause a significant decrease in HMG-CoA reductase protein levels after 24 hours and 48 hours post-treatment. There was a small decrease in HMG-CoA reductase protein levels at 48 hours, although not significant (Figure 34).

Western blot analysis however, showed that MOE treatment decreases GAPDH protein level. This result indicates a possible effect of MOE treatment on the translation or stability of GAPDH protein (Figure 34). Importantly, after 48 hours, there was a further decrease (46%, p=0.028). Therefore, we opted to compare protein levels with another housekeeping β-actin.

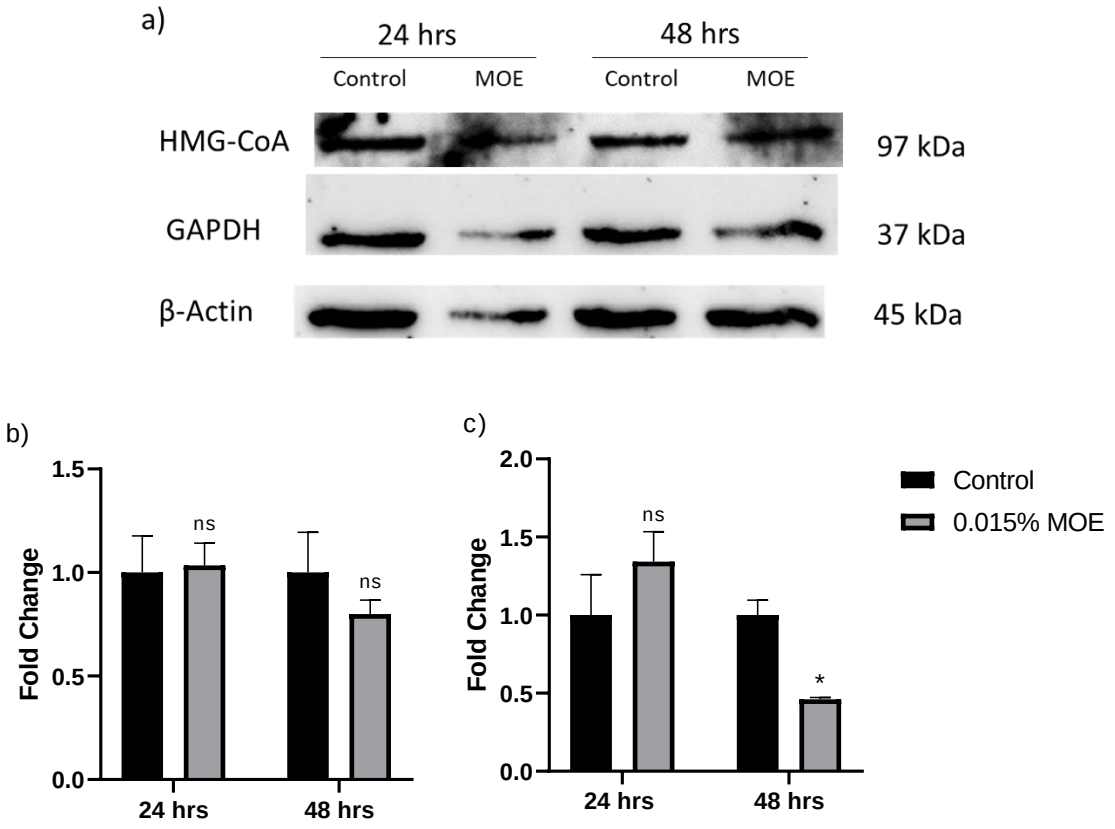


Figure 34: Western blot of GAPDH and HMG-CoA reductase protein in HepG2 cells a) Western blot of HMG-CoA and GAPDH protein levels b) Quantification of HMG-CoA reductase protein levels c) Quantification of GAPDH protein levels MO treated HepG2 cells. Images were quantified using ImageJ program. Results represent the average ± SEM of determinations from 3 different experiments for the HMG-CoA reductase and 2 different experiment for GAPDH. (*) correspond to P < 0.05.

D. Intracellular cholesterol levels in MOE treated HepG2 cells

To examine the effect of MOE on intracellular cholesterol level. Lipids were extracted from MOE treated HepG2 cells and assessed for cholesterol level normalized to protein level. No significant change in total cholesterol levels (free-cholesterol and cholesterol esters) was found upon 24 hours and 48 hours of treatment with MOE (p=0.993 and 0.993, respectively) as represented in figure 35.

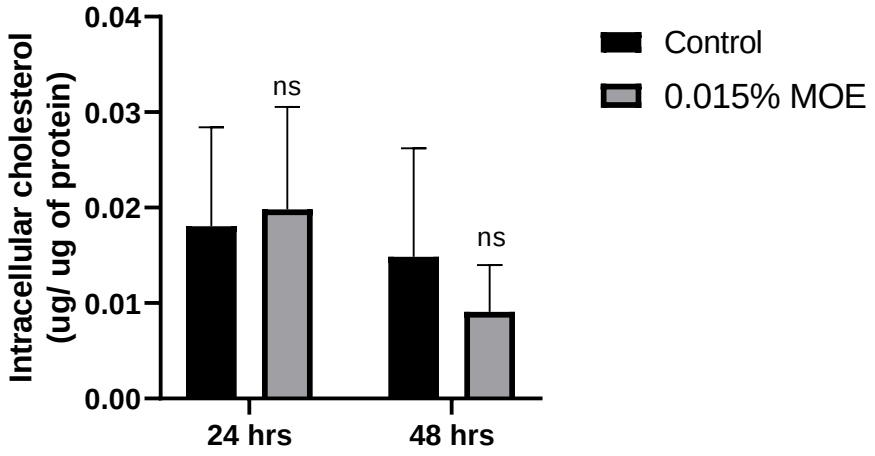


Figure 35: Level of cholesterol in MOE treated HepG2 cells after 24, 48 hours treatment. Results represent the average ± SEM of 2 determinations from 3 different experiments. Asterisks on bars represent the statistical significance compared to the control. Control values are 0.018±0.017 for 24 hours and 0.0148±0.019 at 48 hours.

E. Direct effect of MOE on HMG-CoA reductase activity

Variation of absorption with time showed an early inhibitory effect of MOE treatment on HMG-CoA reductase enzymatic activity which a decrease with time, without restoring the control value (~16% inhibition) as per figure 36.

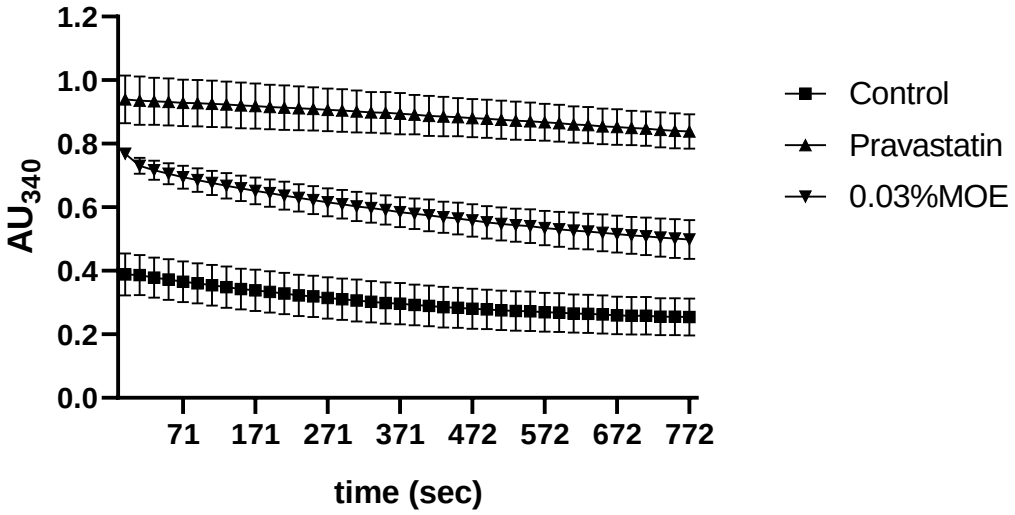


Figure 36: Inhibition of HMG-CoA reductase (HMGR) activity in MOE treated HepG2 cells. Results represent the average ± SEM of determinations of 3 different experiments.

Table 3: Effect of sub-lethal dose of MOE concentrations on purified HMG-CoA reductase activity

	HMG-CoA Mean Activity	**p-value**
Control	100%	-
Pravastatin	22%	0.0123
0.03% MOE	84%	0.321

CHAPTER IV

DISCUSSION

Medicinal plants provide a rich source of phytochemicals that are recognized by WHO and NIH for their low-cost preventive or curative therapeutic potentials. Use of plant extracts is advantageous over the conventional artificial drugs for their ability to target multiple sites simultaneously. Among the many recognized medicinal plants is Moringa oleifera known for having a wide spectrum of biological activities in all its parts in traditional medicine.

A recent review reported the nutraceutical and pharmacologic benefits of MOE in treatment of chronic diseases including but not limited to anticancer, hepatoprotective, neuroprotective, hypoglycemic, and hypolipidemic.

In the current study we investigated the potential effect of M. Oleifera water extract in regulating cholesterol levels. We hereby report the in vitro effect of MOE on controlling cholesterol homeostasis in HepG2 cells. MOE treated HepG2 cells resulted in a) an increase in the expression of LDL receptors, hence uptake of cholesterol into HepG2 cells; and b) a decrease in the transcription of HMG-CoA reductase, consequently decreasing endogenous cholesterol biosynthesis. No significant variation was noted in total intracellular cholesterol. Our findings suggest a preventive therapeutic hypocholesterolemic potential of MOE

Moringa oleifera, the miracle tree is widely consumed as raw, cooked or as hot drink with no reports of toxic effects (Stohs & Hartman, 2015). The therapeutic potential of

MO has demonstrated its possible role in treating cancer (Al-Asmari et al., 2015; Berkovich et al., 2013; Fahey et al., 2018; Khor et al., 2018; Madi et al., 2016; Reda et al.; Sreelatha et al., 2011), hyperglycemia, hyperlipidemia and hypercholesterolemia (Adisakwattana & Chanathong, 2011; Almatrafi et al., 2017; Chumark et al., 2008; Cordier et al., 2020; Edoga et al., 2013; Ghasi et al., 2000; Gupta et al., 2012; Jain et al., 2010; Khan et al., 2017; Reddy et al., 2017; Tabboon et al., 2016; Waterman et al., 2015). Most studies attributed pharmacologic effects for the different parts of MO extracted with organic solvents (ethyl acetate); hydro alcoholic (methanol- water); and phenolic enriched extracts (Mbikay, 2012; Njan et al., 2014; Prabakaran et al., 2018; Saini et al., 2016; A.K. Singh et al., 2019; Tshabalala et al., 2020). Being widely consumed as a hot beverage (MO-tea drink) we opted to mimic the effect of the aqueous extract on cholesterol homeostasis in liver cells.

The liver plays a central role in glucose and lipid metabolism including biosynthesis, distribution of endogenous and exogenous (dietary) cholesterol and excretion (bile acids). A well characterized and suitable cell model of the human liver, widely used in biochemical and nutritional studies, is HepG2 cells. Examining the effect of varying MOE concentration (0.01%-0.1%) on viability of HepG2 cells, we estimated 50% cell death to occur at final concentration ranging between 0.02% - 0.03%, which agrees with reported studies (Cordier et al., 2020; Madi et al., 2016; Tsitsi et al., 2008). To mimic the people's, frequent and daily consumption of MO tea, we opted to perform all the subsequent experiments at MOE concentrations < 0.02% and for more extended periods (24, 48 and 72 hours).

Regardless of the treatment time, the cells exhibited a concentration dependent, yet time independent viability (MTT and trypan blue). At any specific concentration, the extent

of cell death did not vary between 24, 48 and 72 hours. Moreover, changing the MOE containing media on daily basis exerted no further cell death (data not shown).

Being a rich source of antioxidants' phytochemicals (flavonoids, polyphenols, quercetin, tannins, etc.) we checked if the obtained viability at 72hrs, post MOE treatment, may be attributed to a decrease in ROS level. Using NBT assay, HepG2 cells treated with MOE, generated ROS in a concentration dependent, but time independent manner. The insignificant change in ROS level with time, may be attributed to the net opposing/ and or synergistic effects of MOE phytochemicals targeting multi- cellular sites/pathways. While controlled level of ROS may serve a signaling role in various redox–dependent processes favoring proliferation, over-production of ROS causes severe damage to macromolecules leading to death.

Previous studies in our lab reported a significant generation of ROS species in MOE treated A549 cells (Madi et al., 2016) and HCT116 cells (Reda et al., 2017), causing depolarization of mitochondrial membranes, a decrease in ATP level, and subsequently cell death.

Examining the intracellular ATP level in MOE treated cells, our findings were also time independent indicating a strictly controlled mechanism maintaining viability and integrity of the cells. The drop in intracellular ATP level remained relatively the same between 24-72 hours while maintaining the viability, and ability to proliferate hence metabolic activity such as cholesterol biosynthesis and metabolism.

Increase in cholesterol level underlie one of the mechanisms leading to atherosclerosis (H.H. Wang et al., 2017). Many of the strategies used to lower cholesterol level have targeted its biosynthesis, absorption, and excretion. Stains are the current drugs

of choice for treating patients at high risk of cardiovascular disease because of imbalanced cholesterol level. This class of drugs competitively inhibit the rate determining step in cholesterol pathway, catalyzed by HMG-CoA reductase decreasing de novo synthesis of cholesterol, hence intracellular level. In addition, statins were found to increase the expression of LDL receptors, stimulating LDL uptake that compensates for the intracellular decrease in cholesterol and reduces its plasma level.

Although statins decrease cholesterol levels, mortality, and morbidity; yet they have adverse side effects including hepatotoxicity and myopathy (Adhyaru & Jacobson, 2018; Lim, 2016; Ward et al., 2019), hence the need to develop or identify safer drugs with less side effects, alternatively dietary components with preventive effect

It is well known that the intracellular cholesterol level is maintained by a class of transcription factors Sterol Regulatory Element-Binding Proteins (SREBP). Proteolytic processing of SREBP favors its migration to the nucleus where it interacts with Sterol Response Elements in the promoter region of both HMG-CoA reductase and LDL receptor genes activating their transcription (Maron et al., 2000; Schonewille et al., 2016).
We next examined the effect of MOE on gene expression of HMG-CoA reductase and LDL receptors. In our study, treatment of HepG2 cells with MOE, upregulated the gene expression of LDL receptor, but down regulated the HMG-CoA reductase gene with no change in the house keeping gene GAPDH. Our results were: a) in line with previous studies using different plant extracts such as: berries, green tea, geraniol, and Phikud navakot (Gayen & Peffley, 1995a; G. Liu et al., 2015; J. Liu et al., 2006; Peffley & Gayen, 2003; Tirawanchai et al., 2018) down regulating HMG-CoA reductase and upregulating LDL-R; but b) partially in agreement with study on HepG2 cells treated with phenolics

enriched extract of MO, in which expression of both genes (LDL receptor and HMG-CoA reductase) increased significantly, the protein expression and binding activity of the LDL receptor was enhanced expectedly. However, the activity of HMG-CoA was inhibited in a dose dependent manner (Tabboon et al., 2016). In another study, red grape juice increased both the mRNA of LDL receptor and the protein level without inhibiting cholesterol biosynthesis (Davalos et al., 2006).

Other mechanisms have attributed the hypocholesterolemic effects of natural products such as Epigallo catechingallate the green tea component to: an increase in SREBP2 activity as well as the expression and stability of LDL-mRNA; a decrease in the HMG-CoA reductase mRNA stability caused by a non -coding micro RNA interacting with HMGCR 3′ untranslated region, leading to a posttranslational inhibition followed by mRNA degradation (G. Liu et al., 2015).

The proper effect, however, of MOE is not limited to the mRNA gene expression, but to the level and more importantly the activity of the translated proteins. Examining the translation level of both proteins post MOE treatment, we obtained:

1) a significant increase in the mature and premature LDL receptor (western blotting and immunostaining) concomitant with the enhanced gene expression.

2) a non-significant decrease in the HMG-CoA reductase protein level, that was not commensurate with the obtained decrease in gene expression.

3) an unexpected decrease in the translated level of the house keeping gene GAPDH while its mRNA did not vary with time; hence in our study, translated proteins were normalized relative to β-Actin.

Our findings pose a question regarding the role of the housekeeping protein

GAPDH. The GAPDH is the only enzyme in the glycolytic pathway that utilizes NAD, generating NADH, and which provides a link between glycolysis and mitochondrial electron transport chain (ETC). Thus, it is plausible to postulate that the MOE-induced decrease in GAPDH protein, will reduce the rate of the NADH generating reaction in glycolysis, decreasing the flow of NADH electrons into ETC at NADH Dehydrogenase (complex-1) thus causing a decrease in ATP, with consequent ROS generation (figure 37).

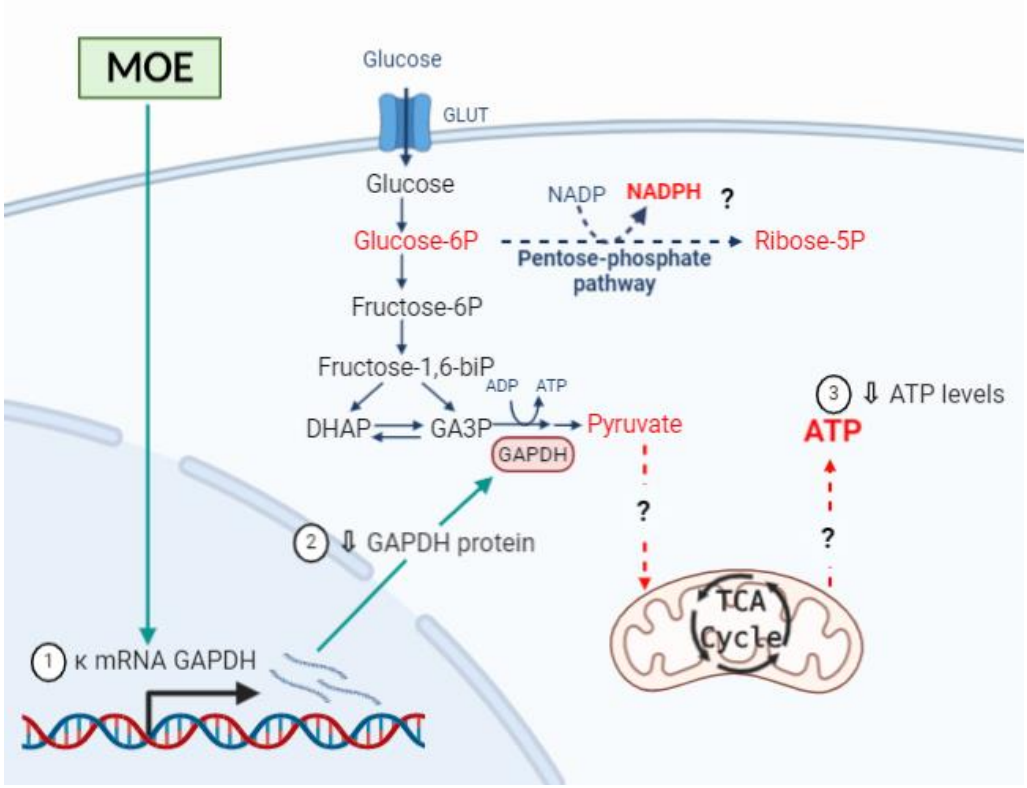


Figure 37: Schematic representation of possible mechanism of action of MOE on the activation of the pentose phosphate pathway and it interference on the ATP production via alteration of the glycolysis and TCA cycle. Our results demonstrated that MOE may decrease GAPDH protein levels which can induce a decrease on the production of ATP from the TCA cycle. Therefore, our next step would be to study the effect of MOE on a) Mitochondrial function; b) levels of NAPDH upon treatment and c) investigate the effect of MOE in the glycolysis and HMP shunt pathway.

On the other hand, the decrease in GAPDH level reduces the glucose flux into glycolytic pathway, diverting it into the hexose monophosphate shunt, the main generator of NADPH (Aziz & Mohiuddin, 2020). The increase in NADPH level, will control the level and hence signaling role of ROS maintaining a constant level over 72 hours while favoring proliferation (viability). Alternatively, the MOE-induced expression of antioxidant enzymes (SOD, glutathione reductase, catalase) may not be excluded and require verification in future studies.

The MOE is composed of many phytochemicals, which target multi-intracellular sites that may exert different effects depending on the relative % composition of the extracted material and key signaling proteins expressed in the treated cell. The direct effect of MOE on pure HMG-CoA reductase activity caused a 16% decrease in activity. However, this may not reflect the actual activity in MOE treated cells, as some of the phytochemicals may induce phosphorylation of the HMG-CoA reductase rendering it inactive. Although the mRNA of HMG-CoA reductase decreased, the protein level was not significantly modified, which has no bearing and is not indicative of the enzyme activity. Regardless of the MOE-induced HMG-CoA reductase status (active /inactive) the increase in LDL receptors allows the uptake of LDL, thus increasing intracellular cholesterol that will exert a negative feedback on the enzyme leading to its inhibition (figure 38).

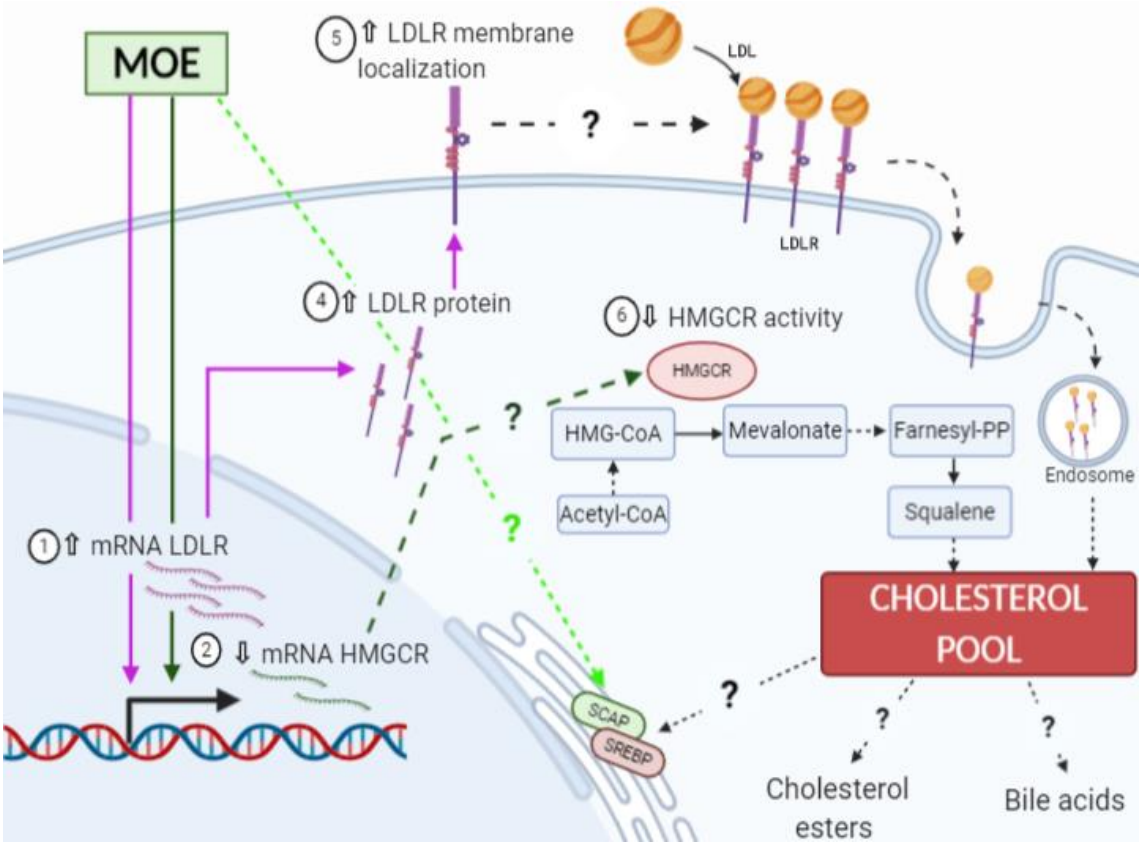


Figure 38: Schematic drawing of the hypothesis of the lowering cholesterol effect of MOE treatment on cells. According to our hypothesis, bioactive agents present on MOE induce increase transcription and translation of LDL-R and decrease expression of HMG-CoA reductase mRNA. Moreover, our results showed an increase localization of LDL-R on the cell surface. Our next step would be assessing if upon MOE-treatment a) increase of LDL activity and internalization occur; b) stability of HMG-CoA reductase mRNA; c)determination of efflux of cholesterol as cholesterol esters or bile acid production; d) Activity of TF SREBP2, which will regulate the transcriptional activity of cholesterol biosynthesis genes (HMG-CoA reductase) and low-density lipoprotein receptor (LDLR)-mediated cholesterol influx

We next examined the level of cholesterol post MOE treatment. Our data shows no variation after 24 hours but decreased after 48 hours though non- significantly.

Further experiments determining the activity of other contributors to the cholesterol pool must be determined, such as the effect of MOE on cholesterol conversion into cholesterol esters, bile acids, and steroids.

To sum up, in this study we provide:

1. A link between dietary practices and disease prevention.

2. A preliminary evidence on the therapeutic hypocholesterolemic potential of MOE on HepG2 cells.

Mimicking people ingestion of MO tea drink, we show the hypocholesterolemic potential of MOE proposing it as preventive therapeutic alternative for people with low risk of cardiovascular diseases.

Treatment with MOE at concentration < EC50 resulted in a significant increase in the expression of LDL-receptor gene and a significant decrease in the HMG-CoA reductase gene with no variation in the gene expression of GAPDH.

On the other hand, the effect of MOE on the translated product showed, an increase in LDL receptor favoring uptake of LDL (uptake of cholesterol), with no significant change in HMG-CoA reductase enzyme levels while a decrease in GAPDH protein level was obtained.

Our results demonstrated the hypocholesterolemic potential of MOE. More importantly, widely consumed as a tea-drink, MOE may be regarded as an alternative preventive therapeutic extract that may help in maintaining cholesterol homeostasis. The findings presented here help our current understanding of the MOE actions in the cholesterol homeostasis. We hope that our results showcase the potential use of natural medicine as an alternative therapy providing patients with a better quality of life and prolonged lifespan.

A. Future studies

Future plans include measuring the effect of MOE on the change in the enzymatic activities of HMG-CoA reductase using cell-based assay (microsomal fractions) and the

effect of MOE in the stability of HMG-CoA reductase mRNA.

In addition, we aim to determine levels of free cholesterol and cholesterol esters in HepG2-treated cells.

Future work could include the assessment of LDL receptor activity after MOE treatment and the effect of MOE on the synthesis and secretion of VLDL.

More importantly, future plan includes the investigation of the effect of MOE treatment on the decrease in GAPDH protein levels. It is important to investigate how this result will impact on other cellular metabolic pathways.

Another future aim would be testing the co-treatment of MOE with statins, investigating potential new therapeutic approaches combining lower statins doses and specific dietary compounds. This may help patients who cannot tolerate high-dose statin therapy.

Finally, it would be of immense importance to investigate the cholesterol-lowering effect of MOE original extract along with effective sub-fractions in vivo animal models.

B. Limitations

Although our results indicate the potential of MOE as a cholesterol-lowering agent, we were able to identify some limitations in our study.

First, the use of in-vitro liver-derived cell (HepG2 cells) is a major limitation of our current study. Although HepG2 has been extensively used in drug-metabolism studies, it would be recommended that a comparative study using primary hepatocytes cells to further confirm the results obtained in this study

Secondly, in the present study we investigated the activity of pure HMG-CoA

reductase using MOE. This non-base cell assay showed us a minimal inhibitor potential of MOE on the enzyme activity of HMG-CoA reductase. However, the use of a cell-based assay could help us further understand the multiple potential mechanism of inhibition of MOE-treatment on the direct or indirect HMG-CoA reductase activity.

Next, although no difference in the total cholesterol levels was observed after MOE treatment, it is better to determine the effect of MOE on free cholesterol- and cholesterol ester levels, independently.

More importantly, it would be relevant to identity the phytochemical composition present in different MOE concentrations. Therefore, we could understand the possible mode of action of the different bioactive compounds on the cell response, as well as identify their specific contributions to the hypocholesterolemic effect.

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