

The antiatherogenic potential of two triterpenes from *Protorhus longifolia* stem bark (Benrh.) Engl.

By

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Thesis

Submitted in fulfilment of the degree

Master of Science (Biochemistry)

In the

Faculty of Natural & Agricultural Sciences

Department of Biochemistry, Genetics and Microbiology

University of Pretoria

Pretoria

January 2024

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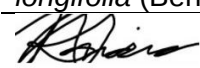
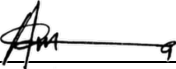
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Dedication

This work is dedicated to God, my ancestors and to myself. I did it!

Acknowledgements

This project was financially supported by the UP postgraduate bursary for masters and doctoral students. This project could not have been completed without the assistance of my supervisor, Dr Rebamang Mosa and everyone in the Diabetes and Vascular Research Group. Special mentions to Tristen Rees and Anja Turner. I would also like to acknowledge the cell biology group, thank you Dr June Serem and Carolynn for the support as well as Raven Wienk (FABI), I would have not been able to finish the second half of my project without you.

I would like to thank everyone in the BGM department from all the different research groups for all the laughs, wisdom and encouragement, a special mention to Retla, TK, Hildsley, Eugene, David, and Clifford, you all helped me out so much. Thank you to Nobuhle Ndlangamandla and Kamogelo Maluleke for your friendship and support throughout this degree, you were my pillars! Thank you to Dr Precious Motshwene for the many insightful conversations and words of advice. I appreciate all that you have shared with me. Thank you to Dr Itumeleng Baloyi, I wish we met sooner but I am grateful for the little moments we have shared.

Lastly, I would like to thank my friends, with special mention to Lindokuhle, Kelebogile, Lesego, Nelisa, Nadene, Mandla and Taze, you have no idea how much your support has carried me through this degree. Thank you for being there for my tears and all, I could not do it without you. To my partner, thank you for listening to my complain and cry throughout the course of this study and for your words of encouragement and motivation. Thank you for celebrating when the experiments worked and for driving up to see me on the days that I was not alright, I appreciate it. To my siblings, cousins, aunts, and uncles, thank you for your prayers and support and the constant check-ins, I am so grateful to have a family that cares so much about my well-being in all aspects. Finally, to my parents (Mr and Mrs Ramasia) words cannot express how much you have helped me throughout this degree, financially, mentally, and emotionally. You are the best parents anyone could ask for. Thank you for supporting my wish to pursue my MSc, I am so thankful.

Summary

Atherocardiovascular diseases (ACVDs) are considered among the major causes of death in South Africa and worldwide. Hypercholesterolemia, which is the main major risk factor of atherosclerosis, increases the occurrence of ACVDs. Despite the use of the current drugs to treat ACVDs, the continuous increase in morbidity and mortality rates associated with atherocardiovascular events indicate limitations of the current treatment regime. Medicinal plants are known to contain numerous compounds with various bioactivities including antioxidant capacity and lipid lowering properties. The triterpenoid compounds (ARM-2 and RA-5) isolated from *Protorhus longifolia* (Benrh.) Engl. stem bark have shown promising bioactivities such as inhibiting dietary cholesterol digestion and potential to regulate the endogenous pathway of cholesterol biosynthesis. These properties play a vital part in the amelioration of hypercholesterolemia. This study investigated the antiatherogenic potential of ARM-2 and RA-5. The computational and wet lab experiments were implemented to investigate the preventative properties of the compounds against oxidative modification of LDL and foam cell formation that underlie the progression of atherosclerotic plaque. The *in silico* molecular docking of the compounds against some proteins involved in cholesterol trafficking, and their ADMET properties were determined. *In vitro* analysis of potential bioactivities of the compounds was determined through antioxidant assays (ABTS, DPPH, ferric reducing power and ORAC), and inhibition of the CuSO₄ mediated LDL oxidation. The oxidized LDL-induced foam cell formation experiment was carried out to determine the inhibitory activity of the compounds against foam cell formation in RAW 264.7 cells. Molecular docking of the compounds indicated poor binding affinity against CD36, SR-A1, ABCA-1 and sPLA₂ in contrast to strong binding affinities against ABCG-1, ACAT-1 and LOX-1. Both compounds were predicted to have promising ADMET properties and drug-likeness depicted by high intestinal absorption and no inhibition observed for the cytochrome P450 isoform. The triterpenes showed poor free radical scavenging activity (ABTS and DPPH) and reducing power but indicated good activity against peroxy free radicals. Inhibition of oxidative modification of LDL was observed by decreased CD and MDA formation by both compounds at 500 µM. However, the compounds were unable to inhibit sPLA₂ activity. Except for RA-5 at 25 µM, high viability of the cells was observed following their exposure to both triterpenes. The compounds decreased intracellular lipid droplet formation, ultimately resulting in decreased foam cell formation by 14% and 10% for ARM-2 (25 µM) and RA-5 (10 µM) respectively. Overall, the results from the study revealed that both triterpenes possess bioactive properties to inhibit oxidative modification of LDL and decrease foam cell formation, highlighting their antiatherogenic potential.

Table of Contents

DECLARATION OF ORIGINALITY	ii
Dedication	iii
Acknowledgements	iv
Summary	v
Table of Contents	vi
List of Figures	viii
List of Tables	x
List of Abbreviations	xii
1.0 Introduction	1
1.1 Overview of atherosclerosis and its global impact	1
1.2 Atherogenesis: Overview of biochemical events	2
1.3 Hypercholesterolemia: a major risk factor of atherosclerosis	6
1.4 Atherogenic effect of LDL	7
1.5 HDL and its antiatherogenic effects	9
1.6 Current treatment of atherosclerosis and specific drug targets	11
1.7 Alternative sources of new antiatherogenic drugs: Medicinal plants	12
1.8 Strategies for identifying or discovering new drug candidates from traditional medicinal plants	14
1.9 Rationale of the undertaken study	17
2.0 Aim	18
2.1 Hypothesis	18
2.2 Objectives	18
3.0 Material and Methods	19
3.1 Chemicals and reagents	19
3.2 <i>In silico</i> studies: molecular docking, ADMET properties and molecular target prediction of the test compounds	19

3.3 <i>In vitro</i> analysis of potential bioactivities of the test compounds	20
3.4 <i>In vitro</i> antiatherogenic activity of the compounds in RAW264.7 cells	24
4.0 Results	28
4.1 Chemical structures of the compounds/ligands used in the docking study	28
4.2 Molecular Docking of the triterpenoids against some proteins involved in cellular cholesterol trafficking	30
4.3 ADMET properties of ARM-2 and RA-5	33
4.4 Drug-Likeness and Target prediction of the plant triterpenoids.....	34
4.5 Antioxidant activity of the compounds <i>in vitro</i>	36
4.6 Inhibition of LDL oxidation.....	39
4.7 Inhibitory effect of the triterpenes on sPLA ₂	41
4.8 <i>In vitro</i> antiatherogenic activity of the compounds in RAW264.7 cells	41
5.0 Discussion	46
5.1 Molecular docking of the compounds.....	46
5.2 ADMET properties, drug-likeness, and Swiss target predictions of the triterpenoids	47
5.3 Antioxidant capacity of the triterpenoids.....	48
5.4 Inhibitory activity of the compounds on LDL modification.....	49
5.5 Inhibitory effect of the compounds on sPLA ₂	50
5.6 Cytotoxicity, intracellular lipid accumulation and foam cell formation	50
6.0 Conclusion	52
7.0 References	53

List of Figures

<i>Figure 1: Diagram indicating the pathophysiology of atherogenesis.</i>	2
<i>Figure 2: The endogenous pathway of LDL particles and the reverse cholesterol transport.</i>	7
<i>Figure 3: Proteins involved in foam cell formation of macrophage cell.</i>	9
<i>Figure 4: Chemical structure of 3α,26-dihydroxytirucalla-7,24-dien-21-oic acid, (ARM-2), 3β-hydroxylanosta9,24-dien-21-oic acid (RA5) and methyl-3βhydroxylanosta-9,24-dien-21-oate (RA3).</i>	14
<i>Figure 5: Drug discovery and development processes</i>	15
<i>Figure 6: The chemical structures of the compounds used in the molecular docking of the target proteins.</i>	29
<i>Figure 7: Molecular docking interactions (hydrogen bonding) between ACAT-1 and (A) Terpendole C, (B) ARM-2 (C) RA-5.</i>	32
<i>Figure 8: Molecular docking interactions (hydrogen bonding) between sPLA₂ and (A) Varespladib, (B) ARM-2 (C) RA-5</i>	33
<i>Figure 9: Free radical scavenging activity and reducing potential of the triterpenes on (A) ABTS and (B) DPPH radicals, and (C) ferric ions, respectively.</i>	37
<i>Figure 10: Oxygen radical absorbance capacity of the plant compounds</i>	38
<i>Figure 11: Inhibitory effect of the triterpenes on copper sulphate mediated LDL and HDL lipid oxidation.</i>	40
<i>Figure 12: Cytotoxic potential of ARM-2 and RA-5 on the RAW264.7 cells.</i>	42
<i>Figure 13: Effects of the triterpenes on lipid accumulation induced by oxLDL in RAW264.7 macrophage cells.</i>	44
<i>Figure 14: Effect of RA-5 on the ABCA-1 mRNA levels in RAW264.7 macrophage cells.</i>	45
<i>Figure A1: The chemical structures of the compounds used in the molecular docking of the target proteins.</i>	66
<i>Figure A2: Molecular docking interactions (hydrogen bonding) of the compounds against ABCA-1.</i>	69
<i>Figure A3: Molecular docking interactions (hydrogen bonding) of the compounds against ABCG-1</i>	71

<i>Figure A4: Molecular docking interactions (hydrogen bonding) of the compounds against ACAT-1</i>	<i>73</i>
<i>Figure A5: Molecular docking interactions (hydrogen bonding) of the compounds against CD-36.....</i>	<i>75</i>
<i>Figure A6: Molecular docking interactions (hydrogen bonding) of the compounds against LOX-1</i>	<i>77</i>
<i>Figure A7: Molecular docking interactions (hydrogen bonding) of the compounds against sPLA₂.....</i>	<i>79</i>
<i>Figure A8: Molecular docking interactions (hydrogen bonding) of the compounds against SR-A1</i>	<i>81</i>

List of Tables

<i>Table 1: Gene primers for some of the proteins involved in cholesterol trafficking.....</i>	<i>27</i>
<i>Table 2 A: Molecular docking scores (XP score) and glide energies (kJ/mol) of the plant triterpenes (ARM-2 and RA-5) on some target proteins involved in cellular cholesterol trafficking.</i>	<i>30</i>
<i>Table 2 B: Molecular docking scores (XP score) and glide energies (kJ/mol) of the plant triterpenes (ARM-2 and RA-5) on some target proteins involved in cellular cholesterol trafficking.</i>	<i>31</i>
<i>Table 3: The ADMET properties of the plant triterpenoids and lovastatin</i>	<i>34</i>
<i>Table 4: Physiochemical and medicinal chemistry properties of the plant triterpenoids and lovastatin.....</i>	<i>35</i>
<i>Table 5: Some of the SwissTarget Predictions indicated by probability scores of the triterpenoids and Lovastatin</i>	<i>36</i>
<i>Table 6: Table indicating the ORAC values ($\mu\text{M TE}$) of ARM-2 and RA-5 at different concentrations.....</i>	<i>38</i>
<i>Table 7: sPLA2 (secretory phospholipase A2) inhibition of the plant compounds ARM-2 and RA-5.</i>	<i>41</i>
<i>Table A1: Molecular docking scores (XP score) and glide energies (kJ/mol) of the plant triterpenes (ARM-2 and RA-5) on some target proteins involved in cellular cholesterol trafficking.....</i>	<i>67</i>
<i>Table A2: Molecular docking scores (XP score) and glide energies (kJ/mol) of the plant triterpenes (ARM-2 and RA-5) on some target proteins involved in cellular cholesterol trafficking.</i>	<i>67</i>
<i>Table A3: ADMET properties of ARM-2</i>	<i>82</i>
<i>Table A4: ADMET properties of RA-5</i>	<i>83</i>
<i>Table A5: ADMET properties of RA-3</i>	<i>84</i>
<i>Table A6: Drug-likeness and medicinal chemistry of ARM2, RA5, lovastatin and RA3.....</i>	<i>85</i>
<i>Table A7: Swiss Target predictions for ARM-2.....</i>	<i>86</i>
<i>Table A8: Swiss Target predictions for RA-5.....</i>	<i>90</i>

<i>Table A9: Swiss Target prediction of RA-3.....</i>	<i>94</i>
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List of Abbreviations

AA	Arachidonic acid
ABCA-1	ATP-binding cassette transporters A1
ABCG-1	ATP-binding cassette transporters G1
ACAT-1	Acyl co-enzyme A: cholesterol acetyltransferase-1
ACVDs	Atherosclerotic cardiovascular diseases
ANOVA	One-way analysis of variance
ApoA-1	Apolipoprotein A1
ApoB-100	Apolipoprotein B100
CD	Conjugated dienes
CD-36	Cluster of differentiation 36
CE	Cholesterol esters
CVDs	Cardiovascular diseases
FE	Free cholesterol
HDL	High-density lipoproteins
LDL	Low-density lipoproteins
LOX-1	Lectin-like oxidized low-density lipoprotein receptor 1
MDA	Malondialdehyde
nCEH	Neutral cholesteryl ester hydrolase
RCT	Reverse cholesterol transport pathway
ORAC	Oxygen radical absorbance capacity
ox-HDL	Oxidised high-density lipoproteins
ox-LDL	Oxidised low-density lipoproteins
ROS	Reactive oxygen species

SMILES	Simplified Molecular Input Line Entry System
sPLA ₂	Secretory phospholipase A ₂
SR-A1	Scavenger receptor class A1
SR-B1	Scavenger receptor B1
WHO	World health organisation

1.0 Introduction

1.1 Overview of atherosclerosis and its global impact

Atherosclerosis is a chronic inflammatory disease that involves the formation of arterial plaques in the intima layer of the artery (Han *et al.*, 2019; Kavitha *et al.*, 2019). It is characterized by infiltration of inflammatory cytokines and lipid (cholesterol) accumulation in the intima arterial vascular walls (Han *et al.*, 2019; Filipek *et al.*, 2020;). Among the various pathophysiological factors, foam cell formation, which is caused by the difference between cellular cholesterol uptake and removal, has a central role in the occurrence and progression of atherosclerosis (Wang *et al.*, 2015). Depending on the artery affected, the dreadful and very common complications of atherosclerosis include peripheral artery disease, myocardial infarction, ischemic stroke, dementia, and heart failure, all generally referred to as atherosclerotic cardiovascular diseases (Libby, 2021).

Diabetes and hypercholesterolemia are among the major risk factors for ACVDs. While diabetes related complications are connected to oxidative stress and hyperglycaemia induced inflammation, hypercholesterolemic effects are linked to the plasma cholesterol transport and modification of LDL and HDL. Transport and oxidative modification of LDL (commonly referred to as bad cholesterol) are among the major underlying molecular events of atherosclerosis and its associated cardiovascular events (Linton *et al.*, 2000; Nguyen *et al.*, 2019). Oxidative, enzymatic, or non-enzymatic modification of HDL (referred to as good cholesterol), mainly involved in reverse cholesterol transport and cellular removal of oxidized LDL particles, is also reported to predispose affected individuals to ACVDs (Bergt *et al.*, 2003; Ito and Ito, 2020). Various other lifestyle factors such as smoking, lack of regular exercise and an unhealthy diet (diet that consists of increased intake of refined sugars, saturated fats, and trans-fats) contribute to the increasing incidence and prevalence of ACVDs. Understanding the pathophysiological events leading to atherosclerosis is of paramount importance in the search for new therapies and management strategies for atherosclerosis.

Atherosclerosis continues to be among the leading common cause of death in the whole world (Filipek *et al.*, 2020). Approximately 17 million deaths from atherosclerosis were reported in 2016 and this number is expected to rise to 25 million by 2030, thus making it a cause for concern (Aziz and Yadav, 2016). ACVDs remain the cause of death worldwide, with an estimated 18 million deaths annually (World Health Organization, 2023). ACVDs and CVDs remain the top three and four leading causes of death in South Africa respectively, after tuberculosis and diabetes mellitus (Statistics South Africa, 2018). Although a global decline in

mortality caused by CVDs has been observed in the past 30 years, the majority decline was observed in countries with a high socio-demographic index. However, the CVD related mortality remained the same in other regions of the world with a barely noticeable change, if any at all (Vogel *et al.*, 2021). These high CVD related mortality rates in countries with low socio-demographic index are partly due to inaccessibility to medications (Yusuf *et al.*, 2011; Thomas *et al.*, 2018;). Given the current state of the world having dealt with the covid-19 pandemic and currently facing its financial repercussions, it can be confidently stated that ACVDs and CVDs continue to be a global issue that impacts our lives.

1.2 Atherogenesis: Overview of biochemical events

Atherogenesis is the process of plaque formation in the intima layer of arteries. The development of the atherosclerotic plaque indicates the clinical complication of atherosclerosis. The pathological phases of atherogenesis can be separated into four steps namely, endothelial dysfunction, migration of leukocytes into the intima layer of the artery, foam cell formation, and plaque formation (Figure 1). Understanding the molecular and biochemical events of atherogenesis can assist in identifying key players in the process as well as targets that could lead to the treatment of atherosclerosis.

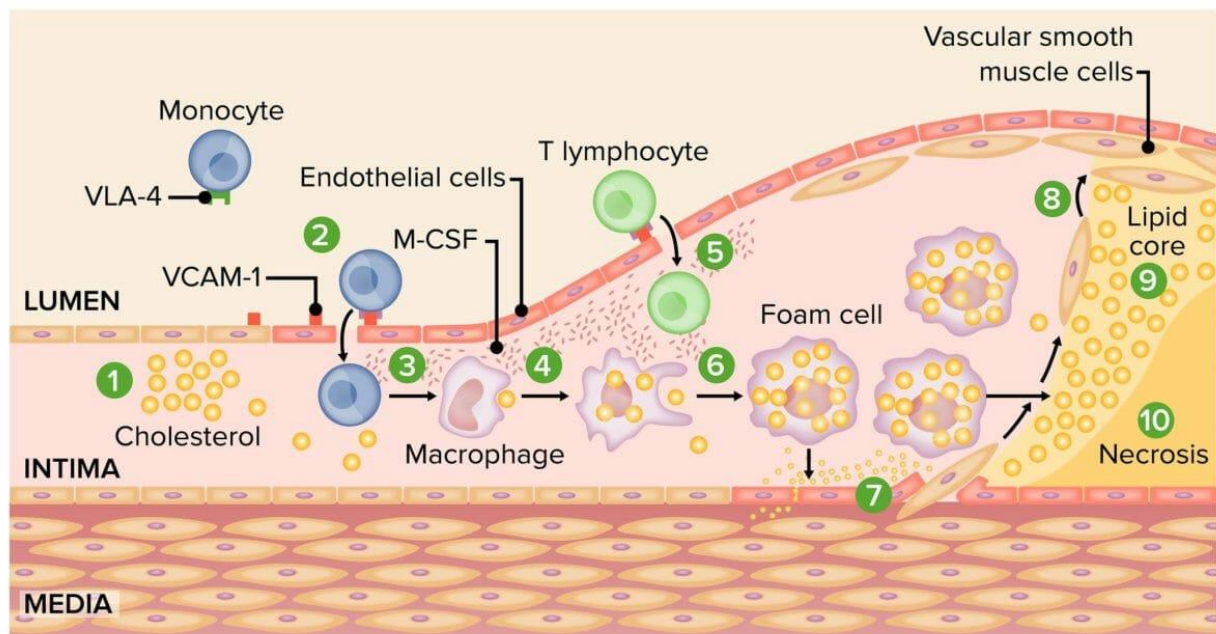


Figure 1: Diagram indicating the pathophysiology of atherogenesis. (1) Activation of the endothelial layer is a consequence of hypercholesterolemia, causing in an increased influx of LDL, resulting in the oxidation of the particles by ROS. (2 & 3) Endothelial dysfunction triggers the immune response followed by increased expression of leukocyte adhesion molecules and differentiation of monocytes to macrophages. (4 & 5) Uptake of ox-LDL by macrophages and increased presence of other leukocytes occurs simultaneously. (6) Macrophages become foam cells due to lipid droplet formation by high cholesterol concentrations. (7, 8 & 9) Accumulation of dead foam cells

and migration of smooth muscles, form the necrotic core leading to plaque formation. (10) Necrosis begins and results in increased plaque formation, causing plaque instability which can result in heart failure and or heart attack. (Minelli and Montinari, 2019).

1.2.1 Endothelial dysfunction

The vascular endothelium is a diverse monolayer created by endothelial cells that line the vascular arterial, venous, and lymphatic vessels, which become the initial barrier that pathogens, cells, and molecules interact with within the bloodstream (Xu *et al.*, 2021; Mussbacher *et al.*, 2022;). The luminal layer (intima) of the blood vessel is comprised of collagen, endothelial, and elastic fibres (Jebari-Benslaiman *et al.*, 2022). This layer of cells has a significant part in the transportation of blood constituents (cellular and acellular) from the arterial bed to the vascular wall (Markin *et al.*, 2020). The integrity and proper functioning of this layer are crucial in the maintenance of cardiac functions such as fluctuations in circulating blood which later mediates signal transduction to the other layers of the vascular wall (Hosseinkhani *et al.*, 2018; Xu *et al.*, 2021). Hypercholesterolemia in conjunction with the release of ROS and stimulation of the LOX-1 enzyme cause endothelial dysfunction of the intima layer in an arterial blood vessel (Figure 1). The production and release of ROS activate the inflammatory process, releasing proinflammatory cytokines. These proinflammatory cytokines create an atherogenic environment (Badimon and Vilahur, 2014; Markin *et al.*, 2020). Endothelial dysfunction is one of the first signs of atherogenesis and is complemented by LDL retention and its alteration in the luminal layer (Shafi, 2020; Mussbacher *et al.*, 2022). LDL particles in the intima are targets for oxidative and enzymatic modifications. LDL oxidation is defined by the loss of the antioxidant component of LDLs which leads to the degradation of polyunsaturated fatty acids (PUFAs) ultimately resulting in the formation of conjugated dienes and hydroperoxides (Friedman *et al.*, 2002; Khatana *et al.*, 2020). The ApoB-100 also undergoes modifications that inhibit LDL-LDL-R (LDL Receptor) recognition causing an increased uptake of LDL particles via uncontrolled receptors (Jebari-Benslaiman *et al.*, 2022).

1.2.2 Migration of leukocytes into the vessel wall

An activated or dysfunctional endothelium stimulates the selective recruitment of monocytes within the intima of the arterial wall. In the intima layer of the artery, monocytes differentiate into macrophages that either form into the M1 (pro-inflammatory, disease progression) or M2 (anti-inflammatory, disease regression) phenotype (Badimon and Vilahur, 2014; Jebari-Benslaiman *et al.*, 2022). M1 macrophages release proinflammatory cytokines and produce nitric oxide (NO) and ROS, which are known to encourage monocyte recruitment and enhance the inflammatory response, functioning similarly to an activated endothelium (Jiang *et al.*, 2022; Xu *et al.*, 2021). Most importantly, macrophages produce plenty of receptors that facilitate the internalization of oxidised and non-oxidised LDLs leading to the formation of lipid droplets within the macrophages (Figure 1).

The inflammatory response results in the secretion of proinflammatory enzymes including sPLA₂ and Leukotriene A₄ hydrolase (LTA₄H) as well as inflammatory cytokines namely vascular cell adhesion molecule 1 (VCAM-1) and chemokines (CXCL1, CXCL2, CXCL4, and CCL5). VCAM-1 assists in the binding of the monocytes to the endothelial cells. The transmigration and activation of the monocytes (Figure 1) is supported by the chemokines that assist by increasing monocyte adhesiveness (Shafi, 2020). The most important function of the secretory phospholipases involves the initiation of inflammatory mediation by prostanoids and leukotrienes through degradation of AA and converting it into bioavailable eicosanoid compounds such as prostaglandins (Autilio *et al.*, 2019; Dore and Boilard, 2019). The hydrolytic action of sPLA₂s leads to the release of AA to nearby tissues. However, overexpression of sPLA₂ results in uncontrolled production of AA leading to inflammatory illnesses such as atherosclerosis, asthma, and Alzheimer's disease, to name a few (Alasmary *et al.*, 2017).

1.2.3 Foam cell formation

During atherogenesis, the cellular influx of cholesterol exceeds the efflux and disproportionate amounts of cholesterol accumulate mainly in macrophage and endothelial cells (peripheral cells), resulting in foam cell formation (Sobenin, 2020; Waldie *et al.*, 2020). Foam cell formation occurs when the macrophages can no longer absorb the ox-LDL and become overwhelmed (Figure 1). An increased concentration of the ox-LDL particles results in the increased ox-LDL particles uptake by the macrophages. When there is a large amount of CE found in the macrophage cells, they begin to accumulate and form lipid droplets (Figure 1). The presence of lipid droplets overwhelms the macrophage cell and causes it to take on a foam-like structure (foam cell formation) and then it dies. When the foam cell dies, it remains

in the blood vessel and releases its contents which include CE, resulting in an increased concentration of cellular cholesterol thus signalling more macrophages. This process takes place in many macrophages hence the accumulation of the foam cells can form a plaque which leads to atherosclerotic lesions.

Cholesterol trafficking must be balanced to avoid or decrease the chance of plaque formation. The proteins that participate in the cellular (macrophage) uptake of ox-LDL include CD-36, LOX-1, and SR-A1 (Filipek *et al.*, 2020; Jiang *et al.*, 2022). CD-36 receptor has a high affinity for ox-LDL, taking in over 75% of ox-LDL particles compared to its counterpart, the SR-A1 receptor (Yu *et al.*, 2013; Filipek *et al.*, 2020). Literature states that the CD-36 receptor plays a significant part in the formation of atherosclerotic lesions with its capability to attach to and uptake ox-LDL into the artery wall (Filipek *et al.*, 2020). Due to its high affinity for ox-LDL particles, CD36 can induce macrophage foam cell formation. SR-B1, ABCA-1 and ABCG-1 are proteins involved in the cellular efflux of cholesterol (Yu *et al.*, 2013; Chistiakov *et al.*, 2017). The atherogenic environment of atherosclerotic lesions inhibits the function of ABCA-1 and ABCG-1 on the macrophages, thus also encouraging foam cell accumulation which promotes plaque development.

1.2.4 The development and formation of plaque

As the plaque continues to evolve, it begins to accumulate cellular debris and free cholesterol leading to the breakdown of the extracellular matrix. This can cause haemorrhage and/ or the plaque can begin to calcify (Badimon and Vilahur, 2014; Markin *et al.*, 2020). In order to stabilise the plaque, the necrotic core (lipid rich area) is enclosed by fibres, consequently, a fibrous cap is formed. The fibrous cap and necrotic core are the biomarkers of advanced atherosclerosis (Xu *et al.*, 2020). The main function of the fibrous cap is to provide structural assistance in avoidance of exposure to the prothrombotic constituents of the necrotic core, that usually cause the onset of thrombosis (Jebari-Benslaiman *et al.*, 2022). The necrotic core on the other hand continues to increase in size due to the atherogenic environment. This ultimately results in decreased plaque stability. Narrowing of the blood vessel which causes ischemic symptoms, is an effect of plaque progression. Smooth cells begin to migrate to the plaque, proliferate and release extracellular matrix macromolecules. The continuous stimulation of inflammation and activated apoptosis pathways lead to the death of foam and muscle cells (Khatana *et al.*, 2020). These developments compromise plaque integrity and stability by weakening the fibrous cap, making it unstable and prone to bursting when exposed to the different dynamics of blood flow in the vessel. Once the plaque has ruptured, thrombogenic components are released and begin to accumulate at the rupture site, forming

a thrombus. Thrombosis under atherogenic conditions increases the chances of major clinical complications of atherosclerosis due to an increased risk of blood vessel obstruction (Khatana *et al.*, 2020; Negre-Salvayre *et al.*, 2020). An embolus is formed from a thrombus detachment from the arterial wall that moves around the cardiovascular system. Finally, the attachment of the embolus in the distal arteries impedes blood flow resulting in potential myocardial infarction, local ischemia, or organ dysfunction (Jebari-Benslaiman *et al.*, 2022).

1.3 Hypercholesterolemia: a major risk factor of atherosclerosis

Hypercholesterolemia is a lipid metabolic disorder that is defined by abnormally raised blood levels of total cholesterol and LDL, and low levels of HDL cholesterol. It is the foundation of the development of atherosclerosis. While LDL is recognised for its involvement in cholesterol transport from the liver to extrahepatic tissues, HDL transports cholesterol from extrahepatic cells to the liver for metabolism and clearance (Figure 2). The cells in the human body can easily assimilate LDL from the bloodstream via LDL receptors (Perego *et al.*, 2019; Xu *et al.*, 2020). Increased levels of LDL in the intima layer of the artery and subsequent accumulation of ox-LDL, are major components in the initiation and accumulation of atheromatous lesions (Majdalawieh *et al.*, 2021).

The blood level of cholesterol is influenced by both exogenous (dietary) and endogenous sources (cholesterol biosynthesis). Hypercholesterolemia could be caused by a diet that is high in saturated fats, dietary cholesterol or defects in genes associated with plasma cholesterol clearance. Recent studies have indicated that a diet that is rich in saturated and/or trans fats is directly proportional to increased levels of LDL in the blood (Idris *et al.*, 2018; Soliman, 2018; Carson *et al.*, 2020). Saturated and/or trans fats contain a high concentration of TGs. Increased levels of TGs are directly proportional to the concentration of VLDL particles found in our blood, thus increasing the amount of LDL found in the blood. Therefore, abnormally increased levels of LDL in the blood due to a diet that is rich in TGs results in hypercholesterolemia, triggering the early onset of atherogenesis.

In addition to cholesterol, LDL also carries triglycerides (TGs). TGs are found in higher concentrations within the very low-density lipoprotein (VLDL) particles and lower concentrations in intermediate low-density lipoproteins (IDL) particles. As shown in Figure 2 following the endogenous pathway of cholesterol metabolism, the FC gets converted into CE by the ACAT-1 in the liver. The CE is transported out of the liver via the VLDL particles. The IDL particles have two fates, they either travel back to the liver or get converted into LDL particles. A large percentage (~70%) of LDL particles travel back to the liver while a small

percentage (~30%) gets assimilated by extrahepatic tissues (Mineo *et al.*, 2006). Thus, indicating the importance of a well-functioning endogenous pathway that consists of the required amounts and concentration of LDL particles.

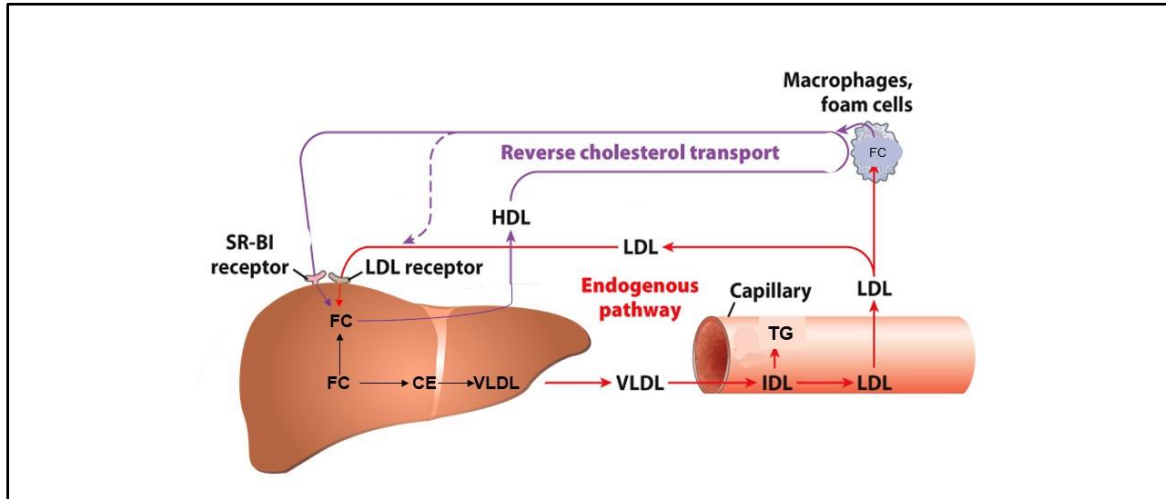


Figure 2: The endogenous pathway of LDL particles and the reverse cholesterol transport. In the endogenous pathway, FC converts into CE in the liver and is transported out via the VLDL particles. VLDL become IDL particles and the remaining IDL travel back to the liver or get converted into LDL. Macrophages uptake LDL contents and over time form foam cells. Through the reverse cholesterol transport pathway, HDL particles uptake FC from the macrophages/foam cells and deposit it back to the liver via the hepatic SR-BI receptor (Adapted. W.H. Freeman *et al.*, 2017).

1.4 Atherogenic effect of LDL

LDL cholesterol is a large lipid rich lipoprotein with an average particle diameter of 22 nm. In addition to the lipid core, the LDL particle also consists of a single copy of apolipoprotein-B100 and some lipophilic antioxidants (Esterbauer *et al.*, 1992; Hevonoja *et al.*, 2000). The crucial function of ApoB-100 is observed in the position it plays in the maintenance of structural integrity of the lipoprotein as well as overseeing its (LDL) interactions. Several functional domains have been identified on the ApoB-100. For example, the binding site for the LDL receptor is residues 3359-3369 (Hevonoja *et al.*, 2000).

Various structural modifications of LDL and other lipoproteins result in their functional loss. These modifications could be enzymatic or non-enzymatic. Non-enzymatic modifications include glycation, desialylation and oxidation. HDL and LDL are highly sialylated particles, as a result, the desialylation of these lipoproteins results in them being proatherogenic (Sukhorukov *et al.*, 2019). Chronic hyperglycemia results in uncontrolled non-enzymatic glycation and the development of advanced glycation end-products (Vlassara and Uribarri, 2014; Heidari *et al.*, 2020). Advanced glycation end-products (AGEs) bind to the lipoproteins,

forming glycated lipoproteins and delaying their clearance in the bloodstream (Al Saudi *et al.*, 2018). This can increase the formation of oxidised lipoproteins that are a result of these particles being attacked by reactive oxygen species (Al Saudi *et al.*, 2018). The focus of this study was on the oxidative modification of the lipoproteins.

Lipid peroxidation of LDL (ox-LDL) results in the break-down of polyunsaturated fatty acids (PUFAs), mainly arachidonic and linoleic acids, which can be modified into lipid hydroperoxides (Friedman *et al.*, 2002; Khatana *et al.*, 2020). The ox-LDL particles form heterogeneous groups of particles that differ immensely in composition, size, and structure (Hevonoja *et al.*, 2000). The lipid hydroperoxides (LOOH) lead to conjugated dienes formation and further modification into short-chain aldehydes like MDA. MDA is a known biomarker for oxidative stress and interacts with protein-bound Lys residues to produce dihydropyridine (DHP)-type of adducts (Ito and Ito, 2020). Modification of ApoB-100, the main protein of the LDL, also occurs due to an oxidative environment. Under normal conditions, aldehydes produced from lipid oxidation form adducts with the lysine residues of ApoB-100. However, in atherogenic conditions, it is possible for the direct modification of ApoB-100 to occur, specifically by oxidizing agents at tyrosine residues (Jebari-Benslaiman *et al.*, 2022).

Alteration of the ApoB-100 reduces the affinity of ox-LDL particles for LDL receptors (LDL-R), resulting in an increased uptake of ox-LDL particles by non-regulated receptors such as CD-36 and SR-A1 (Negre-Salvayre *et al.*, 2020; Xu *et al.*, 2021). Once the contents of the ox-LDL particle (mainly free cholesterol and cholesterol esters) have been transferred into the macrophage cells as seen in Figure 3, two main events occur. Firstly, the FC can freely exit the macrophage cell through the ABCA-1 and ABCG-1 located on the surface of the cell via the Apo-A1 found on the HDL molecule. This free cholesterol is transported back to the liver where it enters through the SR-B1 receptors. The free cholesterol that is not taken up by the HDL molecule can be converted into CE facilitated by the ACAT-1 enzyme. This is considered the most significant role ACAT-1 plays in cholesterol homeostasis (Filipek *et al.*, 2020). The second fate of the ox-LDL content ultimately has the same result as the first one however, it must undergo an important process facilitated by the nCEH, whereby the CE gets converted into FC in the endoplasmic reticulum. After the de-esterification of CE, the FC can exit the macrophage cell and travel back to the liver to get metabolised.

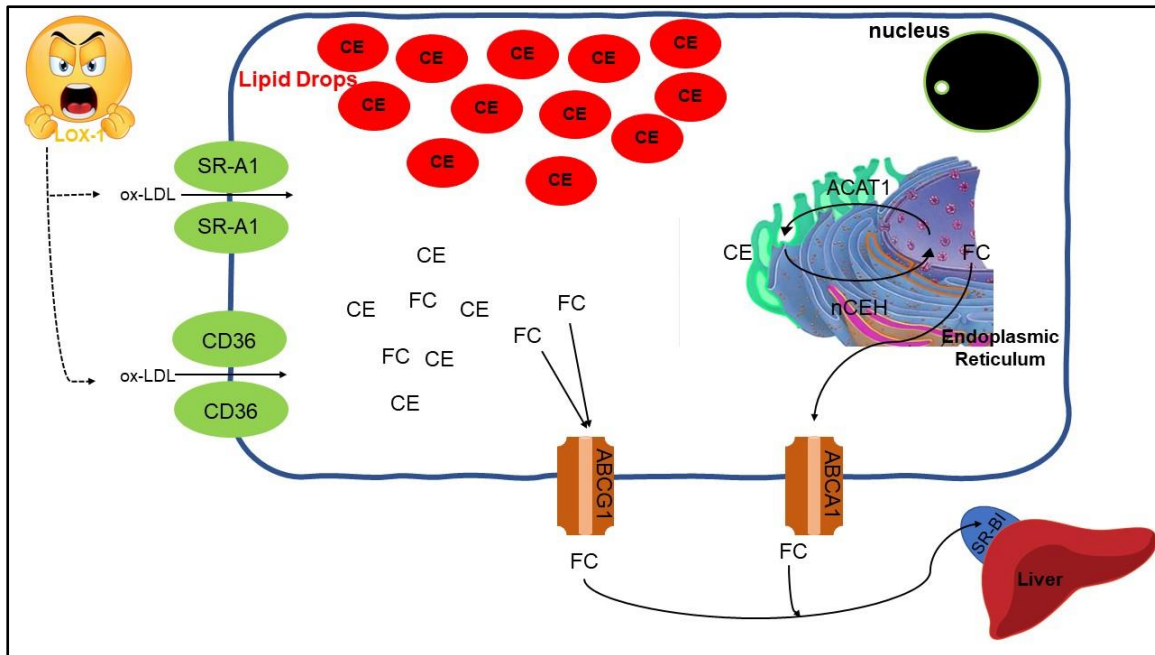


Figure 3: Proteins involved in foam cell formation of macrophage cell. *LOX-1* induces endothelial dysfunction forming ox-LDL particles. *SR-A1* and *CD-36* are liable for the intake of ox-LDL particles into the macrophage cell. The intake of ox-LDL into the macrophage cell results in FC and CE accumulating in the cell. *ABCA-1* and *ABCG-1* proteins are responsible for the efflux of FC from the macrophage. The enzyme *nCEH* is involved in the de-esterification of cholesterol, *ACAT-1* is important in the esterification of cholesterol. FC- free cholesterol; CE- cholesterol ester.

It is observed that an increased concentration of LDL particles in an atherogenic environment results in the unregulated formation of ox-LDL particles. These ox-LDL particles ultimately result in foam cell formation (Figure 3). This is due to the overexpression of *ACAT-1* and suppression of *nCEH*. Overexpression of *ACAT-1* can lead to the accumulation of esterified cholesterol, and macrophage-derived foam cell formation (Yu *et al.*, 2013). Foam cell formation is one of the many biomarkers of atherosclerosis and is important in the progression of the disease. Even though some mice models gave controversial results regarding the inhibition of *ACAT-1* to prevent foam cell formation (Farese, 2006), *ACAT-1* inhibition has been proven to suppress the build-up of total cholesterol in human macrophages by lessening the internalization of acetylated LDL (acLDL) and assisting in free cholesterol outflow (An *et al.*, 2008). Therefore, inhibition of foam cell formation could be vital to halt the atherogenesis process.

1.5 HDL and its antiatherogenic effects

HDL is the lipoprotein associated with health benefits as it transports cholesterol to the liver from extrahepatic tissues. HDL is a small protein-rich lipoprotein with a particle size of 6–12.5 nm (Eren, 2012). Similar to LDL, HDL molecules have a highly heterogeneous chemical,

structural and biological composition. HDL is extremely diverse and contains many different proteins, with ApoA-1 making up approximately 70 % of total HDL protein and being the main structural and functional component of the lipoprotein (Witztum and Schonfeld, 2015). Interactions with cellular receptors, inhibition of LOOH accumulation in both lipoproteins (LDL and HDL), activation of lecithin/cholesterol acyltransferase (LCAT), as well as providing HDL with many anti-atherogenic activities, are all important functions of ApoA-1 (Linton *et al.*, 2023).

The ApoA-1 obtains cholesterol and other lipids that are removed by hepatocytes and macrophages (Zhang *et al.*, 2017; Loving and Bruce, 2020). This is known as the RCT pathway. The RCT pathway assists in maintaining cholesterol balance and is also how the peripheral cells rid the body of unwanted cholesterol. The scavenger receptors found on the macrophages and the Apo-A1 found on the HDL particle assist in the transportation of cholesterol to the liver when there are elevated concentrations of cholesterol in the blood. Therefore, modification of ApoA-1 results in the functional loss (antiatherogenic property) of HDL.

HDL also functions to prevent the oxidation of the LDL particle as well as assist in the removal of the ox-LDL particles. HDL particles prevent oxidation of the LDL particles by promoting endothelial function. It does this by signalling and stimulating endothelial nitric oxide synthase (Shaul and Mineo, 2004; Mineo *et al.*, 2006; Bardagjy and Steinberg, 2019). These actions assist in the preservation of the health and strength of the vascular endothelium (Seetharam *et al.*, 2006; Bardagjy and Steinberg, 2019). However, if the HDL particles fail to prevent the oxidation of LDL, they will attempt to remove the ox-LDL particles. HDL carries out this process by binding onto the scavenger receptors (ABCG-1 and ABCA-1) of the foam cells, as an immature HDL particle, to take up the cholesterol. Once matured, the HDL particle takes the cholesterol or lipid droplets from the foam cells and transports the cholesterol to the liver (Figure 2) as well as to the steroidogenic organs (Mineo *et al.*, 2006). The atheroprotective properties of HDL are also associated with the ability to reduce the chances of thrombosis (Bardagjy and Steinberg, 2019).

Although HDL is considered antiatherogenic based on its functions, the atheroprotective or antiatherogenic qualities that are associated with HDL are highly dependent on the functional capabilities of the particle and not solely on the plasma concentration of lipoprotein particles (Bardagjy and Steinberg, 2019). Similar to LDL, HDL particles are also susceptible to both enzymatic and/or non-enzymatic modifications consequently leading to their functional loss. Human genetics studies have indicated that exceedingly decreased or elevated HDL levels

are not automatically associated with high risk or protection from CVDs, respectively (Ito and Ito, 2020). This implies that HDL levels are not the only contributory factor to its antiatherogenic property.

1.6 Current treatment of atherosclerosis and specific drug targets

Atherosclerosis offers pathological information in the progression of cardiovascular diseases (CVD) (Zhang *et al.*, 2017). Preventive measures for atherosclerosis include lifestyle changes such as practising regular physical exercise and low consumption of high caloric food. However, in the case of clinical health complications, therapeutic drugs against atherosclerosis and related complications need to be administered. To properly treat any disease, identifying specific drug targets is vital. Literature has identified key enzymes and proteins that are involved in lipoproteins and cholesterol metabolism. These proteins include HMG-CoA reductase and SQLE. Targeting genes that have a role in the expression of these proteins may bring us a step closer to finding the best treatment for atherosclerosis with limited or no side effects.

Treatment of atherosclerosis and related complications currently includes the use of cholesterol lowering and anti-inflammatory drugs. Anti-inflammatory drugs and statins are normally prescribed together to combat ACVD or CVDs. Statins, the commonly used lipid lowering drugs, function by inhibiting HMG-CoA reductase which is an important enzyme catalysing the rate-limiting step of cholesterol biosynthesis (Ness and Chambers 2000). This enzyme is activated by insulin and suppressed by high amounts of intercellular cholesterol as well as glucagon. The constant increase in specific pro-inflammatory biomarkers (C-reactive protein, cyclooxygenase-1 and cyclooxygenase-2) can end in a chronic state of subclinical or low-grade inflammation, that is recognized as a crucial constituent in the progression of ACVDs and CVDs (Kosmas *et al.*, 2019). Therefore, the anti-inflammatory drugs target C-reactive protein (CRP), cyclooxygenase-1 (COX -1) and cyclooxygenase-2 (COX-2) activities.

Since both inflammation and ROS have significant roles in the atherogenic process, the use of anti-inflammatory and antioxidant therapy is also crucial in atherosclerosis treatment. Antioxidants are molecules that are capable of inhibiting oxidation of other molecules, through scavenging oxidants or reducing the production of ROS (Wang and Kang, 2020). The mechanism of inhibition is based on the ROS-producing systems. For example, one of the approaches that statins utilize in atherosclerosis treatment is the inhibition of NADPH oxidase activation (Poznyak *et al.*, 2020). Novel antioxidants are separated into three categories: the stimulants of endogenous antioxidant defence systems, the inhibitors of the formation of

oxidative stress, and molecules that permit functional restoration of ROS-induced impairment (Wang and Kang, 2020).

Despite the efficacy of the medicine currently used to treat ACVDs, they are linked to some health complications, therefore limiting their clinical uses (Wengrofsky *et al.*, 2019). Long-term use of cholesterol lowering medications such as statins can damage the liver (Saha and Garg, 2021), and in some other patients cause myopathy as well as increased risk of haemorrhage stroke, especially in patients with prior cerebrovascular disease (Ribe *et al.*, 2020). Asian people are known to be at high risk for haemorrhage stroke (Pinal-Fernandez *et al.*, 2018). Statins have also been reported to be less effective in elderly people aged 70 years and above (Horodinschi *et al.*, 2019). The use of anti-inflammatory medications that fall under the NSAIDs category can result in elevated blood pressure due to sodium and water retention, and a higher chance of developing thrombosis. This is due to their non-specific inhibition of the COX-2 and COX-1 enzymes thus, compromising other physiological processes of the body (Varga *et al.*, 2017). These limitations indicate a need to search for alternative drugs, preferably of natural origin.

1.7 Alternative sources of new antiatherogenic drugs: Medicinal plants

Medicinal plants have, for ages, served as rich sources of bioactive compounds giving rise to many therapeutically active drugs in clinical use today. These compounds can be found in a multitude of plants thus, providing researchers with an array of options to help increase the number of therapeutic drugs that are being used today. Due to the wide chemical diversity in plants, medicinal plant-derived compounds possess numerous bioactivities (medicinal properties) and some are yet to be explored (Rifaioğlu *et al.*, 2019).

There are several plants or herbs that have been studied for antiatherogenic or atheroprotective potentials. For instance, Chinese danshen or sage (*Salvia miltiorrhiza*) has been reported to have the potential to inhibit foam cell formation by decreasing CD-36 expression (involved in cholesterol influx) and up-regulation of ABCA-1 (involved in cholesterol efflux) which are the receptor proteins found on the surface of the macrophage cells (Kirichenko *et al.*, 2020). This plant has also shown antihypertensive activity by increasing nitric oxide production which can assist in atherosclerosis treatment (Kirichenko *et al.*, 2020). The atheroprotective activity of *Allium sativum* (garlic) oil has been associated with the inhibition of lipid peroxidation in isoproterenol (ISO)-induced myocardial infarction (MI) rats (Senthilkumar *et al.*, 2009). The antiatherogenic or atheroprotective potential of other medicinal plant extracts has been linked to their anti-inflammatory, antihyperlipidemic and

antioxidant properties (Saqib et al., 2021; Markina et al., 2022; Yang et al., 2023). Triterpenes are among the classes of plant compounds that have gained the interest of researchers across the world due to their diverse bioactivities (Furtado *et al.*, 2017; Ma *et al.*, 2018). These compounds have been studied and proven to hold many prominent medicinal potentials.

1.7.1 Triterpenes as potential targets in drug discovery and development

Triterpenes are a large and structurally diverse class of natural compounds commonly referred to as triterpenoids. This large class of lipid organic compounds have important functional and structural roles with origins in plants, animals, and fungi (Chudzik *et al.*, 2015; Sohag *et al.*, 2022). These compounds are naturally found in plants as free acids or aglycones (Yin, 2015). Triterpenoids from vegetable oils, cereals, and fruits contain various health benefits including cholesterol-lowering effects (Sohag *et al.*, 2022). The most common type of triterpenes comprises pentacyclic and tetracyclic triterpenes (Nazaruk and Borzym-Kluczyk, 2015). Plant derived triterpenes have recently gained much attention from researchers as novel targets in drug development because of varied bioactivities including atheroprotective effects and cardioprotective properties (Wang et al., 2015; Sangweni et al., 2019). Triterpenoids are known to exhibit anti-inflammatory (Mosa *et al.*, 2016; Sohag *et al.*, 2022), anti-diabetic (Sangweni et al., 2019; Perera et al., 2021), and anti-oxidative properties (Chudzik *et al.*, 2015; Khlebnicova *et al.*, 2019).

The antiatherogenic effects of plant triterpenes include the ability to inhibit inflammation, LDL oxidation, and foam cell formation which are the key factors that lead to atherosclerosis. Their anti-inflammatory mechanism of action is exerted through the inhibition of NF- κ B and related signalling pathways, STAT and GATA-3 (Renda *et al.*, 2022). These pathways are responsible for the regulation of immune response and inflammation. Triterpenoids isolated from *Zizyphus jujuba* exhibited inhibition of acetylated LDL induced foam cell formation in macrophage cells (Fujiwara *et al.*, 2011). Additionally, triterpenoids consisting of a carboxylic group at C-28 have been shown to significantly inhibit foam cell formation in human macrophages, proposing that these compounds could have an important role in the prevention of atherosclerosis (Fujiwara *et al.*, 2011).

Other studies have indicated that tetracyclic triterpenes such as lanostanes from *Ganoderma* species have numerous bioactivities including anti-inflammatory activity, and HMG-CoA reductase inhibitory activity (Wang et al., 2015; Ghiulai et al., 2020). Some lanosteryl triterpenes (Figure 4) from *Protorhus longifolia*, a plant commonly used by traditional healers in Kwa-Zulu Natal in the treatment of cardiovascular ailments, have also shown some

promising bioactivity relevant to atherosclerotic amelioration. Specifically, the bioactivities of RA-3 and RA-5 previously studied, include cardioprotective potential (Sangweni *et al.*, 2019), anti-inflammatory (Mosa *et al.*, 2011, 2015, 2016), antihyperglycemic (Mabhida *et al.*, 2017) and antihyperlipidemic activities (Machaba *et al.*, 2014). RA-5 and ARM-2, a newly isolated triterpenoid from *P. longifolia*, have displayed antihypercholesterolemic potential through the regulation of cholesterol biosynthesis and stimulation of LDL uptake in HepG2 Cells and were the focus compounds of this study (Ndlovu *et al.*, 2023).

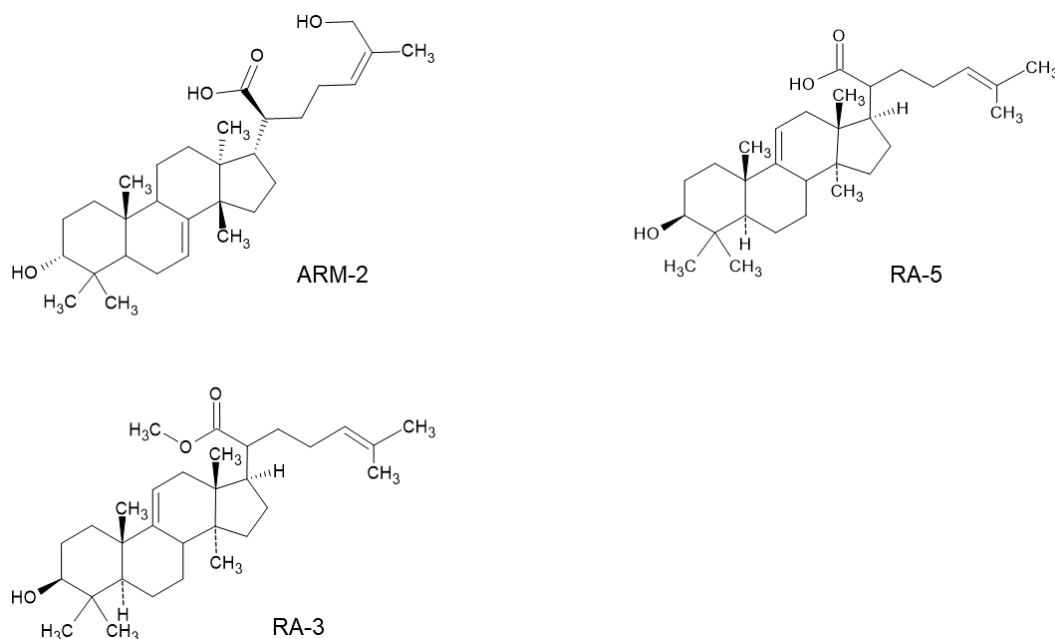


Figure 4: Chemical structure of 3 α ,26-dihydroxytirucalla-7,24-dien-21-oic acid, (**ARM-2**), 3 β -hydroxylanosta-9,24-dien-21-oic acid (**RA5**) and methyl-3 β -hydroxylanosta-9,24-dien-21-oate (**RA3**).

1.8 Strategies for identifying or discovering new drug candidates from traditional medicinal plants

There are a variety of strategies that are available to assist in identifying and discovering new drug candidates from plants. These include *in silico*, *in vitro* and *in vivo* strategies. A combination of all these strategies assists in the study of novel compounds which are usually abundant in plants, helping researchers in narrowing down to the focus compounds. Traditionally, drug discovery and development begin with finding biomolecular targets for a proposed treatment. The chosen biomolecular targets are developed with the use of high-throughput screening experiments. These experiments assist in determining the bioactivity of the target compounds. Bioactive compounds are chosen because of their desired targets alongside their corresponding bioactivity levels. As vital as these high throughput screening experiments are, unfortunately, they are expensive, time consuming, and of extensive

chemical and biological libraries (Rifaioğlu *et al.*, 2019). Most importantly, it is impractical to perform high-throughput screening experiments for all known compounds and every protein that is expressed in the human genome. Lastly, high-throughput screening experiments are known to have high failure rates, which limits and puts a strain on the identification of novel drugs. This further worsens the issue when considering the development of drugs, resulting in the failure of clinical trials by the majority of drug candidates attributed to unanticipated side effects and toxicity (Sun *et al.*, 2022).

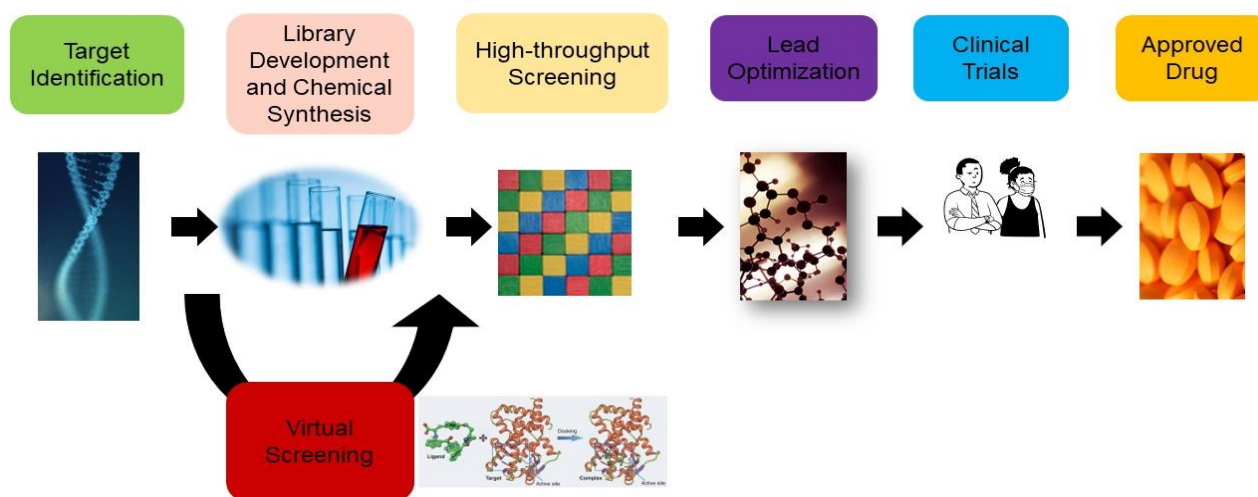


Figure 5: Drug discovery and development processes

The aforementioned difficulties have been addressed by the development of computational methods, which now come before high throughput experiments as shown in Figure 5. The main goal of computational drug discovery approaches, known as virtual screening (VS), is to predict the biological interactions among compounds with drug-like potential and prospective target proteins for the discovery of new drugs (Rifaioğlu *et al.*, 2019; de Souza Neto *et al.*, 2020). VS can significantly lower the financial expense and time desired for high-throughput screening, drug repurposing as well as off-target effect identification (Gorgulla *et al.*, 2020). The main purpose of off-target effect identification is to find novel applications for already approved drugs (Rifaioğlu *et al.*, 2019). This computational approach is divided into structure-based, proteochemometric modelling (non-structure) and ligand-based (non-structure).

The non-structure-based virtual screening methods are mostly preferred due to the limited structural information of most proteins and compounds. Structural information is limited because of the challenges associated with the experimental identification of the 3D structures resulting in unreliable target structures (Vázquez *et al.*, 2020). An example of non-structure-based drug discovery is Vemurafenib (Zelboraf™), which treats melanoma, moved quite

rapidly (6 years) throughout the steps of research and development (R&D) until it attained Food and Drug Association (FDA) approval (de Souza Neto *et al.*, 2020). This proved that fragment-based drug discovery (FBDD) has desirable opportunities to offer for the drug discovery field (de Souza Neto *et al.*, 2020).

To further enhance VS, an addition of molecular dynamics, molecular docking and ADMET property prediction are implemented. Molecular docking is a computational method applied to predict the position, orientation, and binding scores of small molecules to target proteins (Torres *et al.*, 2019; de Souza Neto *et al.*, 2020). The binding scores, also known as docking scores, are numerical values. A good docking score can be interpreted by a large negative value as it indicates a strong and stable binding complex between a ligand and receptor. ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties of new compounds are predicted, because of the drawbacks that were previously presented due to poor synthetic feasibility (estimated ease of synthesis) and/or undesirable biological properties (Yang *et al.*, 2019; de Souza Neto *et al.*, 2020). SwissADME is a tool that uses predictive models for the physicochemical properties, and lipophilicity of the target compounds and provides rapid ADME properties among others (de Souza Neto *et al.*, 2020). There is a lot of data that is generated under each property therefore ADMET prediction data is interpreted by looking at the predicted value of the target compound in conjunction with the unit. However, the downside of VS is that for the results to be reliable, a combination of different computational methods needs to be used. There is no known reliable computational software that can provide molecular dynamics, molecular docking and ADMET property prediction simultaneously.

In vitro and *in vivo* experiments offer significant information throughout biomedical research and have been present throughout drug discovery long before the implementation and discovery of VS. *In vitro* studies provide a good foundation by providing a regulated control of the chemical and physical environment, they are much cheaper, have a higher throughput, and decrease animal use (Graudejus *et al.*, 2018). Decreasing the use of animals in the laboratory experiments with high throughput screening, assists in the production of more reliable results. This is because high throughput screening results are highly sensitive. These kinds of studies occur in controlled environments such as test tubes and microplates and allow researchers to investigate different biological systems in a more detailed manner without the variables present in whole organisms. This same advantage is a disadvantage, which is why this data needs to be interpreted with restraint as it does not represent accurate findings that one would observe in the whole organism. This is a foundation on which *in vivo* studies are built. Literature has indicated that in human clinical trials, around 30% of drugs have failed, as

a consequence of adverse side effects despite favourable pre-clinical studies, and an additional 60% fail because of lack of efficacy (Tagle, 2019). The above statement highlights the importance of *in vivo* studies.

The abovementioned strategies for identifying or discovering new drug candidates from traditional medicinal plants have highlighted both the advantages and disadvantages of *in silico*, *in vivo* and *in vitro* studies. Overall, each strategy on its own is important and has a vital role in contributing to the drug discovery and development process (Figure 5). However, a combination of all the strategies is what can contribute to a better understanding of the potential drug candidate. Obtaining all possible information (through experimentation) on the potential drug candidate increases its chances of reaching clinical trials.

1.9 Rationale of the undertaken study

Atherosclerosis continues to be among the leading common cause of death in the whole world (Filipek *et al.*, 2020). Hypercholesterolemia which is a major risk factor for atherosclerosis increases the occurrence of atherocardi vascular diseases. Despite the use of current drugs to treat atherosclerosis and its related vascular complications, an increase in morbidity and mortality rates associated with atherosclerosis indicates challenges and limitations of the current treatment regime. A search for alternative drugs with improved efficacy and novel mechanisms of action is necessary. Considering the promising results from our laboratory on the bioactivities of the *P. longifolia* isolated triterpenes (ARM-2 and RA-5), the current study focused on the potential of these compounds to inhibit atherogenesis.

2.0 Aim

To investigate the potential of two triterpenoids from *P. longifolia* to prevent oxidative modification of LDL and formation of foam cells that underly development of atherosclerotic plaque.

2.1 Hypothesis

H₁: The compounds will significantly inhibit MDA formation.

H₀: The compounds will not significantly inhibit MDA formation.

H₁: The compounds will significantly inhibit foam cell formation.

H₀: The compounds will not significantly inhibit foam cell formation.

2.2 Objectives

- i. *In silico* molecular docking of the plant compounds against some proteins involved in cellular cholesterol trafficking.
- ii. In silico analysis of ADMET properties of the compounds.
- iii. Investigate the antioxidant capacity, LDL oxidation inhibitory activity of the plant compounds.
- iv. Determine the inhibitory potential of the compounds on secretory phospholipase A2 (GIIA).
- v. Determine the effect of the compounds on oxidized LDL-induced intracellular cholesterol accumulation in macrophage cells (RAW 264.7 cells).

3.0 Material and Methods

3.1 Chemicals and reagents

The fluorescein sodium salt, 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8- tetramethylchromane-2-carboxylic acid (Trolox), 2-thiobarbituric acid (TBA), L-Ascorbic acid, gallic acid, trichloroacetic acid (TCA), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), EDTA disodium salt dihydrate, potassium persulfate, high density lipoprotein (HDL), low density lipoprotein (LDL), dialysis tubing cellulose membrane, were purchased from Sigma Aldrich (Missouri, United States of America). 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), phenol red, sodium taurodeoxycholate hydrate and xanthine were purchased from Glentham Life Sciences (Corsham, England). Recombinant human secretory phospholipase A2 protein (ab63843), Oligo primers (Inqaba Biotech, South Africa), SV Total RNA Isolation (Z3100), and Go Taq qPCR Master Mix (A6001) (Promega) (Wisconsin, United States of America) were used.

3.1.1 Plant compounds tested in the study

The two triterpenoids (3 β -hydroxylanosta9,24-dien-21-oic acid and 3 α ,26-dihydroxytirucalla-7,24-dien-21-oic acid (Figure 4) used in this study were received as a donation from Mr Musawenkosi Ndlovu, a senior member of our research group (University of Pretoria). The compounds have been previously isolated and characterized from the chloroform extract of *Protorhus longifolia* stem bark (Mosa *et al.*, 2011; Ndlovu *et al.*, 2023).

3.2 *In silico* studies: molecular docking, ADMET properties and molecular target prediction of the test compounds

3.2.1 Molecular docking of the test compounds against some proteins involved in cellular cholesterol trafficking

Molecular docking of the plant compounds (ARM-2 and RA-5) against some proteins that participate in cellular cholesterol trafficking was assessed by utilizing Maestro from Schrodinger. Three-dimensional crystal structures of the target receptors (proteins) were acquired from the RCSB Protein Data Bank (PDB). The following proteins with their respective PDB IDs were docked: SR-A1 (7DPX), CD-36 (3C64), ABCA-1 (5XJY), ABCG-1 (7R8C), LOX-1 (6TL9), ACAT-1 (6P2J), and sPLA2 (1DCY). The ligands (test compounds) and proteins were prepared using Maestro. The structures of the plant triterpenoids and other test compounds were constructed/drawn through the utilization of the ChemDraw Ultra 6.0 software, where they were further converted into 3D formats. An open-source chemical

toolbox was used to perform all file conversions mandatory for the docking study. The molecular docking was carried out using Maestro, thereafter the 2D structures of the binding site on the targeted proteins were identified. The glide energy and docking score of the provided inhibitor conformation in the macromolecular structures were determined. A good docking score is indicated by a large negative value (≤ -5) with respect to the XP scores. The docking score can also be considered 'good' or 'bad' by comparison to the docking score of the reference ligand (Faiza, 2022).

3.2.2 Drug-likeness and target prediction of ARM-2 and RA-5

SwissTargetPrediction was used to predict the drug-likeness and potential targets of the compounds (ARM-2 and RA-5). SwissTargetPrediction is a free online server (<http://www.swisstargetprediction.ch/>) which is centred around the observation that bioactive compounds that are similar in structure, are more liable to share similar biological targets (Keiser et al., 2007; Armstrong et al., 2011). The structures of ARM-2 and RA-5 were constructed by using the ChemDraw Ultra 6.0 software and were further converted into SMILES files.

3.2.3 ADMET profiling prediction of the compounds

ADMET studies play a vital part in drug discovery and research. The online server, pkCSM (<https://biosig.lab.uq.edu.au/pkcsml/>), was used to determine the ADMET properties of the compounds (ARM-2 and RA-5). pkCSM is a well-trusted, secure, and free online server, with a very easy-to-use interface. The structures of ARM-2 and RA-5 were constructed by using the ChemDraw Ultra 6.0 software and were further converted into SMILES files.

3.3 *In vitro* analysis of potential bioactivities of the test compounds

3.3.1 Sample preparations

A stock solution (5000 μ M) of ARM-2 and RA-5 was prepared in methanol and was refrigerated at 4°C until needed. Depending on the requirements of each experiment, the desired working solutions were prepared from the stock solutions.

3.3.2 Antioxidant activity of the compounds *in vitro*

Antioxidant assays test the ability of a compound to inhibit oxidation of other molecules or compounds. This antioxidant activity can be based on an electron or hydrogen transfer system, metal ion chelating, and reduction power. The free radical scavenging activity

(electron transfer system) of the compounds was determined on 2,2-diphenyl- 1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radicals. The ORAC assay was performed to test the antioxidant potential of the plant compounds in a hydrogen transfer system. The ferric reducing power assay was also used to test the ability of the compounds to reduce ions. The free radical scavenging activity of the compounds was calculated using the following formula:

$$\% \text{ Scavenging activity} = [(Abs_{\text{control}} - Abs_{\text{test}}) / Abs_{\text{control}}] \times 100 \quad (3.1)$$

Where, Abs_{control} and Abs_{test} represent the absorbance of the sample without inhibitor (control sample) and sample with inhibitor, respectively.

3.3.2.1 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay described by Afshari and Sayyed-Alangi (2017) was adhered to, to investigate the free radical scavenging capacity of the plant triterpenes. Equal volumes (1:1) of both the DPPH (30 μM in methanol) and test compound (25, 50, 100, 200, and 500 μM in methanol) were mixed and then incubated in the dark at room temperature (30 min). The sample control consisted of an equal volume of methanol in place of the test compound. Ascorbic acid was the standard antioxidant. The absorbance of the samples was measured at 517 nm utilizing the SpectraMax Paradigm Microplate Reader (Molecular Devices, California, United States of America). The percentage radical scavenging activity was calculated using the formula given in 3.1.

3.3.2.2 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) scavenging activity

The method of Re *et al.* (1999) was adapted for the evaluation of ABTS scavenging activity of the plant compounds. The ABTS radical stock solution was made up by mixing (1:1 v/v) 7 mM ABTS and 2.4 mM potassium persulphate. The mixture was incubated in the dark at room temperature (16 hours). ABTS working solution was made by diluting (1:10 v/v) the ABTS radical stock solution with methanol. To determine the ABTS radical scavenging capacity of the triterpenes, equal volumes (1:1) of both the ABTS radical and test compound (25, 50, 100, 200, 500 and 1000 μM in methanol) were mixed. Ascorbic acid was the standard antioxidant. The sample control consisted of an equal volume of methanol in place of the test compound. The reaction mixture stood at room temperature for 7 min. The absorbance of the samples was measured at 734 nm using the SpectraMax Paradigm Microplate Reader. The percentage free radical scavenging activity was determined using the formula in 3.1.

3.3.2.3 Reducing power assay

The reducing power of the plant compounds was studied on ferric iron (III) following the modified method of Yildirim *et al.* (2001). The reaction mixture was comprised of 315 μL each of the test compounds (25, 50, 100, 200, and 500 μM), potassium phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$; 1 %). The mixture was briefly vortexed and incubated for 30 minutes at 50°C. Then, 315 μL of trichloroacetic acid (10 % w/v) was added and vortexed. The mixture was then centrifuged (Eppendorf Minispin Personal Microcentrifuge) at 3000 rpm for 10 min. The supernatant (315 μL) was collected and diluted with an equal volume of distilled water. This was followed by the addition of 65 μL of ferric chloride (FeCl_3 ; 0.1 %) and mixed well. Ascorbic acid was the standard for the experiment and the negative control contained methanol in place of the test compound. The absorbance was read at 700 nm using the SpectraMax Paradigm Microplate Reader. The results were expressed as absorbance vs compound concentration. A higher absorbance reading is directly proportional to a higher reducing power.

3.3.2.4 Oxygen radical absorbance capacity assay

The ORAC assay (Jiménez *et al.*, 2015) was also done to determine the antioxidant activity of the plant compounds. The working solutions (50, 100, 200, 500 and 1000 μM) of the test compounds were prepared in methanol. Fluorescein (1 μM) and AAPH (250 mM) were prepared in a potassium phosphate buffer (75 mM, pH 7.4). An amount of 25 μL of each test compound was added to 100 μL of fluorescein in a black 96-well microplate and mixed well. The mixture was incubated in the dark at 37°C (30 minutes). Thereafter, 75 μL of AAPH was added to the mixture of fluorescein and the test compound. The fluorescence intensity of fluorescein was kinetically measured every 2 minutes for 120 minutes at excitation and emission wavelengths of 485 nm and 520 nm, respectively, using the SpectraMax Paradigm Microplate Reader. Trolox was used as the standard for the experiment. The fluorescence decaying curves were determined by measuring the area under the decay curves and the relative ORAC values were expressed as micromoles of Trolox equivalents ($\mu\text{M TE}$).

3.3.3 Inhibition of LDL oxidation

The antioxidant activity of the triterpenes was further determined using the CuSO_4 -mediated LDL oxidation method described by Kim *et al.* (2020), with slight adjustments. The inhibition of LDL (and/or HDL) oxidation was confirmed by monitoring the formation of conjugated dienes and determining the amount of thiobarbituric acid reactive substances (TBARS) formed as described below.

3.3.3.1 Measurement of conjugated dienes formation

For measuring the formation of dienes by the chain reaction of lipid peroxidation, LDL (100 µg protein/ml) was mixed with CuSO₄ (25 µM) in the presence and absence of the triterpenes (500 µM). The above were prepared to their final concentrations and the final volume was made up with potassium phosphate buffer (10 mM, pH 7.4). Ascorbic acid was the positive control. The reaction mixtures were incubated at 37 °C for 180 minutes. For the period of incubation, the formation of conjugated dienes was monitored uninterruptedly every 2 minutes by measuring absorbance at 234 nm using the SpectraMax Paradigm Microplate Reader.

3.3.3.2 Determination of TBARS formation

Native LDL and native HDL (each at 500 µg protein/ml) were separately mixed (at their final concentration) with CuSO₄ (25 µM) in the presence or absence of the triterpenes (ARM-2 and RA-5) and gallic acid (at final concentrations of 100 and 500 µM). The final volume was made up to 100 µL with a potassium phosphate buffer (10 mM, pH 7.4). The reaction mixture was incubated at 37 °C (5 hours). An equal volume (1:1) of 0.5 mM EDTA was added to stop the reaction (oxidation). Thereafter, 100 µL of 20% trichloroacetic acid (TCA) and 200 µL of 0.67% thiobarbituric acid (TBA, prepared in 0.05 M of NaOH), were added to the mixture. The final mixture was placed in a water bath at 95°C (1 hour) and cooled to room temperature (15 min). The mixture was then centrifuged (Eppendorf Minispin Personal Microcentrifuge) and the supernatant was collected (3000 rpm for 15 minutes). The optical density of the supernatant (in 96-well clear bottom plates) was measured at 532 nm with the SpectraMax Paradigm Microplate Reader. The amount of TBARS (nM) formed was determined from the standard curve of MDA (See Appendix B). The MDA standard curve was prepared using the lipid peroxidation (MDA) assay kit (Sigma-Aldrich) protocol. The MDA concentration was calculated according to the kit instructions and percentage inhibition of MDA formation was calculated using the formula shown below.

$$\% \text{ MDA inhibition} = (C_{\text{MDAC}} - C_{\text{MDAS}}) / C_{\text{MDAC}} \times 100 \quad (3.2)$$

Where, C_{MDAC} and C_{MDAS} represent the MDA concentration in the control sample (without inhibitor) and the sample with the inhibitor, respectively.

3.3.4 sPLA₂ inhibition assay

The method described by Alasmary *et al.* (2017) was followed to investigate the inhibitory activity of the triterpenes against sPLA₂. For this experiment, Varespladib (positive control), ARM-2 and RA-5 working solutions were 25 µM and 50 µM. The salt mixture consisted of 100

mM NaCl, 10 mM CaCl₂ and 3mM NaTDC (Sodium taurodeoxycholate) at final concentrations, made up in 100 ml with Tris buffer (1 mM, pH 8). The stock solutions of phosphatidylcholine (10 mM, dissolved in absolute ethanol) and phenol red (1000 µM, dissolved in dH₂O) were prepared separately. The concentration of the working enzyme solution (400 µg/µL) was made up with distilled water. Lastly, a 20 mM Tris buffer (pH 7.9) was prepared. The reaction mixture was prepared fresh for every experiment and consisted of 0.055 mM phenol red and the salt mixture.

A volume of 5 µL of enzyme (sPLA₂), 5 µL Tris buffer (20 mM), and 10 µL of test compound (25 and 50 µM) was added to 170 µL of reaction mixture. The mixture was pre-incubated at room temperature (10 min). After incubation, the reaction began through the addition of 20 µL phosphatidylcholine (10 mM, final concentration) and the kinetics of its hydrolysis was carried out by measuring the optical density (OD) at 558 nm for 10 min at 1 min intervals using the SpectraMax Paradigm Microplate Reader. The blank contained 3 µL of Tris buffer (20 mM) in place of the test compound and enzyme, the rest was made up with the reaction mixture. The negative control was made up of buffer in the place of the test compound. The percentage enzyme inhibition was calculated using the formula below.

True absorbance (TA) = Abs_{Blank} - Abs_{Sample}

$$\% \text{ Inhibition} = (TA_{\text{negative}} - TA_{\text{sample}}) / TA_{\text{negative}} \times 100 \quad (3.3)$$

Where, TA_{negative} and TA_{sample} represent the true absorbance of the control sample and the sample with the inhibitor, respectively.

3.4 *In vitro* antiatherogenic activity of the compounds in RAW 264.7 cells

3.4.1 Preparation of oxidized LDL

The copper mediated oxidation of LDL method described by Kim *et al.* (2020) was revised with minor alterations to also prepare the oxidized LDL for use in the cell culture studies. The native LDL (5 mg protein/mL) was mixed and incubated with 250 µM of copper sulphate (prepared in a 10 mM potassium phosphate buffer pH 7.4) for 20 hours at 37°C. This was followed by the addition, to the reaction mixture, of an equal volume (1:1) of 0.5 mM of EDTA to terminate the reaction (oxidation). The LDL oxidation, indicated by lipid peroxidation, was confirmed by determining the thiobarbituric acid-reactive substance (TBARS) content as described in 3.3.3.2. The oxidized LDL (oxLDL) was dialysed in potassium phosphate buffer (10 mM, pH 7.4) for 24 hours at 4°C and its concentration was determined using Nanodrop ND-1000 Spectrophotometer (Marshall Scientific, New Hampshire, United States of America).

3.4.2 Preparation of test compounds

A stock solution (100 mM) of each test compound was prepared in 100% DMSO and was further diluted with 1X PBS to a 1000 μ M stock and stored at -80°C until required for use. The desired working solutions were prepared from the 1000 μ M stock solutions in 1X PBS to a final volume of 1000 μ L. The compounds were plated at their final concentrations.

3.4.3 Cell culture and maintenance

Murine macrophage RAW 264.7 cells were cultured and preserved by adhering to standard practices and techniques (Segeritz and Vallier, 2017). The cells were cultured in Dulbecco's modified minimal essential medium (DMEM) alongside 10% (v/v) of fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin). Standard cell culture maintenance was followed (incubation at 37°C with 5% CO₂ and 95% humidity) until the cells were 70-80 % confluent.

3.4.4 Assessment of cytotoxicity of the compounds on RAW 264.7 cell line

The crystal violet assay was adapted to determine the cytotoxic potential of the plant compounds in RAW 264.7 cells. The crystal violet stain binds to the DNA and proteins present in the cells, where the quantification of DNA correlates to the viability of healthy cells. The cells were cultured as in 3.4.2 and were seeded at 5×10^4 cells/mL in each well. The cells were then exposed to different concentrations (1, 10 and 25 μ M) of the compounds and lovastatin (1 μ M; reference drug). While the normal control cells were exposed to an equivalent volume of PBS only, 1% DMSO and Triton X were used as negative and positive controls respectively. Another set of cells was exposed to oxLDL (50 μ g/mL) in the presence and absence of the test compounds at the different concentrations. The control cells were exposed to oxLDL (50 μ g/mL) in the presence and absence of 1% DMSO. A blank control group (normal control) consisted of only the cells and PBS. All the cells were then incubated for 48 hours (37°C with 5% CO₂ and 95% humidity). Cells were fixed with a final concentration of 2% formaldehyde for 1 hour at 37°C. They were then stained with crystal violet (0.1% w/v) for 30 minutes at room temperature. Background staining was removed by rinsing the cells three times under running water. A 1% final concentration of acetic acid was added to the cells to extract the crystal violet dye for quantitative analysis. The cell viability was determined by measuring absorbance at 630 nm using the FLUO star Omega microplate reader (BMG Lab Tech, Ortenberg, Germany).

3.4.5 Intracellular lipid content and foam cell formation in RAW264.7 cells

The method described by Wang *et al.* (2015) was followed to investigate the inhibitory effect of the plant compounds against oxLDL-induced foam cell formation. Briefly, the RAW 264.7 cells were seeded in at 5×10^4 cells/mL in each well. They were then exposed to 50 $\mu\text{g/mL}$ of oxLDL in the presence and absence of the plant compounds at various concentrations (1, 10 and 25 μM). Lovastatin (1 μM) was used as a reference drug. The model control cells were exposed to oxLDL (50 $\mu\text{g/mL}$) in the presence of 1% DMSO and Triton X as negative and positive controls, respectively. The normal control cells (not exposed to oxLDL) were exposed to an equivalent volume of PBS only. All the cells were then incubated for 48 hours (37°C with 5% CO_2 and 95% humidity). After the incubation phase, the cells were fixed with a final concentration of 2% formaldehyde for 1 hour at 37°C. The Oil Red O staining method described by Theerakittayakorn and Bunprasert (2011), was followed to determine the amount of intracellular accumulation. Briefly, the cells were then stained with Oil Red O (0.3 mg/mL) for 30 minutes at room temperature. Background staining was eliminated by rinsing the cells three times with PBS (Theerakittayakorn and Bunprasert, 2011). Foam cell formation (Intracellular lipid droplets) was observed under the Olympus IX71 microscope (Olympus Life Science, Massachusetts, United States of America). A 6% final concentration of isopropanol was added to the cells to extract oil red o stain for quantitative analysis and the extent of intracellular lipid accumulation was quantified by measuring absorbance at 405 nm. The cells were also stained with Nile red (1 μM) and were left to incubate in the dark at room temperature (3 hours), followed by fluorescence reading at 544/590 with a 1300 gain. The fluorescence and absorbance were measured using the FLUO star Omega microplate reader.

3.4.6 Real-Time Quantitative Reverse-Transcriptase PCR Analysis

Following treatment of the cells (RAW 264.7) as described in section 2.3.3, the mRNA levels of cholesterol trafficking related genes (ACAT-1, CD36, ABCA-1, and LOX-1) were investigated by real-time quantitative PCR (Wang *et al.*, 2015). Total RNA extraction from the cells was carried out through the usage of the SV Total RNA Isolation kit (Promega) and reverse transcriptase synthesis was done using SensiFast cDNA synthesis kit (Meridian Bioscience) following manufacturer's instructions. Quantitative PCR (qPCR) analysis was carried out through the use of synthesised cDNA and GoTaq qPCR Master Mix kit (Promega) according to the manufacturer's instructions. Every real-time PCR reaction consisted of a 1 μL cDNA (1 μg , final concentration) template, GoTaq qPCR Master Mix (10 μL), forward (200 nM, final concentration) and reverse (200 nM, final concentration) primer each. The PCR protocol consisted of an initial denaturation of 2 min at 95°C subsequently, 40 cycles of denaturation for 15 s at 95°C, annealing and extension for 1 min at 60°C. The samples were

measured using the BioRad CFX96 Real-Time PCR Detection System (BioRad, California, United States of America) and the Bio-Rad CFX Maestro software was used to analyse the results. The cycle threshold (CT) was calculated through the default setting (real-time sequence detection software). The gene-specific primers (Inqaba Biotech) indicated in Table 1 were utilized for the qPCR and were created from the online server, BLAST NCBI, by the National Institute of Health (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). All mRNA expression levels were set to relative quantity and the obtained data was expressed as a fold-change.

Table 1: Gene primers for some of the proteins involved in cholesterol trafficking

Protein/Enzyme	Forward Sequence (5' → 3')	Reverse Sequence (5' → 3')
ABCA-1	GGGGGAAAGAGGGAGAGAACA	CGACACTGTCAGGACGACCA
ACAT-1	GACGTCGCAAGCTCTGCGGTGG	CTTCTTGGTAGTAATTTTACACCCAAC
CD-36	TCAGAGACCTATATTTATCTCCTAGTAGGC	CACCCTTTGCTACAGTAATTTTAGTCTAAA
LOX-1	GTTGCTTATTCATTCAGAGTGGG	GGAGGACGGACCGTTTCGATG
β-actin	CAGGTCATCACTATTGGCAA	AGGTCTTTACGGATGTCAAC

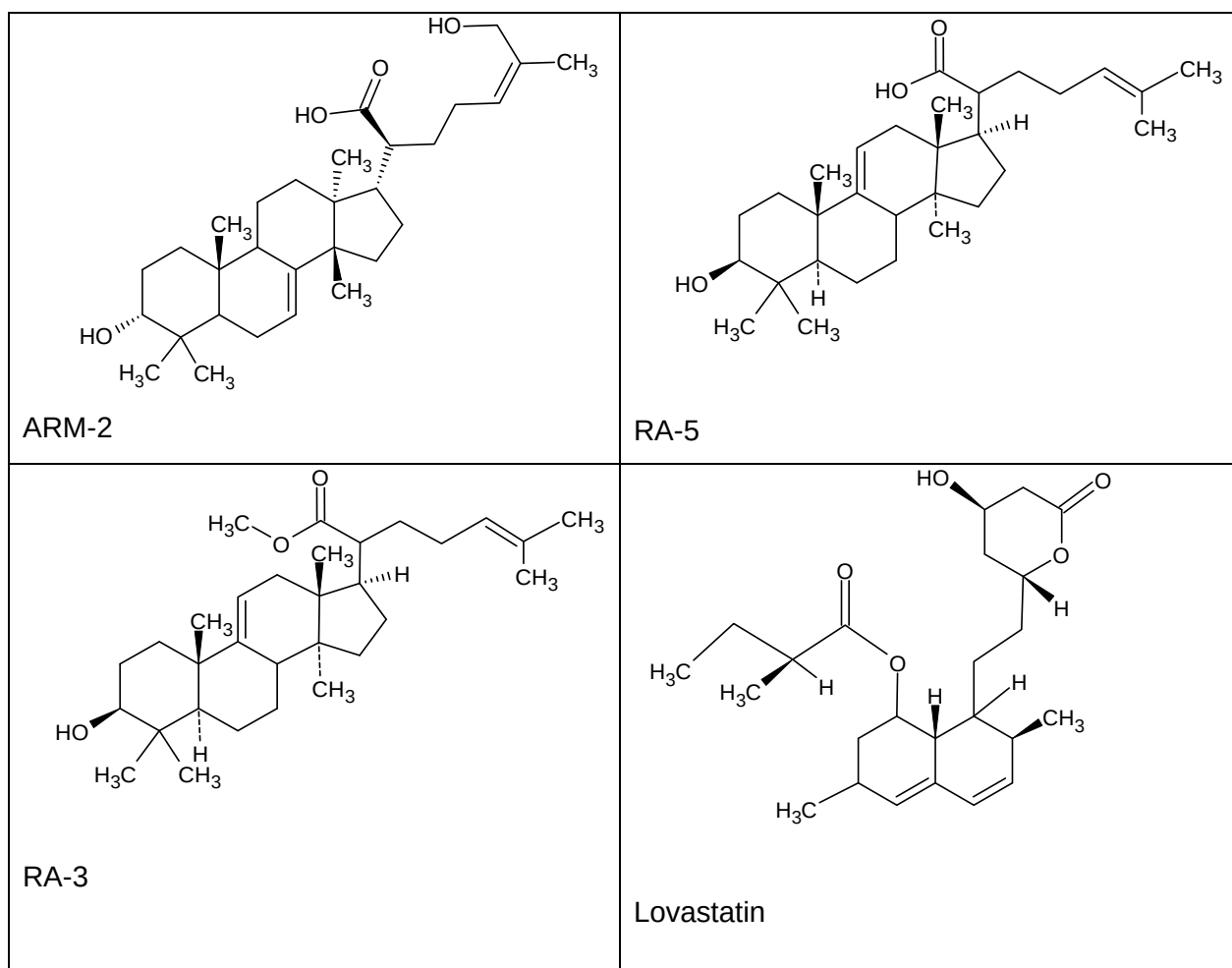
3.5 Data analysis

Unless stated otherwise, each of the individual experiments was carried out in triplicate for at least three independent biological repeats. The results obtained were expressed as mean ± standard error mean (SEM) unless stated otherwise. The results were further studied by ANOVA followed by Dunett's multiple comparisons test utilizing GraphPad Prism version 9 (V9). The statistical difference was deemed significant where $p < 0.05$.

4.0 Results

4.1 Chemical structures of the compounds/ligands used in the docking study

The compounds with the chemical structures shown in Figure 6 were used in the molecular docking study. While ARM-2 and RA-5 are the compounds of interest for the study, the other compounds were selected as reference compounds or known inhibitors of the targeted proteins (LOX-1, ACAT-1, CD-36, ABCA-1 and ABCG-1) involved in cellular cholesterol trafficking. See Appendix A for additional data.



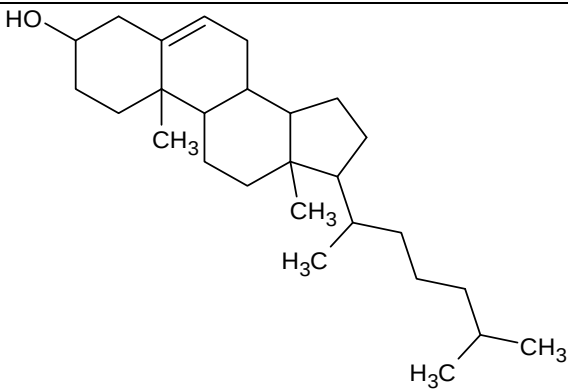
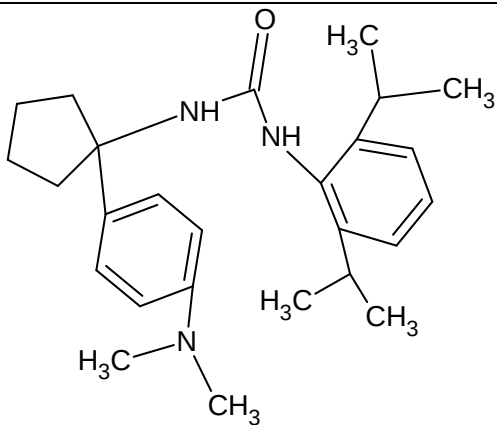
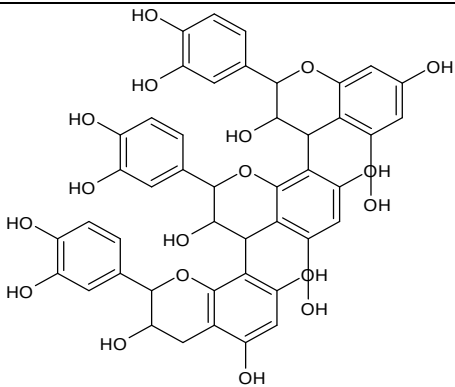
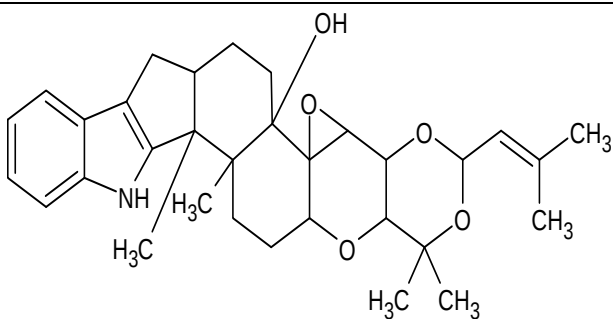
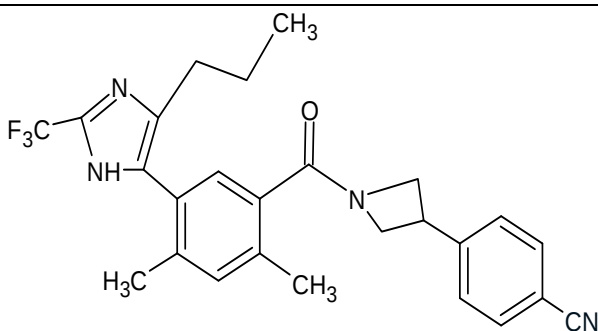
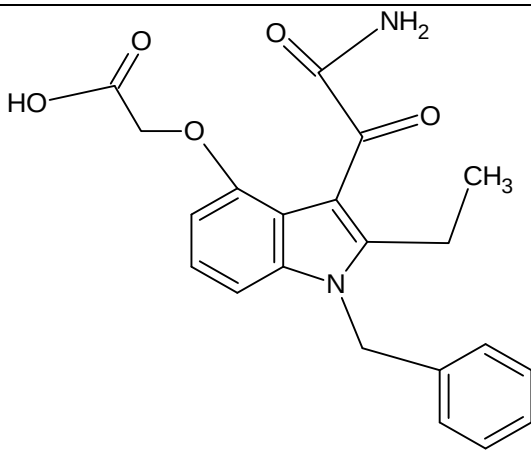
 Cholesterol	 Nevanimibe
 Procyanidin C1	 Terpendole C
 TVB-3664	 Varespladib

Figure 6: The chemical structures of the compounds used in the molecular docking of the target proteins.

4.2 Molecular Docking of the triterpenoids against some proteins involved in cellular cholesterol trafficking

Molecular docking is carried out to computationally determine a stable ligand-receptor complex between the compound and protein of choice. A good docking score predicts a good binding receptor (protein) ligand (compound) complex. ARM-2 and RA-5 can accept 4 and 3 hydrogens respectfully and both have carboxyl functional groups. In contrast to the triterpenes, lovastatin can accept 5 hydrogen bonds and has an ester functional group. Table 2 A and Table 2 B show the docking scores of the compounds against CD-36, SR-A1, ABCA-1, ABCG-1, LOX-1, ACAT-1 and sPLA₂, some proteins involved in cellular cholesterol trafficking. See Appendix A for more information. Low to moderate docking scores by the compounds were observed against CD-36, SR-A1, ABCA-1, ABCG-1 and sPLA₂. However, both triterpenes displayed high docking scores against ACAT-1. ARM-2 also showed a good docking score (-8.40) against LOX-1.

Table 2 A: Molecular docking scores (XP score) and glide energies (kJ/mol) of the plant triterpenes (ARM-2 and RA-5) on some target proteins involved in cellular cholesterol trafficking.

Ligand/Inhibitor	CD-36		SR-A1		ABCA-1		ABCG-1	
	Score	Energy	Score	Energy	Score	Energy	Score	Energy
#Lovastatin	-3.54	-29.07	-4.38	-30.73	-5.62	-34.90	-7.49	-27.67
ARM-2	-3.19	-26.68	-4.13	-28.93	-4.83	-36.59	-7.12	-11.86
RA-5	-3.02	-23.58	-4.17	-25.18	-4.29	-30.24	-7.82	-14.95
RA-3	-2.54	-22.77	-3.20	-24.45	-4.89	-33.70	-8.89	-18.63
Cholesterol	-2.62	-24.55	-3.06	-26.20	-5.28	-28.05	-9.36	-28.33
*TVB-3664	-3.57	-34.04						
*Nevanimibe					-5.96	-37.59	-8.49	-42.66

#reference drug; * inhibitor

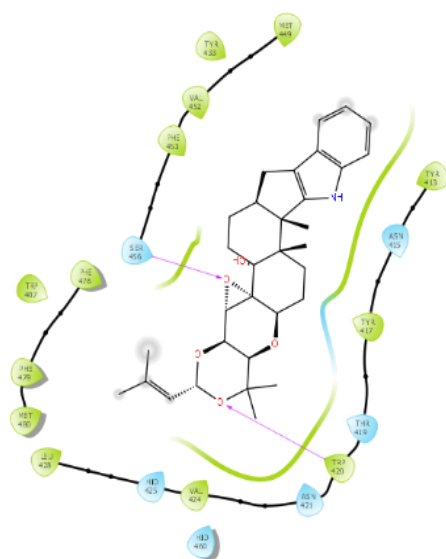
Table 2 B: Molecular docking scores (XP score) and glide energies (kJ/mol) of the plant triterpenes (ARM-2 and RA-5) on some target proteins involved in cellular cholesterol trafficking.

Ligand/Inhibitor	LOX-1		ACAT-1		sPLA ₂	
	Score	Energy	Score	Energy	Score	Energy
#Lovastatin	-7.79	-41.11	-10.10	-43.06	-8.34	-42.35
ARM-2	-8.40	-34.67	-11.40	-47.01	-5.77	-37.89
RA-5	-6.23	-37.83	-9.62	-36.65	-3.46	-24.95
RA3	-6.67	-32.82	-8.41	-38.66	-4.30	-31.22
Cholesterol	-8.07	-34.47	-8.46	-33.83	-5.90	-30.17
*Procyanidin C1	-14.85	-67.36				
*Terpendole C			-10.18	-49.93		
*Varespladib					-10.27	-58.18

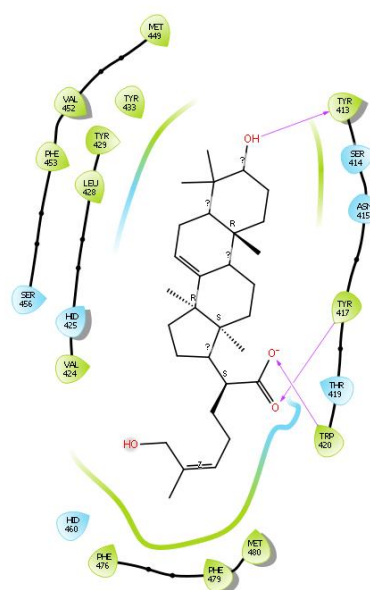
#Reference drug; * inhibitor

Figures 7 and 8 show the molecular docking interactions between the triterpenes (ARM-2 and RA-5) and ACAT-1 and sPLA₂ respectively. See Appendix A for additional data. A strong binding affinity is predicted by a high number of hydrogen bonds (H-bonds). In Figure 7, terpendole C can accept 5 H-bonds, and ARM-2 and RA-5 can accept 4 and 3 H-bonds, respectively. ARM-2 donated 1 hydrogen bond and accepted 2 from ACAT-1 in comparison to terpendole C and RA-5 which only accepted 2 hydrogen bonds. This indicates the increase in ligand-receptor stability between ARM-2 and ACAT-1.

A



B



C

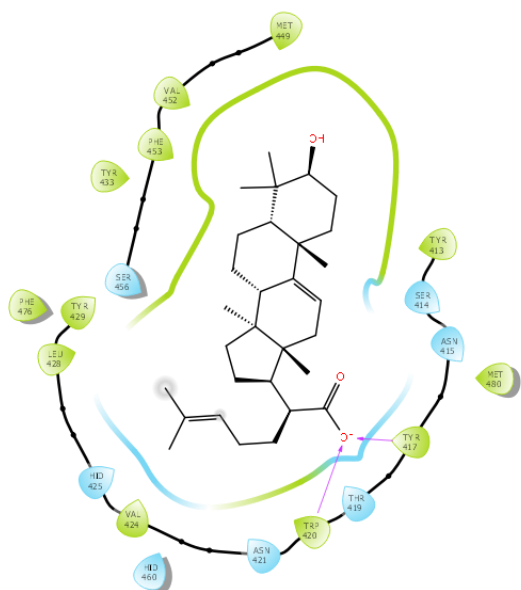
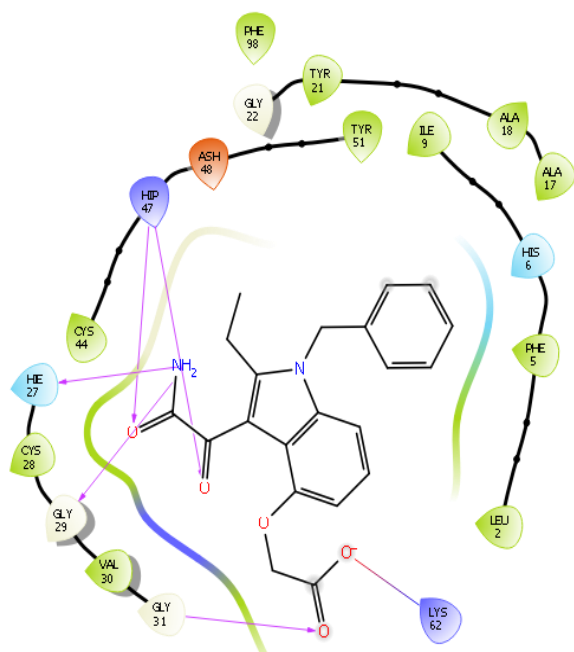


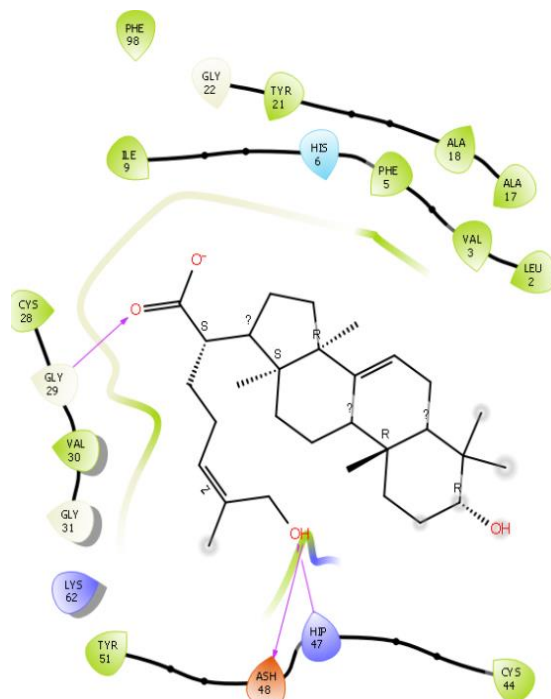
Figure 7: Molecular docking interactions (hydrogen bonding) between ACAT-1 and (A) Terpendole C, (B) ARM-2 (C) RA-5

In Figure 8, the number of H-bonds varespladib can accept is 5 and it was able to accept all 5 H-bonds in comparison to ARM-2 and RA-5. The inability of the compounds to accept the maximum number of H-bonds indicated possible decrease in the ligand-receptor stability.

A



B



C

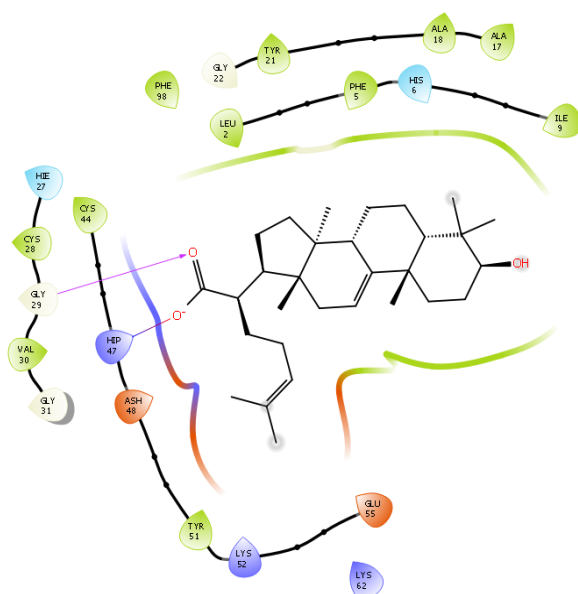


Figure 8: Molecular docking interactions (hydrogen bonding) between sPLA₂ and (A) Varespladib, (B) ARM-2 (C) RA-5. HIE and ASP are ligands found on the protein.

4.3 ADMET properties of ARM-2 and RA-5

ADMET properties of ARM-2 and RA-5 were determined using the online webserver, pkCSM. The results presented in Table 3 (See Appendix A for additional data) predict that despite the water insoluble nature of the two plant triterpenes, both compounds have high human intestinal absorption potential with 95% and 94% absorbability for ARM-2 and RA-5, respectively. These results are similar to those observed for lovastatin. The physiological distribution properties of the triterpenes, predicted by the blood-brain barrier (BBB) and central nervous system (CNS) permeability, showed no permeability by both compounds. Additionally, both ARM-2 and RA-5 were predicted to be substrates and non-inhibitors of CYP3A4. The compounds were also predicted to not be substrates for renal OCT2. However, both compounds were predicted to have potential hepatotoxicity.

Table 3: The ADMET properties of the plant triterpenoids and lovastatin

Property	Model Name	Predicted Value for Lovastatin	Predicted Value for ARM-2	Predicted Value for RA-5	Reference
Absorption	Water solubility (log mol/L)	-4.74	-4.11	-4.45	>0 soluble <-4 insoluble
	Intestinal absorption (human) (% Absorbed)	95.96	95.28	93.64	<30% poor absorption
	Skin Permeability (log Kp)	-3.38	-2.73	-2.74	>-2.5 low skin permeability
Distribution	BBB permeability (log BB)	-0.38	-0.70	-0.40	>0.3 permeability <-1 no permeability
	CNS permeability (log PS)	-2.84	-1.63	-1.27	>-2 permeability <-3 no permeability
Metabolism	CYP3A4 substrate	Yes	Yes	Yes	Yes/No
	CYP3A4 inhibitor	Yes	No	No	Yes/No
Excretion	Total Clearance (log ml/min/kg)	0.93	0.40	0.32	>1 high
	Renal organic cation transporter 2 (OCT2) substrate	No	No	No	Yes/No
Toxicity	Hepatotoxicity	No	Yes	Yes	Yes/No
	Skin Sensitisation	No	No	No	Yes/No

4.4 Drug-Likeness and Target prediction of the plant triterpenoids

The drug-likeness and target prediction of the plant compounds were both determined using the SWISS-ADME online webserver. The drug-likeness of compounds is defined by their physiochemical properties and medicinal chemistry. Both ARM-2 and RA-5 obey Lipinski's rule of five. No PAINS alerts were predicted for both compounds and lovastatin, used as a reference drug (Table 4). RA-5 showed the highest oral bioavailability score (0.85), in

comparison to ARM-2 with a score similar to that of lovastatin (0.55). See Appendix A for additional data.

Table 4: Physiochemical and medicinal chemistry properties of the plant triterpenoids and lovastatin

Compounds	TPSA (Å²)	Lipinski	PAINS	Oral Bioavailability Score	Synthetic Accessibility
Lovastatin	72.83	Yes	0 alert	0.55	5.76
ARM-2	77.76	Yes*	0 alert	0.56	6.13
RA-5	57.53	Yes*	0 alert	0.85	6.02

*1 violation: MLOGP>4.15

The SwissTargetPrediction was used to predict the potential protein targets of the plant compounds. The potential interaction between the compound and its potential protein target is expressed as a probability. The closer the value is to 1, the more likely that an interaction will occur. As shown in Table 5, both triterpenoids (ARM-2 and RA-5) and lovastatin were predicted to have no interaction with phospholipase A2 group IIA. Low or no probability scores were predicted for both ARM-2 and RA-5 against cyclooxygenase-2, the cytochromes, nitric oxide synthase, phospholipase A2 group 1B and HMG-CoA reductase. Despite its predicted interaction with HMG-CoA reductase, Lovastatin showed a similar pattern as the plant triterpenoids. Unlike ARM-2 (0.25) and lovastatin (0), RA-5 displayed a higher probability (0.59) of interacting with prostaglandin E synthase.

Table 5: Some of the SwissTarget Predictions indicated by probability scores of the triterpenoids and Lovastatin

Target protein	Lovastatin	ARM-2	RA-5
Cytochrome P450 19A1	No hit	0.16	0.12
Cytochrome P450 51	No hit	0.12	0.13
Cytochrome P450 2C19	0	0.12	0.10
Nitric oxide synthase	No hit	0.15	0.10
Phospholipase A ₂ group 1B	No hit	0.12	0.13
Prostaglandin E synthase	0	0.25	0.59
Phospholipase A ₂ group IIA	No hit	No hit	No hit
Cyclooxygenase-1	No hit	No hit	0.10
Cyclooxygenase-2	0	0.12	0.10
HMG-CoA reductase	0.85	0.12	0.12

No hit: data not obtained.

4.5 Antioxidant activity of the compounds *in vitro*

4.5.1 Free radical scavenging activity and reducing potential

The free radical scavenging activity and reducing potential of the compounds (ARM-2 and RA-5) were determined on ABTS and DPPH radicals, and ferric ions respectively. Both compounds showed poor reducing power and free radical scavenging activity as they were unable to scavenge ABTS and DPPH radicals and reduce ferric ions. In contrast, the reference standard (ascorbic acid) was able to reduce ferric ions and scavenge ABTS and radicals in a concentration dependent manner (Figure 9).

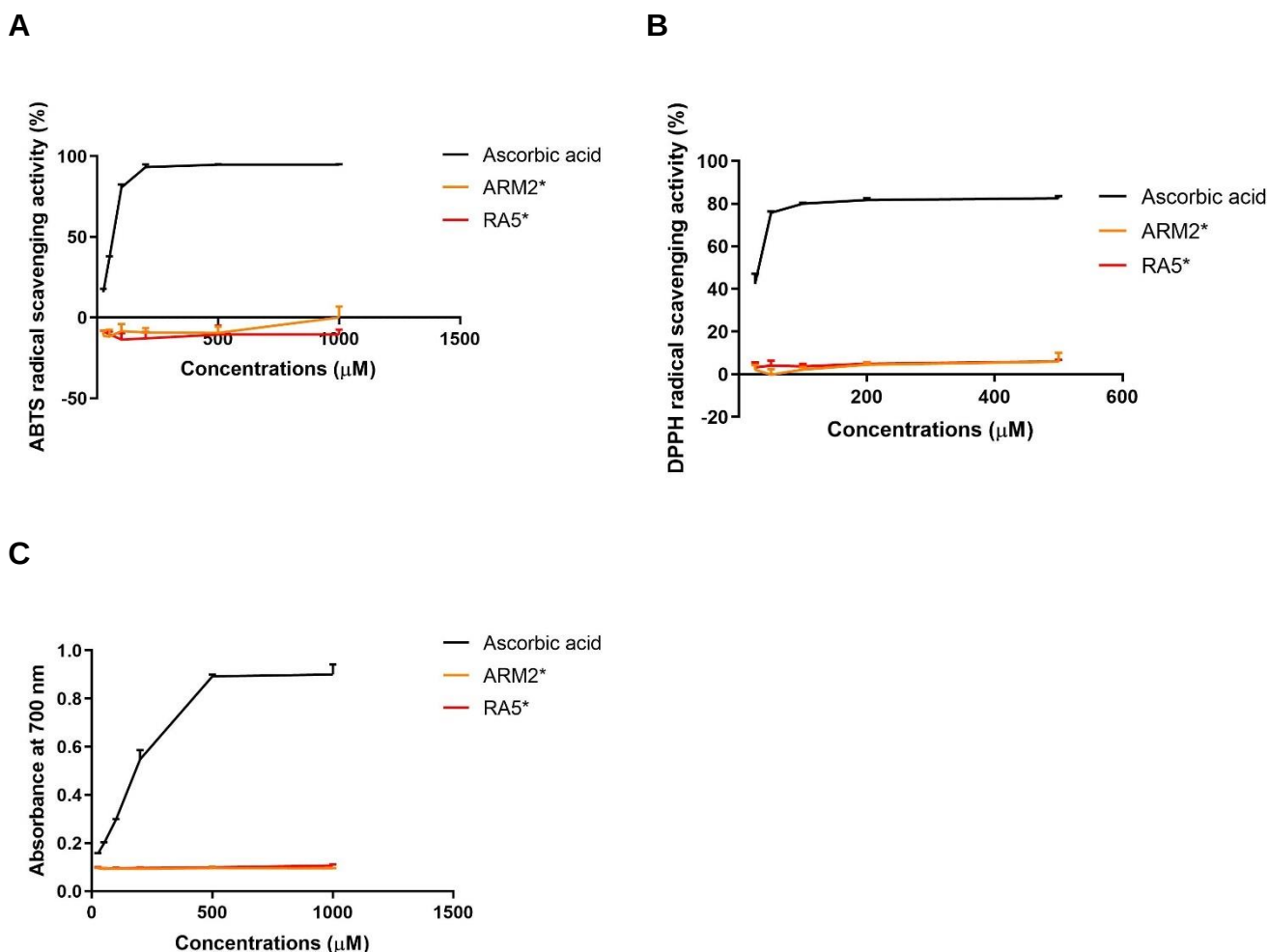


Figure 9: Free radical scavenging activity and reducing potential of the triterpenes on (A) ABTS and (B) DPPH radicals, and (C) ferric ions, respectively. AA was used as the reference standard in the experiments. The data is expressed as the mean $\pm$ SEM of three independent repeats in duplicate ($n=3$), $*p < 0.0001$ vs ascorbic acid.

4.5.2 Oxygen radical absorbance capacity of ARM-2 and RA-5

The ORAC of the compounds was determined and the results are shown in Figure 10 and Table 6. A decrease in fluorescence is directly proportional to the antioxidant capacity/potential of a compound. Both compounds showed oxygen radical absorbance capacity in a concentration dependent manner. ARM-2 and RA-5 indicated the best delayed fluorescent decay at 1000 μM in a manner similar to Trolox (Figure 10). This was further supported by their high ORAC values (Table 6), with ARM-2 displaying better ORAC when compared to RA-5.

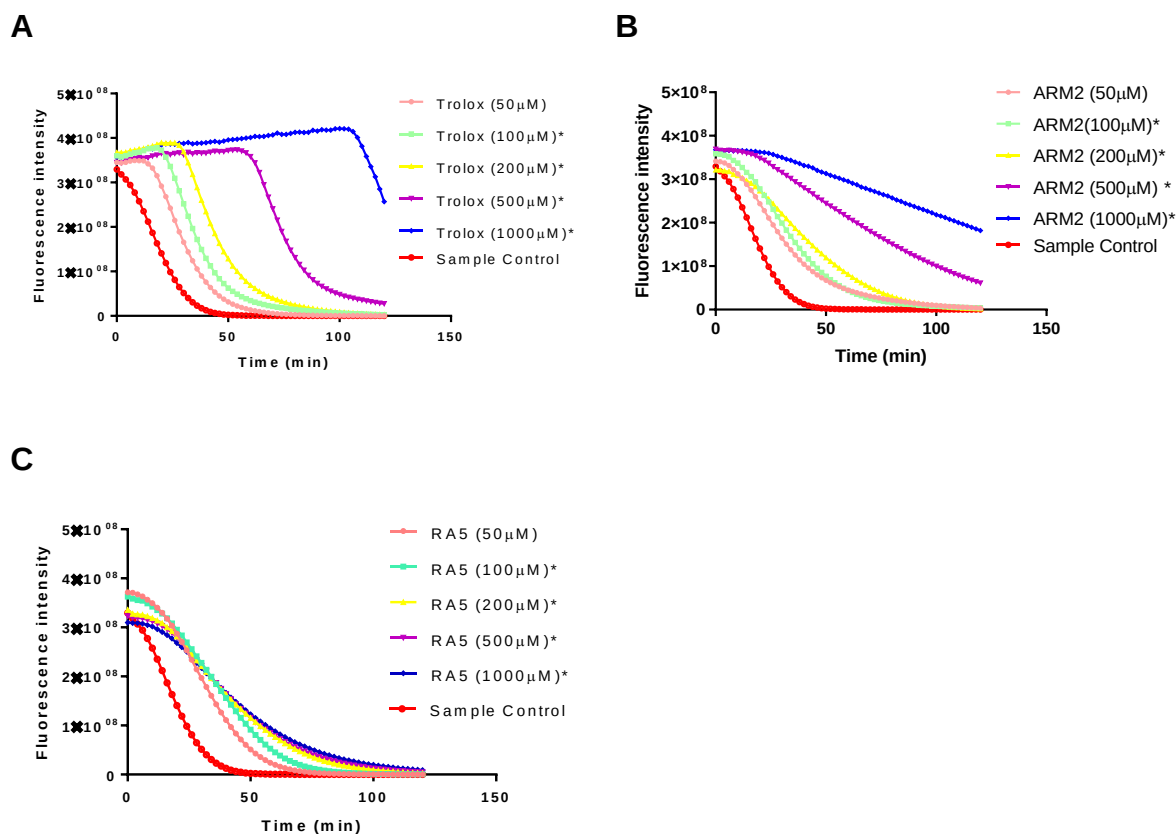


Figure 10: Oxygen radical absorbance capacity of the plant compounds. *Fluorescence decaying curves of fluorescein in the presence of (A) Trolox, (B) ARM-2 and (C) RA-5, were monitored at Ex 485 nm and Em 520 nm every 2 minutes for 2 hours and 30 minutes. The data is expressed as the mean $\pm$ SEM of four independent repeats in triplicate (n=4), where * $p < 0.001$.*

Table 6: Table indicating the ORAC values ($\mu\text{M TE}$) of ARM-2 and RA-5 at different concentrations

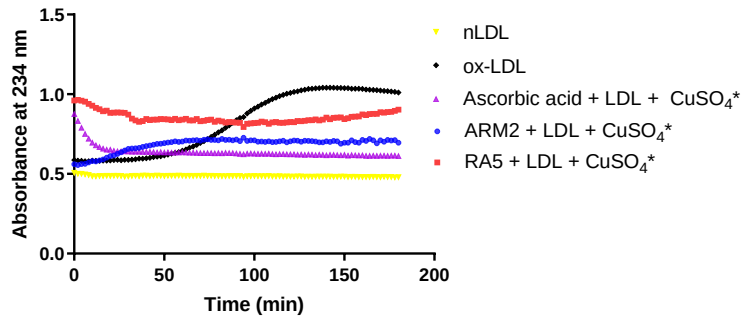
Concentration (μM)	ORAC Value ($\mu\text{M TE}$)	
	ARM-2	RA-5
50	65.85 $\pm$ 39.47	41.50 $\pm$ 8.89
100	86.90 $\pm$ 31.84	81.44 $\pm$ 21.85
200	133.34 $\pm$ 47.81	128.69 $\pm$ 47.02
500	332.12 $\pm$ 108.62	172.22 $\pm$ 55.73
1000	516.12 $\pm$ 156.74	206.69 $\pm$ 64.45

The data is expressed as the mean $\pm$ SEM of four independent repeats in triplicate (n=4).

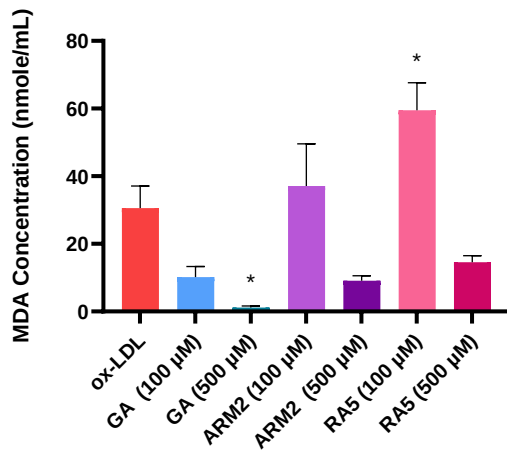
4.6 Inhibition of LDL oxidation

The measurement of conjugated dienes (CDs) and determination of TBARS content is a common method used to determine lipid peroxidation of lipoproteins *in vitro*. The antioxidant activity of the triterpenes was further established by evaluating their lipid peroxidation inhibitory activity in CuSO₄ induced LDL (ox-LDL) and HDL (ox-HDL) oxidation. A significantly higher CD formation with an increased lag phase was observed in the LDL exposed to CuSO₄ only in comparison to the native LDL (nLDL). However, there was a significant decrease in CD formation in the LDL exposed to CuSO₄ in the presence of ARM-2 and RA-5, respectively (Figure 11A). Ascorbic acid (AA, positive control) displayed results similar to nLDL. The LDL oxidation inhibitory activity of the compounds was further supported by the observed significantly lower MDA concentration in the presence of the triterpenes at 500 µM (Figure 11B) when compared to the control (ox-LDL only). The compounds inhibited MDA formation by up to 62% and 45% for ARM-2 and RA-5 respectively (Figure 11C). However, this was less comparable to the inhibitory activity exhibited by gallic acid (85%) (Figure 11C). Surprisingly, HDL treated with the compounds did not indicate any noteworthy inhibition of MDA formation (Figure 11E) which was indicated by the high MDA concentrations observed (Figure 11D).

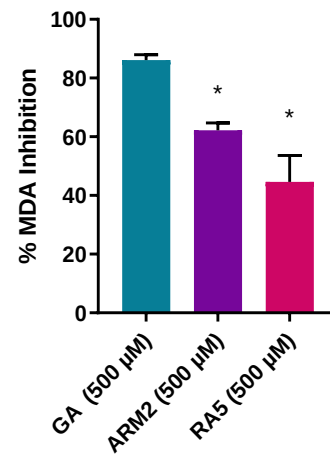
A



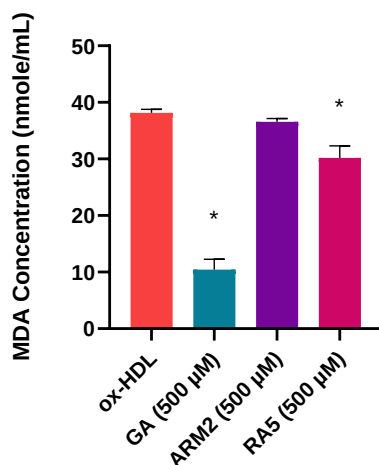
B



C



D



E

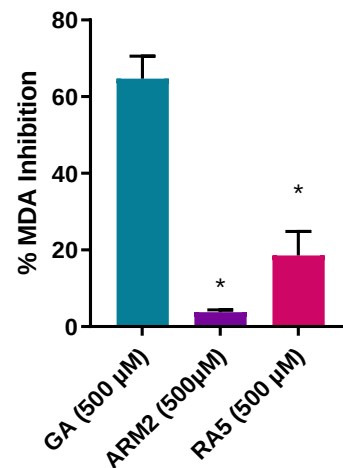


Figure 11: Inhibitory effect of the triterpenes on copper sulphate mediated LDL and HDL lipid oxidation. (A) The diene formation of LDL in the presence and absence of the plant compounds at 500 µM. (B & D) The TBARS (MDA) production of LDL (or HDL) in the presence and absence of the plant compounds. (C & E) % MDA inhibitory activity of the compounds. Ascorbic acid (AA) and gallic acid (GA) were used as positive controls. The data was expressed as the mean $\pm$ SEM of three independent repeats in duplicate ($n=3$), $*p < 0.0001$ with regards to oxLDL or oxHDL.

4.7 Inhibitory effect of the triterpenes on sPLA₂

The enzyme inhibitory activity of the compounds was screened on secretory phospholipase A₂ (sPLA₂), an enzyme known to enhance the inflammatory response by converting phospholipids into arachidonic acid. While varespladib (used as positive control) at the highest concentration of 50 μ M, exhibited up to 63 % inhibition of the enzyme, no enzyme inhibitory activity was observed in the presence of both plant triterpenes at the tested concentrations (Table 7).

Table 7: sPLA₂ (secretory phospholipase A₂) inhibition of the plant compounds ARM-2 and RA-5.

Compound	% Inhibition (25 μ M)	% Inhibition (50 μ M)
Varespladib	50 $\pm$ 5.01	63 $\pm$ 4.40
ARM-2	-48 $\pm$ -36.76	-36 $\pm$ -24.79
RA-5	-23 $\pm$ -27.63	-10 $\pm$ -32.37

The data is expressed as the mean $\pm$ SEM of three independent repeats in duplicate (n=3).

4.8 *In vitro* antiatherogenic activity of the compounds in RAW264.7 cells

4.8.1 Cell toxicity of the compounds on RAW264.7 cell line

The crystal violet assay was used to determine the cytotoxic potential of the plant compounds in RAW 264.7 cells. The crystal violet stain binds to the DNA of the cells subsequently quantifying DNA which correlates to the number of viable cells. Results revealed high cell viability in the presence of ARM-2 (1, 10 and 25 μ M), RA-5 (1, 10 μ M) and lovastatin (Figure 12A) when compared to the normal control cells (exposed to PBS only). However, a decrease (35%) in the cell viability was observed in the presence of RA-5 at 25 μ M. Triton X is a known cytotoxic agent to cells and indicated 30% cell viability. Interestingly, a high cell viability was also observed when the cells were exposed to oxLDL in the absence and presence of the triterpenoids or lovastatin (Figure 12B).

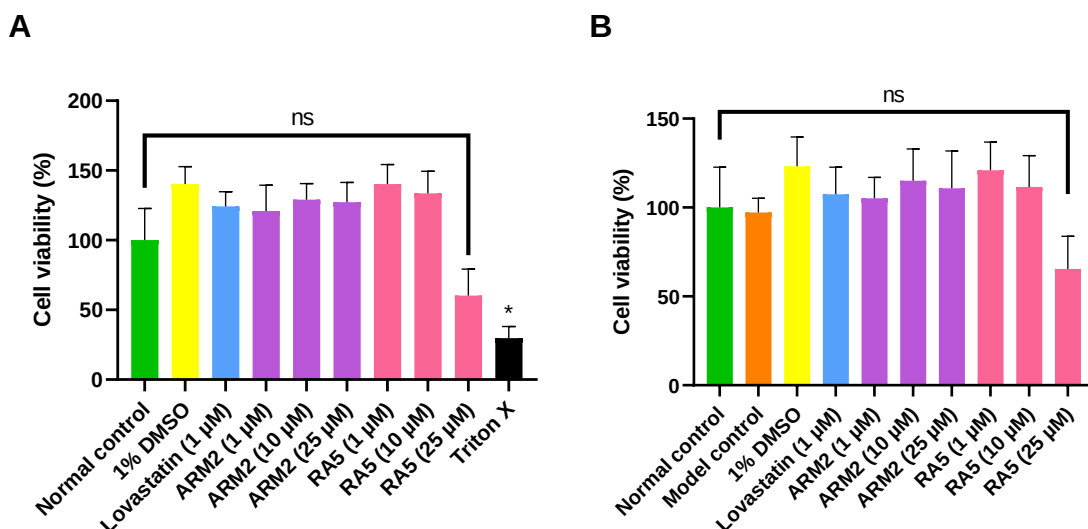


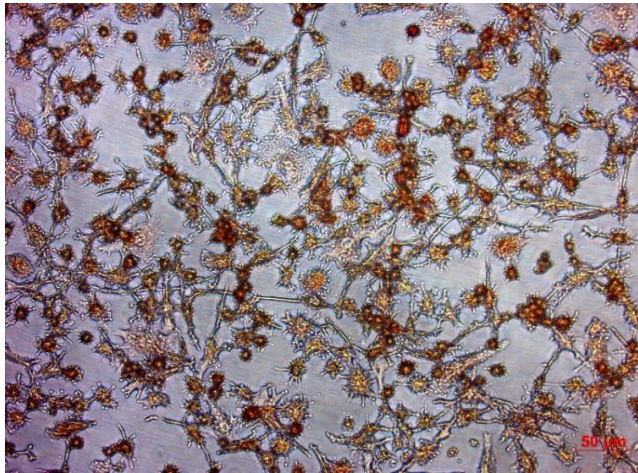
Figure 12: Cytotoxic potential of ARM-2 and RA-5 on the RAW264.7 cells. The cytotoxicity potential was determined by the crystal violet assay. Cells were exposed to (A) the test compounds (1-25 μ M) only and (B) oxLDL in the presence and absence of the test compounds (1-25 μ M). While lovastatin was used as a reference drug, DMSO (1%) and Triton X were used as negative and positive controls respectively. The data is expressed as the mean $\pm$ SEM of four independent repeats in triplicate ($n=4$). * $p < 0.0001$ with regards to normal control

4.8.2 Lipid content and foam cell formation

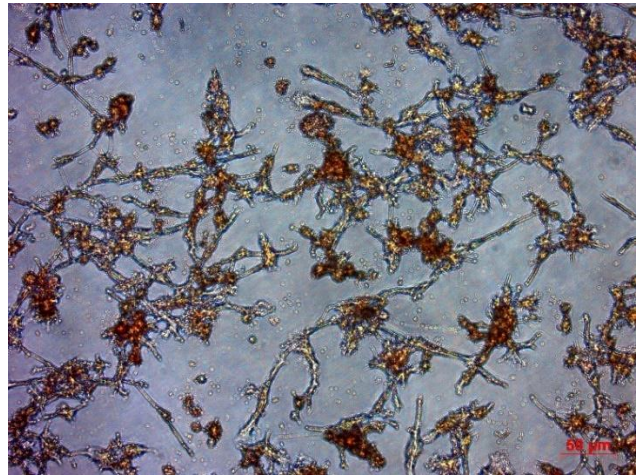
Oil red O and Nile red staining were used to quantify the amount of intracellular lipid accumulated in RAW264.7 cells following their exposure to oxLDL in the presence and absence of the triterpenoids (ARM-2 and RA-5). See Appendix B for additional data. An increase in the intracellular lipid content (100%) was observed in the cells exposed to oxLDL only. However, the cells exposed oxLDL and treated with ARM-2 or RA-5 showed a decrease in lipid droplets (foam cell formation) across all concentrations in comparison with the cells exposed to oxLDL only (Figure 13 A-D). The compounds ARM-2 (10 and 25 μ M) and RA-5 (1, 10 and 25 μ M) displayed a decrease in lipid content when stained with oil red O (Figure 13E) and Nile red, respectively (Figure 13F). The Oil red O data showed a lower intracellular lipid content of 88 and 90 % following exposure of the cells to ARM-2 (25 μ M) and RA-5 (10 μ M), respectively (Figure 13E). Similarly, the Nile Red results indicated 86 % lipid content for ARM-2 at 25 μ M and 90 % for RA-5 at 10 μ M (Figure 13F). The decreased lipid content for RA-5 at 25 μ M could also be a result of its weak cytotoxicity effect on the cells (Figure 13 E-F). However, the difference between the model control (cells exposed to oxLDL exposed only) and the test compounds was not statistically significant.

A oxLDL Treated/ Cells only

oxLDL (model control)

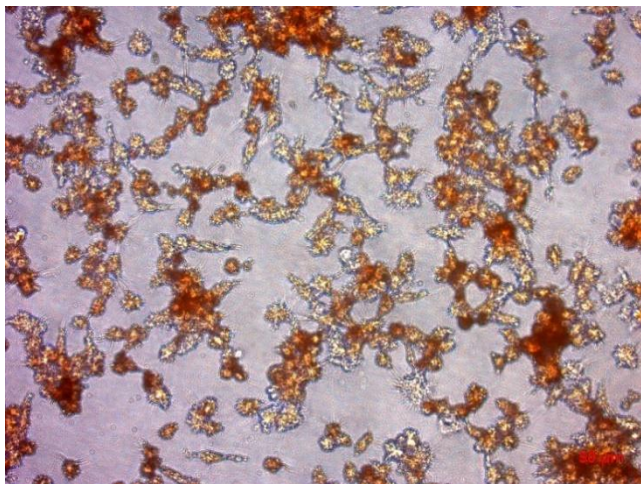


Normal control (cells + PBS)



B Compounds at 1 µM

oxLDL + 1 µM ARM-2

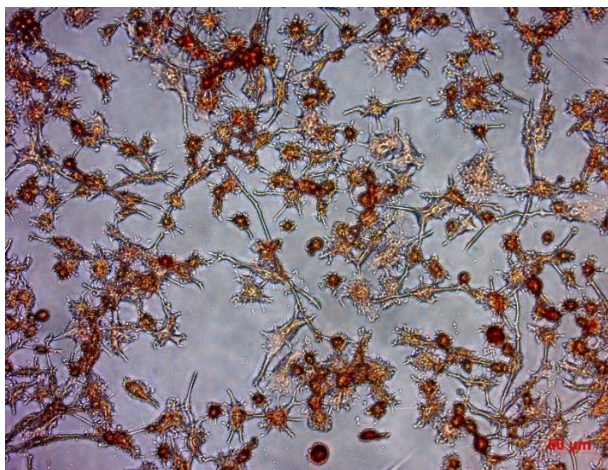


oxLDL + 1 µM RA-5

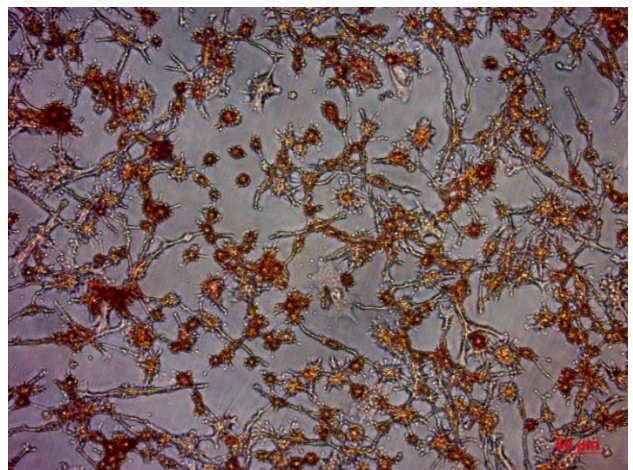


C Compounds at 10 µM

oxLDL + 10 µM ARM-2

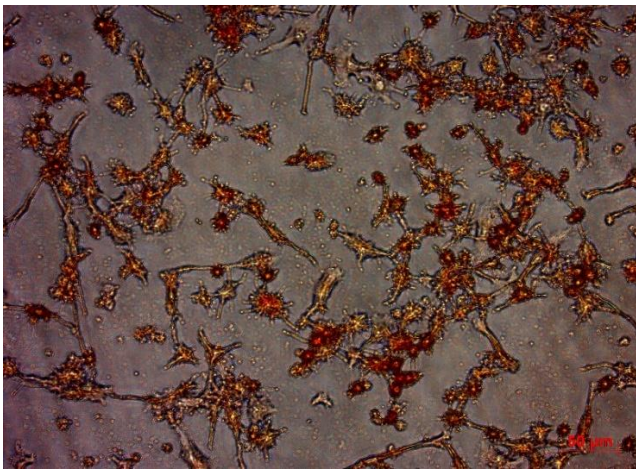


oxLDL + 10 µM RA-5

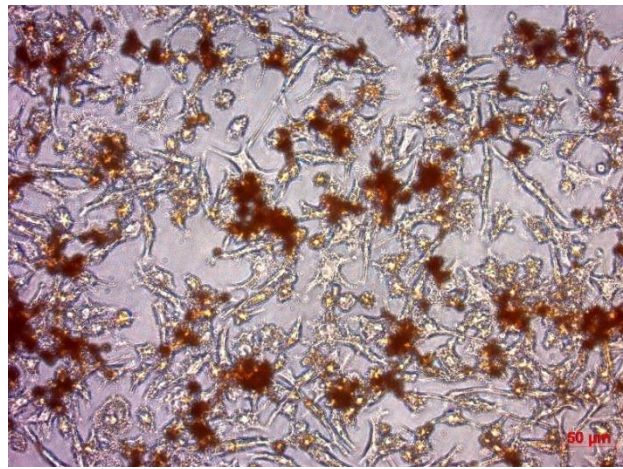


D Compounds at 25 μ M

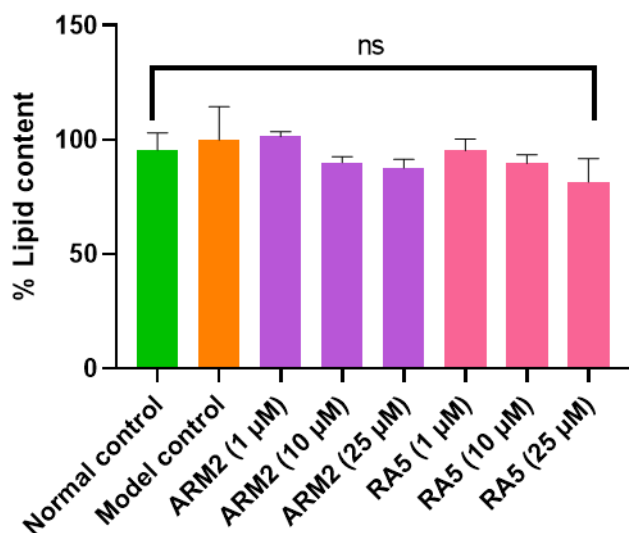
oxLDL + 25 μ M ARM-2



oxLDL + 25 μ M RA-5



E



F

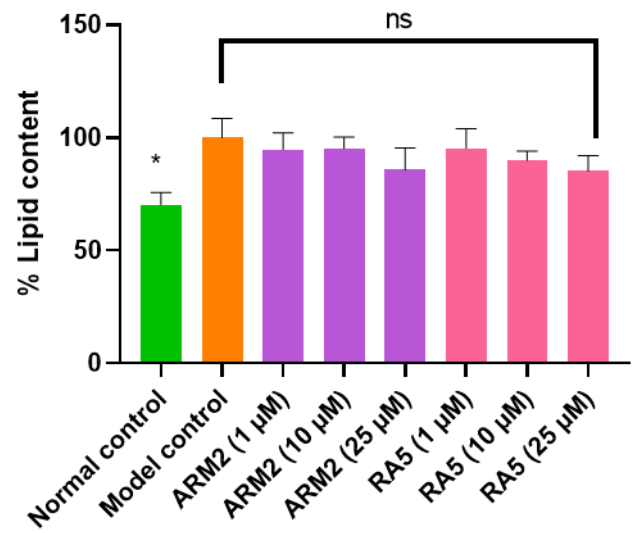


Figure 13: Effects of the triterpenes on lipid accumulation induced by oxLDL in RAW264.7 macrophage cells. (A-D) Images of oil-red O-stained lipid droplets (foam cell formation). Micrograph scale is 10 μ m (E) Lipid content of oil red o-stained cells measured at 405nm. (F) Lipid content of cells stained with Nile red, fluorescence measured at Ex 544nm and Em 590nm (1300 gain). The data is expressed as the mean $\pm$ SEM of four (oil red o, $n=4$) and three (Nile red, $n=3$) independent repeats in triplicate. * $p < 0.05$ with regards to model control.

4.8.3 Effect of the terpenes on the expression of some genes involved in cholesterol trafficking

The effect of the triterpenes on the mRNA levels of some cholesterol trafficking related genes (ACAT-1, CD36, ABCA-1, and LOX-1) was investigated through real-time quantitative PCR. This experiment had many inconsistencies to give conclusive results. As a result, only the effects of the oxLDL and RA-5 (at 10 μ M) on mRNA levels of ABCA-1 are reported in this study. As seen in Figure 14, high mRNA levels (1.6-fold) of the ABCA-1 gene were observed in the normal control cells in comparison to the cells exposed to 50 μ g/ml of oxLDL (1-fold). However, an increase in the mRNA levels of the ABCA-1 gene was observed in the cells treated with RA-5 and oxLDL. Relative to the cells exposed to the oxLDL only, RA-5 (at 10 μ M) showed a 0.29-fold increase in mRNA levels of the gene.

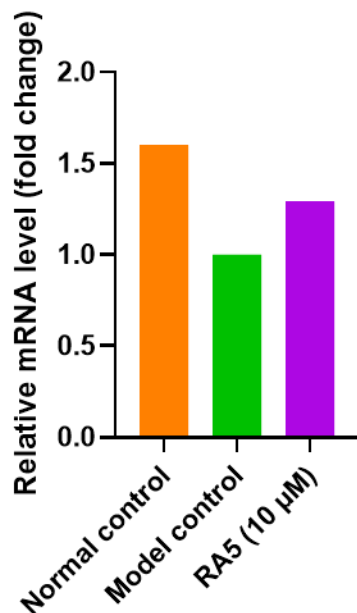


Figure 14: Effect of RA-5 on the ABCA-1 mRNA levels in RAW264.7 macrophage cells. *The cells were exposed to 50 μ g/ml of oxLDL (for 48 h at 37 $^{\circ}$ C) in the presence and absence of RA-5 at 10 μ M. The data is expressed as mean $\pm$ SD of one independent experiment (n=2).*

5.0 Discussion

Hypercholesterolemic effects linked to plasma cholesterol transport and modification of lipoproteins (LDL and HDL) increase the chances of atherosclerosis development. Due to the limitations associated with the current atherosclerosis treatment regime, the search for alternative drugs with improved efficacy and novel mechanisms of action continues. The wide chemical diversity in plants has fuelled an interest in searching for new plant-derived bioactive compounds with hypocholesterolemic and antiatherogenic properties. Literature has reported the atheroprotective properties of some plant compounds (Vahdat-Lasemi *et al.*, 2021). The triterpenoids (ARM-2 and RA-5) from the stem bark of *Protorhus longifolia* have also been investigated and studies show that they have bioactivities crucial to the amelioration of hypercholesterolemia. These include inhibition of HMGCR, upregulation of the expression of the LDL-R gene and downregulation of the expression of the HMGCR gene in hepatic cells (Ndlovu *et al.*, 2023). The current study aimed to discover, using both *in silico* and *in vitro* techniques, the potential of these triterpenoids to prevent oxidative modification of LDL as well as foam cell formation that underlies the development of atherosclerotic plaque.

5.1 Molecular docking of the compounds

The main objective of computational drug discovery approaches, known as virtual screening (VS), is to predict the biological interactions among compounds with drug-like properties and prospective target proteins for the discovery of new drugs. Molecular docking is carried out to assist in the prediction of the ligand receptor complex stability. In the current study, the plant triterpenoids were docked against enzymes and proteins involved in cholesterol trafficking (CD-36, SR-A1, ABCA-1, ABCG-1, LOX-1 and ACAT-1) and the inflammatory response (sPLA₂). Large negative docking scores and a high number of hydrogen bonds predict the potential the compounds have, to strongly bind with the enzyme/protein and inhibit its activity. The predicted inability of the compounds to potentially inhibit CD-36 and SR-A1, proteins crucial in the uptake of ox-LDL, suggests that the compounds may not inhibit the uptake of ox-LDL by macrophage cells. However, the predicted moderate binding affinity by the compounds against ABCA-1 and ABCG-1 suggests the potential of the compounds to mediate the release of free cholesterol from the macrophage cells. This is favourable as ABCA-1 plays a significant role in the maintenance of efflux of phospholipids and cellular cholesterol.

The low and high docking scores of the compounds are attributed to the number of hydrogen bonds (H-bonds) they share with the proteins. Hydrogen bonding is necessary for most high-affinity ligands as they have a vital role in influencing the ligand binding specificity (Wade and

Goodford, 1989; Pantsar and Poso, 2018). In a study conducted by Raschka *et al.*, 2018, they discovered that proteins prefer to act as hydrogen donors (Raschka *et al.*, 2018). Therefore, the ability of a compound to act as a hydrogen acceptor would increase the potential to have a more stable ligand receptor binding complex. The compound ARM-2 displayed this ability against ACAT-1 (Figure 7) where accepting 2 and donating 1 H-bond resulted in the best docking score in comparison to terpendole C and RA-5 (Table 2 B). However, the inability of the compounds to accept the maximum number of H-bonds (Figure 8), resulted in their low docking scores against sPLA₂ (Table 2 B).

Interestingly, despite the low to moderate docking scores observed against CD-36, SR-A1, ABCA-1, ABCG-1 (Table 2 A), and sPLA₂ (Table 2 B), the high docking scores against ACAT-1 and LOX-1 (for ARM-2) (Table 2 B) predicted the potential of these compounds to inhibit endothelial dysfunction and foam cell formation. Endothelial dysfunction which results in the release of macrophages and activation of immune response, commonly known as the first step of atherogenesis, is triggered by the release of LOX-1. Therefore, the predicted inhibitory potential of ARM-2 against LOX-1 could result in a decreased expression of the enzyme (Jebari-Benslaiman *et al.*, 2022). Since over-expression of ACAT-1 can result in the accumulation of esterified cholesterol and macrophage-derived foam cell formation (Reiss and Cronstein, 2012; Yu *et al.*, 2013; Sobenin, 2020), the potential of the compounds to also inhibit the expression of ACAT-1 could be crucial in the prevention of foam cell formation.

5.2 ADMET properties, drug-likeness, and Swiss target predictions of the triterpenoids

ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties, drug-likeness and protein target predictions of new compounds are carried out due to their major role in drug discovery (Yang *et al.*, 2019; de Souza Neto *et al.*, 2020). It is important to incorporate virtual screening in drug discovery as it notably assists drug repurposing via off-target effect identification and the optimisation of lead compounds (Rifaioğlu *et al.*, 2019). The ADMET properties, drug-likeness and protein target predictions of the compounds were compared to lovastatin which is used in the treatment of ACVDs and CVDs. The compounds did not display any noteworthy probability against phospholipase A2 group IIA or any of the other targets in Table 5, except for RA-5 against prostaglandin E synthase (0.59). Prostaglandin E synthase has a significant role in the inflammatory response, fever, and pain by converting cyclooxygenase (COX)-derived prostaglandin (PG) H₂ to PGE₂. This means that RA-5 is predicted to have the potential to inhibit this enzyme resulting in the possibility of relieving inflammatory symptoms. Interestingly, in a recent study carried out by Ndlovu and colleagues, they obtained data displaying the inhibitory potential that ARM-2 and RA-5 have on HMG-CoA

reductase and their ability to downregulate the expression of the gene that codes for the enzyme (Ndlovu *et al.*, 2023).

ARM-2 and RA-5 have zero PAINS alerts, implying that there should be no false positives in the high-throughput screening data. The compounds obey Lipinski's rule which states that orally active drugs should have no more than one violation of the following conditions: no more than 5 H-bond donors and no more than 10 H-bond acceptors (Lipinski *et al.*, 2001). The high lipid solubility and desired TPSA value range of the compounds indicate good drug absorption in the human intestines and drug bioavailability, similar to lovastatin (Table 3 & Table 4). ARM-2 and RA-5 are substrates and not inhibitors for CYP3A4 which means that they can be metabolised by cytochrome P450. The cytochrome P450 enzymes contribute to cellular metabolism, the detoxification of xenobiotics as well as homeostasis (Manikandan and Nagini, 2018). As a result, they would most likely not negatively interfere with the P450 pharmacokinetics. Total clearance is a characteristic of the excretion of drugs and is a great contributor in the control of overall drug exposure in the blood (Lagorce *et al.*, 2017). The predicted lower total clearance of the compounds ARM-2 (0.404) and RA-5 (0.317) could partly support the predicted hepatotoxicity of both compounds (Table 3). It is also worth noting that despite the *in silico* predicted hepatotoxicity potential of both ARM-2 and RA-5, the *in vitro* results by Ndlovu *et al.*, 2023 revealed no hepatotoxicity of the compounds at up to 100 μ M. The virtual screening findings indicate that the compounds do not show many adverse effects in humans thus suggesting their potential in drug development.

5.3 Antioxidant capacity of the triterpenoids

Oxidative stress is implicated in various pathophysiological procedures including atherogenesis. Endothelial dysfunction results in the synthesis and release of ROS, which triggers the inflammatory process releasing proinflammatory cytokines thus creating an atherogenic environment (Badimon and Vilahur, 2014; Markin *et al.*, 2020). Therefore, the scavenging or neutralisation of free radicals is vital in preventing an atherogenic environment. The observed poor radical scavenging activity of the triterpenes on ABTS and DPPH radicals as well as poor reducing potential on ferric ions suggested the compounds lack antioxidant properties (Figure 9). However, their good ORAC values (Figure 10 & Table 6) indicated their potential as hydrogen donors instead of electron donors. The contrasting observations may be a result of the different systems each assay uses. The DPPH, ABTS and ferric reducing assays all use an electron-transfer system in contrast to the ORAC, which makes use of the hydrogen-transfer system.

Antioxidant activity is partly dependent on the number of hydroxyl (-OH) functional groups present in the chemical structure of the compound and this relates to the number of hydrogens that can be donated. A strong antioxidant has a high number of -OH groups and, RA-5 and ARM-2 have only 2 and 3 respectively. Carboxyl (-COOH) functional groups are also found in both ARM-2 and RA-5, and these groups are known to be electron withdrawing and polar. This means that the compounds will not give up their electrons, but they can participate in hydrogen bonding, which could be the reason why their potential antioxidant capacity was observed in the ORAC assay in comparison to the other three systems that use an electron-transfer system. Therefore, the compounds can prevent or decrease oxidative processes, more so in polar environments.

5.4 Inhibitory activity of the compounds on LDL modification

The lipid hydroperoxides, which come from the modification of HDL and LDL followed by minor degradation of polyunsaturated fatty acids (PUFAs), lead to CD formation, and are further modified into short-chain aldehydes like MDA. Increased MDA (biomarker for oxidative stress) levels and CD formation result in continued oxidation of the lipids (*Ito and Ito, 2020; Kim et al., 2020*). The observed ability of both ARM-2 and RA-5 to significantly decrease the CD formation as well as inhibit MDA formation in the CuSO₄-induced LDL oxidation (Figure 4 A&C), indicated that compounds could potentially prevent oxidative modification of LDL, making the environment (intima layer of the artery) less atherogenic. However, the same was not observed in Figure 11 E for the peroxidation of HDL, where both compounds failed to successfully inhibit MDA formation at the highest concentration of 500 µM.

The difference in the results of the compounds between HDL and LDL could be due to the structural composition of the lipoproteins. HDL is far easier to oxidise in comparison to LDL. This is because it has less antioxidant composition (lower vitamin E content) and the composition of its PUFA's has more fatty acids with 3 to 6 double bonds in comparison to LDL (*Nagyová et al., 2001*). The fact that the compounds have a similar backbone structure to cholesterol could also contribute to the poor results obtained for HDL. This is because it is possible that the compounds were oxidised after the quick oxidation of the native HDL. The results obtained in this study are consistent with other literature reports on the capability of triterpenes to inhibit LDL oxidation (*Fatima et al., 2022; Silva et al., 2022*).

5.5 Inhibitory effect of the compounds on sPLA₂

Inflammation plays a major role in creating and maintaining an atherogenic environment where the oxidative modification of LDL is favoured. The inflammatory response results in the secretion of proinflammatory enzymes such as sPLA₂ and Leukotriene A₄ hydrolase (LTA₄H). sPLA₂ is an enzyme that initiates inflammatory mediation by degrading arachidonic acid (AA) and converting it into prostaglandins (Dore and Boilard, 2019; Casale *et al.*, 2023). Prostaglandins are important substrates in enhancing and stimulating the inflammatory response. Therefore, inhibition of sPLA₂ is also important in preventing the creation and maintenance of an atherogenic environment that favours oxidative modification of LDL. The observed inability of both triterpenoids to inhibit sPLA₂ at the concentrations of 25 and 50 µM (Table 7) is possibly due to their poor interactions with the enzyme. The results of this *in vitro* assay confirmed the predicted computational results in which the compounds displayed weak ligand-receptor binding stability (Table 2 B). The lack of failure of the compounds to this enzyme is further supported by the predicted zero (0) probability scores of the compounds against the enzyme as shown in Table. Even though the tested triterpenes (ARM-2 and RA-2) did not inhibit sPLA₂, other triterpenes (pentacyclic triterpenes) such as maslinic acid and ursolic acid have been reported to have inhibitory activity on the enzyme (Yap *et al.*, 2018; Luan *et al.*, 2021).

5.6 Cytotoxicity, intracellular lipid accumulation and foam cell formation

5.6.1 Cytotoxicity potential of the compounds in RAW 264.7 cells

Cytotoxicity assays are usually carried out for drug screening purposes to determine the result of target compounds on cell proliferation. The proliferation of cells is an important factor in understanding the pathways that are affected in cell survival or apoptosis, following the exposure of the cells to toxic agents (Adan *et al.*, 2016). RAW 264.7 cells have been used in macrophage cell culture studies for over 40 years making them a common and appropriate model for macrophages (Taciak *et al.*, 2018). Even though the cytotoxicity potential of ARM-2 and RA-5 has previously been tested in other types of cell lines (Ndlovu *et al.*, 2023), their cytotoxicity potential on RAW 264.7 cells has not been tested. The standard value for good cell viability is any value above 80%, within 60% - 80% is weak cytotoxicity, within 40% - 60% is moderate cytotoxicity and below 40% is strong cytotoxicity (Iso, 2009; López-García *et al.*, 2014). The observed high viability of the cells (Figure 12 A-B) exposed to ARM-2 (1, 10 and 25 µM) and RA-5 (1 and 10 µM) indicated that the compounds do not possess cytotoxic properties. Interestingly, the compounds were shown to stimulate the growth of cells. However, the observed low cell viability after exposure of the cells to RA-5 (at 25 µM) indicated

a moderate to weak cytotoxicity effect (Figure 12 A-B) of the compound at concentrations of 25 μ M and higher.

5.6.2 intracellular lipid accumulation and foam cell formation

The recruitment of monocytes and the formation of foam cells are important steps in the progression of atherosclerotic plaque. Foam cell formation is caused by the imbalance between cellular cholesterol influx and efflux. Increased cellular cholesterol influx into macrophage cells results in the formation of lipid droplets which ultimately results in foam cell formation. The exposure to ox-LDL in the macrophages increased the formation of lipid droplets (Figure 13A), which is supported by literature and similar studies (Orekhov and Myasoedova, 2019; Kim *et al.*, 2020). The decrease in lipid droplets in the cells exposed to ox-LDL in the presence of the test compounds (Figure 13 B-D) indicates the potential that the compounds possess in lessening foam cell formation. The atheroprotective potential of the compounds may have not shown statistical significance however, the decrease in lipid droplet formation was biologically significant. RA-5 showed better inhibitory activity in comparison to ARM-2, with RA-5 (10 μ M) having the best results at a lower concentration (Figure 13E). The findings by Fujiwara *et al.* (2011) have also suggested that the triterpenes found in *Zizyphus jujuba* have the potential to inhibit foam cell formation.

Though sufficient conclusive data could not be obtained, the increased expression of the ABCA-1 gene in the cells exposed to ox-LDL in the presence of RA-5 suggests the possible mechanism of the compounds in inhibiting foam cell formation. The gene expression levels of ABCA-1 in the normal cells and cells exposed to RA-5 and ox-LDL were upregulated in comparison to the cells that were only treated with ox-LDL (Figure 14). This is supported by literature that states that an increased concentration of ox-LDL suppresses the expression of ABCA-1 and increases the expression of ACAT-1 (Yu *et al.*, 2013; Chistiakov *et al.*, 2017). The findings are also in agreement with the poor *in silico* docking scores obtained against ABCA-1. The ability of ARM-2 and RA-5 to decrease the formation of lipid droplets could potentially be beneficial in preventing atherogenesis and slowing down the progression of atherosclerosis.

6.0 Conclusion

Based on the findings obtained in this study, it is concluded that both triterpenoids (ARM-2 and RA-5) possess antiatherogenic properties. Their bioactivity is supported by their ability to inhibit oxidative modification of LDL and foam cell formation. The antioxidant mechanism of the compounds could be associated with their ability to scavenge peroxy radicals and chelate metal (copper) ions. Thus, suggesting that there are other possible mechanisms that these compounds could use to decrease or prevent further development and progression of atherogenesis (atherosclerotic plaque). Since the compounds were unable to show a statistically significant inhibition of foam cell formation, the first hypothesis was rejected. However, the observed decrease in foam cell formation in the cells treated with the compounds was biologically significant.

6.1 Limitation(s) of the study

The conclusive results on the effect of the compounds on the expression of some genes involved in cholesterol trafficking could not be obtained. There are a few possible errors that could have led to the unsuccessful RT-qPCR experiments. First is the quality and integrity of the cDNA. During the extraction of RNA, DNase-1 was used to digest genomic DNA (gDNA) from the samples and RNA purity and concentration were determined using a nanodrop. However, a gel was not run to further confirm the absence of the said gDNA. Consequently, after cDNA synthesis, the cDNA presence, quality, and integrity were not confirmed. The second error could have been with the primers themselves. The primer concentrations and annealing temperatures were not optimized by running gradient PCR assays. The above hypothesis could not be proven, or errors corrected due to the lack of available reagents, finances, and time constraints.

6.2 Future work/ Recommendations for further study

- The negative/positive effects of the compounds on other non-enzymatic modifications such as glycation and desialylation of lipoproteins (LDL) are recommended.
- The effect of the compounds on the gene expression of the proteins involved in cellular cholesterol trafficking, such as CD-36, SR-A1, nCEH, and ACAT-1.
- The activity of the compounds on RAW cells differentiated into both the M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes, to further study their anti-inflammatory properties in an atherosclerotic environment.

7.0 References

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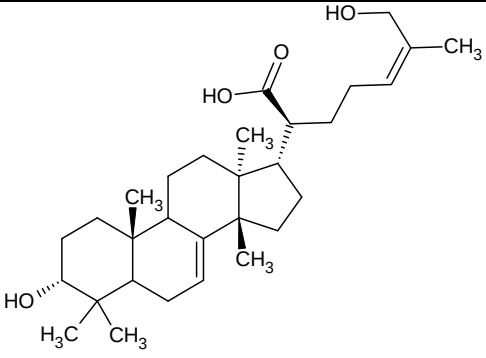
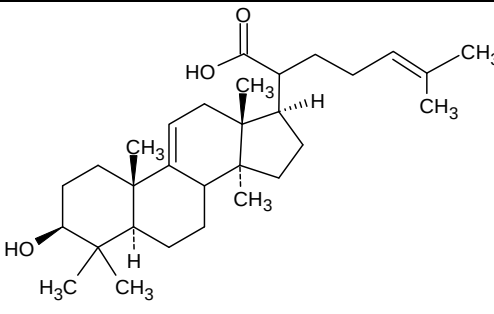
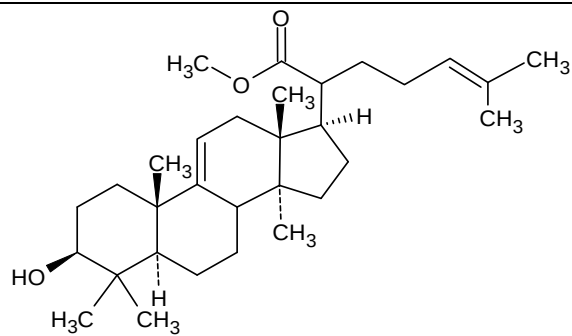
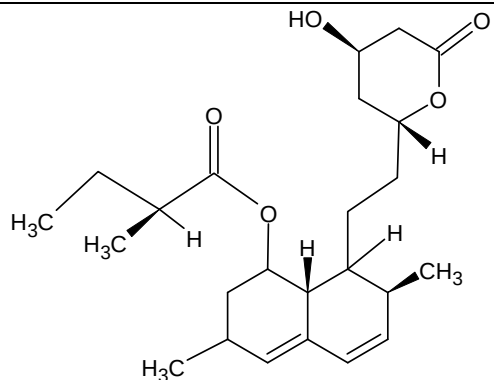
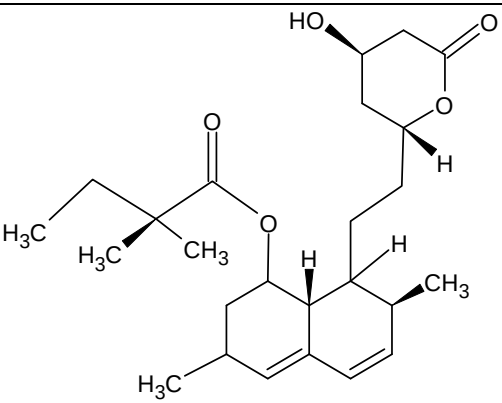
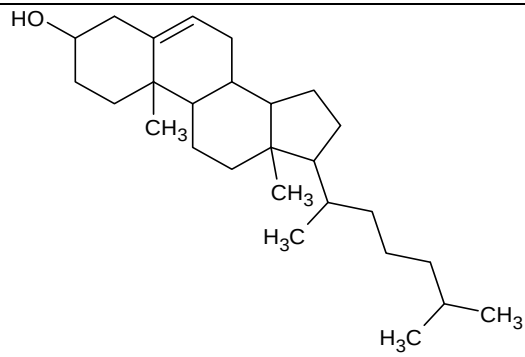
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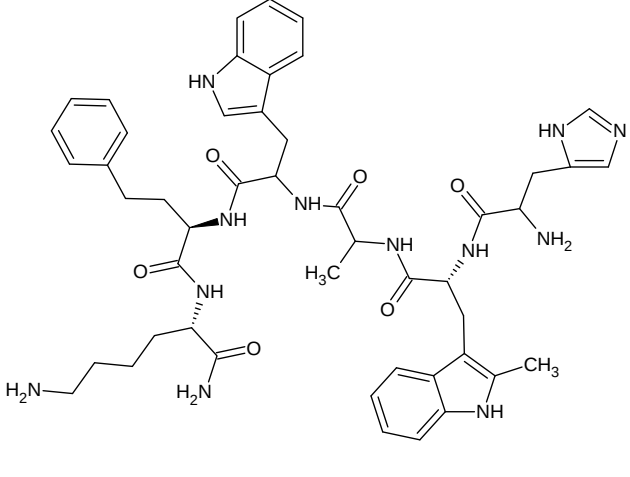
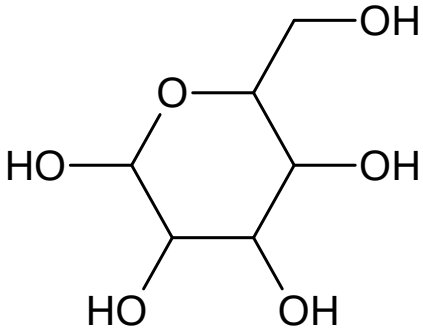
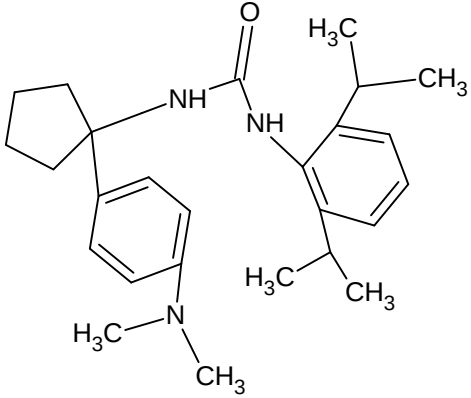
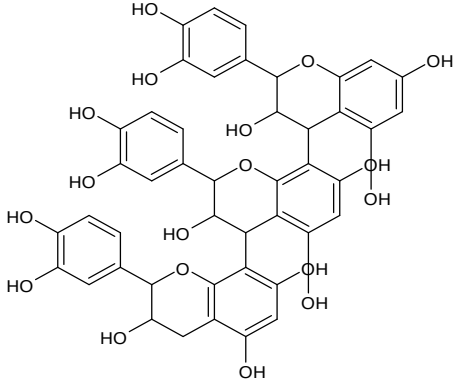
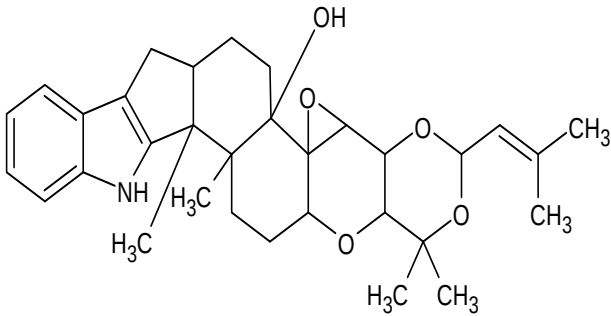
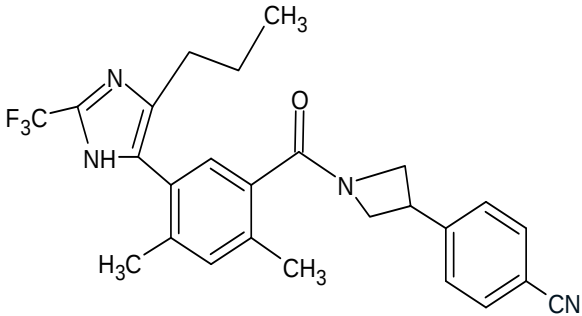
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Appendix A: *In silico* studies

 ARM-2	 RA-5
 RA-3	 Lovastatin
 Simvastatin	 Cholesterol

 Hexarelin	 Glucose
 Nevanimibe	 Procyanidin C1
 Terpendole C	 TVB-3664

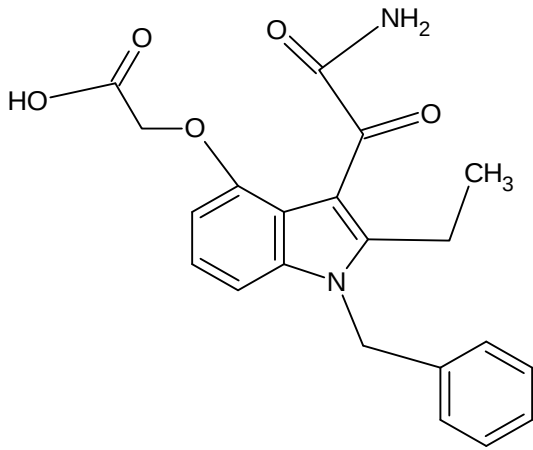
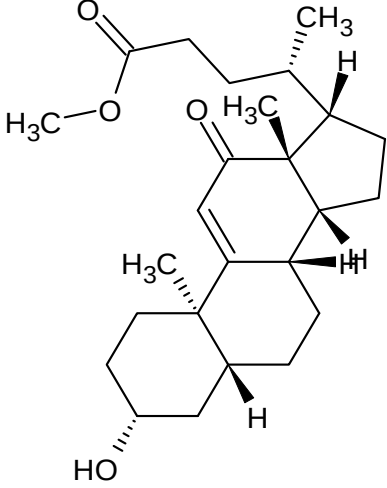
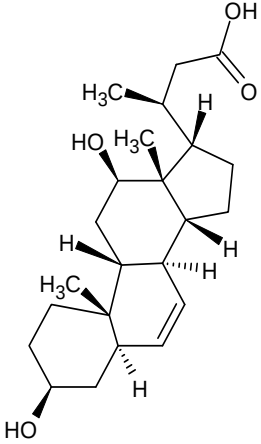
 Varespladib	 ZINC257357466
 ZINC253610271	

Figure A1: The chemical structures of the compounds used in the molecular docking of the target proteins.

Table A1: Molecular docking scores (XP score) and glide energies (kJ/mol) of the plant triterpenes (ARM-2 and RA-5) on some target proteins involved in cellular cholesterol trafficking.

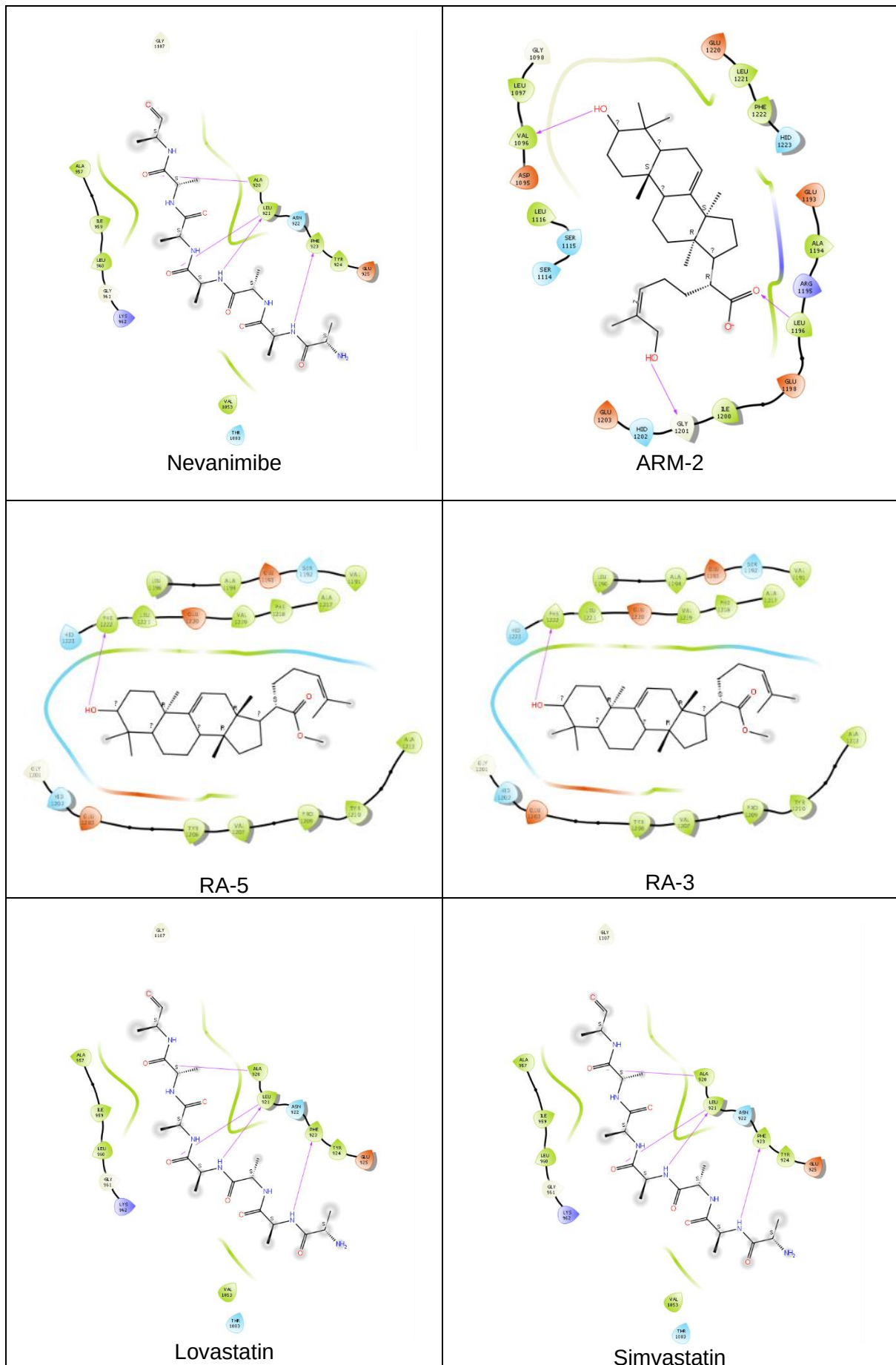
Ligand/Inhibitor	CD36		SR-A1		ABCA-1		ABCG-1	
	Score	Energy	Score	Energy	Score	Energy	Score	Energy
#Simvastatin	-3.63	-28.57	-3.67	-29.45	-5.28	-37.18	-8.13	-32.39
#Lovastatin	-3.54	-29.07	-4.38	-30.73	-5.62	-34.90	-7.49	-27.67
ARM-2	-3.19	-26.68	-4.13	-28.93	-4.83	-36.59	-7.12	-11.86
RA-5	-3.02	-23.58	-4.17	-25.18	-4.29	-30.24	-7.82	-14.95
RA-3	-2.54	-22.77	-3.20	-24.45	-4.89	-33.70	-8.89	-18.63
Cholesterol	-2.62	-24.55	-3.06	-26.20	-5.28	-28.05	-9.36	-28.33
ZINC253610271	-2.14	-23.60	-0.64	-23.62	-4.67	-27.62	-6.80	-23.67
ZINC257357466	-2.74	-28.15	-1.84	-25.54	-3.67	-28.45	-6.37	-31.59
*Hexarelin	-5.83	-56.87						
*TVB-3664	-3.57	-34.04						
*Nevanimibe					-5.96	-37.59	-8.49	-42.66

#reference drug; * indicates activator; *indicates inhibitor

Table A2: Molecular docking scores (XP score) and glide energies (kJ/mol) of the plant triterpenes (ARM-2 and RA-5) on some target proteins involved in cellular cholesterol trafficking.

Ligand/Inhibitor	LOX-1		ACAT-1		sPLA ₂	
	Score	Energy	Score	Energy	Score	Energy
#Simvastatin	-8.18	-39.74	-10.89	-41.78	-7.49	-40.01
#Lovastatin	-7.79	-41.11	-10.10	-43.06	-8.34	-42.35
ARM2	-8.40	-34.67	-11.40	-47.01	-5.77	-37.89
RA5	-6.23	-37.83	-9.62	-36.65	-3.46	-24.95
RA3	-6.67	-32.82	-8.41	-38.66	-4.30	-31.22
Cholesterol	-8.07	-34.47	-8.46	-33.83	-5.90	-30.17
ZINC253610271	-8.23	-34.44	-8.85	-38.90	-6.39	-28.90
ZINC257357466	-6.17	-36.05	-9.13	-35.74	-5.00	-34.32
*Procyanidin C1	-14.85	-67.36				
*Glucose	-7.75	-24.82				
*Terpendole C			-10.18	-49.93		
*Varespladib					-10.27	-58.18

#reference drug; * indicates activator; *indicates inhibitor



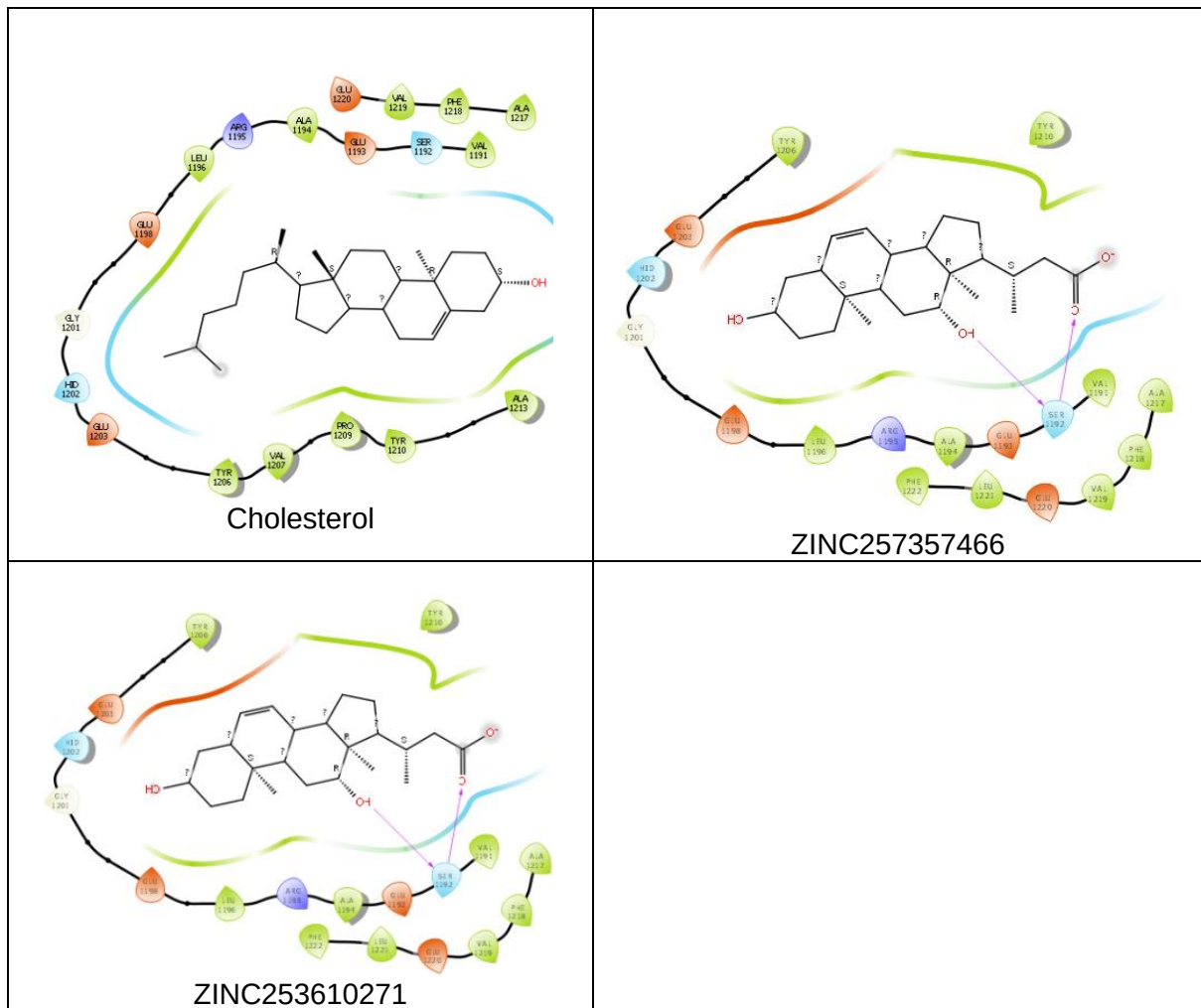
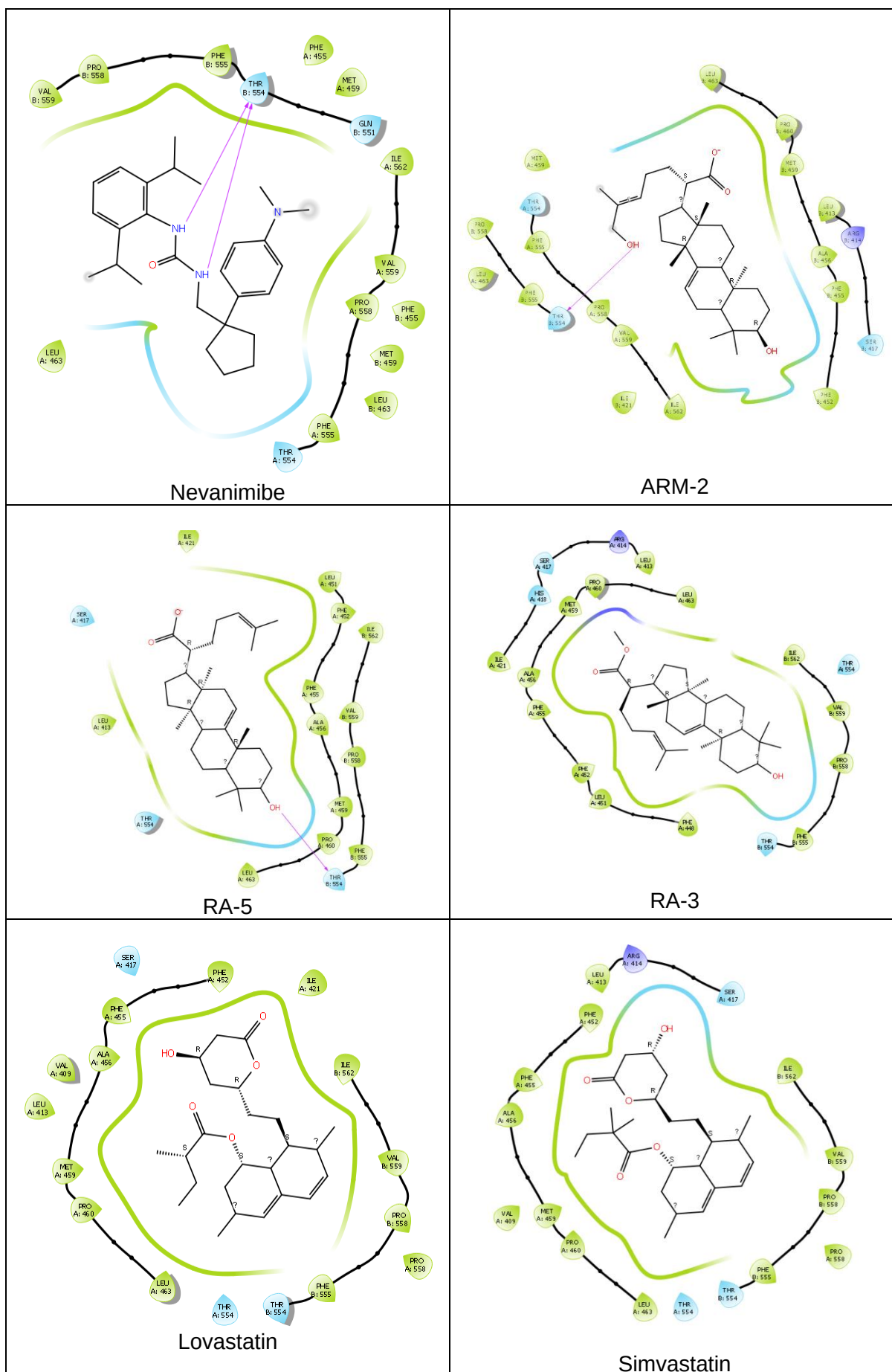


Figure A2: Molecular docking interactions (hydrogen bonding) of the compounds against ABCA-1



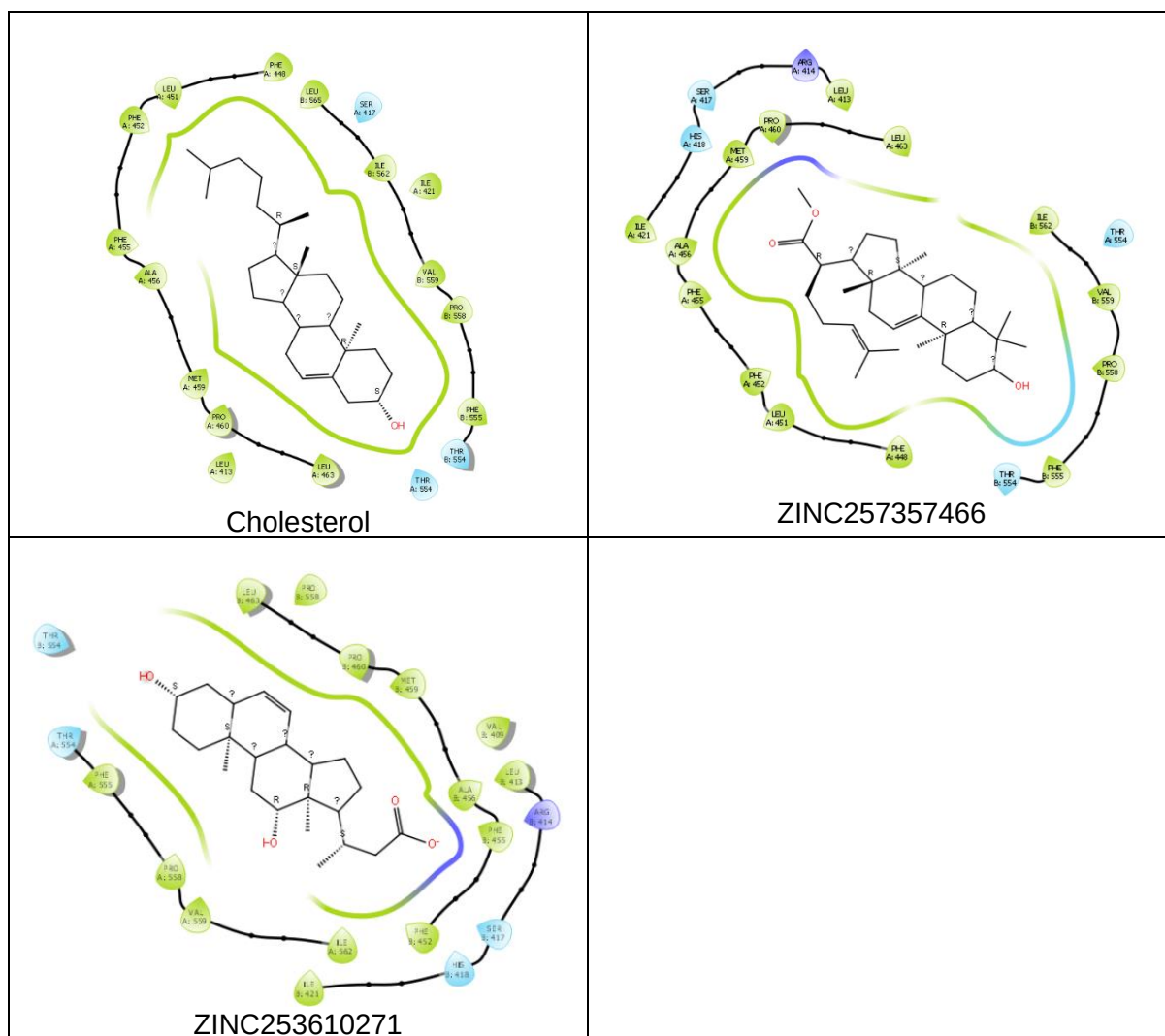
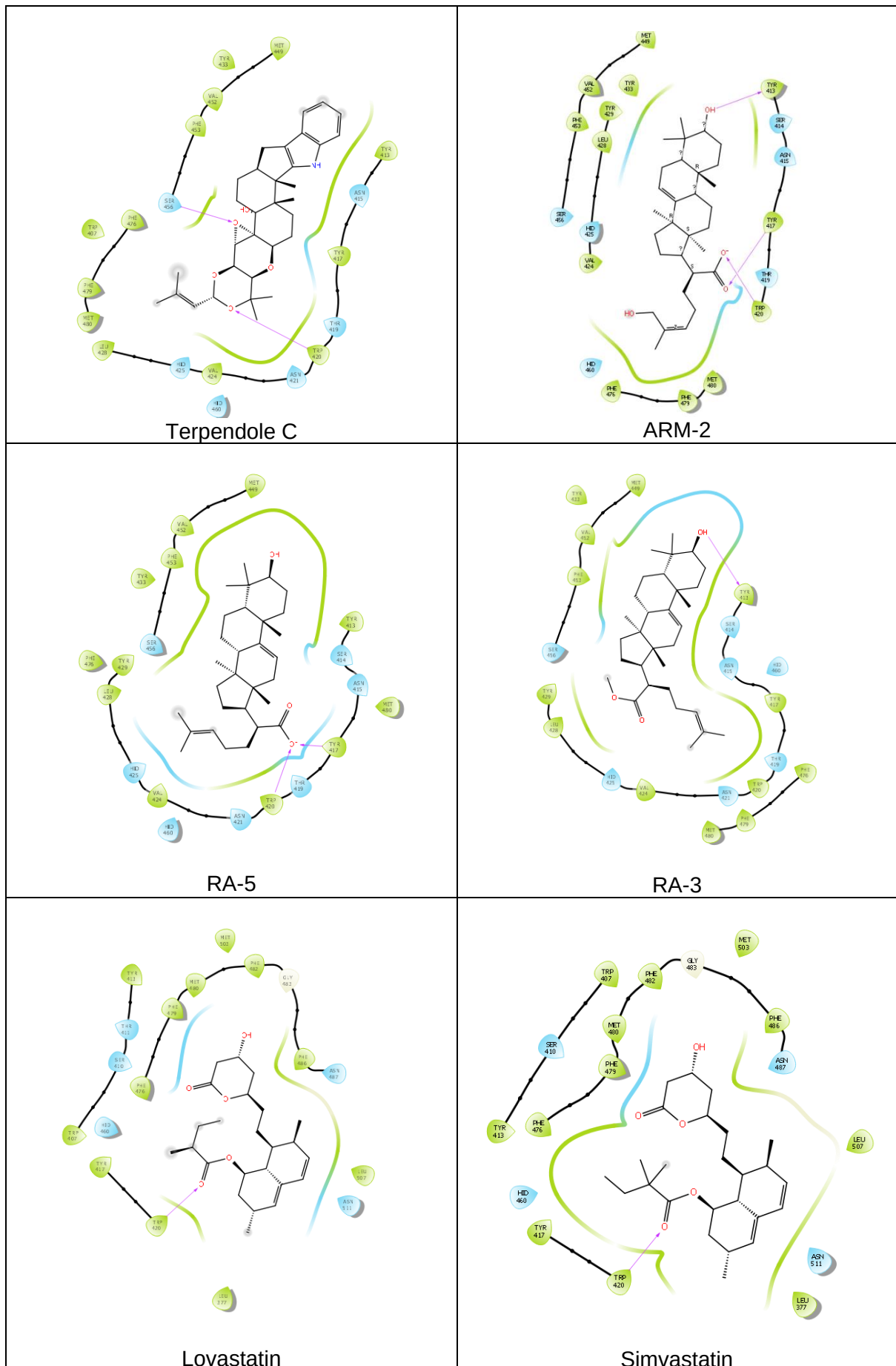


Figure A3: Molecular docking interactions (hydrogen bonding) of the compounds against ABCG-1



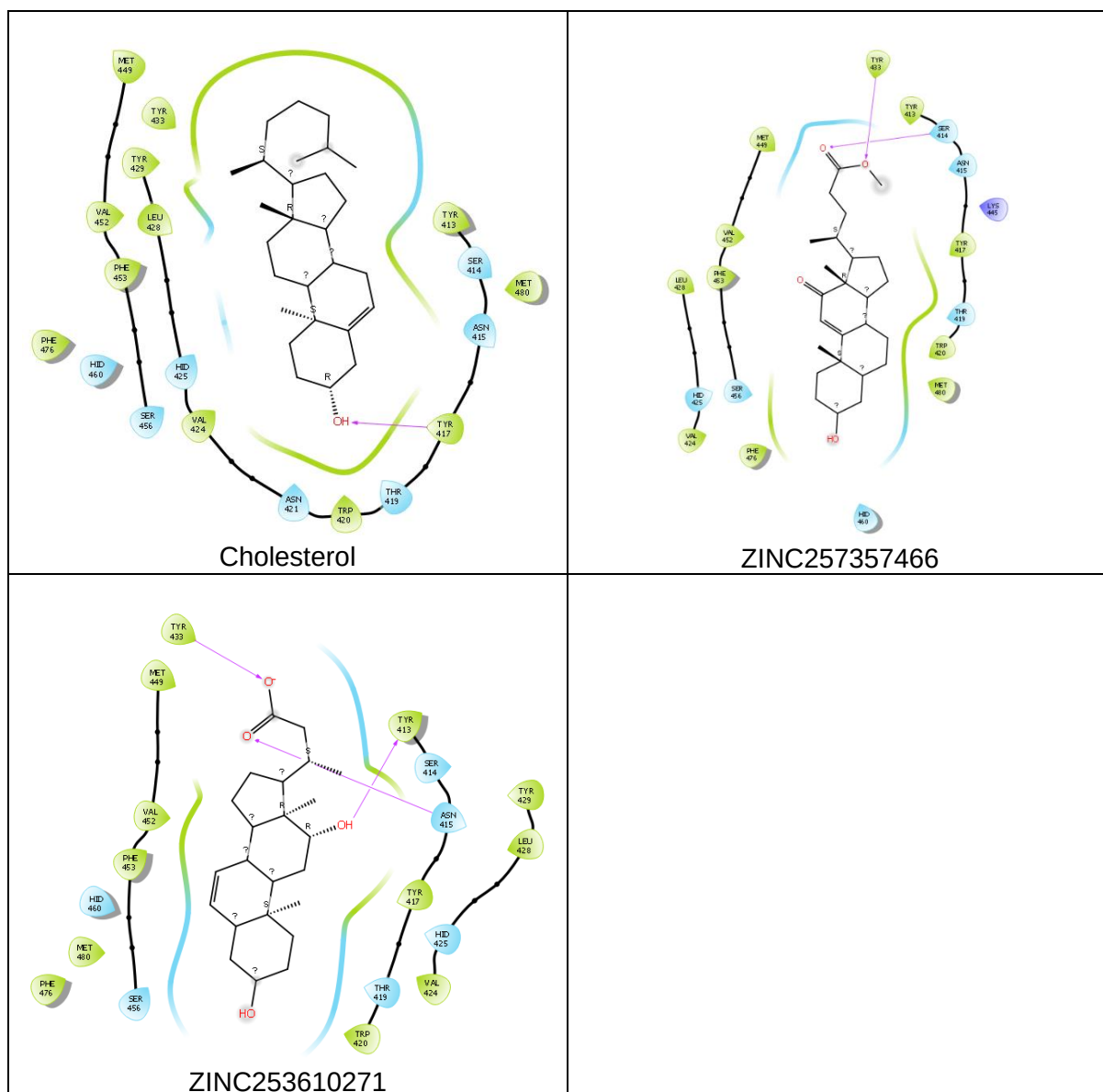
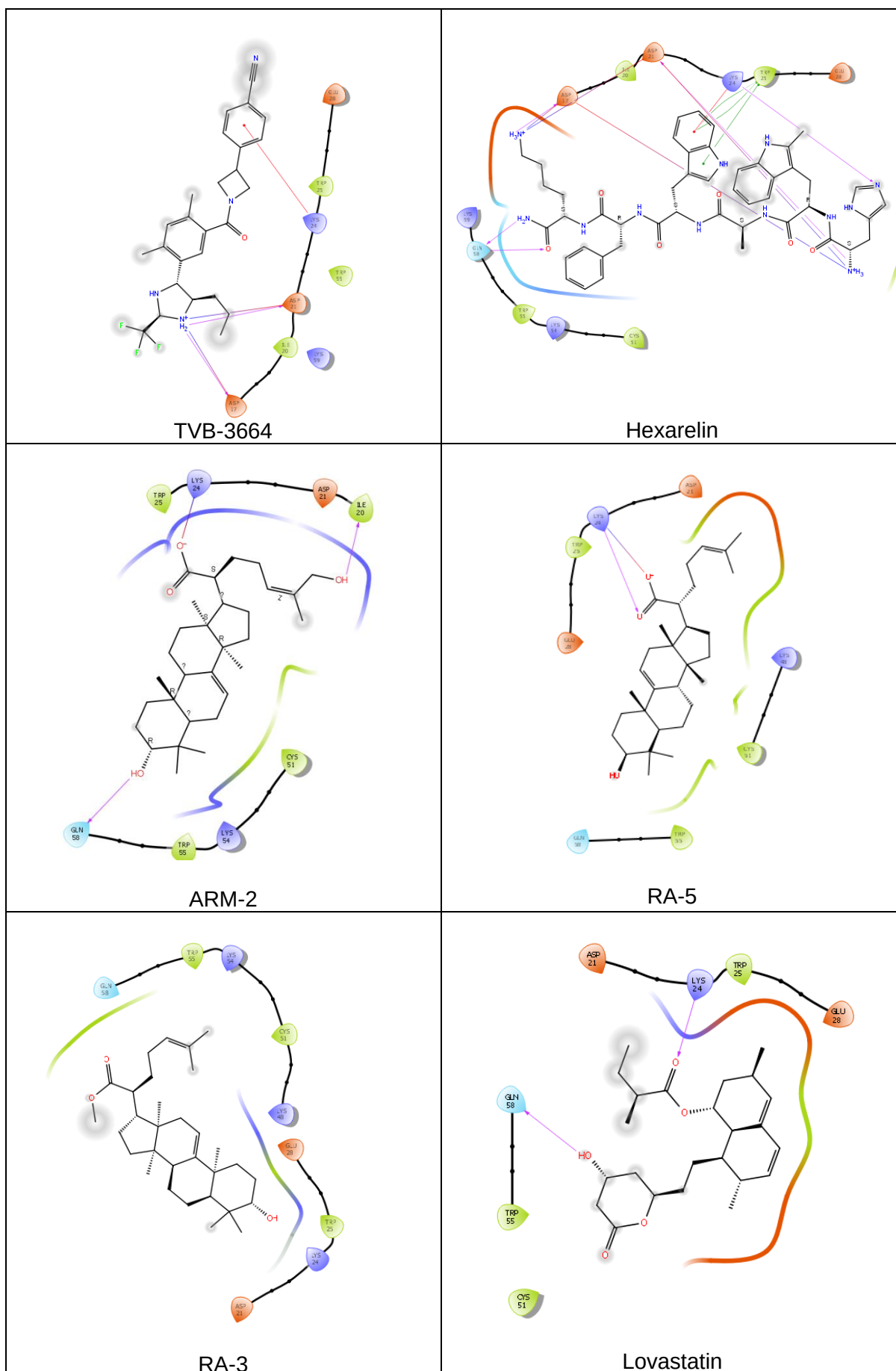


Figure A4: Molecular docking interactions (hydrogen bonding) of the compounds against ACAT-1



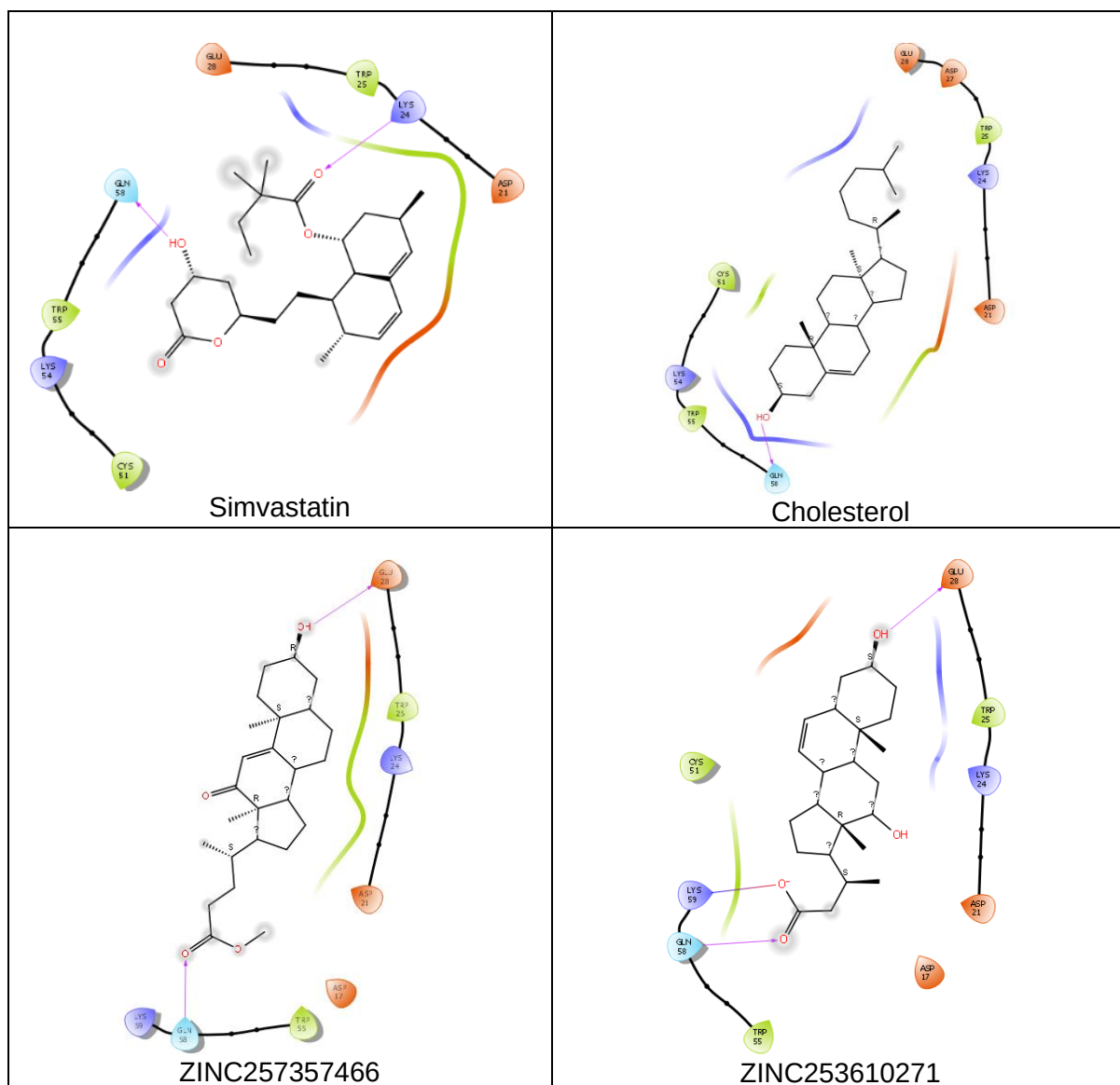
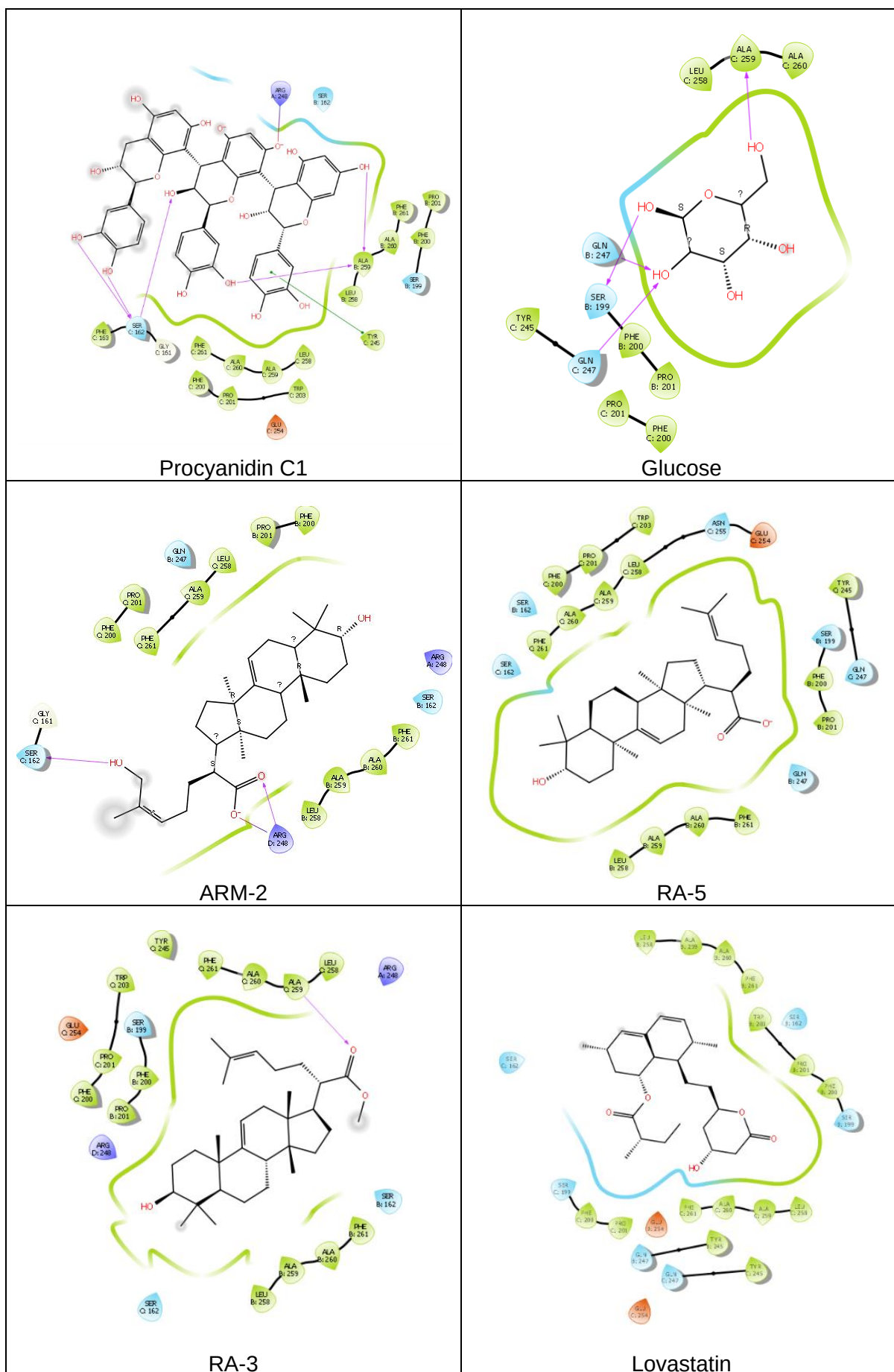


Figure A5: Molecular docking interactions (hydrogen bonding) of the compounds against CD-36



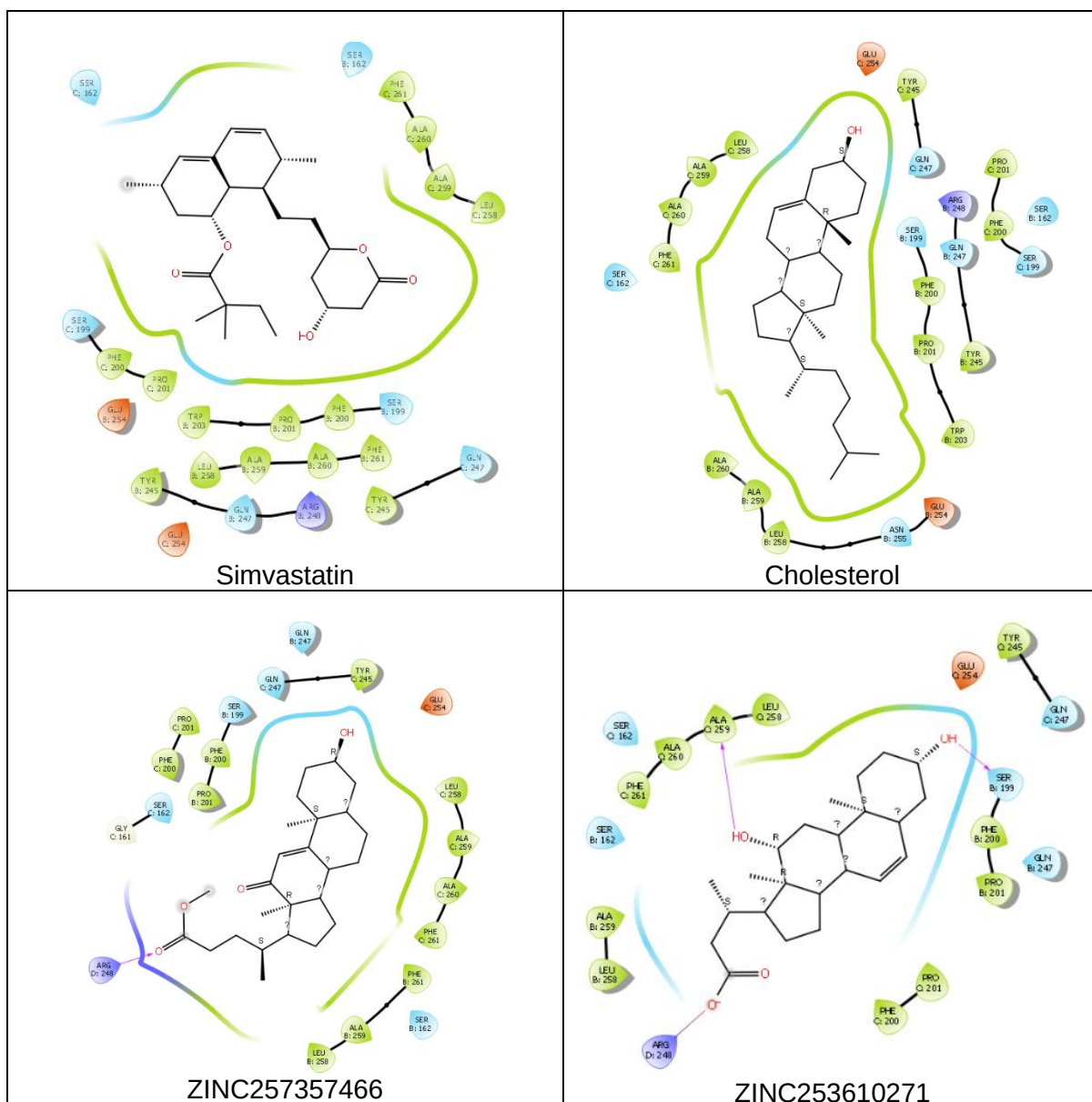
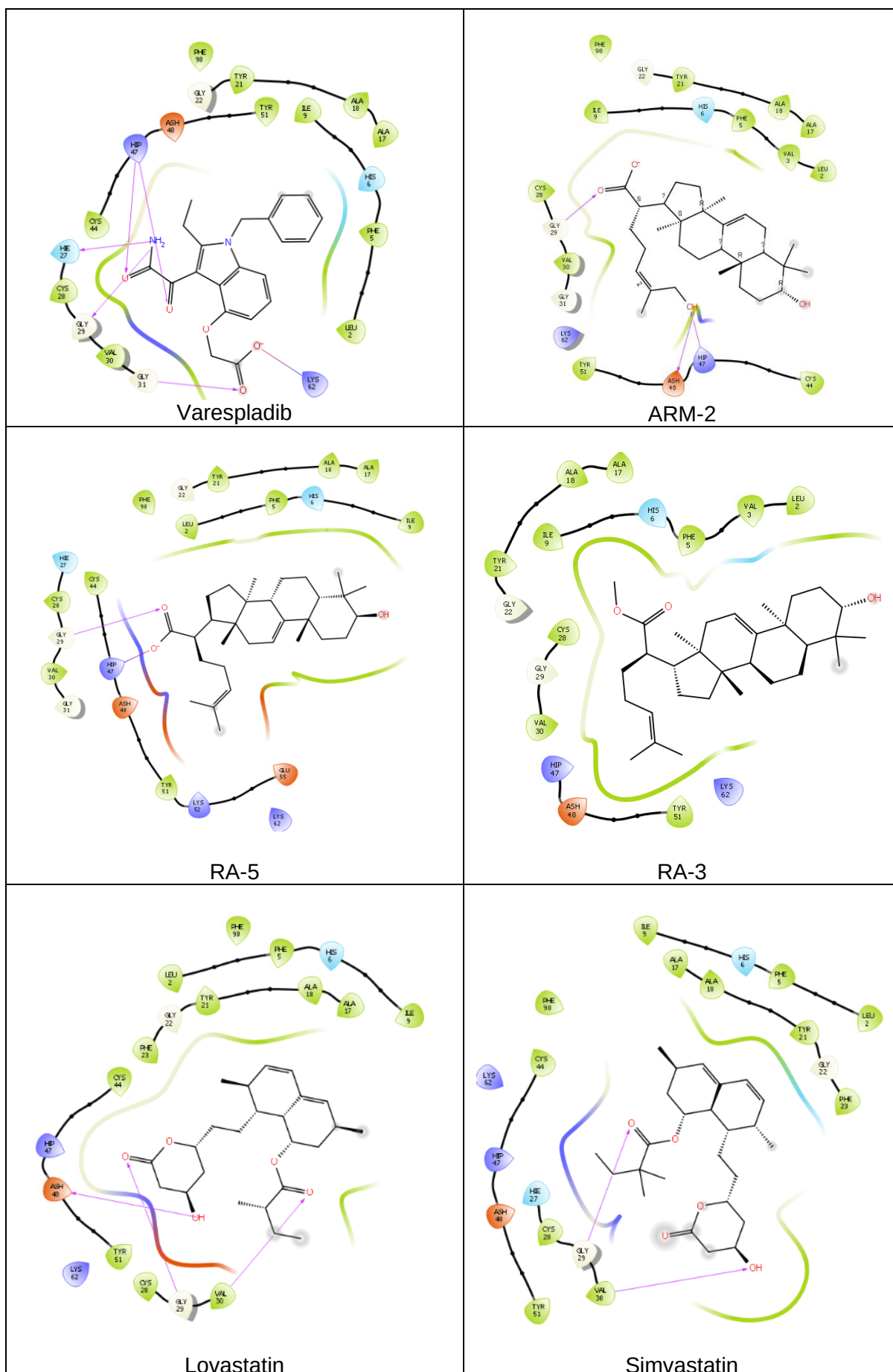


Figure A6: Molecular docking interactions (hydrogen bonding) of the compounds against LOX-1



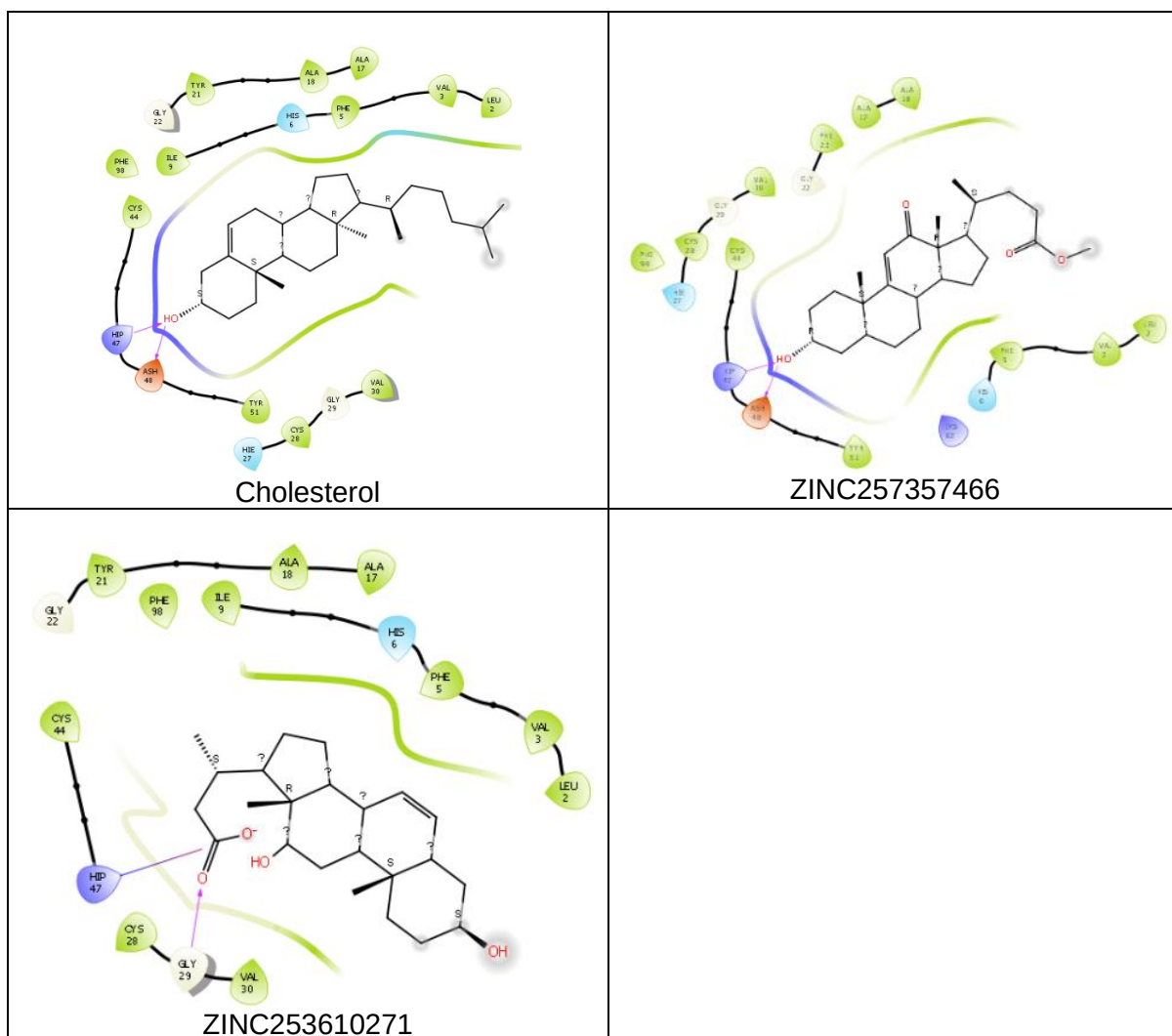
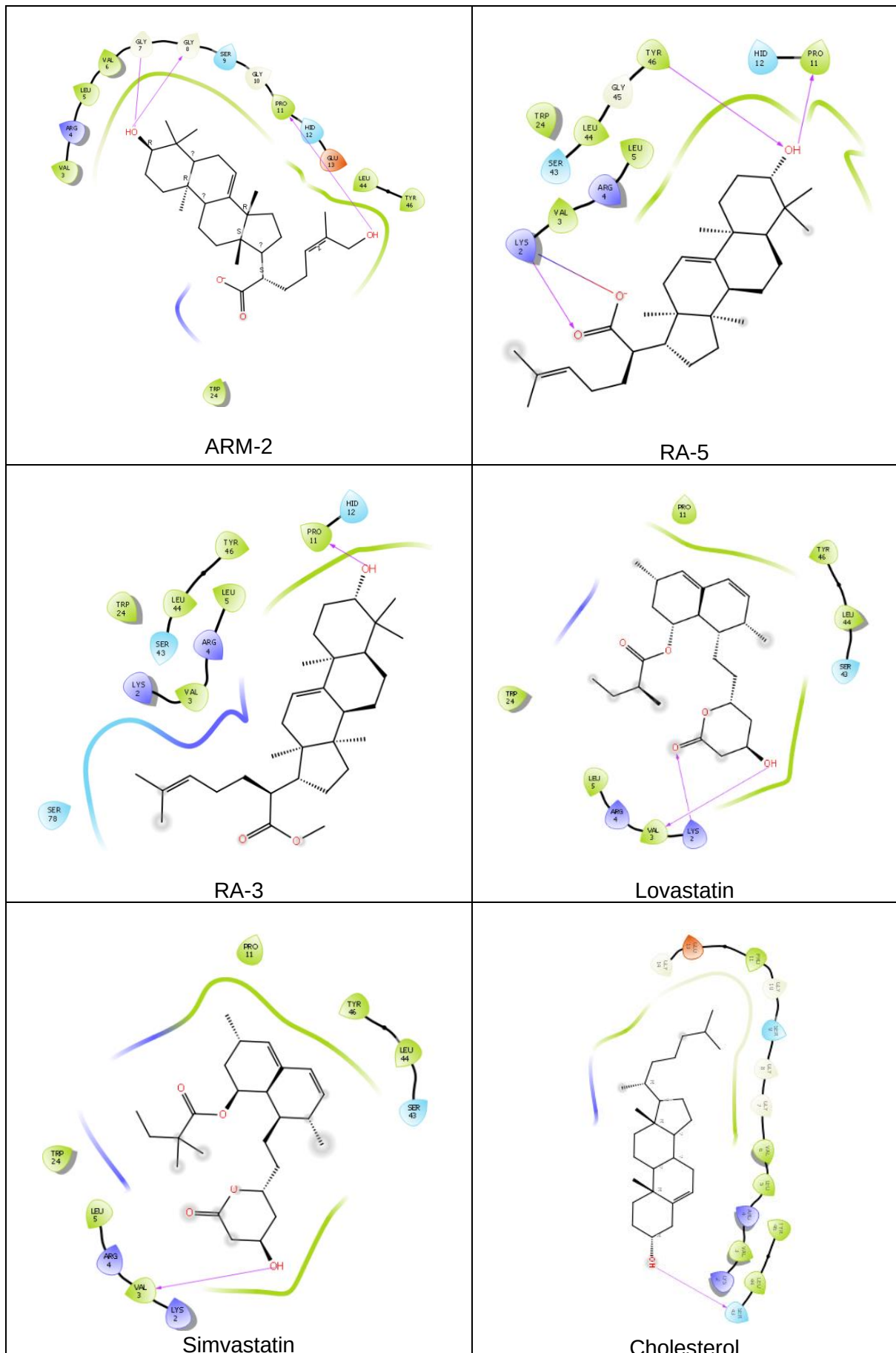


Figure A7: Molecular docking interactions (hydrogen bonding) of the compounds against SPLA₂



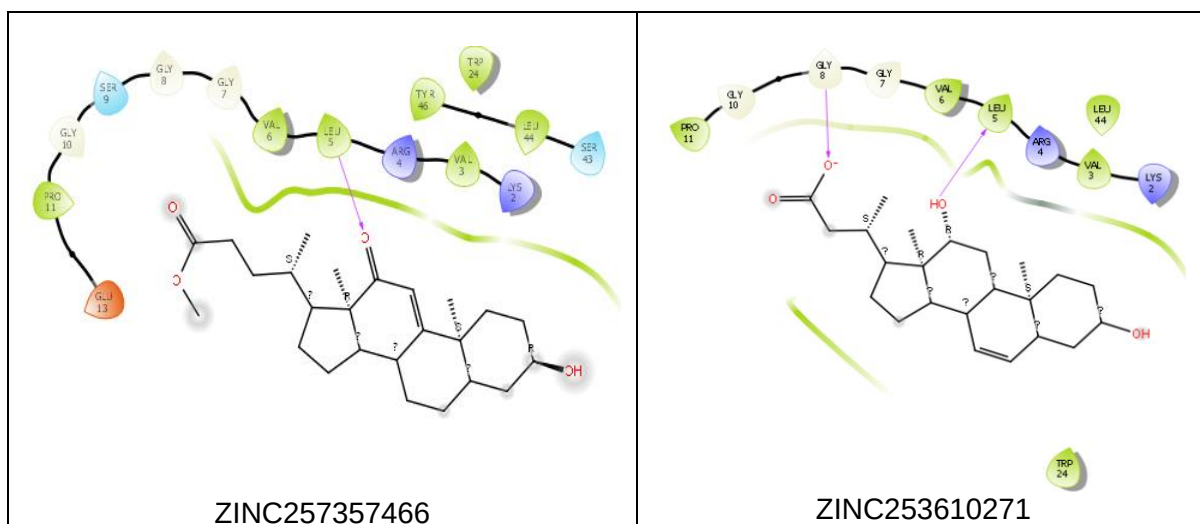


Figure A8: Molecular docking interactions (hydrogen bonding) of the compounds against SR-A1

Table A3: ADMET properties of ARM-2

Property	Model Name	Predicted Value	Unit
Absorption	Water solubility	-4.11	Numeric (log mol/L)
Absorption	Caco2 permeability	0.73	Numeric (log Papp in 10-6 cm/s)
Absorption	Intestinal absorption (human)	95.28	Numeric (% Absorbed)
Absorption	Skin Permeability	-2.73	Numeric (log Kp)
Absorption	P-glycoprotein substrate	Yes	Categorical (Yes/No)
Absorption	P-glycoprotein I inhibitor	No	Categorical (Yes/No)
Absorption	P-glycoprotein II inhibitor	Yes	Categorical (Yes/No)
Distribution	VDss (human)	-0.60	Numeric (log L/kg)
Distribution	Fraction unbound (human)	0.02	Numeric (Fu)
Distribution	BBB permeability	-0.70	Numeric (log BB)
Distribution	CNS permeability	-1.63	Numeric (log PS)
Metabolism	CYP2D6 substrate	No	Categorical (Yes/No)
Metabolism	CYP3A4 substrate	Yes	Categorical (Yes/No)
Metabolism	CYP1A2 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2C19 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2C9 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2D6 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP3A4 inhibitor	No	Categorical (Yes/No)
Excretion	Total Clearance	0.40	Numeric (log ml/min/kg)
Excretion	Renal OCT2 substrate	No	Categorical (Yes/No)
Toxicity	AMES toxicity	No	Categorical (Yes/No)
Toxicity	Max. tolerated dose (human)	-1.71	Numeric (log mg/kg/day)
Toxicity	hERG I inhibitor	No	Categorical (Yes/No)
Toxicity	hERG II inhibitor	No	Categorical (Yes/No)
Toxicity	Oral Rat Acute Toxicity (LD50)	4.68	Numeric (mol/kg)
Toxicity	Oral Rat Chronic Toxicity (LOAEL)	2.02	Numeric (log mg/kg_bw/day)
Toxicity	Hepatotoxicity	Yes	Categorical (Yes/No)
Toxicity	Skin Sensitisation	No	Categorical (Yes/No)
Toxicity	T.Pyriformis toxicity	0.31	Numeric (log ug/L)
Toxicity	Minnow toxicity	0.56	Numeric (log mM)

Table A4: ADMET properties of RA-5

Property	Model Name	Predicted Value	Unit
Absorption	Water solubility	-4,45	Numeric (log mol/L)
Absorption	Caco2 permeability	1,48	Numeric (log Papp in 10-6 cm/s)
Absorption	Intestinal absorption (human)	93,64	Numeric (% Absorbed)
Absorption	Skin Permeability	-2,74	Numeric (log Kp)
Absorption	P-glycoprotein substrate	Yes	Categorical (Yes/No)
Absorption	P-glycoprotein I inhibitor	No	Categorical (Yes/No)
Absorption	P-glycoprotein II inhibitor	Yes	Categorical (Yes/No)
Distribution	VDss (human)	-0,56	Numeric (log L/kg)
Distribution	Fraction unbound (human)	0	Numeric (Fu)
Distribution	BBB permeability	-0,40	Numeric (log BB)
Distribution	CNS permeability	-1,27	Numeric (log PS)
Metabolism	CYP2D6 substrate	No	Categorical (Yes/No)
Metabolism	CYP3A4 substrate	Yes	Categorical (Yes/No)
Metabolism	CYP1A2 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2C19 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2C9 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2D6 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP3A4 inhibitor	No	Categorical (Yes/No)
Excretion	Total Clearance	0,32	Numeric (log ml/min/kg)
Excretion	Renal OCT2 substrate	No	Categorical (Yes/No)
Toxicity	AMES toxicity	No	Categorical (Yes/No)
Toxicity	Max. tolerated dose (human)	-1,40	Numeric (log mg/kg/day)
Toxicity	hERG I inhibitor	No	Categorical (Yes/No)
Toxicity	hERG II inhibitor	No	Categorical (Yes/No)
Toxicity	Oral Rat Acute Toxicity (LD50)	3,86	Numeric (mol/kg)
Toxicity	Oral Rat Chronic Toxicity (LOAEL)	2,16	Numeric (log mg/kg_bw/day)
Toxicity	Hepatotoxicity	Yes	Categorical (Yes/No)
Toxicity	Skin Sensitisation	No	Categorical (Yes/No)
Toxicity	T.Pyriformis toxicity	0,34	Numeric (log ug/L)
Toxicity	Minnow toxicity	-0,97	Numeric (log mM)

Table A5: ADMET properties of RA-3

Property	Model Name	Predicted Value	Unit
Absorption	Water solubility	-6,10	Numeric (log mol/L)
Absorption	Caco2 permeability	1,43	Numeric (log Papp in 10-6 cm/s)
Absorption	Intestinal absorption (human)	97,01	Numeric (% Absorbed)
Absorption	Skin Permeability	-2,61	Numeric (log Kp)
Absorption	P-glycoprotein substrate	No	Categorical (Yes/No)
Absorption	P-glycoprotein I inhibitor	Yes	Categorical (Yes/No)
Absorption	P-glycoprotein II inhibitor	Yes	Categorical (Yes/No)
Distribution	VDss (human)	-0,20	Numeric (log L/kg)
Distribution	Fraction unbound (human)	0	Numeric (Fu)
Distribution	BBB permeability	-0,26	Numeric (log BB)
Distribution	CNS permeability	-2,13	Numeric (log PS)
Metabolism	CYP2D6 substrate	No	Categorical (Yes/No)
Metabolism	CYP3A4 substrate	Yes	Categorical (Yes/No)
Metabolism	CYP1A2 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2C19 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2C9 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2D6 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP3A4 inhibitor	No	Categorical (Yes/No)
Excretion	Total Clearance	0,38	Numeric (log ml/min/kg)
Excretion	Renal OCT2 substrate	No	Categorical (Yes/No)
Toxicity	AMES toxicity	No	Categorical (Yes/No)
Toxicity	Max. tolerated dose (human)	-0,41	Numeric (log mg/kg/day)
Toxicity	hERG I inhibitor	No	Categorical (Yes/No)
Toxicity	hERG II inhibitor	No	Categorical (Yes/No)
Toxicity	Oral Rat Acute Toxicity (LD50)	2,61	Numeric (mol/kg)
Toxicity	Oral Rat Chronic Toxicity (LOAEL)	1,93	Numeric (log mg/kg_bw/day)
Toxicity	Hepatotoxicity	No	Categorical (Yes/No)
Toxicity	Skin Sensitisation	No	Categorical (Yes/No)
Toxicity	T.Pyriformis toxicity	0,44	Numeric (log ug/L)
Toxicity	Minnow toxicity	-1,60	Numeric (log mM)

Table A6: Drug-likeness and medicinal chemistry of ARM2, RA5, lovastatin and RA3

Drug-likeness						
Compounds	Lipinski	Ghose	Veber	Egan	Muegge	Oral Bioavailability Score
Lovastatin	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
ARM2	Yes; 1 violation: MLOGP>4.15	No; 3 violations: WLOGP>5.6, MR>130, #atoms>70	Yes	No; 1 violation: WLOGP>5.88	No; XLOGP3>5	0.56
RA5	Yes; 1 violation: MLOGP>4.15	No; 3 violations: WLOGP>5.6, MR>130, #atoms>70	Yes	No; 1 violation: WLOGP>5.88	No; XLOGP3>5	0.85
RA3	Yes; 1 violation: MLOGP>4.15	No; 3 violations: WLOGP>5.6, MR>130, #atoms>70	Yes	No; 1 violation: WLOGP>5.88	No; XLOGP3>5	0.55
Medicinal Chemistry						
Compounds	PAINS	Brenk	Leadlikeness	Synthetic Accessibility		
Lovastatin	0 alert	1 alert: more_than_2_esters	No; 2 violations: MW>350, XLOGP3>3.5	5.76		
ARM2	0 alert	1 alert: isolated_alkene	No; 2 violations: MW>350, XLOGP3>3.5	6.13		
RA5	0 alert	1 alert: isolated_alkene	No; 2 violations: MW>350, XLOGP3>3.5	5.76		
RA3	0 alert	1 alert: isolated_alkene	No; 2 violations: MW>350, XLOGP3>3.5	6.06		

Table A7: Swiss Target predictions for ARM-2

Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability	Known actives (3D/2D)
Prostaglandin E synthase	PTGES	O14684	CHEMBL5658	Enzyme	0,24	74/14
Protein farnesyltransferase	FNTA FNTB	P49354 P49356	CHEMBL20941 08	Enzyme	0,18	148/15
Nitric oxide synthase, inducible (by homology)	NOS2	P35228	CHEMBL4481	Enzyme	0,15	10/21
Carboxylesterase 2	CES2	O00748	CHEMBL3180	Enzyme	0,15	0/14
Cytochrome P450 19A1	CYP19A1	P11511	CHEMBL1978	Cytochrome P450	0,15	2/276
Glucocorticoid receptor	NR3C1	P04150	CHEMBL2034	Nuclear receptor	0,14	26/36
Protein-tyrosine phosphatase 1B	PTPN1	P18031	CHEMBL335	Phosphatase	0,14	93/65
T-cell protein-tyrosine phosphatase	PTPN2	P17706	CHEMBL3807	Phosphatase	0,14	28/19
Leukotriene B4 receptor 1	LTB4R	Q15722	CHEMBL3911	Family A G protein-coupled receptor	0,14	43/6
Peroxisome proliferator-activated receptor gamma	PPARG	P37231	CHEMBL235	Nuclear receptor	0,13	262/25
DNA polymerase beta (by homology)	POLB	P06746	CHEMBL2392	Enzyme	0,13	6/12
Prostanoid EP4 receptor	PTGER4	P35408	CHEMBL1836	Family A G protein-coupled receptor	0,13	211/18
Peroxisome proliferator-activated receptor alpha	PPARA	Q07869	CHEMBL239	Nuclear receptor	0,13	106/17
Aldo-keto reductase family 1 member B10	AKR1B10	O60218	CHEMBL5983	Enzyme	0,12	9/7
Peroxisome proliferator-activated receptor delta	PPARD	Q03181	CHEMBL3979	Nuclear receptor	0,12	51/11
Estrogen receptor alpha	ESR1	P03372	CHEMBL206	Nuclear receptor	0,12	62/39
Estrogen receptor beta	ESR2	Q92731	CHEMBL242	Nuclear receptor	0,12	13/47
11-beta-hydroxysteroid dehydrogenase 1	HSD11B1	P28845	CHEMBL4235	Enzyme	0,12	42/41
Progesterone receptor	PGR	P06401	CHEMBL208	Nuclear receptor	0,12	9/50
Prostanoid EP2 receptor	PTGER2	P43116	CHEMBL1881	Family A G protein-coupled receptor	0,12	153/29
Steroid 5-alpha-reductase 2	SRD5A2	P31213	CHEMBL1856	Oxidoreductase	0,12	6/34
Glucose-6-phosphate 1-dehydrogenase	G6PD	P11413	CHEMBL5347	Enzyme	0,12	0/7
Cytochrome P450 51 (by homology)	CYP51A1	Q16850	CHEMBL3849	Cytochrome P450	0,12	0/2

11-beta-hydroxysteroid dehydrogenase 2	HSD11B2	P80365	CHEMBL3746	Enzyme	0,12	10/20
HMG-CoA reductase	HMGCR	P04035	CHEMBL402	Oxidoreductase	0,12	173/32
Dual specificity phosphatase Cdc25A	CDC25A	P30304	CHEMBL3775	Phosphatase	0,12	7/19
Prostanoid EP1 receptor	PTGER1	P34995	CHEMBL1811	Family A G protein-coupled receptor	0,12	58/15
Prostanoid EP3 receptor	PTGER3	P43115	CHEMBL3710	Family A G protein-coupled receptor	0,12	37/6
TNF-alpha	TNF	P01375	CHEMBL1825	Secreted protein	0,12	8/6
Solute carrier family 22 member 6 (by homology)	SLC22A6	Q4U2R8	CHEMBL1641347	Electrochemical transporter	0,12	0/1
Fatty acid-binding protein, liver	FABP1	P07148	CHEMBL5421	Fatty acid binding protein family	0,12	0/3
Cytochrome P450 17A1	CYP17A1	P05093	CHEMBL3522	Cytochrome P450	0,12	0/44
Prolyl endopeptidase	PREP	P48147	CHEMBL3202	Protease	0,12	3/2
Beta-secretase 1	BACE1	P56817	CHEMBL4822	Protease	0,12	34/2
CD81 antigen	CD81	P60033	CHEMBL1075180	Surface antigen	0,12	1/1
Prostanoid DP receptor	PTGDR	Q13258	CHEMBL4427	Family A G protein-coupled receptor	0,12	81/1
Protein-tyrosine phosphatase 1C	PTPN6	P29350	CHEMBL3166	Phosphatase	0,12	4/1
Androgen Receptor	AR	P10275	CHEMBL1871	Nuclear receptor	0,12	6/133
Anandamide amidohydrolase	FAAH	O00519	CHEMBL2243	Enzyme	0,12	22/17
Prostanoid FP receptor	PTGFR	P43088	CHEMBL1987	Family A G protein-coupled receptor	0,12	18/8
Acyl-CoA desaturase	SCD	O00767	CHEMBL5555	Enzyme	0,12	0/1
Fatty acid binding protein adipocyte	FABP4	P15090	CHEMBL2083	Fatty acid binding protein family	0,12	8/4
Prostanoid IP receptor	PTGIR	P43119	CHEMBL1995	Family A G protein-coupled receptor	0,12	14/11
MAP kinase ERK1	MAPK3	P27361	CHEMBL3385	Kinase	0,12	2/1
Protein-tyrosine phosphatase 2C	PTPN11	Q06124	CHEMBL3864	Phosphatase	0,12	35/1
Nuclear receptor ROR-gamma	RORC	P51449	CHEMBL1741186	Nuclear receptor	0,12	2/10
Receptor-type tyrosine-protein phosphatase F (LAR)	PTPRF	P10586	CHEMBL3521	Membrane receptor	0,12	10/1
Low molecular weight phosphotyrosine protein phosphatase	ACP1	P24666	CHEMBL4903	Phosphatase	0,12	1/1
Phospholipase A2 group 1B	PLA2G1B	P04054	CHEMBL4426	Enzyme	0,12	5/1

Dual specificity phosphatase Cdc25B	CDC25B	P30305	CHEMBL4804	Phosphatase	0,12	12/6
LXR-alpha	NR1H3	Q13133	CHEMBL2808	Nuclear receptor	0,12	3/20
Corticosteroid binding globulin	SERPINA6	P08185	CHEMBL2421	Secreted protein	0,12	1/23
Testis-specific androgen-binding protein	SHBG	P04278	CHEMBL3305	Secreted protein	0,12	1/54
SUMO-activating enzyme	SAE1 UBA2	Q9UBE0 Q9UBT2	CHEMBL2095174	Enzyme	0,12	2/1
Protein kinase C eta	PRKCH	P24723	CHEMBL3616	Kinase	0,12	2/1
Nuclear receptor ROR-alpha	RORA	P35398	CHEMBL5868	Nuclear receptor	0,12	0/4
Serotonin transporter	SLC6A4	P31645	CHEMBL228	Electrochemical transporter	0,12	4/5
Fatty acid binding protein muscle	FABP3	P05413	CHEMBL3344	Fatty acid binding protein family	0,12	3/5
Fatty acid binding protein epidermal	FABP5	Q01469	CHEMBL3674	Fatty acid binding protein family	0,12	1/2
Phosphodiesterase 4D	PDE4D	Q08499	CHEMBL288	Phosphodiesterase	0,12	12/6
DNA topoisomerase II alpha	TOP2A	P11388	CHEMBL1806	Isomerase	0,12	1/2
Telomerase reverse transcriptase	TERT	O14746	CHEMBL2916	Enzyme	0,12	4/1
Arachidonate 5-lipoxygenase	ALOX5	P09917	CHEMBL215	Oxidoreductase	0,12	68/21
Dopamine transporter	SLC6A3	Q01959	CHEMBL238	Electrochemical transporter	0,12	4/1
Adenosine A3 receptor	ADORA3	P0DMS8	CHEMBL256	Family A G protein-coupled receptor	0,12	4/3
Type-1 angiotensin II receptor	AGTR1	P30556	CHEMBL227	Family A G protein-coupled receptor	0,12	138/0
Thromboxane A2 receptor	TBXA2R	P21731	CHEMBL2069	Family A G protein-coupled receptor	0,12	127/4
Matrix metalloproteinase 3	MMP3	P08254	CHEMBL283	Protease	0,12	118/0
Matrix metalloproteinase 1	MMP1	P03956	CHEMBL332	Protease	0,12	91/0
Matrix metalloproteinase 2	MMP2	P08253	CHEMBL333	Protease	0,12	154/0
Cyclooxygenase-2	PTGS2	P35354	CHEMBL230	Oxidoreductase	0,12	17/6
Niemann-Pick C1-like protein 1	NPC1L1	Q9UHC9	CHEMBL2027	Other membrane protein	0,12	1/12
Sigma opioid receptor	SIGMAR1	Q99720	CHEMBL287	Membrane receptor	0,12	1/5
Sphingosine 1-phosphate receptor Edg-5	S1PR2	O95136	CHEMBL2955	Family A G protein-coupled receptor	0,12	25/0

Endothelin receptor ET-B	EDNRB	P24530	CHEMBL1785	Family A G protein-coupled receptor	0,12	146/0
Endothelin receptor ET-A	EDNRA	P25101	CHEMBL252	Family A G protein-coupled receptor	0,12	351/0
Cannabinoid receptor 1	CNR1	P21554	CHEMBL218	Family A G protein-coupled receptor	0,12	13/8
p53-binding protein Mdm-2	MDM2	Q00987	CHEMBL5023	Other nuclear protein	0,12	83/0
Cytochrome P450 2C19	CYP2C19	P33261	CHEMBL3622	Cytochrome P450	0,12	0/4
AMP deaminase 2	AMPD2	Q01433	CHEMBL2997	Enzyme	0,12	13/0
Bile acid receptor FXR	NR1H4	Q96RI1	CHEMBL2047	Nuclear receptor	0,12	30/8
Squalene synthetase (by homology)	FDFT1	P37268	CHEMBL3338	Enzyme	0,12	47/3
Mineralocorticoid receptor	NR3C2	P08235	CHEMBL1994	Nuclear receptor	0,12	2/27
C-C chemokine receptor type 1	CCR1	P32246	CHEMBL2413	Family A G protein-coupled receptor	0,12	10/0
5-lipoxygenase activating protein	ALOX5A P	P20292	CHEMBL4550	Other cytosolic protein	0,12	84/0
Butyrylcholinesterase	BCHE	P06276	CHEMBL1914	Hydrolase	0,12	1/2
DNA topoisomerase I	TOP1	P11387	CHEMBL1781	Isomerase	0,12	3/2
Cysteinyl leukotriene receptor 1	CYSLTR 1	Q9Y271	CHEMBL1798	Family A G protein-coupled receptor	0,12	19/0
G protein-coupled receptor 44	PTGDR2	Q9Y5Y 4	CHEMBL5071	Family A G protein-coupled receptor	0,12	308/0
Integrin alpha-V/beta-3	ITGAV ITGB3	P06756 P05106	CHEMBL19075 98	Membrane receptor	0,12	338/0
HERG	KCNH2	Q12809	CHEMBL240	Voltage-gated ion channel	0,12	14/0
Voltage-gated T-type calcium channel alpha-1G subunit	CACNA1 G	O43497	CHEMBL4641	Voltage-gated ion channel	0,12	2/0
ADAMTS5	ADAMTS 5	Q9UNA 0	CHEMBL2285	Protease	0,12	11/0
Histamine H1 receptor	HRH1	P35367	CHEMBL231	Family A G protein-coupled receptor	0,12	46/0
Matrix metalloproteinase 14	MMP14	P50281	CHEMBL3869	Protease	0,12	23/0
Sterol regulatory element-binding protein 2	SREBF2	Q12772	CHEMBL17951 66	Unclassified protein	0,12	0/1
Thyroid hormone receptor alpha	THRA	P10827	CHEMBL1860	Nuclear receptor	0,12	108/0
Thyroid hormone receptor beta-1	THRB	P10828	CHEMBL1947	Nuclear receptor	0,12	118/0
Integrin alpha-4/beta-1	ITGB1 ITGA4	P05556 P13612	CHEMBL19075 99	Membrane receptor	0	388/0
Angiotensin II receptor	AGTR2	P50052	CHEMBL4607	Family A G protein-coupled receptor	0	28/0

Table A8: Swiss Target predictions for RA-5

Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Prostaglandin E synthase	PTGES	O14684	CHEMBL5658	Enzyme	0,59	32/14
Protein-tyrosine phosphatase 1B	PTPN1	P18031	CHEMBL335	Phosphatase	0,40	52/67
T-cell protein-tyrosine phosphatase	PTPN2	P17706	CHEMBL3807	Phosphatase	0,40	15/21
Aldo-keto reductase family 1 member B10	AKR1B10	O60218	CHEMBL5983	Enzyme	0,29	7/7
11-beta-hydroxysteroid dehydrogenase 1	HSD11B1	P28845	CHEMBL4235	Enzyme	0,23	32/43
DNA polymerase beta	POLB	P06746	CHEMBL2392	Enzyme	0,17	6/8
Carboxylesterase 2	CES2	O00748	CHEMBL3180	Enzyme	0,16	1/14
SUMO-activating enzyme	SAE1 UBA2	Q9UBE0 Q9UBT2	CHEMBL2095174	Enzyme	0,14	2/1
Nuclear receptor ROR-gamma	RORC	P51449	CHEMBL1741186	Nuclear receptor	0,14	1/10
Receptor-type tyrosine-protein phosphatase F (LAR)	PTPRF	P10586	CHEMBL3521	Membrane receptor	0,14	1/1
Low molecular weight phosphotyrosine protein phosphatase	ACP1	P24666	CHEMBL4903	Phosphatase	0,14	1/1
Peroxisome proliferator-activated receptor gamma	PPARG	P37231	CHEMBL235	Nuclear receptor	0,14	64/22
Corticosteroid binding globulin	SERPINA6	P08185	CHEMBL2421	Secreted protein	0,13	0/23
Testis-specific androgen-binding protein	SHBG	P04278	CHEMBL3305	Secreted protein	0,13	0/57
Glucose-6-phosphate 1-dehydrogenase	G6PD	P11413	CHEMBL5347	Enzyme	0,13	0/9
Cytochrome P450 51 (by homology)	CYP51A1	Q16850	CHEMBL3849	Cytochrome P450	0,13	0/2
Phospholipase A2 group 1B	PLA2G1B	P04054	CHEMBL4426	Enzyme	0,13	1/1
Dual specificity phosphatase Cdc25B	CDC25B	P30305	CHEMBL4804	Phosphatase	0,13	3/2
Phosphodiesterase 4D	PDE4D	Q08499	CHEMBL288	Phosphodiesterase	0,13	4/2
Fatty acid-binding protein, liver	FABP1	P07148	CHEMBL5421	Fatty acid binding protein family	0,13	0/3
Cytochrome P450 19A1	CYP19A1	P11511	CHEMBL1978	Cytochrome P450	0,12	0/296

Niemann-Pick C1-like protein 1	NPC1L1	Q9UHC9	CHEMBL2027	Other membrane protein	0,12	0/12
Sigma opioid receptor	SIGMAR1	Q99720	CHEMBL287	Membrane receptor	0,12	0/5
Cytochrome P450 17A1	CYP17A1	P05093	CHEMBL3522	Cytochrome P450	0,12	0/53
HMG-CoA reductase	HMGCR	P04035	CHEMBL402	Oxidoreductase	0,12	64/66
Glucocorticoid receptor	NR3C1	P04150	CHEMBL2034	Nuclear receptor	0,12	14/38
Androgen Receptor	AR	P10275	CHEMBL1871	Nuclear receptor	0,12	5/133
Protein-tyrosine phosphatase 2C	PTPN11	Q06124	CHEMBL3864	Phosphatase	0,12	8/1
Protein-tyrosine phosphatase 1C	PTPN6	P29350	CHEMBL3166	Phosphatase	0,12	2/2
Acyl-CoA desaturase	SCD	O00767	CHEMBL5555	Enzyme	0,12	0/1
LXR-alpha	NR1H3	Q13133	CHEMBL2808	Nuclear receptor	0,12	2/20
Telomerase reverse transcriptase	TERT	O14746	CHEMBL2916	Enzyme	0,12	1/1
Protein kinase C eta	PRKCH	P24723	CHEMBL3616	Kinase	0,12	2/1
11-beta-hydroxysteroid dehydrogenase 2	HSD11B2	P80365	CHEMBL3746	Enzyme	0,12	9/22
Protein farnesyltransferase	FNTA FNTB	P49354 P49356	CHEMBL2094108	Enzyme	0,12	32/14
Progesterone receptor	PGR	P06401	CHEMBL208	Nuclear receptor	0,12	5/50
DNA topoisomerase I	TOP1	P11387	CHEMBL1781	Isomerase	0,12	0/3
Prolyl endopeptidase	PREP	P48147	CHEMBL3202	Protease	0,12	2/3
Peroxisome proliferator-activated receptor alpha	PPARA	Q07869	CHEMBL239	Nuclear receptor	0,10	7/17
Peroxisome proliferator-activated receptor delta	PPARD	Q03181	CHEMBL3979	Nuclear receptor	0,10	7/10
CD81 antigen	CD81	P60033	CHEMBL1075180	Surface antigen	0,10	1/1
Butyrylcholinesterase	BCHE	P06276	CHEMBL1914	Hydrolase	0,10	0/2
Prostanoid EP2 receptor (by homology)	PTGER2	P43116	CHEMBL1881	Family A G protein-coupled receptor	0,10	70/24
Dual specificity phosphatase Cdc25A	CDC25A	P30304	CHEMBL3775	Phosphatase	0,10	4/12
Fatty acid binding protein adipocyte	FABP4	P15090	CHEMBL2083	Fatty acid binding protein family	0,10	6/4
Estrogen receptor beta	ESR2	Q92731	CHEMBL242	Nuclear receptor	0,10	7/47
Nuclear receptor ROR-alpha	RORA	P35398	CHEMBL5868	Nuclear receptor	0,10	0/4
Anandamide amidohydrolase	FAAH	O00519	CHEMBL2243	Enzyme	0,10	10/17
Prostanoid EP1 receptor (by homology)	PTGER1	P34995	CHEMBL1811	Family A G protein-coupled receptor	0,10	29/12

Nitric oxide synthase, inducible (by homology)	NOS2	P35228	CHEMBL4481	Enzyme	0,10	11/21
Beta-secretase 1	BACE1	P56817	CHEMBL4822	Protease	0,10	11/2
Fatty acid binding protein muscle	FABP3	P05413	CHEMBL3344	Fatty acid binding protein family	0,10	1/5
Fatty acid binding protein epidermal	FABP5	Q01469	CHEMBL3674	Fatty acid binding protein family	0,10	1/2
DNA topoisomerase II alpha	TOP2A	P11388	CHEMBL1806	Isomerase	0,10	1/2
Cyclooxygenase-1	PTGS1	P23219	CHEMBL221	Oxidoreductase	0,10	0/3
Prostanoid EP4 receptor (by homology)	PTGER4	P35408	CHEMBL1836	Family A G protein-coupled receptor	0,10	125/9
MAP kinase ERK1	MAPK3	P27361	CHEMBL3385	Kinase	0,10	1/1
Dopamine transporter	SLC6A3	Q01959	CHEMBL238	Electrochemical transporter	0,10	1/2
Muscarinic acetylcholine receptor M2	CHRM2	P08172	CHEMBL211	Family A G protein-coupled receptor	0,10	0/1
Acetylcholinesterase	ACHE	P22303	CHEMBL220	Hydrolase	0,10	0/3
Norepinephrine transporter	SLC6A2	P23975	CHEMBL222	Electrochemical transporter	0,10	0/3
Cytochrome P450 2C19	CYP2C19	P33261	CHEMBL3622	Cytochrome P450	0,10	0/4
Adenosine A3 receptor	ADORA3	P0DMS8	CHEMBL256	Family A G protein-coupled receptor	0,10	1/3
Arachidonate 5-lipoxygenase	ALOX5	P09917	CHEMBL215	Oxidoreductase	0,10	23/23
Bile acid receptor FXR	NR1H4	Q96RI1	CHEMBL2047	Nuclear receptor	0,10	26/10
Estrogen receptor alpha	ESR1	P03372	CHEMBL206	Nuclear receptor	0,10	47/39
Sterol regulatory element-binding protein 2	SREBF2	Q12772	CHEMBL1795166	Unclassified protein	0,10	0/1
Cyclooxygenase-2	PTGS2	P35354	CHEMBL230	Oxidoreductase	0,10	3/5
G-protein coupled bile acid receptor 1	GPBAR1	Q8TDU6	CHEMBL5409	Family A G protein-coupled receptor	0,10	14/14
Indoleamine 2,3-dioxygenase	IDO1	P14902	CHEMBL4685	Enzyme	0,10	0/1
Mineralocorticoid receptor	NR3C2	P08235	CHEMBL1994	Nuclear receptor	0,10	1/28
Steroid 5-alpha-reductase 2	SRD5A2	P31213	CHEMBL1856	Oxidoreductase	0,10	2/32
5-lipoxygenase activating protein	ALOX5AP	P20292	CHEMBL4550	Other cytosolic protein	0,10	26/0
Pregnane X receptor	NR1I2	O75469	CHEMBL3401	Nuclear receptor	0,10	0/3
Estradiol 17-beta-dehydrogenase 3	HSD17B3	P37058	CHEMBL4234	Enzyme	0,10	0/12
Leukotriene B4 receptor 1	LTB4R	Q15722	CHEMBL3911	Family A G protein-coupled receptor	0,10	18/6
Solute carrier family 22 member 6 (by homology)	SLC22A6	Q4U2R8	CHEMBL1641347	Electrochemical transporter	0,10	0/2

Dual specificity phosphatase Cdc25C	CDC25C	P30307	CHEMBL2378	Phosphatase	0,10	0/1
Cannabinoid receptor 1 (by homology)	CNR1	P21554	CHEMBL218	Family A G protein-coupled receptor	0,10	8/17
Prostanoid DP receptor	PTGDR	Q13258	CHEMBL4427	Family A G protein-coupled receptor	0,10	37/1
Nuclear receptor subfamily 1 group I member 3 (by homology)	NR1I3	Q14994	CHEMBL5503	Nuclear receptor	0,10	0/2
Thyroid hormone receptor alpha	THRA	P10827	CHEMBL1860	Nuclear receptor	0,10	52/0
Thyroid hormone receptor beta-1	THRB	P10828	CHEMBL1947	Nuclear receptor	0,10	54/0
Free fatty acid receptor 1	FFAR1	O14842	CHEMBL4422	Family A G protein-coupled receptor	0,10	1/3
p53-binding protein Mdm-2	MDM2	Q00987	CHEMBL5023	Other nuclear protein	0,10	51/0
Prostanoid IP receptor	PTGIR	P43119	CHEMBL1995	Family A G protein-coupled receptor	0,10	6/8
Vanilloid receptor	TRPV1	Q8NER1	CHEMBL4794	Voltage-gated ion channel	0,10	0/2
Matrix metalloproteinase 3	MMP3	P08254	CHEMBL283	Protease	0,10	36/0
Matrix metalloproteinase 1	MMP1	P03956	CHEMBL332	Protease	0,10	22/0
Matrix metalloproteinase 2	MMP2	P08253	CHEMBL333	Protease	0,10	49/0
Cathepsin D	CTSD	P07339	CHEMBL2581	Protease	0,10	4/1
Serotonin transporter	SLC6A4	P31645	CHEMBL228	Electrochemical transporter	0,10	1/5
Prostanoid EP3 receptor	PTGER3	P43115	CHEMBL3710	Family A G protein-coupled receptor	0	17/3
Type-1 angiotensin II receptor	AGTR1	P30556	CHEMBL227	Family A G protein-coupled receptor	0	53/0
Squalene synthetase	FDFT1	P37268	CHEMBL3338	Enzyme	0	21/4
Integrin alpha-4/beta-1	ITGB1 ITGA4	P05556 P13612	CHEMBL19075 99	Membrane receptor	0	85/0
Vitamin D receptor	VDR	P11473	CHEMBL1977	Nuclear receptor	0	9/40
Arachidonate 12-lipoxygenase	ALOX12	P18054	CHEMBL3687	Enzyme	0	0/2
TNF-alpha	TNF	P01375	CHEMBL1825	Secreted protein	0	5/8
Toll-like receptor (TLR7/TLR9)	TLR9	Q9NR96	CHEMBL5804	Toll-like and IL-1 receptors	0	1/0

Table A9: Swiss Target prediction of RA-3

Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
LXR-alpha	NR1H3	Q13133	CHEMBL2808	Nuclear receptor	0,15	7/19
Prostaglandin E synthase	PTGES	O14684	CHEMBL5658	Enzyme	0,15	0/14
Carboxylesterase 2	CES2	O00748	CHEMBL3180	Enzyme	0,15	5/14
11-beta-hydroxysteroid dehydrogenase 1	HSD11B 1	P28845	CHEMBL4235	Enzyme	0,14	203/49
Protein-tyrosine phosphatase 1B	PTPN1	P18031	CHEMBL335	Phosphatase	0,13	11/74
Cyclooxygenase-2	PTGS2	P35354	CHEMBL230	Oxidoreductase	0,13	16/8
Cytochrome P450 17A1	CYP17A 1	P05093	CHEMBL3522	Cytochrome P450	0,12	55/45
DNA topoisomerase II alpha	TOP2A	P11388	CHEMBL1806	Isomerase	0,12	1/2
Androgen Receptor	AR	P10275	CHEMBL1871	Nuclear receptor	0,12	42/140
Estradiol 17-beta-dehydrogenase 2	HSD17B 2	P37059	CHEMBL2789	Enzyme	0,12	0/2
Protein-tyrosine phosphatase 1C	PTPN6	P29350	CHEMBL3166	Phosphatase	0,12	0/2
T-cell protein-tyrosine phosphatase	PTPN2	P17706	CHEMBL3807	Phosphatase	0,12	0/24
Cytochrome P450 19A1	CYP19A 1	P11511	CHEMBL1978	Cytochrome P450	0,12	14/286
11-beta-hydroxysteroid dehydrogenase 2	HSD11B 2	P80365	CHEMBL3746	Enzyme	0,12	10/23
Prostanoid EP2 receptor	PTGER2	P43116	CHEMBL1881	Family A G protein-coupled receptor	0,12	0/20
Prostanoid IP receptor	PTGIR	P43119	CHEMBL1995	Family A G protein-coupled receptor	0,12	0/6
Nitric oxide synthase, inducible (by homology)	NOS2	P35228	CHEMBL4481	Enzyme	0,12	2/21
Dual specificity phosphatase Cdc25A	CDC25A	P30304	CHEMBL3775	Phosphatase	0,12	5/25
Protein-tyrosine phosphatase 2C	PTPN11	Q06124	CHEMBL3864	Phosphatase	0,12	0/1
Aldo-keto reductase family 1 member B10	AKR1B1 0	O60218	CHEMBL5983	Enzyme	0,12	0/7
HMG-CoA reductase	HMGCR	P04035	CHEMBL402	Oxidoreductase	0,12	127/90
Corticosteroid binding globulin	SERPIN A6	P08185	CHEMBL2421	Secreted protein	0,12	0/23
Glucose-6-phosphate 1-dehydrogenase	G6PD	P11413	CHEMBL5347	Enzyme	0,12	0/7
Cytochrome P450 51 (by homology)	CYP51A 1	Q16850	CHEMBL3849	Cytochrome P450	0,12	10/2
Prolyl endopeptidase	PREP	P48147	CHEMBL3202	Protease	0,12	0/3

Testis-specific androgen-binding protein	SHBG	P04278	CHEMBL3305	Secreted protein	0,12	1/54
Fatty acid binding protein adipocyte	FABP4	P15090	CHEMBL2083	Fatty acid binding protein family	0,12	0/4
Fatty acid binding protein muscle	FABP3	P05413	CHEMBL3344	Fatty acid binding protein family	0,12	0/5
Fatty acid binding protein epidermal	FABP5	Q01469	CHEMBL3674	Fatty acid binding protein family	0,12	0/2
Peroxisome proliferator-activated receptor delta	PPARD	Q03181	CHEMBL3979	Nuclear receptor	0,12	0/11
Fatty acid-binding protein, liver	FABP1	P07148	CHEMBL5421	Fatty acid binding protein family	0,12	0/3
Glucocorticoid receptor	NR3C1	P04150	CHEMBL2034	Nuclear receptor	0,12	56/40
Protein kinase C eta	PRKCH	P24723	CHEMBL3616	Kinase	0,12	8/1
Aldo-keto reductase family 1 member C2	AKR1C2	P52895	CHEMBL5847	Enzyme	0,12	0/1
Aldo-keto reductase family 1 member C1	AKR1C1	Q04828	CHEMBL5905	Enzyme	0,12	0/1
Niemann-Pick C1-like protein 1	NPC1L1	Q9UHC9	CHEMBL2027	Other membrane protein	0,12	3/16
MAP kinase ERK1	MAPK3	P27361	CHEMBL3385	Kinase	0,12	0/1
Butyrylcholinesterase	BCHE	P06276	CHEMBL1914	Hydrolase	0,12	5/3
Beta-secretase 1	BACE1	P56817	CHEMBL4822	Protease	0,12	125/1
DNA polymerase beta	POLB	P06746	CHEMBL2392	Enzyme	0,12	0/18
Receptor-type tyrosine-protein phosphatase F (LAR)	PTPRF	P10586	CHEMBL3521	Membrane receptor	0,12	0/1
Phospholipase A2 group 1B	PLA2G1B	P04054	CHEMBL4426	Enzyme	0,12	0/1
Low molecular weight phosphotyrosine protein phosphatase	ACP1	P24666	CHEMBL4903	Phosphatase	0,12	0/3
Dual specificity phosphatase Cdc25B	CDC25B	P30305	CHEMBL4804	Phosphatase	0,12	5/12
DNA topoisomerase I	TOP1	P11387	CHEMBL1781	Isomerase	0,12	0/3
Nuclear receptor ROR-alpha	RORA	P35398	CHEMBL5868	Nuclear receptor	0,12	0/3
Phosphodiesterase 4D	PDE4D	Q08499	CHEMBL288	Phosphodiesterase	0,12	5/6
Protein farnesyltransferase	FNTA FNTB	P49354 P49356	CHEMBL2094108	Enzyme	0,12	5/12
NACHT, LRR and PYD domains-containing protein 3	NLRP3	Q96P20	CHEMBL1741208	Unclassified protein	0,12	0/1

SUMO-activating enzyme	SAE1 UBA2	Q9UBE0 Q9UBT2	CHEMBL2095174	Enzyme	0,12	0/1
Nuclear receptor ROR-gamma	RORC	P51449	CHEMBL1741186	Nuclear receptor	0,12	11/9
Arachidonate 5-lipoxygenase	ALOX5	P09917	CHEMBL215	Oxidoreductase	0,12	11/38
Mineralocorticoid receptor	NR3C2	P08235	CHEMBL1994	Nuclear receptor	0,12	3/27
Progesterone receptor	PGR	P06401	CHEMBL208	Nuclear receptor	0,12	11/51
Trypsin I	PRSS1	P07477	CHEMBL209	Protease	0,12	0/5
Chymotrypsin C	CTRC	Q99895	CHEMBL2386	Protease	0,12	0/3
CD81 antigen	CD81	P60033	CHEMBL1075180	Surface antigen	0,12	0/1
Dopamine transporter	SLC6A3	Q01959	CHEMBL238	Electrochemical transporter	0,12	10/3
Sigma opioid receptor	SIGMAR1	Q99720	CHEMBL287	Membrane receptor	0,12	19/5
Telomerase reverse transcriptase	TERT	O14746	CHEMBL2916	Enzyme	0,12	3/2
Estrogen receptor beta	ESR2	Q92731	CHEMBL242	Nuclear receptor	0,12	7/47
Free fatty acid receptor 1	FFAR1	O14842	CHEMBL4422	Family A G protein-coupled receptor	0,12	0/3
Adenosine A3 receptor	ADORA3	P0DMS8	CHEMBL256	Family A G protein-coupled receptor	0,12	3/1
Prostanoid EP4 receptor (by homology)	PTGER4	P35408	CHEMBL1836	Family A G protein-coupled receptor	0,12	0/6
C-C chemokine receptor type 1	CCR1	P32246	CHEMBL2413	Family A G protein-coupled receptor	0,12	84/0
Anandamide amidohydrolase	FAAH	O00519	CHEMBL2243	Enzyme	0,12	6/18
Thrombin	F2	P00734	CHEMBL204	Protease	0,12	24/7
Peroxisome proliferator-activated receptor gamma	PPARG	P37231	CHEMBL235	Nuclear receptor	0,12	1/25
Peroxisome proliferator-activated receptor alpha	PPARA	Q07869	CHEMBL239	Nuclear receptor	0,12	1/17
Aldo-keto-reductase family 1 member C3	AKR1C3	P42330	CHEMBL4681	Enzyme	0,12	4/1
Beta amyloid A4 protein	APP	P05067	CHEMBL2487	Membrane receptor	0,12	22/0
Neurokinin 1 receptor	TACR1	P25103	CHEMBL249	Family A G protein-coupled receptor	0,12	37/0
Cytochrome P450 2C19	CYP2C19	P33261	CHEMBL3622	Cytochrome P450	0,12	3/7
Thromboxane A2 receptor	TBXA2R	P21731	CHEMBL2069	Family A G protein-coupled receptor	0,12	4/10
Cathepsin S	CTSS	P25774	CHEMBL2954	Protease	0	32/0
Cathepsin L	CTSL	P07711	CHEMBL3837	Protease	0	8/0

Endothelial PAS domain-containing protein 1	EPAS1	Q99814	CHEMBL1744522	Unclassified protein	0	0/1
Phospholipase A2 group IIA	PLA2G2A	P14555	CHEMBL3474	Enzyme	0	0/3
Vasopressin V1a receptor	AVPR1A	P37288	CHEMBL1889	Family A G protein-coupled receptor	0	15/0
Gamma-secretase	PSEN2 PSENEN NCSTN APH1A PSEN1 APH1B	P49810 Q9NZ42 Q92542 Q96B13 P49768 Q8WW43	CHEMBL2094135	Protease	0	31/0
Cannabinoid receptor 1	CNR1	P21554	CHEMBL218	Family A G protein-coupled receptor	0	242/23
Cannabinoid receptor 2	CNR2	P34972	CHEMBL253	Family A G protein-coupled receptor	0	199/7
Leukotriene B4 receptor 1	LTB4R	Q15722	CHEMBL3911	Family A G protein-coupled receptor	0	0/6
Protein kinase C alpha	PRKCA	P17252	CHEMBL299	Kinase	0	55/15
LXR-beta	NR1H2	P55055	CHEMBL4093	Nuclear receptor	0	7/1
Protein Mdm4	MDM4	O15151	CHEMBL1255126	Unclassified protein	0	7/0
Monocarboxylate transporter 1	SLC16A1	P53985	CHEMBL4360	Electrochemical transporter	0	17/0
p53-binding protein Mdm-2	MDM2	Q00987	CHEMBL5023	Other nuclear protein	0	84/0
Acyl-CoA desaturase	SCD	O00767	CHEMBL5555	Enzyme	0	4/1
Serotonin 2b (5-HT2b) receptor	HTR2B	P41595	CHEMBL1833	Family A G protein-coupled receptor	0	3/0
Adrenergic receptor alpha-2	ADRA2C	P18825	CHEMBL1916	Family A G protein-coupled receptor	0	2/0
Indoleamine 2,3-dioxygenase	IDO1	P14902	CHEMBL4685	Enzyme	0	0/1
Cholecystokinin B receptor (by homology)	CCKBR	P32239	CHEMBL298	Family A G protein-coupled receptor	0	41/0
C5a anaphylatoxin chemotactic receptor	C5AR1	P21730	CHEMBL2373	Family A G protein-coupled receptor	0	20/0
GABA transporter 1 (by homology)	SLC6A1	P30531	CHEMBL1903	Electrochemical transporter	0	2/0
Phosphodiesterase 4A	PDE4A	P27815	CHEMBL254	Phosphodiesterase	0	3/0
Phosphodiesterase 4B	PDE4B	Q07343	CHEMBL275	Phosphodiesterase	0	6/0
Phosphodiesterase 4C	PDE4C	Q08493	CHEMBL291	Phosphodiesterase	0	2/0
Calcium sensing receptor	CASR	P41180	CHEMBL1878	Family C G protein-coupled receptor	0	58/0
Estrogen receptor alpha	ESR1	P03372	CHEMBL206	Nuclear receptor	0	8/38

Appendix B: *In vitro* studies

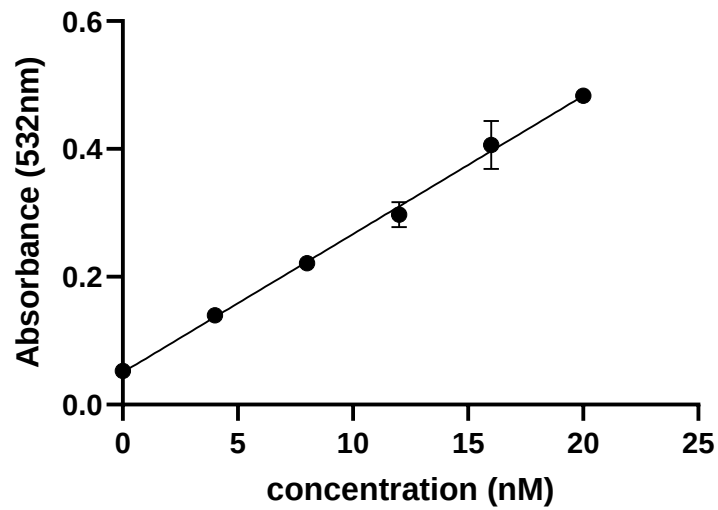
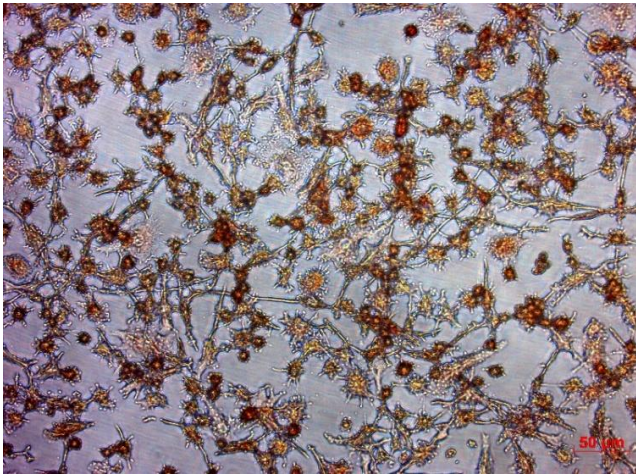


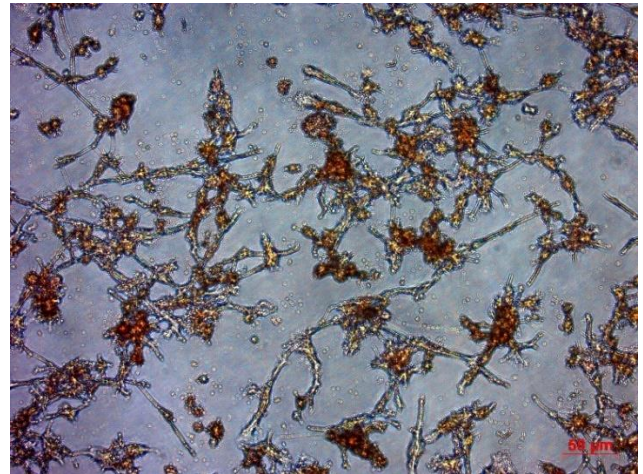
Figure B1: Standard curve of malondialdehyde (MDA). The data is expressed as the mean $\pm$ SEM of two independent repeats in triplicate. $R^2=0.99$; $p<0.0001$.

A oxLDL Treated/ Cells only

oxLDL (model control)

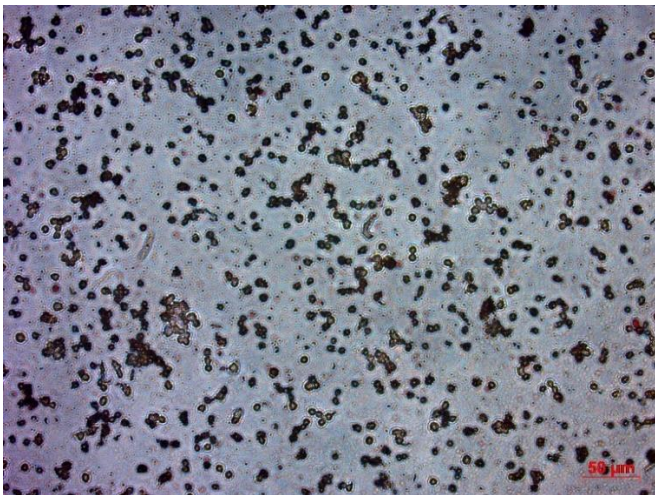


Normal control (cells + PBS)

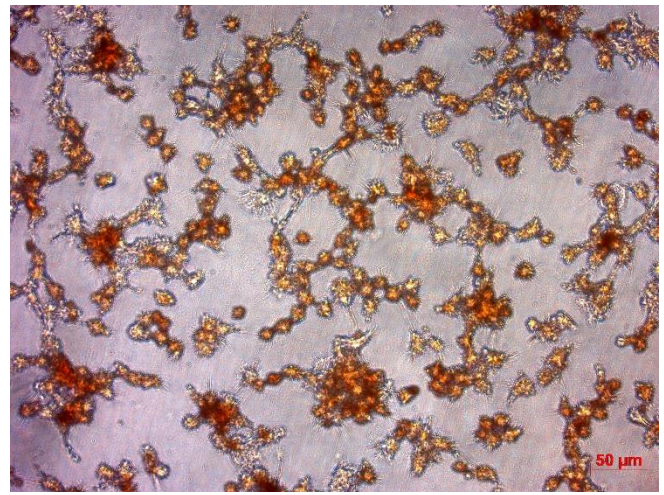


B Triton X/ 1% DMSO

Triton X + PBS

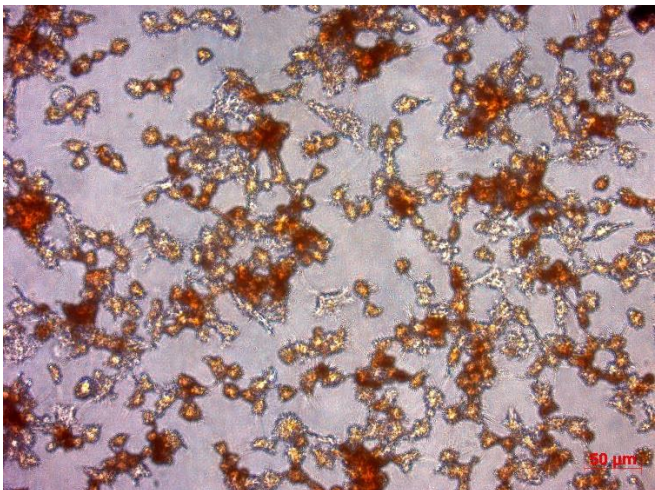


1% DMSO + PBS

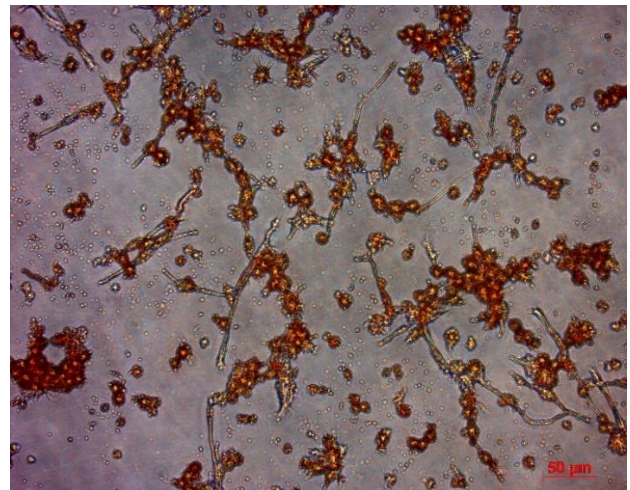


C 1% DMSO/ Reference Drug

oxLDL + 1% DMSO



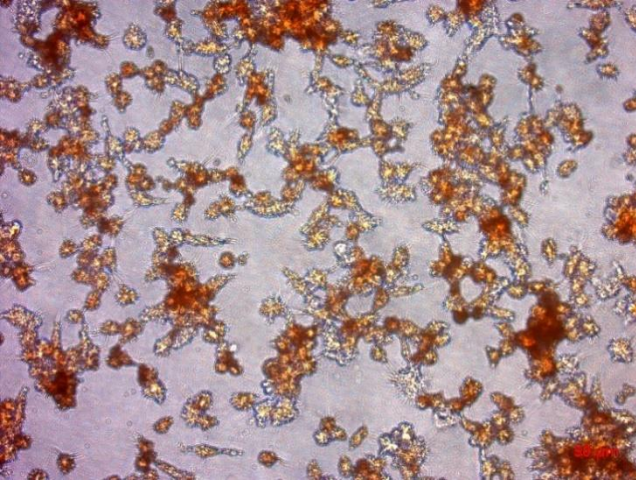
oxLDL + 1 µM Lovastatin



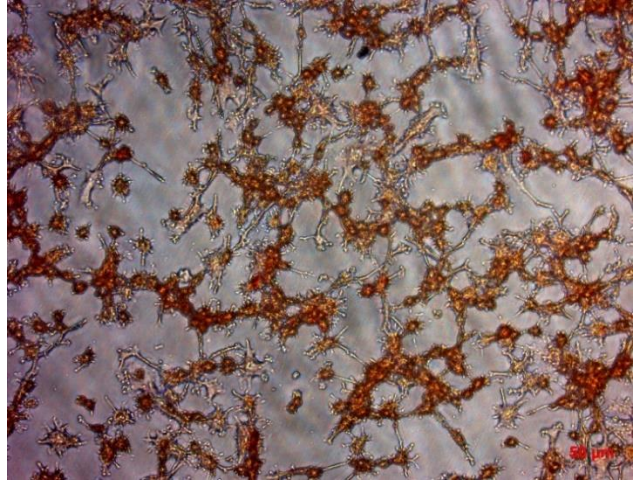
D

Compounds at 1 μ M

oxLDL + 1 μ M ARM-2



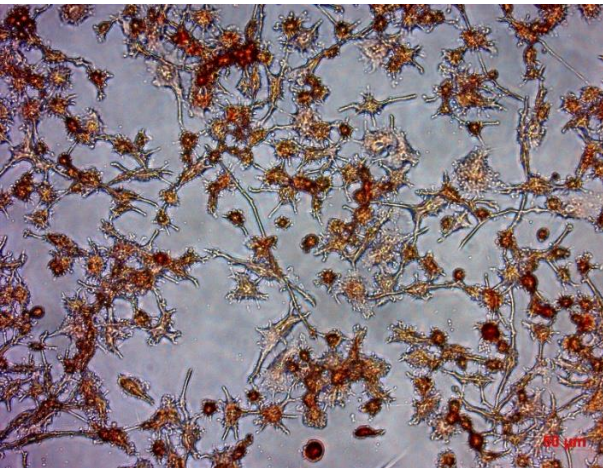
oxLDL + 1 μ M RA-5



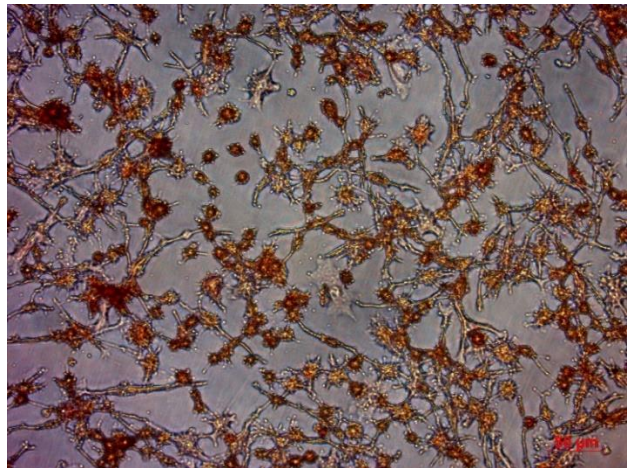
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Compounds at 10 μ M

oxLDL + 10 μ M ARM-2



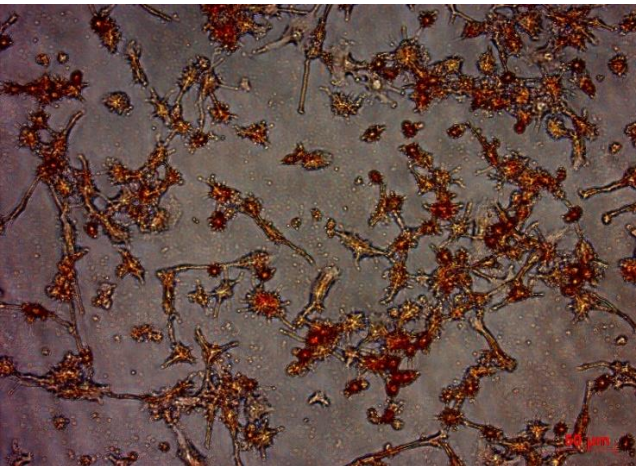
oxLDL + 10 μ M RA-5



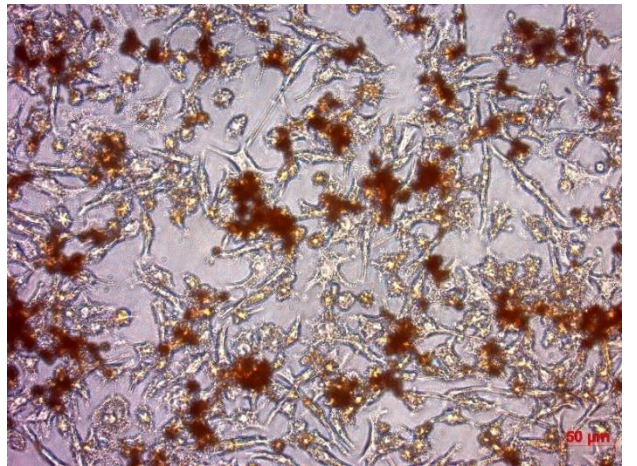
F

Compounds at 25 μ M

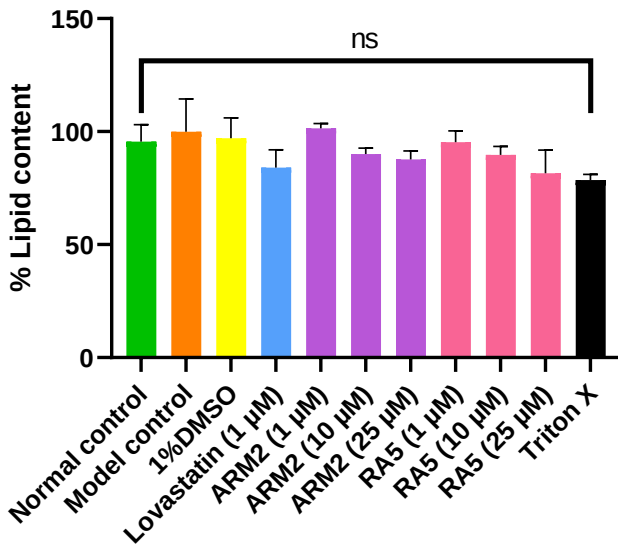
oxLDL + 25 μ M ARM-2



oxLDL + 25 μ M RA-5



G



H

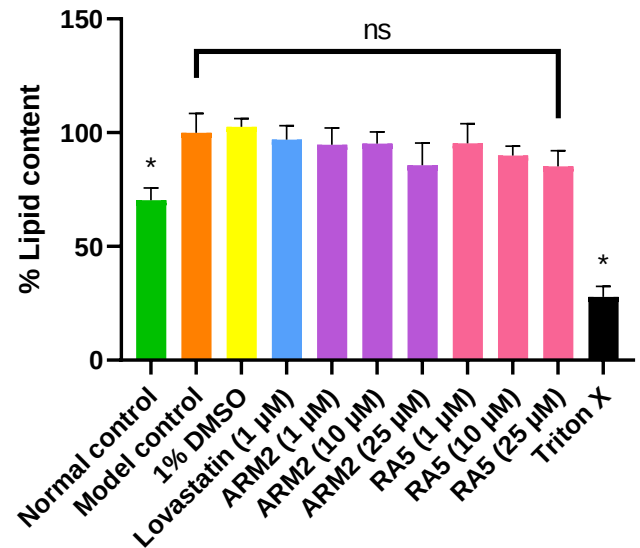


Figure B2: Effects of ARM-2 and RA-5 on oxLDL induced lipid accumulation in RAW264.7 macrophage cells. (A-F) Images of oil-red O-stained lipid droplets (foam cell formation). Micrograph scale is 10 µM. (F) Lipid content of oil red o-stained cells measured at 405nm. (H) Lipid content of cells stained with nile red, fluorescence measured at Ex 544nm and Em 590nm (1300 gain). The data is expressed as the mean ± SEM of four (oil red o) and three (nile red) independent repeats in triplicate. * $p < 0.05$ with regards to model control.

Appendix C: Ethical Clearance



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Natural and Agricultural Sciences
Ethics Committee
E-mail: ethics.nas@up.ac.za

6 June 2022

ETHICS SUBMISSION: LETTER OF APPROVAL

Ms NN Ramasia
Department of Biochemistry, Genetics and Microbiology
Faculty of Natural and Agricultural Science
University of Pretoria

Reference number: NAS280/2021

Project title: Antiatherogenic potential of the lanosteryl triterpenes from *Protorhus longifolia* stem bark (Benrh) Engl

Dear Ms NN Ramasia,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Natural and Agricultural Sciences Research Ethics Committee.

Please note the following about your ethics approval:

- Please use your reference number (NAS280/2021) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
- Please note that ethical approval is granted for the duration of the research (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
- The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
- **If Applications using GM permits:** If the GM permit expires before the end of the study, please make an amendment to the application with the new GM permit before the old one expires
- **If Applications using Animals:** NAS ethics recommendation does not imply that Animal Ethics Committee (AEC) approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Post approval submissions including application for ethics extension and amendments to the approved application should be submitted online via the Ethics work centre.

We wish you the best with your research.

Yours sincerely,

Prof VJ Maharaj
Chairperson: NAS Ethics Committee