Effects of polyphenols on nuclear receptor activation and their role in modulating inflammation

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Abstract

Polyphenols are associated with numerous biological activities and health benefits for human health. However, the exact mechanism of how polyphenols act on a cellular level is not well known. It has recently been suggested that some members of the flavonoid subgroup of polyphenols might have the ability to act as ligands for nuclear receptors and thereby impact on transcriptional regulation of metabolic pathways, such as cell development, energy metabolism, and inflammation. Meanwhile, LXRα is a ligand-dependent nuclear receptor that plays an important role in the control of lipid and cholesterol metabolism, as well as inflammatory disease making LXRα an interesting target. Therefore, this project aimed to assess the role of LXRα in the anti-inflammatory properties of polyphenols. Representatives of each polyphenol class were tested for their ability to act as a ligand for LXRα in MDA-MB-231 stably transfected with LXRα target gene as the cell model, followed by the evaluation of LXRα target genes modulation in vitro.

Quercetin appears to have the potency as a partial agonist of LXRα by showing the ability to modulate LXRα activity on both MDA-MB-231 and HepG2 cell lines. This study demonstrated that there is a structure-function relationship between polyphenol and ligand-activated function of LXRα as shown by the ability of tamarixetin but not isorhamnetin, both methylated form metabolites of quercetin, to induce the activity of LXRα. Furthermore, in the hepatic inflammation model, quercetin, tamarixetin, and GW3965 as LXRα ligand, failed to suppress inflammatory cytokine production following TNF-α induced inflammation.

Meanwhile, GW3965 and quercetin showed anti-inflammatory activities in RAW264.7 macrophages. Both compounds inhibit inflammation by interfering with the NF-κB signaling pathway and inhibit pro-inflammatory cytokines production. The anti-inflammatory properties of quercetin are independent of LXRα. This study showed that LXRα is partially involved in the anti-inflammatory property of quercetin. Moreover, the ability of quercetin to suppress inflammation is not dependent on ABCA1/LXRα pathway. More studies are needed to understand the relationship between polyphenol structure and nuclear receptor activation and its biological function.

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Abbreviations

OHC	: hydroxycholesterol
LXR	: liver X receptor
PPAR	: peroxisome proliferated-activated receptor
NR	: nuclear receptor
GR	: glucocorticoid receptor
ER	: estrogen receptor
VDR	: vitamin D receptor
STAT	: signal transducer and activator of transcription
IRF	: interferon regulatory factor
NF-κB	: nuclear factor kappa-light-chain-enhancer of activated B cells
IL	: interleukin
CRP	: C-reactive protein
LPH	: lactase-phlorizin hydrolase
SGLT	: sodium-glucose transport protein
GLUT	: glucose transporters
UGT	: uridine glucuronosyltransferase
ERK1/2	: extracellular signal-regulated kinases 1/2
JNK	: c-Jun N-terminal kinase
TNF	: tumor necrosis factor
LPS	: lipopolysaccharides
MAPK	: mitogen-activated protein kinase
TLR	: toll like receptors
CREB	: cAMP response element-binding protein
ATF	: activating transcription factor
PGE2	: prostaglandin E2
LTB4	: leukotriene B4
ROS	: reactive oxygen species
AP-1	: activator protein-1
RLR	: RIG-I like receptor

NLR : NOD-like receptor
PRR : pattern recognition receptor
CLR : C-type lectin receptor
PAMP : pathogen-associated molecular pattern
DAMPs : damage-associated molecular patterns
PR : progesterone receptor
TR : thyroid receptor
DEPC : deionized, diethylpyrocarbonate
DBD : DNA binding domain
LBD : ligand binding domain
DNA : deoxyribonucleic acid
mRNA : micro ribonucleic acid
RXR : retinoid X receptor
RNA : ribonucleic acid
AR : androgen receptor
COX2 : cyclooxygenase 2
iNOS : inducible nitric oxide synthase
EGCG : epigallocatechin gallate
H_2O_2 : hydrogen peroxide
q-RT-PCR : quantitative real time polymerase chain reaction
PBS : phosphate buffer saline
SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST : tris-buffered saline-tween
ABCA1 : ATP-binding cassette transporter-1
APOE : apolipoprotein E
TSS : transcription start site

Chapter 1. Introduction

Polyphenols are naturally occurring plant bioactive compounds that have been associated with some health benefits for humans (Del Bo' *et al.*, 2019). A recent review demonstrated that polyphenol consumption has been linked to human health due to its numerous biological effects such as anti-inflammatory activity as shown by quercetin by inhibiting the inflammatory signalling during atherosclerotic development. Moreover, resveratrol also found to inactivate the activity of peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear receptor that regulate energy homeostasis and metabolic function (Cosme *et al.*, 2020). Nuclear receptors are defined as a family of ligand-regulated transcription factors that regulate various physiological functions including general metabolism, reproduction, development as well as inflammation (Sever and Glass, 2013). Conflicting results of anti-inflammatory properties of polyphenol are observed in both in vitro and in vivo studies indicating that inflammation is a complex mechanism that has not been fully understood. Numerous studies demonstrate that polyphenol regulates inflammation by inflammatory cytokines modulation and inhibits the signalling pathway of transcription factors such as NF-κβ, AP-1, and Nrf2 (Lawrence, 2009; Saha *et al.*, 2020). The role of transcription factors in inflammation as regulators of inflammatory target gene expression has gained much interests. Signal transducers and activators of transcription (STATs), interferon regulatory factors (IRFs), and nuclear factor κB (NFκB) are three main transcription factors known to regulate inflammatory transcriptional response (Platanitis and Decker, 2018). Since polyphenols are prominent in suppressing inflammatory cytokines production, their expression may be mediated through nuclear receptor signaling. However, apart from the anti-inflammatory activity of polyphenols, it is still unclear whether their action is preceded by modulation of gene transcription involved during inflammation. The previous study revealed that flavonoids may act as a dietary regulator and as a ligand for numerous nuclear receptors such as CAR, ERα, Erβ, and LXRα hence modulate their biological activity in regulating metabolic function (Avior *et al.*, 2013), however, studies lacking that address this aspect in detail. Studies on polyphenols as activators of nuclear receptors may shed more light on cellular targets of polyphenols which may support the development of novel therapies.

1.1 A review on polyphenols: classification and beneficial effects

Polyphenols are naturally occurring bioactive compounds widely found in plants. A polyphenol-rich diet has been linked with numerous health benefits, especially in the prevention of chronic diseases (Knekt *et al.*, 2002). The strong antioxidant and anti-inflammatory properties of polyphenols are considered as the key for their benefits towards human health although recently increasing evidence suggests that polyphenols may have more beneficial effects on health (D'Archivio *et al.*, 2010; Kim, Quon and Kim, 2014; Del Bo' *et al.*, 2019).

Dietary intake of fruits, vegetables, and beverages such as tea, coffee, and wine are the main source of polyphenols in the diet. Since the health benefits of polyphenols are highly associated with their bioavailability (D'Archivio *et al.*, 2010), it is important to consider numerous factors including food matrix, gut microbiota, food processing, and initial content in foods (Arfaoui, 2021). These may be the reasons for differences in the daily average polyphenol intake among the population. On average, the intake of polyphenols among the French adult population is about 377.5 mg/day (Perez-Jimenez *et al.*, 2011) and around 332.7 mg/day in older adults based on a study from Mallorca island (Karam, Bibiloni, and Tur, 2018). Meanwhile, the average flavonoid intake in UK and Ireland populations was about 176.8 and 182.2 mg/day respectively (Beking and Vieira, 2011). A cross-sectional study was launched to determine the association between the intake of polyphenols related to the reduced risk for chronic diseases. For example, anthocyanin and flavone intake was associated with lower insulin concentrations and peripheral insulin resistance (Jennings *et al.*, 2014). Furthermore, a cohort study revealed that polyphenol intake has also been linked with low-grade inflammation marked by lower CRP levels and white blood cells count (Pounis *et al.*, 2016). Although several epidemiological studies demonstrated a positive correlation of polyphenol consumption with health outcomes (Dauchet, Amouyel, and Dallongeville, 2005; Ghosh and Scheepens, 2009), many questions remain such as dose-response relationships in vivo and efficacy of individual polyphenols on specific mechanisms.

1.1.1 Polyphenol classification and dietary sources

In general, polyphenols are a group of plant bioactive compounds characterized by at least two phenolic rings with one or more hydroxyl groups attached (Pandey and Rizvi, 2009). In plants, polyphenols are mainly existing in the form of glycosides, with one or more sugar residues linked to the hydroxyl groups. Polyphenols are typically classified into different subgroups based on their structure. The difference in chemical structure may affect their stability, bioavailability, and beneficial function related to human health (Tsao, 2010).

Simple classification categorizes polyphenols into four groups as shown in Figure 1.1. This classification is based on the number of phenol rings where flavonoids contain two phenol rings and non-flavonoid only have one phenol ring. All flavonoids share the same basic structure of diphenyl propane (C6-C3-C6), where both phenolic rings (A and B) are linked by a heterocyclic closed pyran ring (C) (Figure 1.2). Flavonoids can further be classified based on the oxidation of central carbon. Most flavonoids have their ring B attached to the C2 position of ring C (Figure 1.3) including flavanols, flavonols, and flavonols, but not isoflavones where its ring B is connected at the C3 position of the C ring. Flavonoids are the most abundant polyphenols available in the human diet. Online databases such as the Phenol-Explorer (http://phenol-explorer.eu/) provide details on comprehensive polyphenol content in a range of foods as shown in Table 1 (Neveu *et al.*, 2010; Rothwell *et al.*, 2012, 2013).

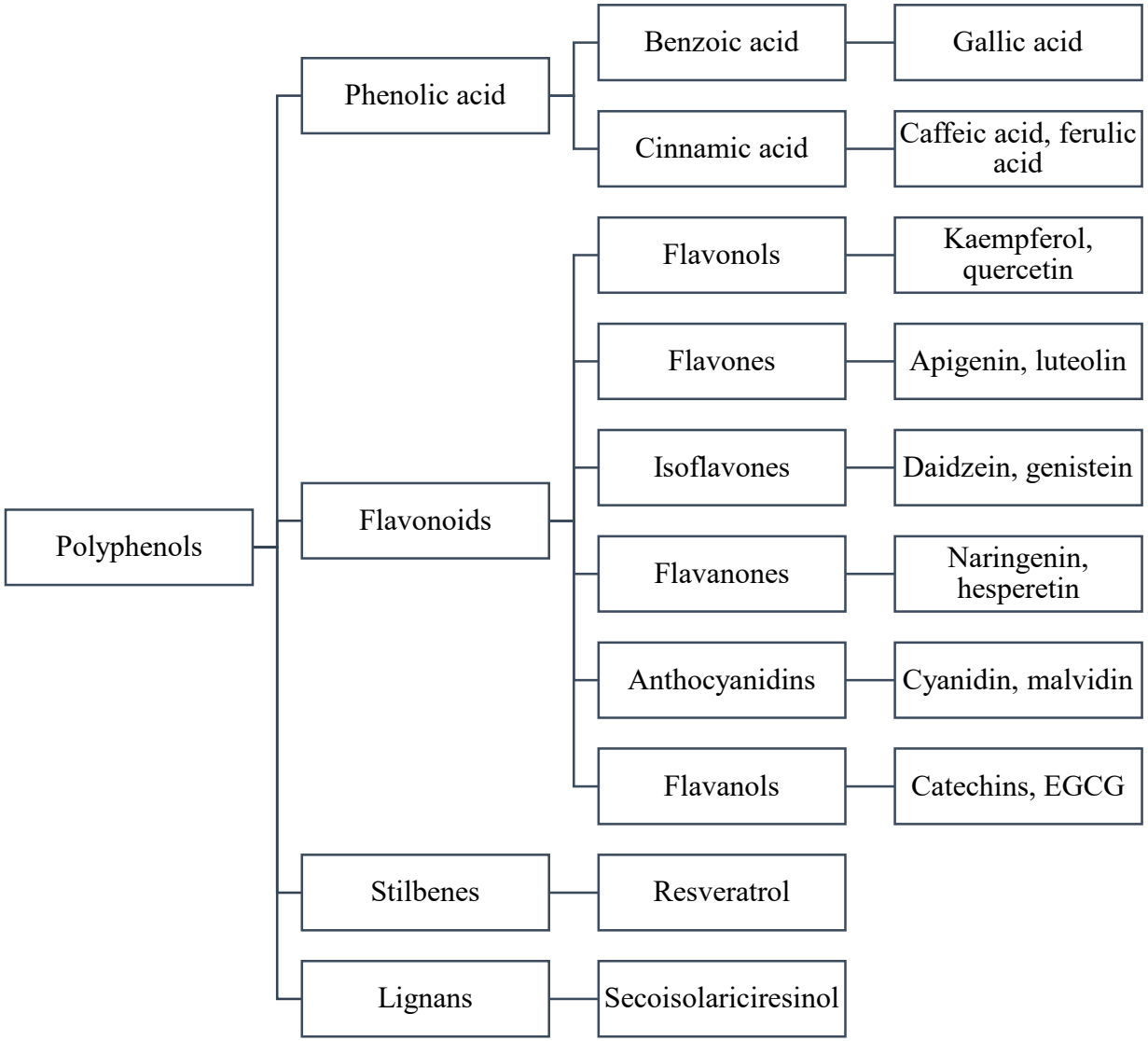
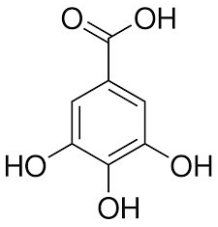
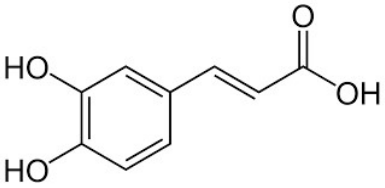


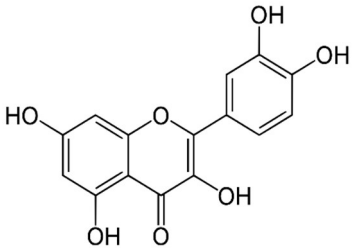
Figure 1. 1. Classification of polyphenols based on the chemical structure



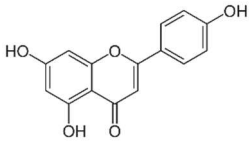
(A) Gallic acid



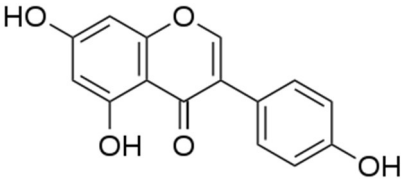
(B) Caffeic acid



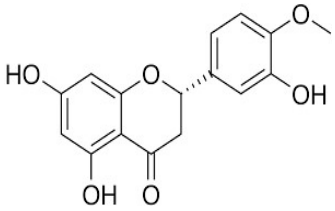
(C) Quercetin



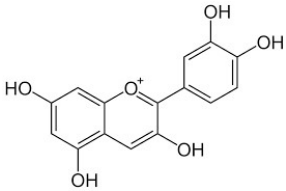
(D) Apigenin



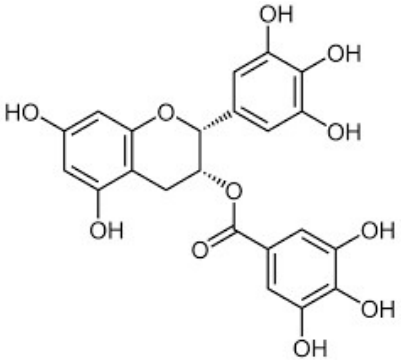
(E) Genistein



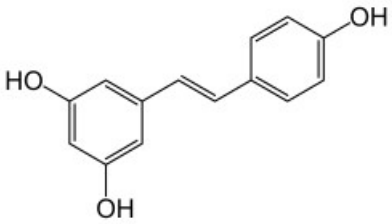
(F) Hesperetin



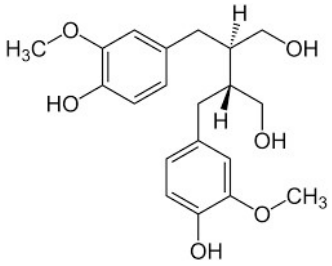
(G) Cyanidin



(H) EGCG



(I) Resveratrol



(J) Secoisolariciresinol

Figure 1. 2 Chemical structures of most common examples from each of the polyphenol subclasses

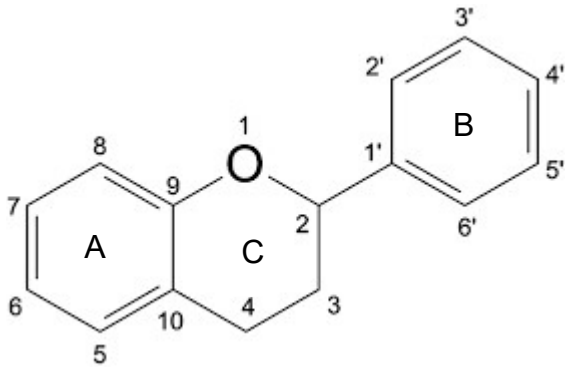


Figure 1.3 The basic backbone of flavonoids

Table 1. 1 Food and beverages as sources of polyphenols*

Flavonoid	Class	Typical dietary sources
Gallic acid	Phenolic acid	Chestnut, black tea, cloves
Caffeic acid	Cinnamic acid	Black chokeberry, common sage, dries spearmint
Quercetin	Flavonols	Capers, onion, shallot, black elderberry
Apigenin	Flavones	Dried marjoram, Italian oregano, olive oil
Genistein	Isoflavones	Soybean, tofu, tempe, soy paste
Hesperetin	Flavanones	Grape wines, fresh welsh onion
Cyanidin	Anthocyanidins	Res raspberry, strawberry, common bean
EGCG	Flavanols	Tea, avocado, kiwi
Resveratrol	Stilbene	Red wine, lingonberry, cranberry
Secoisolariciresinol	Lignans	Cocoa, blackberry, apricot

**Data derived from Phenol-Explorer (www.phenol-explorer.eu)*

1.1.2 Quercetin absorption, metabolism, bioavailability

Quercetin is one of the most abundant flavonoids found in edible plants such as onions, grapes, and berries where it is frequently found in glycosylated (attached to a sugar moiety) form. Monosaccharides, glucose, rhamnose, galactose, arabinose, rutinose, and xylose are the most common sugars found attached to quercetin (Dabeek and Marra, 2019). For example, quercetin is present in onion attached to glucose as quercetin-3-ucoside (Kaşıkcı and Bağdatlıoğlu, 2016), quercetin-4'-glucoside, and quercetin 7,4'-

diglucoside (Kwak *et al.*, 2017) whereas quercetin found in tea and apples is usually attached to rutinose to form quercetin-3-O-rutinoside or rutin (Kaşıkcı and Bağdatlıoğlu, 2016). Studies comparing the absorption of quercetin from different food sources revealed that the form of sugar moieties significantly affects its absorption and bioavailability (de Vries *et al.*, 1998). For instance, a study revealed that quercetin-3-rutinoside only has 20% of that of quercetin-4'-rutinoside in humans after quercetin supplementation (Olthof *et al.*, 2000).

Quercetin glycoside, such as rutin, from the diet, is unable to be absorbed in the small intestine due to its attachment to sugar moieties that increased its hydrophilicity. Hence, it is assumed to pass the small intestine and directly enter the cecum and colon and be subjected to hydrolyzation into quercetin aglycone, mediated by lactase-phlorizin hydrolase (LPH). On the other hand, quercetin aglycone is easily absorbed in the epithelial cells of the large intestine by the sodium-dependent glucose transporter-1 (SGLT-1), enter the circulation, and is subjected to *O*-methylation, glucuronidation, and/or sulphation (Murota and Terao, 2003; Ulusoy and Sanlier, 2020).

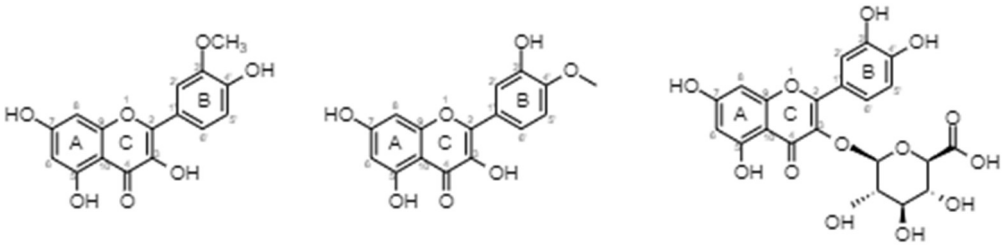
Following absorption, quercetin undergoes phase II metabolism in the small intestine. This process includes conjugation reactions facilitated by several enzymes such as sulfotransferases (SULTs), uridine-5'-diphosphate glucuronosyl transferases (UGTs), and catechol-*O*-methyl-transferases (COMTs) to form glucuronidated, sulfated, or methylated metabolites in different combinations. For quercetin, glucuronidation by UGTs is considered as the primary metabolic pathway both in the intestine and in the liver, with reactivity to attach glucuronic acid moieties at the 7-, 3-, 3'- or 4'-OH positions. With glucuronidation occurring at different positions and multiple times, also in combination with methylation and sulfation, a range of metabolites are possible that could be found in the circulation. As main metabolites of quercetin, quercetin-7-glucuronide, quercetin-3-glucuronide, quercetin-7'-glucuronide, and quercetin-3'-glucuronide have been identified in human (Hai *et al.*, 2020).

Quercetin and its metabolites produced in the gut are transported into the liver to undergo additional reactions, such as methylation, glucuronidation, and sulphation. A study conducted by O'Leary *et al* (2002) showed that quercetin-3'- and quercetin-7-glucuronide can further be processed in the liver by methylation of the catechol functional group or

hydrolysis of the glucuronide by endogenous hepatic β-glucuronidase followed by sulfation to quercetin-3'-sulfate (O'Leary *et al.*, 2003). This research suggests that metabolically active tissue, like the liver, can further metabolize quercetin and/or its metabolites that were generated in the gut, into different forms of metabolites. Some quercetin metabolites produced in the liver will be secreted into bile and excreted in feces. Quercetin can also be excreted through urine in the form of quercetin-diglucuronide, isorhamnetin-3-glucuronide (Figure 1.4 C), and -glucuronide sulfate (Dabeek and Marra, 2019).

Quercetin and its metabolites are also shown to accumulate in the organs involved in metabolism and excretion. A study conducted in rats fed with quercetin for 11 weeks demonstrated that there is an accumulation of quercetin, isorhamnetin (Figure 1.4 A), and tamarixetin (Figure 1.4 B) with the highest concentration found in the lungs and the lowest in the brain, white fat, and spleen (De Boer *et al.*, 2005). In contrast, a study in pigs treated with 50 mg/kg/day quercetin for 4 weeks showed that accumulation only occurred in the organs involved in flavonol metabolism and excretion, such as the small intestine, liver, and kidney (Bieger *et al.*, 2008) and that mitochondria are the main compartment within cells where quercetin accumulates (Fiorani *et al.*, 2010).

Recent studies have suggested that quercetin can accumulate in the nucleus (Walle, Vincent and Walle, 2003; Nifli *et al.*, 2007) and mitochondria (Fiorani *et al.*, 2010) emphasizing their importance for metabolic function and gene expression, respectively. For example, a study revealed that quercetin treatment at 3μM induces accumulation in nucleoplasmic structure in HepG2 cell line followed up and downregulation of over 15,000 modified genes (Notas *et al.*, 2012).



(A) Isorhamnetin (B) Tamarixetin (C) Quercetin-3-glucuronide

Figure 1.4 Chemical structure of quercetin metabolites (A) isorhamnetin (B) tamarixtin and (C) quercetin-3-glucuronide

Quercetin is known for its low bioavailability due to its insolubility in water. Experiments in pigs showed that dietary fat may enhance quercetin bioavailability by increasing its solubility and absorption through lipid micelles (Lesser, Cermak, and Wolffram, 2004). Other factors may contribute to quercetin bioavailability including glucose moieties (Arts *et al.*, 2004), interindividual variation (Almeida *et al.*, 2018), vitamin c status (Guo, Mah and Bruno, 2014), and food matrix (Petersen *et al.*, 2016).

1.1.3 Anti-inflammatory properties of quercetin and its metabolites

The anti-inflammatory properties of quercetin have been demonstrated in different cell types, both in human and animal cell lines, including murine macrophages (Boesch-Saadatmandi *et al.*, 2011; Xue *et al.*, 2017), human alveolar epithelial cells (Günay *et al.*, 2016), and human mast cell line (Min *et al.*, 2007). Numerous in vitro studies revealed that quercetin treatment suppressed LPS-induced activation of NF-κβ (Lee *et al.*, 2018) and STAT3, a signaling pathway that involved in inflammatory genes expression, in murine RAW264.7 macrophages (Xue *et al.*, 2017). Moreover, in the human retinal pigment epithelial cell line, quercetin inhibited the production of pro-inflammatory cytokines and chemokines induced by IL-1β through inhibiting phosphorylation and translocation of several inflammatory signaling including NF-κB and MAPK pathway (Cheng *et al.*, 2019). Quercetin has also been shown to prevent TNF-α from activating inflammatory inducers such as extracellular signal-related kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and NF-κB (Li *et al.*, 2016).

Table 1. 2 Summary of the effects of quercetin and its metabolites on inflammation in vitro

Concentration	Compound	Cell lines	Proposed mechanism	Reference
30 µg/mL	Quercetin	Human umbilical vein (HUVECs)	Inhibition of TNF-α induced apoptosis and inflammation by blocking NF-κB and AP-1 signaling pathway	(Chen *et al.*, 2020)
10-30 µg/mL	Quercetin	Murine RAW264.7 macrophages	Suppression of TLR-3 expression and inhibition of inflammatory transcriptional factors NF-κB and IRF3 induced by herpes simplex virus-1 (HSV-1)	(Lee *et al.*, 2017)
0.03-15 µg/mL (quercetin)	Quercetin	Murine RAW264.7 macrophages	Reduction in LPS-stimulated inflammatory factors (TNF-α-IL-6, and IL-1β) and ROS production	(Tang *et al.*, 2019)
6.25-25 µM	Quercetin	Murine RAW264.7 macrophages	Inhibition of nuclear translocation of NF-κB and activation suppression of Erk1/2 and JNK in LPS-induced inflammatory response	(Lee *et al.*, 2018)
10-100 µmol/L	Quercetin	Murine RAW264.7 macrophages	Attenuation of NF-κB signaling, activation of Nrf2 pathway, and regulation of miR-155	(Boesch-Saadatmandi *et al.*, 2011)
2.5-20 µM	Quercetin	Human retinal pigment epithelial cell line (ARPE-19 cells) Human monocytic leukemia cell line (THP-1 cells)	Inhibition of signaling pathways including MAPKs, IKKα/β, c-Jun, CREB, ATF2, and NF-κB p65	(Cheng *et al.*, 2019)

Concentration	Compound	Cell lines	Proposed mechanism	Reference
1-10 μM	Quercetin-3'-*O*-sulfate, quercetin-3-*O*-glucuronide, 3'-*O*-methylquercetin-3-*O*-glucuronide	Peripheral blood mononuclear cells (PBMC)	Quercetin-3'-*O*-sulfate and 3'-*O*-methyl quercetin-3-*O*-glucuronide retain the ability to inhibit LTB_4 and PGE_2, structural modification affect metabolites bioactivity	(Loke *et al.*, 2008)
2-10 μmol/L	Quercetin-3'-sulfate, quercetin-3-glucuronide, 3'-methylquercetin-3-glucuronide	Human umbilical vein (HUVECs)	Quercetin conjugates retain the ability to inhibit the expression of vascular endothelial and chemokines in LPS induced inflammation	(Tribolo *et al.*, 2008)
10 μmol/L	Quercetin-3-glucuronide, isorhamnetin	Murine RAW264.7 macrophages	Isorhamnetin but not quercetin-3-glucuronide suppressed inflammatory genes and downregulate microRNA-155 expression	(Boesch-Saadatmandi *et al.*, 2011)

Recently, quercetin metabolites have gained interest since they showed similar or higher biological effects compared to their parent compounds. Plasma metabolites of quercetin, such as quercetin-3'-*O*-sulphate, quercetin-4'-*O*-sulphate, quercetin-3-*O*-glucuronide, and isorhamnetin-3-*O*-sulphate showed potential antioxidant activity (Justino *et al.*, 2004; Dueñas *et al.*, 2011). Isorhamnetin, a methylated metabolite of quercetin, has been shown to have biological activity especially in suppressing inflammation of similar magnitude to quercetin in RAW264.7 macrophages stimulated with LPS (Boesch-Saadatmandi *et al.*, 2011). Research exploring the anti-inflammatory properties of quercetin metabolites is shown in Table 1.2.

1.2 Mechanisms of the inflammatory response

Inflammation is a natural response from the immune system to fight against infection caused by bacteria or tissue injury. As a tightly controlled mechanism, inflammation is needed to maintain homeostasis by regulating the immune system and returning the system to a homeostatic state to prevent prolonged inflammation which may lead to tissue damage. During the inflammatory process, the innate immune system has a pivotal role that involves immune cells, for instance, macrophages, dendritic cells, mast cells, neutrophils, and lymphocytes (Libby, 2007; Ahmed, 2011).

Inflammation consists of three stages, namely initiation, regulation, and resolution. Initiation started when immune cells recognize the inflammatory stimuli through certain transmembrane receptors which are called pattern-recognition receptors (PRRs) including toll-like receptors (TLRs), RIG-I-like receptors (RLR), NOD-like receptors (NLR), and C-type lectin receptors (CLR) (Takeuchi and Akira, 2010). PRRs are responsible for sensing the cause of inflammation and the damage that might happen. PRRs can identify inflammation stimuli from microbes through pathogen-associated molecular patterns (PAMPs), for example, lipopolysaccharide (LPS), as well as stimuli from internal injuries, called the danger-associated molecular patterns (DAMPS) (Ahmed, 2011).

The communication between stimuli with the correct receptors results in signaling to the nucleus where gene transcription involved in inflammation is produced which marks the beginning of the second stage, regulation. In this step, the signal from PRRs activates transcription factors and induces the production of proinflammatory cytokines and

chemokines. NF-kB is well-known as is the major transcription factor that regulates the mechanism of inflammatory gene expression involved during inflammation. Besides NF-kB, the transcription factor AP-1 also plays an important role during the modulation of inflammatory genes production (Ahmed, 2011). NF-κB is a family of transcription factors that consist of RelA/p65, c-Rel, RelB, p50, and p52. The most abundant form of activated NF-κB is the heterodimer of p50 and p65. Phosphorylation plays an important role in the activation of NF-κB and its downstream signaling. In inactivated states, NF-κB is residing in the cytosol, bound to an inhibitory protein, IkB family of inhibitor protein, such as IκBα. Activation of e.g. macrophage inflammatory signaling by stimuli such as LPS leads to the activation of the IKK complex by phosphorylation of its subunit that consists of two IkB kinases, IKKα and IKKβ. Phosphorylation of IkBα by the IKK complex followed by ubiquitination and proteasomal degradation releases the NF-κB p65:p50 dimers from the inhibitory complex. The dimer then translocates into the nucleus where it binds to a specific response element and triggers gene transcription such as IL-1 and TNF-α (Lawrence, 2009; Christian, Smith and Carmody, 2016; Giridharan and Srinivasan, 2018).

1.3 Overview of nuclear receptors

Nuclear receptors (NRs) are a family of ligand-activated transcription factors that regulate numerous gene expressions related to a range of biological processes including metabolism, reproduction, and inflammation (Tenbaum and Baniahmad, 1997; Weikum, Liu and Ortlund, 2018). NRs can be classified according to different criteria. Based on their DNA-binding properties and dimerization, NRs are groups into four subfamilies: (1) retinoid X receptor (RXR) heterodimers, such as pregnane X receptor (PXR), vitamin D receptor (VDR), and liver X receptor (LXR), (2) steroid receptors, such as glucocorticoid receptor (GR), estrogen receptor (ER), and androgen receptor (AR), (3) dimeric orphan receptors, such as retinoid X receptor (RXR), hepatocyte nuclear receptor 4 (HNF-4), and testicular receptor 2 (TR-2), (4) monomeric orphan receptor, such as retinoid-related orphan receptor (ROR), and steroidogenic factor 1 (SF-1) (Olefsky, 2001; Porter *et al.*, 2019). Meanwhile, based on the sequence alignment and phylogenetic tree, nuclear receptor subfamily are classified into seven subgroups (Weikum, Liu and Ortlund, 2018) as shown in Table 1.3.

Table 1.3 Nuclear receptor superfamily

Family	Nuclear receptor	Family	Nuclear receptor
0B	Dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome, Gene 1	2C	Testicular receptor 2 Testicular receptor 4
1A	Thyroid hormone receptor-α Thyroid hormone receptor-β	2E	Tailess homolog orphan receptor Photoreceptor-cell-specific nuclear receptor
1B	Retinoic acid receptor-α Retinoic acid receptor-β Retinoic acid receptor-γ	2F	Chicken ovalbumin upstream promoter-transcription factor α Chicken ovalbumin upstream promoter-transcription factor β Chicken ovalbumin upstream promoter-transcription factor γ
1C	Peroxisome proliferator-activated receptor-α Peroxisome proliferator-activated receptor-β Peroxisome proliferator-activated receptor-γ	3A	Estrogen receptor-α Estrogen receptor-β
1D	Reverse-Erb-α Reverse-Erb-β	3B	Estrogen-related-receptor-α Estrogen-related-receptor-β Estrogen-related-receptor-γ
1F	Retinoid acid-related-orphan-α Retinoid acid-related-orphan-β Retinoid acid-related-orphan-γ	3C	Androgen receptor Glucocorticoid receptor Mineralcorticoid receptor Progesterone receptor
1H	Farnesoid X receptor Farnesoid X receptor-β Liver X receptor-α Liver X receptor-β	4A	Nerve growth factor 1B Nurr-related factor 1 Neuron-derived orphan receptor 1
1I	Vitamin D receptor Pregnane X receptor Constitutive androstane receptor	5A	Steroidogenic factor 1 Liver receptor homolog 1
2A	Hepatocyte nuclear factor-4-α Hepatocyte nuclear factor-4-γ	6A	Germ cell nuclear factor
2B	Retinoid X receptor-α Retinoid X receptor-β Retinoid X receptor-γ		

In general, NRs consist of two main conserved domains, a DNA-binding domain (DBD) and a ligand-binding domain (LBD) (Figure 1.4) that are linked together by the hinge region (Nikolenko and Krasnov, 2007).

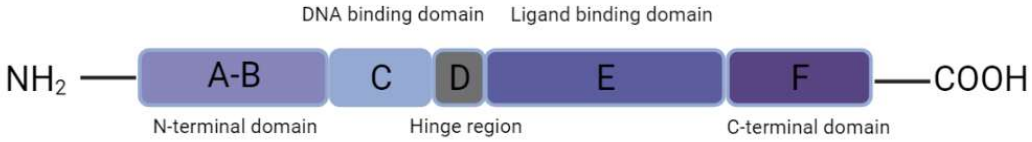


Figure 1.5 Classical structure of the nuclear receptor

Ligand binding is needed for ligand-induced nuclear receptor activation. Several hydrophobic compounds are known to be nuclear receptors ligands such as oxysterols for LXRs, fatty acids for PPARs, derivatives of retinoids for RXRs, thyroid hormones for TRs, and 1α,25-dihydroxyvitamin D3 for VDR, as well as xenobiotics for CAR (Weikum, Liu and Ortlund, 2018). Nuclear receptor ligands are defined as ones that can induce conformational changes and trigger a cascade of effects (Sladek, 2011). Ligands can be synthetic or endogenous, activate (agonists) or block (antagonists) the activity of nuclear receptors. The term partial agonist or antagonist is often used to describe the ability of a certain compound to change only partially the activation of transcription (Flaveny *et al.*, 2014).

Nuclear receptors modulate gene transcription in response to ligand binding, the release of co-repressor, and the recruitment of cofactors that are important for transcription activation, also called transactivation. In addition, nuclear receptors also affect gene transcription by binding to other transcription factors rather than binding to DNA. This mechanism is also known as trans-repression (Gomperts, Kramer, and Tatham, 2003). An example of transrepression is the ability of LXR to suppress inflammatory cytokines production, such as COX-2, by antagonizing the NF-κβ signaling pathway (Joseph *et al.*, 2003).

1.4 Overview of nuclear receptor liver X receptor alpha (LXRα)

The liver X receptor (LXRs) is a member of the NRs superfamily and is known as the major regulator of cholesterol and lipid intracellular homeostasis. Another known health property of LXRs is the anti-inflammatory activity by linking lipid metabolism and

inflammation, especially in macrophages (Schulman, 2017). LXRs consists of two isoforms, LXRα (NR1H3) and LXRβ (NR1H2). Both share 77% similarity in the DBD and LBD regions but their expression patterns are quite different. A review by Bilotta *et al (*2020) stated that LXRα is expressed mainly in the liver, intestine, macrophages, and adipose tissue, LXRβ is more ubiquitously expressed in many tissues. Besides the similarities, both isoforms showed distinct features in lipid metabolism where activation of LXRβ alone could benefit in raising HDL level without causing accumulation of triglycerides in the liver (Lund *et al.*, 2006).

LXRs are activated by oxysterols as the major endogenous LXR agonist. Some examples of oxysterols also known as LXRs ligands are 20(S)-, 22(R)-, 24(S)-, 25-, and 27-hydroxycholesterol, 24(S),25-epoxycholesterol. In addition, LXRs can also be activated by cholesterol biosynthesis intermediates, such as desmosterol (Fessler, 2018). To this date, T0901317 and GW3965 have been identified as synthetic LXR agonists with high affinity to both LXR isoforms (Figure 1.5) (Houck *et al.*, 2004) although T0901317 also found to activate other nuclear receptors (Mitro, Vargas, *et al.*, 2007). Synthetic antagonists of LXRs have also been identified, known as GSK2033, where it has the ability to suppressed LXR activity (Griffett and Burris, 2016).

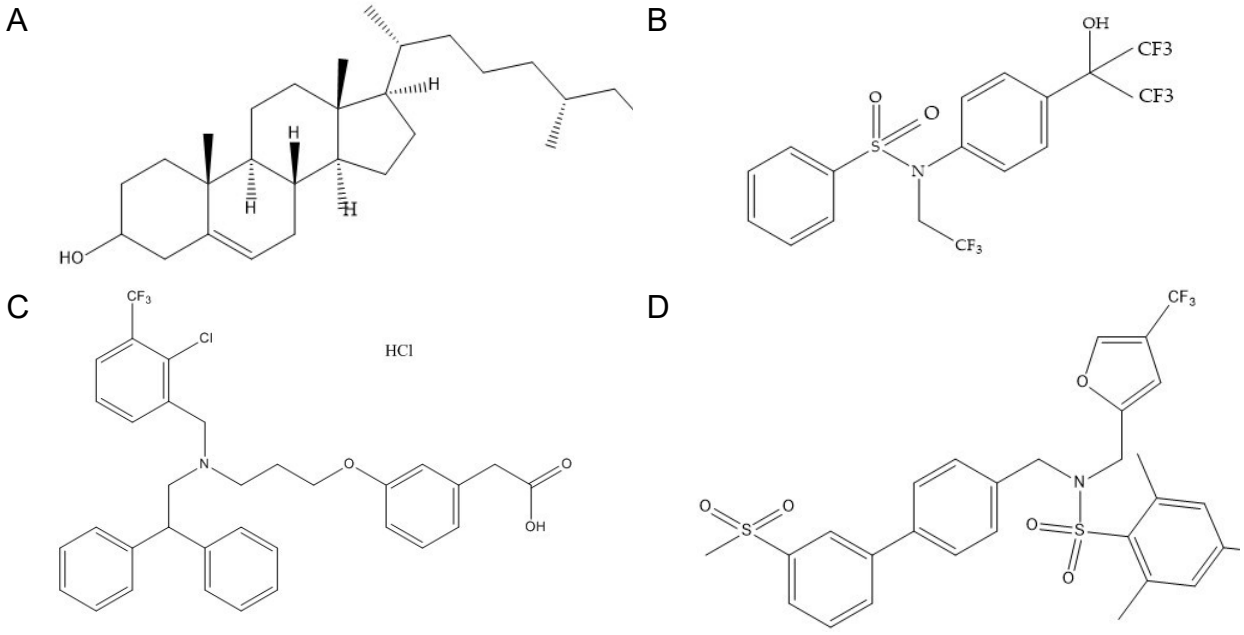


Figure 1.6 Chemical structure of LXR ligands: (A) 27-OHC, (B) T0901317, (C) GW3965, and (D) GSK2033

LXRα forms an obligatory heterodimer with RXR (Figure 1.6). LXRα regulates transcription activity by several pathways: ligand-independent repression, direct activation, ligand-dependent activation, and trans-repression (Komati *et al.*, 2017). In the ligand-dependent model, LXRα-RXR heterodimer interacts with co-repressor, which silenced transcription, followed by the release of co-repressor (for example nuclear receptor corepressor/NcoR) and recruitment of co-activator (for example activating signal cointegrator-2/ASC-2), leading to increased transcription of the target genes (Viennois *et al.*, 2011, 2012).

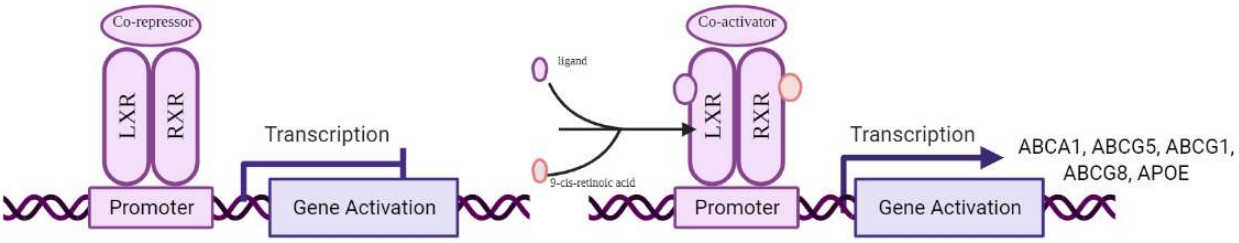


Figure 1.7 Schematic representation of LXRs activation

1.5 Liver x receptor alpha (LXRα) role in inflammation

The main function of nuclear receptors is to regulate gene transcription positively or negatively. At a cellular level, inflammatory response and cytokines production are under NF-kB or AP-1 control. Since nuclear receptors can affect the activity of other transcription factors involved in inflammation through trans-repression, it is expected that nuclear receptors activation will decrease the inflammatory response. Activation of several nuclear receptors such as GR, PPAR, LXRα, ER, AR, PR, VDR can interfere with NF-kB signaling during inflammation (Beck, Haegeman and De Bosscher, 2010). Out of the nuclear receptors mentioned above, LXRα, PPAR, and GR are considered in this project since activation of these three resulted in synergistic and additive anti-inflammatory effects (Glass and Ogawa, 2006; Pascual and Glass, 2006; Beck, Haegeman and De Bosscher, 2010).

In terms of inflammation, LXRα has been shown to negatively impact the expression of inflammatory genes such as IL-6, IL-1β, and COX2 through inhibition on NF-kB and AP-1 (Komati *et al.*, 2017). For example, a study revealed that GW3965 inhibits translocation

of p65, a subunit of NF-κβ, into the nucleus in HUVEC cells treated with lysophosphatidylcholine (LPC), resulting in the decrease of IL-8 expression (Bi *et al.*, 2016). In an in vitro inflammatory model induced by bacterial pathogens, LXRα activation diminished NF-kB-dependent cytokine production such as IL-1β, IL6, iNOS, and TNF (Joseph *et al.*, 2003). Meanwhile, in murine RAW 264.7 macrophages cells, LXRα activation not only induced genes involved in reverse cholesterol transport, but also inhibited expression of pro-inflammatory target genes following LPS, TNFα, or IL-1β stimulation (Noelia and Castrillo, 2011).

1.6 Polyphenols as dietary regulators of nuclear receptor activity

In general, the anti-inflammatory effects of polyphenols can be explained through several mechanisms, including suppression of enzymes associated with pro-inflammatory activities, direct inhibition of IKK activity, and inhibition of nuclear translocation of NF-κB (Hussain *et al.*, 2016). A previous review emphasized that the pharmacological activity of flavonoids may at least be partially mediated through nuclear receptors based on the structural similarities between flavonoids and nuclear receptor ligands (Avior *et al.*, 2013). There is an increasing interest in the potential interaction of polyphenols with nuclear receptors in recent years. As summarized by (Delfosse *et al.*, 2015), genistein binds to both ERα and ERβ, allowing them to activate or inhibit their action. Flavanols, especially catechin and EGCG, have been demonstrated to activate the constitutive androstane receptor, a nuclear receptor that regulates genes involved in the metabolism and excretion of xenobiotic compounds (e.g. drugs) (Yao *et al.*, 2010).

Interaction between polyphenols with NF-κβ transcription factors is the most known pathway to explain the anti-inflammatory property of polyphenols. So far, it is known that certain polyphenols mimic ligands for nuclear receptors. For example, the similarity between estrogen and isoflavone structures provides the ability for isoflavones to act as estrogen agonists or antagonists (Fraga *et al.*, 2010), indicating that polyphenols might be capable of activation of other nuclear receptors alike although further research is needed to better understand the action of polyphenols in nuclear receptors modulation related to its beneficial health effects.

1.7 Research gap

LXRα is a nuclear receptor that plays an important role in the modulation of cell metabolism, especially in lipid homeostasis, cholesterol metabolism, and inflammation. LXRα is activated by endogenous ligands, oxysterols, and synthetic ligands such as T0901317 and GW3965, although research showed that synthetic ligands may have unwanted effects such as inducing triglyceridaemia and fatty liver in an animal model (Peng *et al.*, 2011). Polyphenols, naturally occurring bioactive compounds which can be found ubiquitously in the daily diet, have been pointed out to have structural similarity to steroids (Zand, Jenkins and Diamandis, 2000) therefore considered as good candidates for targeting nuclear receptors. A previous study by Fouache *et al* (2019) revealed that flavonoids from Algerian propolis such as quercetin, naringenin, galangin, and apigenin have different effects on LXRs activations. Although there is some evidence on a few compounds demonstrating potency to modulate nuclear receptors activation, there is limited investigation available into the potential of polyphenols to bind and activate LXRα as well as its effect on downstream signaling. Despite previous research that demonstrates the anti-inflammatory properties of polyphenols, the information regarding LXRα activation and its consequences on the regulation of inflammation is scarce. Numerous previous researches demonstrated that polyphenols have anti-inflammatory properties, however, little is known about the molecular mechanisms by which LXRα impacts the anti-inflammatory activity of polyphenol in suppressing pro-inflammatory cytokines expression. Besides, the evidence for the cellular targets of polyphenols and the direct interaction between polyphenols and nuclear receptors is still lacking.

1.8 Aim and objectives

This research aimed to investigate the molecular mechanism of nuclear receptor activation by polyphenols and its effect on cell metabolism and inflammation.

The objectives were:

- Screening of a range of polyphenols from different classes in order to establish structure-function relationships using a reporter cell model.
- Selection of most promising polyphenols and analysis of LXRα activation in this polyphenol and related metabolites.

- Investigating the role of selected polyphenol in activation of LXRα and its impact on hepatic inflammation.
- Analysis of LXRα role in selected polyphenol's anti-inflammatory effects in murine RAW264.7 macrophages.
- Analysis of the interaction between polyphenols and LXRα ligands on inflammation.

1.9 Hypothesis

Based on the literature review, this study hypothesizes that the health benefits of polyphenols are mediated by their ability to act as a ligand for LXRα and that LXRα activation may contribute to the anti-inflammatory effects of polyphenols.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Polyphenols and LXRα ligands

Polyphenols used in this experiments were obtained from different manufacturers, as follows: Sigma-Aldrich, Dorset, UK for hesperetin (W431300-5G, purity ≥95%), quercetin (Q4951-10G, purity ≥95%), EGCG (E4143-60MG, purity ≥95%); Extrasynthase, Genay, France for isorhamnetin (1120S, purity ≥99%), tamarixetin (1140S, purity >99%), quercetin-3-glucuronide (1315, purity >99%); Cayman Chemical, Michigan, USA for resveratrol (CAYM70675-50, purity ≥98%), and Fluorochem, Hadfield, UK for genistein M01798-1g, purity ≥98%). All polyphenol compounds were dissolved in molecular biology grade DMSO from Sigma-Aldrich (D4818-100ML). T0901317 as a synthetic ligand for LXRα was obtained from Cayman Chemical (CAYM71810-10) and was dissolved in DMSO. GSK2033 (SML1617-5MG) and oxysterol (27-hydroxycholesterol and 22R-hydroxycholesterol) were obtained from Sigma (700021P-1MG; H9384), dissolved in nitrogen-flushed ethanol. All polyphenols and LXRα ligands were prepared as 50- and 10-mM stock solutions, respectively, and diluted further as needed.

2.1.2 General reagents

Reagents used in this study were purchased from different manufacturers, as follows: VWR, Poole, UK, for molecular biology grade ethanol, methanol, and chloroform. Phosphate buffered saline tablet, Ponceau S solution, fetal bovine serum, Tween 20, NP-40, protease, and phosphatase inhibitor cocktails, Oil Red O solution, and neutral red dye were purchased from Sigma-Aldrich. DEPC treated water, Amplex Red Cholesterol Assay, Dulbecco's modified Eagle's medium (DMEM), and Pierce BCA assay kit were obtained from Thermo Fisher Scientific, Loughborough, UK. TRIsure and SensiFAST SYBR Hi-ROX were purchased from Bioline, Tennessee, USA. Luciferase assay system was bought from Promega, Southampton, UK. Dried skimmed milk powder was obtained from Premier Foods Group Ltd, London, UK. 4X Laemmli Sample Buffer, Precision Plus Protein Western C Blotting Standards, Precision Streptactin HRP, Clarity ECL Western

Blotting Substrates, and Trans-Blot Turbo Transfer System RTA Transfer Kits were purchased from BioRad, California, USA.

2.2 Methods

2.2.1 Cell culture treatment

Murine RAW264.7 macrophages, human triple negative breast cancer cell MDA-MB-231, and human liver cancer cell HepG2. Murine RAW264.7 macrophages and human liver cancer cell HepG2 were purchased from the European Collection of Authenticated Cell Cultures (ECACC), meanwhile human triple negative breast cells MDA-MB-231 was kindly provided by a collague. All cell lines were routinely cultivated in Dulbecco's modified Eagle's (DMEM) medium, high glucose (4.5 g/L, sodium pyruvate (110 mg/l), and supplemented with 10% (v/v) fetal bovine serum (heat-inactivated). All cells were grown in a humidified incubator at 37°C and 5% CO_2. Cells used in this study were cultivated for no more than twenty in-house passages.

All cells were cultivated in T75 flasks until they reached ±80% confluence. For MDA-MB-231 and HepG2, cells were harvested by adding 3 mL pre-warmed 1X trypsin solution to the side wall of the flask, followed by 3-5 minutes incubation to fully detach the cells. Once cells detached, 7 mL of warm complete growth media was added to inactivate trypsin. RAW264.7 macrophages were harvested by adding 5 mL of warm media followed by gentle scraping using a cell scraper to lift them off the flask surface. For experiments, cells were plated in 6-well plates (MDA-MB-231: $1.5x10^4$ cells/cm^2, HepG2: $1.2x10^5$ cells/cm^2, murine RAW264.7 macrophages: $6.75x10^5$ cells/ cm^2) unless stated otherwise, before start of experiments.

2.2.2 Cell viability assay

Cytotoxic effects of test compounds were determined by neutral red (NR) assay as described in the previous publication (Repetto, Del Peso et al. 2008). Briefly, a neutral red stock solution (4 mg/ml) was prepared by dissolving 40 mg neutral red dye in 10 mL ethanol and stored at room temperature, protected from light. Neutral red destain solution was made from 50% ethanol (96%), 49% deionized water, and 1% glacial acid.

Cells were treated with compounds of interest for 24 hours before incubation with a medium containing neutral red dye (40 mg/mL) and incubated for another 2 hours. Afterward, cells were washed with DPBS and then 1 mL destain solution was added per well. The plate was left on a plate rocker for 15 minutes to dissolve the dye from the cells and to form a homogenous mixture. The mixture was then transferred to 96-well plates, with 200 µL for each well pipetted in triplicate. The absorbance was read at a wavelength of 540 nm using a Tecan plate reader with destain solution as a blank reference.

2.2.3 Hydrogen peroxide (H_2O_2) measurement

H_2O_2 production was measured in cell culture media after 24 hours of polyphenol incubation. The importance of H_2O_2 measurement in cell culture study using polyphenols is that H_2O_2 sometimes acts as a messenger for the signaling pathway (Kim, Quon et al. 2014). Pierce quantitative peroxide assay kit (aqueous) was obtained from Thermo-Fisher to measure H_2O_2 level. FOX (ferrous oxidation-xylenol orange) assay detects hydrogen peroxide-based on the oxidation of ferrous to ferric ion in the presence of xylenol orange. In this assay, the conversion of Fe2+ to Fe3+ at acidic pH with the xylenol orange dye yields a purple colour that represents the H_2O_2 level. H_2O_2 was measured according to the manufacturer's protocol. Briefly, 20 µL culture media were mixed with 200 µL FOX reagents. The mixture was then incubated for 15-20 minutes at room temperature. Absorbance was read at wavelength 595 nm using a plate reader. The H_2O_2 level was corrected and calculated using a standard curve as shown in Figure 2.1. All reagents, cell culture media, and H_2O_2 standards were prepared freshly before the measurement and kept on ice during the measurement since H_2O_2 is highly unstable.

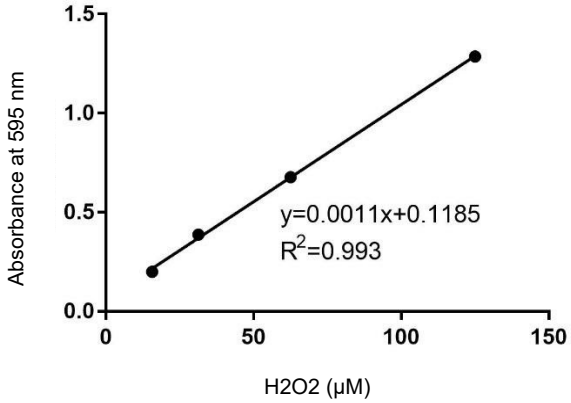


Figure 2.1 Standard curve for H_2O_2 quantification. H_2O_2 quantification is based on the equation shown in the picture above.

2.2.4 Luciferase assay

MDA-MB-231 and HepG2 LXRα reporter cells were kindly provided and developed by a colleague, Dr. Samantha Hutchinson, University of Leeds (Hutchinson and Thorne 2019). The generation of a stable cell line had been performed by transducing these cell lines with lentivirus particles containing firefly luciferase genetic sequence. Subsequently, the cells were cultured under antibiotic puromycin selection conditions to select antibiotic-resistant cell clones stably expressing LXRα.

MDA-MB-231-Luc cells were plated in 6-well plates with a cell density of $1.5x10^5$ cells/well. Meanwhile, for HepG2-Luc, cells were plated in 96-well flat clear bottom white polystyrene microplates with cell density $1.0x10^4$ cells/well. All cells were incubated overnight before being treated with polyphenols and LXRα ligands in increasing concentrations (2.5-50 μM) or VC. After 16 hours of incubation, the medium was discarded, and cells were washed once with DPBS. Afterward, 100 μL of 1X passive lysis buffer (Promega, E1910) was added to each well. The lysis buffer was prepared by adding 4 parts of double distilled water to 1 part of 5X passive lysis buffer. The plate was left on a plate rocker for 15 minutes at 1000 rpm. For MDA-MD-231, lysis buffers containing cells were then transferred into a 96-white-opaque well microplate in duplicate.

Activation of LXRAα was reflected through an increase in signal intensity detected by luminescence, by injecting 30 μL of the substrate (Promega, E4550) on each well through an autoinjector. Signal was measured two seconds after the substrate was added using

a Tecan Spark 10M plate reader. To calculate the activation on MDA-MB-231, the luminescence signal was normalized to total cell protein.

2.2.5 Protein quantification

Pierce BSA quantification assay was used to measure total protein concentration in the cell lysates. Since luminescence readings from luciferase assay varied from passage to passage, it was considered necessary to normalize the luminescence signal to the cellular protein content. Cell protein is representing cell mass at the end of the experiment. Hence, total protein was measured in the remaining cell lysate samples from luciferase measurement.

Total protein quantification was done according to the manufacturer's protocol. Briefly, 10 µL of cell lysate were diluted with 23 µL double-distilled H_2O and mixed well. 10 µL of diluted cell lysate were mixed with 200 µL working reagent and then incubated for 30 minutes at 37ºC. Each sample was measured in triplicate. The absorbance was read at wavelength 570 nm, and the total protein concentration was calculated against a standard curve with bovine serum albumin (working range 20-2.000 µg/mL) after subtraction of the blank value.

2.2.6 RNA isolation and cDNA synthesis

Test compounds were incubated for 24 and/or 6 hours concentration in order to establish the LXRα target genes and cytokines transcriptional changes, respectively, following experimental treatments. At the end of the incubation, cells were washed with DPBS, lysed with 600 µL of Trisure and frozen for subsequent RNA isolation. Briefly, cells were resuspended in Trisure reagent by pipetting up and down and transferred into 2 mL RNA/DNA free Eppendorf tubes. 100 µL of pure chloroform was then added into the tubes and vortexed vigorously for 10 seconds. Samples were incubated at room temperature for 3 minutes and centrifuged at high speed (12,000 x g) for 15 minutes at 2-8°C to separate the aqueous from the organic phase. The chloroform layer (aqueous phase) was then transferred into a new 2 mL RNAse/DNAse free Eppendorf tube, without disturbing the other phase. Afterward, 300 µL of ice-cold molecular grade isopropanol were added to the chloroform layer and vortexed vigorously for 10 seconds. Samples

were then incubated at room temperature for 10 minutes. Then, samples were centrifuged at high speed (12,000 x g) for 10 minutes at 2-8°C. Later, the supernatant was discarded without disturbing the cells pellet. The cell pellet was then washed with 600 µL of 75% molecular grade ethanol and vortexed gently followed by centrifuged (7500 x g) for 5 minutes at 2-8°C. Supernatants were discarded and 25-50 µL of DEPC treated water was then added depending on the size of the pellet. To dissolve the RNA, the tubes were flicked and pipetted gently. The tubes were vortexed and centrifuged quickly to spin down the liquid to the bottom of the tubes. RNA concentration and purity were quantified by using TECAN Spark 10M reader. All samples tested must have a ratio of A260/A280 by 1.8-2.1 for further experiment. Samples were stored at -80°C for further analysis.

2.2.7 cDNA synthesis

All steps for cDNA synthesis must be done on ice to prevent RNA degradation. Mastermix was made fresh previously by mixing 1 part of the iScript RT enzyme and 4 parts of 5x iScript buffer in a 2 mL RNAse/DNAse free tube. RNA samples were thawed by placing the tubes on ice. Briefly, 500 ng of RNA were added into a 0.5 mL RNAse/DNAse free tube and mixed with DEPC treated water to make up a final volume of 7.5 µL. 2.5 µL of the master mix was then added into the tubes and mixed gently by pipetting up and down several times. All the samples were then put into a thermo block for incubation. The incubation cycle consisted of 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, and then hold on 4°C or put on ice. Samples were then diluted 1:20 by adding 190 µL of DEPC treated water and mixed gently to homogenize the solution. Samples were stored at -20°C for later use.

2.2.8 Quantitative real-time PCR (q-RT PCR)

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using SensiFAST SYBR Hi-ROX following the manufacturer's instructions. The primers used for RT-PCR are described in Table 2.1 below. The results were normalized to the expression of HPRT, as the housekeeping gene. All primer sequences used in this experiment were designed using the NCBI Primer-BLAST (ncbi.nlm.nih/gov/tools/primer-

blast) and were tested for specificity and efficiency. All primer used have efficiency between 90-110%.

Table 2. 1 List of primer sequences used for RT-PCR analysis

Human		
Target gene	Forward	Reverse
ABCA1	GCACTGAGGAAGATGCTGAAA	AGTTCCTGGAAGGTCTTGTTCAC
LXRα	CAAGAGGAGGAACAGGCTCA	TCTCGATCATGCCCAGTTGT
ApoE	GTTGCTGGTCACATTCCTGG	GGTAATCCCAAAAGCGACCC
HPRT	GACCAGTCAACAGGGGACAT	CCTGACCAAGGAAAGCAAAG
Murine		
Target gene	Forward	Reverse
ABCA1	AACAGTTTGTGGCCCTTTTG	AGTTCCAGGCTGGGGTACTT
Actin	CCTCTATGCCAACACAGTGC	CCTGCTTGCTGATCCACATC
IL6	AGTTGCCTTCTTGGGACTGA	CAGAATTGCCATTGCACAAC
IL-1β	CAGGCAGGCAGTATCACTCA	AGCTCATATGGGTCCGACAG
APOE	ACAGATCAGCTCGAGTGGCAAA	ATCTTGCGCAGGTGTGTGGAGA
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
p65	AGGCTTCTGGGCCTTATGTG	TGCTTCTCTCGCCAGGAATAC
NF-κβ	ATGGCAGACGATGATCCCTAC	TCTTCACAGTGGTATTTCTGGTG
Iκβ	TGAAGGACGAGGAGTACGAGC	TTCGTGGATGATTGCCAAGTG

The master mix of SensiFAST SYBR Hi-ROX was prepared by mixing 10 parts of DEPC treated water, 1 part of primer forward, 1 part of primer reverse, and 20 parts of 2X SensiMix reagents. Mastermix was prepared fresh, kept on ice, and protected from direct light. Briefly, 16 µL pf master mix was added into 96 PCR well plate to each well, followed by 4 µL of the sample. Each sample was performed in duplicate. The plate was then closed by adhesive film and centrifuged at low speed for 10 seconds. The plate was then put into a cycler using conditions below:

- Enzyme activation: 95°C for 10 minutes
- Cycling (40x): 95°C for 15 seconds, 57°C for 15 seconds, and 72°C for 15 seconds
- Melt curve: 95°C for 15 seconds, 60°C for 60 seconds, and 95°C for 15 seconds

The results were determined by calculating the ΔC_T value ratio between target gene and housekeeper as follows ΔC_T= C_T *target*-C_T *housekeeper*. The fold change was then calculated from the ΔC_T obtained before with the equation (2- ΔC_T).

2.2.9 Extraction of nuclear and cytoplasmic protein

Protein fractions were extracted using NE-PER nuclear and cytoplasmic extraction reagents from ThermoFisher. The isolation consists of two-part separation and three reagents, cytoplasmic extraction reagent I (CER I), cytoplasmic extraction reagent II (CER II), and nuclear extraction reagent (NER). The master mix was prepared by maintaining the volume ratio of CER I:CER II: NER by 200:11:100 µL. Phosphatase and protease inhibitor cocktail was added immediately to the mixture before use and kept on ice to maintain the temperature.

Briefly, cells were grown in a 100 mm petri dish until they reach ±70% confluence. Afterward, the old medium was replaced with the fresh medium containing test compounds. Cells were left for 24 hours incubation with test compounds followed by 6 hours incubation with LPS. To harvest the cells, cells were washed twice using 5 ml ice-cold PBS. Next, 1 mL of ice-cold PBS was added gently into the sidewall of the petri dish. Scrape the cells using a plastic cell scraper and transfer them into a 2 ml Eppendorf tube, followed by centrifugation for 15 minutes at the highest speed in a pre-cooled centrifuge at 4°C. The supernatant was removed carefully by aspiration and isolation was done according to the manufacturer's instruction.

During isolation, all tubes and reagents were always kept on ice. Protein samples were measured using BCA assay as mentioned above. All samples were kept in aliquots at -80°C for further experiments.

2.2.10 Western blot

SDS-PAGE was used to determine changes in protein level following 24 hours of treatment of test compounds. A 7.5% gel was used to detect high molecular size protein,

such as ABCA1 (molecular size 254 kDa). Acrylamide gels were made manually with 4% stacking gel and 7.5% resolving gel. All gels had 1.00 mm thickness. The composition for both gels is shown in Table 2.2 below.

Table 2.2 Recipes for stacking and resolving gels

	Stacking gel	Resolving gel
	4%	7.5%
30% Acrylamide/bis	1.98 mL	3.75 mL
0.5M Tris-HCl, pH 6.8	3.78 mL	-
1.5M Tris-HCl, pH 8.8	-	3.75 mL
10% SDS	150 µL	150 µL
diH2O	9 mL	7.28 mL
TEMED	15 µL	7.5 µL
10% APS	75 µL	75 µL
Total Volume	15 mL	15 mL

The resolving solution was then poured gently into a cassette and set aside to polymerize for about 20 minutes. After that, the stacking solution was poured on top until covered all the cassettes were. Slowly put the comb into the cassette to avoid any bubble formation and set it aside to allow the gel to polymerize for 30-45 minutes. The cassette was stored in the fridge, covered in wet tissues, for one week, or used directly for electrophoresis.

Before detecting the target proteins, initial tests were performed to determine the amount of protein needed for western blot as well as antibody performance. Increasing amounts of protein (10-40 µg) were loaded into the individual slots. Based on the initial tests, 40 µg of protein was suitable to use for further assays. Therefore, 40 µg of protein sample was mixed with 6X SDS sample buffer non-heated for ABCA1 protein, before loading onto the gel. 5 µL of Precision Plus Protein Western C Blotting Standards was used as the loading control and placed on the first line before the samples.

Electrophoresis was run at 80V for around 2 hours until the band was observed at the bottom of the cassette. Once the electrophoresis step was finished, the cassette was then removed and washed gently using ddH_2O. Running buffer and TBS stock solution were

prepared in 10X concentration and stored at room temperature for up to 6 months. The recipe for running buffer and TBS is shown in Table 2.3 below.

Table 2.3 Recipes for running buffer, TBS, and TBST solution

	Composition	Amount	
10X Tris glycine buffer (running buffer stock)	250 mM Tris base (MW 121.114)	30.3 g	pH should be at 8.3 Do not adjust pH
	1.9 M glycine (MW 75.07)	142.6 g	
	1% SDS	10 g	
	ddH_2O	1 L	
10X TBS	Tris base (MW 121.1)	24 g	Adjust pH to 7.6 with 12 N HCl
	NaCl (MW 58.4)	88 g	Add distilled water to a final volume of 1 L
	ddH_2O	900 mL	
TBST	Dissolve 1 L of 10X TBST and add pure water to 1 L Add 1 mL Tween 20		

Once the electrophoresis step was finished, the gel was removed from the cassette for blotting. 0.2 µM PVDF membranes from BioRad were used and prepared according to the manufacturer's instructions. Firstly, the transfer buffer was prepared by the 5X transfer buffer with ultrapure water and molecular grade ethanol. Pre-cut membranes and transfer stacks were then immersed in 100% methanol until they became translucent and were then transferred to a tray containing transfer buffer for 3 minutes. On a Trans-Blot Turbo Transfer System cassette, a sandwich was formed by placing one transfer stack at the bottom, followed by a membrane, gel, and another transfer stack. The sandwich was then rolled to remove air bubbles and then inserted into the blotting cassette. The transfer process was done by using the High MW protocol provided in the instrument for the detection of ABCA1.

Next, the membrane was immersed in Ponceau Red stain to ensure the transfer process and followed by de-stain the membrane using water until all the stain was removed. The membrane was then blocked by incubating it on a tray filled with 3% milk in TBST for one hour at room temperature. After that, the milk solution was removed and followed by primary antibody diluted in TBST incubation overnight at 4°C with gentle agitation and protected from light. The secondary antibody incubation was done the day after at room temperature for one hour, protected from light. The secondary antibody was also diluted in 3% milk in TBST mixed with 1 µL of Precision Streptactin HRP to target the marker. The list of antibodies used in this experiment can be seen in Table 2.4 below.

Table 2.4 Primary and secondary antibodies for western blotting

Target antibody		Company	Size (kDa)	Dilution
Primary	ABCA1	Merck (MAB10005)	254	1:500
	B-actin	Santa Cruz (SC-1616)	43	1:1000
Secondary	Goat anti-rabbit	Santa Cruz (sc-516102)	-	1:1000

After the secondary antibody incubation, the membrane was then washed three times with TBST. For imaging, the membrane was incubated briefly in the dark with Clarity ECL Western Blotting Substrates by mixing the same amount of Clarity Western Peroxide Reagent and Clarity Western Luminol/Enhancer Reagent. The membrane was then exposed using a ChemiDoc gel imaging system. The band intensity was then quantified using the software provided by ChemiDoc.

2.2.11 Transcription factor binding site detection by TRANSFAC

In silico binding site analysis was done using the TRANScription Factor (TRANSFAC) database GeneXplain platform. In this present study, the transcription factors involved in the expression of inflammatory cytokines and LXRα target genes both in humans and mouse were done by using the Match tools. Best supported promoter and high-quality matrices in the region between were selected for the intended genes and scanned for all

the promoters in the region from -10000 to +1000 bp relative to the transcription start sites (TSS) with core score and matrix score was set at 0.8-1.0.

2.3 Data analysis

All results are presented as the mean of at least three biological replicates that were performed in duplicate. Data are shown as mean ± standard deviation. All statistical analyses and graphs were performed using Graphpad Prism 7. Data normality was tested using Shapiro-Wilk test. Statistical differences between means of different treatment groups were analyzed using one-way ANOVA followed by Dunnett's test for normally distributed data and Kruskal Wallis followed by Dunn's test for the data that not normally distributed. An unpaired t-test was also used in order to compare the means of two independent groups. Data were considered significant if the p-value < 0.05.

Chapter 3. Identification of dietary polyphenols as modulators of LXRα

Abstract

The liver X receptors are ligand-inducible transcription factors that play an important role in numerous biological activities, especially in lipid and cholesterol metabolism. The LXRα form is activated by oxysterols as the endogenous ligand as well as synthetic ligands, although the latter showed some limitations. Therefore, the development of naturally occurring LXRα ligands from the diet may be beneficial. Recent research showed that some natural products including flavonoids have the potential to act as ligands for LXRα although some of the results were conflicting. This present study was aimed to demonstrate whether different polyphenols could regulate the LXRα pathway. Six polyphenols from different classes were tested towards the modulation of LXRα activity using the MDA-MB-231 cell line stably transfected with the LXRα reporter gene. Hesperetin, genistein, resveratrol, and quercetin were reported to modulate LXRα activity. Further investigation into downstream LXRα target genes revealed that only quercetin induces ABCA1 expression on mRNA level. This study also demonstrated that LXRα antagonist, GSK2033, diminished quercetin activity in modulating LXRα suggesting that quercetin may be a weak LXRα modulator. Altogether, the results indicate that each polyphenol has different effects in the regulation of LXRα. The data also support further research into the development of quercetin as a natural modulator of LXRα.

3.1 Introduction

The liver x receptor alpha (LXRα) is a ligand-dependent nuclear transcription factor, that once activated will induce the expression of several target genes, especially those that are involved in lipid metabolism, including ABC transporters. LXRα is mainly found in lipid-related tissues, such as liver and adipose tissue. It is also expressed in macrophages, kidneys, lungs, and intestines (Lund, Peterson et al. 2006, Patel, Oza et al. 2008).

Oxysterols were found as the major endogenous ligands found in humans with the most abundant found in human serum are 27-, 24(S)-, 7α-, and 4β-hydroxycholesterol (OHC). Meanwhile, 24(S), 25-EPOX, 24(S)-OHC, 22(R)-OHC, 20(S)-OHC, and 27-OHC are considered to be the most physiologically important endogenous ligands for LXRα (Olkkonen, Béaslas and Nissilä, 2012). Although oxysterols are present in very low concentrations they can activate and modulate LXRα activity even in nanomolar concentrations (Janowski *et al.*, 1999).

Activation of LXRα is crucial for various physiological processes including glucose homeostasis (Laffitte *et al.*, 2003; Mitro, Mak, *et al.*, 2007), cholesterol metabolism (Zhao and Dahlman-Wright, 2009; Zhu *et al.*, 2012), and inflammatory response (Thomas *et al.*, 2018; Sohrabi *et al.*, 2020). LXRα also has been linked to several disease progressions such as breast cancer (Hutchinson *et al.*, 2021), atherosclerosis (Watanabe *et al.*, 2005), and fatty liver disease (Ahn *et al.*, 2014; Becares *et al.*, 2019).

ABCA1, the main target gene of LXRα, has been pointed out to be critical in the development of LXRα ligands as drug target since ABCA1 function is useful especially in removing excess cholesterol from cells hence could lead to potential effects for dyslipidemia and atherosclerosis (Lund, Menke, and Sparrow, 2003). A synthetic ligand of LXRα, such as T0901317 and GW3965 has been developed to treat metabolic disorders in the animal model. (Cao *et al.*, 2003; Laffitte *et al.*, 2003; Baranowski *et al.*, 2014). However, several side effects such as liver steatosis and hyperlipidemia were reported which become the major drawback for the development of LXRα ligand as a therapeutic agent (Schultz *et al.*, 2000; Quinet *et al.*, 2006; Bischoff *et al.*, 2010; Zhang *et al.*, 2012; Ni *et al.*, 2019).

Meanwhile, polyphenols are naturally occurring plant bioactive compounds that have been associated with health benefits for humans. The rationale underlying this hypothesis

is based on the structural similarity between polyphenols and cholesterol derivatives suggests that polyphenols may have acted as a ligand for nuclear receptors, especially LXRα (Avior, Bomze et al. 2013).

Previous publications demonstrated that hesperetin managed to reduce foam cell formation and promote cholesterol efflux on THP macrophages by upregulating LXRα and ABCA1 protein expression (Iio *et al.*, 2012; Chen *et al.*, 2020, 2021). Furthermore, hesperetin also enhances LXR and ABCA1 activity on THP-1 macrophages transiently transfected with LXR and ABCA1 promoter (Iio *et al.*, 2012). Naringenin, a flavonoid found in grapefruit, was also found to induce not only LXRα mRNA and protein expression, but also the expression of LXRα target genes in THP-1 macrophages (Saenz *et al.*, 2017). Polyphenols extract from *Hibiscus sabdariffa* leaf (Chen *et al.*, 2013) and Shanxi-aged vinegar extract (Song *et al.*, 2022) were also shown to inhibit foam cell formation and reduce lipid accumulation on J774A1 and HepG2 cells, respectively, through upregulation of LXRα/ABCA1 pathway.

Screening for nuclear receptor novel ligand could be done using several methods. Computational methods including the ligand-based methods and structure-based modeling methods (Buñay *et al.*, 2020) such as molecular docking has also been as it can be used to predict binding models and also study the interaction between receptors and ligands (Wang *et al.*, 2018). A previous study based on transfection assay, measurement of LXRs target genes modulation and molecular docking revealed that cyanidin and quercetin showed the ability to fit into the ligand-binding pocket of both LXRα and LXRβ (Fouache *et al.*, 2019), suggesting their potency as novel LXR ligand.

The molecular mechanisms behind the health benefits of polyphenols have not yet been completely elucidated. Many researchers suggest that it's associated with their direct antioxidant activity (Leri *et al.*, 2020). However, most polyphenols are heavily metabolized and have poor bioavailability that might impact their activity on different cell lines. Besides, to show antioxidant activity, polyphenols must be of high concentration which may be difficult if obtained from dietary intake (Tsao, 2010). Recently, some polyphenols have been reported to activate other metabolic regulators, the nuclear receptors (Avior *et al.*, 2013). Nuclear receptors directly regulate the transcription of genes that help with many biological processes such as metabolism, cell proliferation, development, and immunity

response (Sever and Glass, 2013). Therefore, this chapter will be focused on the investigation of polyphenols to act as a ligand for LXRα and the ability of polyphenols to modulate LXRα activity as well as its effect on downstream signaling. For that purpose, a representative from each polyphenol class will be tested for its ability to modulate LXRα activity and its target genes on the cell model.

3.2 Methodology

The focus of this chapter was for screening polyphenols from different classes for their ability to modulate LXRα activity on the MDA-MB-231 cell line stably transfected with the LXRα reporter gene. The MDA-MB-231-Luc cell line was provided by Dr. Samantha Hutchinson and its generation (Hutchinson and Thorne, 2019) and application to assess LXR ligand function (Hutchinson, Lianto, Moore, *et al.*, 2019; Hutchinson, Lianto, Roberg-Larsen, *et al.*, 2019; Hutchinson *et al.*, 2021) have been reported previously. Representatives of each polyphenol class were chosen, namely resveratrol, hesperetin, genistein, EGCG, quercetin, and isorhamnetin for their ability to induce LXRα activity on the MDA-MB-231-Luc cell line. Polyphenols that showed modulation, were further analyzed for their ability to modulate the LXRα target gene, ABCA1. Furthermore, the ability of selected polyphenols will also be assessed in combination with known LXRα ligands to evaluate its interaction in the presence of LXRα agonist and antagonist on LXRα activation. Selected polyphenol will be further used as the main compound for the later experiments. Figure 3.1 below shows a summary of the steps used.

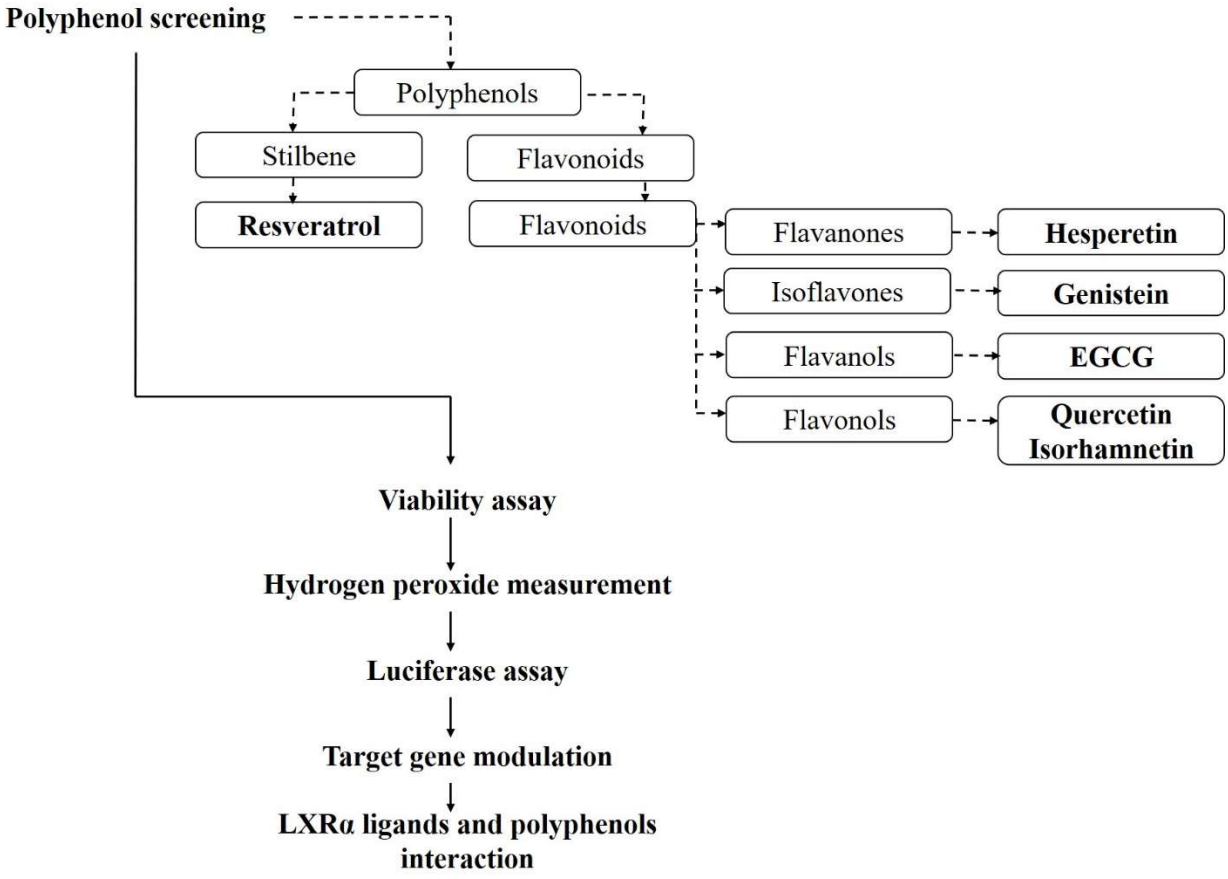


Figure 3.1 Overview on polyphenol selection and experimental workflow

3.3 Results

3.4.1 Polyphenol effects on MDA-MB-231 cell viability

The cytotoxicity effects of polyphenols were determined by the neutral red assay. The concentration tested in this experiment was based on the effective concentration to suppress pro-inflammatory markers on numerous cell lines as shown in previously published research (Boesch-Saadatmandi, Pospissil et al. 2009, Boesch-Saadatmandi, Loboda et al. 2011, Lanzilli, Cottarelli et al. 2012, Wu, Choi et al. 2017, Tong, Chen et al. 2020, Teng, Li et al. 2021, Wu, Qian et al. 2021).

The control treatment was vehicle control (DMSO 0.1%). During incubation, the cell culture medium contained only 0.1% DMSO as it is the safest concentration and has proven not to interfere with in vitro cell-based assay as tested for 20 hours of incubation in five different cell culture systems, including PBMC, peripheral blood mononuclear cells

(PBMC), peripheral leukocytes, the human monocytic cell line Mono Mac 6 (MM6), the murine monocytic cell line RAW 264.7 and the HL-60 cell line (Timm *et al.*, 2013). As shown in Figure 3.2, quercetin 50 µM, resveratrol 50 µM, isorhamnetin 10 µM, and EGCG 10 µM reduced cell viability to 80, 86, 81, and 88% respectively compared to the vehicle control. Meanwhile, there was no cytotoxicity effect shown on cells incubated with hesperetin and genistein as shown in Figure 3.3. Based on this result, those concentrations are considered as sub cytotoxic since there are still sufficient viable cells for further measurement that will be used for further experiments.

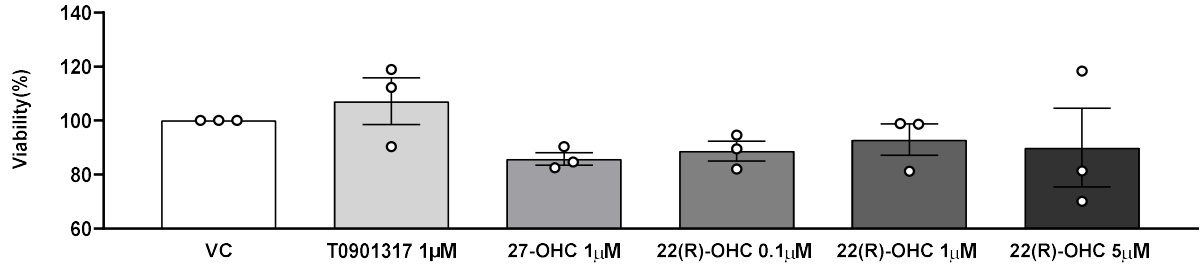


Figure 3.2 Effects of LXRα ligands on the viability of triple-negative breast cancer cells. MDA-MB-231 cells were incubated with different LXRα agonists in increasing concentrations for 24 hours. Cytotoxicity was assessed using a neutral red assay. Data are mean with SEM of three independent experiments performed in duplicate. *, ** and *** indicate significant differences to the control group (Dunnett's test), $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

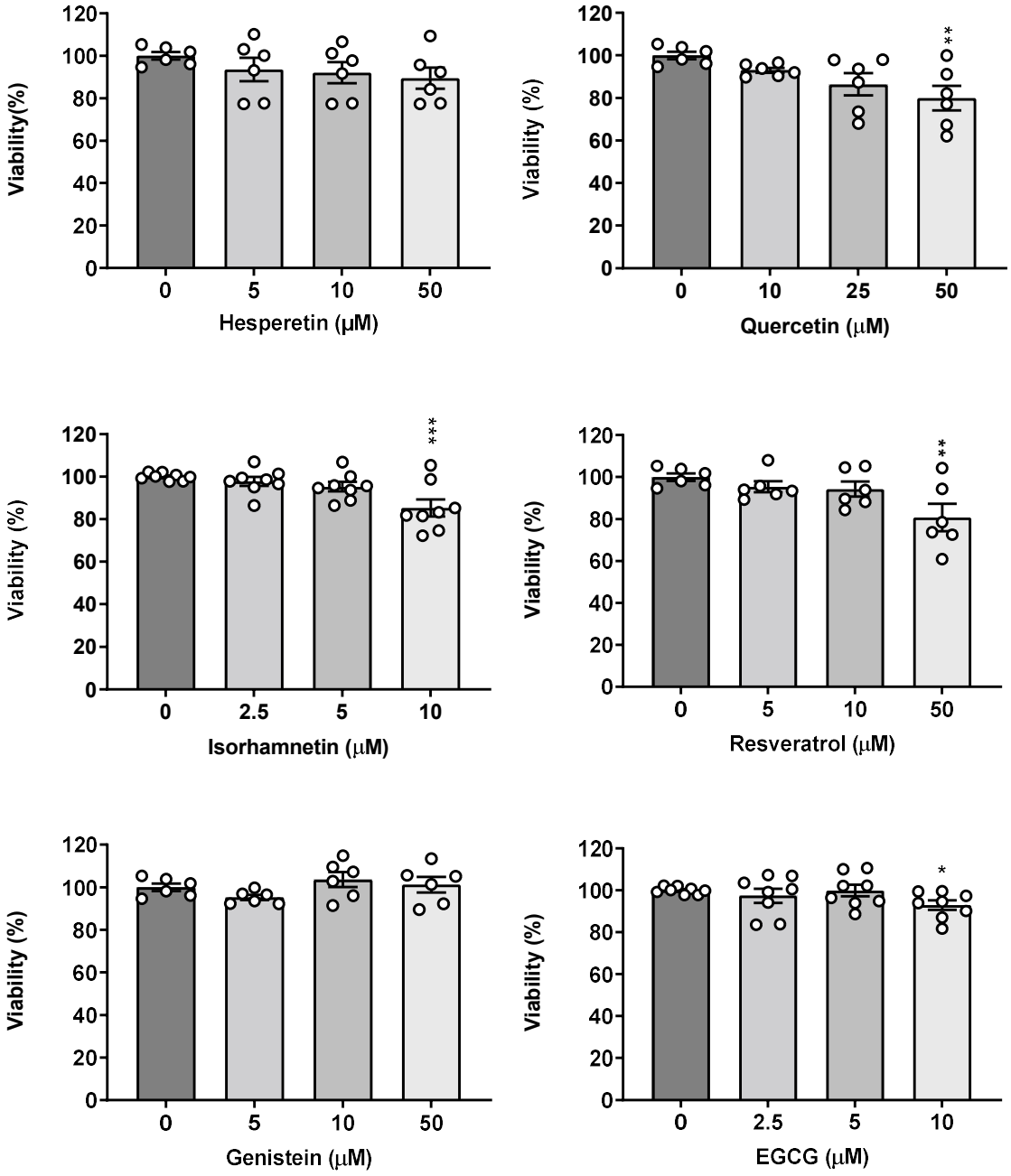


Figure 3.3 Effects of polyphenol test compounds on viability of triple-negative breast cancer cells. MDA-MB-231 cells were incubated with increasing concentrations of (A) LXRα agonists, and (B) hesperetin, quercetin, resveratrol, genistein (0, 5, 10, 50 μM) and isorhamnetin and epigallocatechin gallate (EGCG) (0, 2.5, 5, 10 μM). Cytotoxicity was assessed using a neutral red assay. Data are mean with SEM (n>3). *, ** and *** indicate significant differences to the control group (Dunnett's test), $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

3.4.2 Screening of polyphenols on LXRα reporter cell

In order to normalize reporter data, the reporter activity for a particular sample is divided by a second value specific to the same sample. Normalization removes sample-to-sample variations caused by factors that aren't the factors tested in the experiment, such as variations in cell plating, transfection efficiency, pipetting inconsistencies, and toxicity. In this study, protein normalization was chosen as it can improve results from cells that are stably transfected. Protein quantification was measured using BCA assay as explained further in the Methods section (Chapter 2).

According to Figure 3.4, both endogenous and synthetic ligands of LXRα have a strong activation at 1μM concentrations. Figure 3.5 shows LXRα activation by polyphenols where the result showed that polyphenols tested in this experiment modulate LXRα to a certain degree. Compared to other polyphenols tested in this experiment, EGCG shows no effect on LXRα modulation activity since it shows no difference compared to the unstimulated cells. Quercetin, hesperetin, resveratrol, and genistein showed moderate ability in modulating LXRα. Furthermore, the result from the LXRα reporter gene shows that resveratrol and genistein are prospective compounds to enhance LXRα activity.

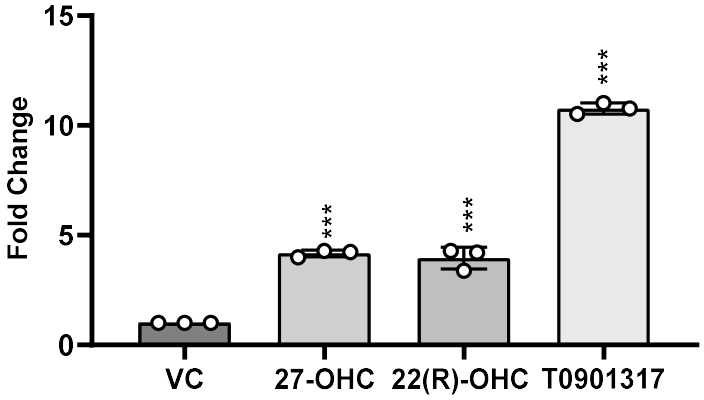


Figure 3.4 Effects of natural (oxysterol; 22R and 27-OHC) and synthetic (T0901317) ligand of LXRα activation. MDA-MB-231 cells were incubated with different LXRα agonists at 1μM. Data are mean with SEM of three independent experiments performed in duplicate. *, ** and *** indicate significant differences to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

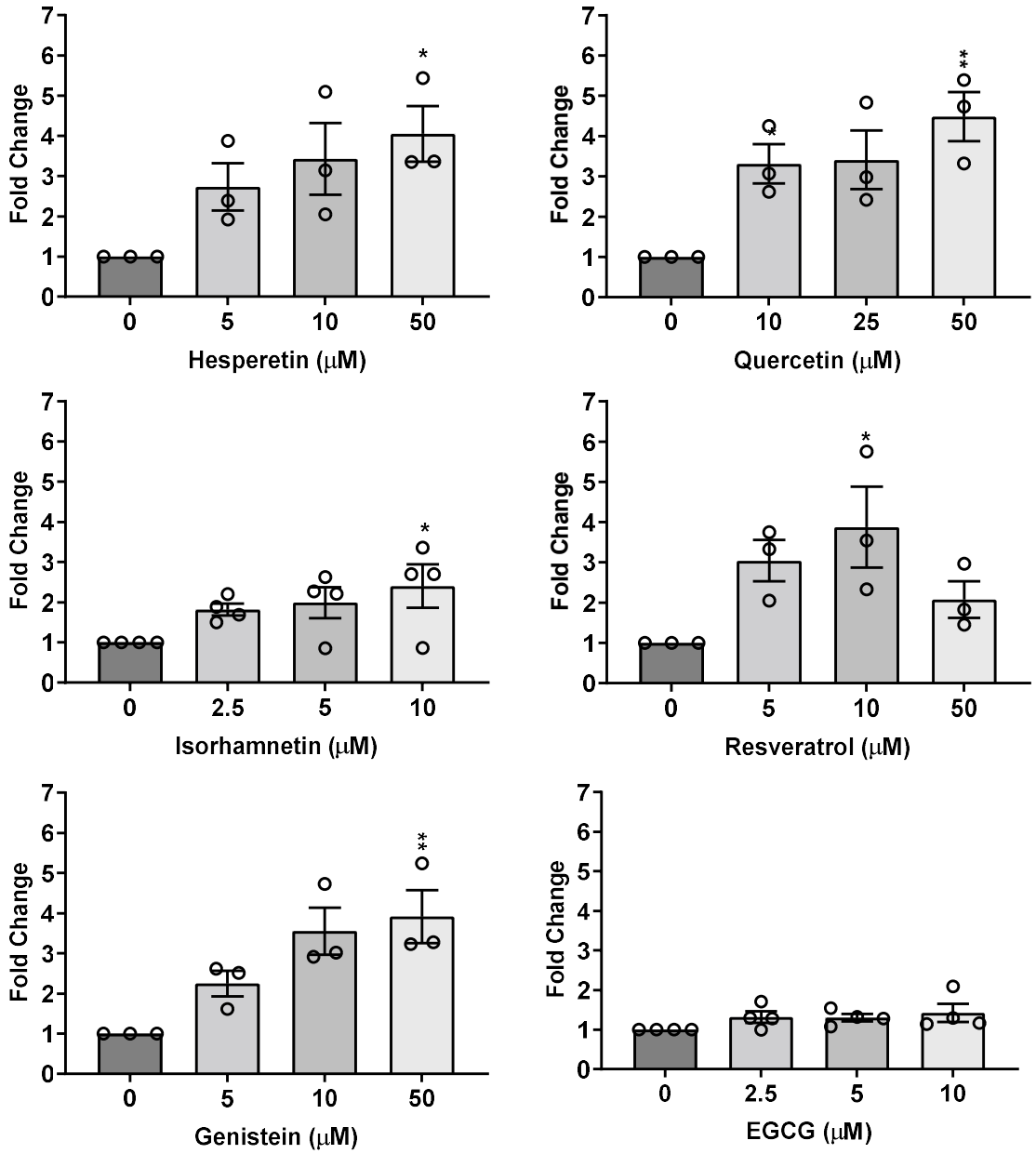


Figure 3.5 Activation of LXRα following incubation with selected polyphenols to represent different subclasses of flavonoids. Data were normalised to the amount of cell protein and fold change was calculated relative to vehicle control. Data are mean with SEM from three independent experiments performed in duplicate. *, **, and *** indicate significant differences to the control group (Dunn's test: hesperetin, quercetin, resveratrol, genistein; Dunnett's test: isorhamnetin and EGCG), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

3.4.3 Polyphenols and LXRα ligand interaction in modulating its target gene

Based on the result from the reporter cell, it was decided to use quercetin, resveratrol, and genistein for further experiments. In theory, activation of LXRα following ligand binding will lead to modulation of its target genes. In this experiment, the expression of ABCA1 as a canonical LXRα target gene was explored. The cells were incubated for 24 hours with selected polyphenols in increasing concentration. Figure 3.6 demonstrated activation of LXRα in reporter cell is followed by modulation of ABCA1 on mRNA level. Quercetin in high concentration (50 μM) generated the highest modulation compared to other concentrations even though quercetin 50 μM decreased the cell viability. On the contrary, resveratrol and genistein do not cause significant modulation of ABCA1 on mRNA level even though their activation in the reporter cells is statistically significant. This indicates that not all activation on reporter cells will cause modulation on mRNA level.

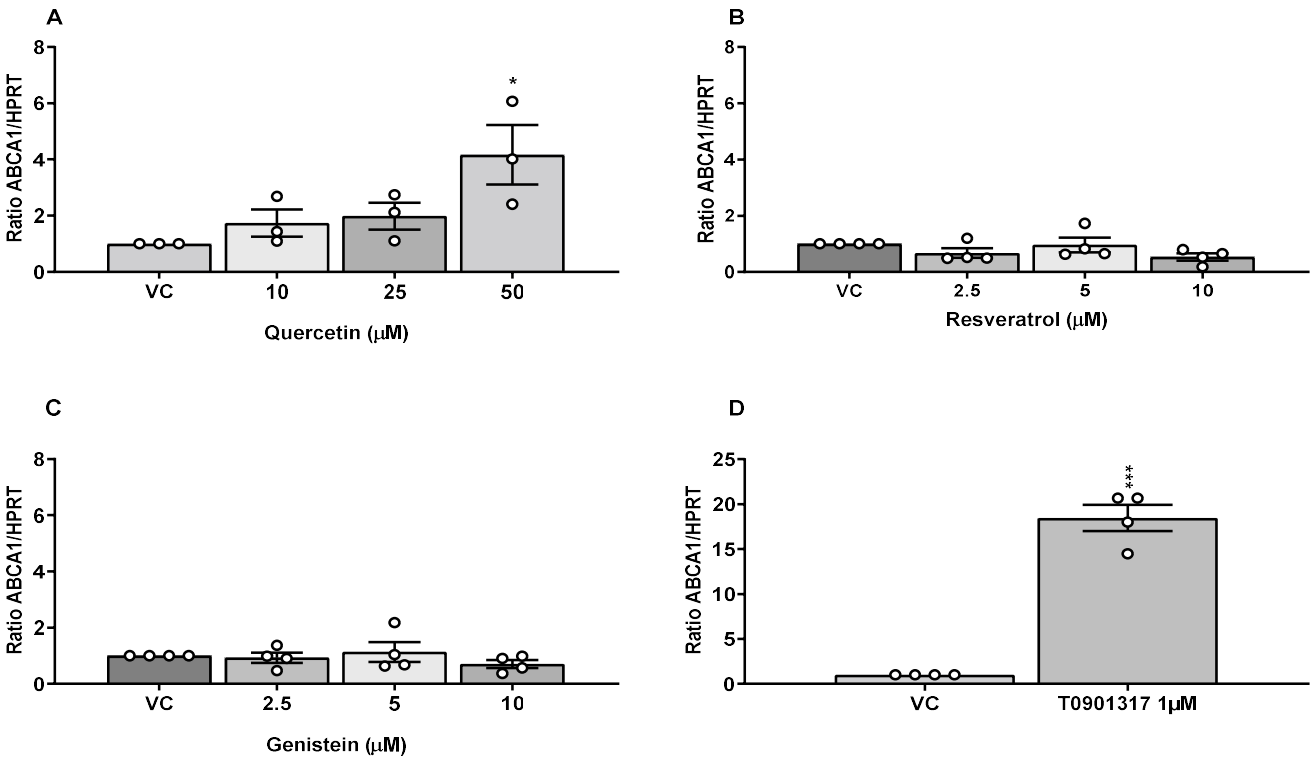


Figure 3.6 ABCA1 mRNA expression on MDA-MB-231 cells following test compounds incubation. Cells were treated with (A) quercetin, (B) resveratrol, (C) genistein, and (D) T0901317 for 24 hours. Fold changes are shown relative to HPRT as a housekeeper. Data are mean with SEM from three independent experiments performed in duplicate. *, **, and ***, indicate significant differences to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

In order to evaluate that quercetin modulates ABCA1 expression through the LXRα pathway, further experiments involving LXRα antagonists, GSK2033, were conducted. As shown in Figure 3.7, co-treatment between quercetin and GSK2033 diminished any activation caused by quercetin. Furthermore, Figure 3.8 further showed the weakened effect of quercetin on ABCA1 modulation. Taken together, both data suggest that GSK2033 and quercetin bind to the same ligand-binding domain of LXRα and that GSK2033 has a stronger binding affinity to LXRα compared to quercetin.

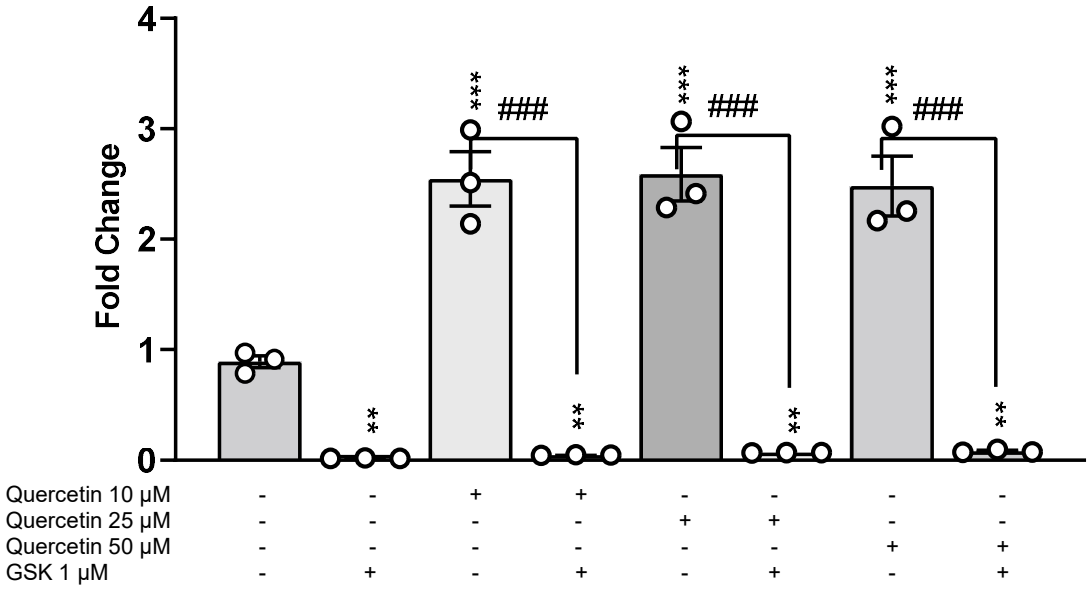


Figure 3.7 The activation of LXRα by quercetin and LXRα antagonist. Activation of LXRα is abolished by co-incubation with antagonist GSK2033. Experiments were conducted in human breast cancer cells, MDA-MB-231, stably transfected with LXRα reporter. Data presented as mean with SEM from three independent experiments performed in duplicate. *, **, and ***, indicate significant differences to the control group (ANOVA followed by Dunnett's t-test), meanwhile #, ##, and ### represent significance by t-test, $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

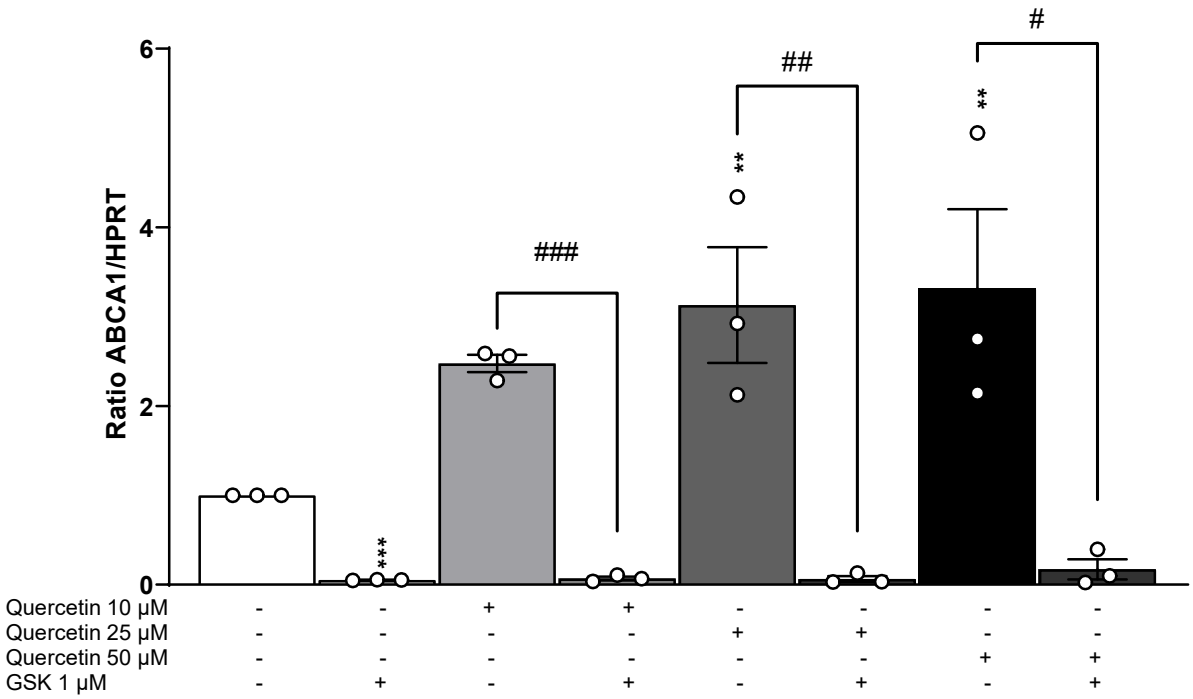


Figure 3.8 Inhibition of ABCA1 mRNA expression in human breast cancer cells by LXRα antagonist. The cells were treated with quercetin in increasing concentration in combination with GSK2033 for 24 hours. Fold changes are shown relative to HPRT as a housekeeper. Data are mean with SEM from three independent experiments performed in duplicate. *, **, and *** indicate significant differences to the control group (Dunnett's test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

After evaluating the interaction between quercetin and antagonist, it was decided to test the effect on co-treatment between quercetin and agonist, 27-OHC, to find if there was a specific effect on LXRα activation on the reporter cell model. 27-OHC was chosen as the endogenous agonist since it is one of the most abundant cholesterol metabolites in the body (Olkkonen, Béaslas and Nissilä, 2012). Later, the data showed that a combination of both does not have a significant effect compared to 27-OHC alone (Figure 3.9). This also indicates that 27-OHC has a higher binding affinity compared to quercetin.

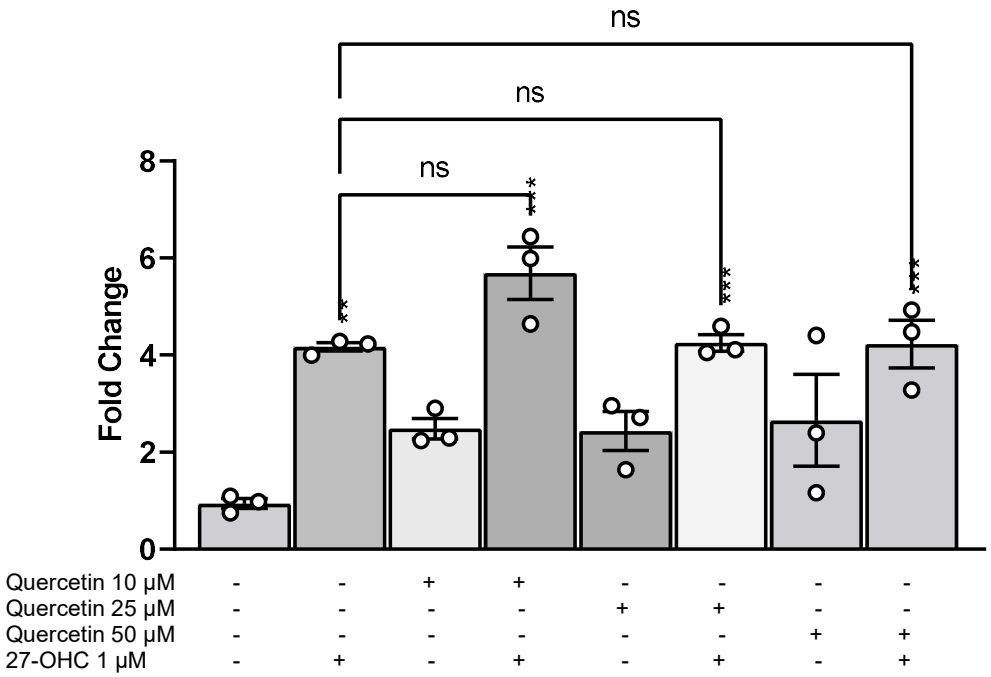


Figure 3.9 Combination effect in the activation of LXRα by quercetin and 27-hydroxycholesterol. The cells were treated with quercetin in increasing concentration in combination with 27-OHC for 16 hours. Data presented as mean with SEM from three independent experiments performed in duplicate. *, **, and ***, indicate significant differences to the control group (ANOVA followed by Dunnett's test) $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

3.4 Discussion

In this present study, the data suggest that quercetin showed activity to modulate LXRα activity better than other polyphenols tested. Several methods could be used to investigate appropriate ligand that functions as a specific nuclear receptor modulator, such as computational, biochemical, and cellular methods. Cellular reporter gene assay for NRs ligand screening is widely used since it has many advantages including its straightforwardness and versatility since it can measure transcriptional activity caused by ligand binding to the nuclear receptor. It also can evaluate the dose-dependent effect of a certain compound (Bunay, Fouache et al. 2020).

In this present study, selected polyphenols that were tested showed that hesperetin, genistein, resveratrol, and quercetin, but not isorhamnetin showed modulation on LXRα activity. To further prove the ability of polyphenols to act as a ligand for LXRα, quercetin,

resveratrol, and genistein was chosen to determine ABCA1 modulation on mRNA level, as the primary target of LXRα. According to the results obtained in this study, only quercetin increased the mRNA expression of ABCA1, whilst quercetin and resveratrol showed no changes (Figure 3.6). Further analysis using co-incubation between quercetin and GSK2033 confirms that quercetin is a weak ligand for LXRα. Co-incubation between quercetin and LXRα antagonist, GSK2033, both on reporter and wild-type cells fully eliminates quercetin action. Meanwhile, co-treatment between quercetin and LXRα natural agonist, 27-OH, as shown in Figure 3.9, showed no difference in response compared to 27-OHC alone. This indicates that both GSK2033 and 27-OHC have a strong affinity to bind to LXRα, suggesting that quercetin action as a ligand for LXRα is weak.

Interestingly, although isorhamnetin is one of the metabolites from quercetin that has high similarity in chemical structure, it shows different abilities in activating LXRα. As suggested by Fouache et al (2019), slight changes in hydroxyl group position may change the action from agonist to the antagonist (Fouache, Zabaiou et al. 2019). In this case, the difference between quercetin and isorhamnetin lies in the replacement of the hydroxyl group with the methyl group. This present study confirms the theory that structural changes play an important role in ligand binding to a nuclear receptor.

The ability of oxysterols as LXRα modulators has been proven previously (Hutchinson, Lianto, Roberg-Larsen, *et al.*, 2019). As expected, both natural and synthetic ligands of LXRα showed statistically significant effects on LXRα activity in lower concentration compared to quercetin (Figure 3.4) although the concentration used in this study is higher than physiological concentrations found in the human body (~0.01-0.1μM in plasma) (Schroepfer Jr, 2000).

Although polyphenols have been researched for their ability to modulate nuclear receptors activity, their specificity to a certain nuclear receptor is uncertain. In this present study, resveratrol, quercetin, and genistein were shown to modulate LXRα activity. Meanwhile, a previous study revealed that the same polyphenols tested in this study also modulate other nuclear receptors such as quercetin upregulates the expression of PPARγ (Lee *et al.*, 2013), meanwhile, resveratrol and genistein induces ERα expression

(Levenson *et al.*, 2003; Liao *et al.*, 2014). These data suggest that polyphenols might act as a modulator for several nuclear receptors.

As a transcription factor, LXRα is responsible for the transcription of its target genes, including ABCA1. Present work demonstrated the complexity of gene regulation. For example, genistein and resveratrol that showed modulation of LXRα on reporter cells did not accompany the modulation of ABCA1 expression on mRNA level suggesting that those compounds are not eligible as novel LXRα ligand. Besides, although the reporter cell system is a powerful method for screening possible ligands, it is still a synthetic system that needs further verification.

Despite efforts in polyphenol research and polyphenol's beneficial effects on human health, the evidence for polyphenols as a ligand for nuclear receptors, molecular mechanisms, and effect on cell metabolism is scarce. A study conducted in 2019 by Fouache showed a similar result to this study by showing that flavonoids have distinct effects on both LXRs isoforms where agonistic activities were shown on quercetin in both LXRs isoforms, meanwhile, apigenin only induced LXRβ. Furthermore, they also showed that galangin antagonizes LXRs action (Fouache *et al.*, 2019). The difference between these two studies is in the selection of polyphenols tested. While they were focused on flavonoids, this study also included resveratrol as the representative of stilbene. Both studies agree that quercetin showed agonistic activity on LXRα and induces its main target genes, ABCA1.

The current study demonstrated that among polyphenol tested, quercetin showed the most ability to act as a ligand for LXRα. To further prove quercetin’s potential as a ligand, the next chapter will focus to explore the possible mechanisms mediated by LXRα activation on the cellular level, especially in suppressing the inflammatory response, both in acute and chronic models. To ensure that there is no effect on H2O2 production due to polyphenols treatment on inflammatory signaling and proinflammatory cytokines production, hydrogen peroxide was measured beforehand, and no significant effects were found that might interfere with further experiments (Appendix 1). In this present study, only modulation of ABCA1 was observed as the main target gene of LXRα. By studying a single target gene, it is not possible to fully understand how LXR signaling works. Meanwhile, this study did not consider the LXRβ role in ABCA1 modulation. Furthermore,

the data also showed that in order for quercetin to modulate the expression of the LXRα target gene, it needs to be in relatively high concentration, above the level that could be achieved from the diet.

Even though T0901317 is known as a potent LXRα and LXRβ modulator, its action is not specific to LXRs. It also activates both FXR and LXR even in low concentrations (Chuu and Lin 2010, Raksaseri, Chatsudthipong et al. 2013). Hence for further experiments, GW3965 will be used as a positive control instead of T0901317.

3.5 Conclusion

The chemical structure is crucial for the development of LXRα selective ligands. Among polyphenols tested in this study, only quercetin shows the ability to activate LXRα in the reporter cell model and modulates the LXRα target gene, ABCA1. Experiments involving LXRα agonist and antagonist indicate that quercetin is a weak ligand for LXRα. This study also demonstrates that the reporter cell model is a good tool to screen potential ligand candidates. However, not all activation was found to lead to target gene modulation in wild-type cells.

CHAPTER 4. The effects of quercetin and its metabolites on LXRα activation and inflammatory cytokines production in liver cells

Abstract

In vitro and some animal studies revealed that quercetin displays a broad range of health benefits, including an anti-inflammatory property. However, the health benefits of quercetin are affiliated with the constituents and concentrations of its metabolites. LXRα is a ligand-dependent transcription factor that plays a crucial role in cholesterol metabolism and immunity. LXRα is mainly expressed in organs involved in lipid metabolism, including liver, kidney, and macrophages. Here, the ability of quercetin and its methylated and glucuronidated metabolites was evaluated, namely tamarixetin, isorhamnetin, and quercetin-3-glucuronide, in suppressing inflammation by acting as a ligand for liver X receptor alpha (LXRα). HepG2, a human liver cancer cell line, was used as a cell model since quercetin is mainly metabolized in the liver. Besides, LXRα is highly expressed in the liver, making HepG2 a suitable cell model for this experiment. In the present study, both quercetin and tamarixetin, a methylated derivative of quercetin, activated LXRα in the LXRα reporter cell model in a dose-dependent manner. Whilst tamarixetin induced LXRα target genes ABCA1 and APOE on the transcriptional level, quercetin incubation did not lead to changes in target gene expression. Furthermore, the results also showed that both quercetin and tamarixetin did not show inhibitory effects on TNF-α induced inflammation in HepG2 cells. In conclusion, this study showed that tamarixetin is more potent than quercetin in having the ability to act as a ligand for LXRα. Interestingly, quercetin, tamarixetin, and LXRα synthetic agonist did not show any anti-inflammatory activity in suppressing hepatic inflammation.

4.1 Introduction

Polyphenols have been proposed to exert several physiological health benefits including antioxidant and anti-inflammatory activities. Epidemiological studies and associated systematic review revealed that long-term intake of dietary polyphenols from fruits and vegetables have favorable effects on the pathogenesis and incidence of several chronic and metabolic diseases, such as cardiovascular diseases, diabetes mellitus, and neurodegenerative disease (Cory *et al.*, 2018; Del Bo' *et al.*, 2019; Luca *et al.*, 2020).

Studies regarding polyphenol health benefits were mostly derived from in vitro experiments or animal models, however little is known about the exact mechanisms underlying their health benefits (D'Archivio *et al.*, 2010). The main drawback of those experiments is that the compounds tested were polyphenol aglycones rather than their active metabolites. Moreover, concentrations used were much higher than what would be achievable through a diet. Importantly, polyphenol bioavailability is low (Luca *et al.*, 2020). For example, the bioavailability of quercetin in a human was about 44.8% following 100 mg administered orally (Walle, Walle and Halushka, 2001).

Quercetin is one of the most abundant dietary flavonoids found in fruits and vegetables, such as broccoli, apples, onions, and berries (David, Arulmoli, and Parasuraman, 2016). Average daily quercetin intake from food varies among countries. In the US, the daily intake of quercetin among the adult population is 3.5 mg/day (Dabeek and Marra, 2019) meanwhile in Finland and China the intake is 3.3 (Knekt *et al.*, 2002) and 20.9 mg/day, respectively (Yao *et al.*, 2019).

Most quercetin in foods is attached to a sugar molecule known as a glycoside. Following ingestion, quercetin aglycones are absorbed in the intestine as aglycones via deglycosylation by enterobacteria. After absorption, quercetin suffers metabolism in several organs, such as the small intestine, liver, colon, and kidney. Quercetin metabolites are mainly formed in the small intestine and liver which are the results of phase II metabolism by biotransformation enzymes including catechol-O-methyl-transferases (COMTs), sulfotransferases (SULTs), and UDP-glucuronosyltransferases (UGTs) (Hai *et al.*, 2020; Ulusoy and Sanlier, 2020).

A study using Sprague-Dawley rats to estimate the distribution of quercetin and its metabolites after oral and intravenous administration demonstrated that 93% and 3.1%

of quercetin were metabolized in the intestine and liver, respectively (Chen *et al.*, 2005). Meanwhile, a previous study in mice fed with high quercetin diet revealed that 48% of quercetin was methylated to isorhamnetin while this particular metabolite was found in human plasma in low amounts (Boesch-Saadatmandi *et al.*, 2010). Furthermore, quercetin and its metabolites were also distributed in rat tissues following the quercetin diet. The highest concentration was found in the lungs, and the lowest concentration was in the brain, white fat, and spleen (De Boer *et al.*, 2005).

Meanwhile, in humans, the major quercetin metabolites found in plasma after 1.5 hours of onion consumption were quercetin-3-glucuronide, 3'-methyl-quercetin-3-glucuronide, and quercetin-3'-sulfate (Day *et al.*, 2001). Another study revealed that several metabolites of quercetin, namely quercetin-3'-glucuronide, quercetin-7'-glucuronide, quercetin-7-glucuronide, and quercetin-3-glucuronide were detected following 500 mg quercetin supplementation with quercetin-3'-glucuronide as the main metabolite (Chalet *et al.*, 2018). Isorhamnetin, tamarixetin, isorhamnetin-3-O-glucoside, are also proven to have anti-inflammatory activity by decreasing the production of 12-HHT, PGE2, and 12-HETE in vivo (Lesjak *et al.*, 2018).

The research exploring the health properties of quercetin metabolites is limited. Experimental studies have revealed that quercetin metabolites showed the anti-inflammatory property in HUVEC and murine RAW264.7 macrophages cell lines (Tribolo *et al.*, 2008; Boesch-Saadatmandi *et al.*, 2011). According to Morand et al, a 0.2% quercetin-supplemented diet in rats led to the formation of circulating metabolites such as isorhamnetin, tamarixetin, and quercetin glucuronides. These studies suggested that the health benefits of quercetin might be due to the circulating metabolites instead of the parent compound.

In the previous chapter, it was shown that some polyphenols including quercetin were able to increase LXRα activation in breast cancer cell-based LXRα reporter system. To consider the aspect of metabolism to polyphenol potential, quercetin and some of its in vivo metabolites were selected and exposed to liver cells. Although the human liver is known as a non-immunological organ, it is continuously exposed to numerous inflammatory stimuli including microbial products, toxic products, and hepatotropic pathogens resulting in persistent inflammation. During acute inflammation in the liver,

several inflammatory mediators are released as a response such as IL-6, IL-1, and TNF-α (Robinson, Harmon, and O'Farrelly, 2016).

LXRα as a nuclear receptor has a pivotal role in lipid metabolism as the cholesterol sensors that transport excess cholesterol to the liver and biliary excretion. LXRα is mainly expressed in the liver, adipose tissue, and kidney (Wójcicka *et al.*, 2007). Another biological role of LXRα is linking lipid metabolism and inflammation, especially in immune cells such as macrophages by contributing to the reverse cholesterol transport pathway (Noelia and Castrillo, 2011; Schulman, 2017). Chapter 5 showed that LXRα synthetic agonist suppressed pro-inflammatory cytokines production in murine RAW264.7 macrophage cell. On the contrary, LXRα's role in hepatic inflammation is remain scarce. This chapter will be focused on the ability of quercetin metabolites to activate LXRα in liver cells and its impact on inflammatory cytokines production following TNF-α induction.

4.2 Methodology

The primary focus of this chapter was to explore the ability of quercetin and some of its metabolites on LXRα activation on liver cells. In this chapter, quercetin, quercetin-3-glucuronide, tamarixetin, and isorhamnetin were tested using HepG2 cell line stably transfected with LXRα reporter system. Later, to further ensure that the LXRα-induced activation by quercetin and its metabolites leads to target genes modulation, HepG2 cell wild type was treated with selected compounds for a certain period. Furthermore, to evaluate the role of selected compounds on cells metabolism, HepG2 cells were incubated with TNF-α to induce hepatic inflammation. Optimization was done first to determine the appropriate concentration and time point of TNF-α incubation to induce hepatic inflammation. Pro-inflammatory cytokines production was measured to analyze the inflammatory suppression of LXRα-quercetin/metabolites activation. Detailed procedures for methods used in this chapter are described in Chapter 2.

4.3 Results

4.3.1 Cytotoxicity of test compounds on HepG2 cells

To determine the effect of LXRα agonists as well as quercetin and its metabolites on human liver cells, HepG2 cells were treated with quercetin and its metabolites, as well as

LXRα synthetic agonists, T0901317 and GW3965, in concentrations ranging from 1.0^{-5} to 1.0^{2} μM for 24 hours. Results (Figure 4.1) showed that the synthetic LXRa ligands T0901317 and GW3965 are toxic for the cells at high concentrations. In particular, T0901317 was more toxic for liver cells compared to GW3965 since 4 μM of T0901317 decreased cell viability at an average of 79.1 while the same concentration of GW3965 reduce the viability to 85% (P<0.05).

Meanwhile, quercetin as the parent compound started to decrease cell viability starting from concentration 6.25 μM and significantly reduced cell viability at 50 and 100 μM (P<0.05) by 19.9% and 23.8%, respectively. In contrast, among quercetin conjugates, none of them showed a cytotoxicity effect (Figure 4.2). Cells treated with 100 μM isorhamnetin significantly increased cells growth 27.6% higher compared to the controlled cells. According to the literature, if the relative cell viability compared to the control group is more than 70%, then the concentration used is considered non-cytotoxic (ISO, 2009). In the present study, concentrations chosen for further experiments have viability higher than 80%. Compared to the concentration used in murine RAW264.7 macrophage cell (Chapter 5), HepG2 show higher tolerance to quercetin. For example, 25μM of quercetin reduced viability in RAW264.7 cells to 78% whilst in HepG2 cells, it decreased the viability to 84%.

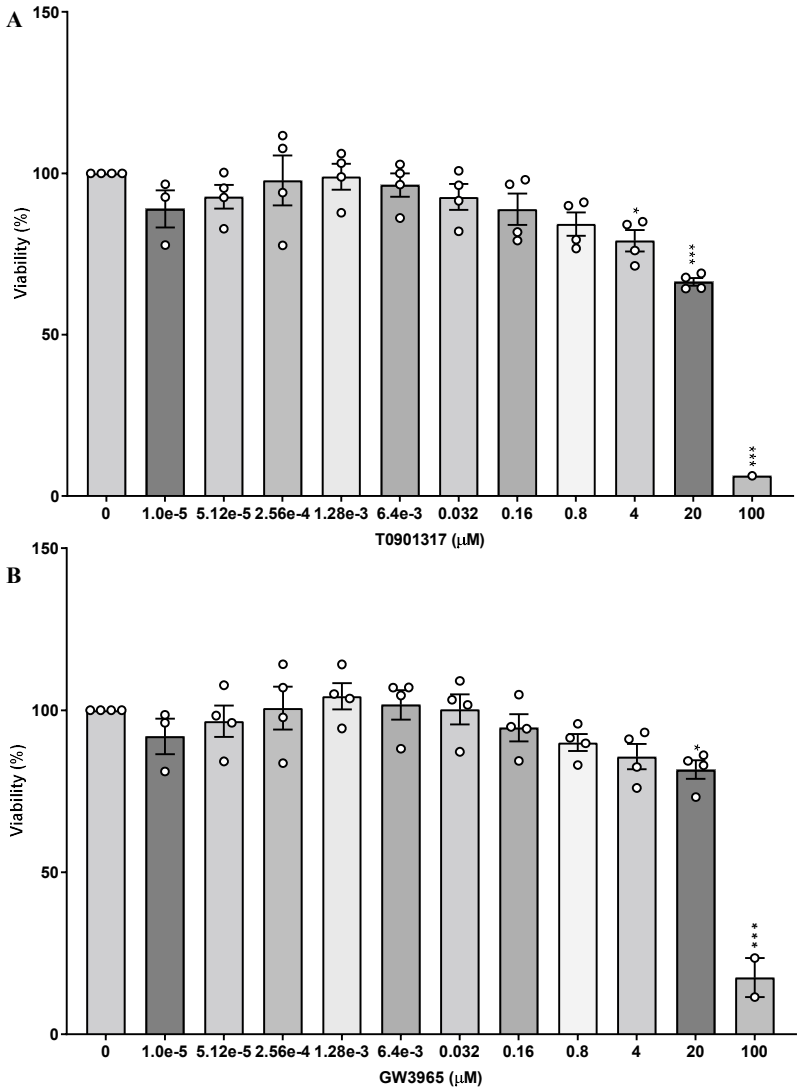


Figure 4.1 Cytotoxicity of LXRα synthetic ligands on HepG2 cell line. Cells were treated with (A) T0901317 and (B) GW3965 at increasing concentrations determined by the Neutral Red assay following 24 hours of test compound incubation. Data shown were mean ± SEM from three independent experiments performed in quadruplicate *, **, and *** indicate significant differences relative to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

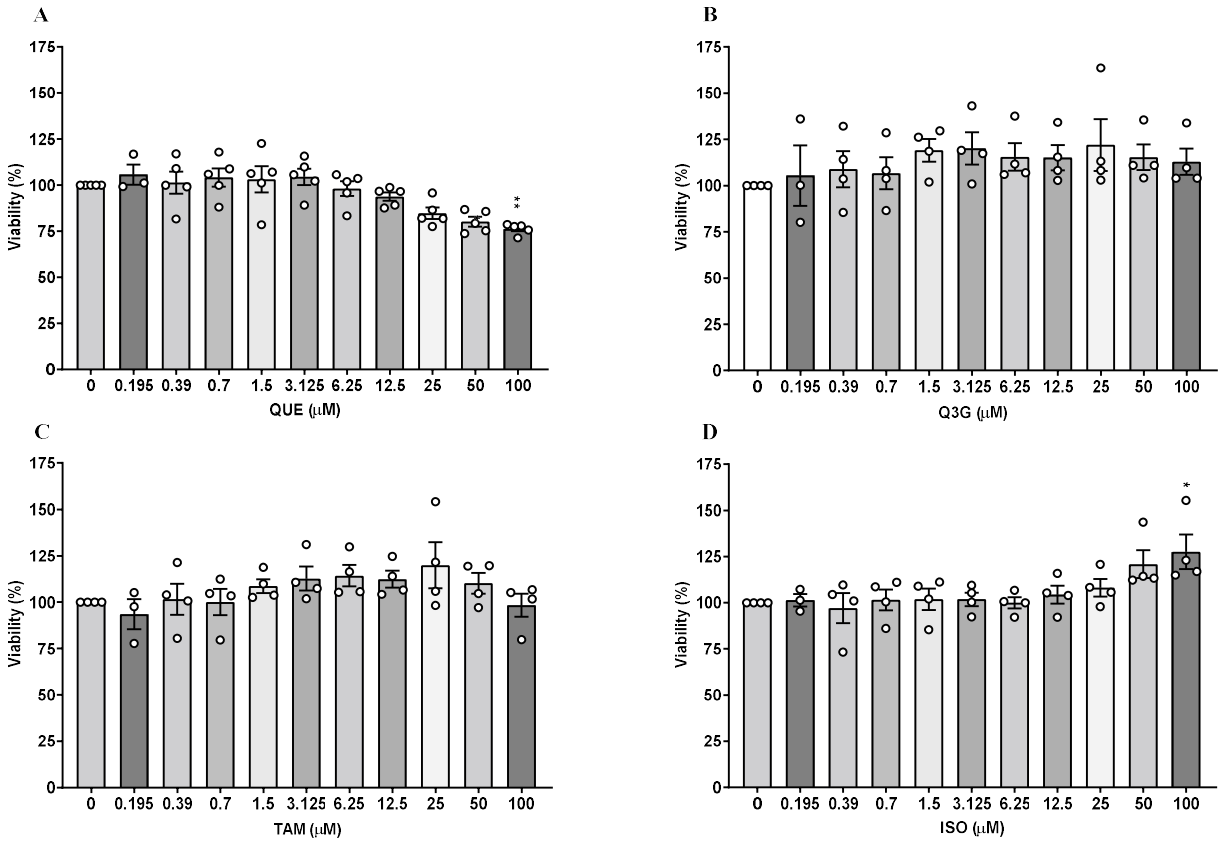


Figure 4.2 The cytotoxicity of quercetin and its metabolites on HepG2 cell line. Cells were treated with (A) quercetin (QUE) and its metabolites, (B) quercetin-3-glucuronide (Q3G), (C) tamarixetin (TAM), and (D) isorhamnetin (ISO) on HepG2 cells at an increasing concentration determined by the Neutral Red assay after 24 hours incubation. Data shown were mean ± SEM from three independent experiments performed in quadruplicate. *, **, and *** indicate significant differences relative to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

4.3.2 LXRα activation in HepG2 reporter cells by quercetin and its metabolites

In the previous chapter, quercetin has been shown to modulate LXRα in breast cancer cell lines stably transfected with LXRα reporter. Based on that result, further experiments were designed to explore the ability of quercetin and its metabolites in a more relevant cell line. Apart from quercetin aglycone, the metabolites chosen in this study were representatives from glucuronidation (quercetin-3-glucuronide), and methylation (tamarixetin and isorhamnetin) that were commercially available.

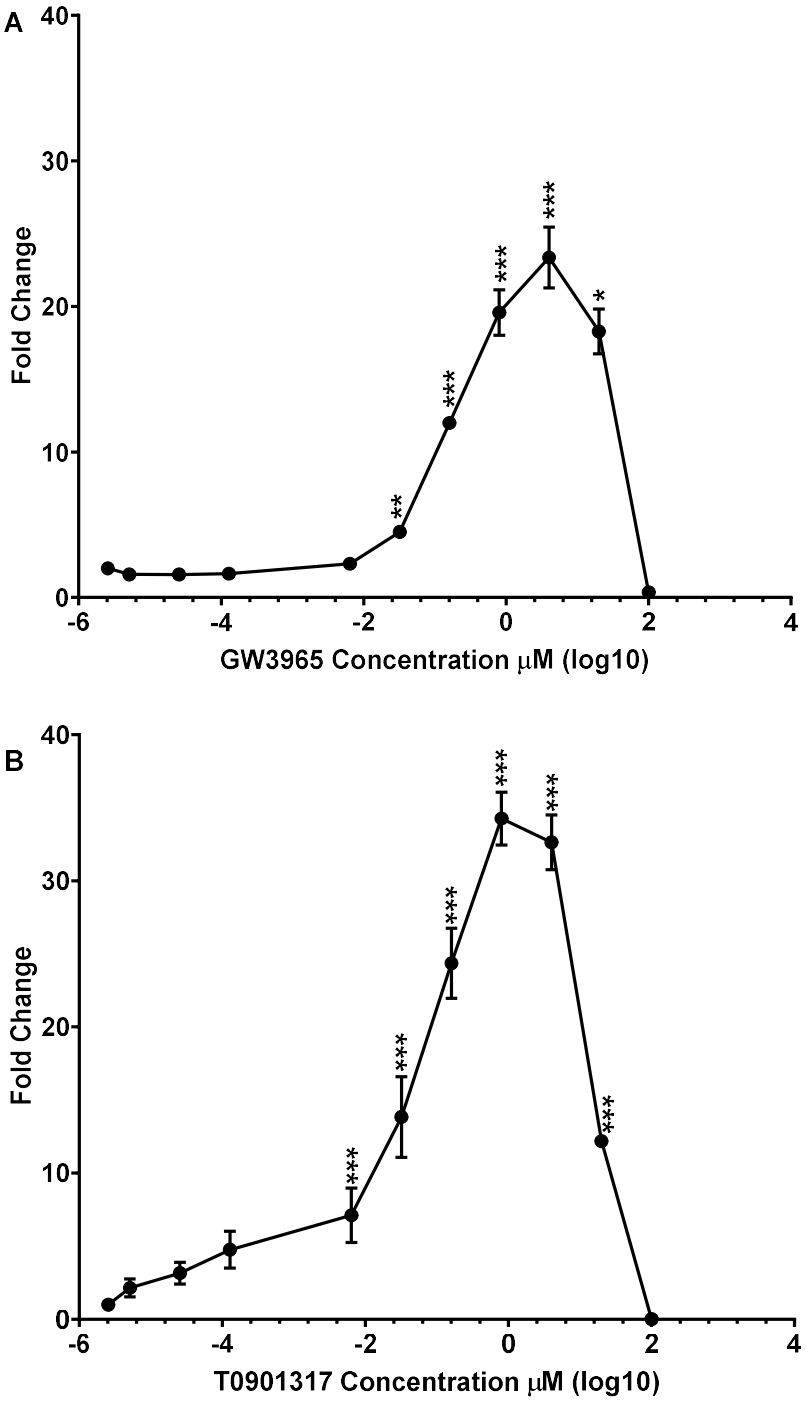


Figure 4.3 Dose-dependent effect of LXRα activation by its synthetic agonists. Cells were treated with (A) GW3965 and (B) T0901317on the LXRα reporter cell model following 16 hours of compounds incubation. Data shown were mean ± SEM from three independent experiments performed in quadruplicate. *, **, and *** indicate significant differences to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

Two known synthetic agonists of LXRα were tested at increasing concentrations starting from 0 to 100 μM (Figure 4.3). The results showed that both agonists worked well in activating the LXRα reporter gene cell model by increasing the activation in a dose-dependent manner. The loss signal of activation from both agonists on high concentration (100 μM) was caused by the cytotoxicity of both compounds as shown in Figure 4.1. A previous study using LXRα synthetic ligands on MDA-MB-468 and MCF7 cell lines stably transfected with the LXRα reporter system also showed activation in a dose-dependent manner (Hutchinson, Lianto, Roberg-Larsen, *et al.*, 2019).

According to figure 4.4, the results demonstrated that only quercetin and tamarixetin, a methylated conjugate, was able to show LXRα activation potential on reporter cells. Quercetin in increasing concentration showed the ability to activate LXRα in a dose-dependent manner. Meanwhile, isorhamnetin and quercetin-3-glucuronide showed no activation on LXRα (Figure 4.4 B and 4.4 D).

Meanwhile, the result also proved that among metabolites tested, only tamarixetin activates LXRα. Significant activation was shown by tamarixetin at concentrations 6.25 and 12.5 μM by 2.17 and 2.16-fold, respectively. Interestingly, both tamarixetin and isorhamnetin are the methylated forms of quercetin but only one of them shows the ability as an LXRα ligand.

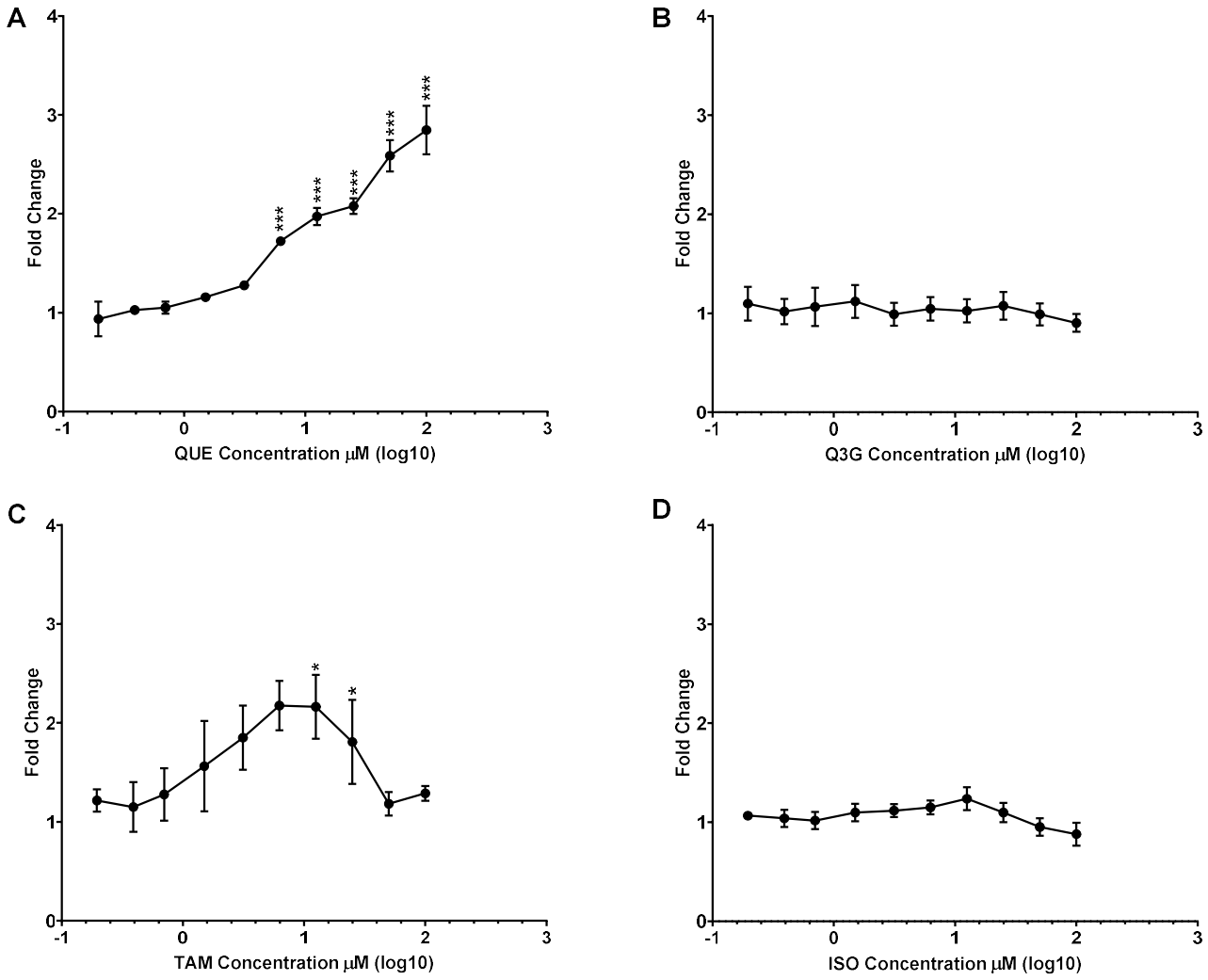


Figure 4.4 Dose-dependent effect of quercetin and its metabolites on LXRα activation. Cells were treated with quercetin (A) and its metabolites, quercetin-3-glucuronide (B), tamarixetin (C), and isorhamnetin (D) on LXRα activation in reporter cell model following 16 hours of test compounds incubation. Data shown were mean ± SEM from three independent experiments performed in quadruplicate. *, **, and *** indicate significant differences relative to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

4.3.3 Modulation of LXRα target genes by quercetin and its metabolites

LXRα as a nuclear receptor can repress or activate transcription of its target genes. The functional state of LXRα is changed following the binding of a ligand (Steinmetz, Renaud and Moras, 2001). To further analyze the possibility of quercetin and its metabolites as a ligand for LXRα, the modulation of ABCA1 and APOE on mRNA level (Figure 4.5) was measured. Based on the data from viability assay and activation on reporter cell line, concentration 25μM was decided to use for further experiment for all polyphenols compounds and 1μM for the positive and negative control.

The two-time point, 6 and 24 hours were used to observe the modulation of ABCA1 and APOE. The result showed that GW3965 treatment both at 6 and 24 hours, modulated ABCA1 and APOE expression on mRNA level. Cells treated with GW3965 for both time points gave 2.67 and 2.34-fold modulation compared to the controlled cells as shown in Figure 4.5. Meanwhile, APOE expression also increased following GW3965 treatment although the modulation was weaker compared to the ABCA1 expression.

Interestingly, GSK2033, which has been identified as an agonist for LXRα and display high binding affinity and blocked LXR target gene expression in cell culture (Zuercher *et al.*, 2010), showed no effect on antagonizing ABCA1 expression on HepG2 cells although a small reduction was shown on APOE after GSK2033 treatment. This result is contradictive compared to the previous result in murine RAW264.7 macrophage where GSK2033 effectively diminished both ABCA1 and APOE expression on mRNA and protein level.

Meanwhile, although quercetin shows some activation on the LXRα reporter gene (Figure 4.4.A), it does not affect modulating LXRα target genes. Similar results were also shown following quercetin-3-glucuronide and isorhamnetin incubation although this result is expected since both compounds showed no effect previously on the reporter gene model. Furthermore, tamarixetin further proved its ability as a ligand for LXRα by modulating ABCA1 and APOE expression 24 hours after compound incubation. This result demonstrates that gene expression modulation or repression by the nuclear receptor is preceded by ligand binding.

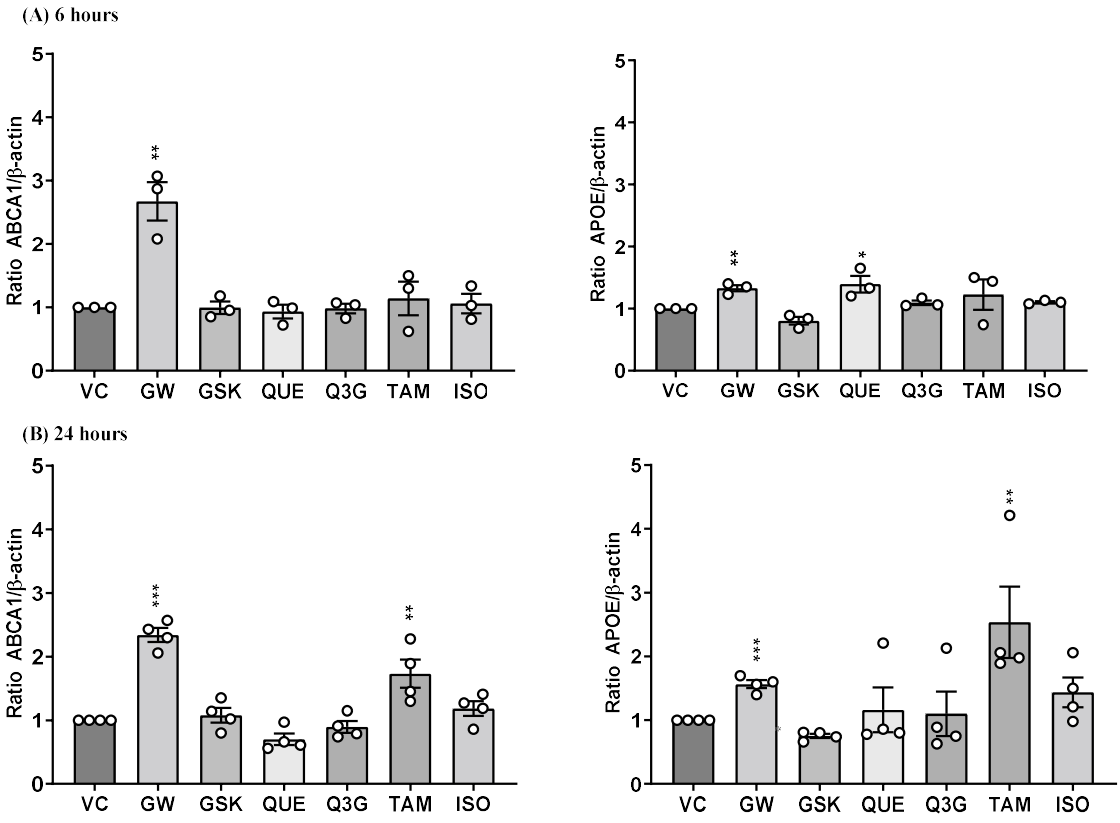


Figure 4.5 The modulation of LXRα target genes. ABCA1 and APOE at mRNA level at two different time points, (A) 6 hours and (B) 24 hours. Cells were incubated with LXRα agonist, GW3965, and antagonist, GSK2033 both at 1μM, and quercetin and its metabolites at 25μM concentration. Data shown were mean ± SEM from three independent experiments performed in duplicate. *, **, and *** indicate significant differences relative to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

4.3.4 Inflammatory stimulation in HepG2 cells using TNF-α

To evaluate the potential role of hepatocytes in response to an inflammatory signal, HepG2 cells were treated with TNF-α in response to LXRα agonist, antagonist, quercetin, and tamarixetin. First, the effect of TNF-α was tested at different time points and concentrations (Figure 4.7) to select the optimum stimulation condition by measuring ICAM1 and TNF-α expression on mRNA level. the cytotoxicity was also tested using Neutral Red assay on cells following TNF-α incubation for 24 hours to make sure that the concentration used won't kill the cells (Figure 4.6). Based on the results below, 6 hours stimulation time with TNF-α at 10 ng/mL concentration was used for further experiment.

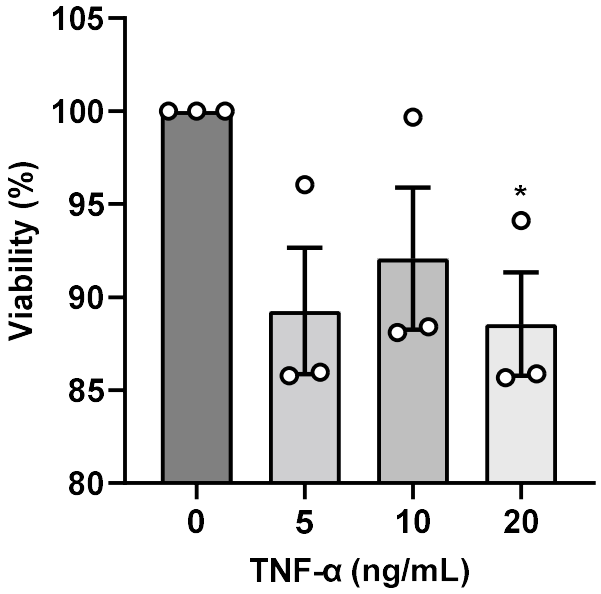


Figure 4.6 Viability of HepG2 cells following TNF-α stimulation. Cytotoxicity was determined by the Neutral Red assay following 24 hours of test compounds incubation. Data shown were mean ± SEM from three independent experiments performed in duplicate. *, **, and *** indicate significant differences relative to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

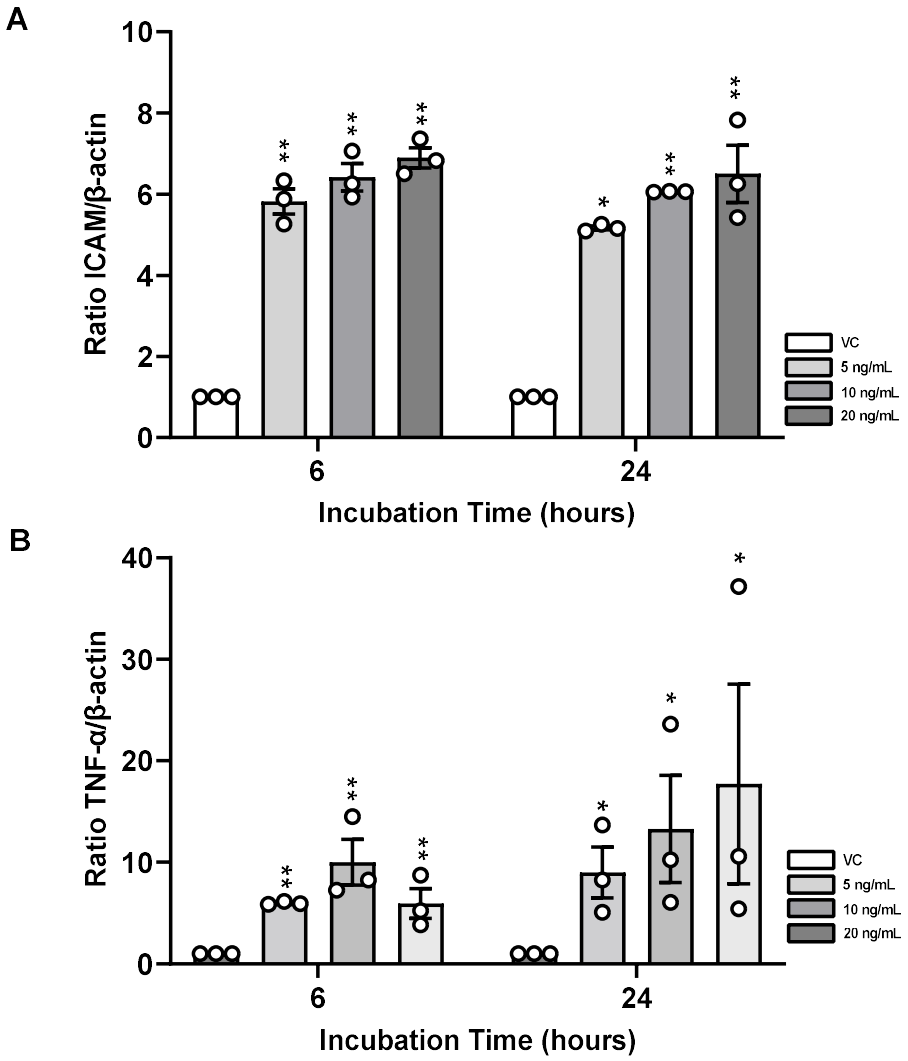


Figure 4.7 ICAM1 and TNF-α expression on mRNA level after TNF-α stimulation. Data were collected at two-time points and concentrations. Data shown were mean ± SEM from three independent experiments performed in duplicate. *, **, and *** indicate significant differences relative to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

4.3.5 LXRα target genes expression under TNF-α stimulation

The effect of TNF-α induced inflammation on ABCA1 and APOE on mRNA level expression is presented in Figure 4.8 (A and B). Based on the result above, ABCA1 expression is not affected by inflammatory signals. Only cells treated with GW3965 showed modulation in ABCA1 expression, but this effect is not related to inflammation. Although the statistic result shows that other test compounds tested did not show any significant result, all cells that received antagonist treatment lower ABCA1 expression approximately by 60% compared to the control cells. Furthermore, cells treated with quercetin and tamarixetin along with TNF-α have a different response. A slight reduction was shown by cells treated with TNF-α and tamarixetin by 50%. In contrast, there is no effect on ABCA1 expression in cells treated with TNF-α and quercetin.

A similar result was also observed in APOE expression following test compounds treatment. APOE did not respond to the inflammatory signal by TNF-α. As expected, both LXRα agonist and antagonist could not modulate APOE expression in HepG2 cells. Quercetin and tamarixetin also did not show any modulation on both LXRα target genes following TNF-α stimulation (Figure 4.8).

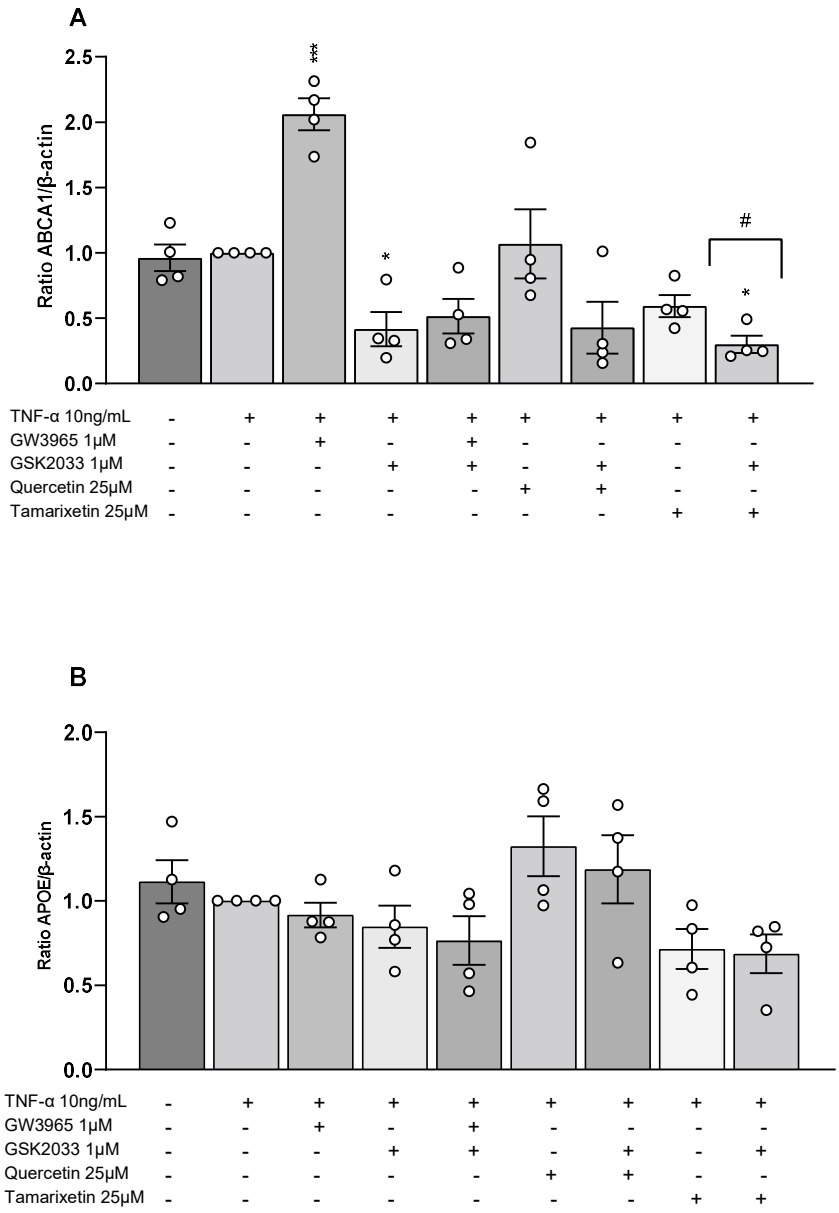
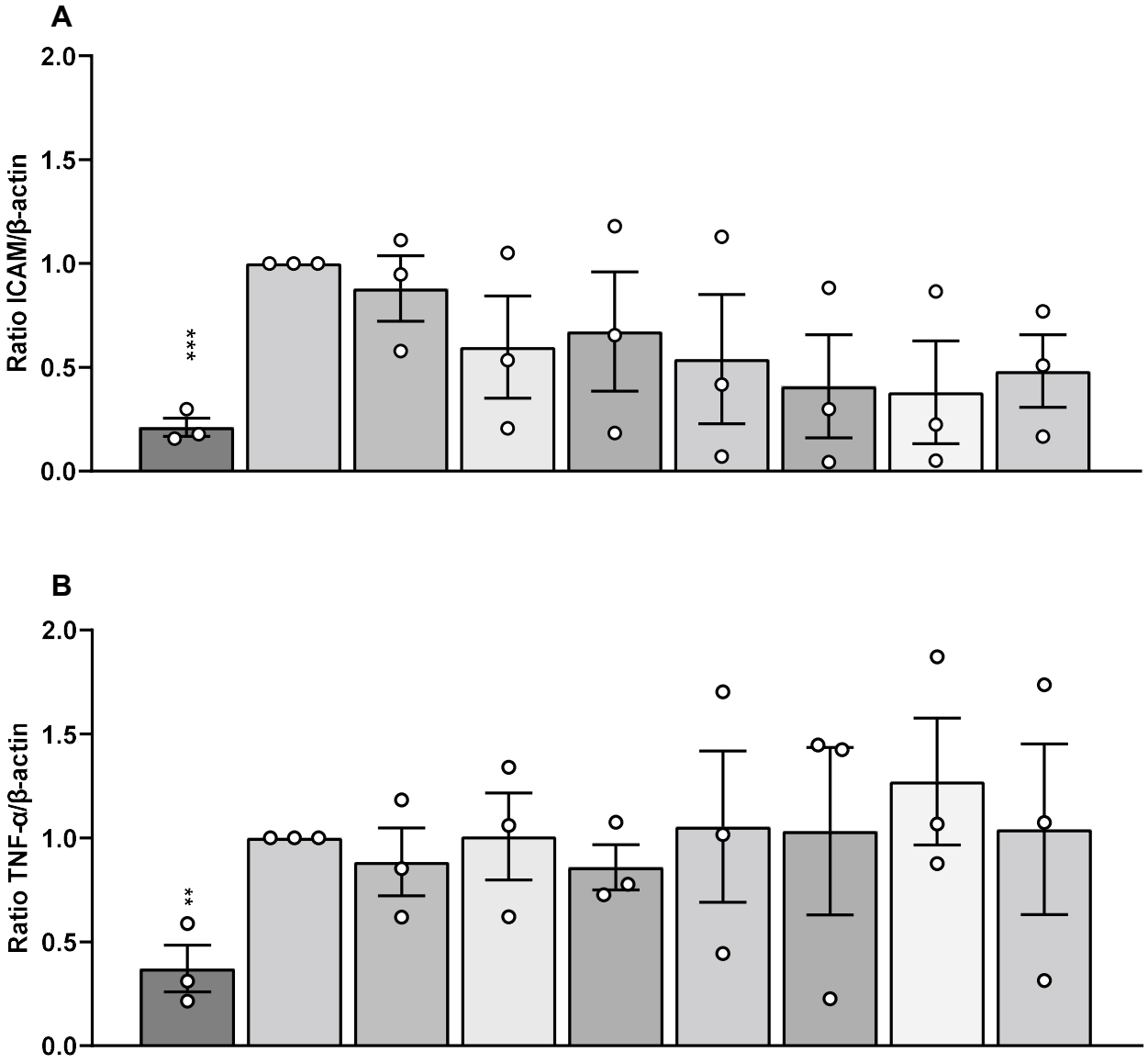


Figure 4.8 LXRα target genes expression after inflammatory stimulation. ABCA1 (A) and APOE (B) mRNA expression were measured following test compounds incubation and TNF-α stimulation for 1 and 6 hours respectively. Data shown were mean ± SEM from three independent experiments performed in duplicate. *, **, and *** indicate significant differences relative to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

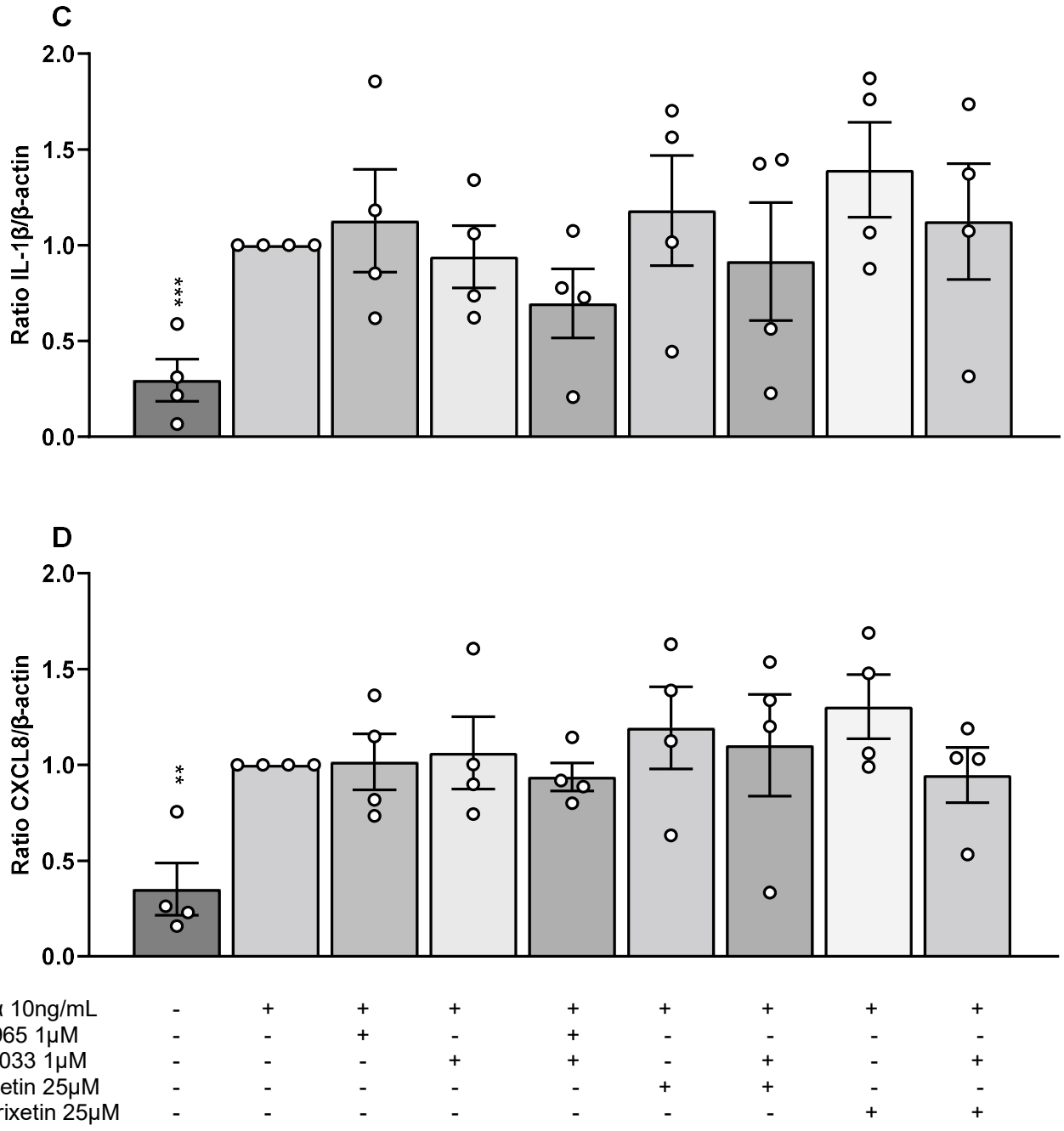
4.3.6 Evaluation of inflammatory genes expression following LXRα activation by ligands and quercetin and its metabolites

Although liver cells are not considered immune cells, they still can express numerous proinflammatory cytokines in response to inflammatory signals such as bacterial infection and toxins. In this study, the expression of both chemokines and proinflammatory cytokines following TNF-α stimulation was measured. Here the result demonstrated that cells treated with TNF-α have higher mRNA expression of TNF-α, ICAM1, IL-1β, and CXCL8 by more than 2-fold compared to the unstimulated cells.

Meanwhile, cells treated with LXRα agonist, showed no significant anti-inflammatory on both ICAM1 and TNF-α levels as shown in Figure 4.8 (P>0.05). In addition, IL-1β and CXCL8 also showed no decrease in its level. As expected, the LXRα antagonist also does not affect all inflammatory cytokines production. Besides, both quercetin and tamarixetin did not show any anti-inflammatory effect on every proinflammatory cytokine tested showing that quercetin and tamarixetin do not have an anti-inflammatory effect on liver cells stimulated with TNF-α.



TNF-α 10ng/mL	-	+	+	+	+	+	+	+	+
GW3965 1μM	-	-	+	-	+	-	-	-	-
GSK2033 1μM	-	-	-	+	+	-	+	-	+
Quercetin 25μM	-	-	-	-	-	+	+	-	-
Tamarixetin 25μM	-	-	-	-	-	-	-	+	+


C
Ratio IL-1β/β-actin
2.0
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0.5
0.0
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D
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2.0
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0.0
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TNF-α 10ng/mL - + + + + + + + +
GW3965 1μM - - + - + - - - -
GSK2033 1μM - - - + + - + - +
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Tamarixetin 25μM - - - - - - - + +

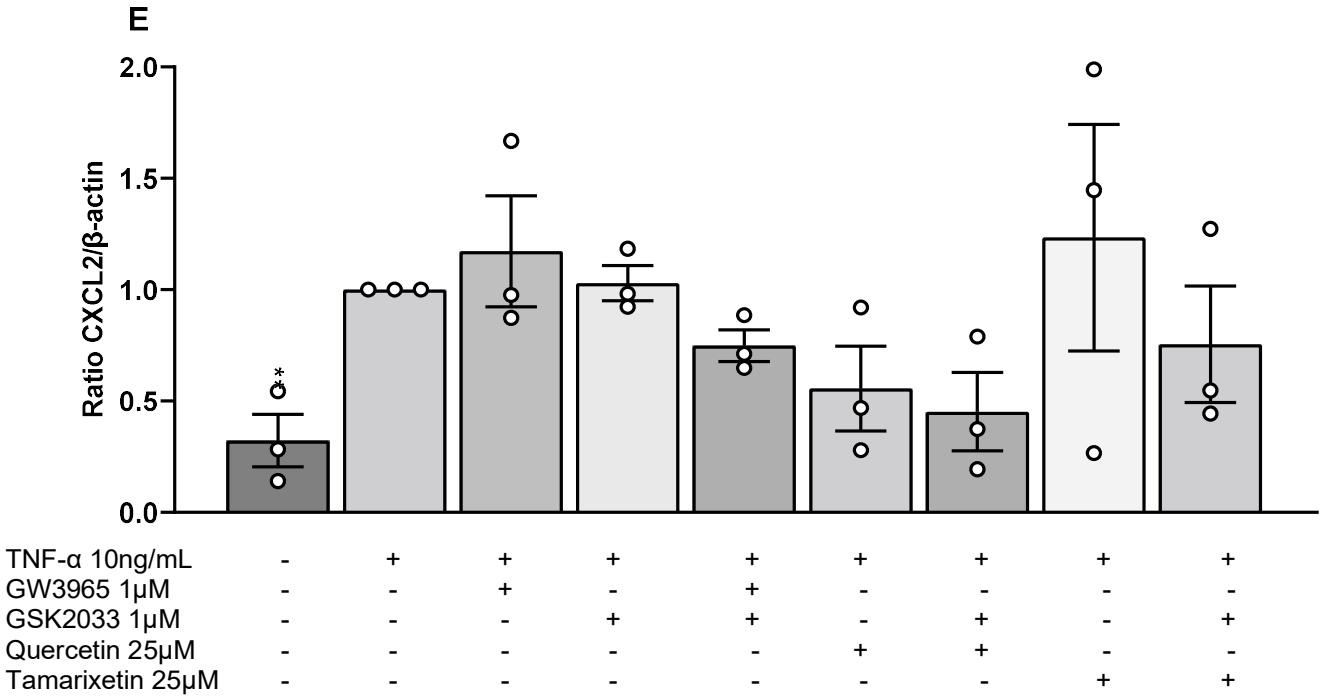


Figure 4.9 Inflammatory cytokines expression following TNF-α and test compounds incubation. mRNA expression of (A) ICAM1, (B) TNF-α, (C) IL-1β, (D) CXCL8, and (E) CXCL2 following test compounds incubation and TNF-α stimulation for 1 and 6 hours respectively. Data shown were mean ± SEM from three independent experiments performed in duplicate. *, **, and *** indicate significant differences relative to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

4.4 Discussion

Quercetin is partly metabolized in the liver and suffers further conjugation to produce sulfated and glucuronidated derivatives. Catechol O-methyl transferases (COMTs) present in the liver will further methylate quercetin and its metabolites (Luca *et al.*, 2020). Although quercetin has relatively poor bioavailability, the rate of elimination of its metabolites is quite slow, ranging from 11 to 28 hours, which might lead to accumulation in some tissues (Liu and Hu, 2007) and exert some beneficial health effects.

Inflammation is a complex reaction to destroy harmful stimuli and maintain tissue homeostasis. Acute inflammation happened when inflammation could be regulated properly and tightly, resulting in an immediate response to inflammatory stimuli and

quickly resolved. Meanwhile, chronic inflammation is caused by the inability of the body to remove and control the inflammation process. Chronic inflammation could last for several months to years and in general is characterized by monocyte/macrophages and lymphocyte infiltration to the site of inflammation (Ashley, Weil and Nelson, 2012; Chen *et al.*, 2018).

Both acute and chronic inflammation are observed in many organ systems, such as the heart, lung, intestinal tract, and liver. Inflammation in the liver has been linked to numerous diseases, including hepatitis, non-alcoholic fatty liver disease, and non-alcoholic steatohepatitis. As the largest solid organ in the body, the human liver is a high-risk target for inflammatory pathogens (Ashley, Weil and Nelson, 2012; Chen *et al.*, 2018). HepG2 cell line is widely used mainly to study drug metabolism, cholesterol and triglycerides metabolism, bile acid synthesis, and lipoprotein metabolism and transport since its expressed many of the genotypes of normal liver cells. It also able to express several acute phase inflammatory cytokines including IL-6, TNF-α, IL-1β, IL-4, IL-5, ICAM1, IL-6R, IL-7, IL-10, IL-11, Il-12, and IFN-γ (Gutiérrez-Ruiz *et al.*, 1999; Stonans *et al.*, 1999; Robinson, Harmon and O'Farrelly, 2016).

Meanwhile, research by Im and Osborne (2011) suggests that LXRα has a unique role in suppressing inflammatory signals in macrophages by promoting reverse cholesterol transport (Im and Osborne, 2011). Novel LXRα agonist, SR9243, significantly decreases the severity of hepatic inflammation on non-alcoholic steatohepatitis BALB/c mice induced by high cholesterol diet (Huang *et al.*, 2018). Other research also demonstrated that LXRα deletion on hepatic mononuclear cells extracted from LXRα/β knockout mice treated with LPS promotes proinflammatory cytokines production such as IL6, IL-1β, IL6, and IL-12β and that this condition was adjusted by the treatment of LXRs agonists (Endo-Umeda *et al.*, 2018) showing that LXRα also suppressed inflammation in the liver.

Structure similarity between steroids and flavonoids to bind to nuclear receptors has been explored although the link remains unclear. In this study, the result showed that quercetin and its metabolites, tamarixetin, can bind to LXRα in a dose-dependent manner (Figure 4.4). Tamarixetin is a metabolite of quercetin formed in vivo, where it is methylated at position O-4'. Interestingly, another methylated metabolite of quercetin was tested, isorhamnetin, where it's methylated at position O-3', but it showed a different result. Even

though these metabolites have a high structural similarity among them, they showed the contrary result in activating LXRα. Like isorhamnetin, a glucuronidated metabolite of quercetin, quercetin-3-glucuronide, also showed no effect on LXRα activation. Here, the result proved that little changes in hydroxyl group position may affect compound activity in binding to a nuclear receptor. Hence, conformational changes and the chemical structure of conjugates determine the chance of a certain molecule to bind to the ligand-binding domain in the nuclear receptor.

The evidence comparing the biological activity of quercetin and its metabolites in suppressing inflammation is limited. A study by Lesjak *et al* (2018) showed that tamarixetin demonstrated better anti-inflammatory activity compared to isorhamnetin and quercetin. Furthermore, methylated conjugates also showed better antioxidant properties compared to the glucuronidated metabolite of quercetin (Lesjak *et al.*, 2018). Further, Boesch-Saadatmandi *et al* (2011) supported the idea that isorhamnetin and quercetin have better anti-inflammatory property by than quercetin-3-glucuronide downregulating inflammatory gene expression related to Nrf2 transcription factor and miR-155 in murine RAW264.7 macrophages stimulated with LPS (Boesch-Saadatmandi *et al.*, 2011). However, in this present study, the data showed that tamarixetin, not isorhamnetin, was more potent to activate LXRα signaling in the reporter model. This experiment highlights that every polyphenol may have different biological activity and may be pathway and/or target specific.

To further prove that quercetin and tamarixetin can act as a ligand for LXRα, it was decided to evaluate both flavonol's effect on LXRα target genes, ABCA1 and APOE. As expected, LXRα synthetic agonist, GW3965, modulates ABCA1 and APOE expression on mRNA level. GSK2033, as the antagonist, could not block the modulation of both target genes (Figure 5.5). In contrast, other studies showed that 1μM GSK2033 treatment in THP-1 cells completely blocked ABCA1 mRNA expression (Ignatova *et al.*, 2013) and in HEK293 cells co-transfected with ABCA1 reporter (Griffett and Burris, 2016) as well as SREBP-1C expression in HepG2 cells (Zuercher *et al.*, 2010). GSK2033 is known as a potent LXR antagonist with high binding activity. Meanwhile, when GSK2033 was tested in an animal model of fatty liver disease, it showed no effect on hepatic steatosis and showed surprising effect by significantly increased lipogenic enzymes such as FASN and

SREBP-1C. It was also revealed that GSK2033 has an antagonistic activity for 13 other nuclear receptors, including ERα, Erβ, and GR in reporter cell model (Griffett and Burris, 2016). Based on previous studies and results from this experiment, it is indicated that the GSK2033 effect on ABCA1 is cell specific.

Furthermore, even though quercetin showed better activity in activating LXRα in the reporter gene compared to tamarixetin (Figure 4.4), it showed no effect on ABCA1 modulation following 24 hours incubation (Figure 4.5.A) but slight modulation was observed after 6 hours of compound incubation (Figure 4.5.B). On contrary, tamarixetin only showed modulation on ABCA1 after 24 hours incubation, but not after 6 hours compound incubation. Meanwhile, both compounds showed no effect on APOE mRNA expression. Incubation time might play an important role in this study. According to the literature, quercetin has a half-life of 3.5 hours (Li *et al.*, 2016) meanwhile its metabolites have a longer half-life ranging from 11-28 hours (Ulusoy and Sanlier, 2020). Besides, quercetin in liver cells might suffer further metabolism and change into different metabolites. A study by O'Leary *et al* (2003) demonstrated that incubating quercetin with HepG2 cells resulted in the formation of 3'-methyl quercetin, 3'-methylquercetin glucuronide, and quercetin glucuronides (O'Leary *et al.*, 2003). In this experiment, quercetin glucuronide has no ability in activating LXRα in HepG2 reporter cells (Figure 4.4). The fact that quercetin might have lost its initial form after 24 hours added with the possibility of metabolites formed that has no ability in activating LXRα, might be the reason behind the inability of quercetin in modulating ABCA1 and APOE expression.

The reporter gene is a powerful tool for biological imaging and could detect the accumulation of specific signals of interest (Griffett and Burris, 2016). The result showed that although quercetin and tamarixetin showed promising activation in the LXRα reporter gene (Figure 4.4), not all activation leads to the modulation of target genes. Transcriptional regulation is a highly complex process where gene expression or gene repression is not only determined by ligand binding but also involved other factors such as co-regulators recruitment or co-repressor release (Carlberg and Seuter, 2010).

LXRα role in inflammation is important in preventing foam cell formation that could lead to the development of atherosclerosis in macrophages. Here, the possibility of anti-inflammatory activity of LXRα in HepG2 cells treated with TNF-α was explored. TNF-α is

a strong proinflammatory cytokine and can stimulate inflammatory cascade by NF-κB signaling pathway and induce cytokines production including IL-1. IL-6, IL-8, and IFN (Neta, Sayers and Oppenheim, 1992). In this study, the data prove that TNF-α stimulation significantly induced the expression of several proinflammatory cytokines ICAM1, TNF-α, IL-1β, CXCL2, and CXCL8. Unfortunately, none of the compounds tested in this study could repress inflammation.

Several factors might contribute to the unresponsiveness of LXRα agonist, antagonist, and flavonols including the inflammatory stimulation used in this study. Although TNF-α has been proven to induce inflammation in other cell lines (Treede *et al.*, 2009; Zhu *et al.*, 2011; Zhou *et al.*, 2017), its effect to induce inflammation in HepG2 cell line remains scarce (Zhou *et al.*, 2011; Song *et al.*, 2012; Zhang Hua *et al.*, 2015; Huang *et al.*, 2018). Numerous published literatures regarding hepatic inflammation induction were varied depending on the pathway of interest where each stimulation will activate a certain inflammatory pathway, resulting in unique pro-inflammatory cytokines production. For example, high glucose treatment in HepG2 cells was used to mimic hepatocytes' response following chronic exposure to glucose (Panahi *et al.*, 2018). In addition, LPS treatment (Xu *et al.*, 2015; Kanmani and Kim, 2018; Al-Bakheit and Abu-Qatouseh, 2020) as well as fatty acid loading as a model of hepatic inflammation (Tanaka *et al.*, 2020).

In this study, although TNF-α induced inflammation by significantly increasing pro-inflammatory cytokines production, such as TNF-α, ICAM-1, IL-1β, CXCL2, and CXCL8, the compounds used in this experiment showed anti-inflammatory activity against TNF-α stimulation. Even though GW3965 has shown anti-inflammatory activity in macrophages (Chapter 5), it does not affect suppressing inflammation in HepG2 cells, indicating that the activity of GW3965 is cell dependent. The same result was also shown by quercetin and tamarixetin where there was no anti-inflammatory effect following TNF-α stimulation. The results from this study were contrary to previously published experiments where quercetin showed a strong anti-inflammatory effect on other cell lines (Cheng *et al.*, 2019; Verna *et al.*, 2021).

Interestingly, a study by Granado-Serrano *et al* (2012) that was using the same inflammatory stimulation in the same cell line showed that quercetin reducing TNF-α induced inflammation in HepG2 cells by suppressing NF-κβ activation suggesting that

quercetin might have an anti-inflammatory effect in HepG2 cells. Even though it showed that quercetin downregulates NF-κβ, they did not show any detailed data related to the pro-inflammatory production. Besides, it also employed a shorter incubation time and lower TNF-α and quercetin concentration (Granado-Serrano *et al.*, 2012). Here, it can be concluded that incubation time may play an important role in determining the appropriate time point in detecting certain pro-inflammatory cytokines production.

4.5 Conclusion

Quercetin has gained great interest because of its numerous health benefit, especially for its ability in suppressing inflammation. Since quercetin along with its metabolites is difficult to directly cross through the cell membrane, limited available information on how beneficial effects of quercetin is mediated on the cellular level is remains unclear. Structural similarities between polyphenols in general and cholesterol derivatives have gained interest for further investigation to prove that polyphenols might act as a ligand for the nuclear receptor.

As a ligand-dependent nuclear receptor, LXRα needs to bind to its ligand to start the transcription process and exert its biological function. Theoretically, ligand binding of the nuclear receptor will be followed by modulation of its target gene. In the present study, activation of LXRα by quercetin and tamarixetin in reporter cell model doesn't guarantee their ability as a ligand for LXRα since it is not followed by any effect on cell metabolism. While quercetin has been known to suppress inflammation, this study could not confirm the anti-inflammatory of quercetin in hepatic cells. The difference in cell line and concentration used in this study may be the reason why the result came out different. It is important to understand that a certain compound might undergo different pathways in different cell lines depending on the stimulation given.

Chapter 5. Quercetin modulates inflammation partially through LXRα activation

Abstract

The flavonol quercetin, widely found in a variety of dietary foods has been extensively researched for its health benefits. There is extensive evidence, both in vivo and in vitro, that has suggested its ability to suppress inflammation; however, the mechanisms underlying its beneficial effects have not been fully elucidated. Nuclear receptors are a family of ligand-dependent transcription factors that are specifically involved in numerous biological processes including metabolism, inflammation, cell proliferation, and reproduction. Most nuclear receptors are regulated by hydrophobic molecule derivatives such as cholesterol, retinoids, fatty acids, hormones, as well as synthetic drugs. The nuclear receptor LXRα is activated by oxysterols which have been known to play an important role not only in lipid metabolism and transport but also in suppressing inflammation. Initial studies indicated that polyphenols might regulate nuclear receptor activity resulting in changes in gene expression.

This study was aimed to explore the ability of quercetin to act as a ligand for LXRα and inhibit the inflammation-induced transcription mediated by LXRα. In this present study, RAW264.7 murine macrophages were treated with quercetin, LXRα ligands, and combinations of both, followed by stimulation with lipopolysaccharide (LPS) to induce inflammation. The data showed that quercetin moderately increased ABCA1 and this effect was blunted in the presence of LXRα antagonist. Moreover, quercetin treatment significantly decreased inflammatory cytokines at the transcription level, including IL-6, IL-1β, and IL-10. Interestingly, this effect was unaffected in the presence of the LXRα antagonist. Furthermore, even though LXRα antagonist blocked quercetin's ability to modulate ABCA1, the anti-inflammatory effect of quercetin is unaltered. Also, our data demonstrated that quercetin could ameliorate inflammation by reducing the transcriptional activity of the NF-κβ signaling pathway in cultured macrophages but LXRα antagonists blocked this effect. Taken together, our data revealed that quercetin can modulate ABCA1 expression and the anti-inflammatory effect is independent of ABCA1 and LXRα.

5.1 Introduction

Inflammation is a physiological response of the immune system to defend the host from harmful stimuli, such as bacteria, viruses, toxic compounds, and infections. Besides, inflammation also helps to remove those stimuli and overcome the damage. However, persistent and unresolved inflammation could lead to tissue and organ alteration, changes in metabolism and cellular physiology, which can increase the risk of several diseases, such as atherosclerosis, arthritis, and neurodegenerative diseases (Maskrey *et al.*, 2011; Freire and Van Dyke, 2013; Sugimoto *et al.*, 2016).

Inflammation is a complex and strictly regulated process involving numerous signaling molecules. Nuclear receptors as a transcription factor also play an important role in the regulation of inflammation by suppressing or initiating pro-inflammatory cytokines gene expression. Some nuclear receptors such as glucocorticoid receptor (GR), peroxisome proliferator-activated receptors (PPARs), and liver X receptor (LXR) vitamin D receptor (VDR), showed involvement in macrophage responses to suppress inflammation. These nuclear receptors are controlled by their adjacent ligands (Wang and Wan, 2008; Huang and Glass, 2010; Leopold Wager, Arnett and Schlesinger, 2019).

Recent studies revealed that polyphenols, a family of plant secondary metabolites mainly found in fruits and vegetables, can bind and induce the transcriptional activity of several nuclear receptors. Experimental work suggested that daidzein and genistein, known as soy phytoestrogens, are believed to modulate PPARγ signaling in adipocytes (Hall *et al.*, 2019) and also estrogen receptors (ER)-β in ovarian cancer cells (Chan *et al.*, 2018). Moreover, genistein, kaempferol, and luteolin showed to activate ER in reporter gene assay (Puranik *et al.*, 2019). As well, quercetin, quercetin-3-O-glucuronide, genistein, daidzein, and naringenin were found to LXRα target gene, ABCA1, at different concentrations in different cell lines (De Stefano *et al.*, 2007; Goldwasser *et al.*, 2010; Kidani and Bensinger, 2012; Ohara *et al.*, 2013; Fouache *et al.*, 2019). Delfosse *et al* (2015) classified resveratrol and luteolin, flavonoids from stilbene and flavone subclass respectively, as naturally occurring compounds that can also act as ligands of PPAR on MCF-7 cells transfected with luciferase reporter vector containing estrogen response elements (Delfosse *et al.*, 2015). These studies suggest that polyphenols exert their health benefits through targeting the nuclear signaling pathway.

Recently, growing interest has emerged in the similarity structure between natural products such as flavonoids and nuclear receptors (Avior *et al.*, 2013). Molecular docking of flavonoid ligand shows that cyanidin has agonistic activity (Jia *et al.*, 2013), meanwhile, luteolin acts as an antagonist (Francisco *et al.*, 2016). Further study also demonstrates that quercetin also has agonistic activity based on its ability to fit into ligand-binding pockets non both LXR isoforms (Fouache *et al.*, 2019).

It has been shown that LXRα synthetic agonists, T0901317 and GW3965, decreased LPS-induced adhesion molecule expression in HUVEC cells (Morello *et al.*, 2009). Another study demonstrated that LXR activation by its agonist suppressed NF-κβ inflammatory signaling and pro-inflammatory cytokine expression such as IL-1β and IL-6 (Ito *et al.*, 2015). Similarly, it was reported that pretreatment with T0901317 lowered LPS-induced TNF-α, IL-6, and IL-1β expression at mRNA level in THP-1 cells (Xiao *et al.*, 2017). These studies demonstrate the involvement of LXRs in the regulation of inflammatory response.

Quercetin is a major flavonoid from the flavonol subclass and ubiquitously found in dietary foods especially tea, onion, apples, and shallots primarily as glycosides form. Quercetin daily intake from food accounts for 75% of total flavonoid intake although this amount differs among countries with an average intake between 4.37 mg/day to 16.2 mg/day (Li *et al.*, 2016). Studies regarding quercetin safety suggested that quercetin be safe for the healthy population (Okamoto, 2005; Andres *et al.*, 2018); and it has indeed been widely consumed as a dietary supplement with daily doses ranging from 200-1200 mg quercetin (Egert *et al.*, 2008). Therefore, quercetin intake through a supplement is significantly higher than the average intake from food.

Health beneficial effects of quercetin have been extensively investigated in vitro, as well as in animal models and clinical trials. Some of the results suggest that quercetin has potent properties as an antioxidant, anticarcinogenic, and anti-inflammatory (Xue *et al.*, 2017; Lesjak *et al.*, 2018; Xu *et al.*, 2019) although the exact mechanism must be further studied. Among others, the anti-inflammatory activity of quercetin has gained great interest. Quercetin could decrease the production of several inflammatory cytokines including IL-1α, IL-1β, IL-2, IL-10, and other chemokines in macrophage cells induced by LPS, in vitro (Boesch-Saadatmandi *et al.*, 2011; Xue *et al.*, 2017). Furthermore, a review

by Qiaowen Qu (2019) revealed that quercetin supplementation has anti-inflammatory effects in humans by reducing CRP and IL-6 levels (Ou *et al.*, 2020).

It has been reported that quercetin may exhibit its ability in reducing inflammation by regulating several inflammatory transcription factors. A study by Cheng *et al* (2019) showed that quercetin inhibits pro-inflammatory cytokines production in stimulated ARPE-19 cell line by blocking the activation of the NF-κB signaling pathway (Cheng *et al.*, 2019). In RAW264.7 murine macrophages, quercetin inhibits the translocation of p50 and p65 subunits of NF-κB and reduces the expression of iNOS and COX-2 (De Stefano *et al.*, 2007). Meanwhile, Liu *et al* (2015) stated that quercetin protects the cell from inflammation by modulating Nrf2/HO-1 and p38/STAT1/ NF-κB signaling pathway (Liu *et al.*, 2015).

However, despite many studies regarding quercetin and inflammation, the links connecting quercetin and inflammation remain unclear. Since LXRα and macrophages play an important role in inflammation, this chapter focused on the evaluation of the anti-inflammatory effect of quercetin induced LXRα activation using murine RAW264.7 macrophages as the cell model for inflammation.

5.2 Methodology

The focus of this chapter was to explore the anti-inflammatory activity of quercetin induced LXRα activation in murine RAW264.7 macrophages stimulated with LPS. As mentioned in the previous chapter, quercetin increases LXRα activation in both MDA-MB-231 and HepG2 cell lines stably transfected with the LXRα reporter system. Cells were exposed to GW 3965, GSK2033, and quercetin, followed by LPS stimulation. To ensure that the anti-inflammatory property of quercetin is LXRα dependent, cells were treated with the combination of quercetin and GSK2033 to block the quercetin from binding to LXRα. Detailed procedures for methods used in this chapter are described in Chapter 2.

5.3 Results

5.3.1 Cell viability of test compounds on murine macrophage RAW264.7

To evaluate the cytotoxicity effect of solvents and compounds used in this study, cells were exposed to treatments accordingly. Solvents concentration in cell culture media was

0.1% to minimize the toxic effect on cells. No cytotoxicity was observed for both solvents compared to DMEM control (medium control). The cytotoxic effects of quercetin, GSK2033, 27-HC, and GW3965 were investigated by neutral red assay in murine RAW264.7 macrophage cells over 24 hours incubation periods. The results demonstrated that quercetin at concentrations 25 and 50 μM decreased cell viability to 78% and 23% respectively. In the previous chapter, quercetin decreased MDA-MB-231 cells at 50 μM which indicated that the effect of quercetin on cell viability is cell- and dose-dependent. There was no cytotoxicity observed when macrophages were incubated with GSK2033. GW3965 at the evaluated concentration also did not show any toxicity effect. On the other hand, T0901317 at the same was more toxic than GW3965. Following the cell viability results, 10 μM quercetin and 1 μM GSK2033 were used for further experiments. Given the lower toxicity of GW3965, this compound was chosen over T0901317 as a positive control for these experiments.

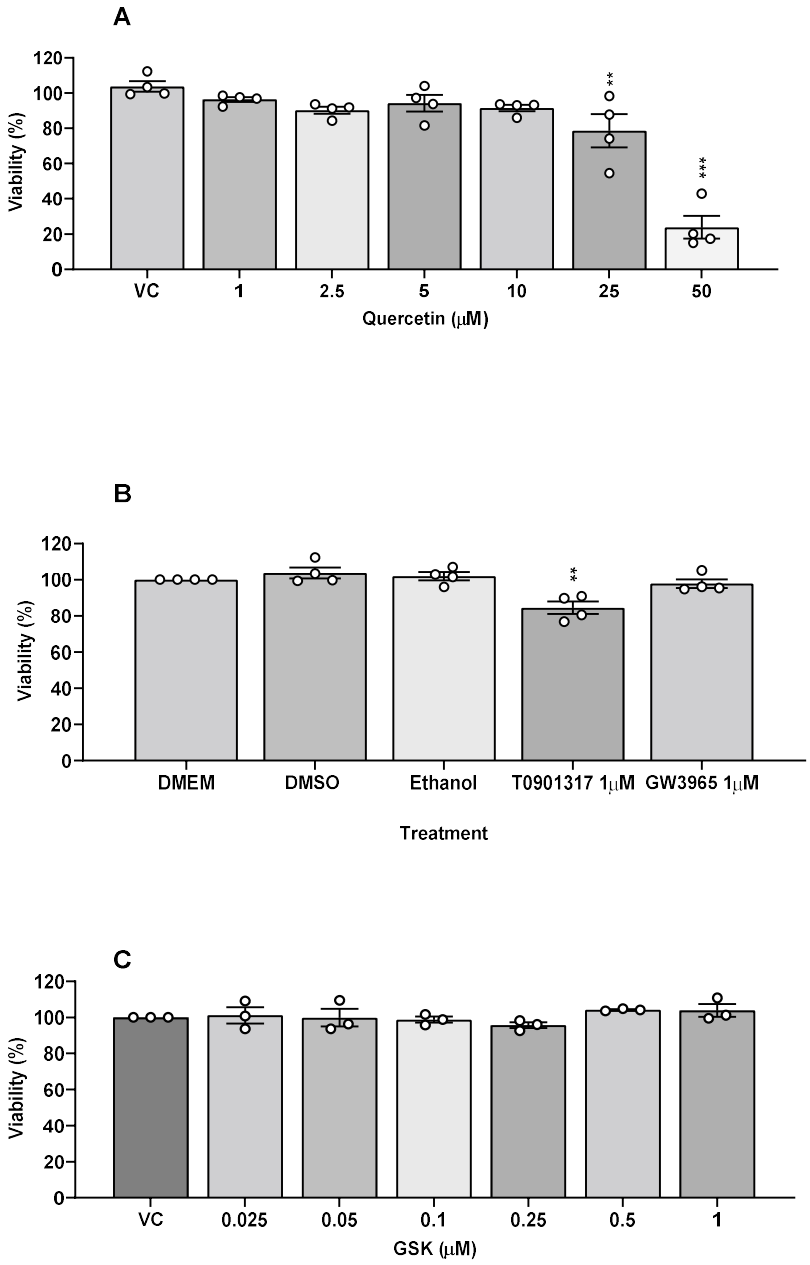


Figure 5.1 Cell viability for test compounds in murine macrophage RAW264.7 cells. Cells were treated with quercetin (A), solvent and LXRα agonists (B), and LXRα antagonist (C) for 24 hours. Cytotoxicity was assessed using a neutral red assay. Data shown were mean ± SEM from three independent experiments performed in duplicates *, **, and *** indicate significant differences relative to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

5.3.2 Activation of LXRα in murine macrophage RAW264.7 by quercetin

To evaluate the activation of LXRα by quercetin in murine RAW264.7 macrophages, the modulation of its target genes, ABCA1 and APOE, was measured on the transcription (Figure 5.3) and protein (Figure 5.5.A) level. An initial test was carried out to establish the proper incubation time for detecting the modulation of LXRα target genes (Figure 5.2). ABCA1 and APOE were chosen as the target genes since both are highly expressed in macrophages and closely related to LXRα in their function to promote reverse cholesterol transport.

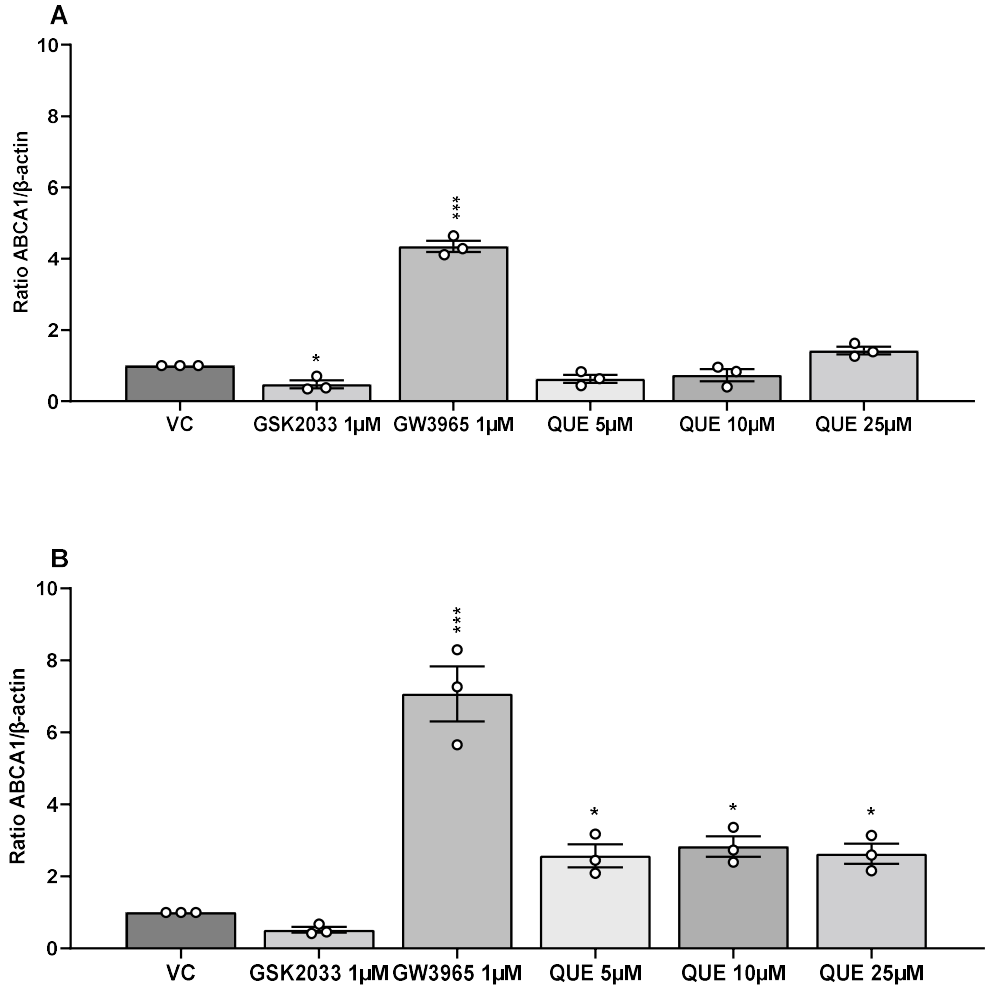


Figure 5.2 ABCA1 mRNA level in murine macrophage RAW264.7 cells. Cells were treated with quercetin, LXRα agonists, and LXRα antagonist for (A) 6 hours and (B) 24 hours. Fold changes are shown relative to β-actin as a housekeeper. Data are mean with SEM from three independent experiments performed in duplicate. *, **, and *** indicate significant differences to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

Different time points were employed to determine the optimal incubation period needed for quercetin to modulate LXRα target genes. As shown in Figure 5.2, 24 hours incubation time showed a higher mRNA level of ABCA1 compared to 6 hours incubation. Longer incubation time also increased ABCA1 mRNA levels by 34% in cells treated with GW3965 (*unpaired t-test, $p<0.05$)*. Since no difference showed in the modulation of ABCA1 despite the concentration, 10 μM quercetin was chosen as the single dose used in this study. Moreover, based on data above and cell viability, the 24-hour incubation time was used in further experiments to observe LXRα target gene modulation.

To confirm that modulation of ABCA1 and APOE is LXRα dependent, quercetin was co-incubated with GSK2033. Figure 5.3 shows that GSK2033 blocked LXRα activation by agonist which resulted in inhibition of ABCA1 modulation. The ability of quercetin to modulate ABCA1 was also completely diminished when cells were co-treated with GSK2033. While there was no significant effect of GW3965 and GSK2033 on APOE, quercetin showed a moderate increase in APOE mRNA levels which was reduced by GSK2033.

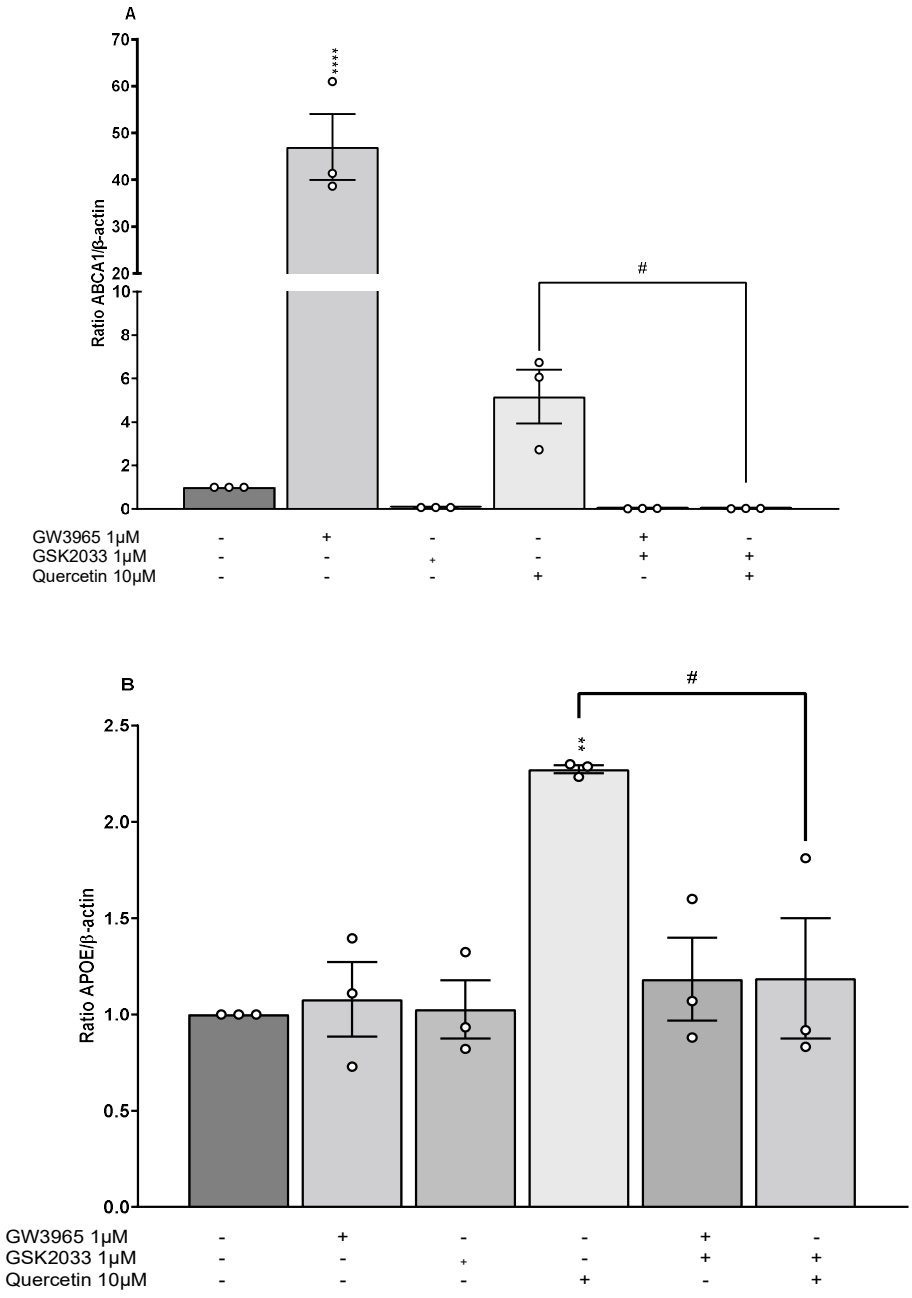


Figure 5.3 LXRα target gene modulation in murine macrophage RAW264.7 cells. ABCA1 (A) and APOE (B) mRNA expression following treatment with quercetin, LXRα agonists, and LXRα antagonist and their combination for 24 hours. Fold changes are shown relative to β-actin as a housekeeper. Data are expressed as mean ± SEM (n=3, *$P<0.05$, **$P<0.01$ relative to control group; #$P<0.05$ unpaired t-test.

5.3.3 LXRα target genes modulation by quercetin under LPS stimulation

As shown in Figure 5.4.A, LPS stimulation significantly induced ABCA1 level compared to the control group by more than 20-fold. Cells treated with GSK2033 and LPS showed no modulation on ABCA1. Meanwhile, GW3954 treatment increased the ABCA1 level by 8-fold compared to LPS control. This effect persists even when the antagonist is added. This demonstrates that the agonist effect is stronger than the agonist in modulating ABCA1. Quercetin treatment also does not affect ABCA1 mRNA levels as shown in Figure 5.4.A, compared to the LPS control. Meanwhile, antagonist addition further reduced the ability of quercetin to modulate ABCA1 in the presence of LPS.

In contrast to ABCA1 responding to inflammation, LPS stimulation decreased APOE mRNA levels compared to medium control (Figure 5.4.B). APOE modulation was increased when cells were treated with quercetin. No effect is seen in agonist and antagonist groups. This demonstrates that quercetin is more potent than GW3965 and GSK2033 in modulating APOE. Similar patterns were observed when cells were treated with quercetin in the absence of LPS stimulation (Figure 5.4.B). It suggests that there might be a different pathway for quercetin to modulate APOE.

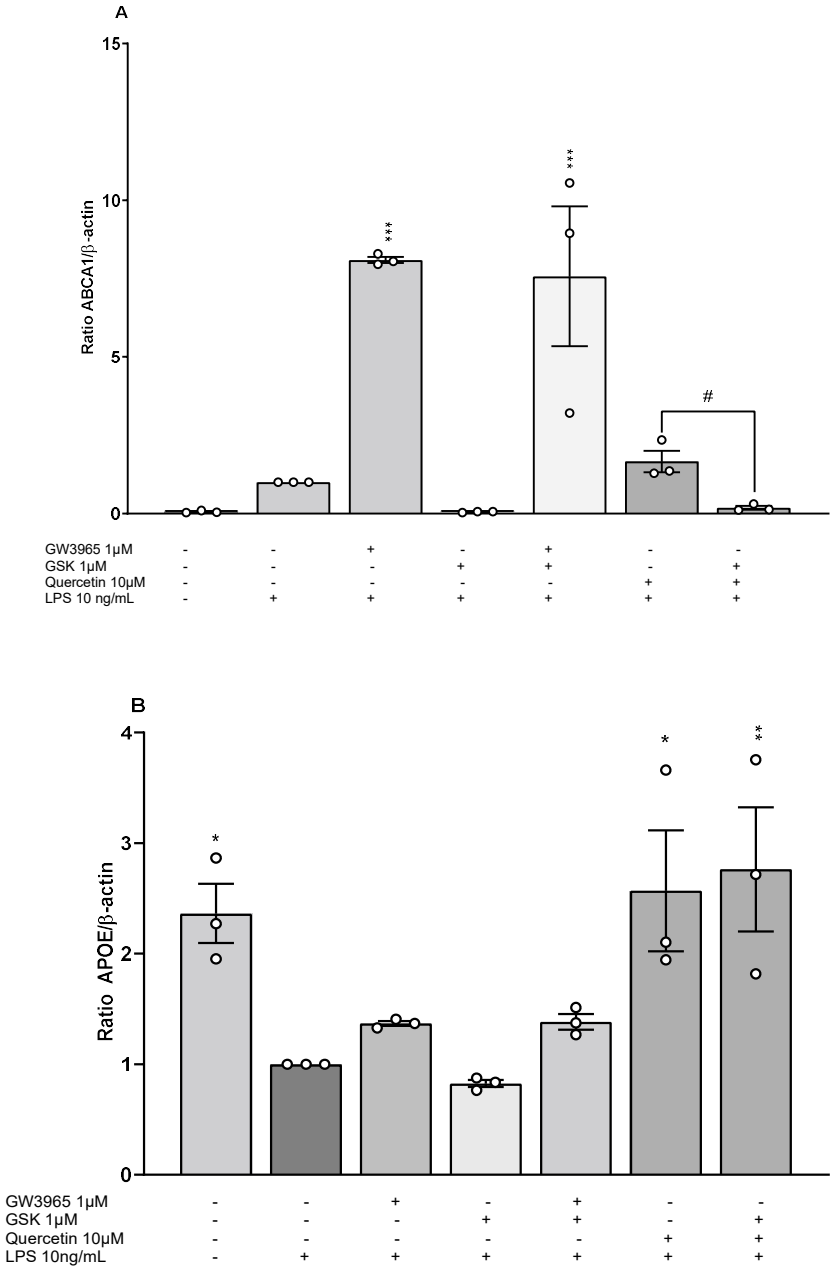


Figure 5.4 LXRα target genes modulation in murine macrophage RAW264.7 cells under LPS stimulation. Cells were treated with quercetin, LXRα agonists, and LXRα antagonist and their combination for 1 hour followed by LPS stimulation for 6 hours for (A) ABCA1 and (B) APOE. Fold changes are shown relative to β-actin as a housekeeper. Data are expressed as mean ± SEM (n=3, *$P<0.05$, **$P<0.01$ relative to LPS group; #$P<0.05$ unpaired t-test).

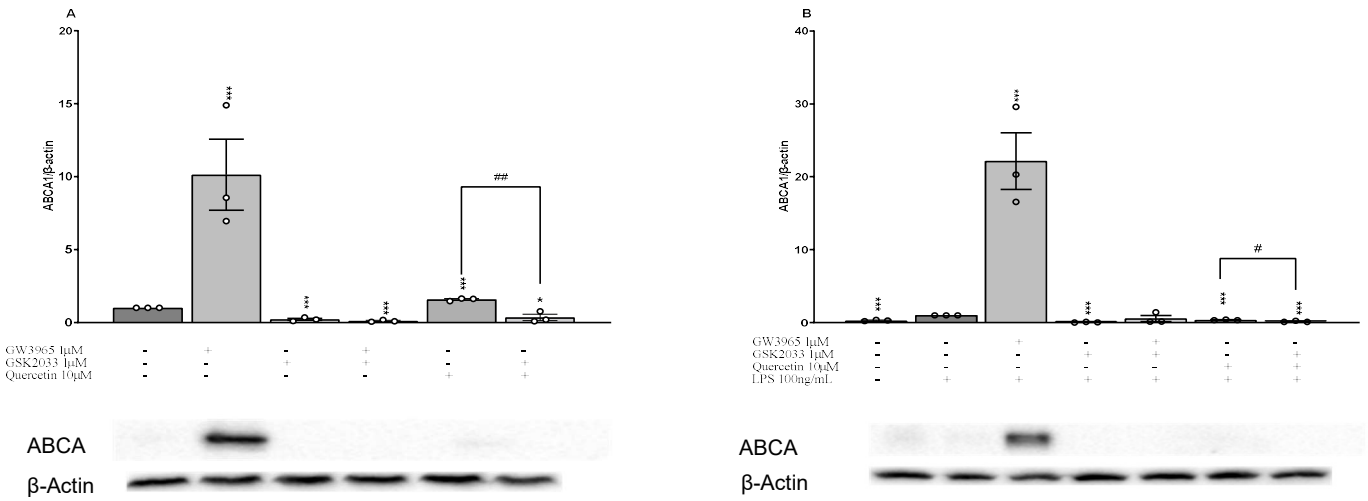


Figure 5.5 ABCA1 protein expression on RAW264.7 murine macrophages. Cells were incubated with test compounds for 24 hours (A) and followed with LPS stimulation for 6 hours (B). All cells were also incubated with DMSO as compounds solvent. Data were analyzed using ANOVA followed by Dunnett's post hoc relative to DMSO control (A) and LPS control (B). Data shown are mean with SEM of three independent replicates. *, **, and *** indicate significant differences to the control group (Dunnett's t-test), *P<0.05*, *P<0.01*, and *P<0.001* respectively.

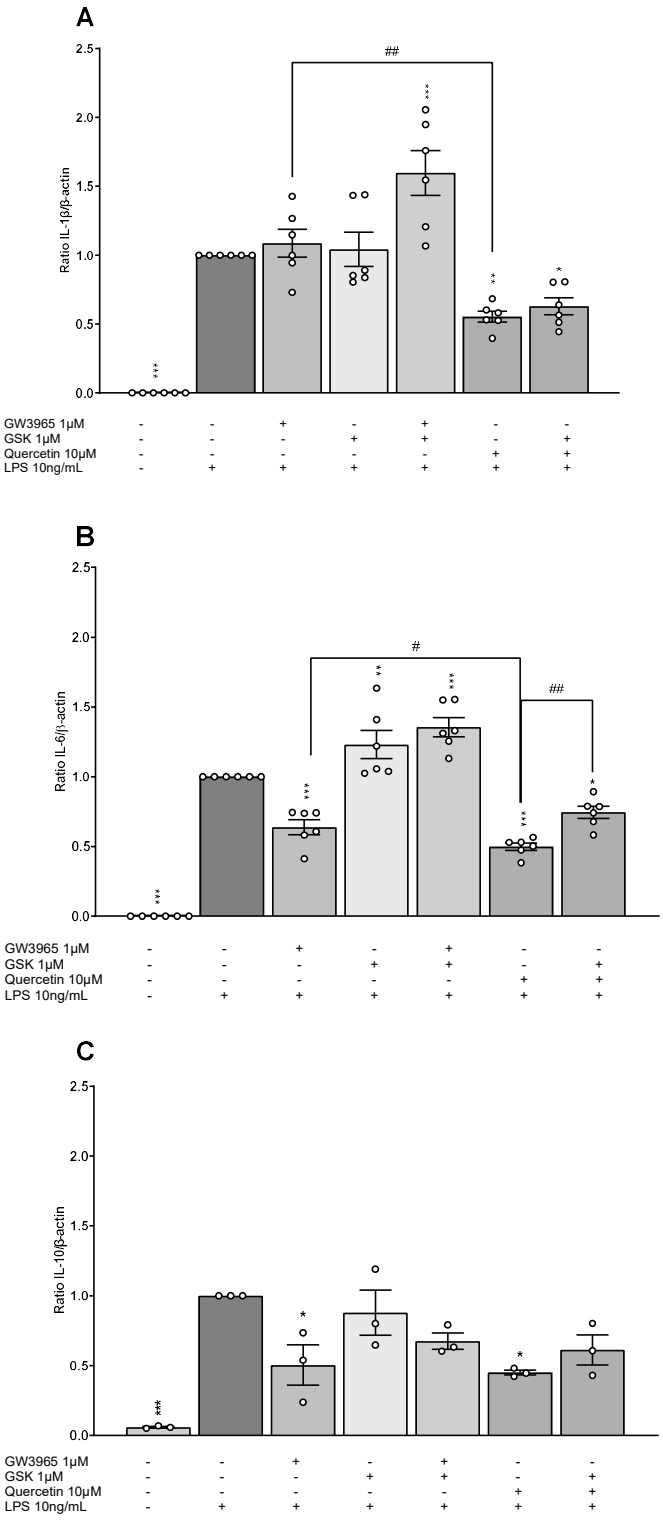
5.3.4 Anti-inflammatory effects of quercetin are LXRα independent

As shown in Figure 5.6, LPS stimulation increased TLR 4 expression on mRNA level (Figure 5.6.E). Subsequently, triggers signaling cascades in the production of pro-inflammatory cytokines, IL-6, IL-1β, and TNF-α, and induces the expression of the anti-inflammatory cytokine, IL-10. Next, the anti-inflammatory property of quercetin is related to LXRα activation was evaluated by co-incubate quercetin with LXRα antagonist. As mentioned previously, GSK2033 blocked LXRα activation both in basal and under inflammation conditions. Here, cells treated with quercetin showed a decrease in inflammation by reducing the production of pro-inflammatory cytokines IL-6, and IL-1β, and also upregulate the expression of potent anti-inflammatory cytokine, IL-10. The percentage of inhibition for IL-6 and IL-1β target genes was 50.3% and 45% respectively when incubating with 10 μM of quercetin. However, co-treatment between quercetin and GSK2033 did not affect quercetin's effects in suppressing inflammation.

LPS treatment has been known to induce IL-10 expression in numerous cell lines (Pengal *et al.*, 2006; Chanteux *et al.*, 2007; Lewkowicz *et al.*, 2016). The anti-inflammatory activity of IL-10 is by suppressing the pro-inflammatory cytokines production such as TNF-α, IL-12, IL-1, and IL-6 (Grütz, 2005) and this activity is mediated through the Akt pathway (Pengal *et al.*, 2006). In this experiment, the expression of IL-10 following LPS and GW3965 and quercetin were lower compared to the cells treated with LPS alone suggesting that GW3965 and quercetin can soften the inflammatory response from LPS. Meanwhile, incubation with antagonist, GSK2033, showed no difference on IL-10 production compared to the LPS-treated cells showing that the ability of LXRα in suppressing inflammation is through promoting the anti-inflammatory cytokine, IL-10.

On the other hand, LXRα agonist effects on inflammation are specific to reducing IL-6 cytokine production by 35%. It is also confirmed that GW3965 lower IL-6 and IL-1β expression better than quercetin (unpaired t-test, $p<0.05$). However, Figure 5.6. A and 5.6.B indicate that LXRα antagonists have promotes the modulation of IL-6 and IL-1β.

Results showed that TNF-α and TLR4 were significantly increased compared to LPS control while GW3965 did not affect both target genes. Meanwhile, 10 μM quercetin treatment increased TLR4 production followed by an increase in TNF-α.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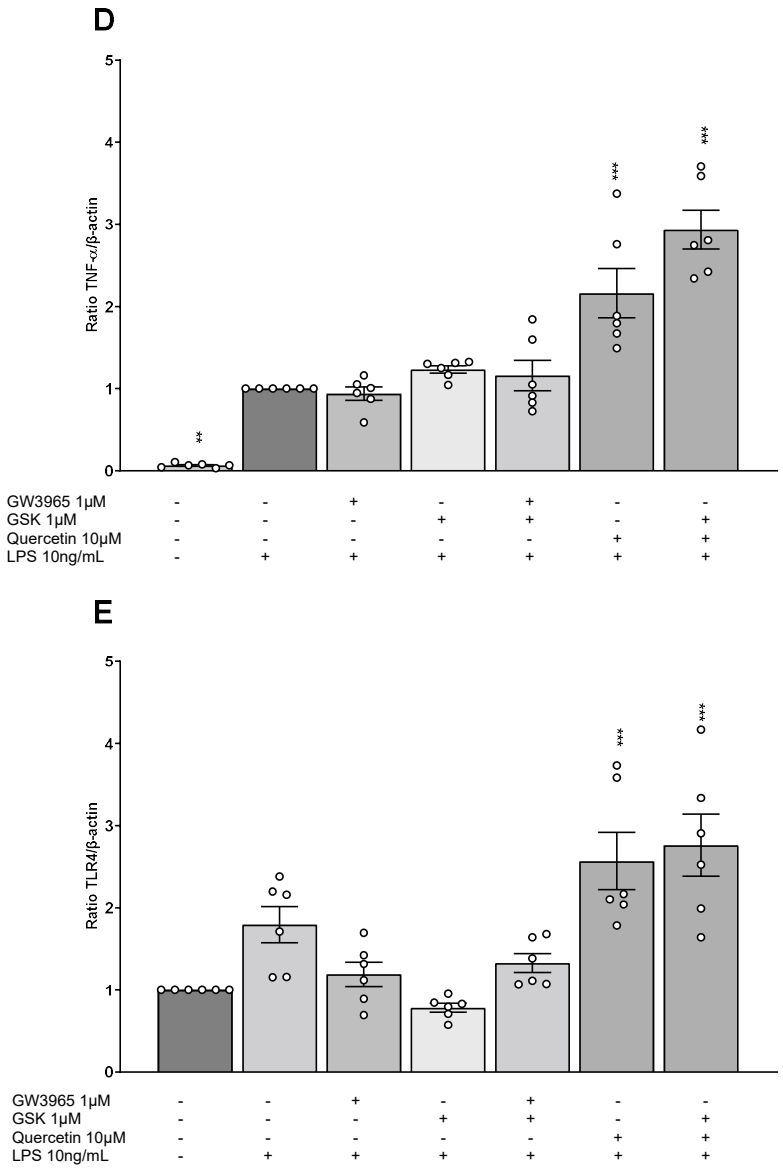


Figure 5.6 The effect of LPS pro-inflammatory cytokine production in murine RAW264.7 macrophages. Cells were treated with quercetin, LXRα agonists, and LXRα antagonist and their combination for 1 hour followed by LPS stimulation for 6 hours for (A) IL-1β, (B) IL-6, (C) IL-10, (D) TNF-α, and (E) TLR4. Fold changes are shown relative to β-actin as a housekeeper. Fold changes are shown relative to β-actin as a housekeeper. Data are expressed as mean ± SEM (n≥3, *$P<0.05$, **$P<0.01$, ***$P<0.001$ relative to LPS group; #$P<0.05$, ##$P<0.01$ from unpaired t-test).

5.3.5 The effects of LXRα activation by quercetin on inflammatory NF-κβ signaling pathway on murine macrophage RAW264.7

The NF-κβ pathway regulates numerous aspects of innate and adaptive immunity and has been known as the major modulator of inflammatory response. Previous research suggests that quercetin demonstrates anti-inflammatory activity by inhibiting the transcriptional activity of NF-κβ. Meanwhile, LXRα suppresses the production of the pro-inflammatory cytokines through the repression of several transcription factors such as NF-κβ, AP-1, or STAT-1. The result in Figure 5.7 demonstrates that both GW3965 and quercetin inhibit the expression of p65, Iκβ, and NF-κβ on mRNA level following LPS stimulation. The anti-inflammatory activity of quercetin in inhibiting NF-κβ signaling is not affected by LXRα.

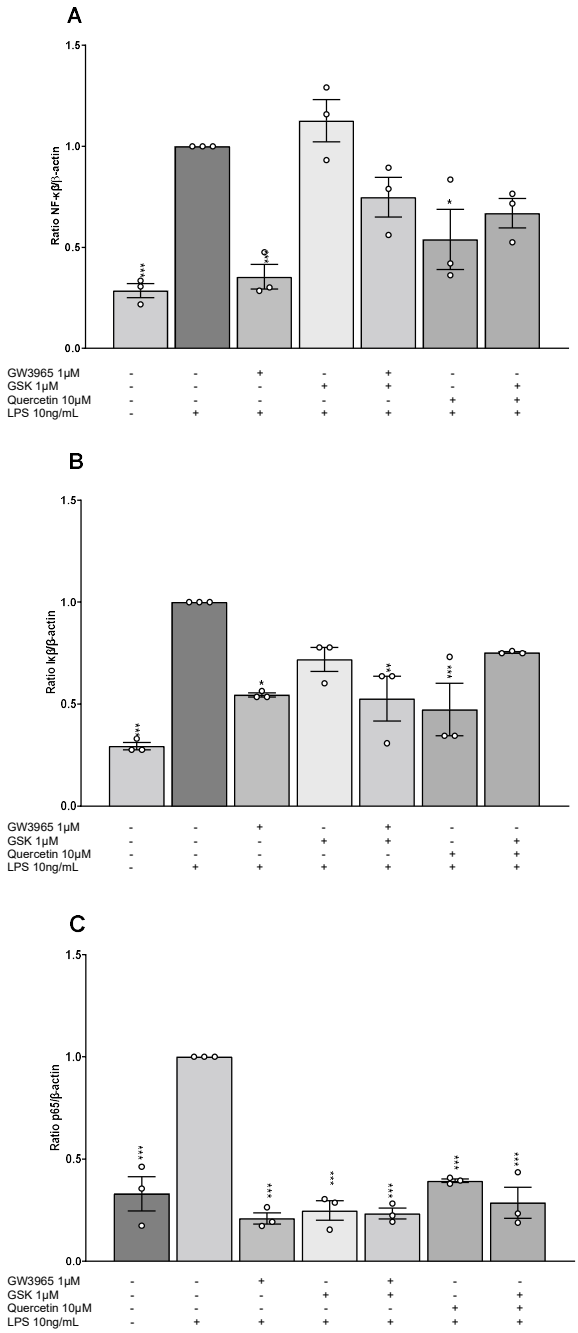


Figure 5.7 The effect of quercetin and LXRα ligands on the NF-κβ signaling pathway. Cells were treated with quercetin, LXRα agonists, and LXRα antagonist and their combination for 1 hour followed by LPS stimulation for 6 hours for (A) NF-κβ, (B) Iκβ, and (C) p65. Data are mean with SEM of three independent experiments performed in duplicate. Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test. *, **, and *** indicate significant differences to the control group (Dunnett's t-test), *P<0.05*, *P<0.01*, and *P<0.001* respectively.

5.4 Discussion

The data in this chapter demonstrated that quercetin could modulate canonical LXRα target genes, ABCA1, in murine RAW264.7 macrophages. Unlike LXRα activation in MDA-MB-231 (Chapter 3) and HepG2 (Chapter 4), macrophages are considered as challenging to transfect since macrophages are equipped with an ability to recognize foreign substances and initiate an immune response against them (Keller *et al.*, 2018). Thus, it was decided to observe the activation of LXRα in RAW264.7 macrophages through the modulation of its primary target genes, both at mRNA and protein levels.

As mentioned, in the results section, GW3965 significantly induced mRNA level of ABCA1 after both, 6 and 24 hours, following incubation start. Similar effects were also seen when cells were treated with quercetin in increasing concentrations. Later it was decided to use 24 hours incubation time to observe the target gene modulation. This decision was made based on modulation on cells treated with quercetin. After 24 hours of incubation, there was no significant difference between the different concentrations tested. According to cytotoxicity and modulation, 10 µM quercetin was used further as it is considered effective and safe. Modulation of LXRα target genes on transcription level by its agonist, GW3965, was varied. ABCA1 level was significantly induced but not APOE. The key function of LXRα in macrophages is to induce the expression of its target genes involved in cholesterol efflux and to limit the limit accumulation that caused atherosclerosis, such as ABCA1, ABCG1, and APOE (Laffitte *et al.*, 2001). Increased free cholesterol levels in macrophages lead to the modulation of APOE (Laffitte *et al.*, 2001). A previous study by Gafencu *et al* (2007) showed that 1 µg/mL of LPS treatment for 18 hours decreased APOE expression in murine RAW264.7 cells (Gafencu *et al.*, 2007). A similar result was also shown in this study where LPS treatment lowered APOE expression. Besides, in this study, the absence of lipid loading might be the reason why APOE expression is not as potent as ABCA1 expression.

To further prove that the activation was caused by LXRα, cells were then treated with a combination between GW3965 and GSK2033 as LXRα antagonists. GSK2033 has high potent inhibition and blocked the activity of both LXRα and LXRβ (Toporova *et al.*, 2020). Interestingly, the modulation was completely diminished in the presence of the antagonist, indicating that the modulation of ABCA1 is LXRα dependent. Yet, this effect was not seen

with both agonist and antagonist of LXRα on APOE which might indicate that the regulation of APOE is not solely controlled by LXRα.

Meanwhile, cells treated with quercetin also showed increases in ABCA1 levels although the strength was lower compared to the agonist effect. It has been reported that quercetin induces ABCA1 on the transcriptional level in HeLa cells (Fouache *et al.*, 2019). On the contrary, quercetin treatment increased APOE expression and the level decreased in the presence of the antagonist. There was no difference in APOE expression following quercetin combined with antagonist and antagonist alone. The modulation showed in quercetin and antagonist group might come from the antagonist itself which indicates that the antagonist binds stronger to LXRα than quercetin. Thus, this demonstrates that quercetin acts as a weak ligand for LXRα in RAW264.7 macrophages.

Under inflammatory conditions, ABCA1 expression was highly increased. Even though the main role of ABCA1 is to maintain lipid metabolism by controlling cholesterol efflux, ABCA1 is also known to have anti-inflammatory properties in disease progression where inflammation is involved including atherosclerosis, metabolic syndrome, and obesity (Babashamsi *et al.*, 2019; He, Gelissen and Ammit, 2020)(He, Gelissen and Ammit, 2020)(He, Gelissen and Ammit, 2020)(He, Gelissen and Ammit, 2020)(He, Gelissen and Ammit, 2020)(He, Gelissen and Ammit, 2020)(He, Gelissen and Ammit, 2020)(He, Gelissen and Ammit, 2020)(He, Gelissen and Ammit, 2020)(He, Gelissen and Ammit, 2020). Agonist treatment combined with LPS stimulation further elevates the ABCA1 level. Again, this effect was blunted when cells were treated with an antagonist.

In contrast, LPS stimulation decreased APOE mRNA expression. This effect was restored by quercetin treatment. Both agonist and antagonist of LXRα failed to show any modulation on APOE under LPS stimulation. Unlike in basal conditions, the combination between agonist and antagonist still modulated ABCA1 in LPS-stimulated cells proving that LPS alone did induce ABCA1 expression. Meanwhile, cells treated with quercetin and LPS also displayed increased ABCA1 levels and this effect was reduced with an antagonist. This indicates that ABCA1 is involved and responded to inflammatory stimulation in RAW264.7 macrophages as described by Kaplan et al (2002). Therefore, it is expected that the anti-inflammatory properties of LXRα are ABCA1 dependent and should be diminished in the presence of an antagonist. When the macrophages were

incubated with GW3965, IL-10 expression was reduced compared to the LPS control accompanied by increase IL-10 anti-inflammatory cytokine compared to non-treated cells but lower compared to the LPS treated cell alone. Meanwhile, no significant change was shown in IL-1β expression. Increased ABCA1 expression under LPS stimulation was accompanied by decreased levels of IL-6 and unchanged in the presence of GSK2033 indicates that a reduction in IL-6 by GW3965 is dependent on ABCA1 expression. However, antagonist treatment significantly increased IL-6 level but does not affect other pro-inflammatory cytokines measured suggesting that GSK2033 mediates pro-inflammatory activity by increasing IL-6 under LPS stimulation.

In contrast, expression of other cytokines, IL-1β, TNF-α, and TLR4 were not related to LXRα since there was no decrease in those cytokines following LXRα agonist treatment. Furthermore, antagonist treatment showed no reduction in pro-inflammatory cytokines measured, but significant increases were seen on the IL-6 level. Co-treatment between LXRα agonist and antagonist significantly induced the expression of IL-6 and IL-1β suggesting that LXRα antagonist blocked the anti-inflammatory activity of both LXRα and ABCA1. In this study, the data demonstrated that LXRα activation by its agonist leads to modulation of ABCA1 which further has an anti-inflammatory activity to reduce IL-6 and modulates the expression of anti-inflammatory cytokine, IL-10 on mRNA levels. Here, the data suggest that the LXRα anti-inflammatory property of GW3965 was mediated through ABCA1 modulation.

On the other hand, quercetin treatment significantly reduced IL-6, and IL-1β, but also induced TNF-α and TLR4 expression. When macrophages were treated with both quercetin and LXRα antagonist, IL-6 level was significantly decreased compared to LPS control. LXRα antagonist reduced quercetin's ability to suppress IL-6 levels compared to the cells treated with quercetin only. Moreover, the LXRα antagonist does not affect IL-1β levels on the anti-inflammatory property of quercetin. This result demonstrates that LXRα antagonist partly blocked quercetin's ability to reduce IL-6 suggesting strong anti-inflammatory activity of quercetin.

Interestingly, a previous study revealed that quercetin decreases TNF-α levels both expressed on the transcriptional level (Li *et al.*, 2019) and TNF-α produced in the cultured supernatant on (Lee *et al.*, 2018) RAW264.7 cells but this study showed the opposite

result. A similar pattern was also seen on TLR4 expression. Quercetin treatment on cells induced TLR4 expression when another research stated otherwise (Li *et al.*, 2019). Based on those results, quercetin still has its anti-inflammatory activity even though the expression of ABCA1 has been blocked by the LXRα antagonist. Thus, the data suggest that quercetin can modulate the LXRα target gene, ABCA1, but its ability to suppress inflammation is LXRα and ABCA1 independent.

To further explore the mechanisms of anti-inflammatory of quercetin-mediated by LXRα, the expression of p65, NF-κβ, and Iκβ on mRNA levels were measured. These genes are related to the NF-κβ signaling pathway. NF-κβ comprises a group of inducible transcription factors, including p65/RelA, RelB, cRel, p106/p50 (NF-κβ-1), and p100/p52 (NF-κβ-2). Under the unstimulated condition, these complexes are in the cytoplasm bound to an inactivator, namely Iκβ (Albensi, 2019). Previous publications demonstrated that GW3965 can antagonize the translocation of p65 subunit into the nucleus in HUVEC cells induced with LPC (Bi *et al.*, 2016), hence GW3965 was used as a positive control. An unexpected result was shown by the antagonist, GSK2033, in the NF-κβ signaling as it significantly decreases p65 and Iκβ mRNA level as GW3965 does. Although GSK2033 performed as expected in blocking ABCA1 expression in murine macrophages and blunted the anti-inflammatory activity of GW3965, it showed unexpected results in NF-κβ signaling, and no effect on pro-inflammatory cytokines production compared to the LPS control.

Here, the results prove that the anti-inflammatory property of quercetin is mediated through the NF-κβ pathway as significant decreased was observed on p65, NF-κβ, and Iκβ. Although LXRα antagonist blocked NF-κβ and Iκβ, quercetin still showed strong inflammatory suppression probably because p65 expression was unaffected by LXRα antagonist. In this study, the LXRα signaling pathway was blocked by GSK2033, therefore the data obtained were assumed as from other signaling cascades. The same pattern was seen on GW3965 anti-inflammatory activity suggesting that there might be other transcription factors involved in the suppression of pro-inflammatory cytokines.

The TRANScription FACtor (TRANSFAC) database is an online resource that contains a comprehensive summary related to the prediction of transcription factor binding sites (Wingender *et al.*, 1996; Matys *et al.*, 2006). As part of TRANSFAC's tools, MATCH

provides the possibility to predict DNA sequences of potential transcription factor binding sites (Kel *et al.*, 2003). Table 5.1 shows the complexity of cytokine production regulation predicted using the TRANSFAC platform and the prediction of LXRα binding sites of the gene of interest. Here, the result showed that several transcription factors are involved in the regulation of one gene of interest.

Table 5.1 List of genes and transcription factors associated with the gene of interests and LXRα binding sites of the gene of interest

Species	Gene of interests	TFs that regulate gene of interests	Number of LXRα binding site	Sequence	Core score	Matrix score
Mouse	IL-6	NF-κβ, NF-κβ1-p50, C/EBPγ:C/EBPγ, RelA-p65: NF-κβ1-p50, AP-1, C/EBP, C/EBPβ:C/EBPγ	1	ggGGTTAagtaagtgca	0.892	0.954
	IL-1β	Iκβ-β:c-Rel:RelA-p65	1	ggGGTCAcctaagacca	0.882	1.000
	IL-10	AhR:arnt, NF-κβ1-p50, c-Rel:RelA-p65, NF-κβ1-p50:c-Rel, RelA-p65:NF-κβ-β1, NF-κβ1:c-Rel				
	ABCA1	LXRα, LXRβ, AP-2α, HNF-3β, LXRβ:RXRβ	1	ggGGTTActaccggtca	0.944	0.954
	APOE	HNF-4alpha				
Human	TNF-α		3	tgGATCAcctgaggtca	0.813	0.875
		AP-1, NF-κβ, NFAT1, LXRα:RXRα, NF-κβ1-p50: NF-κβB1-p50, ATF-2:c-Jun, c-Fos:c-Jun, c-Rel:c-RelA-p65, p50:p50, AP2		cgGATCAcctgaggtca	0.813	0.872
				tgGGTCAcctgtggtca	1.000	0.893
	ABCA1		6	caGGTCAgaacagacca	1.000	0.870

Species	Gene of interests	TFs that regulate gene of interests	Number of LXRα binding site	Sequence	Core score	Matrix score
				ggGGTCAccagagctcg	1.000	0.874
		LXRα:RXRα, LXRβ:RXRα, AP-1α, HNF-3β, SREBP-2-isoform1		gaGGTTActatcggtca	0.954	0.924
				caGCTCAcctcaggtca	0.839	0.864
				gtGATCActtgaggtca	0.813	0.872
				tgacctcaaaTGATCca	0.813	0.891
	IL-1β	NF-κβ, NF-κβB1:p50:RelA-p65, p50:RelA-p65, GR, STAT3, STAT1, IRF-8, C/EBPbeta, PelA-p65, PU.1, PU.1-isoform1	1	tgGTTCAtggaagggca	0.874	0.853

According to the literature, NF-κB, activator protein (AP)-1, CCAAT/enhancer-binding protein (C/EBP), and cAMP response element (CRE)-binding protein (CREB) are known to regulate IL-6 expression (Hershko *et al.*, 2002). Based on the data from Table 5.1, multiple transcription factors modulate IL-6 expression but interestingly LXRα is not one of them. In contrast, this present study showed that IL-6 expression was also regulated by LXRα since LXRα agonist suppressed IL-6 expression, and GSK2033 diminished this effect (Figure 6A). Meanwhile, IL-1β is mainly regulated by C/EBPβ, NRF2, and STAT3 transcription factors (Cornut, Bourdonnay and Henry, 2020). TNF-α is induced by several stimuli, one of them is TLR4. As TLR4 is a receptor for LPS, it is expected that its expression will level up although quercetin and LXRα ligands did not affect TLR4 and TNF-α.

5.5 Conclusion

In this study, quercetin demonstrated a time and dose-dependent increase in ABCA1 through the LXRα signaling pathway. Based on its ability to modulate LXRα target genes, quercetin may be classified as a weak activator compared to its synthetic ligands. The potential of quercetin to suppress inflammation is mediated by the NF-κβ pathway and this effect is partly blunted by LXRα antagonist indicating that there might be other

pathways where quercetin can exert its anti-inflammatory property as shown by the production of pro-inflammatory cytokines that are not affected by LXRα activation.

Chapter 6. General discussion, future work, and conclusion

The present study demonstrates that polyphenols have shown potential to act as a ligand for LXRα. Furthermore, the data also suggest that the anti-inflammatory activity of quercetin is partly mediated through LXRα downstream signaling pathway although there are likely other inflammatory pathways involved. This study also suggests that the cellular targets of quercetin and LXRα in terms of inflammation are determined by numerous factors, including cell type, inflammatory stimulus, and bioactive compounds involved.

Inflammation is a normal physiological response to cellular and tissue damage. Inflammation is characterized by the activation of immune cells to protect the body from bacteria, viruses, or other inflammatory stimuli by eliminating those stimuli and inducing tissue recovery. A normal inflammatory response only lasts for a short time. Failure during the resolution phase of acute inflammation may shift to chronic inflammation characterized by a persistent, non-resolving, and low-grade inflammation.

Research has shown that chronic inflammation is associated with the pathogenesis of several diseases including neurodegenerative disease, cancer, arthritis, and cardiovascular disease (Furman *et al.*, 2019). Numerous approaches have been used to target inflammation including conventional treatment using NSAIDs and alternative treatment based on natural sources, including phenolic compounds (Ambriz-Pérez *et al.*, 2016). Polyphenols have been widely studied as potential alternatives to combat inflammation although the specific mechanisms of the anti-inflammatory activity are not fully understood.

Among polyphenols studied, quercetin has shown strong anti-inflammatory activity as demonstrated in numerous studies. Most studies indicate that the main reason behind the anti-inflammatory properties of quercetin is its antioxidant effects as shown in a study where quercetin lowered ROS-induced oxidative stress and inflammation by inhibiting NOX2 production in A549 lung epithelial cells stimulated with LPS (Sul and Ra, 2021). Moreover, the most known mechanism regarding anti-inflammatory properties of quercetin is the ability of quercetin to inhibit the NF-κB p65 nuclear translocation from cytoplasm to the nucleus, alongside the prevention of IκB degradation, and decrease in

IKK protein levels which will all lead to the reduced transcriptional activity of NF-κB inside the nucleus (Granado-Serrano *et al.*, 2010).

Meanwhile, quercetin has been found to interact with several transcription factors, such as STAT3, NF-κB, Nrf2, and AP1, and also nuclear receptors including PPAR-γ, that play a role in suppressing inflammatory response (Sun *et al.*, 2015; Li *et al.*, 2016; Liao and Lin, 2020). Nuclear receptors are ligand-activated transcription factors that regulate diverse physiological functions including inflammation. For many years, nuclear receptors have been used as a potential drug target for inflammation-related diseases such as inflammatory bowel disease (Klepsch *et al.*, 2019) and rheumatoid arthritis (Shirinsky and Shirinsky, 2011).

The role of LXRα in suppressing inflammation in macrophages is closely related to the early stage of plaque formation in atherosclerosis. LXRα activation on macrophages not only reduces pro-inflammatory cytokine production but also supports the clearance of oxidized lipoprotein via the upregulation of ABC family transporters, which is a critical step in the conversion of macrophages into foam cells. It has been demonstrated that LXRα can be activated by several cholesterol derivatives, including oxysterol (Olkkonen, Béaslas and Nissilä, 2012). Recent studies even showed the potency of some polyphenols to activate LXRα through molecular docking or cell model system. For example, naringenin was shown to have antagonist activity on both LXRs isoforms (Goldwasser *et al.*, 2010; Fouache *et al.*, 2019). Furthermore, hesperetin was also shown to activate LXRα in THP-1 macrophages leading to modulation of its target genes and reduction in lipid accumulation (Chen *et al.*, 2021). Based on that, it is possible that the beneficial effects of quercetin could also be mediated through the nuclear receptor family, i.e. LXRα.

Studies regarding the health benefits of quercetin were mainly based on quercetin in the form of aglycone or glycoside. However, quercetin is heavily metabolized into aglycones conjugates, suggesting that those metabolites may be responsible for quercetin's health benefits. Structural changes in quercetin metabolites may more effective than the parent compound and better candidate as LXRα ligand since many studies have pointed out the similarity between steroids and flavonoids although the link remains unclear (Avior *et al.*, 2013; Fouache *et al.*, 2019). A study by Fouache *et al* (2019) also pointed out that little

changes in the hydroxyl group of flavonoids might change the activity of ligand from agonist to antagonist. This present study supported the previous observation that hydroxyl changes may change polyphenols activity on LXRα activation. The effect on hydroxyl changes was shown between two methylated metabolites of quercetin, isorhamnetin (methylated at 3' carbon position) and tamarixetin (methylated at 4' carbon position), has an impact on the ability to activate LXRα on HepG2 cells stably transfected with LXRα reporter gene.

The effectiveness of quercetin metabolites as an anti-inflammatory agent is intriguing. In vivo and in vitro studies so far showed that quercetin derivatives have potent anti-inflammatory activity by suppressing different inflammatory signaling (Boesch-Saadatmandi *et al.*, 2011; Cho *et al.*, 2016; Lesjak *et al.*, 2018; Bhatt *et al.*, 2021).

6.1 Polyphenols activate LXRα

LXRα is a ligand-activated transcription factor that needs a ligand, either agonist or antagonist to regulate gene expression. Recently, various bioactive compounds have been shown to modulate LXRα activity that might be beneficial and could be developed further as LXRα ligand. The present work showed that some polyphenols were tested to determine if they activate LXRα in the reporter cell system. Three different cell lines, RAW264.7 murine macrophages, HepG2, and MDA-MB-231, were used in this study and the results were consistent between both cell lines, as shown by quercetin that showed increased modulation by 2-3 fold compared to the control cells. The similarity was found between the previous study by Fouache *et al* (2019) and this study that can confirm the agonistic activity of quercetin. The magnitude of LXRα activation and ABCA1 modulation by quercetin observed in this present study is similar to the previous findings.

Table 6.1 Summary of results from polyphenols on LXRα activation and its effect on cell metabolism

Outcome(s)	Cell Line		
	MDA-MB-231	HepG2	RAW264.7
Polyphenols that activates LXRα on reporter cell	Quercetin, hesperetin, resveratrol, genistein	Quercetin, tamarixetin	-
Modulation of LXRα target genes	Quercetin upregulates ABCA1 expression	Tamarixetin weakly upregulates ABCA1 expression	Quercetin induces the expression of ABCA1 and weakly modulates APOE
Effect on inflammation	-	Quercetin and tamarixetin do not show anti-inflammatory activities	Quercetin and GW3965 suppressed inflammation

In general, polyphenols are a group of plant secondary metabolites characterized by at least two phenyl rings and one or more hydroxyl substituents and further classified into subclasses depending on the number of phenol units and substituent groups. The diversity in polyphenol's chemical structure affects their compatibility to bind into LXRα ligand-binding pocket. Screening candidate compounds as ligands for nuclear receptors can be assessed using several methods. A cell-based luciferase reporter gene system, besides molecular docking (Niinivehmas *et al.*, 2016; Fouache *et al.*, 2019), is the common method to test compounds of interest as a ligand for the target nuclear receptors, including polyphenols, as it detects a direct correlation exists between the amount of luciferase produced and its ability to convert D-luciferin into oxyluciferin (Hutchinson and Thorne, 2019). In this study, representation from flavonols, flavones, isoflavones, flavanones, and flavanols were tested and showed to increase activation of LXRα. A study showed that cyanidin is a ligand for both LXRs isoforms with a higher affinity for LXRα than LXRβ (Jia *et al.*, 2013).

The similarity of chemical structure between steroids and polyphenols has been pointed out as the reason why polyphenols may be good candidates as LXRs ligands (Avior *et al.*, 2013). This study supports this idea as shown by quercetin's ability to modulate LXRα activity also upregulates the expression of its target genes. Furthermore, this study also demonstrates that structural modification can change that ability as demonstrated by tamarixetin and isorhamnetin, both methylated conjugates of quercetin, that have a different effect on LXRα modulation. Minor changes in methyl group position on tamarixetin and isorhamnetin may be the reason that affects the binding. Furthermore, the difference in substituent groups in polyphenols may also affect the ability of polyphenols to bind to a nuclear receptor. Here, this study demonstrates that some polyphenols can bind and activate LXRα and it is dependent on the position and constituent groups located. Interestingly, when quercetin was combined with the LXRα antagonist, the effect of quercetin was diminished. This result suggests that quercetin has a weak affinity to LXRα compared to antagonists.

6.2 Evidence for LXRα target gene modulation by polyphenols

The ligand-induced switch from corepressor to coactivator states underlies LXRs-induced transcription. Both LXR isoforms regulate several target genes including ABCA1, SREBP-1C, ABCG1, ABCG5/ABCG8, APOE, and CYP7A1 (Ory, 2004). Among those target genes, ABCA1 is involved in the anti-inflammatory activity of LXRα in macrophages. In this study, the modulation of target genes is used to further confirm the ability of polyphenols as a ligand for LXRα. Interestingly, among polyphenols that showed the ability to activate LXRα in reporter cell system, only quercetin, and tamarixetin could modulate LXRα target genes moderately compared to the synthetic ligand. The inability of resveratrol, genistein, EGCG, isorhamnetin, and quercetin-3-glucuronide in modulating LXRα target genes nullify the probability of those compounds as a ligand for LXRα.

The present study demonstrated that the modulation of ABCA1 by quercetin in murine RAW264.7 macrophages, MDA-MB-231, and HepG2 were quite similar although synthetic ligand, GW3965, modulates ABCA1 level differently indicating that ABCA1 modulation is tissue specific. As the main function of ABCA1 is responsible for transporting cellular cholesterol and phospholipids to lipid-poor apolipoproteins, such as

apoA-I, to create HDL particles and to absorb excess cholesterol from the cells (Liu and Tang, 2012), it is understandable that ABCA1 expression is much higher in macrophages and liver cells than in breast cancer cells since it is not involved in reverse cholesterol transport.

Furthermore, in this present study, the expression of ABCA1 in liver cells is unaffected regardless of the treatment given. According to the literature, ABCA1 is highly expressed in the murine liver both on mRNA and protein level (Wellington *et al.*, 2002) and this study confirmed that ABCA1 is also highly expressed in the human liver. Interestingly, the LXRα antagonist, GSK2033, that pharmacologically inhibits LXRα activation and also its target gene modulation, could not suppress the expression of ABCA1 (Chapter 4) in contrast to the repressed expression of ABCA1 in murine macrophages (Chapter 5) suggesting that it is recommended that ABCA1 be maintained at a certain level in the liver since it is primarily responsible for cholesterol and lipid homeostasis. Since this study did not include lipid loading, repression of LXRα action by antagonist may be unwanted because it is important to control cellular cholesterol level to maintain normal cell function, especially in the liver. As a result, GSK2033 might have tissue-specific effects since it did not produce the expected physiological response in the liver but was effective in macrophages.

Furthermore, although GW3965, quercetin, and tamarixetin modulate APOE expression, the magnitude is lower than ABCA1 level both on macrophages and liver cells. Again, the absence of lipid loading in this study may also contribute to the relatively low modulation of APOE since one of the main functions of APOE is responding to cholesterol efflux and HDL metabolism (Ory, 2004).

This study demonstrates the complexity of interaction between ligands, nuclear receptors, and target genes. Numerous signaling pathways involved make it difficult to explain the exact cause why resveratrol and genistein did not induce LXRα target genes when those compounds showed promising activity in increasing LXRα activation on the reporter cell model. This behavior could also be found in GSK2033 where no antagonist effect was evident in the liver cells when it is widely known as a specific antagonist for LXRα. Despite being widely used to study nuclear receptor systems, luciferase reporter assays are

artificial systems inserted into cells that may not be representative of the metabolism of a whole cell.

6.3 The anti-inflammatory properties of quercetin on murine macrophages and human liver cells

The anti-inflammatory properties of quercetin have been widely researched over the past decades. In vitro, in vivo, and clinical studies experiment demonstrated quercetin's ability to suppress pro-inflammatory cytokine production. Meanwhile, the proposed anti-inflammatory activity of LXRα is closely related to the function of LXRα to regulate lipid metabolism (Zelcer and Tontonoz, 2006; Im and Osborne, 2011; Schulman, 2017). A macrophage is an immune cell that resides in tissues, acting as a sentinel for the immune system. The cells are uniquely suited to detect and respond to infections and tissue injuries mediated by various scavengers, pattern recognition, and phagocytic receptors (Lavin *et al.*, 2015). Besides, macrophages also play an important role in the early stage of atherosclerosis where hypercholesterolemic conditions convert macrophages into foam cells. Several factors control the creation of these cells, including cholesterol influx, esterification, and efflux. When cholesterol inflow and esterification increase and/or cholesterol outflow decreases, macrophages ultimately transform into lipid-laden foam cells, the prototypical cells in atherosclerotic plaques (Yu *et al.*, 2013). Induced transcription of LXRα target genes may be a potential approach to combat atherosclerosis. Hence, the activation of LXRα and the modulation of its target genes, especially ABCA1, are important to suppress inflammation (Ito *et al.*, 2015).

In this present study, as expected, quercetin suppressed pro-inflammatory cytokine production in murine macrophages stimulated by LPS. In contrast, ABCA1 expression did not affect the ability of quercetin to suppress the inflammatory response. Although LXRα antagonist slightly decreased quercetin’s anti-inflammatory action, on both pro-inflammatory cytokine suppression and NF-κB signaling, the effect is negligible (Chapter 5). This study showed that although quercetin activates and modulates LXRα activation in murine macrophages, the anti-inflammatory activity of quercetin is independent of LXRα, suggesting that there are other pathways through which quercetin exerts its anti-inflammatory properties.

In contrast, both quercetin and tamarixetin did not show anti-inflammatory activity in liver cells stimulated with TNF-α. Moreover, LXRα agonist and antagonist also have no effect in modulating any of the pro-inflammatory markers tested in this study. The unresponsiveness of GW3965 and GSK2033 in modulating inflammation is unexpected. As an agonist of LXRα, GW3965 suppressed inflammation as shown by the result from Chapter 5 while GSK2033 inhibited the anti-inflammatory activity by LXRα. Meanwhile, little is known about the effect of both GSK2033 and GW3965 on inflammation since experiments exploring the anti-inflammatory activity by GW3965 were done in immune cells (Scholz *et al.*, 2009; Nunomura *et al.*, 2015).

The results from this study indicate that quercetin, and probably tamarixetin, are selective modulators of LXRα since the anti-inflammatory effects of those compounds seem to be tissue specific. The inflammatory stimulation in liver cells is a more complicated process compared to the macrophages. Since LXRα showed anti-inflammatory activity in macrophages, it is also expected that it would have the same effect in the liver. Surprisingly, this hypothesis is not proven in liver cells. The methods used in this study might be the reason for this finding. In this present study, TNF-α stimulation alone is not sufficient in inducing hepatic inflammation.

A review by Schulman (2010) stated that LXRα responds acutely and more readily in macrophages whereas liver cells are more resistant since they are exposed to cholesterol (and LXRα ligands) consistently in varying concentrations (Schulman, 2017). Consequently, macrophages would be more sensitive to ligand exposure even in small concentrations compared to liver cells. Therefore, a macrophage environment may be more ideal for the development of LXRα synthetic ligands, since the activity is cell-specific and does not affect negatively the liver. Based on the results from this study, quercetin could be considered as a partial agonist of LXRα although further experiments are needed to prove this hypothesis.

6.4 The anti-inflammatory activity of quercetin metabolites on hepatic inflammation

Circulating quercetin metabolites followed by quercetin intake is crucial to explain the health benefits associated with quercetin. Several studies have demonstrated the anti-

inflammatory activity of quercetin metabolites (Tribolo *et al.*, 2008; Boesch-Saadatmandi *et al.*, 2011) when others do not (Suri *et al.*, 2008; Winterbone *et al.*, 2009). Meanwhile, up to the present time, research regarding the potency of quercetin metabolites as a ligand or to regulate the expression of certain nuclear receptors are scarce.

In this study, quercetin and its metabolites tested showed no activity in suppressing inflammation in HepG2 cells subjected to TNF-α stimulation. It was demonstrated that structural modification of dietary flavonoids, including quercetin, from the parent compound to the metabolite forms, can alter the antioxidant and anti-inflammatory activities (Lotito *et al.*, 2011). It is also important to highlight that unlike breast cancer and macrophages cells, HepG2 cells are derived from the liver tissue where the liver itself is a metabolically active tissue that could metabolize quercetin and/or its metabolites into a different form of metabolites. This might be the reason for the different responses of quercetin to inflammatory stimulation in macrophages and liver cells.

According to the literature, quercetin metabolites are found in the human plasma as glucuronide or sulfate (Murota *et al.*, 2007), and the major metabolites found following quercetin food source were quercetin-3-glucuronide, quercetin-3'-sulfate, and 3'-methyl quercetin-3-glucuronide (Day *et al.*, 2001). Methylated conjugates were also found in the plasma in lower amounts as tamarixetin (10-13%) and isorhamnetin (8.5-11%) (Almeida *et al.*, 2018). Meanwhile, this study was using commercially available metabolites namely quercetin-3-glucuronide, tamarixetin, and isorhamnetin that might not reflect the real metabolites available in the liver. The results from this study suggest that structural transformation influenced the bioactivity of quercetin and its metabolites. The preliminary results showed that tamarixetin and quercetin may be good candidates as ligands for LXRα, although the associated downstream changes on hepatic LXRα target gene expression and their association to health benefits, needs further research.

6.5 Limitations of the study

When evaluating the possible health benefits of polyphenols, several aspects must be taken into consideration. First, concentrations used in this study are considered as supraphysiological concentrations where the actual circulating levels found in human plasma are relatively much lower, although some researchers showed that quercetin

accumulates in certain tissue. Nevertheless, cell culture is an artificial system and different concentrations may be required to clearly demonstrate a particular mechanism. Extrapolation in vitro to in vivo may not be accurate, but in vitro studies are useful to demonstrate potential molecular mechanisms which will require validation in vivo. Next, metabolites used in this present study are not the primary circulating metabolites following quercetin ingestion. Besides, the use of liver cells has limitations, although LXRα expression in hepatocytes is prominent, there is high metabolic activity to metabolize and convert quercetin and metabolites into different conjugates therefore the results obtained are the effects of different compounds from the parent compound.

6.6 Conclusion and further work

In conclusion, this study revealed that some classes of polyphenols such as flavonols, isoflavone, flavanones, and stilbenes can modulate LXRα activity. Despite the structural similarity between steroids and polyphenol, they show different effects on the modulation of LXRα target genes. Quercetin and tamarixetin displayed agonistic activity that led to induced expression of ABCA1, while other tested polyphenols did not affect the modulation of LXRα target genes. Interestingly, quercetin and GSK2033 co-treatment resulted in reduced LXRα activation and ABCA1 expression, suggesting that quercetin uses the same binding site as known LXRα ligands. Further, the binding of quercetin into LXRα binding sites induces transcriptional activity leading to the downstream signaling of LXRα especially in suppressing inflammation in macrophages stimulated with LPS by inhibiting the NF-κB signaling pathway

Studies exploring the effectiveness of quercetin and its metabolites in suppressing inflammation mediated by LXRα are limited. As a result, further research is needed to provide a more detailed understanding of the involvement of LXRα on the anti-inflammatory properties of quercetin and its related metabolites. Based on data provided from this study, the following experiment should be conducted in the future:

- Improve the hepatic cell model to investigate the impact of different inflammatory stimuli in combination with fatty acid load, or co-culture systems with stimulated Kupffer cells/conditioned media of these cells as stimulus.

- Investigate further the tissue specific effects on LXRα activation in suppressing inflammation.
- Considering the effect of polyphenol on LXRβ activation and downstream signaling on suppressing inflammation.
- Using the appropriate quercetin metabolites that circulate in human plasma to establish the health benefit of LXRα activating properties of circulating quercetin metabolites.

REFERENCES

Ahn, S. B. *et al.* (2014) 'Expression of liver X receptor correlates with intrahepatic inflammation and fibrosis in patients with nonalcoholic fatty liver disease', *Digestive diseases and sciences*, 59(12), pp. 2975–2982.

Al-Bakheit, A. and Abu-Qatouseh, L. (2020) 'Sulforaphane from broccoli attenuates inflammatory hepcidin by reducing IL-6 secretion in human HepG2 cells', *Journal of Functional Foods*, 75, p. 104210. doi: https://doi.org/10.1016/j.jff.2020.104210.

Albensi, B. C. (2019) 'What is nuclear factor kappa B (NF-κB) doing in and to the mitochondrion?', *Frontiers in cell and developmental biology*, 7, p. 154.

Almeida, A. F. *et al.* (2018) 'Bioavailability of quercetin in humans with a focus on interindividual variation', *Comprehensive reviews in food science and food safety*, 17(3), pp. 714–731.

Ambriz-Pérez, D. L. *et al.* (2016) 'Phenolic compounds: Natural alternative in inflammation treatment. A Review', *Cogent Food & Agriculture*. Edited by F. Yildiz, 2(1), p. 1131412. doi: 10.1080/23311932.2015.1131412.

Andres, S. *et al.* (2018) 'Safety aspects of the use of quercetin as a dietary supplement', *Molecular nutrition & food research*, 62(1), p. 1700447.

Arts, I. C. W. *et al.* (2004) 'The type of sugar moiety is a major determinant of the small intestinal uptake and subsequent biliary excretion of dietary quercetin glycosides', *British Journal of Nutrition*, 91(6), pp. 841–847.

Ashley, N. T., Weil, Z. M. and Nelson, R. J. (2012) 'Inflammation: mechanisms, costs, and natural variation', *Annual Review of Ecology, Evolution, and Systematics*, 43, pp. 385–406.

Avior, Y. *et al.* (2013) 'Flavonoids as dietary regulators of nuclear receptor activity', *Food & function*, 4(6), pp. 831–844.

Babashamsi, M. M. *et al.* (2019) 'ABCA1 and metabolic syndrome; a review of the ABCA1 role in HDL-VLDL production, insulin-glucose homeostasis, inflammation and obesity', *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, 13(2), pp. 1529–1534.

Baranowski, M. *et al.* (2014) 'Insulin-sensitizing effect of LXR agonist T0901317 in high-fat fed rats is associated with restored muscle GLUT4 expression and insulin-stimulated

AS160 phosphorylation', *Cellular Physiology and Biochemistry*, 33(4), pp. 1047–1057.
Becares, N. *et al.* (2019) 'Impaired LXRα phosphorylation attenuates progression of fatty liver disease', *Cell reports*, 26(4), pp. 984–995.
Beking, K. and Vieira, A. (2011) 'An assessment of dietary flavonoid intake in the UK and Ireland', *International Journal of Food Sciences and Nutrition*, 62(1), pp. 17–19.
Bhatt, S. K. *et al.* (2021) 'In vitro anti-inflammatory property of a Quercetin-3-O-diglucoside-7-O-glucoside characterized from fresh leaves of Trigonella foenum-graecum L', *International Journal of Food Properties*, 24(1), pp. 1438–1452.
Bi, X. *et al.* (2016) 'Activation of liver X receptor attenuates lysophosphatidylcholine-induced IL-8 expression in endothelial cells via the NF-κB pathway and SUMO ylation', *Journal of Cellular and Molecular Medicine*, 20(12), pp. 2249–2258.
Bieger, J. *et al.* (2008) 'Tissue distribution of quercetin in pigs after long-term dietary supplementation', *The Journal of nutrition*, 138(8), pp. 1417–1420.
Bilotta, M. T. *et al.* (2020) 'Liver X receptors: regulators of cholesterol metabolism, inflammation, autoimmunity, and cancer', *Frontiers in Immunology*, 11.
Bischoff, E. D. *et al.* (2010) 'Non-redundant roles for LXRα and LXRβ in atherosclerosis susceptibility in low density lipoprotein receptor knockout mice [S]', *Journal of lipid research*, 51(5), pp. 900–906.
Del Bo', C. *et al.* (2019) 'Systematic review on polyphenol intake and health outcomes: is there sufficient evidence to define a health-promoting polyphenol-rich dietary pattern?', *Nutrients*, 11(6), p. 1355. doi: 10.3390/nu11061355.
De Boer, V. C. J. *et al.* (2005) 'Tissue distribution of quercetin in rats and pigs', *The Journal of nutrition*, 135(7), pp. 1718–1725.
Boesch-Saadatmandi, C. *et al.* (2010) 'Effect of quercetin on paraoxonase 1 activity—studies in cultured cells, mice and humans', *Journal of Physiology and Pharmacology*, 61(1), p. 99.
Boesch-Saadatmandi, C. *et al.* (2011) 'Effect of quercetin and its metabolites isorhamnetin and quercetin-3-glucuronide on inflammatory gene expression: role of miR-155', *The Journal of nutritional biochemistry*, 22(3), pp. 293–299.
Buñay, J. *et al.* (2020) 'Screening for Liver X Receptor modulators: where are we and for what use?', *British Journal of Pharmacology*, 178(16), pp. 3277–3293.

Cao, G. *et al.* (2003) 'Antidiabetic action of a liver x receptor agonist mediated by inhibition of hepatic gluconeogenesis', *Journal of Biological Chemistry*, 278(2), pp. 1131–1136.
Carlberg, C. and Seuter, S. (2010) 'Dynamics of nuclear receptor target gene regulation', *Chromosoma*, 119(5), pp. 479–484.
Chalet, C. *et al.* (2018) 'Intestinal disposition of quercetin and its phase-II metabolites after oral administration in healthy volunteers', *Journal of Pharmacy and Pharmacology*, 70(8), pp. 1002–1008. doi: 10.1111/jphp.12929.
Chan, K. K. L. *et al.* (2018) 'Estrogen receptor modulators genistein, daidzein and ERB-041 inhibit cell migration, invasion, proliferation and sphere formation via modulation of FAK and PI3K/AKT signaling in ovarian cancer', *Cancer cell international*, 18(1), pp. 1–14.
Chen, J.-H. *et al.* (2013) 'Hibiscus sabdariffa leaf polyphenolic extract inhibits LDL oxidation and foam cell formation involving up-regulation of LXRα/ABCA1 pathway', *Food chemistry*, 141(1), pp. 397–406.
Chen, L. *et al.* (2018) 'Inflammatory responses and inflammation-associated diseases in organs', *Oncotarget*, 9(6), p. 7204.
Chen, X. *et al.* (2005) 'Pharmacokinetics and modeling of quercetin and metabolites', *Pharmaceutical research*, 22(6), pp. 892–901.
Chen, Xuanjing *et al.* (2020) 'Hesperetin inhibits foam cell formation in macrophages via activating LXRα signal in an AMPK dependent manner', *bioRxiv*.
Chen, Xuanjing *et al.* (2021) 'Hesperetin inhibits foam cell formation and promotes cholesterol efflux in THP-1-derived macrophages by activating LXRα signal in an AMPK-dependent manner', *Journal of physiology and biochemistry*, 77(3), pp. 405–417.
Cheng, S.-C. *et al.* (2019) 'Quercetin inhibits the production of IL-1β-induced inflammatory cytokines and chemokines in ARPE-19 cells via the MAPK and NF-κB signaling pathways', *International journal of molecular sciences*, 20(12), p. 2957.
Cho, Y. *et al.* (2016) 'Anti-inflammatory Potential of Quercetin-3-O-β-D-("2"-galloyl)-glucopyranoside and Quercetin Isolated from Diospyros kaki calyx via Suppression of MAP Signaling Molecules in LPS-induced RAW 264.7 Macrophages', *Journal of food science*, 81(10), pp. C2447–C2456.
Christian, F., Smith, E. L. and Carmody, R. J. (2016) 'The Regulation of NF-κB Subunits

by Phosphorylation', *Cells*, 5(1), p. 12. doi: 10.3390/cells5010012.
Cornut, M., Bourdonnay, E. and Henry, T. (2020) 'Transcriptional Regulation of Inflammasomes', *International Journal of Molecular Sciences*, 21(21), p. 8087.
Cory, H. *et al.* (2018) 'The role of polyphenols in human health and food systems: A mini-review', *Frontiers in nutrition*, 5, p. 87.
Cosme, P. *et al.* (2020) 'Plant phenolics: Bioavailability as a key determinant of their potential health-promoting applications', *Antioxidants*, 9(12), p. 1263.
D'Archivio, M. *et al.* (2010) 'Bioavailability of the polyphenols: status and controversies', *International journal of molecular sciences*, 11(4), pp. 1321–1342.
Dabeek, W. M. and Marra, M. V (2019) 'Dietary Quercetin and Kaempferol: Bioavailability and Potential Cardiovascular-Related Bioactivity in Humans', *Nutrients* . doi: 10.3390/nu11102288.
Dauchet, L., Amouyel, P. and Dallongeville, J. (2005) 'Fruit and vegetable consumption and risk of stroke: a meta-analysis of cohort studies', *Neurology*, 65(8), pp. 1193–1197.
David, A. V. A., Arulmoli, R. and Parasuraman, S. (2016) 'Overviews of biological importance of quercetin: A bioactive flavonoid', *Pharmacognosy reviews*, 10(20), p. 84.
Day, A. J. *et al.* (2001) 'Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin', *Free radical research*, 35(6), pp. 941–952.
Delfosse, V. *et al.* (2015) 'A structural perspective on nuclear receptors as targets of environmental compounds', *Acta Pharmacologica Sinica*, 36(1), pp. 88–101.
Egert, S. *et al.* (2008) 'Daily quercetin supplementation dose-dependently increases plasma quercetin concentrations in healthy humans', *The Journal of nutrition*, 138(9), pp. 1615–1621.
Endo-Umeda, K. *et al.* (2018) 'Liver X receptors regulate hepatic F4/80+CD11b+ Kupffer cells/macrophages and innate immune responses in mice', *Scientific Reports*, 8(1), p. 9281. doi: 10.1038/s41598-018-27615-7.
Fessler, M. B. (2018) 'The challenges and promise of targeting the Liver X Receptors for treatment of inflammatory disease', *Pharmacology & therapeutics*, 181, pp. 1–12.
Fiorani, M. *et al.* (2010) 'Mitochondria accumulate large amounts of quercetin: prevention of mitochondrial damage and release upon oxidation of the extramitochondrial fraction of the flavonoid', *The Journal of nutritional biochemistry*, 21(5), pp. 397–404.

Fouache, A. *et al.* (2019) ‘Flavonoids differentially modulate liver X receptors activity—Structure-function relationship analysis’, *The Journal of steroid biochemistry and molecular biology*, 190, pp. 173–182.
Francisco, V. *et al.* (2016) ‘The flavone luteolin inhibits liver X receptor activation’, *Journal of natural products*, 79(5), pp. 1423–1428.
Freire, M. O. and Van Dyke, T. E. (2013) ‘Natural resolution of inflammation’, *Periodontology 2000*, 63(1), pp. 149–164.
Furman, D. *et al.* (2019) ‘Chronic inflammation in the etiology of disease across the life span’, *Nature medicine*. 2019/12/05, 25(12), pp. 1822–1832. doi: 10.1038/s41591-019-0675-0.
Gafencu, A. V *et al.* (2007) ‘Inflammatory signaling pathways regulating ApoE gene expression in macrophages’, *Journal of Biological Chemistry*, 282(30), pp. 21776–21785.
Ghosh, D. and Scheepens, A. (2009) ‘Vascular action of polyphenols’, *Molecular nutrition & food research*, 53(3), pp. 322–331.
Giridharan, S. and Srinivasan, M. (2018) ‘Mechanisms of NF-κB p65 and strategies for therapeutic manipulation’, *Journal of inflammation research*, 11, p. 407.
Goldwasser, J. *et al.* (2010) ‘Transcriptional regulation of human and rat hepatic lipid metabolism by the grapefruit flavonoid naringenin: role of PPARα, PPARγ and LXRα’, *PloS one*, 5(8), p. e12399.
Granado-Serrano, A. B. *et al.* (2010) ‘Quercetin modulates NF-κ B and AP-1/JNK pathways to induce cell death in human hepatoma cells’, *Nutrition and cancer*, 62(3), pp. 390–401.
Granado-Serrano, A. B. *et al.* (2012) ‘Quercetin attenuates TNF-induced inflammation in hepatic cells by inhibiting the NF-κB pathway’, *Nutrition and cancer*, 64(4), pp. 588–598.
Griffett, K. and Burris, T. P. (2016) ‘Promiscuous activity of the LXR antagonist GSK2033 in a mouse model of fatty liver disease’, *Biochemical and biophysical research communications*, 479(3), pp. 424–428.
Guo, Y., Mah, E. and Bruno, R. S. (2014) ‘Quercetin bioavailability is associated with inadequate plasma vitamin C status and greater plasma endotoxin in adults’, *Nutrition*, 30(11–12), pp. 1279–1286.
Gutiérrez-Ruiz, M. C. *et al.* (1999) ‘Cytokines, growth factors, and oxidative stress in

HepG2 cells treated with ethanol, acetaldehyde, and LPS', *Toxicology*, 134(2–3), pp. 197–207.
Hai, Y. *et al.* (2020) 'Advance on the absorption, metabolism, and efficacy exertion of quercetin and its important derivatives: Absorption, metabolism and function of quercetin', *Food Frontiers*, 1(4), pp. 420–434.
Hall, J. M. *et al.* (2019) 'The Role of Dietary Phytoestrogens and the Nuclear Receptor PPAR γ in Adipogenesis: An in Vitro Study', *Environmental health perspectives*, 127(3), p. 37007.
He, P., Gelissen, I. C. and Ammit, A. J. (2020) 'Regulation of ATP binding cassette transporter A1 (ABCA1) expression: cholesterol-dependent and – independent signaling pathways with relevance to inflammatory lung disease', *Respiratory Research*, 21(1), p. 250. doi: 10.1186/s12931-020-01515-9.
Hershko, D. D. *et al.* (2002) 'Multiple transcription factors regulating the IL-6 gene are activated by cAMP in cultured Caco-2 cells', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 283(5), pp. R1140–R1148.
Houck, K. A. *et al.* (2004) 'T0901317 is a dual LXR/FXR agonist', *Molecular Genetics and Metabolism*, 83(1), pp. 184–187. doi: https://doi.org/10.1016/j.ymgme.2004.07.007.
Huang, P. *et al.* (2018) 'Liver X receptor inverse agonist SR9243 suppresses nonalcoholic steatohepatitis intrahepatic inflammation and fibrosis', *BioMed research international*, 2018.
Huang, W. and Glass, C. K. (2010) 'Nuclear receptors and inflammation control: molecular mechanisms and pathophysiological relevance', *Arteriosclerosis, thrombosis, and vascular biology*, 30(8), pp. 1542–1549.
Hussain, T. *et al.* (2016) 'Oxidative stress and inflammation: what polyphenols can do for us?', *Oxidative medicine and cellular longevity*, 2016.
Hutchinson, S. A., Lianto, P., Roberg-Larsen, H., *et al.* (2019) 'ER-Negative breast cancer is highly responsive to cholesterol metabolite signalling', *Nutrients*, 11(11), p. 2618.
Hutchinson, S. A., Lianto, P., Moore, J. B., *et al.* (2019) 'Phytosterols inhibit side-chain oxysterol mediated activation of LXR in breast cancer cells', *International journal of molecular sciences*, 20(13), p. 3241.
Hutchinson, S. A. *et al.* (2021) 'Liver x receptor alpha drives chemoresistance in response

to side-chain hydroxycholesterols in triple negative breast cancer', *Oncogene*, 40(16), pp. 2872–2883. doi: 10.1038/s41388-021-01720-w.

Hutchinson, S. A. and Thorne, J. L. (2019) 'A Stable Luciferase Reporter System to Characterize LXR Regulation by Oxysterols and Novel Ligands', in *Lipid-Activated Nuclear Receptors*. Springer, pp. 15–32.

Ignatova, I. D. *et al.* (2013) 'Differential regulation of gene expression by LXRs in response to macrophage cholesterol loading', *Molecular endocrinology*, 27(7), pp. 1036–1047.

Iio, A. *et al.* (2012) 'Hesperetin upregulates ABCA1 expression and promotes cholesterol efflux from THP-1 macrophages', *Journal of natural products*, 75(4), pp. 563–566.

Im, S.-S. and Osborne, T. F. (2011) 'Liver x receptors in atherosclerosis and inflammation', *Circulation research*, 108(8), pp. 996–1001.

ISO, I. S. O. (2009) '10993–5: 2009 Biological evaluation of medical devices—part 5: tests for in vitro cytotoxicity', *International Organization for Standardization, Geneva*.

Ito, A. *et al.* (2015) 'LXRs link metabolism to inflammation through Abca1-dependent regulation of membrane composition and TLR signaling', *elife*, 4, p. e08009.

Janowski, B. A. *et al.* (1999) 'Structural requirements of ligands for the oxysterol liver X receptors LXRα and LXRβ', *Proceedings of the National Academy of Sciences*, 96(1), pp. 266–271.

Jia, Y. *et al.* (2013) 'Cyanidin, a natural flavonoid, is an agonistic ligand for liver X receptor alpha and beta and reduces cellular lipid accumulation in macrophages and hepatocytes', *Bioorganic & medicinal chemistry letters*, 23(14), pp. 4185–4190.

Joseph, S. B. *et al.* (2003) 'Reciprocal regulation of inflammation and lipid metabolism by liver X receptors', *Nature medicine*, 9(2), pp. 213–219.

Kanmani, P. and Kim, H. (2018) 'Protective Effects of Lactic Acid Bacteria Against TLR4 Induced Inflammatory Response in Hepatoma HepG2 Cells Through Modulation of Toll-Like Receptor Negative Regulators of Mitogen-Activated Protein Kinase and NF-κB Signaling ', *Frontiers in Immunology* , p. 1537. Available at: https://www.frontiersin.org/article/10.3389/fimmu.2018.01537.

Kaplan, R. *et al.* (2002) 'Bacterial lipopolysaccharide induces expression of ABCA1 but not ABCG1 via an LXR-independent pathway', *Journal of lipid research*, 43(6), pp. 952–

959.
Kaşıkcı, M. B. and Bağdatlıoğlu, N. (2016) 'Bioavailability of quercetin', *Current research in nutrition and food science journal*, 4(Special Issue Nutrition in Conference October 2016), pp. 146–151.
Kel, A. E. *et al.* (2003) 'MATCHTM: a tool for searching transcription factor binding sites in DNA sequences', *Nucleic Acids Research*, 31(13), pp. 3576–3579. doi: 10.1093/nar/gkg585.
Keller, A.-A. *et al.* (2018) 'Transfecting macrophages', in *Macrophages*. Springer, pp. 187–195.
Kidani, Y. and Bensinger, S. J. (2012) 'Liver X receptor and peroxisome proliferator-activated receptor as integrators of lipid homeostasis and immunity', *Immunological reviews*, 249(1), pp. 72–83.
Kim, H.-S., Quon, M. J. and Kim, J. (2014) 'New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate', *Redox biology*, 2, pp. 187–195.
Klepsch, V. *et al.* (2019) 'Nuclear Receptors Regulate Intestinal Inflammation in the Context of IBD ', *Frontiers in Immunology* , p. 1070. Available at: https://www.frontiersin.org/article/10.3389/fimmu.2019.01070.
Knekt, P. *et al.* (2002) 'Flavonoid intake and risk of chronic diseases', *The American journal of clinical nutrition*, 76(3), pp. 560–568.
Komati, R. *et al.* (2017) 'Ligands of therapeutic utility for the liver X receptors', *Molecules*, 22(1), p. 88.
Kwak, J.-H. *et al.* (2017) 'Variation of quercetin glycoside derivatives in three onion (Allium cepa L.) varieties', *Saudi journal of biological sciences*. 2016/05/27, 24(6), pp. 1387–1391. doi: 10.1016/j.sjbs.2016.05.014.
Laffitte, B. A. *et al.* (2001) 'LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes', *Proceedings of the National Academy of Sciences*, 98(2), pp. 507–512.
Laffitte, B. A. *et al.* (2003) 'Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue', *Proceedings of the National Academy of Sciences of the United States of America*.

2003/04/15, 100(9), pp. 5419–5424. doi: 10.1073/pnas.0830671100.
Lavin, Y. *et al.* (2015) 'Regulation of macrophage development and function in peripheral tissues', *Nature Reviews Immunology*, 15(12), pp. 731–744. doi: 10.1038/nri3920.
Lawrence, T. (2009) 'The nuclear factor NF-κB pathway in inflammation', *Cold Spring Harbor perspectives in biology*, 1(6), p. a001651.
Lee, H. N. *et al.* (2018) 'Anti-inflammatory effect of quercetin and galangin in LPS-stimulated RAW264. 7 macrophages and DNCB-induced atopic dermatitis animal models', *International journal of molecular medicine*, 41(2), pp. 888–898.
Lee, S.-M. *et al.* (2013) 'Quercetin up-regulates expressions of peroxisome proliferator-activated receptor γ, liver X receptor α, and ATP binding cassette transporter A1 genes and increases cholesterol efflux in human macrophage cell line', *Nutrition Research*, 33(2), pp. 136–143. doi: https://doi.org/10.1016/j.nutres.2012.11.010.
Leopold Wager, C. M., Arnett, E. and Schlesinger, L. S. (2019) 'Macrophage nuclear receptors: Emerging key players in infectious diseases', *PLoS pathogens*, 15(3), p. e1007585.
Leri, M. *et al.* (2020) 'Healthy Effects of Plant Polyphenols: Molecular Mechanisms', *International journal of molecular sciences*, 21(4), p. 1250. doi: 10.3390/ijms21041250.
Lesjak, M. *et al.* (2018) 'Antioxidant and anti-inflammatory activities of quercetin and its derivatives', *Journal of Functional Foods*, 40, pp. 68–75.
Lesser, S., Cermak, R. and Wolffram, S. (2004) 'Bioavailability of quercetin in pigs is influenced by the dietary fat content', *The Journal of nutrition*, 134(6), pp. 1508–1511.
Levenson, A. S. *et al.* (2003) 'Resveratrol acts as an estrogen receptor (ER) agonist in breast cancer cells stably transfected with ER α', *International journal of cancer*, 104(5), pp. 587–596.
Lewkowicz, N. *et al.* (2016) 'Induction of human IL-10-producing neutrophils by LPS-stimulated Treg cells and IL-10', *Mucosal immunology*, 9(2), pp. 364–378.
Leyva-López, N. *et al.* (2016) 'Protective role of terpenes and polyphenols from three species of Oregano (Lippia graveolens, Lippia palmeri and Hedeoma patens) on the suppression of lipopolysaccharide-induced inflammation in RAW 264.7 macrophage cells', *Journal of Ethnopharmacology*, 187, pp. 302–312.
Li, T. *et al.* (2019) 'Synergistic anti-inflammatory effects of quercetin and catechin via

inhibiting activation of TLR4–MyD88-mediated NF-κB and MAPK signaling pathways', *Phytotherapy Research*, 33(3), pp. 756–767.

Li, Y. *et al.* (2016) 'Quercetin, inflammation and immunity', *Nutrients*, 8(3), p. 167.

Liao, M.-H. *et al.* (2014) 'Genistein induces oestrogen receptor-α gene expression in osteoblasts through the activation of mitogen-activated protein kinases/NF-κB/activator protein-1 and promotes cell mineralisation', *British Journal of Nutrition*. 2013/07/05, 111(1), pp. 55–63. doi: DOI: 10.1017/S0007114513002043.

Liao, Y.-R. and Lin, J.-Y. (2020) 'Quercetin Modulates Cytokine Expression and Inhibits TLR2 Expression and STAT3 Activation in Mouse Activated Inflammatory Macrophages', *Journal of Exploratory Research in Pharmacology*, 5(3), pp. 31–41.

Liu, C.-M. *et al.* (2015) 'Quercetin protects mouse liver against nickel-induced DNA methylation and inflammation associated with the Nrf2/HO-1 and p38/STAT1/NF-κB pathway', *Food and Chemical Toxicology*, 82, pp. 19–26.

Liu, Y. and Tang, C. (2012) 'Regulation of ABCA1 functions by signaling pathways', *Biochimica et biophysica acta*. 2011/09/05, 1821(3), pp. 522–529. doi: 10.1016/j.bbalip.2011.08.015.

Liu, Z. and Hu, M. (2007) 'Natural polyphenol disposition via coupled metabolic pathways', *Expert opinion on drug metabolism & toxicology*, 3(3), pp. 389–406.

Lotito, S. B. *et al.* (2011) 'Metabolic conversion of dietary flavonoids alters their anti-inflammatory and antioxidant properties', *Free Radical Biology and Medicine*, 51(2), pp. 454–463.

Luca, S. V. *et al.* (2020) 'Bioactivity of dietary polyphenols: The role of metabolites', *Critical reviews in food science and nutrition*, 60(4), pp. 626–659.

Lund, E. G. *et al.* (2006) 'Different roles of liver X receptor α and β in lipid metabolism: Effects of an α-selective and a dual agonist in mice deficient in each subtype', *Biochemical pharmacology*, 71(4), pp. 453–463.

Lund, E. G., Menke, J. G. and Sparrow, C. P. (2003) 'Liver X receptor agonists as potential therapeutic agents for dyslipidemia and atherosclerosis', *Arteriosclerosis, thrombosis, and vascular biology*, 23(7), pp. 1169–1177.

Maskrey, B. H. *et al.* (2011) 'Mechanisms of resolution of inflammation: a focus on cardiovascular disease', *Arteriosclerosis, thrombosis, and vascular biology*, 31(5), pp.

1001–1006.
Matys, V. *et al.* (2006) 'TRANSFAC® and its module TRANSCompel®: transcriptional gene regulation in eukaryotes', *Nucleic acids research*, 34(suppl_1), pp. D108–D110.
Mitro, N., Vargas, L., *et al.* (2007) 'T0901317 is a potent PXR ligand: Implications for the biology ascribed to LXR', *FEBS Letters*, 581(9), pp. 1721–1726. doi: https://doi.org/10.1016/j.febslet.2007.03.047.
Mitro, N., Mak, P. A., *et al.* (2007) 'The nuclear receptor LXR is a glucose sensor', *Nature*, 445(7124), pp. 219–223.
Morello, F. *et al.* (2009) 'LXR-activating oxysterols induce the expression of inflammatory markers in endothelial cells through LXR-independent mechanisms', *Atherosclerosis*, 207(1), pp. 38–44.
Murota, K. *et al.* (2007) 'Antioxidant capacity of albumin-bound quercetin metabolites after onion consumption in humans', *The Journal of Medical Investigation*, 54(3, 4), pp. 370–374.
Murota, K. and Terao, J. (2003) 'Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism', *Archives of biochemistry and biophysics*, 417(1), pp. 12–17.
Neta, R., Sayers, T. J. and Oppenheim, J. J. (1992) 'Relationship of TNF to interleukins.', *Immunology series*, 56, pp. 499–566.
Ni, M. *et al.* (2019) 'Biological mechanisms and related natural modulators of liver X receptor in nonalcoholic fatty liver disease', *Biomedicine & Pharmacotherapy*, 113, p. 108778.
Nifli, A.-P. *et al.* (2007) 'Quercetin exhibits a specific fluorescence in cellular milieu: a valuable tool for the study of its intracellular distribution', *Journal of agricultural and food chemistry*, 55(8), pp. 2873–2878.
Niinivehmas, S. P. *et al.* (2016) 'Identification of estrogen receptor α ligands with virtual screening techniques', *Journal of Molecular Graphics and Modelling*, 64, pp. 30–39. doi: https://doi.org/10.1016/j.jmgm.2015.12.006.
Nikolenko, J. V and Krasnov, A. N. (2007) 'Nuclear receptors: Structure and mechanisms of action', *Russian Journal of Genetics*, 43(3), pp. 234–240. doi: 10.1134/S1022795407030027.

Noelia, A. and Castrillo, A. (2011) 'Liver X receptors as regulators of macrophage inflammatory and metabolic pathways', *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1812(8), pp. 982–994.

Notas, G. *et al.* (2012) 'Quercetin accumulates in nuclear structures and triggers specific gene expression in epithelial cells', *The Journal of nutritional biochemistry*, 23(6), pp. 656–666.

Nunomura, S. *et al.* (2015) 'Activation of LXRs using the synthetic agonist GW3965 represses the production of pro-inflammatory cytokines by murine mast cells', *Allergology International*, 64, pp. S11–S17. doi: https://doi.org/10.1016/j.alit.2015.03.001.

O'Leary, K. A. *et al.* (2003) 'Metabolism of quercetin-7-and quercetin-3-glucuronides by an in vitro hepatic model: the role of human β-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism', *Biochemical pharmacology*, 65(3), pp. 479–491.

Ohara, K. *et al.* (2013) 'Quercetin-3-O-glucuronide induces ABCA1 expression by LXRα activation in murine macrophages', *Biochemical and biophysical research communications*, 441(4), pp. 929–934.

Okamoto, T. (2005) 'Safety of quercetin for clinical application', *International journal of molecular medicine*, 16(2), pp. 275–278.

Olefsky, J. M. (2001) 'Nuclear receptor minireview series', *Journal of Biological Chemistry*, 276(40), pp. 36863–36864.

Olkkonen, V. M., Béaslas, O. and Nissilä, E. (2012) 'Oxysterols and their cellular effectors', *Biomolecules*, 2(1), pp. 76–103. doi: 10.3390/biom2010076.

Olthof, M. R. *et al.* (2000) 'Bioavailabilities of Quercetin-3-Glucoside and Quercetin-4′-Glucoside Do Not Differ in Humans', *The Journal of Nutrition*, 130(5), pp. 1200–1203. doi: 10.1093/jn/130.5.1200.

Ory, D. S. (2004) 'Nuclear receptor signaling in the control of cholesterol homeostasis: have the orphans found a home?', *Circulation research*, 95(7), pp. 660–670.

Ou, Q. *et al.* (2020) 'Impact of quercetin on systemic levels of inflammation: A meta-analysis of randomised controlled human trials', *International journal of food sciences and nutrition*, 71(2), pp. 152–163.

Panahi, G. *et al.* (2018) 'High glucose induces inflammatory responses in HepG2 cells

via the oxidative stress-mediated activation of NF-κB, and MAPK pathways in HepG2 cells', *Archives of physiology and biochemistry*, 124(5), pp. 468–474.

Pandey, K. B. and Rizvi, S. I. (2009) 'Plant polyphenols as dietary antioxidants in human health and disease', *Oxidative medicine and cellular longevity*, 2(5), pp. 270–278. doi: 10.4161/oxim.2.5.9498.

Peng, D. *et al.* (2011) 'A novel potent synthetic steroidal liver X receptor agonist lowers plasma cholesterol and triglycerides and reduces atherosclerosis in LDLR(-/-) mice', *British journal of pharmacology*, 162(8), pp. 1792–1804. doi: 10.1111/j.1476-5381.2011.01202.x.

Pengal, R. A. *et al.* (2006) 'Lipopolysaccharide-induced production of interleukin-10 is promoted by the serine/threonine kinase Akt', *Molecular Immunology*, 43(10), pp. 1557–1564. doi: https://doi.org/10.1016/j.molimm.2005.09.022.

Petersen, B. *et al.* (2016) 'Bioavailability of quercetin in humans and the influence of food matrix comparing quercetin capsules and different apple sources', *Food Research International*, 88, pp. 159–165.

Platanitis, E. and Decker, T. (2018) 'Regulatory networks involving STATs, IRFs, and NFκB in inflammation', *Frontiers in immunology*, 9, p. 2542.

Porter, B. A. *et al.* (2019) 'Structure and Function of the Nuclear Receptor Superfamily and Current Targeted Therapies of Prostate Cancer', *Cancers*, 11(12), p. 1852. doi: 10.3390/cancers11121852.

Puranik, N. V *et al.* (2019) 'Determination and analysis of agonist and antagonist potential of naturally occurring flavonoids for estrogen receptor (ERα) by various parameters and molecular modelling approach', *Scientific reports*, 9(1), pp. 1–11.

Quinet, E. M. *et al.* (2006) 'Liver X receptor (LXR)-β regulation in LXRα-deficient mice: implications for therapeutic targeting', *Molecular pharmacology*, 70(4), pp. 1340–1349.

Robinson, M. W., Harmon, C. and O'Farrelly, C. (2016) 'Liver immunology and its role in inflammation and homeostasis', *Cellular & molecular immunology*, 13(3), pp. 267–276.

Saenz, J. *et al.* (2017) 'Grapefruit flavonoid Naringenin regulates the expression of LXRα in THP-1 macrophages by modulating AMP-activated protein kinase', *Molecular pharmaceutics*, 15(5), pp. 1735–1745.

Saha, S. *et al.* (2020) 'An overview of Nrf2 signaling pathway and its role in inflammation',

Molecules, 25(22), p. 5474.
Scholz, H. *et al.* (2009) 'The synthetic liver X receptor agonist GW3965 reduces tissue factor production and inflammatory responses in human islets in vitro', *Diabetologia*, 52(7), pp. 1352–1362.
Schroepfer Jr, G. J. (2000) 'Oxysterols: modulators of cholesterol metabolism and other processes', *Physiological reviews*.
Schulman, I. G. (2017) 'Liver X receptors link lipid metabolism and inflammation', *FEBS letters*, 591(19), pp. 2978–2991.
Schultz, J. R. *et al.* (2000) 'Role of LXRs in control of lipogenesis', *Genes & development*, 14(22), pp. 2831–2838.
Sever, R. and Glass, C. K. (2013) 'Signaling by nuclear receptors', *Cold Spring Harbor perspectives in biology*, 5(3), pp. a016709–a016709. doi: 10.1101/cshperspect.a016709.
Shirinsky, I. V and Shirinsky, V. S. (2011) 'Targeting Nuclear Hormone Receptors: PPARα Agonists as Potential Disease-Modifying Drugs for Rheumatoid Arthritis', *International journal of rheumatology*. 2011/06/21, 2011, p. 937843. doi: 10.1155/2011/937843.
Sohrabi, Y. *et al.* (2020) 'LXR activation induces a proinflammatory trained innate immunity-phenotype in human monocytes', *Frontiers in immunology*, 11, p. 353.
Song, J. *et al.* (2022) 'Polyphenols extracted from Shanxi-aged vinegar exert hypolipidemic effects on OA-induced HepG2 cells via the PPARα-LXRα-ABCA1 pathway', *Journal of Food Biochemistry*, p. e14029.
Song, S. B. *et al.* (2012) 'Inhibition of TNF-α-mediated NF-κB Transcriptional Activity in HepG2 Cells by Dammarane-type Saponins from Panax ginseng Leaves', *Journal of ginseng research*, 36(2), pp. 146–152. doi: 10.5142/jgr.2012.36.2.146.
De Stefano, D. *et al.* (2007) 'Lycopene, quercetin and tyrosol prevent macrophage activation induced by gliadin and IFN-γ', *European journal of pharmacology*, 566(1–3), pp. 192–199.
Steinmetz, A. C. U., Renaud, J.-P. and Moras, D. (2001) 'Binding of ligands and activation of transcription by nuclear receptors', *Annual review of biophysics and biomolecular structure*, 30(1), pp. 329–359.
Stonans, I. *et al.* (1999) 'HepG2 human hepatoma cells express multiple cytokine genes', *Cytokine*, 11(2), pp. 151–156.

Sugimoto, M. A. *et al.* (2016) 'Resolution of inflammation: what controls its onset?', *Frontiers in immunology*, 7, p. 160.

Sul, O.-J. and Ra, S. W. (2021) 'Quercetin Prevents LPS-Induced Oxidative Stress and Inflammation by Modulating NOX2/ROS/NF-kB in Lung Epithelial Cells', *Molecules*, 26(22), p. 6949.

Sun, G. Y. *et al.* (2015) 'Quercetin attenuates inflammatory responses in BV-2 microglial cells: role of MAPKs on the Nrf2 pathway and induction of heme oxygenase-1', *PloS one*, 10(10), p. e0141509.

Suri, S. *et al.* (2008) 'A comparative study of the effects of quercetin and its glucuronide and sulfate metabolites on human neutrophil function in vitro', *Biochemical Pharmacology*, 76(5), pp. 645–653.

Takeuchi, O. and Akira, S. (2010) 'Pattern Recognition Receptors and Inflammation', *Cell*, 140(6), pp. 805–820. doi: https://doi.org/10.1016/j.cell.2010.01.022.

Tanaka, M. *et al.* (2020) 'Gallic acid inhibits lipid accumulation via AMPK pathway and suppresses apoptosis and macrophage-mediated inflammation in hepatocytes', *Nutrients*, 12(5), p. 1479.

Tenbaum, S. and Baniahmad, A. (1997) 'Nuclear receptors: Structure, function and involvement in disease', *The International Journal of Biochemistry & Cell Biology*, 29(12), pp. 1325–1341. doi: https://doi.org/10.1016/S1357-2725(97)00087-3.

Thomas, D. G. *et al.* (2018) 'LXR Suppresses Inflammatory Gene Expression and Neutrophil Migration through cis-Repression and Cholesterol Efflux', *Cell reports*, 25(13), pp. 3774-3785.e4. doi: 10.1016/j.celrep.2018.11.100.

Timm, M. *et al.* (2013) 'Considerations regarding use of solvents in in vitro cell based assays', *Cytotechnology*, 65(5), pp. 887–894.

Toporova, L. *et al.* (2020) 'Assessing the selectivity of FXR, LXRs, CAR, and RORγ pharmaceutical ligands with reporter cell lines', *Frontiers in Pharmacology*, 11, p. 1122.

Treede, I. *et al.* (2009) 'TNF-α-induced up-regulation of pro-inflammatory cytokines is reduced by phosphatidylcholine in intestinal epithelial cells', *BMC Gastroenterology*, 9(1), p. 53. doi: 10.1186/1471-230X-9-53.

Tribolo, S. *et al.* (2008) 'Comparative effects of quercetin and its predominant human metabolites on adhesion molecule expression in activated human vascular endothelial

cells', *Atherosclerosis*, 197(1), pp. 50–56.

Tsao, R. (2010) 'Chemistry and biochemistry of dietary polyphenols', *Nutrients*. 2010/12/10, 2(12), pp. 1231–1246. doi: 10.3390/nu2121231.

Ulusoy, H. G. and Sanlier, N. (2020) 'A minireview of quercetin: from its metabolism to possible mechanisms of its biological activities', *Critical reviews in food science and nutrition*, 60(19), pp. 3290–3303.

Verna, G. *et al.* (2021) 'Quercetin administration suppresses the cytokine storm in myeloid and plasmacytoid dendritic cells', *International journal of molecular sciences*, 22(15), p. 8349.

Viennois, E. *et al.* (2011) 'Targeting liver X receptors in human health: deadlock or promising trail?', *Expert opinion on therapeutic targets*, 15(2), pp. 219–232.

Viennois, E. *et al.* (2012) 'Selective liver X receptor modulators (SLiMs): what use in human health?', *Molecular and cellular endocrinology*, 351(2), pp. 129–141.

Walle, T., Vincent, T. S. and Walle, U. K. (2003) 'Evidence of covalent binding of the dietary flavonoid quercetin to DNA and protein in human intestinal and hepatic cells', *Biochemical pharmacology*, 65(10), pp. 1603–1610.

Walle, T., Walle, U. K. and Halushka, P. V (2001) 'Carbon dioxide is the major metabolite of quercetin in humans', *The Journal of nutrition*, 131(10), pp. 2648–2652.

Wang, K. and Wan, Y.-J. Y. (2008) 'Nuclear receptors and inflammatory diseases', *Experimental Biology and Medicine*, 233(5), pp. 496–506.

Wang, X. *et al.* (2018) 'In silico identification of small molecules as novel LXR agonists for the treatment of cardiovascular disease and cancer', *Journal of molecular modeling*, 24(3), pp. 1–14.

Watanabe, Y. *et al.* (2005) 'Expression of the LXRα protein in human atherosclerotic lesions', *Arteriosclerosis, thrombosis, and vascular biology*, 25(3), pp. 622–627.

Weikum, E. R., Liu, X. and Ortlund, E. A. (2018) 'The nuclear receptor superfamily: A structural perspective', *Protein Science*, 27(11), pp. 1876–1892.

Wellington, C. L. *et al.* (2002) 'ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation', *Laboratory investigation*, 82(3), pp. 273–283.

Wingender, E. *et al.* (1996) 'TRANSFAC: a database on transcription factors and their

DNA binding sites', *Nucleic acids research*, 24(1), pp. 238–241.
Winterbone, M. S. *et al.* (2009) 'Physiologically relevant metabolites of quercetin have no effect on adhesion molecule or chemokine expression in human vascular smooth muscle cells', *Atherosclerosis*, 202(2), pp. 431–438.
Wójcicka, G. *et al.* (2007) 'Liver X receptors (LXRs). Part I: Structure, function, regulation of activity, and role in lipid metabolism Receptory wątrobowe X (LXR). Część I: Budowa, funkcja, regulacja aktywności i znaczenie w metabolizmie lipidów', *Postepy Hig Med Dosw.(online)*, 61, pp. 736–759.
Xiao, J. *et al.* (2017) 'Activation of liver X receptors promotes inflammatory cytokine mRNA degradation by upregulation of tristetraprolin', *Acta Biochimica et Biophysica Sinica*, 49(3), pp. 277–283. doi: 10.1093/abbs/gmw136.
Xu, D. *et al.* (2019) 'Antioxidant activities of quercetin and its complexes for medicinal application', *Molecules*, 24(6), p. 1123.
Xu, J. *et al.* (2015) 'Bilobol inhibits the lipopolysaccharide-induced expression and distribution of RhoA in HepG2 human hepatocellular carcinoma cells', *Oncol Lett*, 10(2), pp. 962–966. doi: 10.3892/ol.2015.3276.
Xue, F. *et al.* (2017) 'Quercetin inhibits LPS-induced inflammation and ox-LDL-induced lipid deposition', *Frontiers in pharmacology*, 8, p. 40.
Yao, Z. *et al.* (2019) 'Estimated daily quercetin intake and association with the prevalence of type 2 diabetes mellitus in Chinese adults', *European journal of nutrition*, 58(2), pp. 819–830.
Yu, X.-H. *et al.* (2013) 'Foam cells in atherosclerosis', *Clinica Chimica Acta*, 424, pp. 245–252. doi: https://doi.org/10.1016/j.cca.2013.06.006.
Zand, R. S. R., Jenkins, D. J. A. and Diamandis, E. P. (2000) 'Steroid hormone activity of flavonoids and related compounds', *Breast cancer research and treatment*, 62(1), pp. 35–49.
Zelcer, N. and Tontonoz, P. (2006) 'Liver X receptors as integrators of metabolic and inflammatory signaling', *The Journal of clinical investigation*, 116(3), pp. 607–614.
Zhang Hua, Y. *et al.* (2015) 'TIPE2 inhibits TNF-α-induced hepatocellular carcinoma cell metastasis via Erk1/2 downregulation and NF-κB activation', *Int J Oncol*, 46(1), pp. 254–264. doi: 10.3892/ijo.2014.2725.

Zhang, Y. *et al.* (2012) ‘Liver LXRα expression is crucial for whole body cholesterol homeostasis and reverse cholesterol transport in mice’, *The Journal of clinical investigation*, 122(5), pp. 1688–1699.

Zhao, C. and Dahlman-Wright, K. (2009) ‘Liver X receptor in cholesterol metabolism.’, *The Journal of endocrinology*, 204(3), pp. 233–240.

Zhou, P. *et al.* (2017) ‘Attenuation of TNF-α-induced inflammatory injury in endothelial cells by ginsenoside Rb1 via inhibiting NF-κB, JNK and p38 signaling pathways’, *Frontiers in Pharmacology*, 8, p. 464.

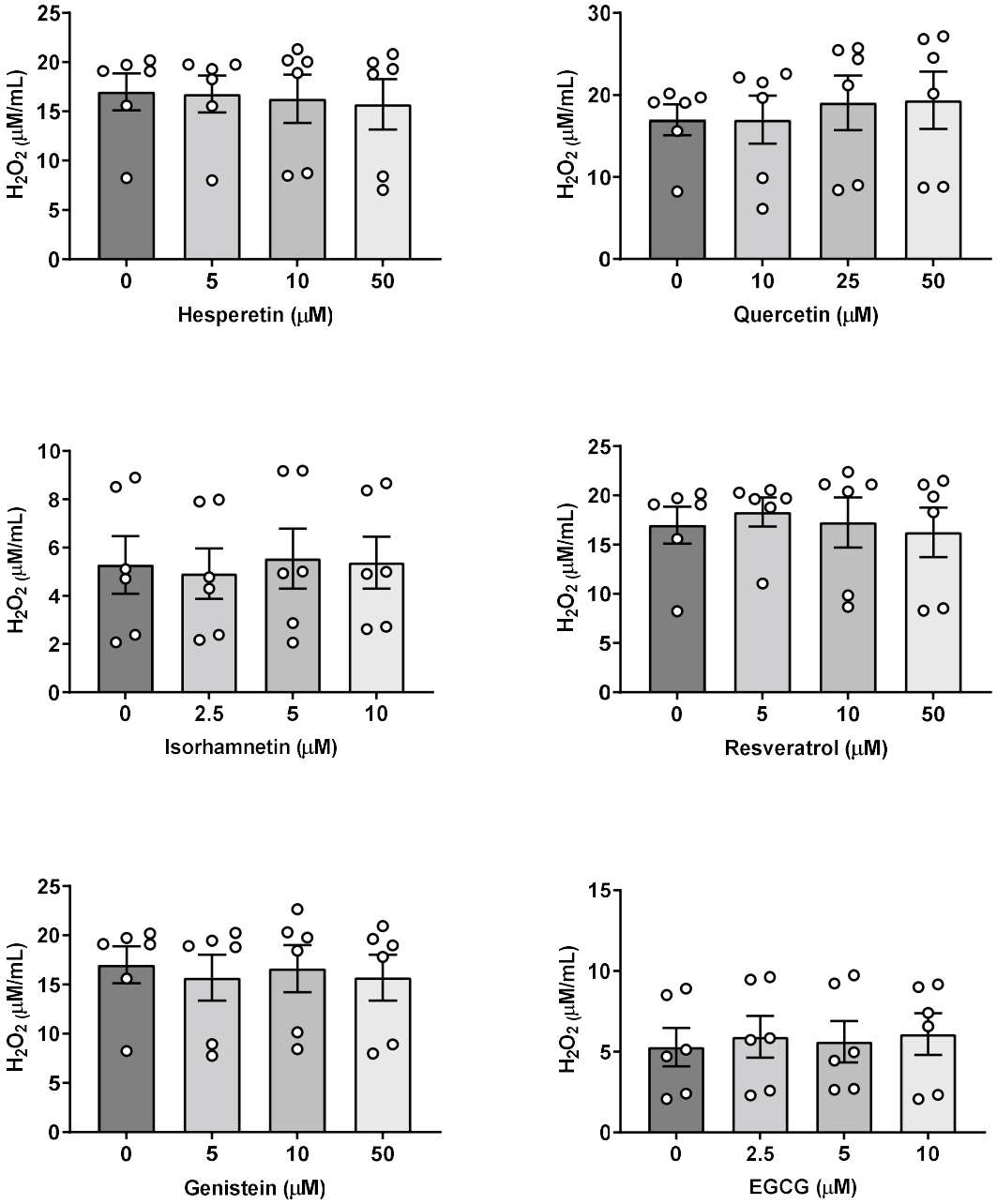
Zhou, R.-R. *et al.* (2011) ‘HMGB1 cytoplasmic translocation in patients with acute liver failure’, *BMC gastroenterology*, 11(1), pp. 1–11.

Zhu, R. *et al.* (2012) ‘Role of liver X receptors in cholesterol efflux and inflammatory signaling (review)’, *Molecular medicine reports*. 2012/01/17, 5(4), pp. 895–900. doi: 10.3892/mmr.2012.758.

Zhu, X. *et al.* (2011) ‘Activation of Sirt1 by Resveratrol Inhibits TNF-α Induced Inflammation in Fibroblasts’, *PLOS ONE*, 6(11), p. e27081. Available at: https://doi.org/10.1371/journal.pone.0027081.

Zuercher, W. J. *et al.* (2010) ‘Discovery of tertiary sulfonamides as potent liver X receptor antagonists’, *Journal of medicinal chemistry*, 53(8), pp. 3412–3416.

Appendix 1



Appendix 1. Hydrogen peroxide (H_2O_2) production following incubation with selected polyphenols to represent different subclasses of flavonoids. Data were normalized to the amount of cell protein and fold change was calculated relative to vehicle control. Data are mean with SEM from three independent experiments performed in duplicate. *, **, and ***, indicate significant differences to the control group (Dunnett's t-test), $p<0.05$, $p<0.01$, and $p<0.001$ respectively.