



Faculty of Medicine and Health Sciences

Investigating the Preventive Effects of *Tinospora crispa* and *Tinospora cordifolia* on Methylglyoxal-induced Cytotoxicity and Metabolic Dysregulation in Osteoblast Cells

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Investigating the Preventive Effects of *Tinospora crispa* and *Tinospora cordifolia* on Methylglyoxal-induced Cytotoxicity and Metabolic Dysregulation in Osteoblast Cells

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Patients with type 2 mellitus (T2DM) typically exhibit significantly increased methylglyoxal (MGO) levels. MGO is a reactive carbonyl compound and a byproduct of glycolysis, serving as a precursor of advanced glycation end products (AGE). AGE enters cells via its receptor of advanced glycation end products (RAGE) leading to oxidative stress, inflammation and consequently death of the cells. In osteoblasts, high AGE level can induce inflammatory response and oxidative stress (OS), as well as reducing osteoblastogenesis. *Tinospora cordifolia* (*T. cordifolia*) and *Tinospora crispa* (*T. crispa*) have been traditionally used to treat diabetes. In modern medicine, metformin serves as the primary treatment to reduce hyperglycaemia when lifestyle changes have failed. Currently, little is known about the effects *T. cordifolia* and *T. crispa* on bone remodelling when bone cells are exposed to high MGO levels. In addition, there is no data available on the interaction of metformin with *T. crispa* and *T. cordifolia* extracts. Therefore, this study aimed to evaluate the effects of *T. crispa* and *T. cordifolia* on bone metabolism in MGO-stimulated osteoblast cells (hFOB1.19) and explore the potential synergistic interactions of these two plants with metformin. The plants underwent extraction using methanol and hot water (aqueous). The phytochemical content of the four extracts were determined using GC-MS, the antioxidant activities were assessed via DPPH assay while anti-glycation properties were assessed via BSA-MGO assay. Then, the effects of the extracts on MGO-stimulated osteoblast cells were assessed with or without metformin for antioxidant capacity, anti-apoptotic activity, and anti-inflammatory properties as well as the impact on the expression and activity of the osteoblastic insulin receptors. The results showed that the plant extracts contained antioxidant and anti-inflammatory bioactive compounds, with notable scavenging activity and anti-apoptotic effects. However, the extracts showed limited anti-glycation activity as

shown in BSA-MGO and AGE formation in MGO-stimulated osteoblasts. Furthermore, the extracts significantly reduced the secretion of IL-6 and IL-18 suggesting their anti-inflammatory properties. The extracts also increased the expression of Runx2 in influencing the osteoblastogenesis. In addition, they increased expression of GLUT4, and insulin receptor in MGO-stimulated hFOB1.19, suggesting positive effects on diabetic bone metabolism. In conclusion, this study highlights the potential of *T. cordifolia* and *T. crispa* extracts in mitigating T2DM-related complications, particularly bone loss.

Keywords: *T. cordifolia*, *T. crispa*, Type 2 diabetes mellitus, bone metabolism

**Menyelidik Kesan Pencegahan *Tinospora crispa* dan *Tinospora cordifolia* Terhadap
Sitotoksiti dan Disfungsi Metabolik yang Disebabkan oleh Metilglioksal dalam Sel
Osteoblas**

ABSTRAK

Pesakit diabetes mellitus jenis 2 (T2DM) biasanya menunjukkan peningkatan yang ketara dalam tahap metilglioksal (MGO). MGO adalah sebatian karbonil reaktif dan produk sampingan glikolisis, yang berperanan sebagai pendahulu produk akhir glikasi lanjutan (AGE). AGE memasuki sel melalui reseptor produk akhir glikasi lanjutan (RAGE) yang menyebabkan stres oksidatif, keradangan dan akhirnya kematian sel. Dalam osteoblas, kadar AGE yang tinggi boleh meningkatkan respons inflamasi dan tekanan oksidatif (OS), serta mengurangkan osteoblastogenesis. *Tinospora cordifolia* (*T. cordifolia*) dan *Tinospora crispa* (*T. crispa*) telah digunakan secara tradisional untuk merawat diabetes. Dalam perubatan moden, metformin berfungsi sebagai rawatan utama untuk mengurangkan hiperglisemia apabila perubahan gaya hidup gagal. Pada masa kini, sedikit maklumat yang diketahui tentang kesan *T. cordifolia* dan *T. crispa* terhadap pembentukan semula tulang dalam sel tulang yang terdedah kepada tahap MGO yang tinggi. Selain itu, tiada data yang tersedia mengenai interaksi metformin dengan ekstrak *T. crispa* dan *T. cordifolia*. Oleh itu, kajian ini bertujuan untuk menilai kesan *T. crispa* dan *T. cordifolia* terhadap metabolisme tulang dalam sel-sel osteoblas (hFOB1.19) yang dirangsang dengan MGO dan menilai potensi interaksi sinergistik dua tumbuhan ini dengan metformin. Tumbuhan-tumbuhan ini telah diekstrak menggunakan metanol dan air panas. Kandungan fitokimia keempat-empat ekstrak ditentukan menggunakan GC-MS, aktiviti antioksidan dinilai melalui ujian DPPH manakala sifat anti-glikasi dinilai melalui ujian BSA-MGO. Kemudian, kesan ekstrak terhadap sel-sel osteoblas yang dirangsang dengan MGO dinilai dengan atau tanpa

metformin untuk kapasitas antioksidan, aktivitas anti-apoptosis, sifat anti-keradangan serta dampak terhadap ekspresi dan aktivitas reseptor insulin di dalam osteoblas. Hasil kajian menunjukkan bahwa ekstrak tumbuhan mengandung sebatian bioaktif antioksidan dan anti-keradangan dan kesan anti-apoptosis yang tinggi. Walau bagaimanapun, ekstrak menunjukkan aktivitas anti-glikasi yang terhad seperti yang ditunjukkan dalam BSA-MGO dan pembentukan AGE dalam osteoblas yang dirangsang dengan MGO. Selain itu, ekstrak secara signifikan mengurangkan pengeluaran IL-6 dan IL-18, menunjukkan sifat anti-keradangan mereka. Ekstrak juga meningkatkan ekspresi Runx2 yang mempengaruhi osteoblastogenesis. Tambahan pula, ekstrak-ekstrak tersebut meningkatkan ekspresi GLUT4 dan reseptor insulin dalam hFOB1.19 yang dirangsang dengan MGO, menunjukkan kesan positif terhadap metabolisme tulang ketika diabetes. Secara kesimpulannya, kajian ini menekankan potensi ekstrak T. cordifolia dan T. crispa dalam mengurangkan komplikasi berkaitan dengan T2DM, terutamanya kesan terhadap osteoblas dan kesihatan tulang.

Kata kunci: T. cordifolia, T. crispa, Diabetes mellitus jenis 2, metabolisme tulang

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AG	Aminoguanidine
AGE	Advanced glycation end products
AO/PI	Acrymidine orange/propidium iodide
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAK	Bcl-2 antagonist killer
BAX	Bcl-2 associated X protein
BSA	Bovine serum albumin
COC	Carboxylated osteocalcin
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ETC	Electron transport chain
FADD	Fas-associated death domain
FRAP	Ferric reducing ability of plasma
GC-MS	Gas chromatography mass spectrometry
HAT	Hydrogen atom transfer
HBSS	Hanks' Balanced Salt Solution
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular Adhesion Molecule 1

IKK	Inhibitor of nuclear factor- κ B (I κ B) kinase
IL	Interleukin
IRS	Insulin receptor substrate
ISO	International Organization for Standardization
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony-stimulating factor
MGO	Methylglyoxal
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NADPH	Nicotinamide-adenine dinucleotide phosphate
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIST-17	National institutes of standard and technology library ver. 2017
NLR	Nucleotide-binding oligomerization domain-like receptors
NY	New York
OC	Osteocalcin
OPG	Osteoprotegerin
OS	Oxidative stress
PBS	Phosphate buffered saline
PRRs	Pattern recognition receptors
RANKL	Receptor activator of nuclear factor kappa-B ligand
RFU	Relative fluorescence unit
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
SET	Single electron transfer
SOST	Sclerostin

T2DM	Type-2 diabetes mellitus
TFC	Total flavonoid content
TNF	Tumour necrosis factor
TPC	Total phenolic content
UCOC	Undercarboxylated osteocalcin
UK	United Kingdom
USA	United States of America
VCAM-1	Vascular cell adhesion molecule 1

CHAPTER 1

INTRODUCTION

1.1 Study Background

In Southeast Asia, *T. crispa* and *T. cordifolia* are traditional plants used for the treatment of diabetes and other illnesses. Both plants belong to the same plant family and therefore have almost the same phytochemical composition. In several cell culture and animal studies, these extracts of these two plants have shown to improve insulin release from pancreatic β -cells and to increase glucose uptake by muscles. Both *Tinospora* species have been studied for their antioxidants, anti-inflammatory as well as antidiabetic properties in different models (Sharma et al., 2015; Sharma et al., 2019; Sulaiman et al., 2016; Upadhyay et al., 2021). However, the effect of these plants towards carbonyl stress caused by diabetic hyperglycaemia in osteoblasts has not been fully elucidated.

Methylglyoxal (MGO) is classified as a reactive carbonyl compound (RCC) which like other RCCs can cause carbonyl stress and diabetic complications. Previous studies revealed that MGO induced prolonged inflammation, oxidative stress, and osteoblast cell death in an *in-vitro* study. Berberine and metformin did reverse the effect partially thus the whole plant extract of *T. crispa* and *T. cordifolia* may be more effective in enhancing these effects.

Osteoblasts produce and secrete osteocalcin (OC), which is vital for binding calcium to the extracellular matrix and therefore contributing to the bone stiffness. In its undercarboxylated form (uOC), acts as a hormone that delivers direct feedback to the pancreatic β cells which regulate insulin secretion. A previous work revealed that MGO

downregulated the expression of insulin receptors situated on the osteoblast cell membrane. So far it is not known if *T. crispa* and *T. cordifolia* could fully restore the insulin signalling pathway in osteoblasts and therefore improve glucose absorption in osteoblasts.

MGO can react with proteins, lipids, and nucleic acids to form advanced glycation end products (AGE), which cause the activation of the NF- κ B pathway which leads to inflammation and consequently the production of pro-inflammatory cytokines. Moreover, the glycation leads to the activation of caspase-3 which then initiate apoptosis. Compromised bone metabolism associated with T2DM indicated through low osteoblastogenesis due to Runx2 suppression and therefore osteocalcin production. *T. crispa* and *T. cordifolia* may help in ameliorating these effects.

In summary, both *Tinospora* species, could show promising protective effects in ameliorating apoptosis and diabetes. Thus, *T. crispa* and *T. cordifolia* may be well suited in protecting osteoblasts from carbonyl stress, restoring osteocalcin production and insulin signalling in osteoblasts which would help to stabilize the whole-body glucose absorption and metabolism

1.2 Problem Statement

In recent years, there has been substantial research into diabetes and its complications, especially due to the rising global prevalence of diabetes and its profound impact across the world. Numerous studies have reported that T2DM leads to complications such as vascular diseases, heart failure, neuropathy and osteoporosis (Deshpande et al., 2008; Farmaki et al., 2021; Tomic et al., 2022). Both *T. cordifolia* and *T. crispa* were reported to possess antioxidants, anti-inflammatory and anti-diabetic properties. Despite these valuable reports, a critical gap remains in order to understand the role of *T. cordifolia* and *T. crispa* in improving and/or ameliorating the diabetes-induced metabolic bone disorder. A causative

agent for developing this bone disorder is excessive glucose in the blood and an associated increase of reactive carbonyl compounds e.g., methylglyoxal (MGO). As these plant extracts have traditionally been consumed by individuals with T2DM, this investigation aims to elucidate whether the combined therapeutic approach could result in synergistic effect which could improve glycaemic level when combined with metformin treatment. Additionally, the study seeks to assess the potential to decrease metformin dosages as its combination of with the extracts may increase the likelihood of hypoglycaemia in T2DM patients. Furthermore, there are limited reports regarding the presence of synergistic activity between the extracts with metformin. This gap necessitates further exploration to enhance the knowledge of *T. cordifolia* and *T. crispa* possess towards T2DM and address challenges such as the development of bone metabolic disorder.

1.3 Objectives

The study aimed to explore the antioxidant, anti-apoptotic, anti-inflammatory effects of the extracts, both in conjunction with metformin and independently, while also analysing their bioactive compounds through GC-MS. Additionally, the study aims to test the extracts' potential in reducing MGO induced osteoblast cell stress and therefore potentially positively influencing bone remodelling when used synergistically with metformin or alone. In detail, the objectives of this study were:

1. to determine the effectiveness of *T. cordifolia* and *T. crispa*, alone or in combination with metformin, in reducing cellular stress in MGO-stimulated hFOB1.19 cells by examining the levels of intracellular ROS and AGE formation.

2. to investigate the anti-apoptotic properties of *T. cordifolia* and *T. crispa*, alone or combined with metformin in ameliorating apoptosis in MGO-stimulated hFOB1.19 cells, by analysing the levels of caspase-3 and Bcl-2 formation.
3. to determine the anti-inflammatory effect of *T. cordifolia* and *T. crispa* with and without metformin in regulating inflammatory action in MGO-stimulated hFOB1.19 through the secretion of IL-6, IL-18 and translocation of NF- κ B.
4. to determine the effect of *T. crispa*, *T. cordifolia* with and without metformin on MGO-induced downregulation of the insulin receptor in osteoblast cells and downregulated Runx2 expression and therefore decreased expression of GLUT4.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to the plants

Tinospora encompasses a genus of flowering plants characterized by succulent, woody climbing shrubs. With a current recognition of thirty-four species, these plants typically develop long aerial roots from host trees and exhibit corky or papery bark. They are primarily distributed across tropical and sub-tropical regions of Asia, Africa, and Australia. Among the most prevalent species are *T. cordifolia* and *T. crispa*. *Tinospora* species demonstrate a diverse range of biological activities, including anti-diabetic, antioxidants, anti-tumour as well as anti-inflammatory effects (Chi et al., 2016).

2.1.1 *Tinospora cordifolia* (*T. cordifolia*)

Tinospora cordifolia (*T. cordifolia*) is commonly known as Guduchi in India. It is a perennial and deciduous climbing shrub. *T. cordifolia* is indigenous to India and holds a significant place in both traditional and Ayurvedic medicinal practices. The historical attributes of *T. cordifolia* have been documented in ancient Ayurvedic texts, such as Rasayana, Sangrahi, Balya and others (Upadhyay et al., 2013). The taxonomical classification of *T. cordifolia* is shown in Figure 2.1. The plant's stem, leaves, and roots have been used for centuries to address various health ailments. Rich in bioactive compounds such as alkaloids, flavonoids, terpenoids, and polysaccharides, *T. cordifolia* exhibits a broad spectrum of medical benefits as shown in Table 2.1. The leaves of *T. cordifolia* are typically quite large, heart-shaped and arranged alternately along the stems shown in Figure 2.2. The leaf margins are either smooth or with small teeth and lack a

central area. The leaves have 3-7 primary veins radiating from the base and 1-3 pairs of secondary veins, with petioles that are angular and swollen near the base. The flowers are greenish-yellow and can be found at higher altitudes (Saha & Ghosh, 2012).

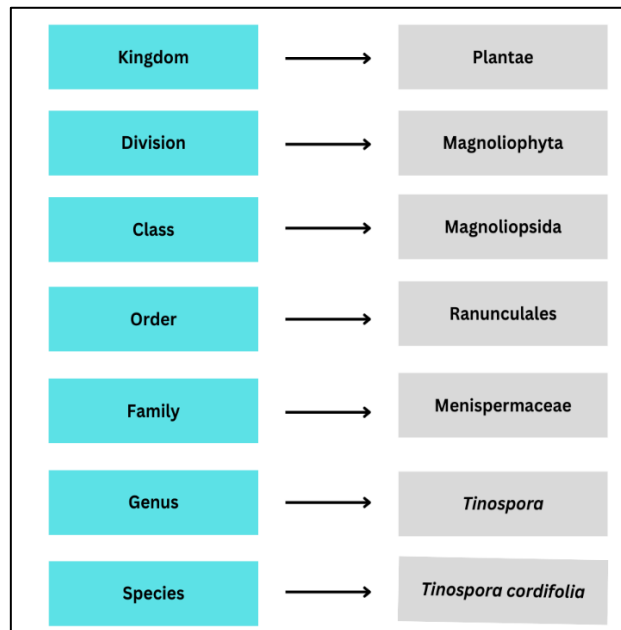


Figure 2.1: The taxonomic classification of *T. cordifolia*. The illustration is adapted from Saeed et al., (2020).

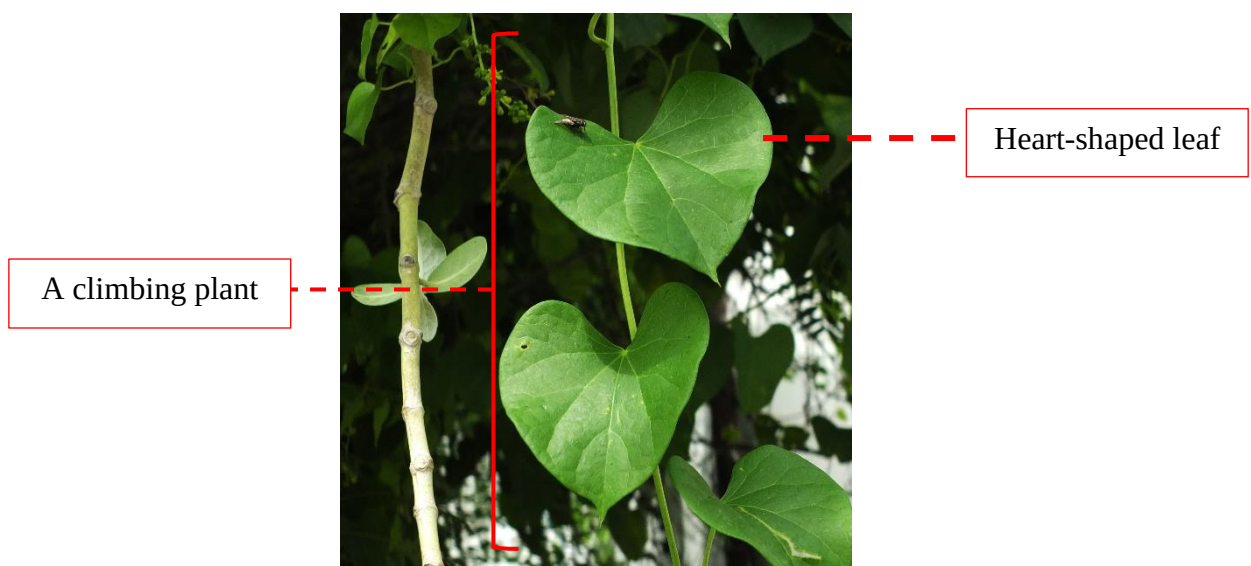


Figure 2.2: The tree of *T. cordifolia* (India Biodiversity Portal, n.d.)

Table 2.1: Different pharmacological activities reported from *T. cordifolia* based on the literature.

Pharmacological activity	Studies conducted	Results	Reference(s)
Antioxidant	Analysing total phenolic content (TPC), total flavonoid content (TFC), reducing power, lipid peroxidation activity, DPPH radical scavenging activity and superoxide radical scavenging activity.	Ethanolic extract of <i>T. cordifolia</i> showed highest TPC and TFC with values of 5.1 ± 0.25 mg/g and 0.52 ± 0.02 mg/g respectively. For reducing power, ethanolic extract showed the value of 41.4 ± 0.45 μ M Fe(II)/g. lipid peroxidation and DPPH showed the EC ₅₀ value of 0.1 and 0.5 mg/mL respectively.	(Premanath & Lakshmidevi, 2010)
	Scavenging effects on DPPH, ABTS radical, hydroxyl radical and ferric reducing antioxidant power (FRAP).	Potent antioxidant nature of methanolic extract of leaf and ethyl acetate extract of stem with respect to inhibition of lipid and protein oxidation.	(Ilaiyaraja & Khanum, 2011)
	Scavenging effects on DPPH and ABTS.	Methanolic stem extract of <i>T. cordifolia</i> showed higher antioxidant activity when compared with methanolic leaf extract.	(Upadhyay et al., 2021)
Anti-inflammatory	In-vitro evaluation was conducted in LPS-stimulated RAW264.7 macrophages. The expression of TNF- α , cytokines such as IL-6, IL-1 β , NF- κ B and p38 MAPK phosphorylation were analysed.	<i>T. cordifolia</i> extract help to downregulates the expression of pro-inflammatory biomarkers in the cells.	(Philip et al., 2018)
	RAW264.7 was pre-treated with <i>T. cordifolia</i> and then stimulated with LPS. The expression of pro-inflammatory cytokines were measured.	<i>T. cordifolia</i> reduced the gene expression of inflammatory cytokines such as TNF- α and IL-1 β and decreased nitrogen oxide production in RAW264.7 cells.	(Ghatpande et al., 2019)

Table 2.1 continued

Antidiabetic	Analysing anti-arthritic activity of <i>T. cordifolia</i> using arthritis-induced rat model by measuring the expression of pro-inflammatory cytokines such as IL-1 β , TNF- α , IL-6 and IL-17.	<i>T. cordifolia</i> has been detected to downregulate the pro-inflammatory cytokines and reduced bone damage.	(Sannegowda et al., 2015)
	Mechanism of <i>T. cordifolia</i> towards the expression of glucose transporter-4 (GLUT4) in differentiated myocytes.	The expression of GLUT4 has been found to be overexpressed when treated with <i>T. cordifolia</i> and palmatine up to 5 fold and 4 folds, respectively.	(Sangeetha et al., 2013)
	A study was done towards anti-hyperglycaemic properties of <i>T. cordifolia</i> had towards in Swiss albino mice.	<i>T. cordifolia</i> showed a distinctive reduction of blood glucose in mice. This study suggests that <i>T. cordifolia</i> has the potential to be developed into drug.	(Sharma et al., 2021)
	Anti-hyperglycaemic effect of <i>T. cordifolia</i> extracts were studied in streptozotocin-induced diabetic rats. The level of HBA1C, serum insulin, C-peptide and liver enzymes were assessed.	Methanolic extract of <i>T. cordifolia</i> significantly reduced the fasting blood glucose and decreased glycosylated haemoglobin level when compared to diabetic group at (p<0.001). insulin and C-peptide were found to improve significantly when treated with <i>T. cordifolia</i> .	(Daisy et al., 2009)

Some of the key phytochemicals that have been reported from *T. cordifolia* include terpenoids such as tinosporaside, sesquiterpene glucoside tinocodifolioside, furanolactone palmatosides C and palmatosides F (Sharma et al., 2019). Another study also revealed the presence of important alkaloids such as berberine, palmatine and choline (Choudhary et al., 2013). In addition, lignans also have been reported in *T. cordifolia* (Sharma et al., 2019). Furthermore, another study by Krupanidhi et al., (2021) revealed the phytochemical constituents of *T. cordifolia* encompass a multitude of constituents, including tinosponone, tinosporin, berberine, palmitine, choline, tembetarine, isocolumbin, tetrahydropalmatine, and various other alkaloids, steroids, lactones, glycosides, and sesquiterpenoids. These phytochemicals possess several important pharmacological properties. For instance, an *in vivo* study showed that palmatine is a therapeutic agent in managing oxidative stress (OS)-mediated neurological diseases (Chaves et al., 2020). In addition, berberine was shown to have anti-inflammatory properties by downregulating the expression of cyclooxygenase 2 (COX-2) and prostaglandin. Berberine also possesses neuroprotective and anti-depressive effects (Och et al., 2022).

The distribution of *T. cordifolia* and *T. crispa* is shown in Figure 2.3. As shown in the figure, *T. cordifolia* is native to India while *T. crispa* is native to Southeast Asia. As *T. cordifolia* is native to India, the plant has been used for traditional medicine such as Ayurveda. Nagral et al., (2021) initially suggested a potential link between liver injury and consumption of traditional medicine *T. cordifolia*, positing autoimmune-like hepatitis or the uncovering of latent autoimmune liver disease as probable causes based on patient observations. However, subsequent investigation and review of *T. cordifolia* fail to confirm this hypothesis. There appears to be no credible evidence supporting a direct causal relationship between *T. cordifolia* consumption and the observed cases of autoimmune-like

hepatitis (Panneer et al., 2023).

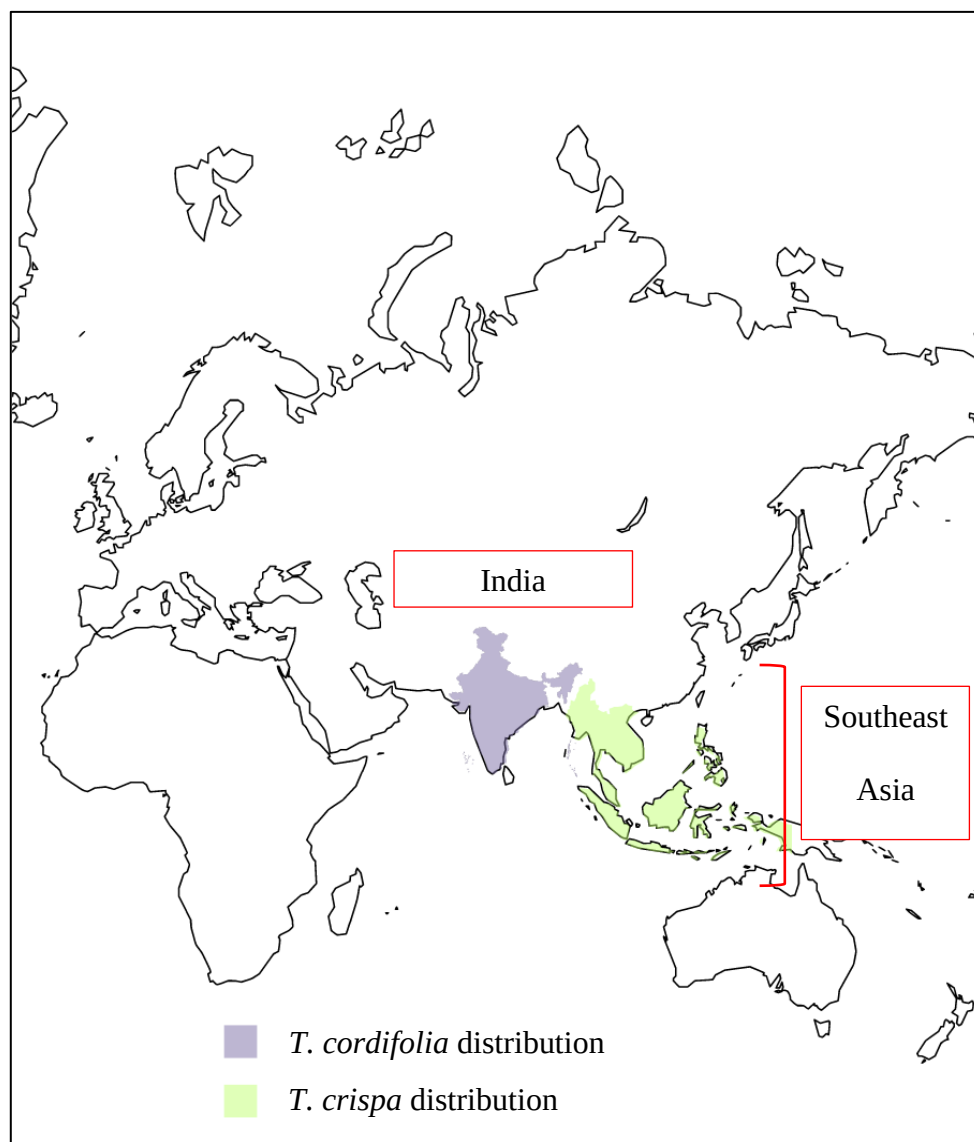


Figure 2.3: The distribution of *T. crispa* and *T. cordifolia*. *T. crispa* is native to Southeast Asian countries while *T. cordifolia* is native to India (ASEAN, 2024; Nair et al., 2019).

2.1.2 *Tinospora crispa* (*T. crispa*)

T. crispa is a perennial vine belonging to the Menispermaceae family. It is native to various regions of Southeast Asia, including Malaysia, Thailand, Indonesia as well as some part in India (Northeastern region of India). This plant has attracted significant attention in the scientific community due to its diverse array of bioactive compounds and potential

medicinal properties (ASEAN, 2024). The plant's distinctive heart-shaped leaves and climbing plant properties make it recognisable in its natural habitat. *T. crispera* exhibits characteristics of succulent woody climbing shrubs, typically manifesting elongated aerial roots that extend from the host trees to facilitate their propagation. The bark possesses a corky or papery texture, which tends to peel upon desiccation. The leaves are simple and alternate, showcasing a broadly ovate shape with a slightly heart-shaped base. The taxonomical classification for *T. crispera* is retrieved from Malaysia biodiversity information system (MyBIS) and is shown in Figure 2.4.

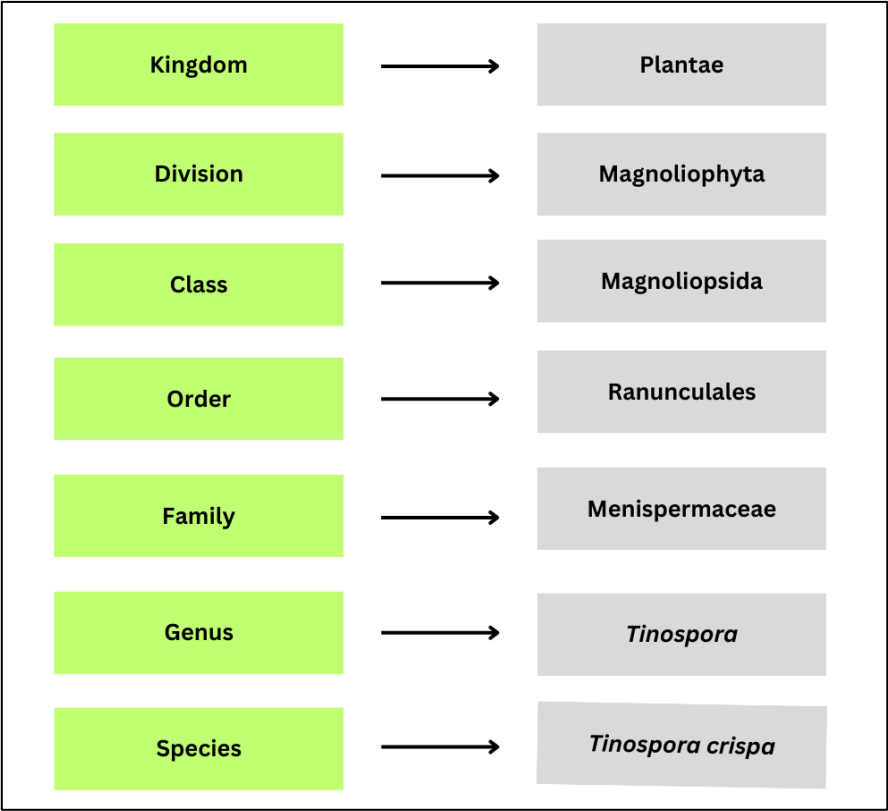


Figure 2.4: The taxonomic classification of *T. crispera* (MyBIS, 2010).

The leaf margins are either smooth or toothed, lacking the characteristic shield-like structure (peltate), while the basal veins range from 3 to 7, accompanied by 1 to 3 pairs of lateral veins. The leaf of *T. crispa* is also love-lobed in shape as shown in Figure 2.5. This climbing plant also has a historical usage in traditional medicine for addressing conditions such as jaundice, rheumatism, urinary irregularities, fever, malaria, diabetes, internal inflammation, fractures, scabies, hypertension, as well as for mitigating thirst, enhancing appetite, moderating body temperature, and promoting overall well-being (Ahmad et al., 2016).

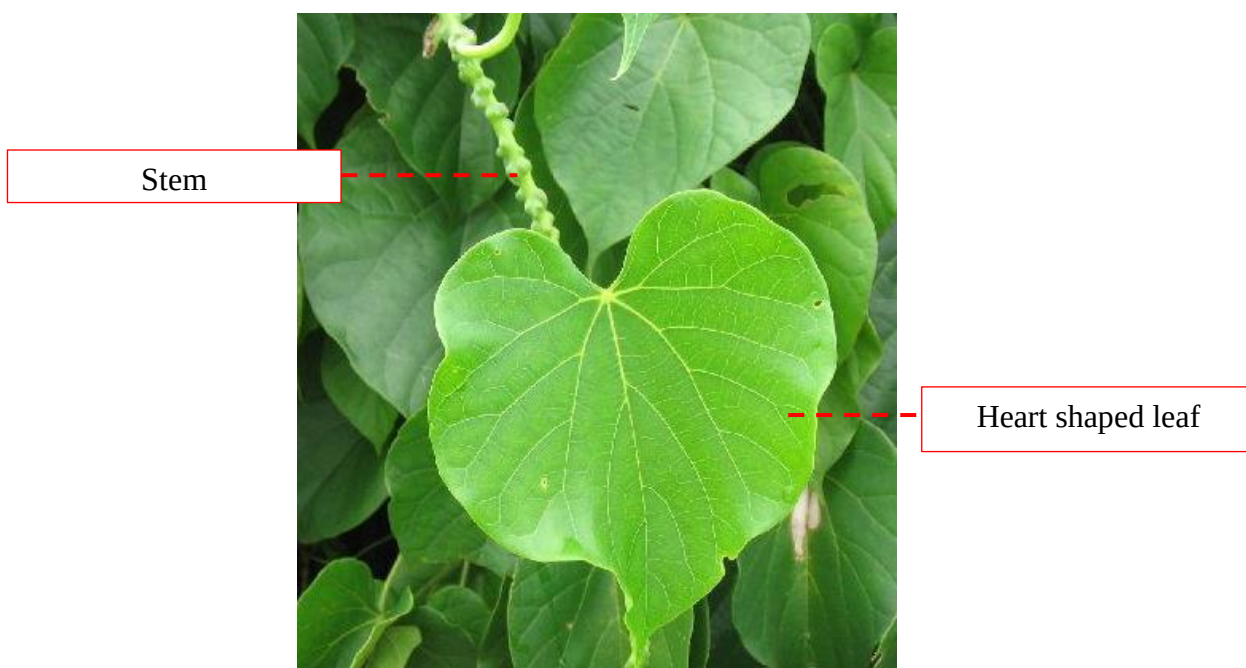


Figure 2.5: The image of *T. crispa* leaves and stem. Similar to *T. cordifolia*, this species also is a climbing plant (India Biodiversity Portal, n.d.-b).

T. crispa is rich with various phytochemicals, which includes diterpenes such as borapetoside A, borapetoside E, borapetoside F and syringin (Ahmad et al., 2016). *T. crispa* also contains alkaloids such as magnoflorine, berberine and palmatine (Abu et al., 2015; Ali & Dixit, 2013). This plant has demonstrated various biological activities, including antioxidant, anti-inflammatory, and antidiabetic effects as shown in Table 2.2. As such, *T.*

crispa holds significant promise as a subject of further research, both to elucidate its phytochemical complexity and to explore its potential applications in modern medicine and healthcare.

Table 2.2: Different pharmacological activities reported from *T. crispa* based on the literature.

Pharmacological activity	Studies conducted	Results	Reference(s)
Antioxidant	DPPH scavenging activity, reducing power and metal chelating assay	Stem extract of <i>T. crispa</i> showed high antioxidant activities in all antioxidant assay. DPPH IC ₅₀ : 0.118 mg/mL, inhibiting metal chelating and reduce Fe ³⁺ to Fe ²⁺ .	(Zulkefli et al., 2013)
	DPPH free radical scavenging antioxidant assay	Methanol extract of <i>T. crispa</i> increased radical scavenging activity with IC ₅₀ of 12 µg/mL.	(Ibahim et al., 2011)
	Quantifying TPC, TFC, DPPH radical scavenging assays of methanolic extract of <i>T. crispa</i>	TPC of <i>T. crispa</i> was 6.12 g GAE/100g of dried extract, while TFC value was 55.58 g QE/ 100g of dried extract. IC ₅₀ of <i>T. crispa</i> was 0.21 mg/mL.	(Sulaiman et al., 2016)
Anti-inflammatory	Human umbilical vein endothelial cells were induced with TNF-α to stimulate inflammation.	Methanolic and aqueous extracts of <i>T. crispa</i> reduced signalling molecules of ICAM-1, MCP-1, VCAM-1 and M-CSF.	(Kamarazaman et al., 2012)
	Carrageenan induced inflammation (oedema) in Sprague-dawley rats.	Methanolic extract reduced the development of oedema in paw of the rats.	(Lourdes et al., 2012)
	Immunostimulatory effect of <i>T. crispa</i> that influences cellular response by studying its impact on various factors including phagocytosis activity of peritoneal macrophages and hypersensitivity response.	Ethanol extract of <i>T. crispa</i> upregulates phagocytosis and lysozymes in Wistar-Kyoto rats.	(Ahmad et al., 2016)

Table 2.2 continued

Antidiabetic	In-vivo approach involves using animal models to simulate insulin deficiency, while the in vitro method employs an assay that tests for the activity of alpha glycosidase inhibition.	The combinations of <i>T. crispa</i> and Anredera cordifolia reduced blood sugar level over a 14-day period in vivo while in vitro test showed an ability to inhibit alpha-glucosidase enzymes. (Kusriani et al., 2023)
	Type 2 diabetes (T2DM) was induced in mice by feeding them a high-fat diet and fructose-sweetened water for 4 weeks. The confirmation of T2DM induction relied on fasting plasma glucose measurements, with diabetes considered present if plasma glucose exceeded 150 mg/dl after the 4-week induction period.	Borapetoside C from <i>T. crispa</i> improves insulin sensitivity in diabetic mice. The study showed that borapetoside C delayed the development of insulin resistance in association with increased insulin sensitivity. (Ruan et al., 2012)
	The effect of glucose transport in L6 myoblast skeletal muscle cells when treated with aqueous <i>T. crispa</i> .	<i>T. crispa</i> at concentration of 4 mg/mL substantially increased the glucose uptake in L6 myotubes with both dose and time dependent, comparable to the effects of metformin and troglitazone. (Klangjareonchai et al., 2015)

In conclusion, both *T. crispa* and *T. cordifolia* have demonstrated noteworthy pharmaceutical activities in various studies. These botanical sources exhibit potential benefits in addressing conditions related to glucose metabolism and diabetes, as evidenced by their ability to enhance glucose transport and reduce blood sugar levels. Furthermore, their antioxidant properties, as observed in different antioxidant assays such as DPPH, reducing power and metal chelating suggest promising applications in enhancing the body's antioxidant capability towards free radicals. Overall, *T. crispa* and *T. cordifolia* hold great promise as sources of bioactive compounds with potential pharmaceutical implications, paving the way for the development of novel therapeutic interventions.

2.2 Cellular stress and apoptotic activity

2.2.1 Introduction to apoptosis

Apoptosis is a vital process in the body where cells are programmed to die in a controlled manner. It helps to maintain the balance and health of tissues by eliminating old, damaged, or unwanted cells. This process is essential during for a normal physiological and metabolism of the body. Various physiological processes such as tissue homeostasis, tissue development and immune system regulation are maintained through cellular apoptosis as it plays a crucial role in maintaining a balance between cell death and division (Jan & Chaudhry, 2019). For instance, apoptosis plays a role in the morphological development such as contributes partially to the digit's development in vertebrae organisms (Smith et al., 2020). Another role of apoptosis is to regulate lymphocyte development. For example, immune cells infected by pathogens are removed through apoptosis to protect the uninfected cells (Nagata & Tanaka, 2017).

Excessive apoptosis, on the other hand, can result in several neurodegenerative diseases such as Alzheimer's disease where neuronal loss is caused by overstimulated cell death (Bank et al., 2017). The generation of reactive oxygen species (ROS) and DNA damage in pathological conditions can further induce cellular apoptosis. Excessive osteoblast apoptosis due to oxidative stress contribute to the progression of bone disorder. As a key regulator of cellular fate, apoptosis highlights the delicate interplay between normal development and disease pathogenesis, providing insights into therapeutic approaches for various disorders.

2.2.2 Intrinsic and extrinsic apoptotic pathways

The mechanism of apoptosis involves two pathways: extrinsic pathway and intrinsic pathway. These two pathways might do lead to the same execution pathway (Vitale et al., 2023).

The extrinsic pathway is initiated when death ligands attach to their death receptors located on the membrane of the cells as shown in Figure 2.6. These attachments further triggered a complex known as death-inducing complex which then activates caspases, such as CASP8. There are several prominent death receptors such as TNFR1 which is activated through TNF ligands, CD95 receptor is activated through CD95L or Fas-ligands (FasL) and DR6 receptor is activated through TL1A (Walczak, 2013). In the extrinsic pathway, ligands from the tumour necrosis factor (TNF) gene superfamily, i.e., TNF- α bind to its receptors on the cell surface, initiating a series of protein interactions that lead to the activation of initiator caspases like CASP8. Subsequent activation of caspases like CASP3 would propel cellular death (Kumar et al., (2005). Cell death triggered by tumour necrosis factor receptor 1 (TNFR1) is believed to occur through the enlistment of the adaptor molecule FADD and

caspase 8 (CASP8) to join the receptor complex which then further activates CASP3, leading the cell to its death (Micheau & Tschopp, 2003).

The activation of CASP8 then leads to the activation of the interactive death domain agonist (FADD) activating BH3-interacting-domain death agonist (tBID) which then activates Bax to allow mitochondrial permeabilization (Wang & Tjandra 2013). Mitochondrial membrane permeabilization enables the release of cytochrome-c into the cytoplasm, where it subsequently combines with apoptotic protease-activating factor-1 to create the apoptosome complex. This complex then initiates the activation of CASP9, which in turn triggers the activation of CASP3 to initiate cell degradation (Jiang et al., 2020).

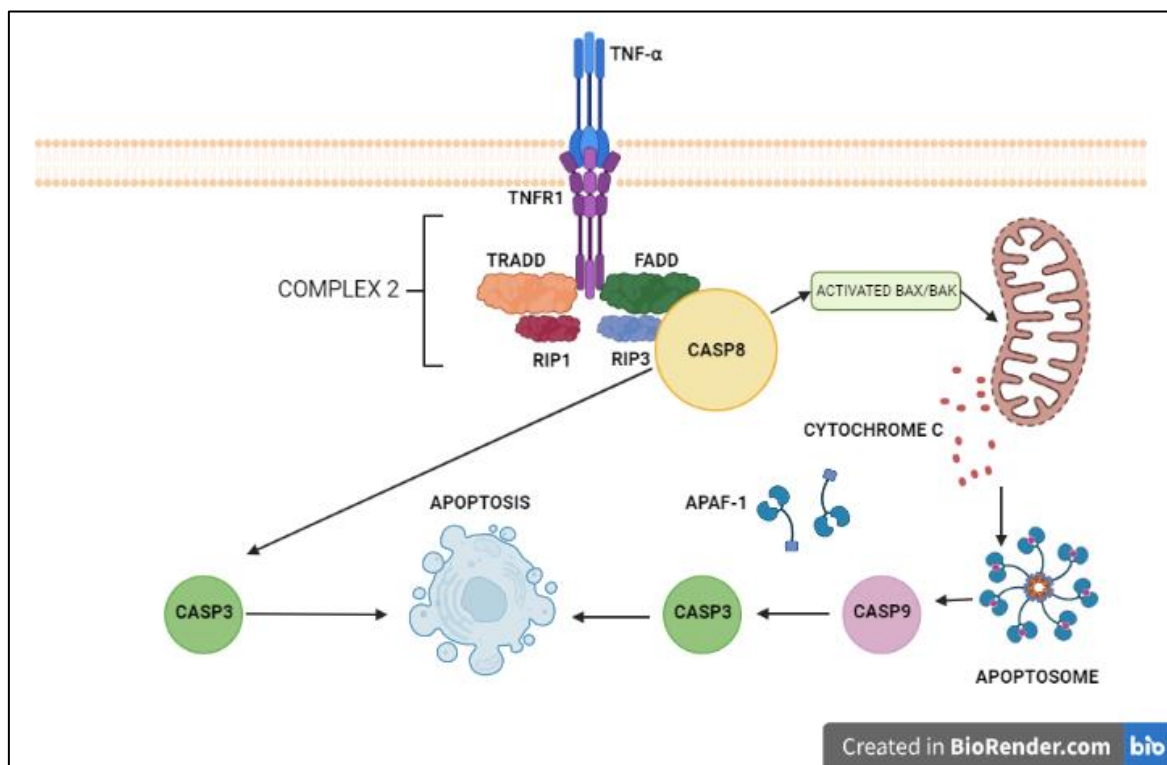


Figure 2.6: The extrinsic pathway of apoptosis when stimulated by TNF- α . The image is created with BioRender.com. Adapted from Kumar et al., (2005); Micheau & Tschopp, (2003).

On the other hand, the intrinsic pathway depends on mitochondrial-mediated signalling as shown in Figure 2.7. Intracellular stressor such as DNA damage can trigger the

intrinsic apoptotic pathway through the p53-dependent pathway (Azzwali & Azab, 2019 ; Hongmei, 2012). Under circumstances such as severe DNA damage, p53, which is a tumour suppressor gene orchestrates cellular apoptosis by suppressing Bcl2, an anti-apoptotic protein, while concurrently upregulating intrinsic apoptotic factors like Bax through transactivation. Upregulation of Bax is followed by activation of CASP9, CASP7 and CASP3 (Shen & White, 2001) culminating in apoptosis. In a pro-proliferative mechanism, Bcl-2 helps in enhancing cell survival (Gundamaraju et al., 2018). Bcl-2 is one of the anti-apoptotic proteins that counteract this action by inhibiting the release of cytochrome-c through binding to pro-apoptotic proteins such as Bax (Jan & Chaudhry, 2019). In addition, p53 also plays the role of enhancing mitochondrial permeability, causing the release of cytochrome-c into the cellular cytosol (Dutordoir & Bates, A. 2016). The p53 gene is responsible for directing BH3 pro-apoptotic proteins that lead to cell cycle arrest (Harris & Levine, 2005).

Moreover, different forms of cellular stress signals have demonstrated the ability to initiate apoptosis. These stressors include chemotherapy drugs, oxidative stress, and endoplasmic reticulum (ER) stress (Fulda et al., 2010). In addition, oxidative stress has the potential to disrupt the balance within the ER, thus interfering with the ER's function for folding proteins. This disruption consequently raises the chance of an increase in misfolded proteins (Gundamaraju et al., 2018). The build-up of misfolded proteins within the ER, along with their clumping together, initiate a condition known as ER stress. The latter disrupts regular cellular operations and has harmful effects that can ultimately result in cell death (Haeri & Knox, 2012). Furthermore, ER stress can induce BAX and BAK localization and calcium ion release from the ER. BAX, BAK and calcium ion can later enter the

mitochondrial matrix, resulting in depolarization of mitochondria and release of cytochrome-c into the cytosol, leading to cell apoptosis (Fribley et al., 2009).

2.2.3 Similarities between intrinsic and extrinsic apoptosis

Cytochrome-c is an important internal signalling component in both the intrinsic and extrinsic pathways. It operates as a pro-apoptotic protein required for programmed cell death through the changes of Bax/Bak turnover and deactivation of Bcl2 proteins (Hongmei, 2012). Cytochrome-c plays a key role in activating the caspase cascade by combining with apoptotic protease activating factor-1 as well as procaspase-9 to synthesise apoptosome. Furthermore, apoptosome activates CASP9 followed by the activation of CASP3 which eventually leads to the degradation of cellular functional proteins (Jan & Chaudhry, 2019; Jiang et al., 2020). Cytochrome-c emerges as a key role as the signal molecules that orchestrate cellular death.

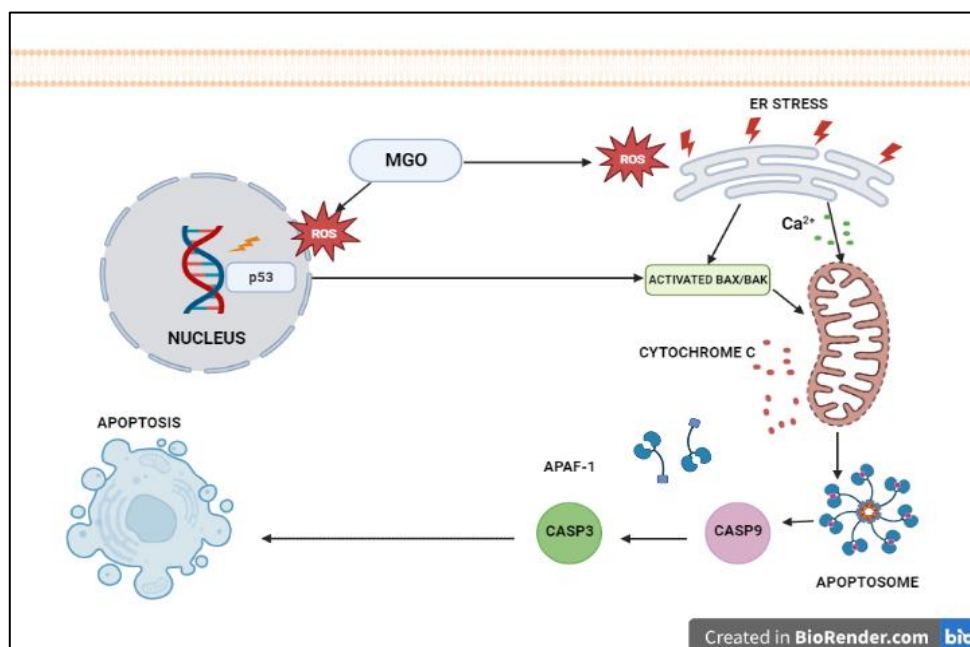


Figure 2.7: The intrinsic pathway of cell apoptosis that is mediated mainly through mitochondrial signalling. Adapted from Azzwali & Azab, (2019); Gundamaraju et al., (2018); Hongmei, (2012).

2.2.4 MGO and apoptosis

Methylglyoxal (MGO) is a reactive carbonyl compound that is produced endogenously in cells via glycolytic pathways, i.e., glucose metabolism. It can also be synthesised exogenously by sugar autoxidation and through the Maillard Reaction during grilling or cooking (Parmar, 2014). The excessive generation of MGO is a symptom of metabolic abnormalities including type 2 diabetes mellitus (T2DM) and oxidative stress (Ramachandra et al., 2019).

MGO can elicit cell apoptosis, cytotoxic effects as well as inflammatory responses through the synthesis of ROS. For instance, MGO has been shown to have the ability to trigger cell apoptosis in HUVEC, pancreatic- β cells as well as HK-2 cells (Wang et al., 2022). Jang et al., (2017) revealed that MGO stimulated apoptosis in HUVEC through increased levels of ROS.

MGO elevated the synthesis of AGE in mesenchymal stem cell-derived osteoblasts (Waqas et al., 2022). In endothelial and melanoma cells, AGE stimulates the production of reactive oxygen species (ROS) and the initiation of the p38 Mitogen-Activated Protein Kinase (MAPK) pathway, which leads to cell apoptosis. Concurrently, AGEs induce apoptosis in fibroblasts by engaging MAP kinases and the transcription factor forkhead box O1 (FoxO1) (Wang et al., 2015).

2.2.5 *T. crispa* and *T. cordifolia* in ameliorating apoptosis

To date, there are limited publications that look at the effect of MGO on hFOB1.19 cells. However, there are numerous publications that look at specific compounds found in both plants e.g., berberine and berberine and their effect on MGO-induced apoptosis and bone homeostasis. For instance, a finding revealed that berberine, extracted from *Coptidis*

rhizoma had a free radical scavenging activity (Jung et al., 2009). Another study also revealed berberine could also stabilise superoxide anions which ameliorates oxidative stress (Choi et al., 2001). A recent study further demonstrated that berberine could promote osteoblast proliferation while inhibiting osteoclast formation to promote bone regeneration (Zhang et al., 2021). Further research had shown that palmatine suppressed osteoclast numbers by reducing RANKL expression, making it as one of the potential agent in restoring excessive bone resorption (Ishikawa et al., 2015).

Meanwhile, in an investigation targeting diabetic peripheral neuropathy, it was discovered that aqueous extract of *T. cordifolia* effectively alleviated hyperalgesia which is a peripheral nerve dysfunction by inhibiting aldose reductase (Nadig et al., 2012). A study reported that *T. cordifolia* manage to ameliorate cancer by inducing cellular apoptosis (Patil et al., 2021). However, *T. cordifolia* was found to regulate titanium dioxide-induced toxicity in *Oreochromis niloticus* by reducing ROS production and pro-inflammatory cytokines secretion (Vineetha et al., 2021)

Literature findings from studies on *T. crispa* and *T. cordifolia* highlight the potential of these plants to possess anti-apoptotic properties. The insights gleaned from *T. crispa* and *T. cordifolia* underscore the potential of these plant to show anti-apoptotic properties. As studies continue to delve into the anti-cancer properties exhibit by these plants, there is a growing recognition of the need for further investigation to extend our understanding of anti-apoptotic properties of these plant extracts.

2.3 Inflammation and its consequences on osteoblast cells

Inflammation is a physiological response that arises from tissue damage or as a reaction to the presence of physical, chemical, or biological stimuli within the body. It is

classified into two types, namely acute inflammation and chronic inflammation. Inflammation occurs in response to harmful triggers like pathogens as well as damaged cells, as the immune system's reaction that serves to eliminate damaging factors and initiated the recovery process (Chen et al., 2018). Furthermore, inflammation is an essential defence mechanism crucial for maintaining good health.

2.3.1 Acute inflammation

Acute inflammation occurs immediately within minutes or hours and generally subsides within a short span of days. During the initial stage of the inflammatory reaction, immune system cells such as neutrophils travel to the injury location following a coordinated series of actions. This process is aided by cytokines, chemokines, and acute-phase proteins (Germolec et al., 2018). Moreover, the redness (erythema) resulting from acute inflammation arises from augmented blood circulation to the impacted region as a consequence of vasodilation due to the migration of leukocytes (Stone et al., 2022). Contributing factors that are responsible for acute inflammation include allergens, irritants as well as non-endogenous materials such as asbestos (Hannoodee & Nasuruddin, 2022). For instance, a cut would trigger cellular stress that stimulates the apoptotic cells to discharge damage-associated molecular patterns (DAMPs), subsequently initiating the activation of the innate immune system by engaging with pattern recognition receptors (PRRs) for the synthesis of pro-inflammatory cytokines (Roh & Sohn, 2018). The study also revealed DAMPs play a crucial role in the development of human diseases by triggering inflammatory responses upon binding with PRRs. PRRs primarily serve to enhance phagocytosis and initiate inflammation by detecting diverse pathogens and molecules originating from compromised cells. Moreover, PRRs are primarily presence within antigen-presenting cells like dendritic cells and macrophages to detect the presence

of pathogens such as viruses and bacteria. PRRs are also present in various other types of both immune and non-immune cells (Zaru, n.d.). Alternatively, DAMPs which can be detected by neutrophils subsequently aiding in the facilitation of neutrophil mobilization towards the injury site (Pittman & Kubes, 2013). This prompts the activation of signalling pathways that promote innate immunity through inflammation. As an example, purine metabolites like Adenosine triphosphate (ATP) and uric acid can also activate the NLR family of PRRs, leading to the production of pro-inflammatory cytokines. The receptor of advanced glycation end products (RAGE) is also known as one of the PRRs which can bind with DAMPs as well as AGE to orchestrate inflammation (Prabhudas et al., 2017).

2.3.2 Chronic inflammation

Chronic inflammation, often termed persistent and prolonged inflammation endures over extended period typically spanning several months to years. According to Roma et al., (2023), the origin of chronic inflammation encompass various scenarios. Firstly, it can be due to an inadequate removal of agents that trigger acute inflammation exemplified by persistent infectious agents such as *Mycobacterium tuberculosis*, certain protozoa, fungi, and other resilient parasites that outlast host defence mechanisms. Secondly, autoimmune disorders, wherein the immune system erroneously targets the body's normal constituents as foreign antigens, consistently attacking healthy tissues and giving rise to certain diseases i.e., rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Lastly, chronic inflammation can also stem from the interplay of inflammatory and biochemical agents that induce oxidative stress and impair mitochondrial function. This is exemplified by the heightened generation of free radicals, AGEs, uric acid (urate) crystals, and various others that contribute to a protracted inflammatory state.

2.3.3 Canonical pathway

The interaction between PRRs and DAMPs triggers a canonical nuclear factor kappa-B (NF- κ B) pathway that initiates cellular inflammation response as shown in **Figure 2.8**. Upon stimulation, a signalling cascade is initiated that leads to the activation of the I κ B kinase (IKK) complex, composed of IKK α , IKK β , and IKK γ (Kojok et al., 2019). This complex phosphorylates I κ B α , targeting it for ubiquitination and subsequent proteasomal degradation (Sun, 2011). As I κ B α is degraded, the retained NF- κ B dimers, primarily the p50/c-Rel (p65) heterodimer, are released and translocated into the nucleus. There, they bind to specific DNA sequences to modulate the expression of target genes involved in immune responses, inflammation, and cell survival (Kojok et al., 2019).

The canonical NF- κ B pathway plays a central role in coordinating the cellular response to external stimuli, contributing to various physiological and pathological processes. Furthermore, following the ligation of PRRs, cells can secrete cytokines such as TNF, IL-6 as well as IL-8 to initiate both innate (neutrophils, macrophages, dendritic) and adaptive cells from the adaptive immune system (Pouwels et al., 2014).

2.3.4 Non canonical pathway

The non-canonical NF- κ B pathway (alternative pathway), represents an alternative mode of NF- κ B activation distinct from the canonical pathway as shown in Figure 2.9. It primarily involves the activation of specific NF- κ B members, notably the RelB/p52 complex, through a mechanism centred on the regulated processing of p100 (Boyce et al., 2015; Novack, 2011). Upon stimulation by select members of the TNF superfamily such as RANK and BAFF, with their receptor RANKL and BAFFR respectively, the non-canonical pathway is initiated. According to Boyce et al., (2015), the initial process of

non-canonical NF- κ B triggers the stabilization and accumulation of NF- κ B-inducing kinase (NIK). NIK subsequently activates IKK α , which triggers the proteasomal processing of p100, converting it into an active p52 subunit (Boyce et al., 2015). The formed p52/b-Rel complex then translocated into the nucleus, where it modulates gene expression profiles distinct from those regulated by canonical NF- κ B dimers. The non-canonical NF- κ B pathway has been implicated in key biological functions like B-cell survival and maturation, and bone metabolism such as osteoclast differentiation and maturation (Boyce et al., 2015; Sun, 2011). Meanwhile, any disturbances in non-canonical NF- κ B signalling have also been linked to lymphoid malignancies, underscoring the importance of comprehending the intricacies of this pathway for therapeutic interventions (Grondona et al., 2018).

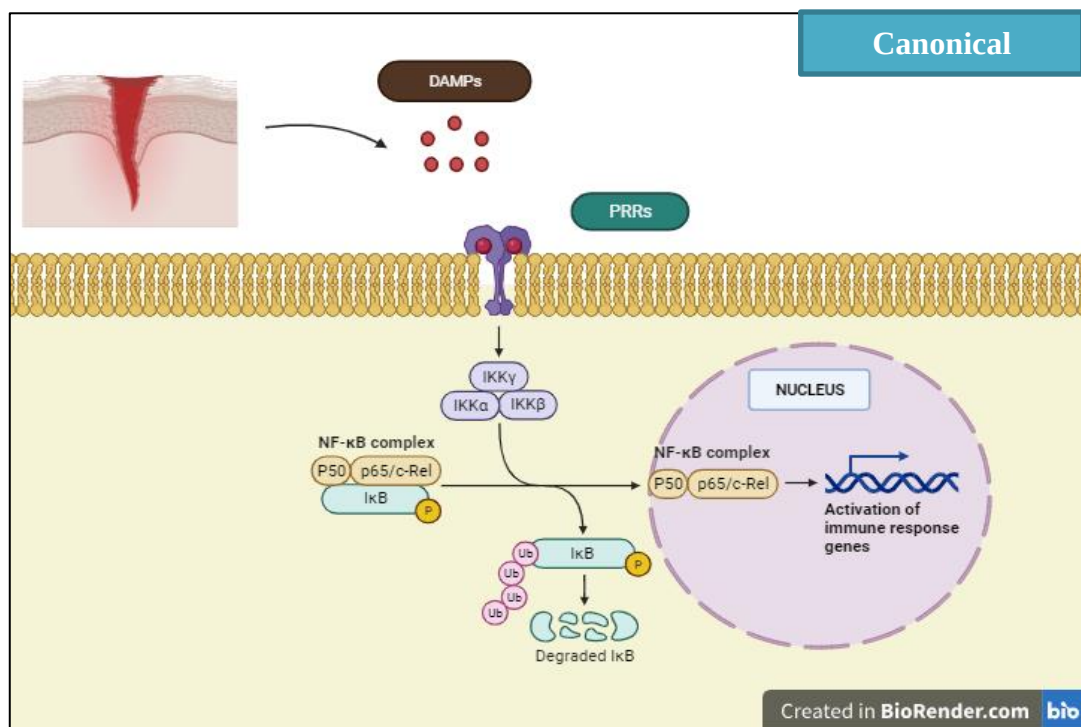


Figure 2.8: NF- κ B canonical pathway is activated when PRRs bind with their respective DAMPs. This pathway witnessed the degradation of I κ B and the translocation of NF- κ B complex into the nucleus, further activating immune response genes. Adapted from Kojok et al., (2019); Pouwels et al., (2014).

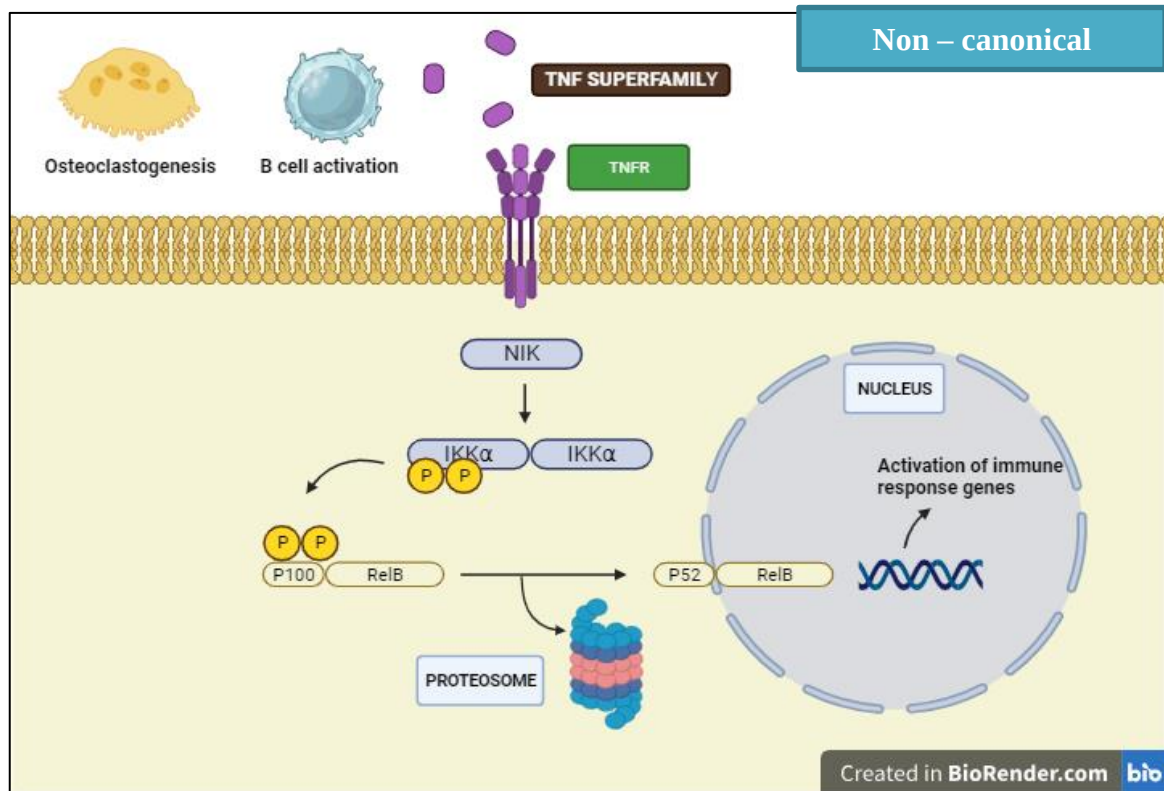


Figure 2.9: NF-κB non-canonical pathway is activated mainly with the binding of TNF superfamily ligand with their respective TNFR. The inactivated dimer RelB/p100 was activated through proteasomal processing before converted into p52/b-Rel and translocate into the nucleus. Canonical pathway of NF-κB is responsible for B-cell maturation as well as osteoclast differentiation. Adapted from Boyce et al., (2015); Novack, (2011); Sun, (2011).

2.3.5 Comparison between acute and chronic inflammation

In comparison, acute inflammation is an immediate and localized response that aims to eliminate threats and initiate healing, while chronic inflammation represents a persistent and systemic process that can be detrimental to tissues and organs (Roma et al., 2023). While acute inflammation is a protective mechanism that plays a vital role in maintaining tissue homeostasis, chronic inflammation can lead to ongoing tissue damage and dysfunction, ultimately contributing to the development and progression of various diseases.

In acute inflammation, various stimuli for example, wound cut or allergen will trigger physiological mechanisms that initiate the healing process. In contrast, in chronic

inflammation, the body continues to trigger inflammatory responses despite the absence of stimulus or trauma. The difference between inflammatory response between acute and chronic inflammation is shown in Figure 2.10.

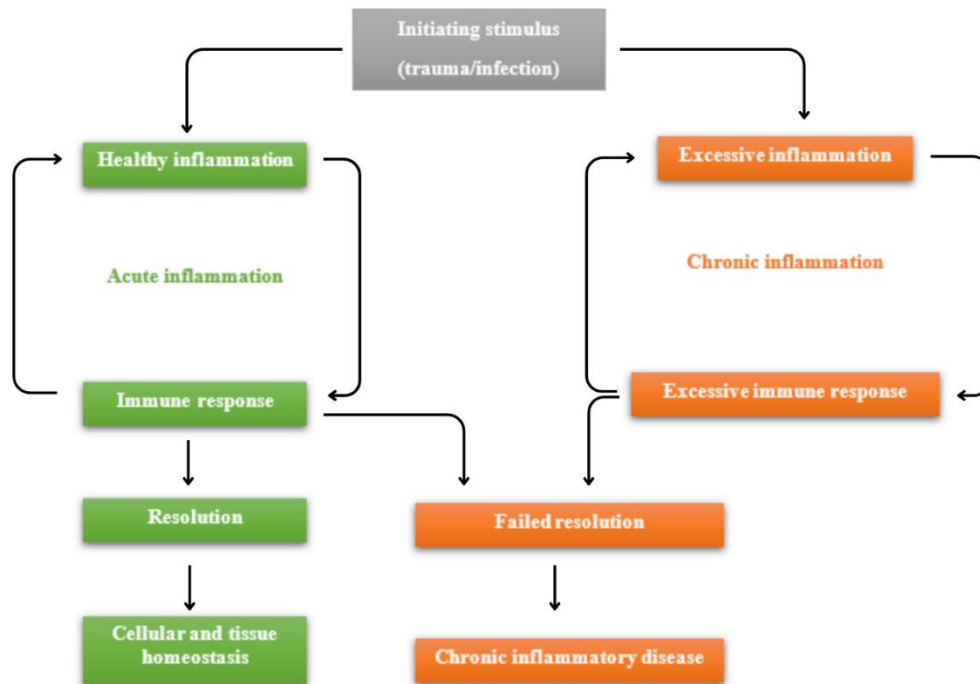


Figure 2.10: Comparison between acute and chronic inflammation. In acute inflammation, the body initiate a physiological mechanism to heal the wound through inflammatory process. However, in chronic inflammation, the body still undergoes inflammatory response despite there is no stimuli.

2.3.6 NF- κ B role in bone cells

A study revealed that NF- κ B induced pathway plays important roles in different bone cells that include osteoblasts, osteoclasts, osteocytes and chondrocytes (Novack, 2011). Different signals including TNF- α and RANKL initiate both the canonical and the non-canonical pathways of NF- κ B, which then stimulate both p50/c-Rel (canonical) and p52/b-Rel (non-canonical) dimers to increase osteoclast survival, osteoblast maturation as well as osteoclast formation. Thus, the deletion of NFKB1 and NFKB2 genes, which

should be expressed into p105/p50 and p100/p52 respectively lead to severe osteoporosis in mice (Novack, 2011).

Osteoclast differentiation is a highly regulated and intricate process that involves the transformation of mesenchymal stem cells into mature, multinucleated and specialized cells that are responsible for bone resorption and remodelling (Huo et al., 2023). The differentiation cascade is orchestrated by a complex interplay of signalling pathways and key regulatory factors. Initially, local microenvironmental cues, such as the presence of cytokines like macrophage colony-stimulating factor (M-CSF) and receptor activator RANKL, trigger the commitment of hematopoietic stem cells toward the osteoclastic lineage (Kim & Kim, 2016). The lack of RANKL as well as its receptor RANK in mice triggered severe osteoporosis – a condition of an excessive bone resorption, due to the imbalance of bone homeostasis (Jimi et al., 2019). In addition, the lack of RANKL decoy receptor in mice, osteoprotegerin (OPG) increased the occurrence of osteoporosis (Jimi et al., 2019).

Meanwhile, a study demonstrated that blocking NF- κ B/p65 led to an increase of osteoblast differentiation in myoblast C2C12 cells (Chang et al., 2009). Additionally, the study also reported that when NF- κ B was inhibited in young transgenic mice, there was a notable increase in trabecular bone density. Furthermore, inhibition of NF- κ B was found to promote the differentiation and mineralization processes of MC3T3 pre-osteoblastic primary murine bone marrow stromal cells (Yamaguchi & Weitzmann, 2009).

2.3.7 Effect of chronic inflammation towards bone

Pathological examples of inflammation encompass conditions where the immune response, which is meant to protect the body, becomes detrimental and contributes to tissue

damage or dysfunction. Two notable examples of inflammation towards bone are osteoarthritis (OA) and osteoporosis.

OA has been recognized as a progressive joint disorder marked by the deterioration of cartilage integrity and limitation of joint movement (Assad & Jackson, 2019). Various factors such as age, physical injury, genetic defect in joint cartilage and obesity are associated with the development of OA (Altman, 2019). In OA patients, the level of high mobility group box 1 (HMGB1) is higher in their synovial fluid when compared to healthy individuals (Shao et al., 2023). The HMGB1 and RAGE levels are upregulated in the knees affected by OA in comparison to the knees of unaffected individuals (Roh & Sohn, 2018). Similar to its role in other inflammatory disorders, extracellular HMGB1, being one of the DAMPs activates the canonical NF- κ B signalling pathway after binding to the PRRs. This action initiates the inflammation and the expression of HMGB1 also correlates with the extent of cartilage degradation (Terada et al., 2011).

Osteoporosis is a disorder characterized by the progressive decline in bone mass and the degradation of bone architecture, resulting in heightened vulnerability to fractures, notably in areas such as the wrist, hip, and spine (Dowson & Lewis, 2010). This condition arises from an imbalance between bone resorption, mediated by osteoclasts, and bone formation, carried out by osteoblasts, resulting in a net loss of bone density and structural integrity. Osteoporosis is commonly associated with ageing and hormonal changes, particularly in post-menopausal women (Cauley, 2013). In addition, osteoporosis is characterized by low bone turnover which refers to the continuous process of bone resorption (breakdown) by osteoclasts and bone formation (building) by osteoblasts (Pouresmaeili et al., 2018). In healthy individuals, bone turnover maintains a balance, ensuring bone strength and renewal. However, in osteoporosis, this balance is disrupted, leading to increased bone

resorption and reduced bone formation. As a result, bones become weaker, more porous, and susceptible to fractures. This disruption in bone turnover is a central factor contributing to the development and progression of osteoporosis. chronic inflammation plays a vital role in stimulating osteoporosis as shown in Figure 2.11.

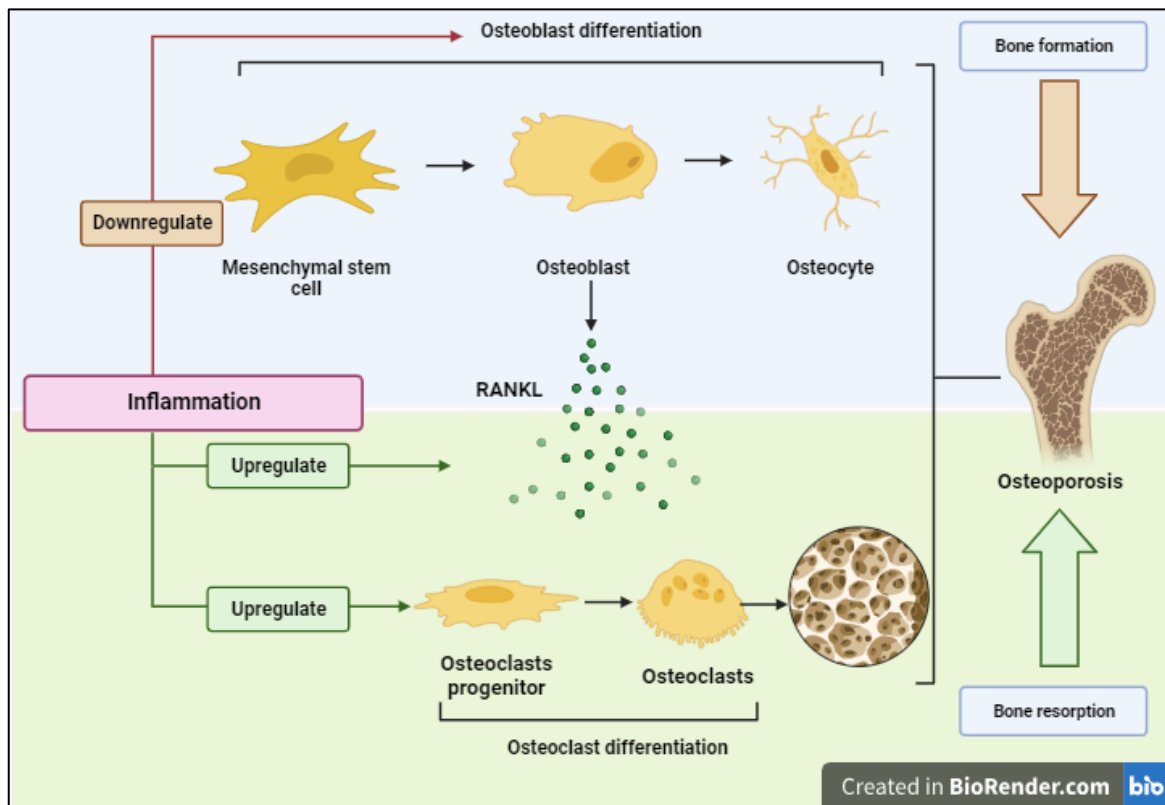


Figure 2.11: Inflammation downregulate osteoblast differentiation and leads to low bone formation. It also upregulates RANKL that binds to osteoclast, leading to an increased bone resorption. Furthermore, it also upregulates osteoclast differentiation that further leads to low bone turnover, leading to osteoporosis. Adapted from Berardi et al., (2021); Epsley et al., (2020); Terkawi et al., (2022).

As inflammatory response triggers NF- κ B which downregulates osteoblast differentiation by reducing the expression of the gene responsible for the process, this affect the bone turnover as bone formation is lesser than resorption (Chang et al., 2009; Yamaguchi & Weitzmann, 2009). Inflammatory responses involve TNF- α , a pivotal cytokine released during inflammation, which plays a crucial role in regulating bone resorption in

inflammatory conditions. This regulation occurs either directly or by stimulating osteoblasts and stromal cells to increase the expression of RANKL and M-CSF (Marahleh et al., 2019).

2.3.8 *T. crispa* and *T. cordifolia* in ameliorating inflammation

T. cordifolia has been used in ayurvedic treatment to treat multiple diseases including diseases that are related to inflammation. A study showed that chloroform extract of *T. cordifolia* reduces the pro-inflammatory markers in RAW264.7 macrophages. In a similar study, their in-vivo result showed that rats pre-treated with *T. cordifolia* had decreased paw oedema (Philip et al., 2018). In another study, *T. cordifolia* provides protection against anaemia linked to inflammation by regulating inflammatory cytokines and the expression of hepcidin in male Wistar rats (Ghatpande et al., 2019). Pre-treatment of RAW264.7 cells with *T. cordifolia* showed a decrease in the expression of TNF- α indicating anti-inflammatory properties of the extract (Ghatpande et al., 2019). Another research involved subjecting THP-cells pre-treated with lipopolysaccharide (LPS) to the chloroform extract of *T. cordifolia*. The study revealed that *T. cordifolia* exhibited the capability to diminish the elevated levels of pro-inflammatory markers within macrophages by hindering the translocation of NF- κ B into the nucleus. Furthermore, in instances of endotoxemia – the elevation of plasma levels of lipopolysaccharides, the chloroform extract of *T. cordifolia* significantly improved survival rates and effectively restrained the tissue expression of pro-inflammatory proteins in the in-vivo module of the study (Philip et al., 2021).

On the other hand, methanol stem extract of *T. crispa* inhibits the important inflammatory molecule signalling such as MCP-1 in TNF- α induced inflammation in HUVEC (Kamarazaman et al., 2012). In another study, methanol extract of *T. crispa* has

been found to significantly reduced the development of oedema in carrageenan-induced inflammation in rats (Lourdes et al., 2012). Furthermore, ethanol extract of *T. crispa* has been studied to upregulate phagocytosis and lysozymes in Wistar-Kyoto rats (Ahmad et al., 2016). Despite its anti-inflammatory properties, *T. crispa* also has been reported to possess pro-inflammatory activities. For example, methanol extract of *T. crispa* has been detected to potentially upregulate the expression of NF- κ B, IL-1 β , TNF- α in U397 human macrophages (Haque et al., 2020). Furthermore, a study by Abood et al., (2014) revealed that ethanol extract of *T. crispa* increased the production of pro-inflammatory cytokines in RAW264.7.

Despite the extensive research on these plants, the number of studies looking at their effects on bone cells is limited, especially immunomodulatory activities. Further comprehensive research is needed to elucidate the underlying mechanisms of these effects and to explore their potential applications in therapeutic interventions for inflammatory conditions.

2.4 Insulin signalling in bone cells for healthy and diabetic individuals

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycaemia, resulting from insufficient insulin secretion, impaired insulin action, or a combination of both (Gopalan & Kirk, 2022). While a fasting blood glucose level of 70–110 mg/dL is considered within the normal range, values ranging from 100 to 125 mg/dL signify prediabetes, with diabetes defined by a blood glucose level of 126 mg/dL or greater. Furthermore, DM serves as a diagnostic term for a cluster of disorders marked by irregular glucose regulation leading to increased blood sugar levels. This encompasses a range of metabolic diseases wherein elevated glucose concentrations arise due to deficiencies in the body's capability to generate and/or effectively employ insulin (Li & Stocum, 2014; Raffel & Goodarzi, 2014).

Diabetes mellitus can be categorized into two main types: type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). T1DM is characterized by autoimmune destruction of pancreatic beta cells, leading to an absolute deficiency of insulin production (Burrack et al., 2017). On the other hand, T2DM is characterized by insulin resistance and progressive insulin deficiency (Fleury-Milfort, 2012).

A study in Malaysia by Akhtar et al., (2022) found that 14.39% of the 103063 participants had T2DM and 11.62% of the participants were prediabetic. In addition, the same study revealed that among the different ethnic groups, diabetes was most prevalent among Indians (25.10 %), followed by Malays (15.25 %) then Chinese (12.87 %), Bumiputera (8.62 %), and others (6.91 %). The outcomes of this investigation indicate a considerable occurrence of prediabetes and diabetes in Malaysia.

T2DM is associated with a spectrum of complications such as diabetic ketoacidosis, hypoglycaemia, hyperglycaemia, diabetic retinopathy, diabetic foot as well as osteoporosis (Farmaki et al., 2021; Lin et al., 2021). Achieving and maintaining glycaemic control is crucial in preventing or delaying the onset of long-term complications. Nonetheless, the intricate pathophysiology and clinical heterogeneity of diabetes necessitate ongoing research to unravel its underlying mechanisms and to develop more targeted and effective therapeutic strategies.

2.4.1 Glucose metabolism in healthy and T2DM individuals

Glucose metabolism within cells is a complex and highly regulated process that plays a fundamental role in providing energy for cellular activities as shown in Figure 2.12. This intricate biochemical pathway involves the uptake, utilization, and storage of glucose molecules. The process of breaking down food into glucose leads to an increase in blood

glucose concentration, which subsequently triggers the release of insulin by the pancreas (Senior & Shapiro, 2007).

Insulin then binds to the alpha subunit of tyrosine kinase insulin receptor (IR) located on the plasma membrane of target cells, which further activates autophosphorylation of the beta subunit of the tyrosine kinases of the IR (Lizcano & Alessi, 2002). Autophosphorylation of the tyrosine kinases further phosphorylates insulin receptor substrate (IRS), before IRS stimulates the p85 subunit to bind with P110, activating phosphoinositide-3 kinase (PI3K) (Ueki et al., 2002). Activated PI3K then phosphorylate phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-biphosphate (PIP3) (Świdarska et al., 2020). Furthermore, increased PIP3 then activates the phosphoinositide-dependent kinase 1 (PDK1) to phosphorylate protein kinase B (Akt). Świdarska et al., (2020) further explained that in cells that are not yet stimulated by insulin, an activating protein known as AS160 continuously inhibits translocation of GLUT4 vesicle. Activated Akt phosphorylates AS160, stopping its inhibiting feature towards glucose transporter (GLUT) e.g., GLUT4 vesicle translocation (Lizcano & Alessi, 2002a; Świdarska et al., 2020). GLUT4 translocate to the plasma membrane, providing a passage for glucose to enter the cell to be metabolized. Glucose that enters the cell will undergo glycolysis and electron transport chain (ETC) to produce energy in terms of adenosine triphosphate (ATP).

T2DM is characterized by a series of disruptions within the intricate insulin signalling pathway, ultimately resulting in diminished response of target cells towards insulin, a condition referred to as insulin resistance. According to Freeman et al., (2023), insulin resistance is recognized as the compromised physiological reaction of target tissues to the stimulation provided by insulin. Insulin resistance hampers the effective management

of glucose, leading to a compensatory rise in insulin production by the beta cells in the pancreas and subsequent hyperinsulinemia. This phenomenon sparks a cascade of metabolic changes that lead to elevated blood glucose levels, otherwise known as hyperglycaemia (Freeman et al., 2023).

As the body's cells become less receptive to insulin, key events in the signalling pathway are disrupted. The phosphorylation of insulin receptor substrates (IRS) is compromised in insulin-resistant states, leading to the impaired activation of downstream signalling pathways. This is because T2DM patients exhibit elevated concentrations of ubiquitination proteins that increase the activity within the ubiquitin/proteasome that could potentially accelerate the breakdown of IRS proteins, possibly intensifying the state of insulin resistance (Z. Liu et al., 2023; White, 2002). Consequently, the translocation of glucose transporter 4 (GLUT4) to the cell surface, a process crucial for glucose uptake, is hindered, contributing to the rise in blood glucose levels. The body's initial countermeasure involves producing higher levels of insulin, known as hyperinsulinemia, in an attempt to override the reduced cellular response (Thomas et al., 2019).

Notably, insulin normally curbs gluconeogenesis, the liver's production of glucose from non-carbohydrate sources (Bhagavan, 2002). In T2DM, this regulatory mechanism falters, leading to an excessive output of glucose by the liver. Enhanced gluconeogenesis in the liver of individuals with T2DM is seen as a big reason for high blood sugar and the resulting harm to organs. Insulin acts as an important hormone that normally inhibits this process and it does not work well in T2DM individuals due to insulin resistance (Hatting et al., 2018).

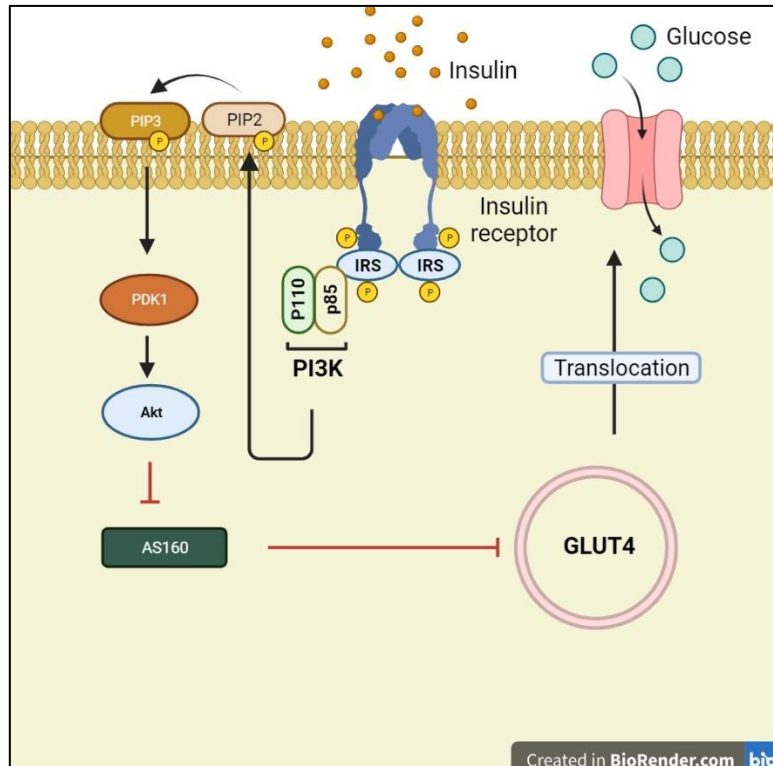


Figure 2.12: The binding of insulin towards its receptor (IR) stimulates PI3K cascade reaction, which phosphorylates PIP2 to PIP3, activating PDK1 and Akt, stopping Akt inhibition towards AS160 protein. The inhibition of AS160 allows GLUT4 to translocate to the membrane, allowing glucose molecules to enter and to be further metabolised into the cells. Adapted from Zakaria et al., (2021).

The complex disturbances in the insulin signalling pathway in T2DM intricately play a role in the development of insulin resistance. This, in turn, sets off a series of metabolic imbalances that result in elevated levels of glucose in the blood.

2.4.2 T2DM and osteoporosis

T2DM and osteoporosis are two prevalent chronic conditions that share complex interrelations, often presenting a challenging clinical scenario. Accumulating evidence suggests that T2DM is associated with an increased risk of osteoporosis and fractures. For instance a study by Lin et al., (2021) revealed an elevated susceptibility to osteoporosis in those with T2DM compared to individuals who are not affected by the condition.

Bone metabolism is a highly intricate and dynamic process orchestrated by a delicate balance between bone-forming cells (OB) and bone-resorbing cells (osteoclast). This regulated interplay is pivotal for maintaining bone structure, density, and strength throughout life. The process begins with the differentiation of mesenchymal stem cells, found in the bone marrow and periosteum, into OB. These precursor cells undergo a series of developmental stages, including proliferation and maturation, characterized by the secretion of extracellular matrix components, including collagen type I and osteocalcin (Rutkovskiy et al., 2016; Amarasekara et al., 2021). Runt-related transcription factor 2 (RUNX2) together with other transcription factors plays important roles towards osteoblastogenesis. The process of osteoblastogenesis is shown in Figure 2.13.

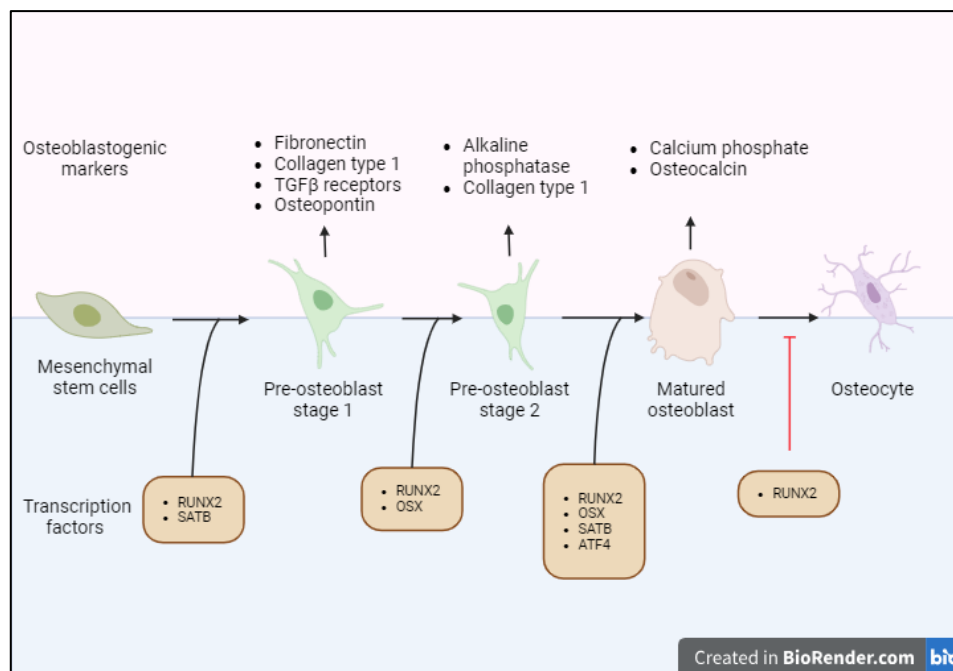


Figure 2.13: Osteoblastogenesis and osteocyte differentiation. Mesenchymal stem cells are regulated by RUNX2 and SATB to be differentiated into pre-osteoblasts. Pre-osteoblast stage 1 and 2 released several osteoblastogenic markers that include collagen type 1, osteopontin as well as alkaline phosphatase. Meanwhile, matured osteoblast release osteocalcin. RUNX2 plays important role in osteoblastogenesis, but it inhibits the differentiation of osteoblasts to osteocytes. SATB, special AT-rich sequence binding protein B. OSX, osterix. ATF4, activating transcription factor 4. Adapted from Amarasekara et al., (2021); Komori, (2018); Rutkovskiy et al., (2016); Soltanoff et al., (2009).

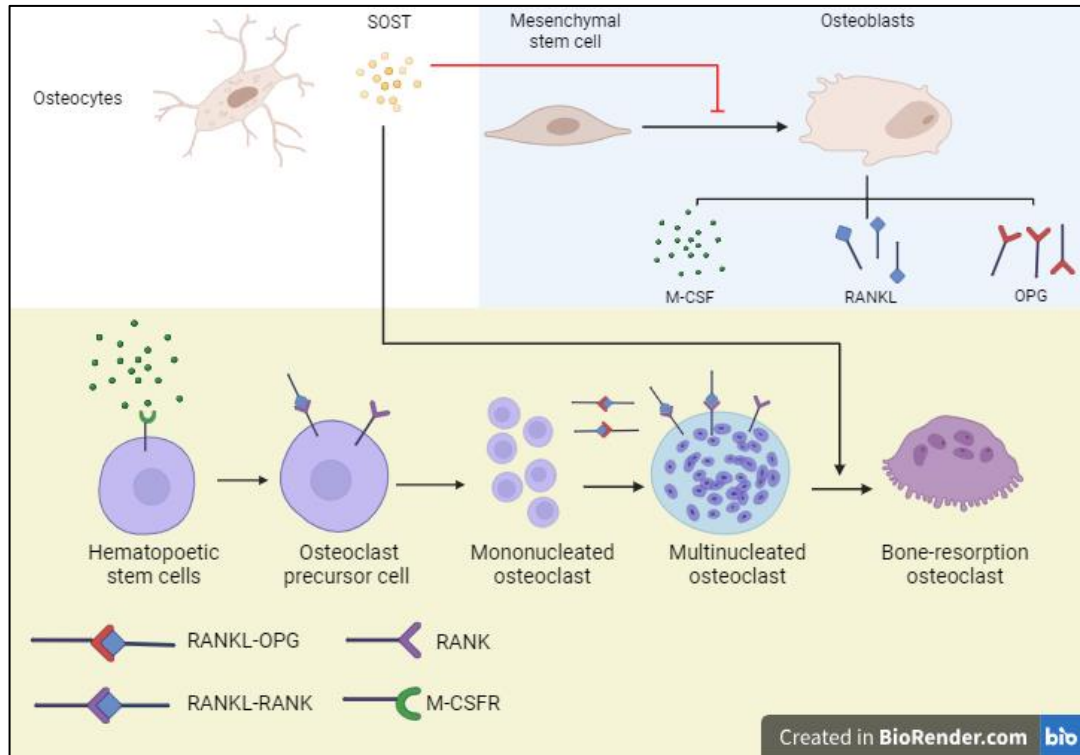


Figure 2.14: The process of osteoclastogenesis. Osteoclast differentiated from hematopoietic stem cells and regulated by M-CSF, which is released by osteoblasts. Osteoclastogenesis also is regulated by RANKL, and counter-regulated by OPG to maintain healthy bone homeostasis. Osteocyte also releases SOST, which helps to stimulate osteoclastogenesis and at the same time prevent osteoblastogenesis. Adapted from Han et al., (2018); Kim et al., (2020); Weivoda et al., (2017).

As osteoblasts and other stromal cells express RANKL, these molecules interact with RANK on the surface of osteoclast precursor cells, triggering a signalling cascade that leads to the differentiation and fusion of mononucleated osteoclast precursors into multinucleated osteoclasts (Han et al., 2018). The balance of RANKL signalling is counteracted by osteoprotegerin (OPG), which serves as a decoy receptor by binding to RANKL and preventing its interaction with RANK, thus downregulating osteoclastogenesis (Kang et al., 2014). Interestingly, osteocytes, the master regulators of bone homeostasis, participate in this intricate process by releasing sclerostin (SOST). Its primary role is to inhibit osteoblastogenesis and bone formation by antagonizing the Wnt signalling pathway (Weivoda et al., 2017). However, it paradoxically contributes to osteoclastogenesis

indirectly by dampening bone formation, which reduces the osteoblast-derived OPG. As OPG decreases, the balance tilts in favour of RANKL-induced osteoclastogenesis, augmenting bone resorption (Zhang et al., 2022). Thus, the dynamic interplay of RANK, RANKL, OPG, and sclerostin orchestrates the finely tuned equilibrium between bone formation and resorption, ultimately maintaining bone integrity and functionality.

In conclusion, bone metabolism involves the orchestrated actions of osteoblasts and osteoclasts, arising from stem cell differentiation to mature functional cells. The dynamic balance between these cell types is crucial for bone health, ensuring proper remodelling, structural integrity, and mineral homeostasis. Understanding this complex process offers insights into treating various bone-related disorders and underscores the essential role bones play in maintaining overall health.

Bone metabolism plays a pivotal role towards insulin secretion by the pancreas as shown in Figure 2.15. The insulin receptor which is a transmembrane protein belonging to the receptor tyrosine kinase family, plays a fundamental role in bone homeostasis (Ferron et al., 2010). Insulin exerts a positive effect on osteoblast differentiation and function, supporting the bone formation and promoting the release of osteocalcin (OC) in mice as reported by Ivaska et al., (2015). This connection between insulin receptor signalling and osteoblast activity establishes a link between glucose metabolism and bone health, suggesting a potential bidirectional relationship (Ferron et al., 2010).

Osteocalcin plays multiple roles in the physiological processes. For instance, ucOC secreted into the bloodstream stimulates the pancreas to release insulin. Then, insulin binds to their receptors, allowing the glucose uptake into the cells (Rosen & Motyl, 2010). Moreover, one of the aspects of OC is that it is essential for brain behaviour in the mouse

since its deficiency resulted in a significant discrepancy in spatial learning and memory and an inducing of anxiety-like activities (Obri et al., 2018).

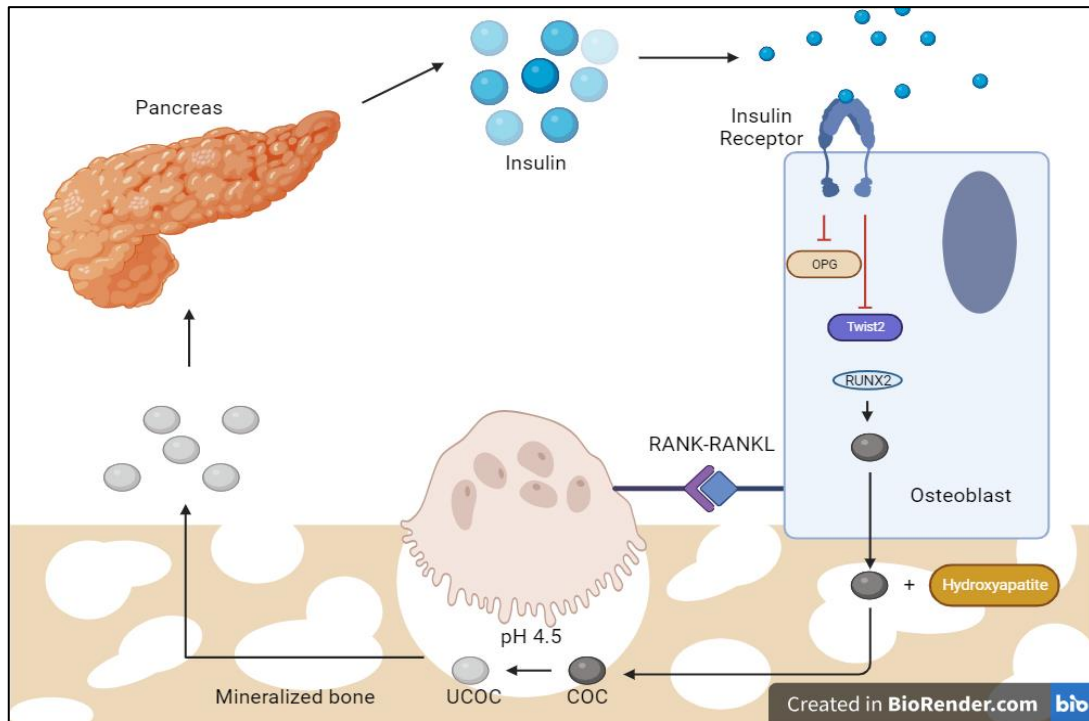


Figure 2.15: The binding of insulin towards its receptor inhibits the function of OPG, and Twist2 which is the transcription factor that downregulate RANKL-RANK and RUNX2 function respectively. Inhibiting the decoy receptor (OPG) and transcription factor (Twist2) lead to the release of UCOC from the matrix to the circulation. UCOC stimulates pancreas to release more insulin, leading to the bidirectional relationship between insulin secretion and bone homeostasis. Adapted from Rosen & Motyl, (2010).

Insulin affects osteoblasts and osteoclasts, the bone-resorbing cells. Osteoblasts express insulin receptors, and insulin signalling can influence their differentiation and mineralization activities. The cross-talk between insulin and bone cells underscores the intricate interplay between metabolic regulation and bone homeostasis. Osteoblasts are tightly regulated by transcription factors such as RUNX2 and Twist2. Twist2, another transcription factor, participates in osteoblast differentiation and also inhibits osteoclastogenesis, the process by which osteoclasts are formed. When insulin binds with its receptor, the function of Twist2 is halted, allowing osteoclastogenesis (Rosen & Motyl,

2010). RUNX2 is a master regulator of osteoblast differentiation, controlling the expression of genes essential for bone matrix synthesis. This dual role of Twist2 in regulating both osteoblast and osteoclast activities establishes a potential molecular link between bone formation and resorption. OPG, a member of the TNF receptor superfamily, plays a critical role in balancing bone remodelling. It acts as a decoy receptor for RANKL, a key regulator of osteoclastogenesis. By binding to RANKL, OPG inhibits its interaction with RANK, thus suppressing osteoclast differentiation and bone resorption (Bengtsson & Ryan, 2002). The OPG/RANKL/RANK system represents a regulatory axis that connects bone formation and resorption, and its interaction with insulin signalling contributes to the broader physiological integration between metabolism and bone health.

The dynamic interplay between insulin, osteoblasts, OPG, and osteoclasts influences bone remodeling and mineralization processes. Osteoclasts resorb bone by dissolving hydroxyapatite, the mineralized component of bone matrix composed primarily of calcium phosphate. The controlled release of calcium and phosphate ions during bone resorption impacts overall mineral homeostasis. Additionally, the intricate molecular connections between insulin signalling, osteoblast differentiation, OPG-RANKL interactions, and osteoclastogenesis emphasize the potential bidirectional communication between the endocrine and skeletal systems, suggesting a coordinated regulation of insulin secretion and bone resorption.

In conclusion, the cross-talk between insulin receptor signalling, insulin, osteoblasts, OPG, Twist2, RUNX2, osteoclast resorption, and hydroxyapatite underscores the complex interrelation between metabolic regulation, bone homeostasis, and the potential influence on insulin secretion and bone remodelling. This integration highlights the multifaceted nature

of physiological systems and encourages further exploration into the intricate connections that govern overall health.

2.4.3 *T. crispa* and *T. cordifolia* in ameliorating diabetes

Studies have suggested that both *T. crispa* and *T. cordifolia* possess bioactive compounds such as alkaloids, glycosides, and terpenoids that exhibit anti-diabetic properties. These compounds have been reported to enhance insulin sensitivity, stimulate glucose uptake in cells, and exert antioxidant effects that mitigate oxidative stress, a hallmark of diabetes. Moreover, these plants have demonstrated the ability to modulate key enzymatic activities involved in carbohydrate metabolism, thereby aiding in the regulation of blood glucose levels. While promising, it is crucial to note that further scientific investigation, including clinical trials, is necessary to fully understand and validate the potential of *T. crispa* and *T. cordifolia* as complementary or alternative therapeutic agents for diabetes management.

T. cordifolia has garnered significant attention in the field of diabetes research, demonstrating potential anti-diabetic effects through various mechanisms. One such mechanism involves the regulation GLUT4 expression in differentiated myocytes. It was observed that *T. cordifolia* extract, along with the bioactive compound palmatine, led to upregulation of GLUT4 expression by up to 5-fold and 4-fold respectively, indicative of enhanced glucose uptake by muscle cells (Sangeetha et al., 2013).

Further investigations into the anti-hyperglycaemic properties of *T. cordifolia* were conducted using Swiss albino mice as a model. The result from Sharma et al., (2021) revealed a distinct reduction in blood glucose levels upon administration of *T. cordifolia*. This finding not only underscores the potential of *T. cordifolia* in managing hyperglycaemia

but also suggests its prospective development as a pharmaceutical agent for treatment of diabetes.

Additionally, the anti-hyperglycaemic effects of *T. cordifolia* were examined in streptozotocin-induced diabetic rats. This research that had been done by Daisy et al., (2009) assessed different parameters such as HbA1c, serum insulin, C-peptide, and liver enzymes. The outcomes of this investigation demonstrated the significant efficacy of the methanolic extract of *T. cordifolia* in reducing fasting blood glucose levels and glycosylated haemoglobin levels when compared to the diabetic control group ($p < 0.001$). Furthermore, improvements in insulin and C-peptide levels were observed following treatment with *T. cordifolia*, further emphasizing its potential to ameliorate the derangements associated with diabetes.

In an *in-vivo* study that utilized animal models to stimulate insulin deficiency, the synergistic application of *T. crispa* in conjunction with *Anredera cordifolia* demonstrated a significant reduction in blood sugar levels over a 14-day period. Furthermore, in the *in-vitro* study, the combination of both extracts highlighted their ability to inhibit alpha-glucosidase enzymes, indicative of potential anti-hyperglycaemic properties (Kusriani et al., 2023).

Additionally, an investigation revealed *T. crispa*'s efficacy in enhancing insulin sensitivity in T2DM-induced rats. Specifically, the compound borapetoside C derived from *T. crispa* exhibited the ability to delay the development of insulin resistance and enhance insulin sensitivity in the context of induced diabetes (Ruan et al., 2012).

Moreover, a focus on in L6 myoblast skeletal muscle cells provided further insights into the potential benefits of *T. crispa*. Through experimentation, it was observed that treatment with aqueous *T. crispa* at a concentration of 4 mg/mL significantly enhanced

glucose uptake in L6 myotubes, showing both dose and time-dependent responses. These effects were comparable to established anti-diabetic agents, metformin, and troglitazone (Klangjareonchai et al., 2015).

In conclusion, *T. crispa* and *T. cordifolia* exhibit promising potential as natural agents for the management of diabetes. Through a variety of scientific investigations, these botanical sources have demonstrated significant anti-diabetic effects by targeting multiple mechanisms associated with hyperglycaemia and insulin resistance. The research has highlighted their ability to enhance glucose uptake, modulate insulin sensitivity, and inhibit key enzymes involved in carbohydrate metabolism. Moreover, both *T. crispa* and *T. cordifolia* have shown efficacy in reducing blood glucose levels in animal models of diabetes, further substantiating their potential therapeutic utility. Nevertheless, while these studies provide compelling evidence, the translation of these botanical agents into mainstream medical practice requires rigorous clinical trials to validate their safety and efficacy in human populations. Therefore, while *T. crispa* and *T. cordifolia* offer exciting prospects, continued research is essential to fully harness their therapeutic benefits for individuals living with diabetes.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

All reagents used were of analytical grade suitable for cellular analysis. Some reagents were diluted using deionized ultra filtered water, produced using the ultrapure water system from ELGA Purelab Medica R-7/15 (UK). The list of materials used for the experiment is shown in Table 3.1.

Table 3.1: List of materials used for the experiment.

Materials and apparatus	Manufacturer
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) kit	R&D System, Minnesota, USA
Acridine orange / Propidium Iodide (AO/PI)	Thermo Fisher, Massachusetts, USA
Aminoguanidine (AG)	Sigma Aldrich, St. Louis, USA
Ascorbic acid	Sigma Aldrich, St. Louis, USA
Bovine Serum Albumin (BSA)	Sigma Aldrich, St. Louis, USA
Dulbecco's Phosphate Buffer Saline (DPBS)	Corning, New York, USA
Hanks Buffer Salt Solution (HBSS)	Applied Biosystems, USA
Metformin	Tocris Bioscience, Bristol, UK
Methanol (analytical grade)	Fisher Scientific, Loughborough, UK
Methylglyoxal (MGO)	Sigma Aldrich, St. Louis, USA
Nuclear Lysate Extraction buffer	Novus Biologicals, USA
RIPA Lysis Buffer	Thermo Fisher, Massachusetts, USA
Serological pipette (5 mL and 10 mL)	Corning, New York, USA,
T-25 flasks	Corning, New York, USA

3.1.2 Cell culture media

The list of materials for cell culture experiments is shown in Table 3.2.

Table 3.2: List of materials for cell culture experiments

Materials	Manufacturer
Dulbecco's Modification of Eagles Medium (DMEM) / HAMS F-12 with L-Glutamine and HEPES	Corning, New York, USA
Foetal Bovine Serum (FBS)	Capricorn Scientific, Ebsdorfergrund, Germany
Penicillin-Streptomycin	Corning, Virginia, USA

3.1.3 Enzyme-linked immunosorbent assay (ELISA) kits

The kits for Enzyme-linked immunosorbent assay are listed in Table 3.3.

Table 3.3: List of ELISA kits

Materials	Manufacturer
Human Bcl-2 ELISA Kit (NBP1-91188)	Novus Biologicals, USA
Human caspase-3 ELISA Kit (CSB-E08856h)	Cusabio, Texas, USA
Human Core Binding Factor Alpha1 CBFA1/Runx2 ELISA Kit (CSB-E12935h)	Cusabio, Texas, USA
Human GLUT4 ELISA Kit Colorimetric (NBP2-82174)	Novus Biologicals, USA
Human IL-6 Immunoassay (D6050)	R&D Systems, Minnesota, USA
Human INSR – Insulin Receptor (NBP2-68039)	R&D Systems, Minnesota, USA
Human Total IL-18 DuoSet ELISA (DY318-05)	R&D Systems, Minnesota, USA
NF- κ B/p65 ActivELISA TM (NBP2-29661)	Novus Biologicals, USA
Oxiselect™ Advanced Glycation End Product (AGE) Competitive ELISA Kit (STA-817)	Cell Biolabs Inc., California, USA
Oxiselect™ Intracellular ROS Assay Kit – Green Fluorescence (STA-342)	Cell Biolabs Inc., California, USA

3.2 Plant powder for extractions

The powdered mixture of leaves and stems of *T. crispa* were purchased from Shine Tech Solutions, Pahang, Malaysia. Meanwhile, powdered leaves and stems of *T. cordifolia* were purchased from Gtee Botanical Extract Pte Ltd, Chennai, India (ISO No. 9001:22000). (Appendix A).

3.3 Preparation of methanolic and aqueous extracts of *T. crispa* and *T. cordifolia*

Twenty g of *T. crispa* plant powder and 20 g of *T. cordifolia* powder were added separately to 400 mL of 90% methanol (Fisher Scientific, UK). Each mixture was left sitting in the dark at 24°C - 25°C environment for 24 hours. Then, the mixture was filtered through filter paper with pore size of 8 – 12 µm (filtraTECH, France) (Appendix B). The solvent was further evaporated using a rotary evaporator. The same procedure was repeated for producing boiled water extracts (aqueous extracts) at 100°C. After rotary evaporation (Appendix C), the extracts underwent freeze-dry procedure to fully eliminate excess water before being stored in a -20 °C freezer for further experimental procedures.

3.4 Non-cells *in-vitro* antioxidant assays

The antioxidant properties of methanolic and aqueous *T. cordifolia* and *T. crispa* were assessed through 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging and bovine-serum albumin – methylglyoxal (BSA-MGO) anti-glycation assay.

3.4.1 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging

DPPH antioxidant assay was performed with slight modifications, adapted from (Zulkefli et al., 2013). The standard used was ascorbic acid (0.0375 mg/mL, 0.125 mg/mL, 0.25 mg/mL, and 0.5 mg/mL). In brief, 100 µL of 0.1 mM DPPH in methanol was added to 800 µL of ascorbic acid standards. Similarly, 100 µL of 0.1 mM DPPH in methanol was

added to 800 μ L of extracts with the same concentrations as the standards. The absorbance was measured at 517 nm after 15 minutes. To get the IC₅₀ value, the percentage of inhibition was calculated, and the graph was produced. The DPPH inhibition in percentage was calculated using the following formula:

$$\text{DPPH inhibition(\%)} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{extracts}}}{\text{Absorbance}_{\text{control}}} \times 100\% \quad \text{Equation 3.1}$$

3.4.2 Bovine serum albumin (BSA) – Methylglyoxal (MGO) glycation assay

The BSA-MGO glycation assay was adapted from the method by Starowicz & Zieliński, (2019). BSA and MGO were supplied by Sigma Aldrich (St. Louis, USA). Then, BSA with the concentration of 2 mg/mL and 400 mg/mL MGO were dissolved in PBS (pH 7.4). Sample tubes containing 300 μ L of BSA, 300 μ L of MGO, or 300 μ L of extract solution ranging from 50 μ g/mL to 400 μ g/mL were prepared. A mixture of 300 μ L of BSA, 300 μ L of MGO and 300 μ L of PBS was considered as a negative control in this assay. Positive control was prepared by mixing 300 μ L of 1 mol/L aminoguanidine (AG) with 300 μ L of MGO and 300 μ L of BSA. AG also served as the positive control. These tubes were incubated at 37°C for 7 days. PBS was considered as blank, and all absorbance values were deducted with the blank absorbance value before proceeding with calculations. The relative fluorescence unit (RFU) was read using fluorescence with the wavelength of (excitation, 340 nm; emission, 420 nm) using microplate reader SpectraMax iD3 (Molecular Devices, USA). The percentage of glycation inhibition was calculated using the following formula:

$$\text{Glycation inhibition (\%)} = \frac{\text{RFU}_{\text{control}} - \text{RFU}_{\text{extracts}}}{\text{RFU}_{\text{control}}} \times 100\% \quad \text{Equation 3.2}$$

3.5 Gas chromatography mass spectrometry (GC-MS) assay

This GC-MS analysis was carried out in accordance with the methodology described by Samling et al., (2021) utilizing a Shimadzu GC-MS-QP2010 Plus (Shimadzu, Japan). The fraction collector (FC) separation was carried out using a BPX-5 fused capillary column (Trajan Scientific and Medical (Australia) coated with fused silica phenyl polysilphenylene-siloxan (5%) with a film thickness of 30 m × 0.25 m. Electron impact with ionization energy of 70 electron volts (eV) with interface temperature of 250°C were applied together with scan mass ranging from 28-400 m/z. Helium was used as carrier gas with the flow rate of 1.0 mL/min. The injection volume was 1.0 µL volume, with a split ratio of 20:1. Using the National Institute of Standards and Technology version 17 (NIST-17) mass spectral library incorporated in the system, the phytochemical compounds such as polyphenols in both methanolic and water extracts were identified.

3.6 Cell culture of hFOB1.19

The human foetal osteoblast (hFOB 1.19) (CRL-3602) cells were a bought from the ATCC (American Type Culture Collection) from Manassas USA after the material transfer agreement form was signed (Appendix F). The vial was removed from dry ice and defrosted in a 37°C water bath for 2 mins. In a Class II biological safety cabinet, the liquid in both vials was then poured into 5.0 mL of Dulbecco's Modification of Eagle's Medium (DMEM) / HAMS F-12 supplemented with L-glutamine, 15 mM HEPES (Corning, NY, USA), 5 % foetal bovine serum (Corning, NY, USA) and 1 % penicillin-streptomycin (Corning, NY, USA) in T-25 cell culture flasks (Corning, NY, USA). For the cell culturing, the cells were kept in 5 % CO₂ incubator at 37 C (Esco Scientific, Singapore). The cell growth was observed until it reached confluency after 3 days. The cell was passaged into another flask and cell concentration was maintained at 0.5 – 1 × 10⁶ cells/mL by changing

media and transferring cells to T-25 flasks after it reached 80 – 90 % confluency. The cell was counted using trypan blue dye cells under a microscope using a haemocytometer. The cells were counted by adding 100 μ L of cells suspension to 100 μ L trypan blue and mounting onto a haemocytometer. Passage 10 of the cells were used for all cell-related assays unless it was stated otherwise.

3.7 Preliminary cell viability assessment

The concentration range for each of the treatments were initiated through series of pilot studies. The largest range of concentrations were tested towards hFOB1.19 before several selected concentrations were confirmed. The experiments were repeated several times to ensure that the concentrations selected were suitable for the upcoming assays.

The IC₅₀ values for MGO, and non-toxic concentrations for metformin and extracts were evaluated using a MTT as well as an AO/PI assay. Table 3.4 shows the treatment concentration for MGO and metformin while Table 3.5 presented the treatment concentration for methanolic plant extracts. Meanwhile, Table 3.6 presented the treatment concentration for aqueous plant extracts.

Table 3.4: Treatment concentrations (μ M) for MGO and metformin

Treatments	Treatment concentrations (μ M)
Methylglyoxal	875
	1225
	1400
	1575
	1750
Metformin	1500
	3000
	6000
	12000

Table 3.5: Treatment concentrations ($\mu\text{g/mL}$) for methanolic *T. crispa* and *T. cordifolia* extracts.

Plant extracts	Treatment concentrations ($\mu\text{g/mL}$)
Methanolic <i>T. crispa</i>	0.00
	30.00
	60.00
	120.00
	240.00
Methanolic <i>T. cordifolia</i>	0.00
	12.50
	25.00
	50.00
	100.00

Table 3.6: Treatment concentrations ($\mu\text{g/mL}$) for aqueous *T. crispa* and *T. cordifolia* extracts.

Plant extracts	Treatment concentrations ($\mu\text{g/mL}$)
Aqueous <i>T. cordifolia</i>	0.00
	15.63
	31.25
	62.50
	125.00
Aqueous <i>T. crispa</i>	0.00
	46.88
	93.75
	187.50
	375.00

3.8 Determination of cell viability through MTT and AO/PI

The cell viability of hFOB1.19 was determined by two types of viability assays. The first one is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay was performed to examine the cellular metabolism, which indirectly measured the cellular viability. Furthermore, cell viability also was assessed through its membrane integrity, and this was measured through Acrymidine Orange/Propidium Iodide (AO/PI) assay.

For MTT assay, the protocol followed the instructions provided by Bio Techne R&D systems MTT kit (Minnesota, USA). The hFOB1.19 cells were seeded in 96 well plate in triplicates with the density of 2×10^4 cells/mL. They were then treated with different concentrations of MGO, ranged from (875 to 1750 μ M) as shown in Table 3.4 for 24 hours. Then, 10 μ L of MTT was added to each well and the plate was incubated for another 24 hours. The treated cells were then added with detergent reagent and let incubated for 6 hours at room temperature. The absorbance reading was measured at a wavelength of 570 nm, with empty media as blank. The cell viability vs. metformin concentration graph was generated and cell viability in percentage was assessed using the following formula :

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of samples} - \text{Absorbance of blank}}{\text{Absorbance of control} - \text{Absorbance of blank}} \times 100\% \quad \text{Equation 3.3}$$

3.8.1 Determination of IC₅₀ value for MGO from MTT assay

The cell viability graph was plotted with the percentage of cell viability (%) on the y-axis against the concentration of MGO (μ M) on the x-axis. Then, a regression (R^2) value that showed a value of equal to or more than 0.95 was determined. Presuming that the dependent variable (cellular viability) was correlated with the independent variable (concentration of MGO) in a linear correlation, then $y = mx + c$ formula was then be

applied. To calculate the IC₅₀, the y-value was replaced with 50, and the x-value was determined. An example of IC₅₀ concentration calculation was calculated according to the formula is shown below:

Assuming a linear correlation between dependent and independent variable from the graph and regression value (R^2) was or more than 0.95, based on the linear formula ($y = mx + c$) generated from the graph,

x = IC₅₀ concentration for MGO (μ M)

y = 50 % viable hFOB1.19

Thus, the derived equation:

$$\text{IC}_{50} \text{ concentration for MGO } (\mu\text{M}) = \frac{50 \% \text{ viable hFOB1.19} - (y - \text{intercept})}{\text{gradient value from the graph}} \quad \text{Equation 3.4}$$

For AO/PI assay, the protocol followed the instructions provided by Invitrogen™ ReadyCount™ Stains AO/PI kit (Thermo Fisher, Massachusetts, USA). Similarly, the hFOB1.9 cells were seeded with the density of 2×10^4 cells/mL in triplicates before they were treated with different concentrations of MGO (875 to 1750 μ M) for 24 hours as shown in Table 3.4. Then, 10 μ L treated cells were mixed with 10 μ L of ReadyCount™ Stain. The mixture was then incubated for 5 minutes at room temperature and away from light. Then, 10 μ L of the stained cells were loaded into a cell counter device (CellDrop by DeNOVIX). The wavelength range used was between 535 to 617 nm. The stained cells were counted automatically through the device for its viability in percentage.

3.8.2 Determination of non-toxic concentration of metformin

This protocol followed the instructions provided by Bio Techne R&D systems MTT kit (Minnesota, USA). The hFOB1.19 cells were seeded in 96 well plate in triplicates with the density of 2×10^4 cells/mL. They were then treated with different concentrations of metformin (Tocris Bioscience (Bristol, UK) ranging from (1500 to 12000 μ M) as shown in Table 3.4 for 24 hours. Then, 10 μ L of MTT was added to each well and the plate was incubated for another 24 hours. The treated cells were then added with detergent reagent and let incubated for 6 hours at room temperature. The absorbance reading was measured at a wavelength of 570 nm, with empty media as blank. The cell viability vs. metformin concentration graph was generated and cell viability in percentage was assessed using the following equation :

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of samples} - \text{Absorbance of blank}}{\text{Absorbance of control} - \text{Absorbance of blank}} \times 100\% \quad \text{Equation 3.5}$$

Then, a graph was plotted with the percentage of cell viability (%) on the y-axis and the concentration of MGO (μ M) on the x-axis. For AO/PI assay, the hFOB1.9 cells were seeded in triplicates, with a density of 2×10^4 cells/mL before they were treated with similar concentrations of metformin (1500 to 12000 μ M) as shown in Table 3.4 for 24 hours. Ten μ L of treated cells were mixed with 10 μ L of ReadyCount™ Stain and incubated for 5 minutes at room temperature. Then, 10 μ L of the mixture was loaded into cell counter device (CellDrop by DeNOVIX). The stained cells were also counted automatically through the device for its viability in percentage.

For both MTT and AO/PI assays, the highest concentration that resulted in ≥ 80 % viable hFOB1.19 when treated with metformin was considered non-toxic towards the cells

and used for cell treatments. 12000 μ M concentration of metformin has been selected for the subsequent assays.

3.8.3 Determination of non-toxic concentrations for plant extracts

For MTT assay, the protocol followed the instructions provided by R&D systems MTT kit. The hFOB1.9 cells were seeded in 96 well plate with the density of 2×10^4 cells/mL in triplicates. They were then treated with different concentrations of methanolic *T. crista* (30 – 240 μ g/mL), methanolic *T. cordifolia* (12.5 – 100 μ g/mL), aqueous *T. crista* (46.88 – 375 μ g/mL) and aqueous *T. cordifolia* (15.63 – 125 μ g/mL) for 24 hours as shown in Table 3.6. Then, 10 μ L of MTT was added to each well and the plate was incubated for another 24 hours. The treated cells were then added with detergent reagent and let incubated for 6 hours. The absorbance reading was measured at a wavelength of 570 nm, with empty media as blank. The cell viability vs. metformin or extracts concentration graph was plotted and analysed.

For AO/PI assay, the hFOB1.9 cells were seeded in triplicates, with a density of 2×10^4 cells/mL before they were treated with different concentrations of plant extract as shown in Table 3.5 and Table 3.6 for 24 hours. 10 μ L treated cells were mixed with 10 μ L of ReadyCount™ Stain. The mixture was then incubated for 5 minutes at room temperature. Then, 10 μ L of the stained cells were loaded into a cell counter device (CellDrop by DeNOVIX). The stained cells were counted through the device for its viability in percentage.

For both MTT and AO/PI assays, two highest concentrations that induced ≥ 80 % viability towards hFOB1.19 when treated with plant extracts were considered non-toxic towards the cells and used for cell treatments (López-García et al., 2014).

3.9 Experimental design

The hFOB1.19 cells were treated according to the experimental groups presented in Figure 3.1.

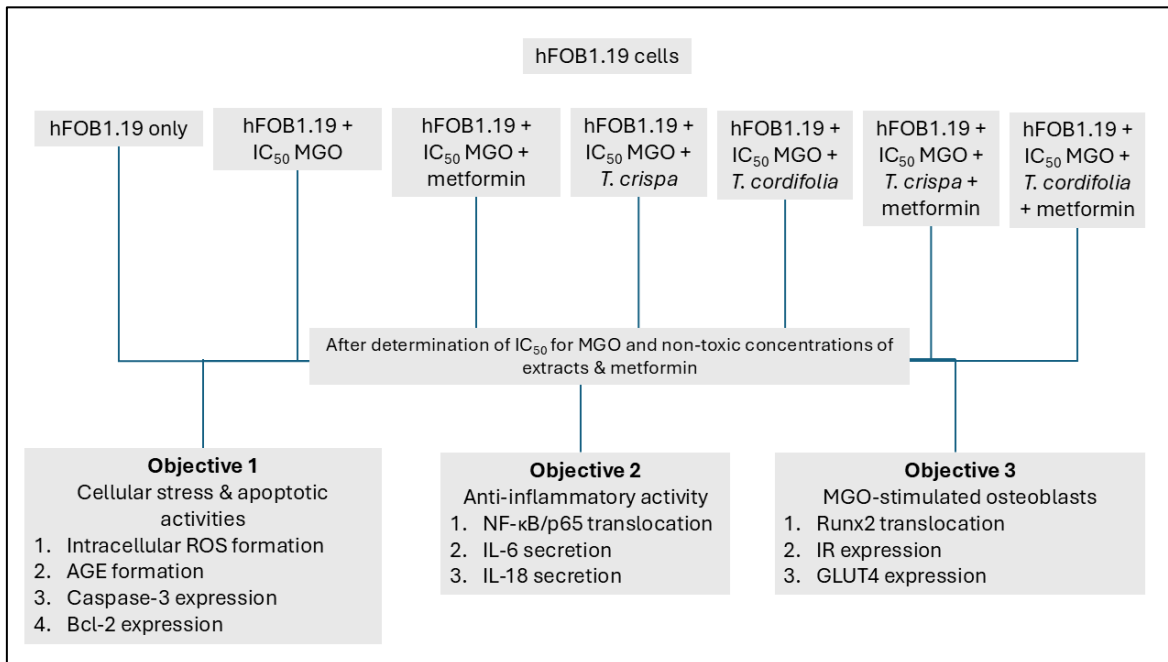


Figure 3.1: Experimental design. The cells were first treated with MGO to analyse its IC₅₀ value, metformin as well as methanolic and aqueous extracts to analyse the non-toxic concentrations. Then, the cellular stress, anti-apoptotic, anti-inflammatory activity and diabetic bone metabolism of the treated cells were analysed through enzyme linked immunosorbent assay (ELISA).

3.10 Incubation of hFOB1.19 with different treatments

The hFOB1.19 cells were seeded into T-25 flasks at a seeding density of 5×10^5 cells/mL. After the cells reached a confluence level of 90 % (approximately 3×10^6 cells/mL), the cells were treated with various treatments: MGO, MGO-metformin, MGO-extracts, and MGO-extracts combined with metformin. Subsequently, they were incubated for 24 hours at 37°C with 5% CO₂ with the humidity of 95 %. The treatments were tabulated in Table 3.7.

Table 3.7: Various treatments of MGO, MGO-metformin, MGO-extracts, and MGO-extracts with metformin for hFOB1.19

Label	Treatments
Control	Untreated (negative control)
MGO	IC ₅₀ of MGO (positive control)
Metformin	IC ₅₀ of MGO + metformin
Methanolic extracts	
TCOR50	IC ₅₀ of MGO + methanolic <i>T. cordifolia</i> 50 µg/mL
TCOR100	IC ₅₀ of MGO + methanolic <i>T. cordifolia</i> 100 µg/mL
TCRIS120	IC ₅₀ of MGO + methanolic <i>T. crispa</i> 120 µg/mL
TCRIS240	IC ₅₀ of MGO + methanolic <i>T. crispa</i> 240 µg/mL
Aqueous extracts	
TCOR62.5	IC ₅₀ of MGO + aqueous <i>T. cordifolia</i> 62.5 µg/mL
TCOR125	IC ₅₀ of MGO + aqueous <i>T. cordifolia</i> 125 µg/mL
TCRIS187.5	IC ₅₀ of MGO + aqueous <i>T. crispa</i> 187.5 µg/mL
TCRIS375	IC ₅₀ of MGO + aqueous <i>T. crispa</i> 375 µg/mL

3.10.1 Cell supernatant samples preparation

The supernatants of hFOB1.19 were collected by simply aspirating the media of the treated cells after 24 hours incubation. Then, the supernatants of different treatments were pipetted into microcentrifuge tubes after centrifugation at 16000×g for 10 minutes at 4 °C. The collected supernatants were kept in a -80°C freezer until their usage for assays. Cell supernatant samples were required to analyse the secretion of IL-6 and IL-18 from the treated hFOB1.19 cells.

3.10.2 Cell lysate samples preparation

Following the collection of the supernatant, the remaining cells in the flasks were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS). Subsequently, 1 mL of RIPA lysis and extraction buffer (Thermo Fisher Scientific, USA) was pipetted into the flask. The flasks were then placed on ice with gentle agitation for 10 minutes. Then, the

cells were detached from the flask using a cell scraper before they were transferred into labelled microcentrifuge tubes. These tubes were centrifuged at 16000×g for 15 minutes at 4°C, leading to the separation of the lysate as supernatant, which was then transferred into new labelled microcentrifuge tubes. Finally, the tubes were stored in a -80°C freezer prior their usage for assays. Cell lysate samples were required to measure the expression AGE, caspase-3, Bcl-2, IR and GLUT4 by ELISA.

3.10.3 Nuclear lysate samples preparation

The pellets left in the microcentrifuge tubes after the collection of cell lysates were nuclear pellets. The pellets were resuspended with 200 µL of nuclear lysis buffer provided by Novus Biologicals (USA) by pipetting up and down. Then, they were incubated at 4 °C on a rocking platform for 30 minutes. Then, the tubes were vortexed for 30 seconds, before they were centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatants, which represented the nuclear fractions, were transferred into pre-chilled microcentrifuge tubes and stored at -80 °C for Runx2 and NF-κB analysis by ELISA. The nuclear lysate samples were required for NF-κB/p65 and Runx2 ELISA.

3.11 Influence of plant extracts with or without metformin towards hFOB1.19 in assessing their effect on cellular stress

The cellular stress of hFOB1.19 was evaluated by the formation of oxidative stress inducers which include intracellular ROS and AGE formation.

3.11.1 Measurement of intracellular ROS formation in treated hFOB1.19

3.11.1.1 Preparation of reagents

i. 1× 2',7'-dichlorofluorescein diacetate (DCFH-DA)/media solution

To prepare 2700 µL of 1× DCFH-DA/media solution, 135 µL of 20× DCFH-DA stock solution was diluted with 2565 µL cell culture media and vortexed to homogeneity. The solution was protected from light by wrapping the solution tube with aluminium foil.

3.11.1.2 Preparation of standards

Dichlorofluorescein (DCF) standards of 1:10 dilution series were prepared in the concentration range of 0 µM to 10 µM by diluting 1 mM DCF stock with DMEM/F-12 cell culture media. Then, 75 µL of each DCF standard was transferred into each well of 96 well plate before adding 75 µL of the 2× cell lysis buffer. The preparation of different standard concentrations is shown in Table 3.8.

Table 3.8: The serial dilution for intracellular ROS standard concentrations

Standard Tubes	DCF Standard (µL)	Culture medium (µL)	DFC (nM)
1	10	990	10,000
2	100 of Tube #1	900	1000
3	100 of Tube #2	900	100
4	100 of Tube #3	900	10
5	100 of Tube #4	900	1
6	100 of Tube #5	900	0.1
7	100 of Tube #6	900	0.01
8	0	1000	0

3.11.1.3 ELISA protocol to determine intracellular ROS production in treated hFOB1.19

This experiment followed the instructions provided by Oxiselect™ Intracellular ROS Green Fluorescence Assay Kit (Cell Biolabs, California, United States). The assay began by infusing the fluorogenic probe 2',7' – dichlorodihydrofluorescein diacetate (DCFH-DA) into the cells which then became oxidised into 2',7' – dichlorodihydrofluorescein by ROS. the fluorescence intensity of ROS levels in the cytosol was measured through excitation and emission wavelengths of 480 nm and 530 nm, respectively.

The hFOB1.9 cells were seeded, in triplicates, with a density of 2×10^4 cells/mL into an opaque, black 96 well plate at 34°C in a 5% CO₂ incubator for 24 hours. After 24 hours, the media was removed from all wells and discarded. The cells were washed gently three times with Hanks Buffered Salt Solution (HBSS) before adding 100 µL of 1× DCFH-DA/media solution to the cells. Then, the cells were incubated for 60 minutes and washed with HBSS three times. Then, the DCFH-DA loaded cells were treated with different treatments in accordance with Table 3.8 for standard and samples. The plate was incubated at room temperature for 1 hour for static analysis. The fluorescence, measured in relative fluorescence unit (RFU) was measured using a microplate reader (Spectramax iD 3) directly after the incubation.

3.11.2 AGE formation in treated hFOB1.19

The AGE formation in hFOB1.19 was determined using an Oxiselect™ Advanced Glycation End (AGE) Product ELISA kit supplied by (Cell Biolabs Inc., California, USA).

The AGE ELISA kit consisted of anti-AGE antibody 1000× with the volume of 10 µL, secondary antibody, HRP conjugate 1000× with the volume of 20 µL, assay diluent with

the volume of 50 mL, 10× wash buffer with the volume of 100 mL, substrate solution with the volume of 12 mL, stop solution with the volume of 12 mL, AGE-BSA standard 1 mg/mL with the volume of 125 µL, AGE conjugate 1 mg/mL with the volume of 50 µL and 100× conjugate diluent with the volume of 300 µL and one 96 well protein binding plate.

3.11.2.1 Preparation of reagents

i. 1× conjugate diluent

1× conjugate diluent was prepared by diluting 50 µL of 100× Conjugate Diluent with 4.95 mL 1× PBS.

ii. 10 µg/mL of AGE conjugate

AGE conjugate of 10 µg/mL was prepared by adding 25 µL of 1.0 mg/mL AGE conjugate with 2.475 mL of 1× PBS.

iii. 1X wash buffer

1× wash buffer was prepared by diluting 50 mL 10× wash buffer with 450 mL of deionized water. The wash buffer was stored in a 500 mL Schott bottle.

iv. 1:1000 diluted anti-AGE antibody and secondary antibody

1:1000 diluted anti-AGE Antibody was prepared by diluting 3.5 µL of 1000× anti-AGE Antibody with 3496.5 of assay diluent. 1:1000 diluted secondary antibody was prepared by diluting 6.5 µL of 1000× secondary antibody with 6493.5 µL assay diluent.

v. AGE conjugate coated plate

Ten µg/mL of AGE conjugate was mixed with 1× conjugate Diluent at 1:1 ratio. The mixture was added to each well and the plate was incubated overnight at 4°C. The

AGE conjugate coating was removed, and the wells were washed twice with 1× PBS. The plate was blotted on paper towels to remove excess fluid. 200 µL of assay diluent was added to each well and the plate was incubated for one hour at room temperature. The plate was transferred to 4°C for further usage.

3.11.2.2 Preparation of standard

The AGE-BSA standard was diluted in assay diluent with the concentration range of 0 to 100 µg/mL as shown in Table 3.9.

Table 3.9: The serial dilution for AGE-BSA standard.

Standard Tubes	1 mg/mL Standard (µL)	AGE-BSA	Assay Diluent (µL)	AGE-BSA (µg/mL)
1	40		360	100
2	200 of Tube #1		200	50
3	200 of Tube #1		200	25
4	200 of Tube #2		200	12.5
5	200 of Tube #3		200	6.25
6	200 of Tube #4		200	3.13
7	200 of Tube #5		200	1.56
8	200 of Tube #6		200	0.78
9	200 of Tube #7		200	0.39
10	0		200	0

3.11.2.3 ELISA protocol to determine AGEs expression in treated hFOB1.19

Fifty µL of either an unknown sample or an AGE-BSA standard was added to the wells of the AGE Conjugate coated plate. The plate was then incubated at room temperature for a duration of 10 minutes on an orbital shaker. Subsequently, 50 µL of the diluted anti-AGE antibody was added to each well and allowed to incubate at room temperature for 1 hour with continuous agitation. After incubation with the antibody, the wells were washed three times with 250 µL of 1× wash buffer, ensuring thorough

aspiration between each wash. After the final wash, excess 1× wash buffer was carefully removed by tapping the microwell strips on an absorbent pad or paper towel. Following this washing step, 100 µL of the diluted Secondary Antibody-HRP conjugate was added to all wells and incubated for another 1 hour at room temperature on orbital shaker. The wells were washed again as described earlier. Subsequently, substrate solution, warmed to room temperature, was introduced at 100 µL per well, and the plate was incubated at room temperature for a period of 2 to 20 minutes on an orbital shaker. The reaction mixture colour in the wells was closely monitored because a rapid colour change indicated a saturation of reaction and therefore, required termination of the reaction. Finally, the enzyme reaction was inhibited by adding 100 µL of stop solution to each well, and immediate reading of absorbance was carried out on a microplate reader using a primary wavelength of 450 nm.

3.12 Influence of plant extracts with or without metformin towards hFOB1.19 in determining apoptotic activity

The apoptotic activity of the treated hFOB1.19 was assessed through the expression of pro-apoptotic protein (caspase-3) and anti-apoptotic protein (Bcl-2) by using human caspase-3 ELISA Kit (CSB-E08856h) (Cusabio, Texas, USA) and human Bcl-2 ELISA Kit (NBP1-91188) (Novus Biologicals, USA) respectively.

Human Bcl-2 ELISA kit consisted of one aluminium pouch with 96-well plate coated with monoclonal antibody to human Bcl-2, 70 µL of biotin-conjugate, 150 µL of streptavidin-HRP, two vials of human Bcl-2 standards, 12 mL of sample diluent, five mL of assay buffer concentrate (20X), 50 mL of wash buffer concentrate (20X), 15 mL of lysis buffer (10X), 15 mL of substrate solution, 15 mL of stop solution and four adhesive films.

3.12.1 Bcl-2 expression in the treated hFOB1.19

3.12.1.1 Preparation of reagents

i. 1× wash buffer

To prepare the 1× wash buffer, 50 mL content of the concentrated 20× wash buffer was poured with 950 mL of deionized water in graduated cylinder. The solution was mixed to prevent foaming. Subsequently, the prepared solution was transferred to a clean wash bottle and was stored within a temperature range of 2 °C to 8 °C.

ii. 1× assay buffer

Five mL content of the 20× assay buffer concentrate was poured into a clean 100 mL graduated cylinder, and 50 mL of distilled water was added. Subsequently, the prepared 1× assay buffer was stored in a 500 mL Schott bottle.

iii. Biotin-conjugate

Sixty µL of biotin-conjugate was added to 5.94 mL assay buffer making a total volume of 6 mL.

iv. Streptavidin-HRP

In a 15 mL centrifuge tube, 120 of streptavidin-HRP with 11.88 mL of assay buffer making a total volume of 12 mL.

3.12.1.2 Preparation of standard

The human Bcl-2 vial standard was added with distilled water, following the reconstitution volume making 64 ng/mL. This mixture was allowed to set for 10-30

minutes, with thorough agitation to homogenize the solution before proceeding with subsequent dilutions. The series of standard concentrations is shown in Table 3.10.

Table 3.10: The serial dilution for Bcl-2 standard

Standard Tubes	Bcl-2 Standard (μL)	Assay Diluent (μL)	Concentration (ng/mL)
1	375 of Tube #1	375	32
2	375 of Tube #1	375	16
3	375 of Tube #2	375	8
4	375 of Tube #3	375	4
5	375 of Tube #4	375	2
6	375 of Tube #5	375	1
7	0	200	0

3.12.1.3 ELISA protocol to determine Bcl-2 expression in treated hFOB1.19

Fifty μL of biotin-conjugate was added to each well and left for incubation at room temperature on the orbital shaker for 2 hours. The wells were then washed three times with 400 μL of 1× wash buffer before adding 100 μL of streptavidin-HRP to each well. Then, the plate was incubated at room temperature for one hour, on the orbital shaker. The wells were washed again three times with 400 μL of 1× wash buffer. This was followed by the addition of 100 μL of TMB substrate to each well and further incubation at room temperature for 10 minutes. The plate was covered entirely with aluminium foil to avoid light. After the incubation, 100 μL of stop solution was added to each well and the absorbance was read at 450 nm using the microplate reader.

3.12.2 Caspase-3 expression in treated hFOB1.19

The caspase-3 protein expression in hFOB1.19 was determined using an ELISA kit supplied by (Cusabio, Texas, USA). Human caspase-3 ELISA kit consisted of one assay

96-well plate, two standard vials, 120 μ L of HRP-conjugate (100X concentrate), 20 mL of HRP diluent, 20 mL of sample diluent, 20 mL of wash buffer (25X concentrate), 10 mL of TMB substrate, 10 mL of stop solution, and adhesive strip.

3.12.2.1 Preparation of reagents

i. 1 $\times$ HRP-conjugate

The vial with the HRP conjugate was centrifuged before opening. 10 μ L of HRP-conjugate was added to 990 μ L HRP-conjugate diluent, making the total volume of 1000 μ L.

ii. 1 $\times$ Wash buffer

The vial containing the 25 $\times$ wash buffer was thawed to room temperature first to dissolve the crystals. The 1 x wash buffer was prepared by adding 20 mL of 25 $\times$ wash buffer with 480 mL of deionized water, resulting in a total volume of 500 mL. The wash buffer was stored in a 500 mL Schott bottle at room temperature.

3.12.2.2 Preparation of standards

The standard vial was centrifuged at 7000 rpm for 30 seconds. Then, it was reconstituted with 1 mL of sample diluent, to make a stock concentration of 20 ng/mL. The stock solution was set to sit for a minimum of 15 minutes with gentle agitation. The serial dilution for caspase-3 is shown in Table 3.11.

Table 3.11: The serial dilution for caspase-3 standard

Standard Tubes	caspase-3 Standard (μL)	Assay diluent (μL)	Concentration (ng/mL)
1	1000	0	20
2	250 of Tube #1	250	10
3	250 of Tube #2	250	5
4	250 of Tube #3	250	2.5
5	250 of Tube #4	250	1.25
6	250 of Tube #5	250	0.625
7	250 of Tube #6	250	0.312
8	0	250	0

3.12.2.3 ELISA protocol to determine casp-3 expression in treated hFOB1.19

The 100 μL of the standards or samples were added into each well and the plate was then incubated for 30 minutes at 37°C. After the incubation, each well was washed three times with 200 μL of 1× wash buffer before adding 100 μL of HRP-conjugate. Then the plate was incubated for another 30 minutes at 37°C. The plate was washed again five times with 200 μL of 1× wash buffer before adding 90 μL of TMB-substrate. Next, the plate was incubated in the dark. After 20 minutes, stop solution was added to each well. Finally, the absorbance was read at 450 nm wavelength.

3.13 Influence of plant extracts with or without metformin towards hFOB1.19 in determining anti-inflammatory activity

The secretion of pro-inflammatory cytokine IL-6 is initiated through the translocation of NF-κB/p65 into the nucleus. The measurement of IL-18, another pro-inflammatory cytokine was also analysed.

3.13.1 NF-κB/p65 translocation in treated hFOB1.19

The translocation of NF-κB/p65 transcription factor into the nucleus in hFOB1.19 was determined using an ELISA kit supplied by (Novus Biologicals, USA). NF-κB/p65

ELISA kit consisted of 200 μ L of capture antibody, 200 μ L of detecting antibody, two bottles of coating buffer with 10 mL volume each, 0.5 g bovine serum albumin (BSA), 50 mL of 20X wash buffer, 10 μ L of AKP-conjugated secondary antibody, 2 bottles of pNPP substrate buffer with 10 mL volume each, 2 vials of recombinant p65 standard with 0.42 μ g each, four vials of pNPP with 5 mg each and two 96 well plates.

3.13.1.1 Preparation of reagents

i. 1 $\times$ wash buffer

The 500 mL of 1 $\times$ wash buffer was prepared by mixing 25 mL of 20 $\times$ wash buffer with 475 mL of deionized water. The wash buffer was stored in a 500 mL Schott bottle.

iii. Blocking buffer

The blocking buffer was made by mixing 0.5 g of BSA with 50 mL of 1 $\times$ wash buffer in a sterile bottle.

3.13.1.2 The coating of wells

First, 100 μ L of capture antibody was diluted with 10 mL of coating buffer. Then, 100 μ L of the diluted antibody was pipetted into the wells. Next, the plate was incubated for 12 hours at 4°C.

3.13.1.3 The blocking of wells

After the 12 hours incubation, the wells were washed twice with 300 μL of 1 $\times$ wash buffer. Then, the plate was incubated for 45 minutes at room temperature.

3.13.1.4 Preparation of standard

First, the recombinant p65 standard was centrifuged at 10000 $\times$ g for 30 seconds. Then, 420 μL of sterile deionized water was added into the vial, creating a stock of 1000 ng/mL. The serial dilution of NF- κ B/p65 standard was prepared as shown in Table 3.12.

Table 3.12: Serial dilution of NF- κ B/p65 standard

Standard Tubes	NF-κB/p65 Standard (μL)	Assay diluent (μL)	Concentration (ng/mL)
1	1000	0	1000
2	100 of Tube #1	100	500
3	100 of Tube #2	100	250
4	100 of Tube #3	100	125
5	100 of Tube #4	100	62.5
6	100 of Tube #5	100	31.25
7	100 of Tube #6	100	15.6
8	0	100	0

3.13.1.5 ELISA protocol to determine NF- κ B/p65 expression in treated hFOB1.19

Firstly, 100 μL of samples and standards were pipetted into the wells and were let to incubate for 4 hours at room temperature. Then, the wells were washed 4 times with 300 μL of 1 $\times$ wash buffer. Next, 100 μL of diluted detecting antibody was pipetted into each well, and the plate was incubated for another 1 hour at room temperature. Next, the wells were washed again for 4 times with 300 μL of 1 $\times$ wash buffer before adding 100 μL of diluted secondary antibody into each well. The plate was incubated again for 1 hour. Next, the secondary antibody was aspirated, and the wells were washed 5 times with 300 μL of

1× wash buffer. Then, 100 µL of pNPP Substrate was added to each well. Lastly, the optical density was measured at 405 nm using the microplate reader.

3.13.2 Measurement of IL-6 secretion by the treated hFOB1.19

The IL-6 expression in hFOB1.19 was determined using an ELISA kit supplied by R&D systems, Biotechne (Minnesota, USA). The IL-6 ELISA kit consisted of one 96 well plate coated with monoclonal antibody specific for human IL-6, two vials of human IL-6 standard in protein base with preservatives, one vial of human IL-6 conjugate with the volume of 21 mL, one vial of assay diluent RD1W with the volume of 11 mL, one vial of calibrator diluent (RD5T) with the volume of 21 mL, one vial of wash buffer concentrate with the volume of 21 mL, one vial of colour reagent A with the volume of 12 mL, one vial of colour reagent B with the volume of 12 mL, one vial of stop solution with the volume of 6 mL and four strips of plate sealers.

3.13.2.1 Preparation of Reagents

i. Wash buffer

The 1× wash buffer was prepared by mixing 20 mL of concentrated wash buffer with 480 mL deionized water. The wash buffer was then stored in a 500 mL Schott bottle.

ii. Substrate Solution

The substrate solution was prepared by mixing colour reagents A and B in equal volumes. The solution was kept in a tube covered with aluminium foil to avoid light.

3.13.2.2 Preparation of Standard

First, the IL-6 standard was reconstituted with 5 mL calibrator diluent RD5T, to make a stock of 300 pg/mL. Then, the standard was let to sit for a minimum of 15 minutes. The serial dilution of IL-6 standards was prepared as shown in Table 3.13.

Table 3.13: Serial dilution of IL-6 standard.

Standard Tubes	IL-16 Standard (µL)	Assay diluent (µL)	Concentration (pg/mL)
1	333 of Stock	667	100
2	500 of Tube #1	500	50
3	500 of Tube #2	500	25
4	500 of Tube #3	500	12.5
5	500 of Tube #4	500	6.25
6	500 of Tube #5	500	31.3
7	0	500	0

3.13.2.3 Determination of IL-6 secretion in treated hFOB1.19 by ELISA

Firstly, 100 µL of assay diluent RD1W was pipetted into each well followed by the addition of 100 µL of standard and samples. The plate was then covered with and incubated for 2 hours at room temperature. After incubation, the wells were washed three times with 400 µL of 1× wash buffer. Then, 200 µL of human IL-6 conjugate was added to each well, before it was incubated for another 2 hours at room temperature. The wells were then washed again with wash buffer before adding 200 µL of substrate solution. The plate was incubated for 20 minutes at room temperature. Then, 50 µL of stop solution was added to each well and a change of colour of the solution in the well from blue to yellow was observed. Finally, the absorbance was measured at 450 nm using a microplate reader.

3.13.3 IL-18 secretion in the treated hFOB1.19

The IL-18 expression in hFOB1.19 was determined using an ELISA kit supplied by R&D systems, Biotechne (Minnesota, USA). The ELISA kit consisted of one vial of human total IL-18 capture antibody, one vial of human total IL-18 detection antibody, two vials of human total IL-18 standard, one vial of streptavidin-HRP B, 500 mL wash buffer, cover strips and 96-well plate.

3.13.3.1 Preparation of reagents

i. Streptavidin-HRP B

Streptavidin-HRP B with the weight of 120 µg of was added with 0.5 mL making a stock of 60 µg/mL, and it was further diluted to a working solution of 2 µg/mL.

ii. Capture antibody

A 1× working capture antibody solution was prepared by mixing 89.09 µL of 40× capture antibody with 10.91 µL of reagent diluent.

iii. 1× wash buffer

The 500 mL of working wash buffer was prepared by mixing 20 mL of concentrated wash buffer with 480 mL of deionized water. The wash buffer was stored in a 500 mL Schott bottle.

3.13.3.2 Preparation of the wells

The 100 µL of capture antibody was pipetted into each well, and the plate was incubated overnight in the dark at room temperature.

3.13.3.3 Preparation of standard

The IL-18 standard was reconstituted with 0.5 mL PBS resulted in a stock with the concentration of 110 ng/mL. Then, the standard was further diluted to 750 pg/mL. The serial dilution of IL-18 standard was prepared as shown in Table 3.14.

Table 3.14: Serial dilution of IL-18 standard

Standard Tubes	IL-18 Standard (μL)	Assay diluent (μL)	Concentration (pg/mL)
1	1000	0	750
2	500 of Tube #1	500	375
3	500 of Tube #2	500	188
4	500 of Tube #3	500	93.8
5	500 of Tube #4	500	46.9
6	500 of Tube #5	500	23.4
7	500 of Tube #6	500	11.7
8	0	500	0

3.13.3.4 Determination of IL-18 secretion by ELISA

Firstly, 100 μL of samples or standards were pipetted into each well and the plate was incubated at room temperature for 2 hours on an orbital shaker. The wells were washed with 400 μL of 1× washing buffer for 2 cycles before adding 100 μL of detection antibody. The plate was again incubated at room temperature for 2 hours on an orbital shaker before washing the wells with 400 μL of 1× washing buffer for 2 cycles. The, 100 μL of streptavidin-HRP-B was pipetted into wells before incubating the plate for 30 minutes at room temperature. The wells underwent the final washing for 2 cycles before adding 100 μL of substrate. After 20 minutes incubation in the dark, stop solution was added into each well and finally the absorbance was measured at 450 nm with a microplate reader.

3.14 Influence of plant extracts with or without metformin towards hFOB1.19 in determining diabetic bone metabolism

The diabetic bone metabolism activity of hFOB1.19 was evaluated by the translocation of transcription factor into the nucleus and expression of diabetic related proteins which include RUNX2, IR and GLUT4 respectively.

3.14.1 Influence of plant extracts with or without metformin towards hFOB1.19 in determining Runx2 translocation

The Runx2 translocation into the nucleus in hFOB1.19 was determined using an ELISA kit supplied by R&D systems, Biotechnie (Minnesota, USA).

Runx2 ELISA kit consisted of one 96-well plate, two vials of Runx2 standard, one vial biotin antibody 100× with the volume of 120 µL, one vial HRP-avidin 100× with the volume of 120 µL, 1 tube of biotin-antibody diluent with the volume of 15 mL, one tube of HRP-avidin diluent with the volume of 15 mL, one tube of sample diluent with the volume of 50 mL, one tube of wash buffer 25× with the volume of 20 mL, one tube of TMB substrate with the volume of 10 mL, one tube of stop solution with the volume of 10 mL and four adhesive strips.

3.14.1.1 Preparation of reagents

i. 1× Biotin-antibody

The vial was centrifuged at 10000 rpm for 30 seconds to ensure the antibody homogeneity. Then, 10 µL of Biotin-antibody was diluted at 100-fold dilution with 800 µL Biotin-antibody diluent, creating a total volume of 79200 µL 1× Biotin-antibody.

ii. 1× HRP-avidin

The vial was centrifuged at 1000 rpm for 30 seconds before opening. 800 µL of HRP-avidin was diluted at 100-fold dilution with 79200 µL HRP-avidin diluent, creating a total volume of 8000 µL 1× HRP-avidin.

iii. 1× wash buffer

Five hundred mL of 1× wash buffer was prepared by mixing 20 mL of 25× concentrated wash buffer with 480 mL deionized water. The wash buffer was stored in a 500 mL Schott bottle.

3.14.1.2 Preparation of Standard

Standard vial was centrifuged at 10000 rpm for 30 seconds. Then, the standard was reconstituted with 1 mL of sample diluent, creating a stock with a concentration of 2000 pg/mL. The reconstituted standard was let sit for 15 minutes before serial dilution was performed according to Table 3.15.

Table 3.15: Serial dilution of Runx2 standard

Standard Tubes	Runx2 Standard (µL)	Assay diluent (µL)	Concentration (pg/mL)
7	1000 of stock	1000	2000
6	250 of Tube #6	250	1000
5	250 of Tube #5	250	500
4	250 of Tube #4	250	250
3	250 of Tube #3	250	125
2	250 of Tube #2	250	62.5
1	250 of Tube #1	250	31.25
0	0	250	0

3.14.1.3 ELISA protocol to determine RUNX2 expression in treated hFOB1.19

Firstly, 100 μ L of standard and samples were pipetted into the wells before it was incubated for 2 hours at 37°C. After incubation, the standard and samples were aspirated before 100 μ L of 1 $\times$ Biotin-antibody was pipetted into each of the wells. The plate was then incubated at 37°C for 1 hour. After incubation, the wells were washed two times with the 1 $\times$ wash buffer at 200 μ L. Next, each of the wells was added with 100 μ L of 1 $\times$ HRP-avidin and the plate was incubated again for 1 hour at 37°C. Then, the wells were washed again with wash buffer before 90 μ L of TMB substrate was added into each well. The plate was incubated for 30 minutes before 50 μ L of stop solution was pipetted into the wells. Lastly, the optical density was measured at 450 nm using a microplate reader.

3.14.2 Insulin receptor (IR) expression in treated hFOB1.19

The IR expression in hFOB1.19 was determined using an ELISA kit supplied by Novus Biologicals (USA).

IR ELISA kit consisted of one 96-well plate, two vials of reference standard, one vial of concentrated biotinylated detection Ab 100 $\times$ with the volume of 120 μ L, concentrated HRP conjugate 100 $\times$ with the volume of 120 μ L, one tube of reference standard and sample diluent with the volume of 20 mL, one tube of HRP conjugate diluent with the volume of 14 mL, one tube of concentrated wash buffer 25 $\times$ with the volume of 30 mL, one tube of substrate reagent with the volume of 10 mL, one tube of stop solution with the volume of 10 mL and five pieces of plate sealer.

3.14.2.1 Preparation of reagents

i. Working wash buffer

Working wash buffer with the volume of 750 mL of was prepared by mixing 30 mL of concentrated wash buffer with 720 mL of deionized water. The wash buffer was stored in a 500 mL Schott bottle.

ii. 1× biotinylated detection Ab working solution

1× biotinylated detection Ab working solution with the volume of 7500 µL was prepared by mixing 75 µL of concentrated 100× biotinylated Ab with 7425 µL of biotinylated Ab diluent.

iii. 1× concentrated HRP conjugate working solution

1× HRP conjugate working solution with the volume of 7500 µL was prepared by mixing 75 µL of 100× concentrated HRP conjugate with 7425 µL of HRP conjugate diluent.

3.14.2.2 Preparation of standard

The standard vial was centrifuged at 10000×g for 1 minute. Then, 1 mL of reference standard and sample diluent was added into the vial, and it was let sit for 10 minutes before serial dilution was performed according to Table 3.16.

Table 3.16: Serial dilution of IR standard

Standard Tubes	IR Standard (µL)	Assay diluent (µL)	Concentration (pg/mL)
7	1000 of stock	1000	100
6	500 of Tube #6	500	50
5	500 of Tube #5	500	25
4	500 of Tube #4	500	12.5
3	500 of Tube #3	500	6.25
2	500 of Tube #2	500	3.13
1	500 of Tube #1	500	1.56
0	0	500	0

3.14.2.3 ELISA protocol to determine IR expression in treated hFOB1.19

Firstly, 100 µL of standard or sample was pipetted to each well before they were incubated for 90 minutes at 37°C. Then, the liquids were aspirated before 100 µL of 1× Biotinylated detection Ab was added into each well. The plate was then incubated again for 1 hour at 37°C. After incubation, the plate was washed for 3 times with 350 µL of 1× wash buffer before adding 100 µL of 1× HRP Conjugate to each well. Next, the plate was incubated for 30 minutes at 37°C before it was washed for 5 times with 350 µL of 1× wash buffer. Then, 90 µL of substrate reagent was added and it was let for its final incubation for 15 minutes at 37°C before 50 µL stop solution was added into each well. Lastly, the optical density was measured at 450 nm using a microplate reader.

3.14.3 Glucose transporter 4 (GLUT4) expression in treated hFOB1.19

The GLUT4 expression in hFOB1.19 was determined using an ELISA kit supplied by Novus Biologicals (USA).

The GLUT4 ELISA consisted of one 96-well plate, two vials of reference standard, one vial of concentrated biotinylated detection Ab 100× with the volume of 120 µL, one

vial of concentrated HRP conjugate 100× with the volume of 120 µL, one tube of reference standard and sample diluent with the volume of 20 mL, one tube of biotinylated detection Ab diluent with the volume of 14 mL, one tube of HRP conjugate diluent with the volume of 14 mL, one tube of concentrated wash buffer 25× with the volume of 30 mL, one tube of substrate reagent with the volume of 10 mL, one tube of stop solution with the volume of 10 mL and 5 pieces of plate sealer.

3.14.3.1 Preparation of reagents

i. Working wash buffer

Working wash buffer with the volume of 750 mL was prepared by diluting 30 mL of concentrated Wash Buffer with 720 mL of deionized water. The wash buffer was stored in a 500 mL Schott bottle.

ii. Biotinylated detection Ab working solution

The working solution of biotinylated detection Ab was prepared with the total volume of 8500 µL. Firstly, the concentrated biotinylated Ab vial was centrifuged at 10000×g for 1 minute. Then, 85 µL of the concentrated biotinylated detection Ab was diluted with 8415 µL of biotinylated detection Ab diluent.

iii. HRP conjugate working solution

The working solution of HRP conjugate was prepared with the total volume of 8500 µL. Firstly, the concentrated HRP conjugate vial was centrifuged at 10000×g for 1 minute. Then, 85 µL of the concentrated HRP conjugate was diluted with 8415 µL concentrated HRP conjugate diluent.

3.14.3.2 Preparation of standard

The standard vial was centrifuged at 10000×g for 1 minute. Then, 1 mL of reference standard and sample diluent was added into the vial and the mixture was let sit for 10 minutes before serial dilution was performed according to Table 3.17.

Table 3.17: Serial dilution of GLUT4 standard

Standard Tubes	GLUT4 Standard (μL)	Standard & Sample diluent (μL)	Concentration (pg/mL)
7	1000 of stock	1000	20
6	500 of Tube #6	500	10
5	500 of Tube #5	500	5
4	500 of Tube #4	500	2.5
3	500 of Tube #3	500	1.25
2	500 of Tube #2	500	0.63
1	500 of Tube #1	500	0.31
0	0	500	0

3.14.3.3 ELISA protocol to determine GLUT4 expression in treated hFOB1.19

Firstly, 100 μL of standard of samples were pipetted into the wells before they were incubated for 90 minutes at 37°C. After incubation, the liquids were removed and 100 μL of Biotinylated Detection Ab working solution were added into the wells before the plate was incubated for 1 hour at 37°C. Next, the wells were washed 3 times using wash buffer with the volume of 350 μL. Then, 100 μL of HRP Conjugate was added into each well, before they were incubated for 30 minutes at 37°C. After the last washing step, 90 μL of substrate reagent was added into each well. The plate was incubated for another 15 minutes at 37°C. Finally, 50 μL of stop solution was added into each well and the plate was read at 450 nm immediately using the microplate reader.

3.15 Statistical analysis

Results shown for GC-MS were calculated in triplicate, which was representative of three separate experiments. The means and standard deviation of the mean (S.D.) shown within each experiment were calculated from triplicate samples. The data were plotted and subjected to statistical analysis using IBM SPSS Statistics software, version 25 for Windows (NY, USA). Significance was confirmed by a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, *indicating a significant difference from the positive control (MGO), ^Δ indicating a significant difference from the negative control (untreated) and Tukey's test, #indicating a significant difference among samples (*p < 0.05 to MGO; ^Δp < 0.05 to untreated cells; #p < 0.05 between treatments). Dunnett's test is used to compare multiple treatment groups against a single control group, effectively assessing the impact of various treatments while controlling the Type I error rate. In contrast, Tukey's test facilitates pairwise comparisons among all treatment groups, allowing for the identification of specific differences between any two groups without a designated control.

CHAPTER 4

RESULTS

4.1 GC-MS analysis of methanolic and aqueous extracts of *T. cordifolia* and *T. crispa*

4.1.1 Methanolic Extracts of *T. cordifolia* and *T. crispa*

The phytochemical analysis using GC-MS revealed the presence of phytochemical compounds in the methanolic extract of *T. cordifolia* extract as shown in Table 4.1 and Figure 4.1. Methylparaben was the major constituent which comprised 44.65 ± 13.02 % of the total phytochemicals in the *T. cordifolia* methanolic extract. The next predominant phytochemicals were l-(+)-ascorbic acid 2,6-dihexadecanoate which is also known as vitamin-c dipalmitate (5.27 ± 4.57 %) and phenol (5.25 ± 2.39 %).

Table 4.1: Phytochemical constituents of methanolic extract of *T. cordifolia* detected by gas chromatography mass spectrometry (GC-MS).

Peak	Compounds	Mean $\pm$ S.D (%)
2	Phenol	5.25 ± 2.39 ^{ab}
3	Guaiacol	3.80 ± 1.11 ^{ab}
4	Benzofuran, 2,3-dihydro-	0.93 ± 0.81 ^b
5	4-vinyl guaiacol	4.17 ± 1.28 ^{ab}
6	Syringol	1.74 ± 0.53 ^a
7	Vanillin	1.50 ± 0.24 ^{ab}
8	Methylparaben	2.09 ± 1.57 ^a
9	Eugenol	1.44 ± 0.23 ^a
10	Methylparaben	0.72 ± 0.62 ^a
11	Methylparaben	44.65 ± 13.02 ^a
12	Apocynin	0.43 ± 0.40 ^b
13	beta.-D-Glucopyranose, 1,6-anhydro-	1.30 ± 1.20 ^a
15	Canolol	1.60 ± 0.21 ^{ab}
16	Eugenol <methoxy->	0.83 ± 0.15
17	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	0.13 ± 0.22

Table 4.1 continued

18	Coniferyl alcohol	2.82 ± 0.98
19	Palmitic acid	1.45 ± 0.37
20	l-(+)-Ascorbic acid 2,6-dihexadecanoate	5.27 ± 4.57^a
21	2,6,8-Trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol	0.38 ± 0.33
22	cis-Sinapyl alcohol	0.51 ± 0.48
23	Methyl 10-trans,12-cis-octadecadienoate	1.05 ± 0.91
24	9-Octadecenoic acid, methyl ester, (E)-	1.06 ± 0.92
25	Phytol	0.52 ± 0.09^{ab}
26	Linoleate	2.12 ± 0.63
27	9-Octadecenoic acid, (E)-	2.43 ± 2.18
28	2-Propanone, 1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-	0.67 ± 0.59
29	Octadecanoic acid	0.79 ± 0.41

Note: ^a important compound reported to possess antioxidant activities; ^b important compound reported to possess anti-inflammatory activities.

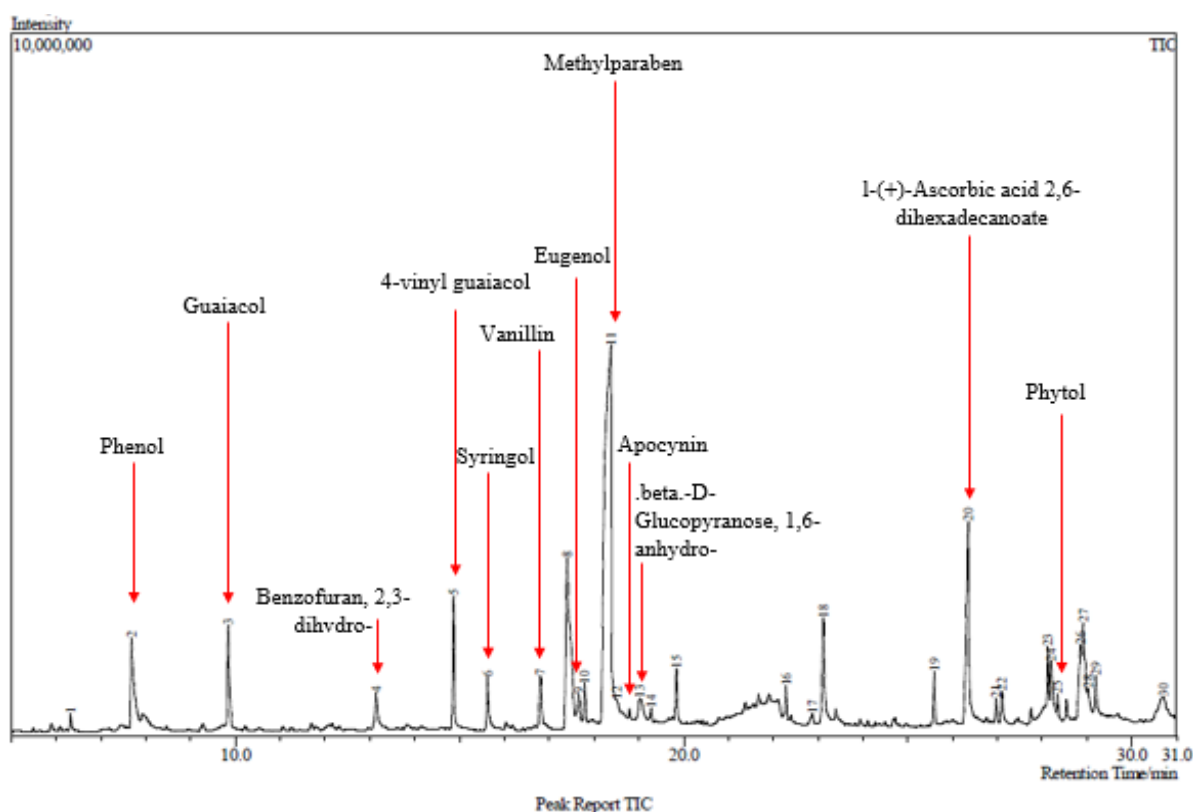


Figure 4.1: Gas chromatography mass-spectrometry (GC-MS) chromatogram of methanolic extract of *T. cordifolia*.

The most predominant phytochemical in methanolic *T. crispa* was methyl palmitate which comprised 24.84 ± 5.60 % of the total phytochemical compositions as shown in Table 4.2. This was followed by methyl stearate (17.22 ± 4.94 %) and octadecanoic acid (6.43 ± 0.17 %).

Table 4.2: Phytochemical constituents of methanolic extract of *T. crispa* detected by gas chromatography mass spectrometry (GC-MS).

Peak	Compounds	Mean $\pm$ S.D. (%)
1	2(5H)-Furanone	0.32 ± 0.43 ^{ab}
2	1,2-Cyclopentanedione	0.93 ± 0.81
3	Guaiacol	4.55 ± 1.08 ^{ab}
5	Benzoic acid, 2-methyl-, methyl ester	0.75 ± 0.65
6	Propanoic acid, 2-methyl-, 2-propenyl ester	0.90 ± 0.11
7	Benzofuran, 2,3-dihydro-	1.55 ± 0.96
8	4-vinyl guaiacol	4.37 ± 1.50 ^{ab}
9	Syringol	2.77 ± 0.39 ^a
11	Vanillin	2.75 ± 0.64 ^{ab}
13	Eugenol	0.78 ± 0.30 ^{ab}
14	2(3H)-Benzofuranone, 3a,4,5,7a-tetrahydro-3a,6-dimethyl-, cis-(.,+.-)-	0.83 ± 0.08
15	Canolol	1.81 ± 0.93 ^{ab}
16	(E)-2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	1.18 ± 1.04
17	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	2.43 ± 0.01
18	Syringylacetone	0.90 ± 0.35
19	Dihydrosyringenin	0.94 ± 0.61
20	2,6,8-Trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol	2.93 ± 0.57
21	2-Pentadecanone, 6,10,14-trimethyl-	1.26 ± 0.37
22	Palmitic acid	2.25 ± 1.96
23	Methyl palmitate	24.84 ± 5.60 ^b
24	Heptadecanoic acid	2.05 ± 1.32 ^{ab}
25	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	1.13 ± 1.01 ^b
26	9-Octadecenoic acid, methyl ester, (E)-	2.66 ± 0.45
27	Methyl stearate	17.22 ± 4.94
29	Octadecanoic acid	6.43 ± 0.17

Note: ^a important compound reported to possess antioxidant activities; ^b important compound reported to possess anti-inflammatory activities.

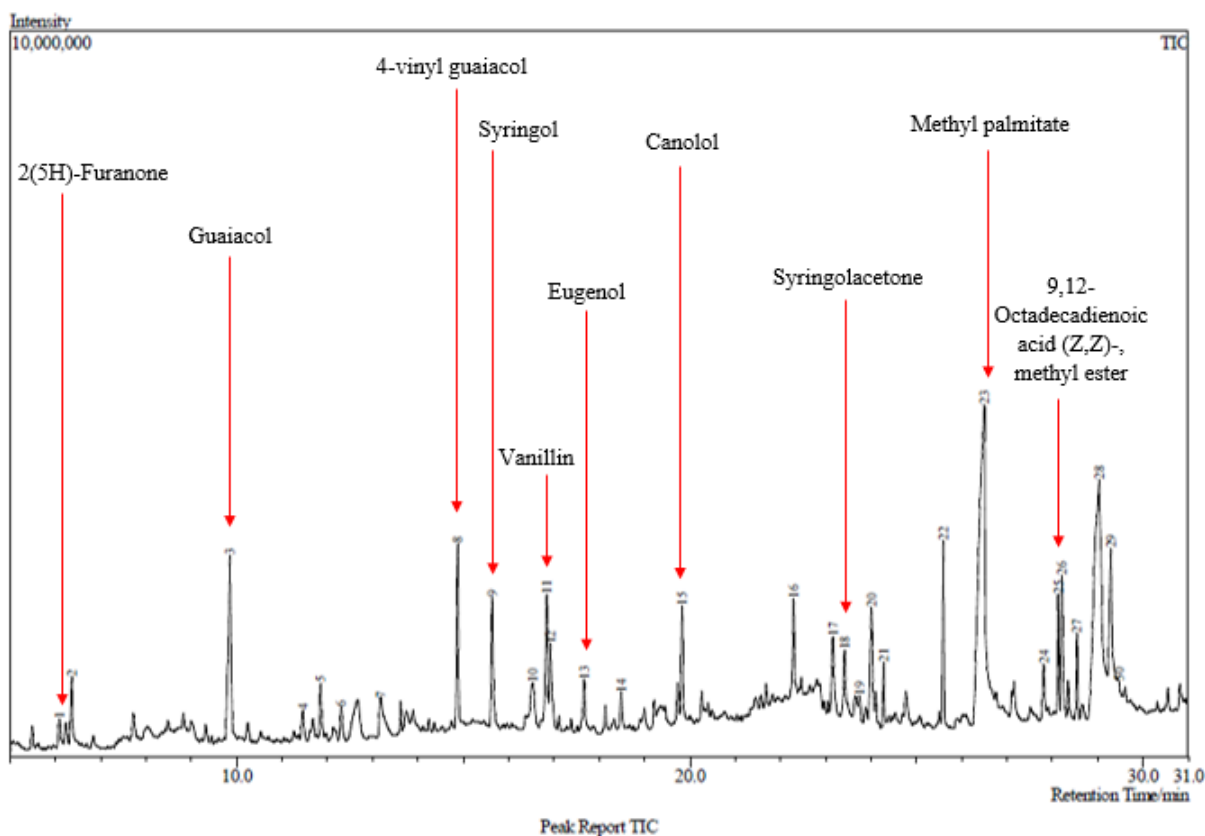


Figure 4.2: Gas chromatography-mass spectrometry (GC-MS) chromatogram of methanolic extract of *T. crista*

4.1.2 Aqueous Extracts of *T. cordifolia* and *T. crista*

The phytochemical analysis using GC-MS revealed the presence of phytochemical compounds in the aqueous extract of *T. cordifolia* extract as shown in Table 4.3 and Figure 4.3. Similar to methanolic extract of *T. cordifolia*, methylparaben was the major *constituent* which comprised 40.77 ± 12.75 % of the total phytochemicals in the aqueous extract of *T. cordifolia*. The next predominant phytochemicals were phenol (10.24 ± 2.90 %) and guaiacol (5.05 ± 2.40 %).

Table 4.3: Phytochemical constituents of aqueous extract of *T. cordifolia* detected by gas chromatography mass spectrometry (GC-MS).

Peak	Compounds	Mean $\pm$ S.D. (%)
1	Cyclopentanone	0.45 $\pm$ 0.02
2	Pyrrolidine	0.85 $\pm$ 0.13
5	Phenol	10.24 $\pm$ 2.90 ^{ab}
6	1,2-Cyclopentanedione, 3-methyl-	0.50 $\pm$ 0.10
7	Guaiacol	5.05 $\pm$ 2.40 ^{ab}
8	Propanoic acid, 2,2-dimethyl-	0.64 $\pm$ 0.23
9	3-[N'-(3H-Indol-3-ylmethylene)-hydrazino]-5-methyl-[1,2,4]triazol-4-ylamine	0.67 $\pm$ 0.00
10	Benzofuran, 2,3-dihydro-	0.61 $\pm$ 0.08
11	4-vinyl guaiacol	2.68 $\pm$ 0.89
12	Syringol	2.23 $\pm$ 1.14 ^a
14	Vanillin	1.34 $\pm$ 1.60 ^{ab}
15	Benzoic acid, 4-hydroxy-, hydrazide	2.59 $\pm$ 1.64
17	Phenol, 2-methoxy-4-(1-propenyl)-	0.60 $\pm$ 0.09
19	Methylparaben	40.77 $\pm$ 12.75 ^a
20	Veratryl alcohol	0.47 $\pm$ 0.01 ^a
21	Canolol	1.06 $\pm$ 0.07 ^{ab}
22	(E)-2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	0.35 $\pm$ 0.30
24	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	0.77 $\pm$ 0.19
25	Syringylacetone	0.51 $\pm$ 0.11
26	2-Cyclohexen-1-one, 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-	0.47 $\pm$ 0.01
27	Hexadecanoic acid, methyl ester	0.39 $\pm$ 0.20
28	(3aR,4R,8R,8aS)-3a,4,7,8a-Tetramethyl-1,2,3,3a,4,5,8,8a-octahydro-4,8-methanoazulene	2.79 $\pm$ 0.10
29	2,6,8-Trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol	1.84 $\pm$ 0.00

Note: ^a important compound reported to possess antioxidant activities; ^b important compound reported to possess anti-inflammatory activities.

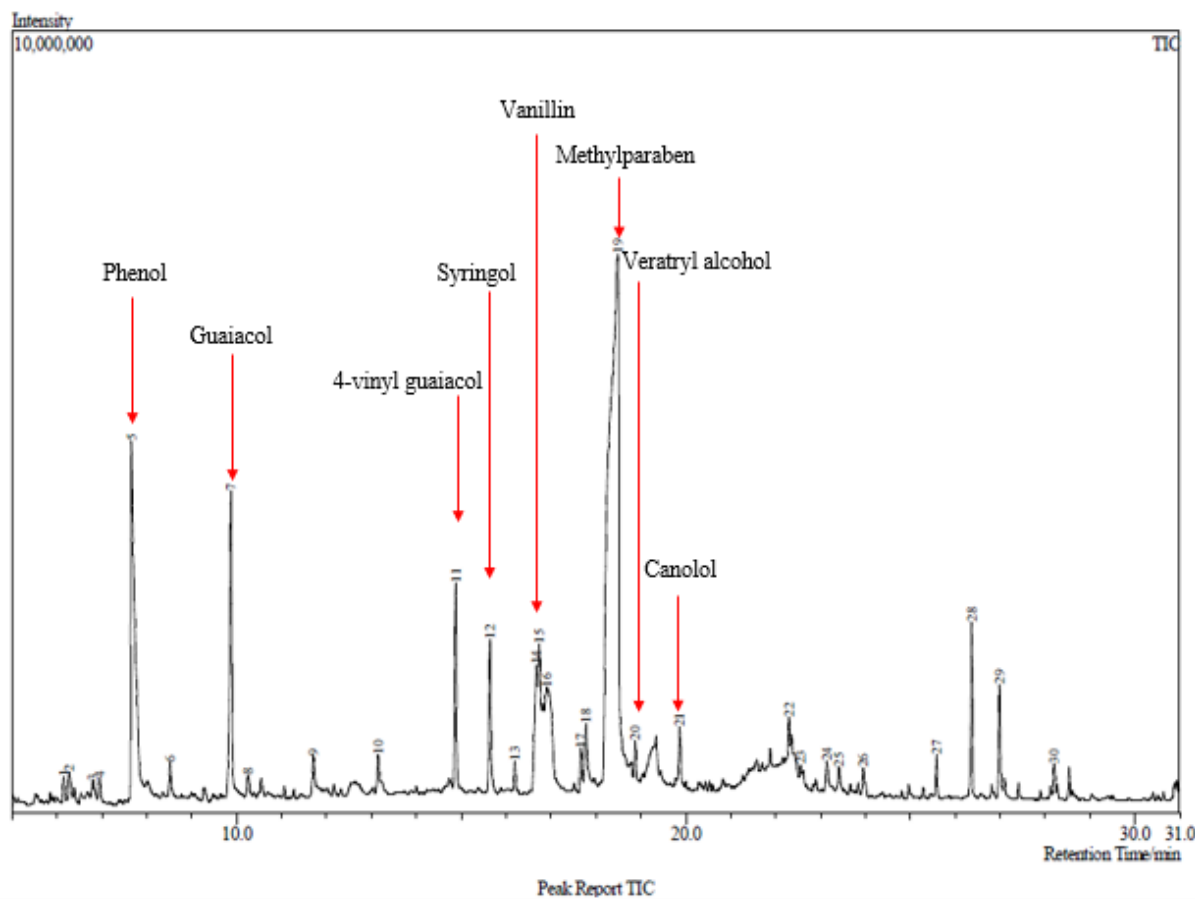


Figure 4.3: Gas chromatography mass spectrometry (GC-MS) chromatogram for aqueous extract of *T. cordifolia*

Moreover, phytochemical constituents were also detected in aqueous extract of *T. crispera*. The most predominant phytochemical was furyl hydroxymethyl ketone which comprised 13.19 ± 3.45 % of the total phytochemical compositions. This was followed by (S)-(+)-2',3'-dideoxyribonolactone (9.44 ± 1.65 %) and sinapyl alcohol (6.04 ± 5.17 %).

Table 4.4: Phytochemical constituents of aqueous extract of *T. crista* detected by gas chromatography mass spectrometry (GC-MS).

Peak	Compounds	Mean $\pm$ S.D. (%)
2	Pyrrolidine	2.39 $\pm$ 0.07
3	1,2-Cyclopentanedione	4.08 $\pm$ 0.13
4	Phenol	3.04 $\pm$ 1.23 ^{ab}
5	1,2-Cyclopentanedione, 3-methyl-	1.48 $\pm$ 0.60
6	3-Hexanol, 2,3-dimethyl-	0.93 $\pm$ 0.02
8	Furyl hydroxymethyl ketone	13.19 $\pm$ 3.45 ^a
9	Propanoic acid, 2,2-dimethyl-	2.09 $\pm$ 0.16
10	1-Methyl-8-nitroisoxazolizidine	4.11 $\pm$ 0.23
11	Propanoic acid, 2-methyl-, 2-propenyl ester	3.61 $\pm$ 0.45
12	(S)-(+)-2',3'-Dideoxyribonolactone	9.44 $\pm$ 1.65
13	Flamenol	3.10 $\pm$ 0.11
14	2(3H)-Furanone, 3-acetyldihydro-3-methyl-	1.87 $\pm$ 0.34
15	4-vinyl guaiacol	5.15 $\pm$ 1.53 ^{ab}
16	Syringol	4.88 $\pm$ 0.43 ^a
17	2-Piperidinemethanol	0.13 $\pm$ 0.23
18	Vanillin	2.22 $\pm$ 0.50 ^{ab}
19	4-Methyl-1-(1,2,2-trimethylcyclopentyl)cyclohex-3-enol	0.83 $\pm$ 1.44
20	Methylparaben	2.62 $\pm$ 0.05 ^a
22	Canolol	3.68 $\pm$ 0.23 ^{ab}
23	1-Methylenespiro[2.4]heptan-4-one	1.46 $\pm$ 0.15
24	Propenyl syringol	2.14 $\pm$ 0.04
26	Syringylacetone	2.05 $\pm$ 0.49 ^a
27	1,2-Diazaspiro[4.4]nonen-3-carboxylic acid, 6,6,9,9-tetramethyl-, methyl ester	1.22 $\pm$ 0.19
28	2,6,8-Trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol	2.92 $\pm$ 0.35
29	Sinapyl alcohol	6.04 $\pm$ 5.17 ^b

Note: ^a important compound reported to possess antioxidant activities; ^b important compound reported to possess anti-inflammatory activities.

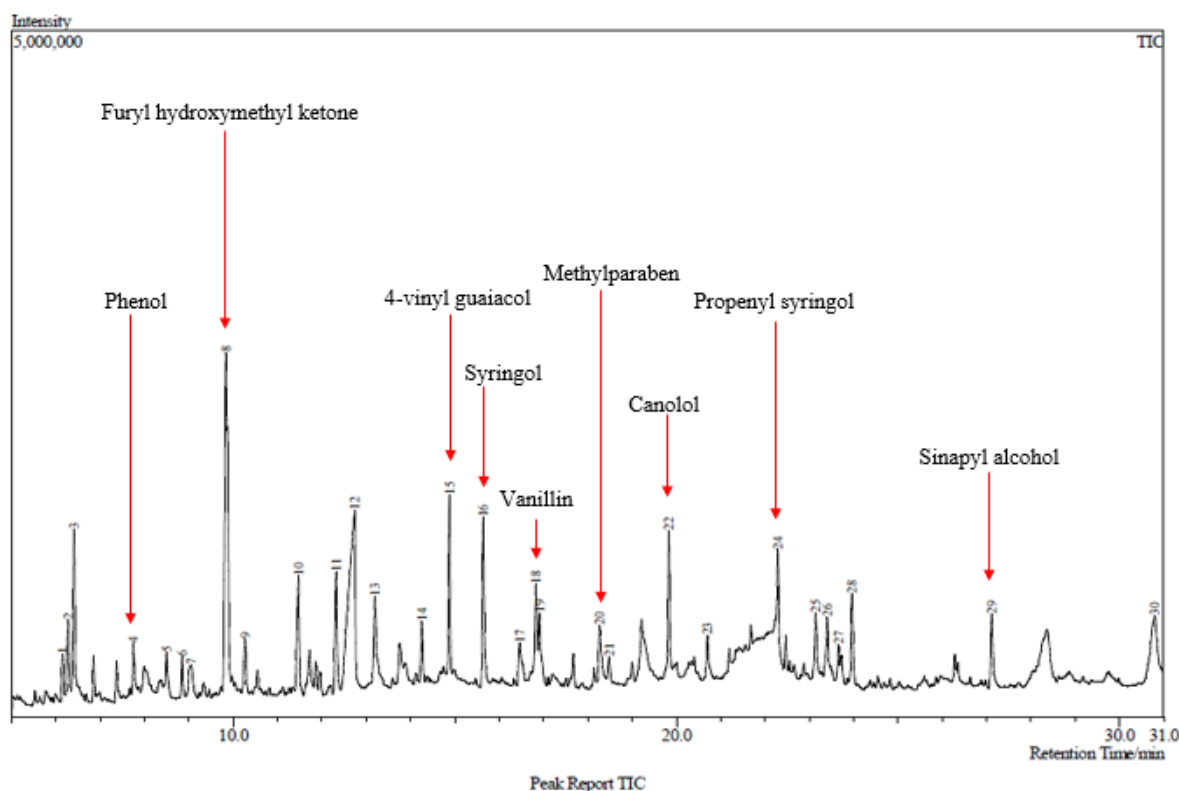


Figure 4.4: Gas chromatography mass spectrometry (GC-MS) chromatogram for aqueous extract of *T. crispa*

4.2 Antioxidant Activities of Methanolic and Aqueous Extracts of *T. cordifolia* and *T. crispa*

The antioxidant activities of methanolic and aqueous extracts of *T. cordifolia* and *T. crispa*, were evaluated using DPPH free radical scavenging activity and BSA-MGO assays. The DPPH assay measures the ability of compounds to scavenge free radicals, where a reduction in the purple DPPH radical solution's colour indicates antioxidant activity. The antioxidant activity of the fraction was compared with ascorbic acid. In the BSA-MGO assay, the protection of bovine serum albumin (BSA) from glycation by MGO reflects the compounds' ability to inhibit protein damage caused by glycation, a process implicated in oxidative stress-related diseases. These assays provided valuable insights into the potential antioxidant properties of *T. crispa* and *T. cordifolia* extracts.

4.2.1 DPPH scavenging antioxidant assay

Ascorbic acid exhibited a maximum effect (plateau effect) at a low concentration of 37.5 µg/mL. Thus, increasing the concentrations of ascorbic acid up to 500 µg/mL did not lead to further increase in the DPPH free radical scavenging activity. Interestingly, the percentage of DPPH free radical scavenging activities of the methanolic extracts of *T. crispa* was similar to the methanolic extracts of *T. cordifolia*. In addition, the percentages of DPPH inhibition of methanolic extracts of *T. crispa* and *T. cordifolia* at 37.5 µg/mL were 51.83 ± 1.57 % and 53.33 ± 0.53 % respectively and maximised at the concentration of 125 µg/mL where further increased in the extract concentrations did not cause further increase in the percentages of DPPH inhibition. This study also shows that the methanolic extracts of both *T. crispa* and *T. cordifolia* exhibited a higher percentage of DPPH scavenging activity than the aqueous extracts (Table 4.5 and Figure 4.5).

Table 4.5: DPPH inhibition by *T. crispa* and *T. cordifolia* crude extracts.

Concentration (µg/mL)	DPPH free radical scavenging activity (%)				Ascorbic Acid standard (control)
	<i>T. crispa</i> (Aqueous)	<i>T. cordifolia</i> (Aqueous)	<i>T. crispa</i> (Methanol)	<i>T. cordifolia</i> (Methanol)	
37.50	12.82±5.56*	19.65±4.74*	51.83±1.57*	53.33±0.53*	86.86±0.25
125.0	16.93±3.66*	39.25±1.65*	78.83±0.54*	81.41±0.55*	86.50±0.53
250.0	26.83±1.96*	47.40±0.80*	79.75±1.68*	76.15±0.32*	86.30±0.78
500.0	42.17±3.50*	72.80±0.66*	79.03±0.66*	81.30±0.28*	85.65±1.70

Values are mean ± standard deviation (n=3). *p < 0.05 indicates that the mean is significantly different when compared with the similar ascorbic acid (control) concentrations respectively.

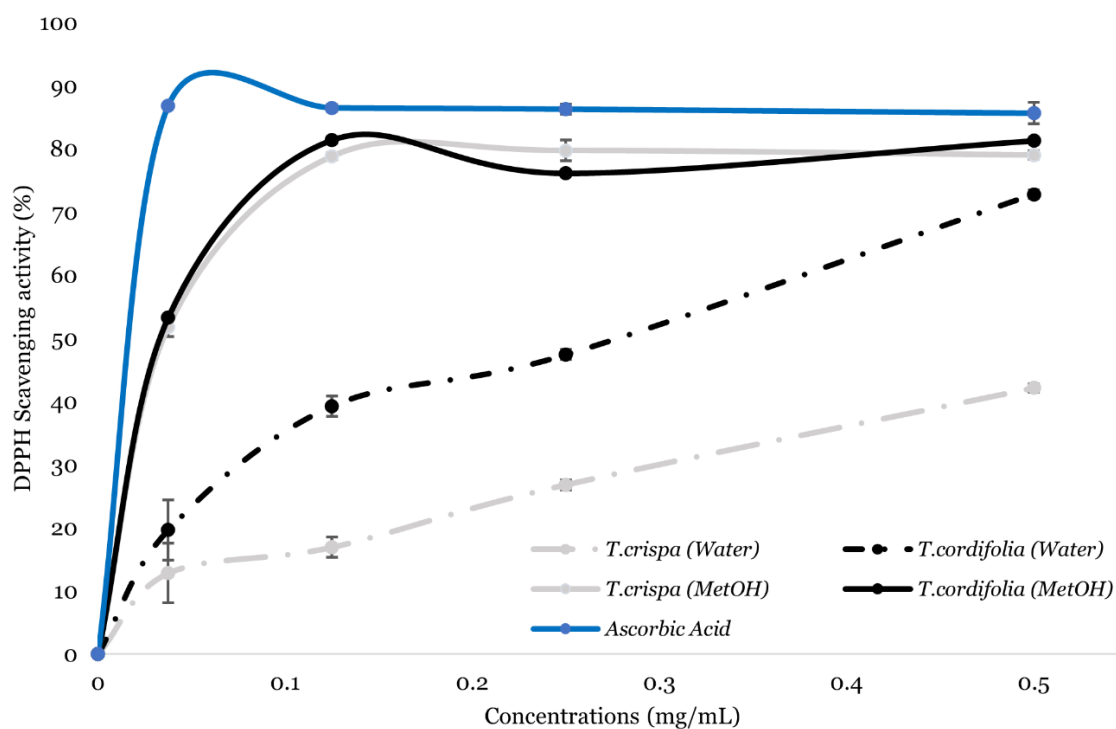


Figure 4.5: The DPPH radical scavenging activity of methanolic and aqueous extracts of *T. crispa* and *T. cordifolia*. Bars represent standard deviation of the means.

The percentage of DPPH scavenging activity for both aqueous extracts of *T. crispa* and *T. cordifolia* increased in the concentration dependent manner. Meanwhile, at the concentration of 500 $\mu\text{g/mL}$, the aqueous extracts of *T. crispa* and *T. cordifolia* did not reach the plateau effect, suggesting that a higher concentration of the aqueous crude extracts for both plants is required to achieve a higher percentage of DPPH radical scavenging.

4.2.2 BSA-MGO anti-glycation assay

The antioxidant activities of the plants extracts were also evaluated using the BSA-MGO anti-glycation assay as shown in Figure 4.6. The positive control, AG (400 mg/mL) inhibited 97.63 ± 0.03 % of glycation between BSA and MGO. All the extracts exhibited anti-glycation activity and were significantly different from the positive control, AG ($p < 0.05$). However, none of the extracts with concentrations ranging from 50 $\mu\text{g/mL}$ to 400 $\mu\text{g/mL}$, prevented the glycation by more than 50 %.

The methanolic extract of *T. crispa* exhibited an increasing trend in its anti-glycation activity in a concentration dependent manner. At 50 µg/mL, the anti-glycation ability of methanolic extract of *T. crispa* was 5.07 ± 0.58 %, followed by 25.93 ± 5.77 % at 100 µg/mL. The anti-glycation ability at 50 µg/mL was significantly different from that at 100 µg/mL, 200 µg/mL and 400 µg/mL ($p < 0.05$). However, there was no significant difference in the anti-glycation ability between 200 µg/mL (20.52 ± 7.42 %) and 400 µg/mL (25.93 ± 5.77 %) ($p > 0.05$). These results showed that increasing the concentration of methanolic extract of *T. crispa* extract from 200 µg/mL to 400 µg/mL did not result in any significant anti-glycation activity.

The methanolic extract of *T. cordifolia* showed an increasing trend in anti-glycation ability from 50 µg/mL (14.06 ± 3.89 %) to 200 µg/mL (17.01 ± 0.16 %) although these concentrations did not show significant differences among each other ($p > 0.05$). Subsequently, the concentrations exhibited a plateau trend from 200 µg/mL (34.38 ± 0.98 %) to 400 µg/mL (34.04 ± 0.67 %) with no significant difference observed between 200 µg/mL and 400 µg/mL ($p > 0.05$). This suggests that increasing the concentration from 200 µg/mL to 400 µg/mL concentration would not result in any significant anti-glycation activity for methanolic extract of *T. cordifolia*.

Overall, methanolic extract of *T. cordifolia* had shown a better anti-glycation ability over methanolic extract of *T. crispa*. At 400 µg/mL, the anti-glycation abilities of both extracts were significantly different from each other, with the methanolic extract of *T. cordifolia* exhibiting the highest level of anti-glycation properties.

Interestingly, the aqueous extract of *T. crispa* showed a plateau in its anti-glycation activity displaying the highest level of anti-glycation, at its lowest concentration, 50 µg/mL (33.83 ± 1.80 %) and continuing up to 400 µg/mL (36.91 ± 2.31 %) among all the extracts.

Furthermore, there were no significant differences observed within its concentration range when compared to each other.

However, the anti-glycation activity for aqueous extract of *T. cordifolia* was observed to increase from 50 µg/mL (24.71 ± 4.87 %) to 100 µg/mL (29.98 ± 3.13 %) although the difference between these two concentrations was not statistically significant ($p > 0.05$). Subsequently, the anti-glycation trend for aqueous extract of *T. cordifolia* showed a consistent trend from 200 µg/mL (34.60 ± 0.81 %) to 400 µg/mL (34.21 ± 0.52 %). In comparison, the aqueous extract of *T. crispa* showed a higher potential to prevent anti-glycation than aqueous extract of *T. cordifolia*. This can be observed through higher anti-glycation ability of aqueous *T. crispa* even at its lowest concentration 50 µg/mL.

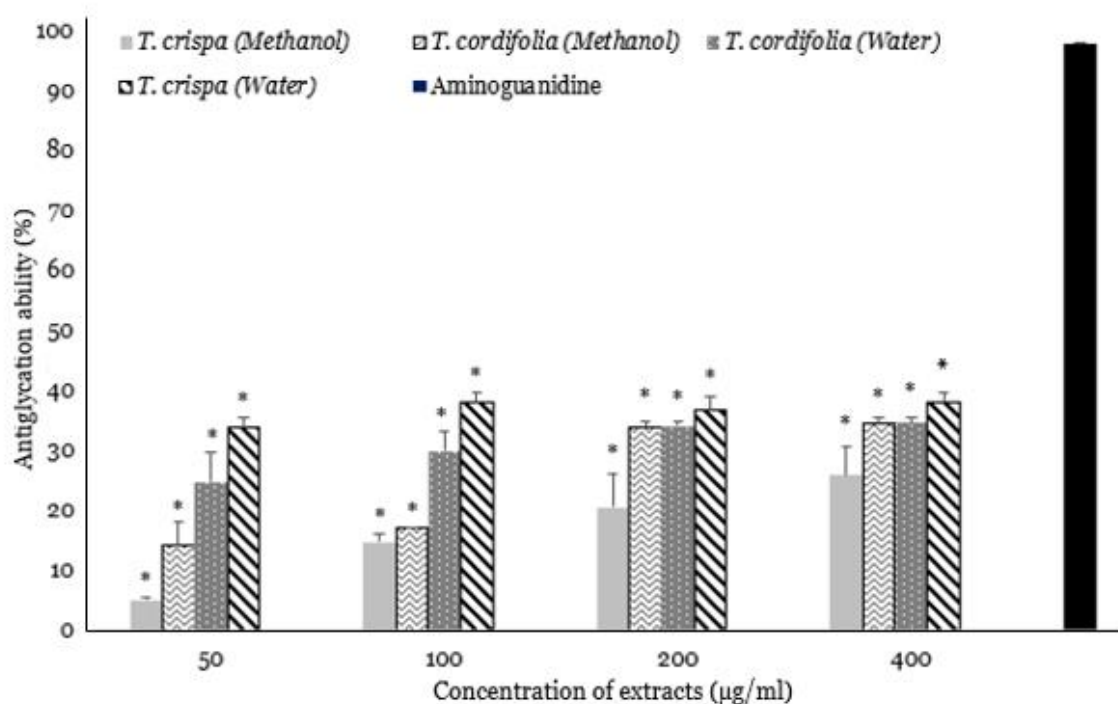


Figure 4.6: BSA-MGO anti-glycation activity of methanolic and aqueous extracts of *T. crispa* and *T. cordifolia*. * $p < 0.05$ as compared to aminoguanidine (positive control) is considered significant.

4.3 Determination of IC₅₀ of MGO, non-toxic concentrations of metformin and extracts

The IC₅₀ of MGO, as well as non-toxic concentrations of metformin and extracts were assessed through MTT and AO/PI assays. These assays operate on different principles: MTT measures viability indirectly by assessing cellular metabolism through mitochondrial enzyme activity that reacts with MTT to form formazan, while AO/PI measures viability directly by evaluating cellular membrane integrity. Nevertheless, both assays can be used to represent hFOB1.19 viability following treatment.

4.3.1 The viability and IC₅₀ of hFOB1.19 after treated with MGO in different concentrations

The effect of MGO on the viability of hFOB1.19 cultured in DMEM/F-12 media was examined through MTT and AO/PI. The result was illustrated in Figure 4.7. Cells were cultured at a density of 2×10^4 cells/mL in each well of the 96 well plate and incubated with MGO for 24 hours.

When assessed via MTT assay, the viability of hFOB1.19 showed decrease in cell viability from 100 ± 7.07 % at 0 $\mu\text{g/mL}$ (negative control) to 40.93 ± 3.49 % when treated with 1750 $\mu\text{g/mL}$ of MGO. Incubation of the cells with 875 $\mu\text{g/mL}$ of MGO caused slight increase in the cell viability (101.52 ± 6.24 %) when compared to untreated cells, however, this increase was not statistically significant ($p > 0.05$). Only with MGO treatment at concentration of 1225 $\mu\text{g/mL}$ and above did the cells showed significant reduction in viability. Moreover, MGO significantly decreased the viability of hFOB1.19 to below 50 % viability at 1750 $\mu\text{g/mL}$.

A similar decreasing trend in cell viability was also observed in the AO/PI assay after treating the cells with MGO for 24 hours. The cell viability decreased from 90.83 ± 4.31 %

at 0 $\mu\text{g/mL}$ (negative control) to $12.3 \pm 1.11\%$ when the cells were treated with 1750 $\mu\text{g/mL}$ of MGO. Through AO/PI assay, MGO at 1750 $\mu\text{g/mL}$ was observed to reduce the viability even lower than with MTT assay at a similar concentration.

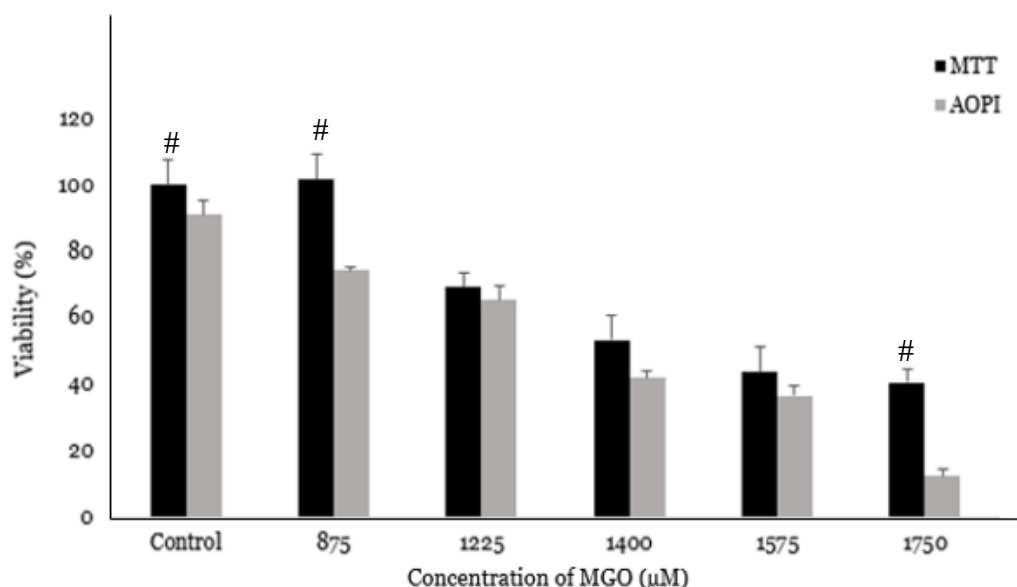


Figure 4.7: The viability of hFOB1.19 (2×10^4 cells/mL) when treated with different concentration of MGO for 24-hour measured through MTT and AO/PI assays. MTT assay only shows the reduction trend of the cell's viability from 1225 μM of MGO. Meanwhile, hFOB1.19 appear to be reduced in viability after treated with 875 μM of MGO. # $p < 0.05$ indicates significance between MTT and AO/PI in determining cellular viability at the respective concentrations.

Based on these results, the variation in cell viability assays did not affect the trend of cellular viability of hFOB1.19 when treated with MGO for 24 hours. The principle underlying the MTT viability assay involves the measurement of mitochondrial dehydrogenase which reduces MTT to form formazan products, indicating living cells undergoing cellular metabolic activity. Thus, a decrease in metabolic activity is associated with the cytotoxic effect, indicating cellular death. Meanwhile, a dual staining AO/PI assay provides a direct approach in measuring cellular viability as viable cells stain green while non-viable or apoptotic cells stain orange. Acridine orange dye can permeate intact cellular membrane, staining the viable cells green, whereas propidium iodide can only permeate

compromised membranes marking cells undergoing cellular death. This study observed a similar trend in both indirect approach (cell metabolism) and direct approach (cell membrane integrity) in measuring hFOB1.19 viabilities when treated with MGO after 24 hours.

The IC_{50} , or half-maximal inhibitory concentration, was determined from the MTT assay, represents the concentration of a substance required to reduce cell viability at least 50 %. The regression (R^2) value and equation for determining the IC_{50} are shown in Figure 4.8. Based on the equation, the calculated IC_{50} for MGO was determined to be 1523.86 μM . Thus, approximately 1523.86 μM of MGO was needed to achieve at least a 50 % reduction in viability of hFOB1.19, after treated for 24 hours.

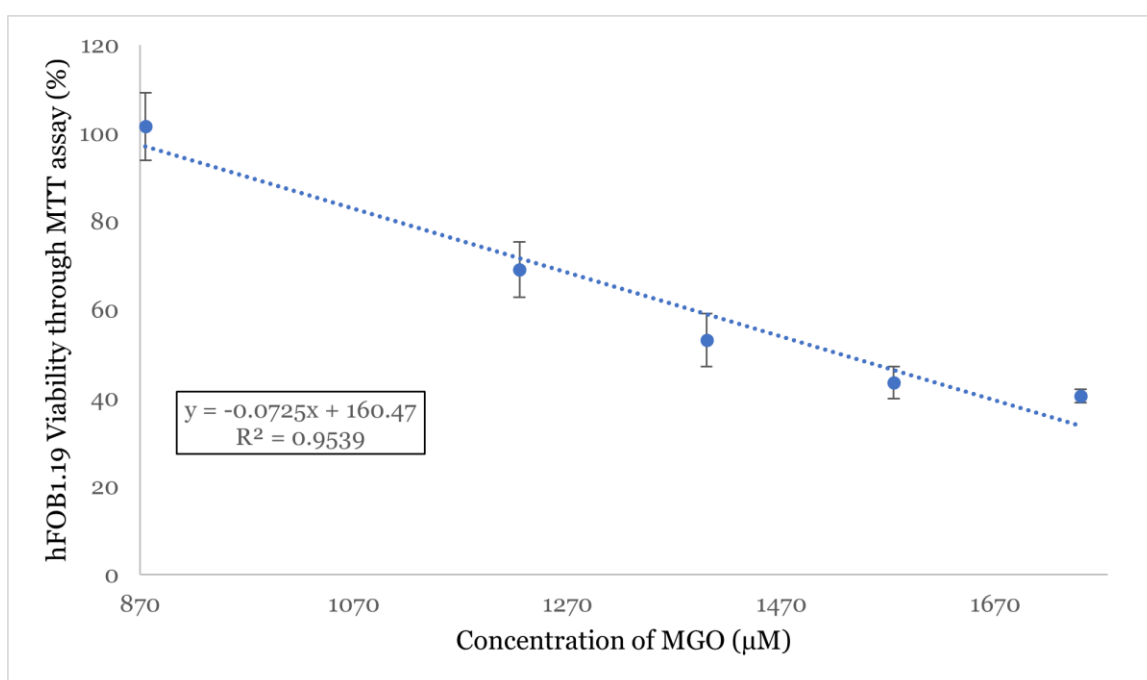


Figure 4.8: IC_{50} determination of MGO for MTT assay. The R^2 was more than 0.90, which indicate strong reliability between the dependent variable (concentration) and the independent (viability of hFOB1.19).

4.3.2 Non-toxic concentration of metformin towards hFOB1.19 viability

The effect of metformin on the viability of hFOB1.19 cultured in DMEM/F-12 media was assessed using MTT and AO/PI as represented in Figure 4.9. Cells were cultured at a density of 2×10^4 cells/mL in each well of a 96 well plate and incubated with metformin concentrations ranging from 1500 μ M to 12000 μ M for 24 hours.

In the MTT viability assay, metformin with the concentrations of 1500 μ M and 3000 μ M showed the cellular viability of 137.30 ± 4.22 % and 118.89 ± 5.27 % respectively. These results showed a significant ($p < 0.05$) increase in the production of formazan crystals reflecting heightened cellular metabolism at these concentrations. At 6000 μ M (104.82 ± 5.40 %), metformin showed a slight increase in cellular metabolism of hFOB1.19, but this was not statistically significant ($p > 0.05$). However, only the concentration of 12000 μ M metformin managed to reduce cellular metabolism to below 100 %, i.e. at 89.89 ± 5.85 % after its treatment for 24 hours with hFOB1.19.

Interestingly, AO/PI assay had revealed a plateau trend of cellular viability after treated with metformin for 24 hours ranging from 1500 μ M to 12000 μ M. At the lowest concentration of 1500 μ M, cell viability exhibited the viability of 84.40 ± 3.92 % and reduced down only until 82.77 ± 2.54 % at 12000 μ M. These observations performed through AO/PI assay revealed that hFOB1.19 treatment with metformin ranging of 1500 μ M to 12000 μ M did not exhibit cytotoxicity properties.

There were significant differences between MTT and AO/PI results for metformin-treated hFOB1.19 cells at concentrations of 0 μ g/mL (negative control), 1500 μ M, 3000 μ M and 6000 μ M. However, there was no difference between MTT and AO/PI results for metformin-treated cells at a concentration 12000 μ M.

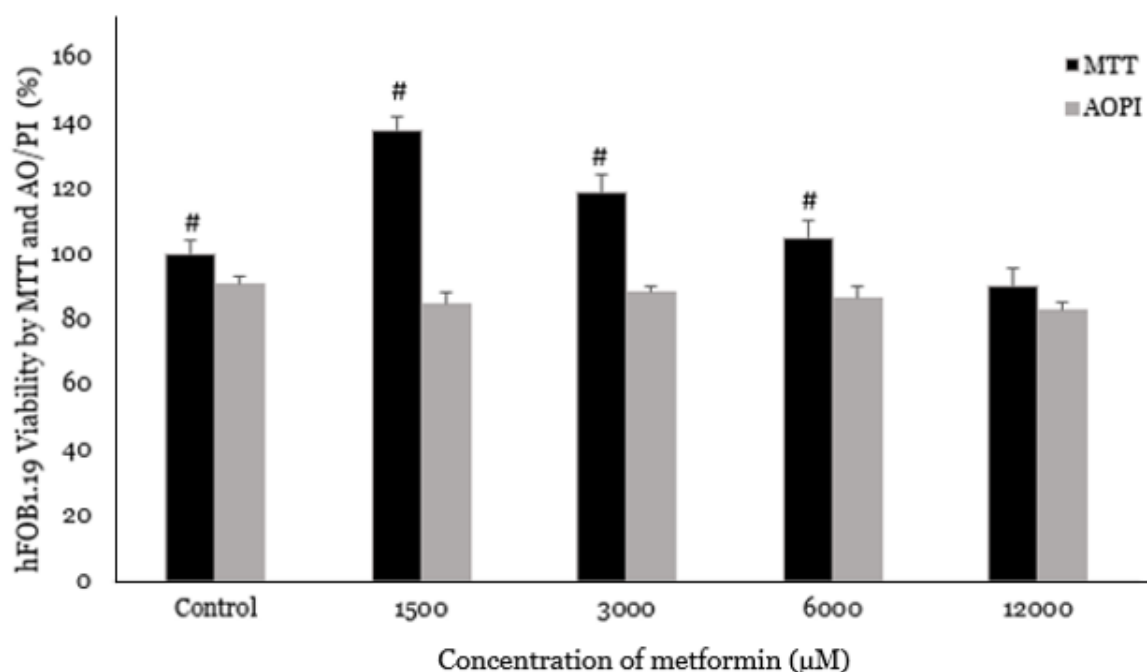


Figure 4.9: The percentage viability of hFOB1.19 (2×10^4 cells/mL) when treated with different concentrations of metformin for 24 hours. 1500 μ M and 3000 μ M significantly increased the cellular metabolism activity when measured through MTT. Meanwhile, AO/PI had shown a plateau trend from 1500 μ M to 12000 μ M in terms of cellular membrane integrity. [#] $p < 0.05$ indicates significance between MTT and AO/PI in determining cellular viability at the respective concentrations.

4.3.3 Non-toxic concentrations of crude plant extracts towards hFOB1.19 viability

In the MTT viability assay conducted with methanolic extract of *T. cordifolia*, the cell viabilities consistently exceeded 80% within the concentration range of 12.5 μ g/mL to 100 μ g/mL as shown in Figure 4.10. At the lowest concentration of 12.5 μ g/mL, cell viability remained at 100 ± 1.53 %, with a slight reduction to 90.23 ± 1.80 % observed only at the highest concentration of 100 μ g/mL. Similarly, the AO/PI assay also showed a similar trend, with cell viability remaining at 100 ± 1.53 % at the lowest concentration of 12.5 μ g/mL and decreasing only to 82.9 ± 2.31 % at 100 μ g/mL. There were no significant differences between MTT and AO/PI results for hFOB1.19 treated with methanolic extract of *T. cordifolia* for concentrations 0 μ g/mL (negative control), 12.5 μ g/mL and 25 μ g/mL.

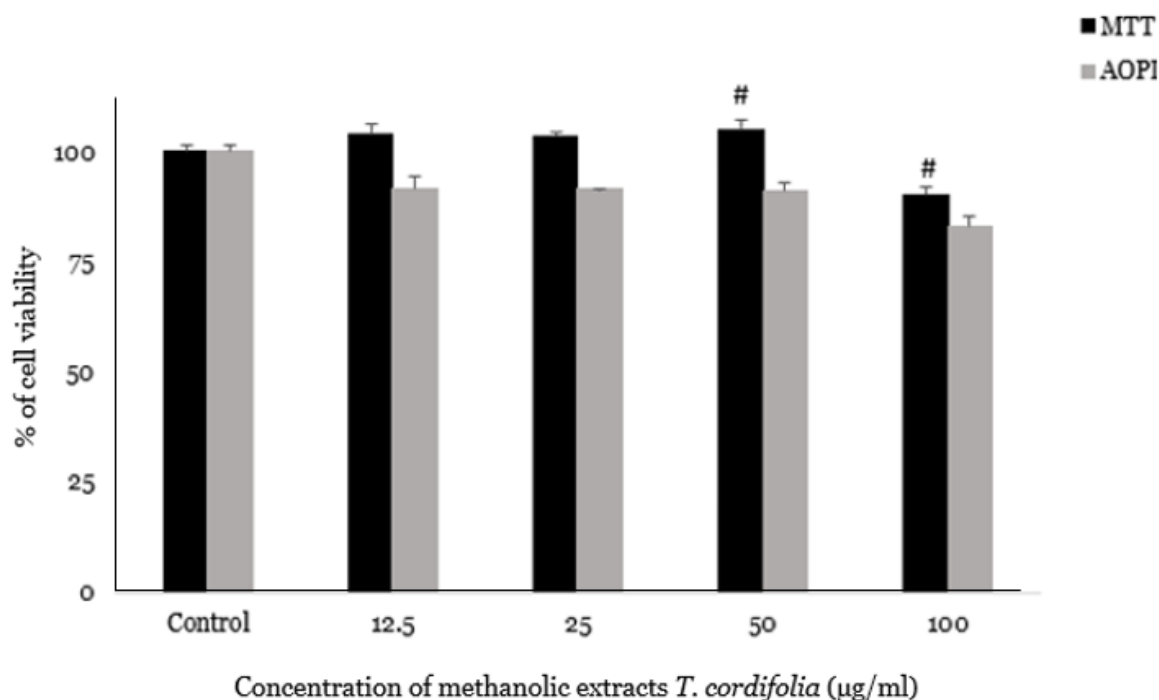


Figure 4.10: The percentage viability of hFOB1.19 (2×10^4 cells/mL) when treated with different concentrations of methanolic extract of *T. cordifolia* for 24 hours. # $p < 0.05$ indicates significance between MTT and AO/PI in determining cellular viability at the respective concentrations.

Moreover, in the MTT viability assay conducted with methanolic extract of *T. cordifolia*, the cell viabilities consistently exceeded 80 % within the concentration range of 30 µg/mL to 240 µg/mL as shown in Figure 4.11. At the lowest concentration of 30 µg/mL, cell viability remained at 100 ± 0.44 %, with a slight reduction to 92.69 ± 3.30 % observed only at the highest concentration of 240 µg/mL. Similarly, the AO/PI assay also represented the similar trend. At the lowest concentration of 30 µg/mL, cell viability remained at 95.33 ± 1.53 % and reduced down only until 82.07 ± 0.15 % at 240 µg/mL. All concentrations, either examined from MTT or AO/PI had shown non-cytotoxic effect when treated with hFOB1.19 cells after 24 hours.

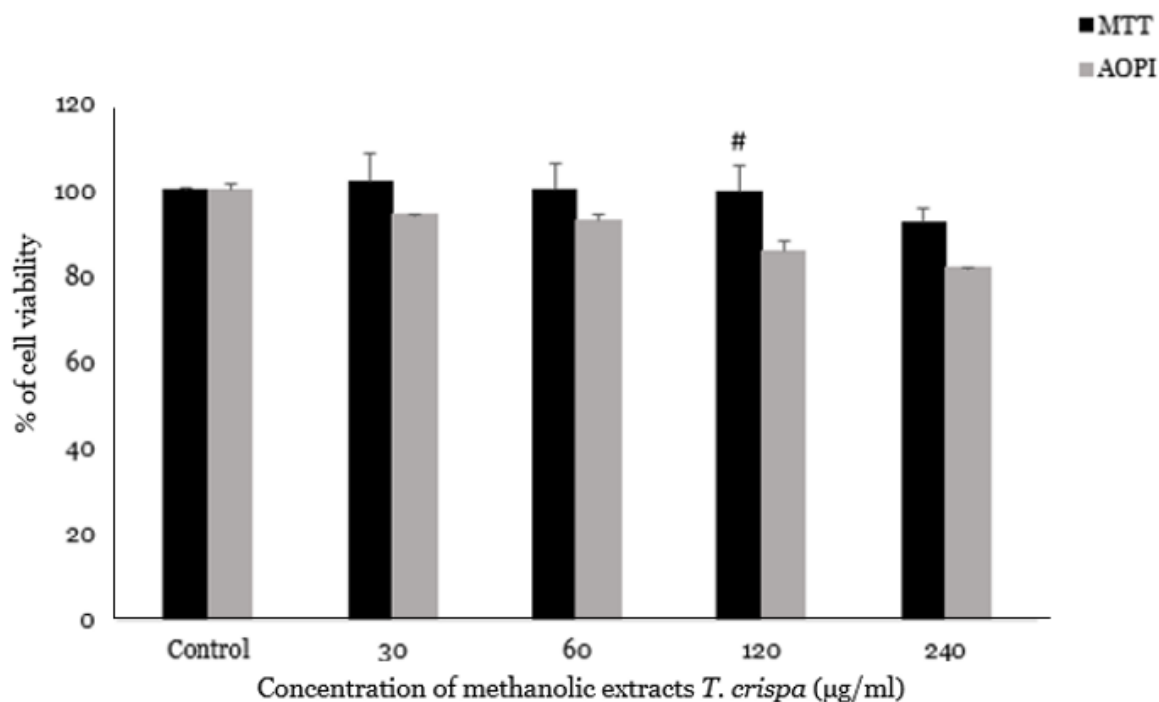


Figure 4.11: The percentage viability of hFOB1.19 (2×10^4 cells/mL) when treated with different concentrations of methanolic extract of *T. crispa* for 24 hours. [#] $p < 0.05$ indicates significance between MTT and AO/PI in determining cellular viability at the respective concentrations.

In the MTT viability assay conducted with aqueous extract of *T. cordifolia*, the cell viabilities consistently exceeded 80 % within the concentration range of 15.63 µg/mL to 125 µg/mL as shown in Figure 4.12. At the lowest concentration of 15.63 µg/mL, cell viability remained at 114.11 ± 1.87 %, with a slight reduction to 101.01 ± 0.01 % observed only at the highest concentration of 125 µg/mL. Similarly, the AO/PI assay also represented a similar trend. At the lowest concentration of 15.63 µg/mL, cell viability remained at 91.63 ± 0.90 % and reduced only to 86.70 ± 3.05 % at 125 µg/mL. All concentrations, either examined from MTT or AO/PI had shown non-cytotoxic effect when treated with hFOB1.19 cells after 24 hours.

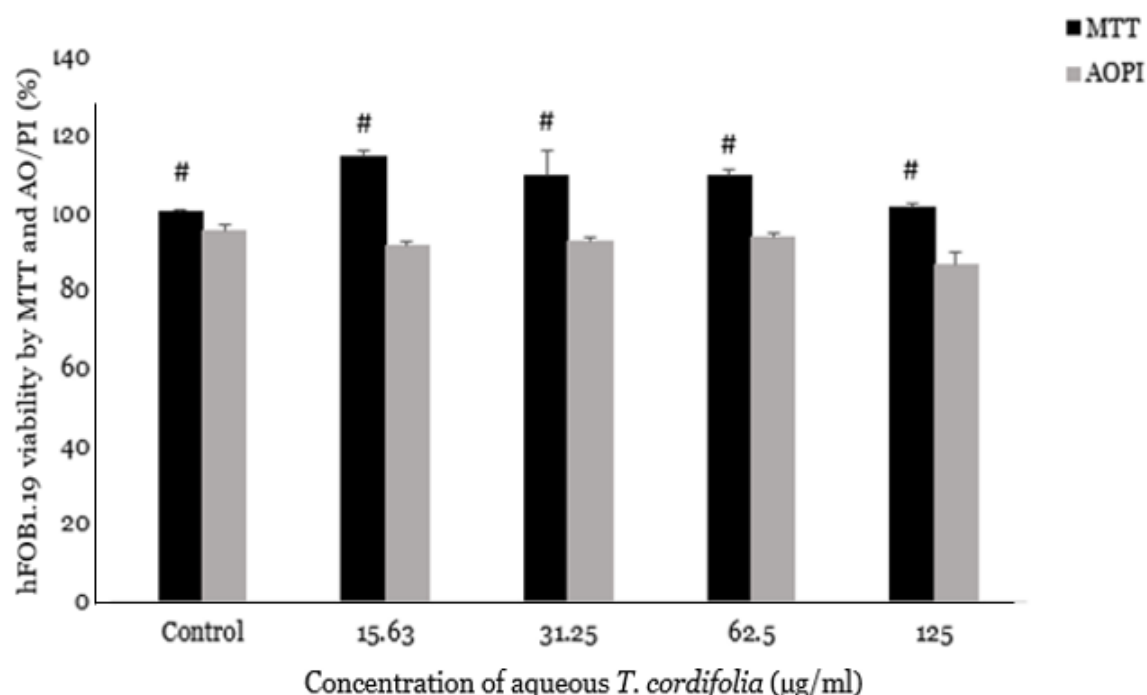


Figure 4.12: The percentage viability of hFOB1.19 (2×10^4 cells/mL) when treated with different concentrations of aqueous extract of *T. cordifolia* after 24-hour treatment. [#] $p < 0.05$ indicates significance between MTT and AO/PI in determining cellular viability at the respective concentrations.

In the MTT viability assay conducted with aqueous extract of *T. crispa*, the cell viabilities consistently exceeded 80 % within the concentration range of 46.88 µg/mL to 375 µg/mL as shown in Figure 4.13. At the lowest concentration of 46.88 µg/mL, cell viability remained at 109.64 ± 1.53 %, with a slight reduction to 103.29 ± 2.94 % observed only at the highest concentration of 375 µg/mL. Similarly, the AO/PI assay also represented the similar trend. At the lowest concentration of 46.88 µg/mL, cell viability remained at 95.33 ± 1.53 % and reduced down only until 89.87 ± 2.97 % at 375 µg/mL. Nevertheless, all concentrations whether examined by MTT or AO/PI had shown a non-cytotoxic effect when treated with hFOB1.19 cells for 24 hours.

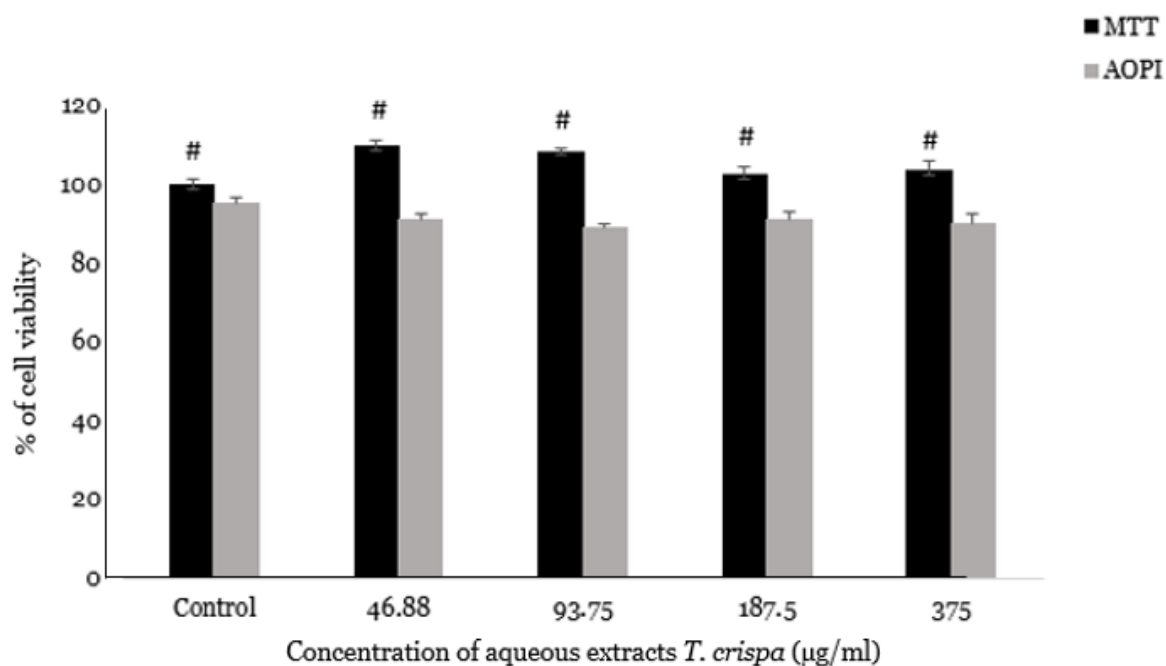


Figure 4.13: The percentage viability of hFOB1.19 (2×10^4 cells/mL) when treated with different concentrations of aqueous extract of *T. crispa* for 24 hours. # $p < 0.05$ indicates significance between MTT and AO/PI in determining cellular viability at the respective concentrations.

This study identified the last two highest concentrations tested as optimal for each extract, as they demonstrated over 80 % viability when exposed to the hFOB1.19 cells suggesting non-cytotoxic properties at these concentrations. Specifically, for methanolic extracts of *T. cordifolia*, the concentrations of 50 µg/mL and 100 µg/mL were chosen, while for methanolic extracts of *T. crispa*, concentrations 120 µg/mL and 240 µg/mL were selected. Similarly, for aqueous extracts of *T. cordifolia*, 62.5 µg/mL and 125 µg/mL were selected. Meanwhile, for aqueous extracts of *T. crispa* concentrations 187.5 µg/mL and 375 µg/mL were found to be suitable. These concentrations were used for the subsequent assays in this study.

4.3.4 Formation of intracellular ROS in MGO-induced hFOB1.19 when treated with crude extracts with or without metformin

The formation of intracellular ROS in MGO-stimulated hFOB1.19 (1523.86 μM) treated with crude methanolic and aqueous extracts with or without metformin (12000 μM) is shown in Figure 4.15 and Figure 4.15 below. It was observed that MGO induced a substantial increase in intracellular ROS levels, signifying oxidative stress. However, the introduction of metformin significantly reduced the intracellular ROS ($p < 0.05$), demonstrating its potential to mitigate cytotoxicity in hFOB1.19. Additionally, the treatment of MGO-stimulated hFOB1.19 with various methanolic crude extracts caused a significant decrease in intracellular ROS levels when compared to the MGO-stimulated cells ($p < 0.05$), indicating the antioxidant properties of these extracts (Figure 4.14). This study also revealed that there was no observed synergistic effect between the methanolic crude extracts and metformin, suggesting that the individual treatments effectively ameliorated the intracellular ROS levels ($p < 0.05$), but no enhanced effect was observed when used in combination.

Notably, all aqueous extracts caused a significant reduction in intracellular ROS levels when compared to MGO-stimulated hFOB1.19 ($p < 0.05$) as shown in Figure 4.15. Additionally, similar to the findings with methanolic extracts, this study did not reveal any synergistic relationships between the aqueous crude extracts and metformin treatments, suggesting that these approaches exerted their effects independently ($p < 0.05$) in alleviating intracellular ROS levels.

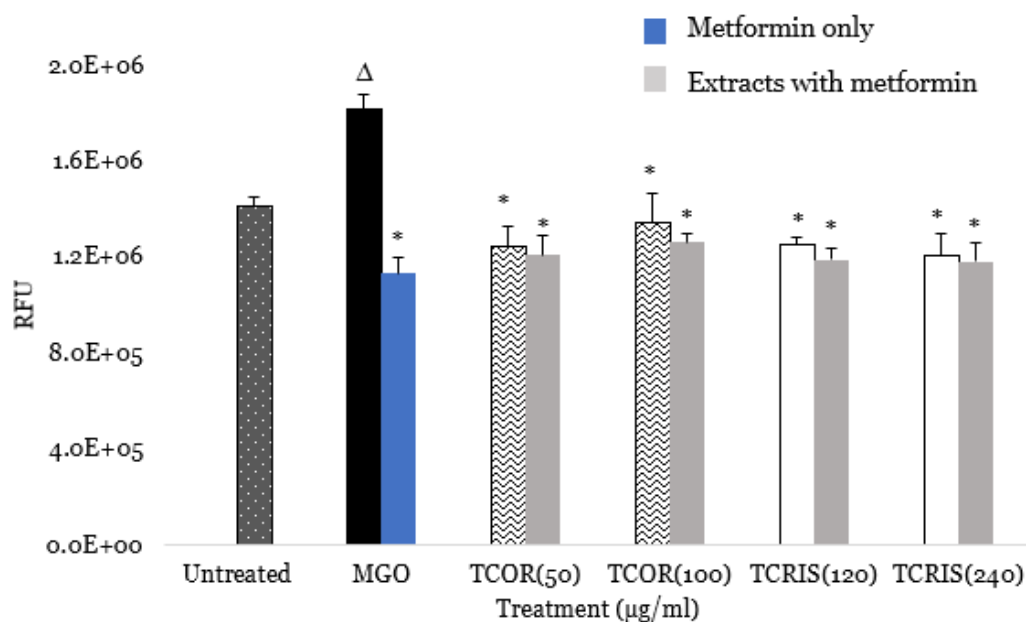


Figure 4.14: Intracellular ROS formation in hFOB1.19 cells when treated with methanolic extracts of *T. crispera* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $\Delta p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

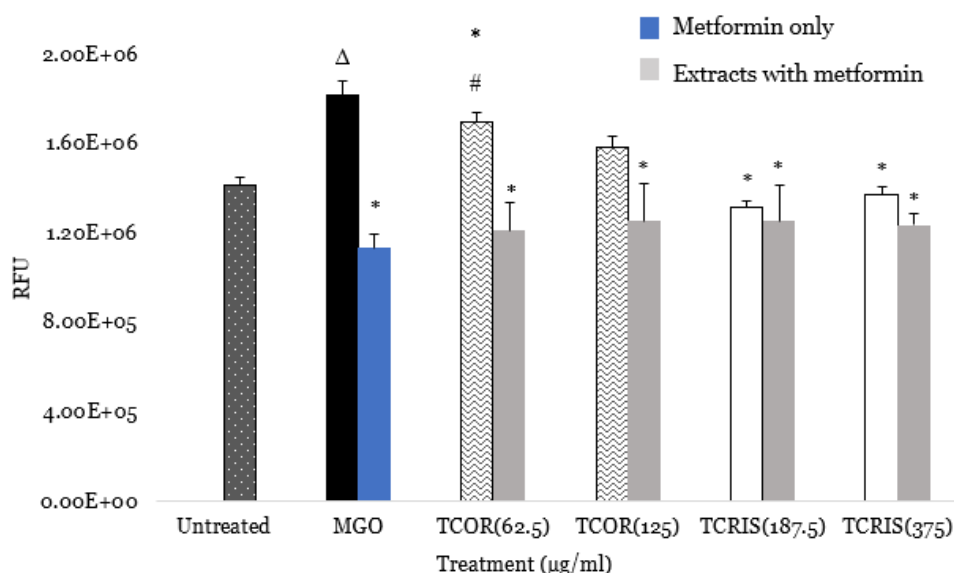


Figure 4.15: Intracellular ROS formation in hFOB1.19 cells when treated with aqueous extract of *T. crispera* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $\Delta p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

4.3.5 Formation of AGE in MGO-induced hFOB1.19 when treated with crude extracts with or without metformin

Generally, the exposure of hFOB1.19 with MGO led to a significant increase in AGE levels when compared to untreated cells ($p < 0.05$), highlighting its role in elevating AGE formation associated with oxidative stress (Figure 4.16). The addition of metformin caused a significant reduction of AGE levels in MGO-stimulated hFOB1.19 cells ($p < 0.05$). In this study, the individual effects of crude extracts did not significantly reduce AGE levels compared to the MGO-stimulated control ($p > 0.05$) except for methanolic extract of *T. cordifolia* at 100 $\mu\text{g/mL}$. However, in combination with metformin, these extracts exhibited a significant reduction in AGE levels, except for methanolic extract of *T. cordifolia* at 50 $\mu\text{g/mL}$ combined with metformin. Although methanolic extract of *T. cordifolia* at 120 $\mu\text{g/mL}$ combined with metformin displayed a higher reduction in AGE levels compared to methanolic extract of *T. cordifolia* at 240 $\mu\text{g/mL}$ combined with metformin, they were not significantly different ($p > 0.05$).

Similarly, aqueous extracts when treated individually did not yield a significant reduction in AGE levels in MGO-stimulated hFOB1.19, with the exception of aqueous extract of *T. crispa* at a concentration of 375 $\mu\text{g/mL}$ ($p < 0.05$) Figure 4.17. However, an intriguing outcome was observed when these extracts were combined with metformin. The combined treatments, involving all the crude aqueous extracts, exhibited a significant reduction in AGE levels, signifying a notable effect in mitigating AGE formation ($p < 0.05$). This underscores the synergistic potential of these crude extracts in combination with metformin to counter AGE expression in MGO-stimulated hFOB1.19 cells.

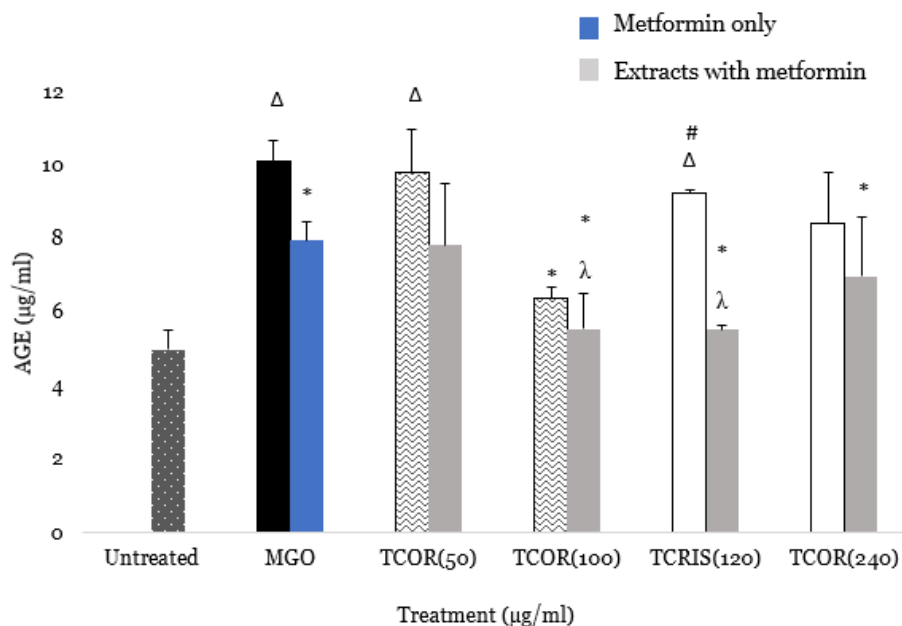


Figure 4.16: AGE formation in hFOB1.19 cells when treated with methanolic extract of *T. crispa* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $\Delta p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

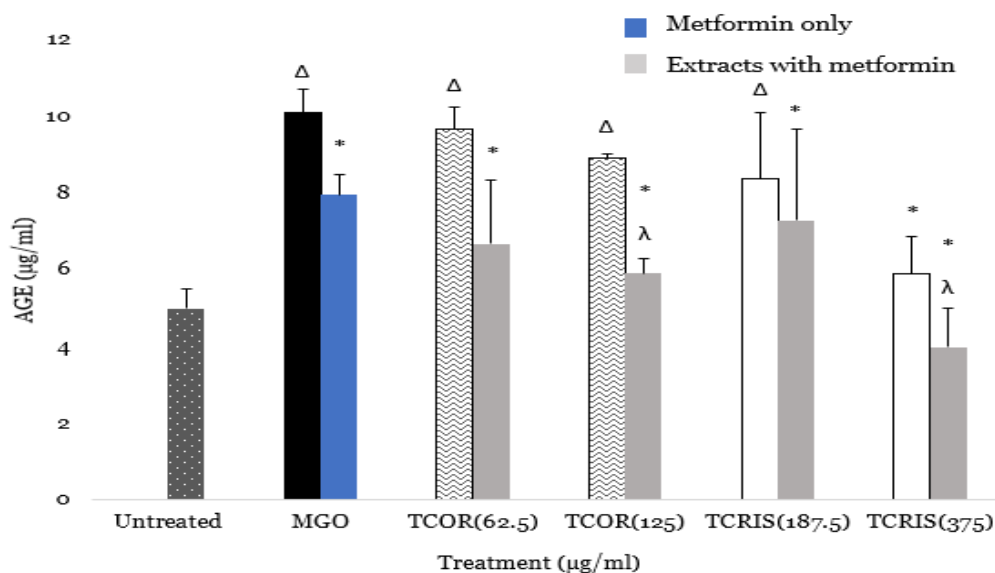


Figure 4.17: AGE formation in hFOB1.19 cells when treated with aqueous extract of *T. crispa* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $\Delta p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

4.4 Impact of crude plant extracts with or without metformin towards hFOB1.19 anti-apoptotic activity

4.4.1 Formation of Bcl-2 in MGO-induced hFOB1.19 when treated with crude extracts with or without metformin

The protein Bcl-2 plays an important role in regulating apoptosis as anti-apoptotic factor that helps cells to evade programmed cell death. In this study MGO significantly reduced Bcl-2 levels in hFOB1.19 which is consistent with its apoptotic-inducing effects ($p < 0.05$) (Figure 4.18). Subsequently, the addition of metformin significantly upregulated Bcl-2 expression in this MGO-stimulated hFOB1.19 ($p < 0.05$). Notably, individual crude methanolic extracts exhibited significantly lower efficacy in upregulating Bcl-2 expression compared to their combined treatments ($p < 0.05$) with metformin. This suggests that a combination of crude methanolic extracts with metformin might induce more pronounced upregulation of Bcl-2 than crude extracts alone, implying synergistic potentials. When aqueous crude extracts were administered individually, only aqueous extract of *T. crispa* at a concentration of 375 $\mu\text{g/mL}$ significantly upregulated Bcl-2 levels in comparison to MGO-stimulated hFOB1.19 cells ($p < 0.05$) as shown in Figure 4.19. Furthermore, all combinations of aqueous crude extracts with metformin managed to significantly upregulate Bcl-2 levels in comparison to MGO-stimulated hFOB1.19 cells ($p < 0.05$). However, the upregulation achieved by the individual crude extracts was notably lower compared to their combined treatments with metformin, suggesting a potential synergistic relationship between these crude aqueous extracts and metformin in increasing the expression of Bcl-2. These results emphasize the potential of metformin and the combined treatments to stimulate the anti-apoptotic activity of hFOB1.19 cells in the presence of MGO-induced apoptotic stress.

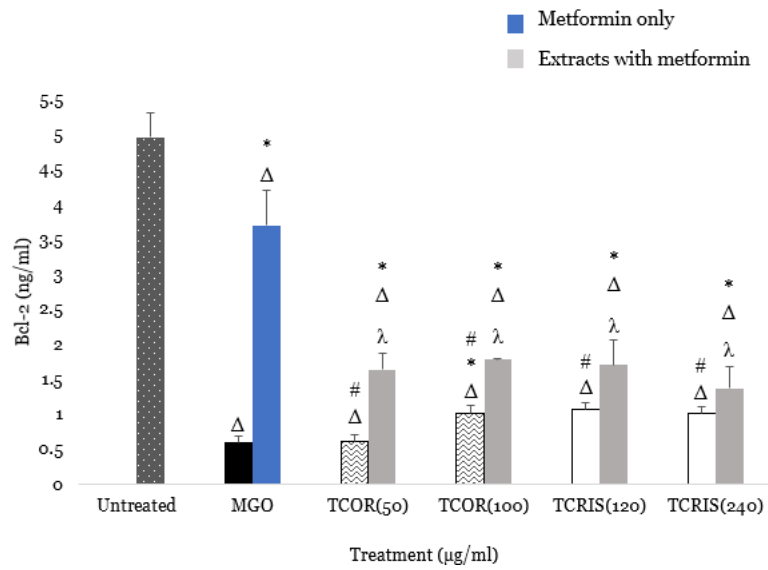


Figure 4.18: Bcl-2 expression in hFOB1.19 cells when treated with methanolic extract of *T. crista* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $\Delta p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

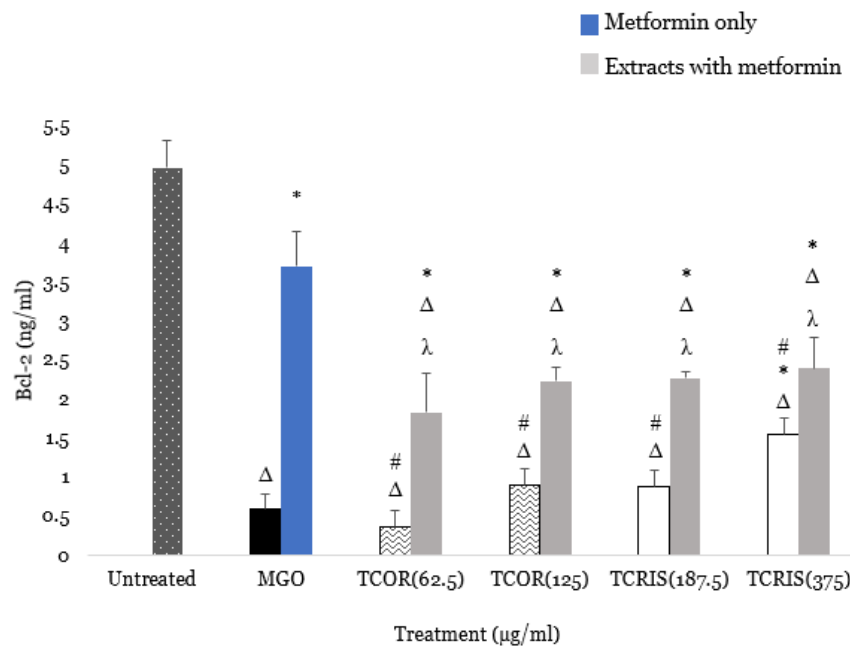


Figure 4.19: Bcl-2 formation in hFOB1.19 cells when treated with aqueous extract of *T. crista* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $\Delta p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

4.4.2 Formation of caspase-3 in MGO-induced hFOB1.19 when treated with crude extracts with or without metformin

Caspase-3 plays a pivotal role in apoptosis as it is responsible for the execution of programmed cell death especially in the final stages of apoptosis that leads to cell death. This study observed that MGO significantly upregulated caspase-3 ($p < 0.05$), consistent with its pro-apoptotic effects, while metformin significantly downregulated caspase-3 ($p < 0.05$), indicating its potential in ameliorating cellular death as shown in Figure 4.20. Moreover, the application of crude methanolic extracts also led to a significant reduction in caspase-3 activity ($p < 0.05$), suggesting their capability to reduce the apoptotic activity in the cells. However, there were no significant differences between the effects of individual crude extracts and their combinations with metformin, implying no synergistic interactions between them. Eventhough methanolic *T. cordifolia* at 50 $\mu\text{g/mL}$ reduced more caspase-3 than 100 $\mu\text{g/mL}$, the difference between them was not significantly different ($p > 0.05$).

These results further revealed a significant reduction in caspase-3 formation when MGO-stimulated hFOB1.19 was treated with crude aqueous extracts ($p < 0.05$), highlighting their capability to reduce apoptotic activity as shown in Figure 4.21. However, only *T. cordifolia* at 62.5 $\mu\text{g/mL}$ did not significantly reduce caspase-3 expression ($p > 0.05$). Remarkably, when crude extracts were combined with metformin, all combinations exhibited a significant reduction in caspase-3 expression when compared to MGO-stimulated hFOB1.19 cells ($p < 0.05$).

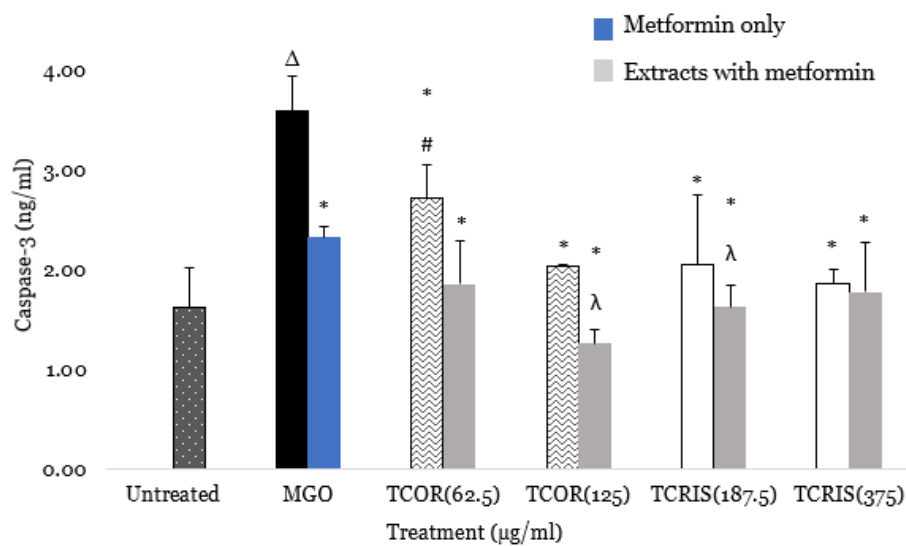


Figure 4.20: Caspase-3 formation in hFOB1.19 cells when treated with methanolic extract of *T. crispa* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $\Delta p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin treatments; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

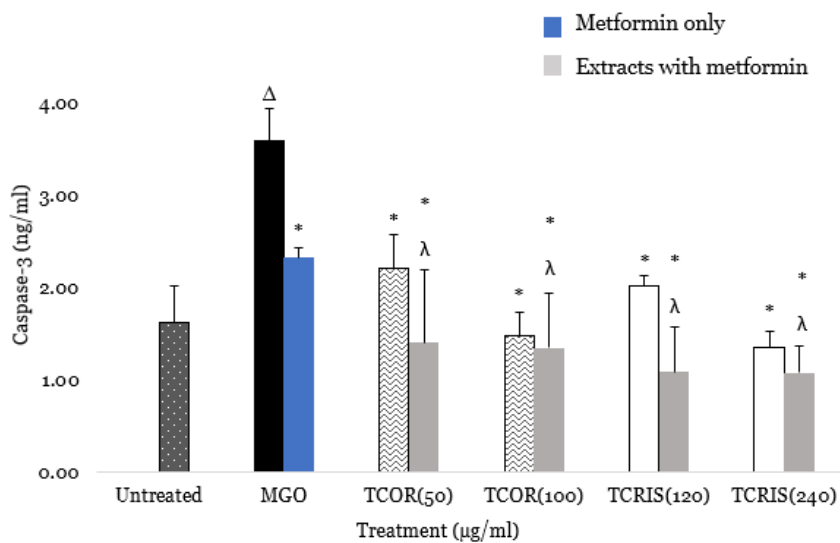


Figure 4.21: Caspase-3 formation in hFOB1.19 cells when treated with aqueous extract of *T. crispa* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control) ; $\Delta p < 0.05$ as compared to the untreated cells (negative control) ; # $p < 0.05$ as compared to combination of crude extract and metformin treatments; $\lambda p < 0.05$ as compared to metformin only at their respective concentrations.

4.5 Impact of crude plant extracts with or without metformin towards hFOB1.19 anti-inflammatory activity

The anti-inflammatory effects of crude extracts with or without metformin on MGO-stimulated hFOB1.19 cells were investigated using a variety of ELISA assays. This study assessed the levels of pro-inflammatory cytokines secretion, including the translocation of NF- κ B/p65 transcription factor into the nucleus.

4.5.1 Translocation of NF- κ B/p65 into the nucleus of MGO-induced hFOB1.19 when treated with crude extracts with or without metformin

NF- κ B/p65 plays a crucial role in the inflammatory response within cells, as it is a transcription factor involved in regulating the expression of numerous inflammatory genes. This study examined the impact of MGO and various treatments on the translocation of NF- κ B/p65 into the nucleus, indicating its activation and involvement in inflammatory processes. Notably, MGO significantly upregulated the translocation of NF- κ B/p65 into the nucleus, indicating its pro-inflammatory influence ($p < 0.05$) as shown in Figure 4.22 below. Subsequently, metformin significantly reduce this translocation ($p < 0.05$), implying its anti-inflammatory potential. Additionally, individual methanolic crude extracts effectively reduced the translocation of NF- κ B/p65 into the nucleus when compared to MGO-stimulated hFOB1.19 cells, suggesting their anti-inflammatory properties. The combination of crude extracts with metformin also yielded a significant reduction in NF- κ B/p65 translocation.

Furthermore, the observed significant differences between the combination of crude extracts with metformin compared to crude methanolic extracts only for *T. cordifolia* at 50 μ g/mL and 100 μ g/mL suggest a potential synergistic effect between metformin and crude methanolic extracts of *T. cordifolia*.

This study also investigated the role of aqueous crude extracts towards the translocation of NF- κ B/p65 into the nucleus. The results revealed that when administered individually, these aqueous extracts were effective in significantly downregulating NF- κ B/p65 translocation when compared to MGO-stimulated hFOB1.19 cells ($p < 0.05$), highlighting their potential as anti-inflammatory agents as shown in Figure 4.23. Moreover, when these aqueous extracts were combined with metformin, significant reduction in NF- κ B/p65 translocation were observed in comparison to MGO-stimulated cells ($p < 0.05$). However, aqueous extract of *T. cordifolia* at 62.5 and 125 μ g/mL, which demonstrated significant differences when compared to their combination treatments with metformin, indicating a possible synergistic effect between crude extracts of *T. cordifolia* with metformin in modulating NF- κ B/p65 translocation, ameliorating pro-inflammatory activities.

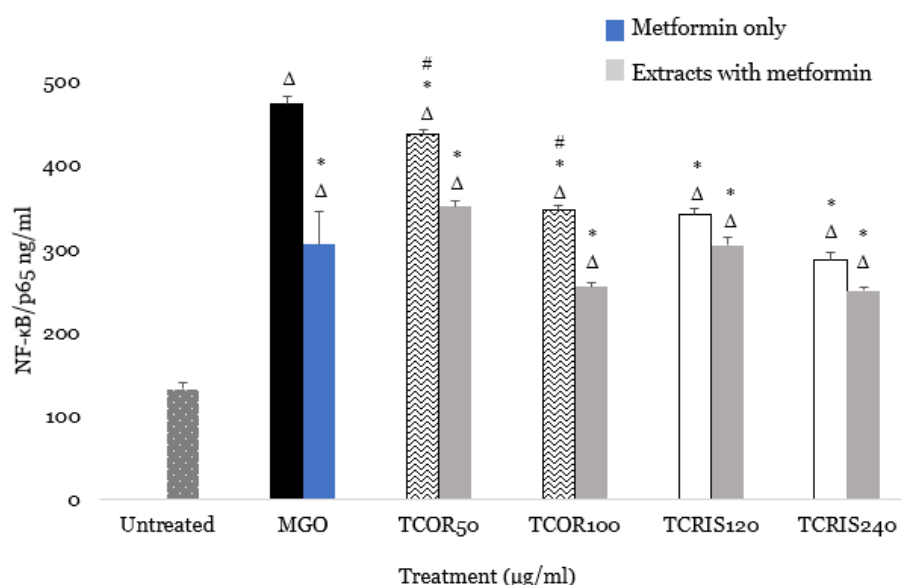


Figure 4.22: NF- κ B/p65 translocation in MGO-stimulated hFOB1.19 cells when treated with methanolic extract of *T. crista* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); ^Δ $p < 0.05$ as compared to the untreated cells (negative control); [#] $p < 0.05$ as compared to combination of crude extract and metformin; ^λ $p < 0.05$ as compared to metformin at their respective concentrations.

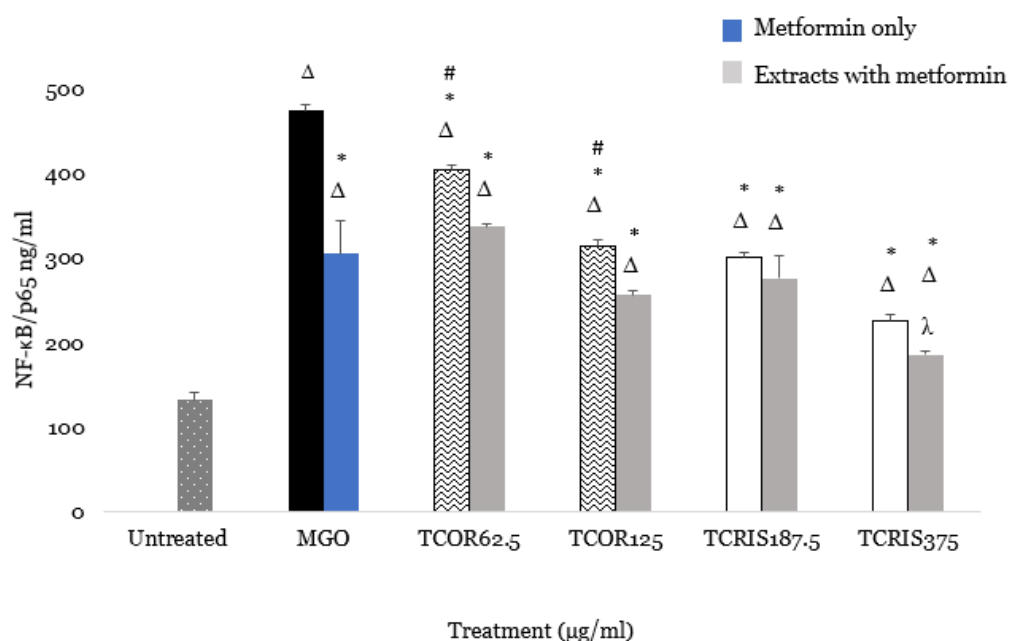


Figure 4.23: NF-κB/p65 translocation in MGO-stimulated hFOB1.19 cells when treated with aqueous extract of *T. crispa* and *T. cordifolia*. *p<0.05 as compared to MGO treated cells (positive control); ^Δp<0.05 as compared to the untreated cells (negative control); [#]p<0.05 as compared to combination of crude extract and metformin; ^λp < 0.05 as compared to metformin at their respective concentrations.

4.5.2 Expression of IL-18 in MGO-induced hFOB1.19 when treated with crude extracts with or without metformin

Interleukin-18 (IL-18) plays a vital role in inflammation as a pro-inflammatory cytokine, contributing to the regulation of the immune response. It serves to promote the activation of various immune cells and the production of other inflammatory mediators. For methanolic extracts, it was observed that MGO significantly upregulated the secretion of IL-18 compared to untreated cells, highlighting its pro-inflammatory role (p<0.05) as shown in Figure 4.24. However, metformin demonstrated its potential to significantly reduce IL-18 secretion, countering the inflammatory effect of MGO (p<0.05). Individual crude methanolic extracts were also effective in significantly downregulating IL-18 secretion when compared to MGO-stimulated cells (p<0.05). Furthermore, the combination of methanolic crude extracts with metformin consistently downregulating the

IL-18 secretion, with the extracts significantly different from their respective combinations with metformin ($p<0.05$), indicating the presence of a potential synergistic effect between the crude methanolic extracts and metformin in mitigating IL-18-mediated inflammation.

For the crude aqueous extracts, all treatments significantly downregulated the secretion of IL-18 in comparison to MGO-stimulated hFOB1.19 cells ($p<0.05$), reflecting their anti-inflammatory properties as shown in Figure 4.25. When combined with metformin, the crude aqueous extracts maintained their ability to significantly reduce IL-18 secretion, further enhancing their anti-inflammatory effects ($p<0.05$). Notably, the extracts significantly attenuated MGO-induced IL-18 in a dose-dependent manner, yet metformin alone, or in combination with either one of the extracts, resulted in a more pronounced reduction IL-18 production.

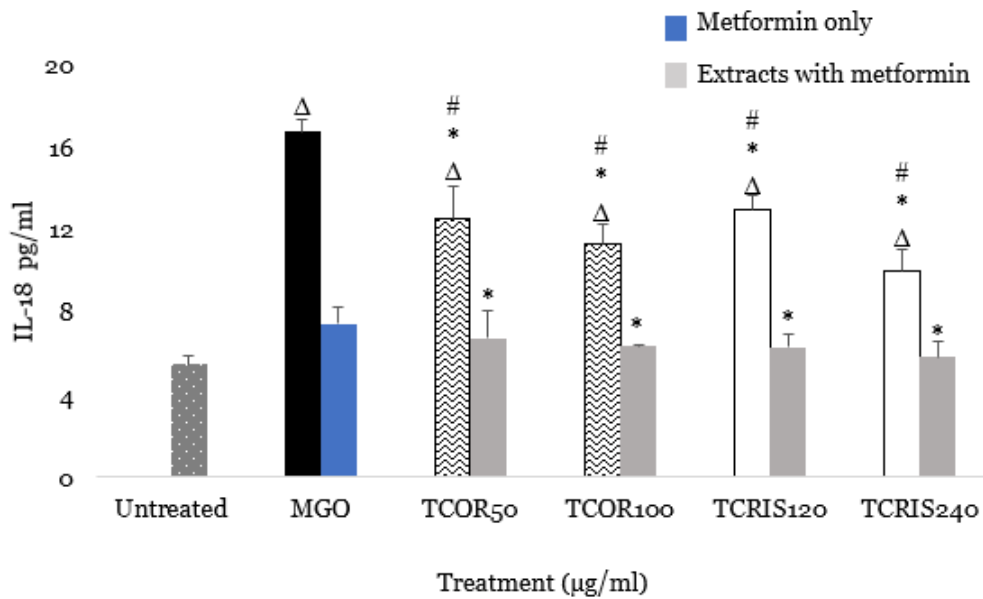


Figure 4.24: The secretion of IL-18 in MGO-stimulated hFOB1.19 cells when treated with methanolic extract of *T. crista* and *T. cordifolia*. * $p<0.05$ as compared to MGO treated cells (positive control); $\Delta p<0.05$ as compared to the untreated cells (negative control); # $p<0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

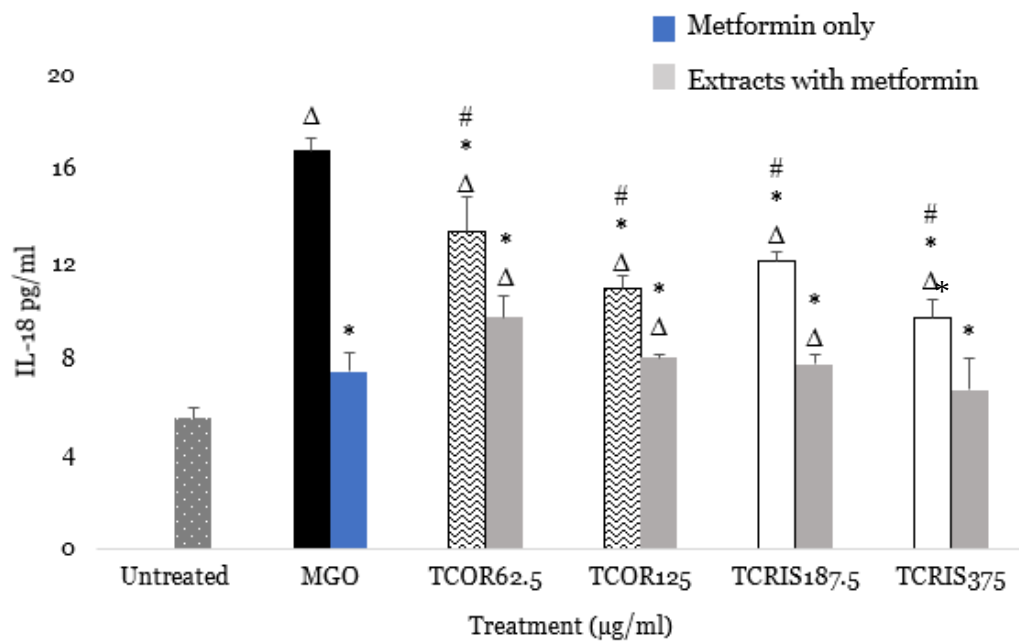


Figure 4.25: The secretion of IL-18 in MGO-stimulated hFOB1.19 cells when treated with aqueous extract of *T. crispa* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control) ; $\Delta p < 0.05$ as compared to the untreated cells (negative control) ; # $p < 0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

4.5.3 Expression of IL-6 in MGO-induced hFOB1.19 when treated with crude extracts with or without metformin

Interleukin-6 (IL-6) plays a pivotal role in inflammation, serving as a pro-inflammatory cytokine involved in various immune responses and acute-phase reactions. It contributes to the regulation of the immune system and is integral in initiating and sustaining inflammation.

This study further explored the IL-6 secretion in MGO-stimulated hFOB1.19 when treated with methanolic extracts focusing on their anti-inflammatory potential in hFOB1.19 cells. This study observed that MGO significantly upregulated the secretion of IL-6 compared to untreated cells, confirming its pro-inflammatory role ($p < 0.05$) as shown in Figure 4.26. However, only methanolic extract of *T. cordifolia* at 100 μg/mL demonstrated

the ability to significantly reduce IL-6 secretion when compared to MGO-stimulated hFOB1.19, indicating its anti-inflammatory properties ($p < 0.05$). The combination of methanolic extract of *T. cordifolia* at 100 $\mu\text{g/mL}$ with metformin further enhanced this effect, significantly reducing IL-6 secretion compared to MGO-stimulated hFOB1.19 ($p < 0.05$). Additionally, methanolic extract of *T. crista* at 240 $\mu\text{g/mL}$, both individually and in combination with metformin, exhibited a significant reduction in IL-6 levels when compared to MGO-stimulated cells, highlighting the potential of these extracts in counteracting MGO-induced inflammation.

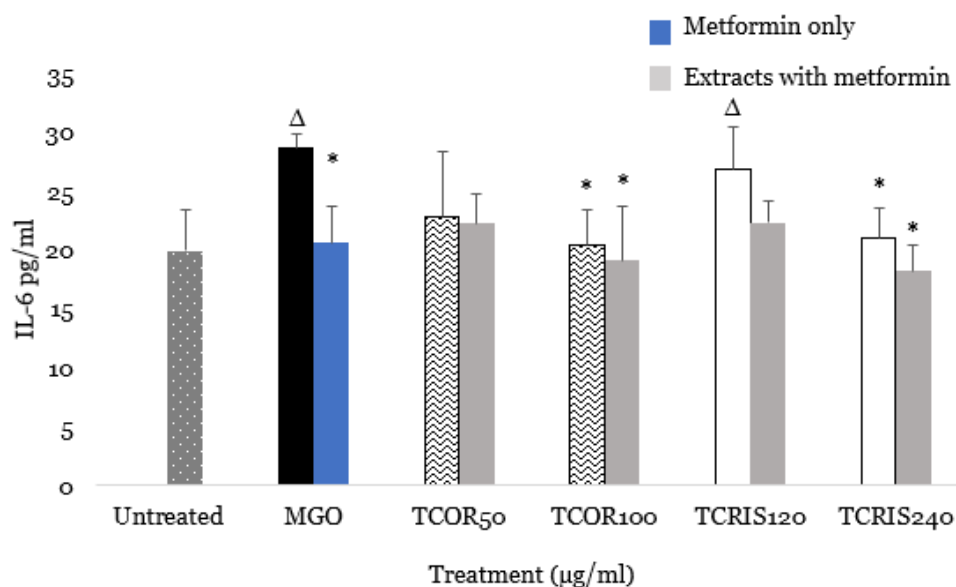


Figure 4.26: The secretion of IL-6 in MGO-stimulated hFOB1.19 cells when treated with methanolic extract of *T. crista* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $^{\Delta}p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin; $^{\lambda}p < 0.05$ as compared to metformin at their respective concentrations.

In contrast, the aqueous crude extracts proved to be more effective in downregulating IL-6 when compared to their methanolic extracts. All aqueous extracts, when administered individually, significantly reduced IL-6 secretion in comparison to MGO-stimulated hFOB1.19 cells ($p < 0.05$), emphasizing their potent anti-inflammatory properties as shown in Figure 4.27. Furthermore, when combined with metformin, the aqueous extracts

significantly reduce IL-6 secretion, further enhancing their anti-inflammatory effects ($p < 0.05$). There is a potential synergistic effect seen between the *T. crista* with metformin in attenuating the secretion of IL-6. As for *T. cordifolia*, the extracts alone had shown a potential in reducing the secretion of IL-6.

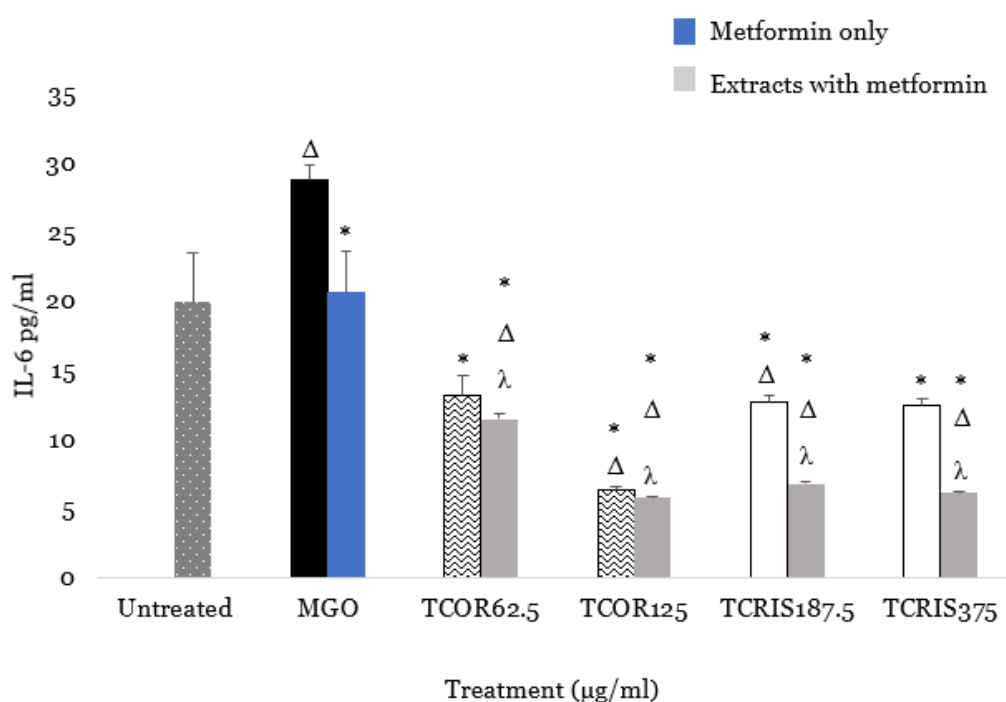


Figure 4.27: The secretion of IL-6 in MGO-stimulated hFOB1.19 cells when treated with aqueous extract of *T. crista* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $\Delta p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

4.6 Impact of crude plant extracts with or without metformin towards MGO stimulated hFOB1.19

The cellular activity of MGO-stimulated hFOB1.19 when treated with crude extracts with or without metformin were investigated using a variety of ELISA assays. This study assessed the expression of transcription factor responsible for bone metabolism, diabetic proteins, including the translocation of Runx2, insulin receptor (IR) and GLUT4.

4.6.1 Production of Runx2 in MGO-induced hFOB1.19 when treated with crude extracts with or without metformin

The production of Runx2 in the MGO-stimulated hFOB1.19 when treated crude methanolic and aqueous extracts with or without metformin is shown in Figure 4.28 and Figure 4.29 below. In bone metabolism, Runx2 plays a crucial role in regulating osteoblast differentiation and bone formation. MGO treatment in hFOB1.19 cells led to a significant downregulation of Runx2 when compared to untreated cells, reflecting its negative impact on osteoblast differentiation ($p < 0.05$) as shown in Figure 4.28. Conversely, metformin demonstrated the ability to counteract MGO's inhibitory effect by significantly upregulating the production of Runx2 in MGO-stimulated hFOB1.19 cells ($p < 0.05$). Moreover, crude methanolic extracts exhibited a positive impact on Runx2 production by significantly upregulating it when compared to MGO-stimulated hFOB1.19 cells ($p < 0.05$). However, when crude extracts were combined with metformin, they did not significantly upregulate the Runx2 production when compared to the crude extracts individually ($p > 0.05$).

Similarly, aqueous crude extracts also showed a significant upregulation of Runx2 when compared to MGO-stimulated hFOB1.19 cells ($p < 0.05$) as shown in Figure 4.29, with the exception of the concentration of 62.5 $\mu\text{g/mL}$. Interestingly, the combination of crude aqueous extracts of *T. cordifolia* at concentrations of 62.5 $\mu\text{g/mL}$ and 125 $\mu\text{g/mL}$ with metformin showed a remarkable ability to significantly upregulate Runx2 ($p < 0.05$). This suggests the potential of this combination to enhance osteoblast differentiation and bone formation. These findings underscore the complex regulatory role of Runx2 in bone metabolism and the potential therapeutic implications of both crude extracts and metformin towards the transcription factor.

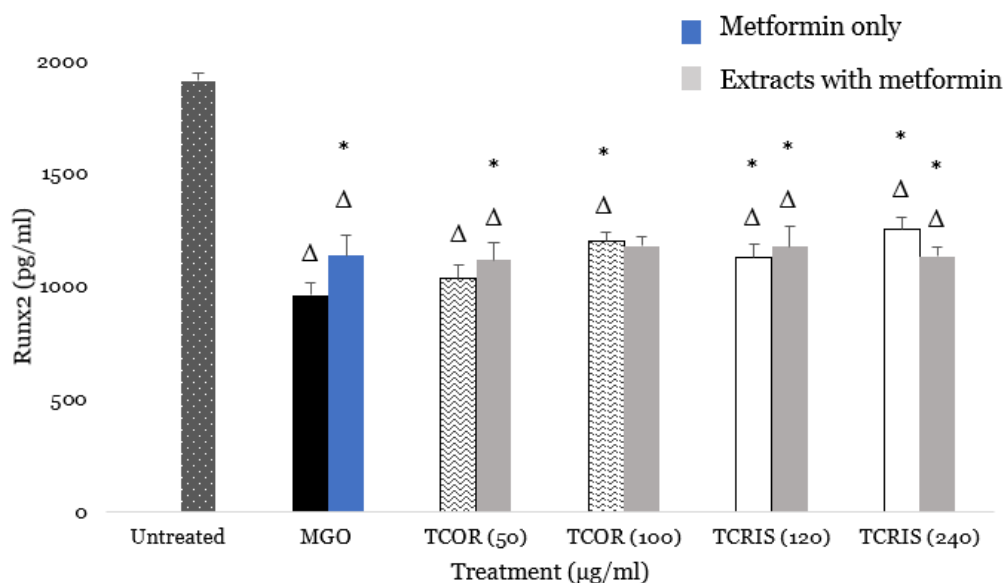


Figure 4.28: The production of Runx2 in MGO-stimulated hFOB1.19 cells when treated with methanolic extract of *T. crista* and *T. cordifolia*. *p<0.05 as compared as MGO treated cells (positive control); Δp< 0.05 as compared to the untreated cells (negative control); #p<0.05 as compared to combination of crude extract and metformin; λp < 0.05 as compared to metformin at their respective concentrations.

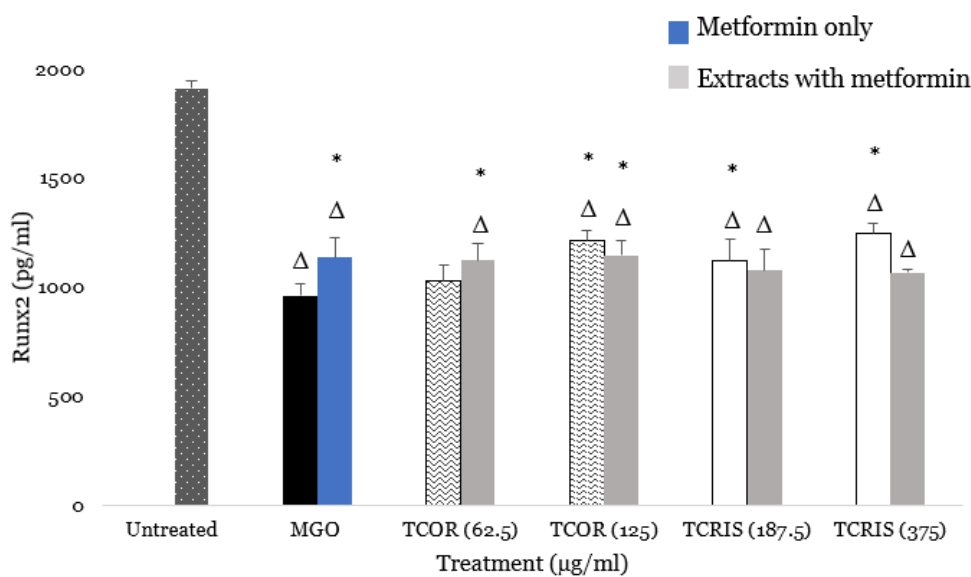


Figure 4.29: The production of Runx2 in MGO-stimulated hFOB1.19 cells when treated with aqueous extract of *T. crista* and *T. cordifolia*. *p<0.05 as compared to MGO treated cells (positive control) ; Δp<0.05 as compared to the untreated cells (negative control) ; #p<0.05 as compared to combination of crude extract and metformin; λp < 0.05 as compared to metformin at their respective concentrations.

4.6.2 Production of insulin receptor (IR) in MGO-induced hFOB1.19 when treated with crude extracts with or without metformin

In this study, MGO treatment led to a significant reduction in IR expression in hFOB1.19 when compared to untreated cells, suggesting a compromised activity of insulin signalling in diabetes ($p < 0.05$) as shown in Figure 4.30. However, the administration of metformin remarkably upregulated IR production, particularly in MGO-stimulated hFOB1.19 cells, indicative of its positive influence on the insulin receptor in a diabetic environment ($p < 0.05$). Additionally, all crude methanolic extracts generally increased IR production in MGO-stimulated hFOB1.19 cells. Furthermore, synergistic effects were observed when methanolic extract of *T. cordifolia* at 100 $\mu\text{g/ml}$, methanolic extract at *T. crispa* at 120 ng/mL and 240 ng/mL were combined with metformin, resulting in a significant upregulation of IR expression.

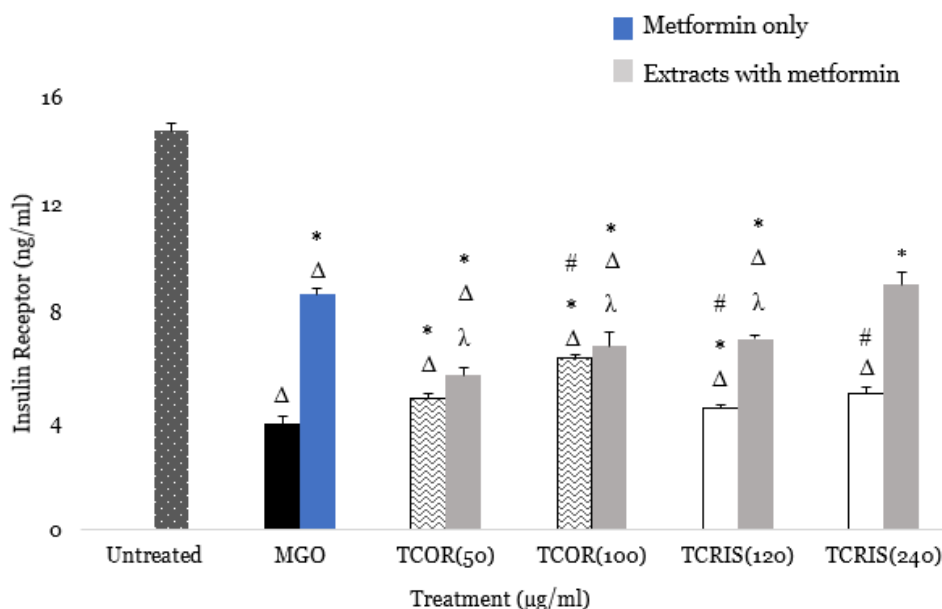


Figure 4.30: The production of IR in MGO-stimulated hFOB1.19 cells when treated with methanolic extract of *T. crispa* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $\Delta p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

Crude aqueous extracts exhibited a significant increase in IR production when compared to MGO-stimulated hFOB1.19 cells, with the exception of *T. crispa* at 375 µg/mL as shown in Figure 4.31. Notably, the combination of aqueous extract of *T. cordifolia* extracts with metformin at concentrations of 62.5 and 125 µg/mL as well as aqueous extract of *T. crispa* at 187.5 µg/mL demonstrated a significant upregulation of IR production compared to MGO-stimulated hFOB1.19 cells. These findings suggest a potential role for crude aqueous extracts, particularly in combination with metformin, in enhancing IR production in the context of elevated MGO level in hFOB1.19.

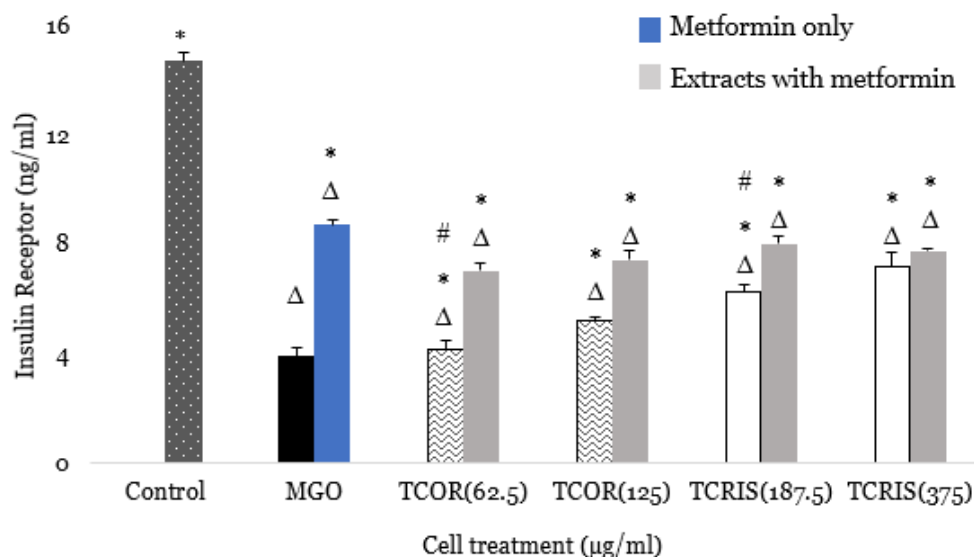


Figure 4.31: The production of IR in MGO-stimulated hFOB1.19 cells when treated with aqueous extract of *T. crispa* and *T. cordifolia*. * $p < 0.05$ as compared to MGO treated cells (positive control); $\Delta p < 0.05$ as compared to the untreated cells (negative control); # $p < 0.05$ as compared to combination of crude extract and metformin; $\lambda p < 0.05$ as compared to metformin at their respective concentrations.

4.6.3 Production of GLUT4 in MGO-induced hFOB1.19 when treated with crude extracts with or without metformin

GLUT4 plays a crucial role in regulating glucose uptake and cellular responses to insulin. This study revealed that MGO led to a substantial downregulation of GLUT4 production compared to untreated cells ($p < 0.05$), indicating a potential impairment in glucose transport mechanisms in a diabetes as shown in Figure 4.32. In contrast, addition of metformin significantly upregulated GLUT4 production, particularly in MGO-stimulated hFOB1.19 cells ($p < 0.05$), highlighting its potential as a therapeutic agent for improving glucose transport in MGO stimulated hFOB1.19. Furthermore, methanolic extracts of *T. crispa* at 120 $\mu\text{g/mL}$ and 240 $\mu\text{g/mL}$ demonstrated a significant upregulation of GLUT4 production when compared to MGO-stimulated hFOB1.19 cells ($p < 0.05$). Notably, all combinations of methanolic crude extracts with metformin exhibited a significant upregulation of GLUT4 ($p < 0.05$), further underlining the potential of combined interventions in enhancing GLUT4 expression in the osteoblasts. Additionally, for crude aqueous extracts, only aqueous extract of *T. crispa* at 125 $\mu\text{g/mL}$ and at 375 $\mu\text{g/mL}$ significantly upregulated GLUT4 compared to MGO-stimulated hFOB1.19 cells ($p < 0.05$), indicating specific efficacy of the crude extract in improving GLUT4 expression as shown in Figure 4.33. Remarkably, all combinations of crude extracts with metformin demonstrated significant upregulation of GLUT4 ($p < 0.05$), emphasizing the potential for combined therapies to enhance GLUT4 expression and potentially ameliorate glucose transport in the cells.

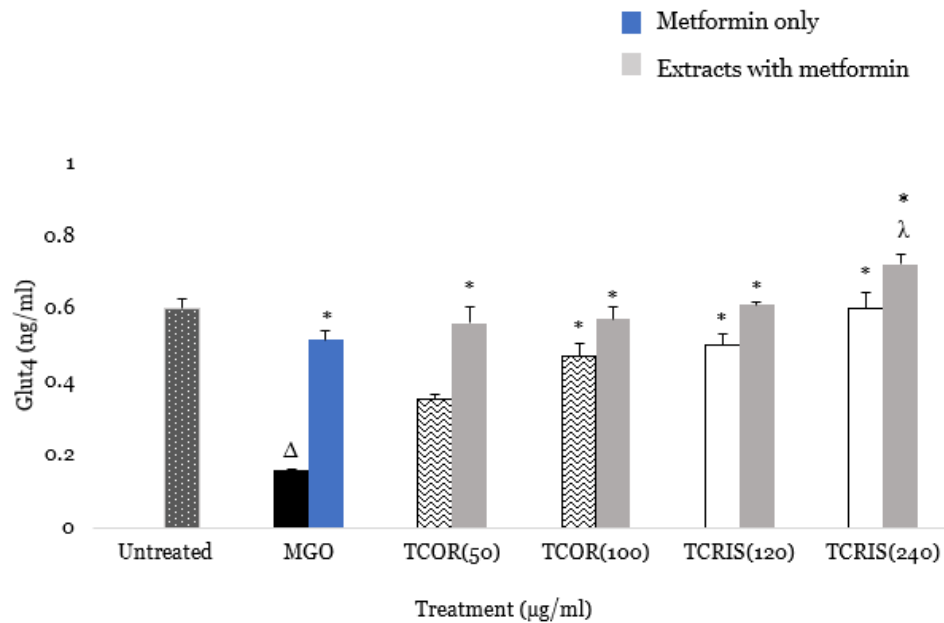


Figure 4.32: The expression of GLUT4 in MGO-stimulated hFOB1.19 cells when treated with methanolic extract of *T. crista* and *T. cordifolia*. *p < 0.05 as compared to MGO treated cells (positive control); Δp < 0.05 as compared to the untreated cells (negative control); #p < 0.05 as compared to combination of crude extract and metformin; λp < 0.05 as compared to metformin at their respective concentrations.

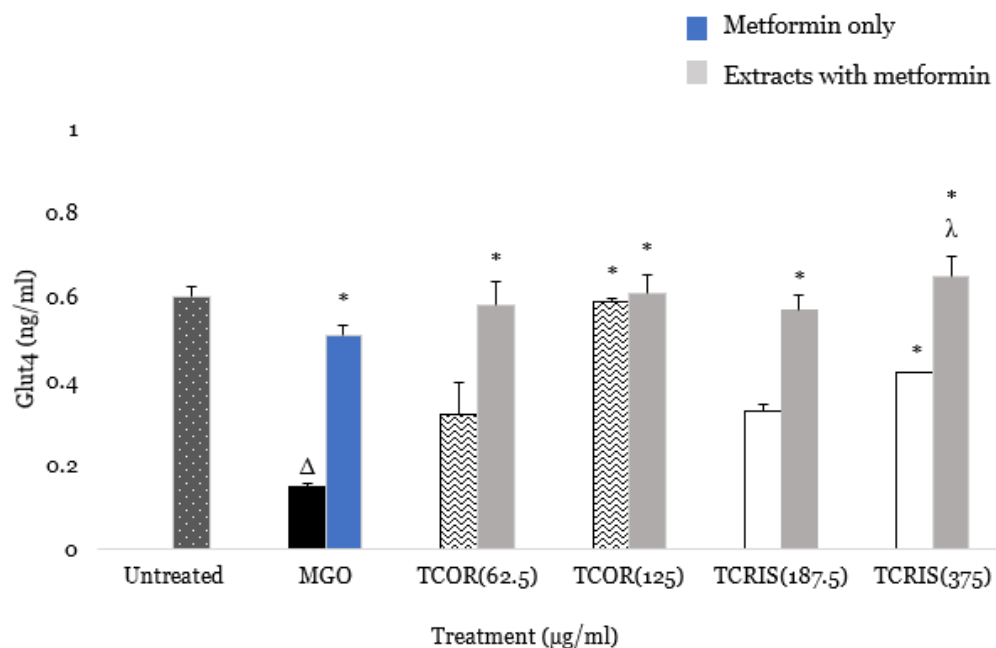


Figure 4.33: The expression of GLUT4 in MGO-stimulated hFOB1.19 cells when treated with aqueous extract of *T. crista* and *T. cordifolia*. *p < 0.05 as compared to MGO treated cells (positive control); Δp < 0.05 as compared to the untreated cells (negative control); #p < 0.05 as compared to combination of crude extract and metformin; λp < 0.05 as compared to metformin at their respective concentrations.

CHAPTER 5

DISCUSSION

5.1 GC-MS analysis of methanolic and aqueous extracts of *T. cordifolia* and *T. crispa*

In this study, GC-MS analysis revealed the presence of several compounds with significant pharmacological properties in both methanolic and aqueous extracts of *T. cordifolia* and *T. crispa*. Compounds such as syringol, eugenol, guaiacol, vanillin and canolol were detected in both methanolic and aqueous extracts of *T. cordifolia* and *T. crispa*. However, 4-vinyl guaiacol was detected in methanolic *T. cordifolia*, methanolic *T. crispa* and aqueous *T. crispa*. Methylparaben was detected in methanolic *T. cordifolia*, aqueous *T. cordifolia* and aqueous *T. crispa*. Phenol was only found in methanolic and aqueous extract of *T. cordifolia*.

The GC-MS analysis for methanolic *T. cordifolia* revealed the presence of four compounds previously identified for their antioxidant properties, three compounds known for their anti-inflammatory effects, and six compounds previously recognized for exhibiting both antioxidant and anti-inflammatory properties in methanolic extract of *T. cordifolia*. For instance, compounds such as methylparaben, l-(+)-ascorbic acid 2,6-dihexadecanoate, syringol (1.74 ± 0.53 %), eugenol (1.44 ± 0.23 %) and beta.-D-glucopyranose, 1,6-anhydro- (1.30 ± 1.20 %) were previously reported to possess antioxidant activities in previous studies. Meanwhile, compounds like benzofuran, 2,3-dihydro- (0.93 ± 0.81 %) and apocynin (0.43 ± 0.40 %) were studied to possess anti-inflammatory effects. Concurrently, compounds such as phenol, guaiacol (3.80 ± 1.11 %), 4-vinyl guaiacol (4.17 ± 1.28 %), vanillin (1.50 ± 0.24 %), canolol

(1.60 ± 0.21 %) and phytol (0.52 ± 0.09 %) were reported to possess both antioxidant and anti-inflammatory activities.

The GC-MS analysis for methanolic extract of *T. crisper* revealed the presence of one compound previously identified for its antioxidant properties, one compound known for its anti-inflammatory effect, and seven compounds previously recognized for exhibiting both antioxidant and anti-inflammatory properties in methanolic extract of *T. crisper*. For example, syringol (2.77 ± 0.39 %) was reported to possess antioxidant effect. Meanwhile, 9,12-octadecadienoic acid (Z,Z)-, methyl ester (1.13 ± 1.01 %) was shown to exhibit anti-inflammatory effects. Compounds such as 2(5H)-furanone (0.32 ± 0.43 %), guaiacol (4.55 ± 1.08 %), 4-vinyl guaiacol (4.37 ± 1.50 %), vanillin (2.75 ± 0.64 %), eugenol (0.78 ± 0.30 %), canolol (1.81 ± 0.93 %) and heptadecanoic acid (2.05 ± 1.32 %) were reported to exhibit both antioxidant and anti-inflammatory properties.

Interestingly, a total of ten compounds were identified in both methanolic extracts of *T. cordifolia* and *T. crisper*. These compounds include guaiacol, benzofuran 2,3-dihydro-, vanillin, syringol, eugenol, canolol, palmitic acid, 9-octadecenoic acid, methyl ester, (E)-, and octadecanoic acid. Remarkably, the majority of these compounds exhibit both antioxidant and anti-inflammatory properties, with the exception of syringol, which was reported solely for its antioxidant effects.

The GC-MS analysis revealed the presence of three compounds in aqueous extract of *T. cordifolia* previously identified for their antioxidant properties and four compounds previously recognized for exhibiting both antioxidant and anti-inflammatory properties in aqueous extract of *T. cordifolia* as shown in Table 4.3. For instance, compounds such as methylparaben, syringol (2.23 ± 1.14 %) and veratryl alcohol (0.47 ± 0.01 %) were previously reported to possess antioxidant activities in earlier studies. Meanwhile, compounds like phenol, guaiacol ($5.05 \pm$

2.40 %), vanillin (1.34 ± 1.60 %) and canolol (1.06 ± 0.07 %) were reported to possess both antioxidant and anti-inflammatory activities.

The GC-MS analysis revealed the presence of four compounds previously identified for its antioxidant properties, one compound known for its anti-inflammatory effect, and found compounds previously recognized for exhibiting both antioxidant and anti-inflammatory properties in aqueous extract of *T. crispera*. For example, feryl hydroxymethyl ketone, syringol (4.88 ± 0.43 %), methylparaben (2.62 ± 0.05 %) and syringylacetone (2.05 ± 0.49 %) were reported to possess antioxidant effect. Meanwhile, sinapyl alcohol was studied to exhibit anti-inflammatory effects. Compounds such as phenol (3.04 ± 1.23 %), 4-vinyl guaiacol (5.15 ± 1.53 %), vanillin (2.22 ± 0.50 %) and canolol (3.68 ± 0.30 %) were reported to exhibit both antioxidant and anti-inflammatory properties.

A total of eleven compounds were identified in both aqueous extracts of *T. cordifolia* and *T. crispera* as shown in Table 4.3. . These compounds include pyrrolidine, phenol, 1,2-cyclopentanedione, 3-methyl-, propanoic acid, 2,2-dimethyl- 2-propenyl ester, 4-vinyl guaiacol, syringol, vanillin, methylparaben, canolol, syringylacetone and 2,6,8-trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol. Remarkably, most of these compounds exhibit both antioxidant and anti-inflammatory properties, with the exception of syringol, methylparaben and syringylacetone which were reported solely for their antioxidant effects. Meanwhile, neither antioxidant nor anti-inflammatory activities were reported from pyrrolidine, 1,2-cyclopentanedione, 3-methyl-, propanoic acid, 2,2-dimethyl-, 2-propenyl ester as well as 2,6,8-trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol.

Although few compounds can be detected in both methanolic and aqueous extracts, there were few compounds that only present exclusively in each extract. Namely, compounds such as l-(+)-ascorbic acid 2,6-dihexadecanoate, beta.-D-glucopyranose, 1,6-

anhydro-, benzofuran, 2,3-dihydro- and apocynin were exclusively detected in methanolic extract of *T. cordifolia*. Additionally, compounds such as 9,12-octadecadienoic acid (Z,Z)-, methyl ester, 2(5H)-furanone and heptadecanoic acid were only found in methanolic extract of *T. crispa*. Moreover, veratryl alcohol was only found in methanolic extract of *T. cordifolia*. Finally, furyl hydroxymethyl ketone and sinapyl alcohol were only found exclusively in aqueous *T. crispa*.

Among these compounds, some of them were reported to possess antioxidant properties. For instance, syringol had been reported for its antioxidant attributes with its ability to scavenge DPPH radicals, neutralized ABTS radical cations, and manifested ferric reducing antioxidant power (FRAP) (Loo *et al.*, 2008). Moreover, eugenol which had been reported as an important component within clove essential oil exhibited apparent antioxidant potential (Silva *et al.*, 2018). It also helped to inhibit both iron and Fenton reagent-mediated lipid peroxidation (Nagababu *et al.*, 2010). Moreover, eugenol inhibited 96.7% ($R^2=0.9319$) lipid peroxidation in a linoleic acid emulsion, possessing the highest antioxidant compound with radical-scavenging activity when compared to Trolox, butylated hydroxytoluene and other antioxidant standards (Gülçin, 2011). In a previous study, *Fraxinus micrantha* plant strongly possessed l-(+)-ascorbic acid 2,6-dihexadecanoate as one of the prominent compounds. *Fraxinus micrantha* also was shown to possess strong antioxidant properties (Kumar & Sati, 2021). Furthermore, *in-silico* finding revealed that l-(+)-ascorbic acid 2,6-dihexadecanoate as one of the top ranked molecules that possessed antioxidant properties (Palaniappan *et al.*, 2022). Meanwhile, a chemical profiling study revealed that beta.-D-glucopyranose, 1,6-anhydro- was detected through GC-MS analysis from *Elytranthe parasitica* also possessed antioxidant properties (Sharath & Naika, 2022). By using voltametric techniques, methylparaben was shown to possess smaller oxidation potentials than unsaturated fatty acids such as oleic,

making it as one of the compounds that can protect against oxidative damage in acetic-acetonitrile solutions (Michalkiewicz, 2013). Veratryl alcohol, which is one of the phenolic compounds has been reported to possess antioxidant properties (Morales & Lucas, 2010). Sinapyl alcohol downregulated nitric oxide, TNF- α in macrophages as well as cyclooxygenase (COX)-2 in dose dependent manner (Choi et al., 2004).

Meanwhile, some of the compounds were reported to possess anti-inflammatory properties. An *in-vitro* study revealed that apocynin inhibited the expression of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α in an allergen-induced experiment, indicating its anti-inflammatory property (Kim et al., 2012). Benzofuran, 2,3-dihydro- had been reported to possess anti-inflammatory activity. A study towards inflammation model of macrophages revealed that benzofuran, 2,3-dihydro- possessed a potential anti-inflammatory as well as anticancer properties (Ayoub et al., 2023). 9,12-Octadecadienoic acid (Z,Z)-, methyl ester has been reported to possess anti-inflammatory properties (Krishnamoorthy & Subramaniam, 2014). Furthermore, an earlier GC-MS analysis revealed that the compound was also detected in *Nigella sativa* and reported to exhibit anti-inflammatory properties (Mohammed et al., 2016).

Some of the detected compounds were reported to possess both antioxidants and anti-inflammatory properties. For example, guaiacol is a commercial antioxidant and has been widely used in food preservation and pharmaceuticals (Anouar et al., 2009; Azadfar et al., 2015). In addition, the antioxidant effects of certain polyphenols are linked to the existence of the guaiacol functional group (Gao *et al.*, 2021). Guaiacol was also studied to significantly mitigated LPS-stimulated inflammation through AMPK-SIRT1 pathway and inhibition of NF- κ B activation. Then, it inhibited the activation of inflammasomes and downregulating the secretion of pro-inflammatory cytokines (Zhao et al., 2019). Moreover,

a study showed that vanillin has significant activity in the ABTS^{•+} scavenging assay through a self-dimerization mechanism and also through oral administration which improved the antioxidant level in mice plasma (Tai *et al.*, 2011). A study towards induced-inflammation on primary human gingival fibroblast revealed vanillin reduced IL-6, IL-8, COX-2 and NO secretion, indicating its anti-inflammatory capability (Costantini *et al.*, 2021). Canolol is a phenol that has considerable promise for its antioxidant and anticancer properties (Nandasiri & Eskin, 2022). Furthermore, it also reduced intracellular OS in H₂O₂-stimulated SW480 cells (Kuwahara *et al.*, 2004). A finding reported that canolol also possessed anti-inflammatory property (Zhang *et al.*, 2017). For instance, a study induced colitis in mouse by administering 2% dextran sulphate sodium (DSS) orally that increased the level of inflammatory cytokines and TNF- α . Then, a canolol rich diet was administered to the mouse resulting in the significant reduction in the level of inflammatory cytokines (Fang *et al.*, 2013). Meanwhile, 2(5H)-Furanone was found to possess antioxidant and anti-inflammatory as reported by (Khabibrakhmanova *et al.*, 2023). Phenol, an organic compound characterized through its hydroxyl group (—OH) attached to a carbon atom is well known for its antioxidant properties. It acts as an antioxidant through SET, HAT as well as metal chelating ability (Zeb, 2020). Moreover, the identification phenolic compounds or extracts from plants has become a major study in the field of medical research especially its antioxidant properties (Dai & Mumper, 2010). Phenol also had been studied to possess anti-inflammatory properties. Based on the GC-MS results, the presence of phenol may be attributed to its formation as a degradation product during the extraction process. However, it is crucial to note that phenol can pose toxicity concerns if consumed in elevated concentration. Furthermore, the anti-inflammatory property of phenolic acids may be due to their ability to interfere with OS signalling and inhibits pro-inflammatory signalling

transduction (Lopez-Corona et al., 2022). In an earlier study, FTIR analysis had revealed the presence of 4-vinyl guaiacol which features hydroxyl and methoxy functional groups. This groups highlights its comparable antioxidant efficacy regarding commercially established antioxidants (Azadfar *et al.*, 2015). In addition, the treatment of murine LPS stimulated RAW264.7 macrophages with 4-vinyl guaiacol resulted in a significant downregulation of IL-6 mRNA (Esatbeyoglu et al., 2015). *Mercurialis annua* had been studied to possess antioxidant properties which contained 12.83 % of heptadecanoic acid. In this study, methanolic *T. crispa* also contains heptadecanoic acid and similarly identified for its antioxidant efficacy, suggesting its role in the observed antioxidant activity (Al-Douri & Shakya, 2019). Moreover, a finding reported that heptadecanoic acid could reduce the secretion of IL-6 *in-vivo* (Venn-Watson et al., 2020).

5.2 Antioxidant properties of methanolic and aqueous extracts without cells and reduction apoptotic activities of MGO-stimulated hFOB1.19 when treated with the extracts

The antioxidant activity exhibited by plant extracts demonstrates their ability to protect and maintain their own physiological and genetic integrity. However, the plant extracts are also increasingly being explored for their potential in mitigating the pathological effects of oxidation contributed by oxidative stress.

Various antioxidant assays have been developed to assess the antioxidant potential of compounds, with three prominent methods being widely utilized. DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay, FRAP (Ferric Reducing Antioxidant Power) assay, and BSA-MGO (Bovine Serum Albumin-Methylglyoxal) assay. The DPPH assay involves measuring the scavenging ability of antioxidants towards the stable free radical DPPH, while the FRAP assay evaluates the reducing capacity of antioxidants by assessing their ability to reduce

ferric ions to ferrous ions. Furthermore, the BSA-MGO assay specifically targets the glycation-induced oxidative stress by measuring the inhibition of methylglyoxal-induced modifications in bovine serum albumin (BSA). For this study, these tests collectively provide information into the antioxidant properties of *T. cordifolia* and *T. crispa*.

In this study, the methanolic extracts had shown higher free radical scavenging activity than aqueous extracts. Higher DPPH scavenging activity seen in methanolic extracts might be reflected by the fact that polyphenols are frequently more soluble in organic solvents that are less polar than water as well as the polar characteristics of the polyphenols that influence their solubility (Haminiuk et al. 2014; Tobgay et al. 2020). A study by Zulkefli et al., (2013) revealed methanol is the most suitable solvent in the extraction of phenolic compounds due to its capability to prevent the reaction of polyphenol oxidase that induces the phenolic oxidation and its evaporating rate when compared to water.

Antioxidants act by neutralizing free radicals due to the hydroxyl groups present in their structure. Additionally, their redox properties enable them to act as redox agents through hydrogen atom transfer (HAT) and single electron transfer (SET). Among these, phenolic acids and flavonoids are noteworthy, representing typical phenolic compounds which can be commonly found in the plant kingdom and recognized for their antioxidant activity. Furthermore, the reaction of DPPH with antioxidants also depends on the chemical structure of the antioxidants (Ulewicz & Wesolowski, 2019). For instance, flavonoids' antioxidant activity is determined by the structure and substitution pattern of their hydroxyl groups. The 3,4-orthodihydroxy structure in ring B and the 4-carbonyl group in ring C are required for efficient radical scavenging. The inclusion of a 3-OH group and 5-OH groups in ring C, resulting in a catechol-like structure, is also helpful to flavonoids' antioxidant activity (Wojdyło et al. 2007).

Previous study showed that *T. crispa* extract consists of hydroxyl groups that contributed an electron to reduce DPPH radicals (Zulkefli et al. 2013). Furthermore, the similar study also reported that *T. crispa* contained a high concentration of phenolic compounds, which served as hydrogen donors and had been widely reported to have antioxidative properties. Another finding has reported that the extract of *T. cordifolia* did show a strong potential as antioxidant agent (Polu et al., 2017).

BSA-MGO anti-glycation assay measured the capacity of the extracts to reduce anti-glycation between protein (BSA) and MGO. Despite showing significant scavenging and reducing power properties, both methanolic and aqueous extracts did not show pronounced anti-glycation properties when tested through BSA-MGO. However, a previous study revealed that *T. crispa* managed to inhibit alpha glucosidase activity, an enzyme that responsible for the breakdown of carbohydrates into glucose. Furthermore, inhibiting alpha glucosidase activity can reduce the postprandial blood glucose, which is one of the main indicators of hyperglycaemia. Strict control of postprandial blood glucose is very important for the prevention and management of DM as continuous hyperglycaemia in diabetic patients can accelerate non-enzymatic glycation (Chang et al., 2015; Zhang et al., 2022). An earlier study also demonstrated *T. cordifolia* ability to inhibit the activity of alpha glucosidase suggesting its potential to lower postprandial glucose levels in individuals with diabetes. A study revealed that rats treated with the *T. cordifolia* extract exhibit a significant reduction in glucose within a two-hour period (Ashok et al., 2009)

An imbalance between ROS production and the cellular antioxidant can induce oxidative stress. The elevated OS can trigger programmed cell death pathway such as apoptosis which compromises cellular integrity and function. The investigation of the extracts with or without metformin in ameliorating OS in MGO-stimulated hFOB1.19 was

measured through intracellular ROS levels and AGE expression. Oxidative stress arises from a persistent imbalance between the generation of ROS and the capacity of the intracellular antioxidant system to neutralize them. The excessive production of ROS by mitochondria through NADPH oxidase pathway is also responsible for OS. High level of ROS can damage cellular components such as proteins, lipids and DNA (Yubero-Serrano et al., 2014). The term 'redox state' summarizes the equilibrium relationship between ROS production and antioxidants. While the body's antioxidant mechanism strives to maintain normal physiological levels of OS, an imbalance can have profound effects on cellular integrity and function. Furthermore, some phytochemicals may hinder sugar attachment to proteins, inhibit the formation of advanced glycation end-products, or prevent the initial formation of Amadori products. Additionally, their ability to scavenge free radicals and break cross-links could contribute to their effectiveness in inhibiting glycation (Younus & Anwar, 2016).

As the oxidative damage can activate signalling pathways leading to programmed cell death, high level of OS due to ROS serves as a crucial sign pro-apoptotic activity, influencing the expression of pro-apoptotic factor i.e., caspase-3. For instance, a finding provided evidence of OS leading to apoptosis in human tendon fibroblasts by activating the caspase-3. Specifically, the study observed that the ROS triggered apoptosis through the mitochondrial cascade pathway. This pathway involves the release of cytochrome-c from the mitochondria into the cytosol, subsequently activating caspase-3, a key executor of apoptosis (Yuan et al., 2003). A more recent study revealed the role of ROS in inducing cell death in the rat osteoblast-like cell line ROS 17/2.8, with a focus on Akt signalling and the involvement of the mammalian target of rapamycin (mTOR) pathway. Furthermore, the study observed an increase in cleaved caspase-3 levels following hydrogen peroxide

treatment, indicating activation of apoptotic signalling in ROS 17/2.8 cells (Zhang et al., 2013).

Consequently, the relationship between OS and apoptosis plays a pivotal role in various physiological and pathological conditions, including low bone density. For instance, OS induced bone loss and osteoporosis by detrimentally impacting the processes of osteoblast proliferation, differentiation, maturation, and apoptosis, while concurrently promoting heightened osteoclastic activity (Iantomasi et al., 2023). For instance, a review by Domazetovic, (2017) reported that OS significantly impacts bone remodelling by enhancing osteoclast differentiation and activity, ultimately leading to increased bone resorption. In addition, ROS promote osteoclastogenesis which disrupts the balance between osteoclasts and osteoblasts. Increased in ROS levels further induce excessive osteocyte apoptosis, intensifying osteoclast-mediated bone resorption. Moreover, ROS-induced apoptosis in osteoblasts and osteocytes not only promotes osteoclastogenesis but also hampers mineralization and osteogenesis.

This study observed a significantly high expression in intracellular ROS and AGE levels in hFOB1.19 cells when exposed to IC₅₀ value of MGO against the untreated cells, indicating its potent inducer of cellular stress. In T2DM patients, elevated MGO levels resulting from hyperglycaemia upregulate the glycation process in comparison to healthy individuals. The glycation resulting from non-enzymatic reactions between sugars and proteins, gives rise to Amadori products, before turning into AGE, an irreversible compound that play an important role in increasing intracellular ROS. Amino acids like lysine, hydroxyl lysine, and arginine, are commonly found on the surface of proteins that include haemoglobin, albumin, osteocalcin, and collagen. The glycation process begins with the aldehyde group of glucose reacting with the (-NH₂) amino group typically of lysine, leading

to the synthesis of glucosyl-lysine. Subsequently, this compound undergoes further reaction that leads to the formation of a Schiff base. This intermediate is unstable and consequently rearranges into an Amadori product. The fate of the Amadori product can either undergoes oxidation to generate AGEs or undergoes dehydration and deamination to yield reactive carbonyl intermediates, such as MGO (Al-Khateeb et al., 2018).

AGEs are linked to elevated OS by orchestrating the generation of ROS, thereby elevating intracellular levels of free radicals such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and nitric oxide (NO). The binding of AGEs with their receptor (RAGE) amplifies OS by initiating ROS production via NADPH oxidase pathway within the mitochondria, influencing cellular metabolism pathologically (Cepas et al., 2020). Generally, NADPH oxidase (NOX) is actively involved in facilitating the electron transfer process from NADPH to oxygen molecules, resulting in the production of either O_2^- or H_2O_2 . As a family of enzymes, NADPH oxidase functions to catalyse the electron transfer to molecular oxygen, thereby generating superoxide or hydrogen peroxide. This enzymatic activity utilizes NADPH as the electron donor, illustrating the central role of NADPH oxidase in the intricate biochemical reactions leading to the production of ROS (Atay et al., 2017; Shen et al., 2020). As the OS increases due to high level of ROS, it affects cellular components such as lipids and proteins, triggers the initiation of antioxidant enzymes, and ultimately activates apoptotic pathways in cells.

In MC3T3-E1 cells, OS triggers mitochondrial dysfunction through the opening of mitochondrial permeability transition pores (mPTPs), leading to the release of reactive oxygen species (ROS) and subsequent impairment of mitochondrial function. The critical regulator for mPTP opening, cyclophilin D (CypD), is implicated in this process (Mao et al., 2023). Subsequent consequences of mitochondrial permeability include the equilibration of

small molecular weight molecules, including cofactors and ions, across the inner membrane of the mitochondria which leads to mitochondrial swelling. As the matrix expands, it exerts pressure on the outer membrane, ultimately resulting in rupture. This rupture, in turn, facilitates the release of cytochrome-c which initiate apoptotic cell death. The complex interplay between elevated MGO, glycation, increased AGE and ROS production, and subsequent mitochondrial permeabilization emphasizes the role of OS influenced by T2DM.

This study observed the role of metformin in significantly reducing the intracellular ROS and AGE when compared to MGO-stimulated hFOB1.19 cells. Metformin is antihyperglycemic drug used for the treatment of T2DM. Its mechanism includes the reduction of hepatic glucose production, mitigation of intestinal glucose absorption, and enhancement of insulin sensitivity. These effects are achieved by augmenting peripheral glucose uptake and utilization (Lazzeroni & Gandini, 2017; Papich, 2016). In addition, metformin has been found to reduce mitochondrial respiration by inhibiting complex I of the electron transport chain. This action increases the AMP/ATP ratio and activates AMPK which ROS production. Consequently, metformin decreases activation of NLRP3 inflammasomes, thereby reducing inflammation. (Lin et al., 2023).

In a study conducted by Marycz et al., (2016), it was reported that metformin treatment to adipose-deprived stem cells led to a decrease in the levels of ROS and Nitric Oxide (NO). These findings provide significant implications for diabetes treatment, as elevated ROS production is associated with the initiation and advancement of vascular complications in individuals with diabetes. Moreover, the antioxidative properties of metformin were evidenced in previous research, wherein it was observed to reduce ROS production in bovine aortic endothelial cells through the inhibition of protein kinase-c activity (Mahrouf et al., 2006).

This investigation further examined the efficacy of crude methanolic and aqueous extracts from both *T. crispa* and *T. cordifolia* in mitigating intracellular ROS and subsequently reducing OS in MGO-stimulated hFOB1.19 cells. This *in-vitro* antioxidative ELISA assay was supported through extracts' capability to scavenge free radicals in DPPH antioxidant assay. This finding through cellular assay supports the capability of the extracts to scavenge free radicals with in *in-vitro* condition in MGO-stimulated hFOB1.19 after examined through DPPH antioxidant assay.

The antioxidant properties of extracts are linked to their phenolic, tannin, and flavonoid content, which are known for their ability to scavenge free radicals and exert reducing power (Manye et al., 2023). Notably, the methanolic extracts of *T. crispa* and *T. cordifolia* demonstrated a more pronounced reduction of intracellular ROS levels compared to their aqueous extracts. The methanolic extracts of *T. crispa* and *T. cordifolia* demonstrated greater antioxidant activity against MGO-stimulated hFOB1.19 cells relatively to aqueous extracts. This enhanced antioxidant effect was supported by the previous GC-MS analysis, which revealed the presence of a higher concentration of antioxidant compounds in the methanolic extracts. Consequently, the methanolic extracts effectively reduced oxidative stress activity in the cells by reducing the intracellular ROS.

Only the aqueous extract of *T. crispa* at 375 µg/mL demonstrated a significant reduction in advanced glycation end product (AGE) expression in MGO-stimulated hFOB1.19 cells. This suggests that the extracts exhibited limited anti-glycation activity. However, the combination of methanolic crude extracts with metformin, as well as aqueous crude extracts with metformin, resulted in a significant reduction in AGE levels in the cells. While the crude extracts insignificantly reduced AGE levels, the addition of metformin to the crude extracts appeared to lower the formation of AGEs. However, no significance

difference was observed between the metformin alone against the combination of extracts with metformin, suggesting the reduced AGEs might be due to only through metformin action, without the synergistic combination with extracts. Metformin has been studied to inhibit the production of AGEs in hyperglycaemic conditions. *In vitro* investigation indicated that metformin could reduce glycation adduct and provide cell protection through antioxidant capability and mitigating glycation-induced cellular damage (Adeshara & Tupe, 2016)

Similarly, the extracts' capability in preventing glycation was previously observed in the BSA-MGO anti-glycation assay, where the extracts demonstrated low anti-glycation activity. Based on previous reports, phytochemical compounds such as baicalin, procyanidins and caffeic acid possess high anti-glycation properties (Froldi et al., 2022; Khan et al., 2022; Zhao et al., 2021). However, none of these compounds can be found through GC-MS in the extracts of methanolic as well as aqueous *T. cordifolia* and *T. crispa*. These extracts may hold promise for exhibiting anti-glycation activity at high concentrations; however, there is a concern regarding their potential to induce cytotoxicity in cells. Thus, future studies are needed to understand the role of anti-glycation of these extracts without compromising the cellular viability.

Osteoblast apoptosis holds a significant role in bone metabolism. In conditions associated with an imbalance between osteoblast and osteoclast activity, such as in metabolic disorder like T2DM, the increased apoptosis of osteoblast disrupts the bone homeostasis. Elevated glucose levels, otherwise known as hyperglycaemia, can induce osteoblast apoptosis through OS and mitochondrial damage. This disruption results in excessive osteoclastic activity, thereby accelerating bone resorption over bone formation by OB. Despite the transformation of osteoblast into osteocytes in bone metabolism, approximately

60 to 80 % of osteoblast still undergo apoptosis, making it as one of contributors for bone loss (Li et al., 2023). This relationship between osteoblast apoptosis imbalance with osteoclastic activity contributes significantly to the pathophysiological bone loss.

The studied crude extracts demonstrated a significant downregulation of caspase-3 formation, indicative of their anti-apoptotic efficacy in the MGO-stimulated hFOB1.19. This anti-apoptotic effect is linked to the extracts' ability to attenuate OS, primarily achieved through their effective reduction of OS through effective reduction of intracellular ROS and AGE. A comprehensive phytochemical analysis through GC-MS of the extracts also unveiled numerous of previously reported phytochemical compounds with antioxidant ability, highlighting their role in OS downregulation. Given that OS can induce DNA and protein damage, consequently promoting apoptosis, the mitigation of OS serves to alleviate these issues, subsequently diminishing the apoptotic rate in the cells.

T. cordifolia has been reported to downregulate caspase-3 in different type of cells. For example, a recent study revealed that ethanolic extract of *T. cordifolia* managed to reduce caspase-3 in rotenone-induced Parkinson's disease mice, offering neuroprotective protection (Dilnashin et al., 2023). An earlier finding also revealed that *T. cordifolia* extract increased the cell proliferation in the fibroblast cell line (MG-63) and primary osteoblast, indicating its pro-proliferative capability (Abiramasundari et al., 2012). Moreover, in a recent *in-silico* study, *T. crista* phytochemical constituents were analysed to possess anti-apoptotic activity. For instance, borapetoside-B bind with TNF which hindered its binding to the receptor TNFR. This not only shows anti-inflammatory effects, but also shows anti-apoptotic as it inhibited the formation of death-inducing signalling complex (DISC), which can activate pro-caspase 8 into caspase, leading to cascade reaction that leads to cytochrome-c released into the cytoplasm (Dai et al., 2015; Zuhri et al., 2022).

When co-administered with metformin, both the methanolic and aqueous extracts exhibited a notable reduction in caspase-3 formation. However, the combinations involving crude extracts with metformin and crude extracts alone did not display any statistically significant differences, indicating the absence of a synergistic effect between crude extracts and metformin in the downregulation of caspase-3. This suggests that the observed reduction in caspase-3 expression is primarily attributed to the individual contributions of metformin and the extracts, without an apparent interaction between the two.

In conclusion, our investigation revealed that the crude extracts exhibited a notable capacity to reduce intracellular ROS. Interestingly, when combined with metformin, there was a further reduction in intracellular ROS, although this upregulation was not statistically significant compared to the respective crude extracts, indicating no synergistic effects between them. Moreover, the crude extracts did not significantly reduce AGE, and it was only in combination with metformin that a reduction in AGE levels was observed, suggesting a potential contribution from metformin alone rather than the crude extracts. The BSA-MGO anti-glycation assay supported this observation, indicating limited anti-glycation activity for the crude extracts. Despite this, the extracts effectively suppressed OS by mitigating intracellular ROS, primarily through scavenging activities validated by the DPPH assay. This suppression of OS factors was further manifested in the downregulation of caspase-3, indicating a reduced apoptotic response. Additionally, the observed upregulation of the anti-apoptotic protein Bcl-2 further emphasized the protective role of the extracts in preventing cytochrome-c release into the cytoplasm from the mitochondria. These findings collectively suggest impact of the crude extracts, underscoring their potential in modulating cellular responses associated with OS and apoptosis.

5.3 Impact of crude plant extracts with or without metformin towards hFOB1.19 anti-inflammatory activity

Upon nuclear entry, NF- κ B binds to specific DNA sequences, leading to the expression of genes responsible for producing chemokines including pro-inflammatory cytokines (Liu et al., 2017). In this study, stimulation with MGO significantly increased NF- κ B translocation from the cytoplasm to the nucleus, which was accompanied by elevated secretion of the pro-inflammatory cytokines IL-6 and IL-18. However, when cells were treated with the combination of MGO with the extracts, a marked reduction in NF- κ B translocation was observed, correlating with a decrease in IL-6 and IL-18 secretion from hFOB 1.19. Earlier, MGO treatment in the synoviocyte cell line (HIG-82) induced the translocation of NF- κ B from the cytoplasm to the nucleus (Lin et al., 2016). In another study, the administration of MGO into 5-month-old male hereditary hypotriglyceridaemic rats resulted in a notable elevation in TNF- α levels, leading to the secretion of pro-inflammatory cytokines through NF- κ B translocation into the nucleus (Hüttl et al., 2020).

Moreover, the reduction in IL-6 secretion was more pronounced when aqueous extracts were used, suggesting that compounds within these extracts may influence other transcription factors such as CCAAT/enhancer binding proteins (C/EBP) involved in IL-6 regulation within osteoblasts (Tseng et al., 2010). This observation raises the possibility of alternative regulatory pathways for IL-6 secretion, independent of NF- κ B, that require further investigation. MGO has been studied previously for their capabilities in inducing inflammation. For instance, an investigation revealed a substantial upregulation of IL-6 messenger RNA (mRNA) expression in myoblast cells that were exposed to MGO (Todoriki et al., 2022). Another study reported that MGO exerted a direct influence on the levels of IL-6 by modulating the NF- κ B pathway in liver cirrhosis. Additionally, the similar study

explained that the interaction between AGE with its receptor, RAGE appeared to enhance IL-6 expression, particularly in T-cells and macrophages (Michel et al., 2021). Particularly, elevated level of IL-6 is also associated with osteoporosis. For instance, a study demonstrated a threefold elevation in IL-6 levels in glucocorticoid-induced osteoporotic mice (Li et al., 2016). Histologically, an 8-week exposure of rats to MGO revealed an upregulated osteoclastic activation and reduced osteoblastic differentiation in the trabecular region of the tibia. Furthermore, their in-vitro analysis using macrophage RAW264.7 cells exhibited a notable increase in IL-6 levels when exposed to MGO compared to the vehicle control (Lee et al., 2019).

As stated earlier, NF- κ B plays an important role in bone metabolism including increasing osteoclast survival and osteoblast maturation (Novack, 2011). Inhibition of NF- κ B in differentiated osteoblasts significantly enhanced trabecular bone mass and bone mineral density in young mice. Moreover, inhibiting NF- κ B in differentiated osteoblasts effectively preserves bone formation, preventing osteoporotic bone loss induced by ovariectomy in adult mice (Chang et al., 2009). Furthermore, another study has observed that in osteoporosis-induced rats, the attenuation of NF- κ B expression contributes to elevated bone density (Li et al., 2020).

This study observed a significant upregulation of IL-18 secretion by hFOB1.19 when exposed to MGO. As previously discussed, the elevation of MGO resulted in an elevated intracellular of ROS and AGE, triggering high level of OS and cellular injury. This cascade of events ultimately leads to release of DAMPs which initiate the inflammasome assembly. IL-18 also has been observed to promote the differentiation of osteoclasts by increasing the expression of soluble and membrane-bound forms of RANKL from T cells (Seong et al., 2016). One of the mechanisms that is responsible for IL-18 secretion is the inflammasome

assembly in response to signals such as PAMPs and DAMPs. In particular, the activation of pro-caspase-1 is facilitated by the assembly of inflammasomes, which is induced by various small molecules originating from infections, tissue damage, or T2DM (Molla et al., 2020). Following activation, caspase-1 enzymatically activated pro-IL-18, leading to their maturation into IL-18 and subsequent release. Once secreted, these pro-inflammatory cytokines play a pivotal role in recruiting and activating immune effector cells, thereby orchestrating the inflammatory response (Puccini & Kumar, 2016). When IL-18 engages with its receptor, IL-18R, it triggers the activation of NF- κ B and facilitating its translocation from the cytoplasm to the nucleus. IL-18, classified as a pro-inflammatory cytokine is secreted by diverse cell types including osteoblasts (Carroll et al., 2008). This study confirmed that IL-18 was indeed secreted by osteoblasts through hFOB1.19. According to a study conducted by Zhang et al., (2013) IL-18 can stimulate osteoclast formation by elevating the production of RANKL in T-cells. Hence, the downregulation of IL-18 expression represents a potential way for contributing to the reduction of osteoclast formation, thereby restoring equilibrium between bone formation and resorption.

5.3.1 Metformin combined with extracts reduced the NF- κ B translocation, downregulated secretion of IL-18 and IL-6

This study observed a notable decrease in the translocation of NF- κ B into the nucleus when the combination of metformin and extracts were administrated into the osteoblast cells. Similarly, the combination of metformin and extracts also reduced the secretion of IL-6 and IL-18 in MGO stimulated osteoblasts. However, there are no significant differences between the combination of metformin with extracts when compared to metformin alone for the level of translocated NF- κ B and secretion of IL-6.

Metformin has been studied previously for their anti-inflammatory properties. A study through real time polymerase chain reaction (rt-PCR) revealed that metformin significantly reduced the expression of NF- κ B genes and proteins when compared high glucose condition in rats (Gu et al., 2014). The observed decrease in IL-6 levels suggests a potential anti-inflammatory effect of metformin in an elevated MGO environment. This finding aligns with existing literature on metformin's anti-inflammatory properties, particularly its role in modulating cytokine secretion. For instance, a finding revealed that metformin suppressed the expression of pro-inflammatory cytokines including IL-6 in cancer cells (Bai & Chen, 2021). However, no synergistic potentials were observed in ameliorating NF- κ B translocation and IL-18 secretion when metformin was added to crude extracts. These observations suggest that though the extracts have the potential in reducing inflammation, adding them with the drugs did not enhance the potential even further.

An interesting observation was observed towards the secretion of IL-6. The combination of metformin with aqueous crude extracts managed to reduce IL-6 significantly, and synergistically when compared to MGO as well as metformin alone. This may indicate that the compounds extracted in the aqueous extracts have the capacity to work together in ameliorating the IL-6 even further. It is important to note that the transcription factor that is responsible for IL-6 secretion in osteoblast is not solely limited to NF- κ B. In fact, C/EBP also had been studied to regulate the secretion of IL-6 in osteoblasts (Tseng et al., 2010).

5.3.2 The extracts reduced the secretion of pro-inflammatory cytokines

The impact of OS on cellular apoptosis within the context of hFOB1.19 was systematically observed by assessing the alterations in caspase-3 formation, an important

pro-apoptotic protein. Methanolic and aqueous extracts reduced the translocation of NF- κ B, secretion of IL-6 and IL-18.

Metformin was reported in downregulating the expression of IL-18. For instance, in a western blot analysis conducted on faecal material obtained from metabolic disorder mice revealed that metformin downregulated the secretion of IL-18 mRNA (Lee et al., 2019). Furthermore, metformin was also found to significantly reduced the secretion of IL-18 in Polycystic ovary syndrome (PCOS) and T2DM (Alzamily et al., 2022). Not only that, Metformin was also investigated to inhibit the IL-18 secretion by activating the AMP-activated protein kinase (AMPK) pathway in T2DM patients with 2-months metformin therapy (Lee et al., 2013). The activation of AMPK pathway provides an antioxidant response through the activation of PGC-1 α , which triggers antioxidant defence mechanisms at cellular level (Rabinovitch et al., 2017; Rius-Pérez et al., 2020). Not only the activation AMPK pathway lead to the regulation of nutrient metabolism, it was also studied to have anti-inflammatory actions (Mancini et al., 2017).

Methanolic extract of *T. cordifolia* was studied to reduce pro-inflammatory cytokines secretion including IL-6 in arthritis-induced rat model, as well as reducing bone damage (Sannegowda et al., 2015). The complex physiological processes in rats are common in the *in vivo* setting, where it could be the reason behind the differences of IL-6 secretion when compared with the *in vitro* results obtained from this study. As *in-vivo* involves a complex physiological mechanism, *in vitro* study was performed in controlled conditions but lacks the full complexity of physiological factors. The dosage of *T. cordifolia* is another possible contribution for the differences. In the *in vivo* study, higher extract dosages may be necessary, but this same dosage in an *in vitro* cellular setting could induce cellular toxicity. Moreover, the cellular types employed in the previous study differ, with the use of spleen

cells in the original research and bone cells (hFOB1.19) in the current investigation. This difference in cell types suggests potential variations in metabolism and cellular responses to IL-6 secretion, highlighting the need to account for these differences in cellular behaviour.

The current research revealed no significant difference in the attenuation of IL-6 secretion from MGO-stimulated hFOB1.19 cells when subjected to methanolic *T. cordifolia* at 50 µg/mL. However, a notable reduction in IL-6 secretion was observed with a higher dosage of *T. cordifolia* at 100 µg/mL. Interestingly, the chloroform extract of *T. cordifolia* at 50 µg/mL also did not exhibit a statistically significant difference in the secretion of IL-6 compared to its positive control—LPS-induced inflammation in RAW246.7 cells as reported from a prior study conducted by (Philip et al., 2018). Furthermore, the similar study also showed that the chloroform extract of *T. cordifolia* at 100 µg/mL demonstrated a significant reduction in IL-6 secretion from RAW246.7 cells stimulated with LPS, also paralleled to finding of this study.

Methanolic *T. crispa* at 120 µg/mL exhibited no significant reduction, but at 240 µg/mL exhibited a significant downregulation in the secretion of IL-6 in MGO-stimulated hFOB1.19 when compared to the positive control. However, all crude aqueous extracts of *T. crispa* and *T. cordifolia* and their combination with metformin significantly when compared to the positive control. Earlier, *T. crispa* was studied to have synergistic effect with cisplatin in downregulating the activation of NF-κB in triple negative breast cancer cells (Al-Rashidi et al., 2016). The downregulation of pro-inflammatory cytokines is associated with the deactivation of NF-κB. This research explored the inhibitory effects of crude methanolic extract of *T. crispa* on the translocation of NF-κB into the nucleus of hFOB1.19 cells. Furthermore, the study demonstrated that *T. crispa*, at the concentration of 240 µg/mL, effectively inhibited the translocation process of NF-κB into the nucleus consequently

suppressing the secretion of IL-6 from the cells. The observed suppression of IL-6 secretion is attributed to the inhibition of NF- κ B activation and translocation, highlighting the potential anti-inflammatory properties of *T. crista*. In previous investigation by Nogueira et al., (2014), tea tree oil (TTO) demonstrated a similar effect by disrupting the NF- κ B pathway, subsequently downregulating the release of IL-6 from LPS-induced macrophages (U937) cells. This highlights the consistency of observations regarding TTO's interference with NF- κ B signalling and its consequential impact on IL-6 secretion when tested *in-vitro*.

Moreover, the present investigation showed a significant decrease in IL-18 secretion from MGO-stimulated hFOB1.19 cells when treated with crude methanolic and aqueous extracts. Given that tissue damage results in the release of DAMPs, the treatment of methanolic extracts to MGO-stimulated hFOB1.19 cells demonstrated efficacy in enhancing cellular viability, signifying its capability to mitigate cell damage. Alleviation of the cell damage observed in MGO-stimulated hFOB1.19 treated with crude methanolic extracts resulted in a reduced release of DAMPs. Consequently, this reduction in DAMPs was reflected in the secretion of IL-18, as reduced DAMPs lead to a decreased assembly of the inflammasome, downregulated the pro-caspase-1 activation, and subsequently, reduced IL-18 secretion (Molla et al., 2020). Interestingly, this study observed a synergistic relationship between crude methanolic and crude aqueous extracts with metformin in ameliorating IL-18 secretion from hFOB1.19.

5.4 Impact of crude plant extracts with or without metformin towards MGO stimulated hFOB1.19

In individuals diagnosed with T2DM, the concentration of plasma MGO exhibited higher level when compared to control subjects. Furthermore, this heightened MGO level was found to be positively correlated with glycosylated haemoglobin (HbA1c) at

correlation coefficient $r = 0.4980$, as indicated by Reyaz et al., (2020), establishing a significant association between elevated plasma MGO levels and HbA1c in T2DM patients.

5.4.1 MGO downregulated the formation of IR, GLUT4 and Runx2

In this study, MGO significantly downregulate the formation of IR. Downregulation of IR formation can lead to reduced cellular sensitivity to insulin, thereby affecting the translocation of GLUT4 to the membrane. This process is critical for facilitating glucose uptake within cells. Earlier study has shown that MGO also downregulated the autophosphorylation of IR which is crucial for the translocation of GLUT4 from cytosol to the membrane observed the rat muscle cells (L6) (Deshmukh et al., 2017). Furthermore, downregulation of IR expression also leads to reduced cellular responsiveness towards insulin that is responsible for the translocation of GLUT4 to the membrane, which is important for glucose uptake in cells (Lewis et al., 2023).

This study also observed the downregulation of GLUT4 in hFOB1.19 when treated with MGO. GLUT4 is expressed in osteoblasts and is required for insulin-stimulated glucose uptake (Li et al., 2016). Hence, GLUT4 is responsible for the glucose uptake in bone cells. Moreover, the downregulation of GLUT4 is often associated with T2DM. For instance, the downregulation of GLUT4 hinders the glucose uptake into the cell, which is necessary for cell metabolism and energy synthesis (Alam et al., 2016). In return, this leads to elevated sugar level in blood known as hyperglycaemia and is one of indicators of T2DM symptoms. In osteoblasts, glucose uptake is not only necessary its cellular metabolism, but also equally important for the regulation of Runx2. A finding by Wei et al., (2015) revealed that glucose inhibiting the AMPK-dependent proteasomal degradation of Runx2 in osteoblasts. Hence,

this process promotes the Runx2 transcription activity, leading to the osteogenesis and bone differentiation.

In addition, this investigation revealed that also downregulated the translocation of Runx2 into the nucleus when compared to untreated hFOB1.19 cells. An *in-vivo* study by Aikawa et al., (2017) revealed that diabetes-induced in male mice led to delayed bone healing, as observed in a femoral bone defect assessment through computed tomography scans. Moreover, their *in-vitro* investigation revealed that the exposure of stromal cells (ST2) to MGO slowed down the osteoblastic differentiation and reduced cell proliferation. In another study by Lee et al., (2019) revealed MGO significantly reduced bone mineral density in MGO-treated mice, indicating its role in initiating imbalance bone remodelling. When tested *in-vitro*, MGO also upregulated osteoclasts bone biomarkers such as cathepsin-k, which is important for its activation. In a recent investigation, the stimulation of human bone marrow-derived stromal cells with MGO was observed to result in a downregulation of Runx2 mRNA expression (Waqas et al., 2022). These findings revealed the impact of MGO on cellular processes, particularly its influence on both the transcriptional and intracellular localization of Runx2. Furthermore, Runx2 regulates the transcription of various genes that are specific to osteoblasts. These genes include those encoding for bone matrix proteins, such as osteocalcin and osteopontin (Amarasekara et al., 2021). As Runx2 serves as a crucial transcription factor facilitating osteoblastogenesis by stimulating the differentiation of mesenchymal stem cells into matured osteoblasts as explained by Komori, (2018), the treatment of hFOB1.19 with MGO resulted in a reduction of Runx2 which consequently reduced the osteoblast formation, creating imbalance in bone turnover.

5.4.2 Metformin elevated the expression of IR, GLUT4 and Runx2 in MGO-stimulated hFOB1.19

In this investigation, metformin was observed to increase the formation of IR in MGO-stimulated hFOB1.19. Previously, an *in-vitro* study towards human granulosa-luteal cells revealed that metformin aided in the translocation of GLUT4 from the cytosol to the plasma membrane by upregulating the expression of insulin receptor substrate-1 (IRS-1) located at the β -subunit of the IR (Rice et al., 2011). In addition, metformin not only helped in the translocation of GLUT4, but this study also observed its capability in upregulating the expression of GLUT4 in MGO-stimulated hFOB1.19.

This study observed the significant upregulation of Runx2 of MGO-stimulated hFOB1.19 when treated with metformin. Metformin has been reported previously to enhance the quality of bone and reduced the risk of fractures in patients with T2DM (Bahrambeigi et al., 2019). On top of that, a recent *in-vivo* study performed towards mice with femoral fracture revealed that metformin accelerated bone fracture healing in both osteoporotic and normal mice (Ruan et al., 2023). A study by Jang et al., (2011) reported that transient transfection assay performed in MC3T3-E1 showed that metformin may promote osteoblast differentiation by transactivating Runx2 via MPK/USF-1/SHP regulatory cascade.

5.4.3 Methanolic and aqueous extracts elevated IR, GLUT4 and Runx2 expression in MGO-stimulated hFOB1.19

In T2DM patients, their cells are highly resistance towards insulin (Taylor, 2012). This study observed the potential of methanolic and aqueous extracts in upregulating the expression of IR to improve insulin sensitivity in MGO-stimulated hFOB1.19. This effect is paralleled to the action of honey in upregulating the expression of IR in HepG2 cells and enhancing the insulin sensitivity (Lori et al., 2019). Additionally, this result also showed a

similar activity as aminoguanidine in upregulating the expression of IR in MGO-stimulated Chinese hamster ovary cells (CHO) (Deshmukh et al., 2017). A study revealed that the restored IR expression was found to be therapeutic in T2DM mice (Wang et al., 2019).

T2DM patients with insulin resistance has been reported to have low GLUT4 expression (Kampmann et al., 2011). Thus, the restoration of GLUT4 expression allows the glucose to enter cells and be metabolised for energy production (Alam et al., 2016). Methanolic and aqueous extracts managed to upregulate GLUT4 expression in MGO-stimulated hFOB1.19. Similarly, *Wisteria sinensis* or commonly known as Chinese wisteria had been studied to upregulate GLUT4 expression in diabetic rats and improved their insulin sensitivity (Huang et al., 2021). Aloperine, an alkaloid from *Sophora alopecuroides* L. also had been reported to upregulate GLUT4 expression in diabetic-induced rats (Song et al., 2021).

This study observed a significant increase in Runx2 expression when MGO-stimulated hFOB1.19 was introduced with methanolic and aqueous *T. cordifolia* and *T. crispa*. These results are aligned with the effect of *Petasites japonicus* in increasing Runx2 expression and regulating osteoblast differentiation in MC3T3-E1 cells (Kim et al., 2021). In another study, *Dimocarpus longan* Lour. had shown a similar effect in upregulating Runx2 in MC3T3-E1 cells (Park et al., 2016). Upregulating Runx2 expression in T2DM patients is crucial as it plays a vital role in osteoblast differentiation and bone formation. Enhanced Runx2 activity can contribute to improved bone mineralization and structural integrity and help to lower the risk of fractures and osteoporosis prevalent in T2DM individuals.

5.5 Advantages of hFOB1.19 and potential limitations

The hFOB1.19 cell line is an immortalized, yet untransformed cells which frequently used to study normal osteoblast function (Oliveira et al., 2021). In addition to its capacity for rapid proliferation, hFOB1.19 cells can be used to measure key markers characteristic of osteoblast activity, such as the transcription factor Runx2, pro-inflammatory cytokines, and caspase-3. The use of immortalized cells offers a significant advantage, as marker expression levels remain stable throughout the experimental period. In comparison to primary osteoblasts, the hFOB1.19 cell line has the benefit of reduced variability, whereas primary cells often exhibit donor-specific differences that can potentially impact experimental outcomes (Richter et al., 2021). For instance, this study observes the upregulation of Runx2 in hFOB1.19 which aligns with findings from Oliveira et al. (2021), who similarly reported increased transcription of osteoblast-specific markers in these cells under osteogenic conditions. However, The limitation of using immortalized cells is that they cannot be regarded as fully "normal," as they possess the ability to divide indefinitely and may exhibit gene expression patterns that are not present in any cell type under physiological conditions (Carter & Shieh, 2010). The direction of this study can be further continued by doing comparative studies. For instance, the study can be expanded by comparing the expression of Runx2 in hFOB1.19 as well as in primary osteoblasts to further validate the differences in terms of marker expressions between both kind of cells.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has revealed that methanolic and aqueous extracts possessed potential antioxidant as well as anti-inflammatory properties. These properties are crucial in regulating OS in osteoblasts due to excessive MGO level especially in T2DM patients. The detrimental effect of MGO in regulating cellular apoptosis, cytokines and insulin signalling is shown in Figure 6.1.

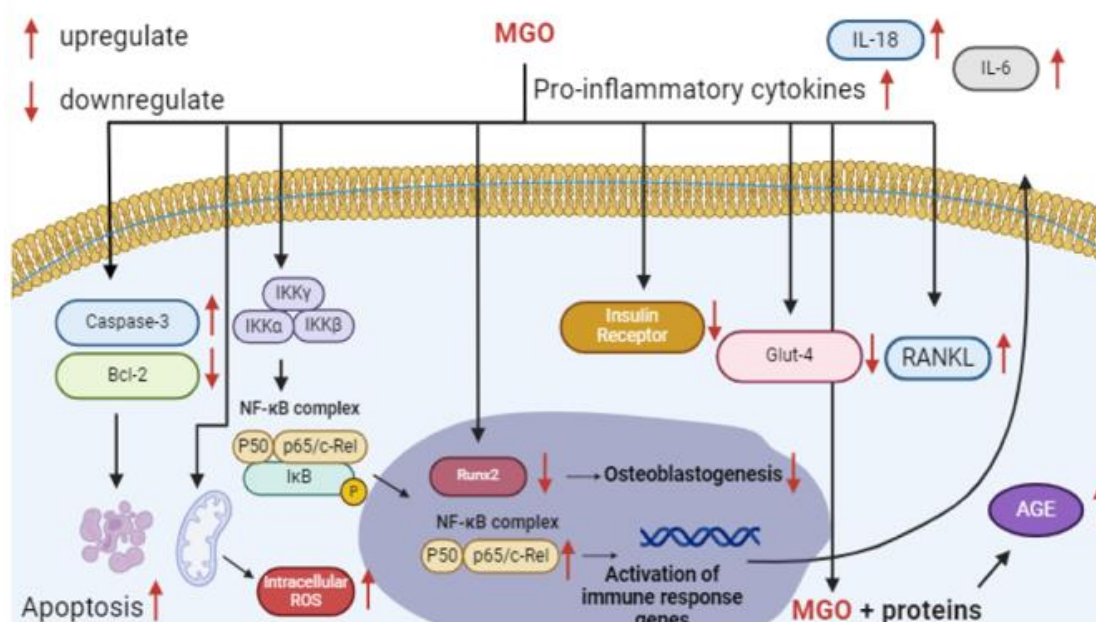


Figure 6.1: The effect of MGO in osteoblasts. MGO stimulates apoptosis, upregulates the release of pro-inflammatory cytokines and reduce the insulin signalling.

In this study, the extracts also have been proved to help in regulating diabetic-bone metabolism. As T2DM patients often experience imbalance bone metabolism, restoring back bone metabolism help to prevent excessive bone loss. GC-MS analysis has uncovered

multiple bioactive compounds that had been previously reported to possess antioxidant and anti-inflammatory properties. These bioactive compounds, detected through GC-MS can be studied further in research for their pharmacological importance individually, rather than using crude extracts.

In addition, the extracts also have shown antioxidant properties when tested through antioxidant assays. In particular, both methanolic and aqueous extracts of *T. cordifolia* and *T. crispa* have shown an excellent free radical scavenging activity in DPPH assay. Then, its antioxidant property has been tested *in-vitro* towards hFOB1.19 cells, by which the extracts managed to downregulate intracellular ROS in MGO-stimulated hFOB1.19 cells. The extracts also exhibit a potential reduction in the glycation activity of protein and MGO to a limited extent when measured through BSA-MGO assay. This is proven again when *in-vitro* analysis revealed that the extracts managed to reduce the AGE expression in MGO-stimulated hFOB1.19. However, it is likely that more pronounced anti-glycation activity could be achieved with higher concentrations.

The extracts have shown an anti-apoptotic property in MGO-stimulated hFOB1.19 cells. Methanolic and aqueous extracts of *T. cordifolia* and *T. crispa* have effectively reduced pro-apoptotic proteins, caspase-3 and upregulate anti-apoptotic proteins, Bcl-2. Additionally, the extracts also have shown anti-inflammatory effects in MGO-stimulated hFOB1.19. Methanolic and aqueous extracts have managed to downregulate the translocation of NF- κ B into the nucleus, IL-6 and IL-18 secretion from the cells, indicating their anti-inflammatory properties. Generally, MGO has suppressed the expression of GLUT4 and IR in hFOB1.19. However, methanolic and aqueous extracts have managed to restore this condition by upregulating GLUT4 and IR expression. Furthermore, the extracts also

managed to restore the expression of Runx2 in MGO-stimulated hFOB1.19, indicating its importance in bone metabolism. The effect of the extracts in restoring back cellular activity in MGO-stimulated hFOB1.19 is summarised in Figure 6.2.

The extracts did not show a potential synergistic effect when combined with metformin. Thus, However, the study observed an additional effect between the extracts and metformin. An interesting result can be seen through the expression of Bcl-2, as the combination of metformin with the drugs further downregulate its expression when compared to metformin alone, suggesting a further study that needed to elucidate this finding.

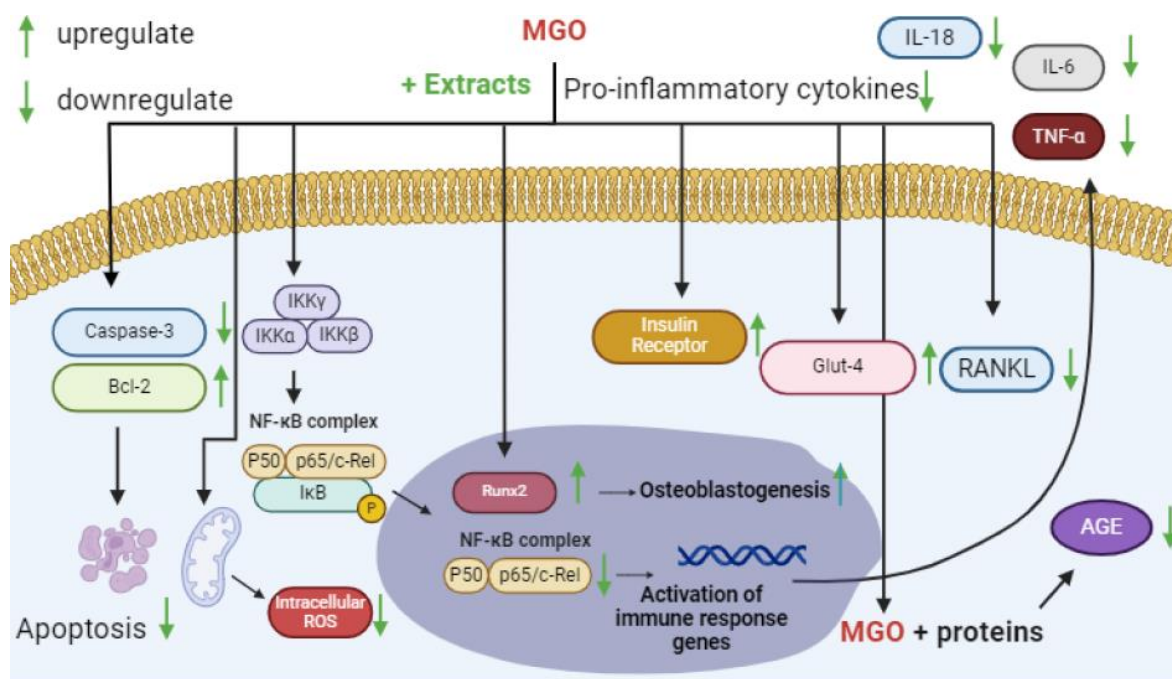


Figure 6.2: The effect of *T. cordifolia* and *T. crispa* in MGO-stimulated hFOB1.19. The extracts helped to regulate apoptosis, reduced pro-inflammatory cytokines released, as well as modulating the insulin signaling.

Furthermore, methanolic and aqueous extracts of *T. crispa* and *T. cordifolia* were found to possess bioactive compounds that have antioxidant and anti-inflammatory activities. In addition, the extracts also have an anti-apoptotic and anti-inflammatory properties towards MGO-stimulated hFOB1.19. Lastly, the extracts also help to upregulate transcription factor Runx2 in osteoblasts which is responsible for matrix proteins as well as attenuates the expression of GLUT4 and IR.

6.2 Recommendations

As this study has highlighted the importance of methanolic and aqueous extracts of *T. cordifolia* and *T. crispa* in *in-vitro* assays, future research may prioritize a more comprehensive study especially involving *in-vivo* study. Naturally, physiological condition in *in-vivo* is far more complex than a controlled environment such as *in-vitro*. Thus, studying the extracts *in-vivo* may open up new findings towards the action of the extracts. Animal models, such as murine models, could provide valuable information on the extracts' bioavailability, dosage optimization, and potential side effects in a living organism.

In the present investigation, financial constraints limited the exploration of additional crucial proteins. Consequently, only a select few pivotal proteins were chosen for analysis. To enhance the comprehensiveness of future studies, it is recommended to focus on assessing the Bax and Bcl-2 ratio for apoptotic activity, providing a more holistic understanding of anti-apoptotic conditions in hFOB1.19. Additionally, for a thorough study of anti-inflammatory activity, emphasis can be placed on investigating pro-inflammatory cytokines, such as TNF- α secretion and IL-8. In the context of diabetic bone metabolism, it is advisable to explore Twist2 and osteocalcin expression to gain insights into pertinent molecular mechanisms

Considering the ethnic and geographic diversity of the *Tinospora* plant species, future studies could investigate variations in the immunomodulatory effects among different *Tinospora* species and geographical locations. This approach could shed light on the impact of environmental factors on the variability of bioactive compounds and their subsequent influence on immune responses.

These suggestions aim to guide future research endeavours, ensuring a more comprehensive exploration of the immunomodulatory potential of *T. crispa* and *T. cordifolia* extracts and their applications in both traditional and modern medicine.

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APPENDICES

Appendix A: : The powder of *T. cordifolia* (a) and *T. crispa* (b)



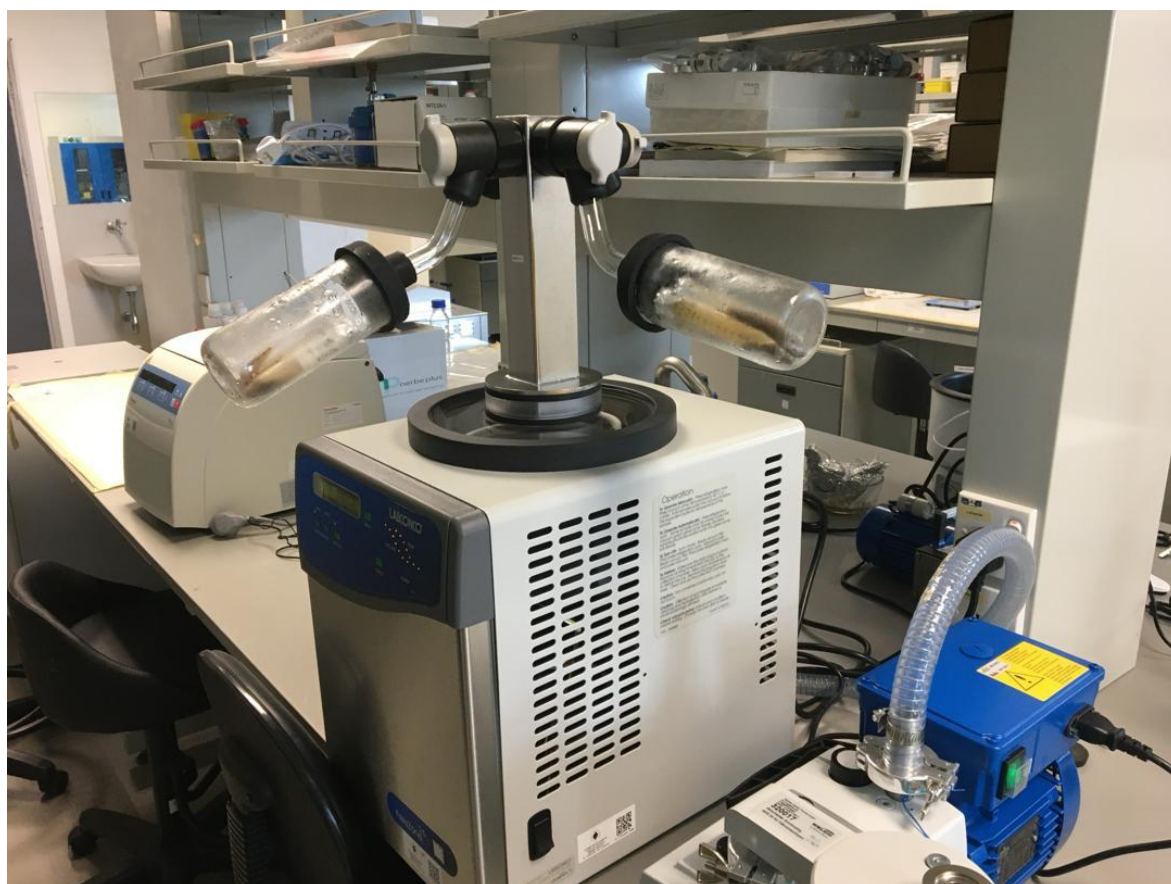
Appendix B: Filtration process



Appendix C: Rotary evaporator



Appendix D: Freeze dry process to evaporate the excess moisture



Appendix E: ATCC biosafety level 2 (BSL-2) form



New Account Application BSL 2

Please submit completed form, lab detail, and Material Transfer Agreement to:

SPD Scientific (M) Sdn Bhd
F-G-45, Block F, Jalan PJU 1A/3, Taipan 2
Damansara, Ara Damansara, 47301, Petaling
Jaya, Selangor Darul Ehsan, Malaysia
Fax: +603 7842 8850
Email: sales.my@spdsscientific.com

New Accounts are subject to approval. Print or type all information to be legible.

All information gathered will be used for ATCC purposes only. Your information will not be shared with any outside organization. Please allow 3 to 5 business days after receipt of completed application for account approval.

Organization Information		
Organization Name Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak,		Company Registration No. (ROC) not applicable
Department Faculty of Medicine and Health Sciences, Biochemistry		Web Site Address https://www.unimas.my
Check type of organization: ☒ University/Education ☐ Research Foundation ☐ U.S. Government ☐ Hospital/Clinic	☐ Diagnostic Lab ☐ Pharmaceutical/Drug Discovery ☐ Biotechnology/Life Science ☐ Contract Laboratory	☐ Industrial ☐ Manufacturing ☐ Food Processing/Agriculture ☐ Environmental ☐ International Government ☐ 3rd Party Purchasing Agent (billing only)
Private, nonprofit organization		
Billing Information		
Choose payment preference (Credit terms net 30 & Prepayment) ☒ Wire Transfer / Telegraphic Transfer (TT) / Automated Clearing House (ACH) ☐ Cheque		
SPD accepts: 1) Malaysian Ringgit by check drawn on any Malaysian bank or international money order made payable to: SPD Scientific (M) Sdn Bhd, F-G-45 Block F, Jalan PJU 1A/3, Taipan 2 Ara Damansara, 47301 Petaling Jaya, Selangor, Malaysia 2) International money wire transfer to our account #3520 3949 9101 at HSBC Bank Malaysia Berhad, Ground Floor Wisma UEP Jalan USJ 10/1A Pusat Perniagaan USJ 10, 47620 Subang Jaya, Selangor, Malaysia. Swift Code HBMBMYKL 3) Automated Clearinghouse (ACH) to our account #3520 3949 9101 at HSBC Bank Malaysia Berhad, Ground Floor Wisma UEP Jalan USJ 10/1A Pusat Perniagaan USJ 10, 47620 Subang Jaya, Selangor, Malaysia.		
Billing Address (Invoices will be sent to this address) Please verify this information with the accounts payable department for your organization.		
First Name Gabriele	Middle Name Ruth, Anisah	Last Name Froemming
Department Faculty of Medicine and Health Sciences	Building FMHS	Room Number Admin office, level 2
Street Address/P.O. Box -		City Kota Samarahan
State/Province Sarawak	Zip/Postal Code 94300	Country Malaysia
Telephone (including Country Code)	Fax (including Country Code)	E-mail (of contact name)
Shipping Address (Complete street address; PO Boxes are not acceptable)		
Department Faculty of Medicine and Health Sciences	Building FMHS	Room Number Admin office level 2
Street Address (PO Boxes cannot be accepted)		City Kota Samarahan
State/Province Sarawak	Zip/Postal Code 94300	Country Malaysia
Telephone (including Country Code) +60 133835613	Fax (including Country Code) +6082665152	E-mail rafgabriele@unimas.my
End User Information (Primary End User)		

Appendix E: Continued

First Name Teknowillie anak	Middle Name	Last Name Singa	Title -
Department FMHS, Biochemistry		Building FMHS	Room Number Biochemistry Lab
Street Address -			City Kota Samarahan
State/Province Sarawak		Zip/Postal Code 94300	Country Malaysia
Telephone (including Country Code) +601115808104		Fax (including Country Code) +6082665152	E-mail teknowillie@gmail.com
Additional End User Information			
First Name Luqman	Middle Name	Last Name bin Jaya	Title -
Department FMHS, Biochemistry		Building	Room Number Biochemistry Lab
Street Address -			City Kota Samarahan
State/Province Sarawak		Zip/Postal Code 94300	Country Malaysia
Telephone (including Country code) +60135893721		Fax (including Country code) +6082665152	E-mail luqmanjaya96@gmail.com
Additional End User Information			
First Name	Middle Name	Last Name	Title -
Department		Building	Room Number
Street Address			City
State/Province		Zip/Postal Code	Country
Telephone (including Country code)		Fax (including Country code)	E-mail

Biosafety Level 2 is suitable for work involving agents that pose moderate hazards to personnel and the environment. It differs from BSL-1 in that 1) laboratory personnel have specific training in handling pathogenic agents and are supervised by scientists competent in handling infectious agents and associated procedures; 2) access to the laboratory is restricted when work is being conducted; and 3) all procedures in which infectious aerosols or splashes may be created are conducted in biological safety cabinets (BSCs) or other physical containment equipment.

The following standard practices, special practices, safety equipment, and facility requirements apply to BSL-2. Please check all that apply.

Access Control
☒ Access to laboratory is controlled with doors and sealed windows present. ☒ All persons entering the laboratory are advised of entry/exit requirements through training and signage, with hazards and responsible parties' information posted. ☒ Policies are in place to prevent consumption of food/beverages. ☐ Personal protective equipment is provided to employees and visitors. Please list the types of PPE (select all that apply): ☒ Laboratory coat/gown ☒ Eye/face protection ☒ Gloves ☐ Respiratory protection ☐ Safety shoes
Training
☒ Are laboratory personnel trained prior to assuming their duties and on an annual basis? Are they trained on a laboratory specific biosafety manual that includes PPE, minimization of splashes, hand washing, sharps and spill training? ☐ Does your organization have an occupational health and medical surveillance program in place which offers appropriate immunizations for the materials handled? Is training provided to employees on this program?
Equipment and Facilities
☒ Are decontamination equipment/procedures in place for the decontamination of surfaces, equipment, and waste? Please list all methods of waste disposal (check all that apply): ☐ Chemical Inactivation ☒ Autoclave – onsite/contractor

Appendix E: Continued

☒ Incineration – onsite/contractor ☒ Are the appropriate sharps containers provided for the laboratories? ☒ Are hand washing facilities provided in the laboratories? Is an eyewash station available? ☒ Is laboratory furniture provided that is designed for easy cleaning and made of appropriate material to withstand the laboratory work being performed? ☒ Are facilities kept free from accumulations of rubbish, unwanted materials and objects that present hazards from tripping, fire, explosion and harbourage of pests?	
☐ Biosafety cabinets (BSC) are required for the manipulation of BSL 2 or higher infectious materials. Please indicate the BSC type and certification schedule: ☐ Class I Explain: ☒ Class II ☐ A1 ☐ A2 ☐ B1 ☒ B2 ☐ Other Explain: ☒ Certification Schedule ☐ Biennial ☒ Annual ☐ Other Explain:	

☒ I acknowledge that the information listed in this facility description is current, complete and accurate to the best of my knowledge. I understand the hazards associated with the material we are requesting and we will work with it under the appropriate laboratory containment as determined by our institutional policies and procedures.

X Prof Dr. Asri bin Said
Biosafety Officer or Environmental Officer (Print)

X  8 Nov 2022
Biosafety Officer or Environmental Officer's Signature and Date

Appendix E: Continued

Intended Use
NOTE: Material purchased from ATCC is usually intended for research use only. For commercial use please contact licensing@atcc.org I will use ATCC Material for (check one): ☒ Research use only ☐ Commercial use ☐ Both research AND commercial use
Product Use
Please provide a scope of use for the materials: The osteoblast cells will be used for a research project under the FRGS scheme investigating the ameliorating effect of t. crista and t cordifolia extract on MGO induced formation of AGE and cell death.
Additional Required Information
• Material Transfer Agreement (MTA) The MTA must be completed and signed by an individual at your organization with the ability to execute legally binding documents on behalf of your organization. The MTA can be found on our website at www.atcc.org/mta. • Curriculum Vitae A Curriculum Vitae may be required.

If you have questions regarding the status of your application, contact us by phone 603-7842 9850, or by e-mail at sales.my@spdsscientific.com

X Prof Dr.Gabriele R.A. Froemming
Applicant First and Last Name (Print)

X  7/11/2022
Applicant Signature and Date

Appendix F: Material transfer agreement (MTA)



Material Transfer Agreement

Version 7.1

This Material Transfer Agreement ("MTA") is by and between the American Type Culture Collection, a District of Columbia non-profit corporation ("ATCC") and the organization listed below ("Recipient").

Background

ATCC has a nonprofit mission to accept biological materials, maintain them with best practices developed over decades of service as the world's leading biological resource center, and make them available to the research community.

Definitions

"ATCC Original Material" means the materials, including but not limited to genetic, proteomic, and metabolomic data relating to the material, received from ATCC by Recipient as described on an ATCC purchase order.

"ATCC Material" means ATCC Original Material along with its Progeny and Unmodified Derivatives including Unmodified Derivatives existing within Modifications.

"Commercial Use" means the use of the ATCC Materials for commercial benefit, including without limitation:

- (i) for sale, license, lease, export, transfer or other distribution for financial purposes or other commercial purposes;
- (ii) to provide a service for financial purposes, including but not limited to proficiency testing, preclinical, clinical, bioproduction/manufacturing services or any other fee-for-service use by a CRO, any university core facility, or any other third-party contractor;
- (iii) to produce or manufacture products for general sale or ultimately intended for general sale, including use in a commercial manufacturing process such as fermentation, bioproduction or isolation processes;
- (iv) in a clinical trial or other testing regulated by a government agency (e.g. FDA, EMEA, EPA, etc.) or in any human;
- (v) to collect and commercially exploit data regarding sequences of nucleic acids, proteins or other biological polymers, or relative amounts of biological substances or biological activities; or
- (vi) to generate a whole or partial genome sequence and use the foregoing for financial purposes.

"Contributor" means the entity depositing material with ATCC.

"CRO" means an organization performing services on behalf of Recipient or other clients, including a contract research organization, a contract manufacturing organization, a contract development and manufacturing organization or other similar entities. Any CRO providing services for a fee should inquire with ATCC regarding its need for a license.

"Modifications" means organisms or biological substances created by or on behalf of Recipient which are not Progeny or Unmodified Derivatives, but which contain or incorporate ATCC Material. By way of a nonlimiting example, Modifications result when ATCC Material is modified through molecular biological technology.

"Non-Commercial Use" means use of the ATCC Materials for purposes not constituting Commercial Use.

"Progeny" means an unmodified descendant from the ATCC Original Materials, such as plasmid from plasmid, virus from virus, cell from cell, or organism from organism.

"Unmodified Derivative(s)" means substances and sequence data native to and characteristic of ATCC Original Material, including without limitation characteristic nucleic acids, proteins, lipids, carbohydrates, metabolites, membranes, exosomes, organelles, and other native substances, characteristic sequence data of nucleotide, amino acid, or other biological polymerizable monomers, biological polymers expressed according to such characteristic sequence data, antibodies secreted by a hybridoma cell line, or purified or fractionated subsets or lysates of any of the above.

Scope of Use

ATCC MATERIALS ARE NOT FOR USE IN HUMANS. ATCC Materials may only be used by Recipient for research purposes and shall not be used for any Commercial Use without first obtaining a Commercial Use license from ATCC. Use may also be subject to restrictions from a Contributor, a patent owner, or a governmental entity and ATCC makes no representation or warranty regarding the existence or the validity of such restrictions. Notwithstanding anything herein to the contrary, ATCC Materials shall not be used in any manner



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that infringes a valid patent in force. Recipient shall have the sole responsibility for identifying and obtaining any third party licenses required.

Transfers

Except as specifically provided in this section, Recipient shall not distribute, sell, transfer or otherwise make available the ATCC Material to any other entity, including its affiliates, without ATCC's prior written approval. Recipient shall ensure that any permitted transfer shall be subject to the permitted transferee agreeing to be bound by terms and conditions of this MTA.

- Core Facility. ATCC Material may not be transferred to or used in any biological material repository or core facility.
- Transfers Within Recipient Organization. Recipient may, for the purposes of the research project make the ATCC Materials available to any Recipient employees solely for use during their employment with Recipient, provided that such employees are made aware of the restrictions against transfer of ATCC Material, including in Modifications, outside of the Recipient organization without ATCC's prior written approval. Recipient accepts responsibility for all violations of such restrictions. Recipient may not (i) use or transfer the ATCC Materials for unrelated projects within Recipient's organization, or (ii) use the ATCC Materials as part of a de facto internal repository or to create a core facility. Recipient shall maintain a record of all such internal transfers and the related projects and shall provide such record to ATCC upon request.
- Non-Commercial Use Research Project Transfers.
 - ATCC Original Material/Progeny. Any party working on a collaborative research project with Recipient shall obtain ATCC Original Material or Progeny only from ATCC.
 - Modifications and Unmodified Derivatives. Modifications and Unmodified Derivatives may only be made and used by Recipient in its facility. Recipient may transfer Modifications and Unmodified Derivatives to (i) CROs, solely for Non-Commercial Use on Recipient's projects, and (ii) Recipient's research collaborators in a Non-Commercial Use research project, in each case, so long as such transferees agree in writing not to further transfer the Modifications or associated Unmodified Derivatives. Upon completion of any collaborative research project, the Recipient shall require transferee to either return or certify destruction of the Modifications and Unmodified Derivatives. Collaborative research projects include, but are not limited to, research sponsored by a for-profit organization carried out at a non-profit organization and by the non-profit organization's personnel. Permitted use hereunder extends only to basic and discovery research related to, directly under, or in direct collaboration with Recipient's research project.
- Transfer of Published Modifications. Recipient may transfer published Modifications for Non-Commercial Use in accordance with the rules of such publication, provided that Recipient notifies ATCC in writing of the transfer.
- Researcher Change of Institution. Should a researcher at Recipient move to another institution, researcher may take Modifications to such institution only if (i) Recipient consents to and notifies ATCC in writing of the transfer and, (ii) Transferee Institution has entered or enters into a material transfer agreement with ATCC.
- Identification of Modifications and Unmodified Derivatives in Transfers. Recipient shall assign and use its own naming scheme when transferring Modifications and Unmodified Derivatives to any transferees and CROs and shall identify such Modifications and Unmodified Derivatives in writing as comprising ATCC Material.
- If the Modifications or Unmodified Derivatives are transferred under any of the conditions described in this Section, Recipient agrees to notify ATCC of the transfer as per the instructions available at www.atcc.org/materialtransfer.

Compliance With Laws

Recipient is solely responsible for and shall ensure compliance with all foreign and domestic, federal, state and local statutes, ordinances and regulations applicable to use of the ATCC Material by Recipient or its transferees, including the U.S. export control laws and related regulations. Recipient is solely responsible for obtaining all permits, licenses or other approvals required by any governmental authority in connection with Recipient's and its transferees' receipt, handling, storage, disposal, transfer and use of the ATCC Materials.



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Material Transfer Agreement

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Indemnification; Limitation of Liability

To the extent permitted by law, Recipient shall indemnify, defend and hold harmless ATCC and its Contributors against all third party claims, losses, expenses and damages, including reasonable attorneys' fees (collectively "Claims") arising out of or relating to Recipient's and its transferees' use, receipt, handling, storage, transfer, disposal and other activities relating to ATCC Materials, provided that Recipient's liability shall be limited to the extent that any such Claim arises out of ATCC's gross negligence or willful misconduct. All non-monetary settlements of any such Claims are subject to ATCC's prior written consent, which consent shall not be unreasonably withheld. If Recipient is the U.S. federal government or a state institution or a foreign equivalent organization, Recipient shall assume all liability for any and all Claims arising out of or relating to Recipient's and its transferees' use, receipt, handling, storage, transfer, disposal and other activities relating to ATCC Materials to the extent provided under the Federal Tort Claims Act, 28 U.S.C. §§ 2671 et seq. or under equivalent applicable State or foreign law.

ALL ATCC ORIGINAL MATERIALS ARE SUPPLIED "AS IS". ATCC MAKES NO REPRESENTATIONS OR WARRANTIES OF ANY KIND, EITHER EXPRESS OR IMPLIED, AND EXPRESSLY DISCLAIMS WARRANTIES OF MERCHANTABILITY, NONINFRINGEMENT OR FITNESS FOR A PARTICULAR PURPOSE. To the maximum extent permitted by law, in no event will ATCC or its Contributors be liable for any indirect, special, incidental or consequential damages of any kind in connection with or arising out of the MTA or ATCC Materials (whether in contract, tort, negligence, strict liability, statute or otherwise) even if ATCC has been advised of the possibility of such damages. In no event shall ATCC's cumulative liability to the Recipient exceed the fees paid by Recipient under this MTA and the applicable ATCC purchase order for the twelve (12) month period preceding the date of the event giving rise to the first such claim. Recipient agrees that the limitations of liability set forth in this MTA shall apply even if a limited remedy provided hereunder fails of its essential purpose.

The provisions of this *Indemnification; Limitation of Liability* section shall survive termination or expiration of this MTA.

Property Rights

ATCC and/or its Contributors shall retain ownership of all right, title and interest in the ATCC Materials, including such ATCC Materials contained or incorporated in Modifications. Recipient retains ownership of: (a) Modifications (except that, as between the parties, ATCC retains ownership rights to ATCC Material included therein and use of ATCC Materials remains subject to Scope of Use, above) and (b) those substances created through the use of ATCC Material, but which do not contain ATCC Material.

Recipient shall cite ATCC as the provider of ATCC Materials and give appropriate credit to ATCC in any publication containing information derived from ATCC Materials.

Recipient expressly acknowledges that all ATCC trademarks are the exclusive property of ATCC and ATCC retains all right, title and interest in and to its trademarks, service marks, trade-names, logos, catalog numbers and ATCC-specific designations of ATCC Materials. Except as mandated by this *Property Rights* section, Recipient shall not use the foregoing in any way without ATCC's prior written agreement.

Miscellaneous

Any disputes arising under this MTA shall be governed by the laws of the State of New York without regard to its conflict of laws principles, and Recipient hereby expressly consents to, submits to and waives any objection to the sole and exclusive jurisdiction and venue of New York courts. If Recipient is a Federal or State non-profit organization or foreign public organization, then any disputes arising under this MTA shall be tried exclusively in a court of competent jurisdiction.

Recipient agrees that any breach of this MTA, including but not limited to any breach of the Scope of Use provisions of this MTA, will entitle ATCC to immediately cease further shipments of ATCC Material without notice to Recipient and ATCC shall have the right to immediately terminate this MTA. Recipient acknowledges that any breach may create such irreparable injury as to entitle ATCC to seek preliminary or permanent injunctive relief in addition to all other equitable and legal remedies available under applicable laws.

Recipient may not assign or otherwise transfer this MTA or any rights or obligations under this MTA, whether by operation of law or otherwise without the prior written



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consent of ATCC and any such attempted assignment or transfer will be void and of no force or effect. This MTA shall be binding on all permitted successors and assigns. This MTA, including all documents incorporated herein by reference, constitutes the entire agreement between ATCC and Recipient with respect to the ATCC Material and supersedes all previous agreements between ATCC and Recipient relating to the ATCC Material. No term or provision contained herein shall be deemed waived or modified and no breach excused unless such waiver or consent shall be in writing and signed by the parties. If any provision of this MTA is for any reason found to be unenforceable, the remainder of this MTA will

continue in full force and effect. The provisions of this MTA which by their nature or implication are intended to survive termination or expiration shall so survive. None of the provisions of this MTA are intended to create, nor shall be deemed or construed to create, any relationship between ATCC or Recipient other than that of independent entities contracting with each other hereunder solely for the purpose of effecting the provisions of this MTA. The undersigned represent that they have full authority to enter into this MTA and to bind the parties for and on behalf of the legal entities set forth below.

AGREEMENT TO THIS MTA witnessed by the following signature as of this effective date: 8/11/2022

Recipient Organization: Faculty of Medicine and Health Sciences (FMHS), Universiti Malaysia Sarawak

Organization tax ID: not applicable

Signed:

By: Dr. Angela anak Siner

Dr Angela Ak Siner
Head of Department
Basic Medical Sciences
Faculty of Medicine and Health Sciences
Universiti Malaysia Sarawak

Its: HOD for Basic Medical Sciences, FMHS, UNIMAS

Email: sangela@unimas.my

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Sarawak, Malaysia

Any correspondence concerning this MTA should be addressed to: ATCC, ATTN: Contracts, 10801 University Boulevard, Manassas, VA 20110. Otherwise, please contact us at contracts@atcc.org.



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Research Article

Antioxidant Marvels : Tapping into *Tinospora crispa* and *Tinospora cordifolia* via Antioxidant assays and GC-MS Analysis

ABSTRACT

Tinospora crispa and *Tinospora cordifolia* are plant species that are commonly used in traditional medicine, such as Ayurvedic medicine, renowned for their therapeutic roles in addressing diverse health issues, including diabetes. These plants are esteemed for their ability to counter oxidative stress through electron donation which is a prominent feature of antioxidant. However, a sole assessment of their antioxidant effectiveness is insufficient to holistically understand their antioxidative capabilities. The primary aim of this study was to study the antioxidative properties exhibited by *T. crispa* and *T. cordifolia*. This evaluation encompassed a range of tests measuring radical scavenging activity (DPPH assay), capacity for reducing ferric ions (FRAP assay), and their anti-glycation potential (BSA-MGO assay). To further enhance this investigation, GC-MS analysis was employed to identify compounds with antioxidative properties within *T. crispa* and *T. cordifolia*. The stems and leaves of *T. crispa* and *T. cordifolia* underwent solvent extraction using 90% methanol and hot distilled water. Notably, the methanolic extract of *T. cordifolia* displayed the most robust radical scavenging activity, evident from its lowest IC₅₀ value, 0.03 ± 0.00 mg/mL in the DPPH assay. Conversely, the methanolic extract of *T. crispa* exhibited the least IC₅₀ value, 0.19 ± 0.00 mg/mL in the FRAP assay. Additionally, the methanolic extract of *T. cordifolia* showcased a minimal IC₅₀ value 0.52 ± 0.18 mg/mL in the BSA-MGO anti-glycation assay. It's worth noting that the methanolic extracts of both *T. crispa* and *T. cordifolia* outperformed their hot water counterparts in terms of antioxidative activity, potentially due to the presence of phytochemical compounds such as phenol, 4-vinyl guaiacol, guaiacol, syringol, and vanillin in the methanolic extracts.

Key words: Antioxidant, GC-MS, *T. cordifolia*, *T. crispa*.

INTRODUCTION

In most parts of the world, herbal products have been used extensively as part of the practice of complementary and alternative medicine (CAM). *T. crispa*, a climbing shrub, is a member of the Menispermaceae botanical family which can be found in Asian and African rainforests. The foliage, stem, and roots of *T. crispa* have been traditionally utilized as alternative remedy for diverse medical conditions. (Ahmad *et al.*, 2016 ; Thomas *et al.*, 2016). Another Menispermaceae family member known as *T. cordifolia* is used in Ayurvedic medicine to address a multitude of disorders including diabetes mellitus (Kumar 2015). A review by Singa *et al.*, (2022) revealed that both *T. crispa* and *T. cordifolia* stems and leaves are high in phytochemical content such as phenols that are widely recognised for their antioxidant and/or hypoglycemic properties. In addition, *in vitro* study showed that the stem extract from *T. cordifolia* could scavenge superoxide anions as well as hydroxyl radicals (Kumar 2015).

Antioxidants are known as free radical scavengers that reduce cell damage caused by reactive oxygen species (ROS). Reactive oxygen species (ROS) play a role in various health conditions. Antioxidants present in plant-based diets have been associated with their ability to counteract conditions such as cardiovascular disorders and type-2 diabetes mellitus (T2DM). This positive effect is attributed to their capacity to neutralize free radicals within the body (Stanner & Weichselbaum, 2012). Antioxidants interact with free radicals through a range of mechanisms. In the mechanism involving hydrogen atom transfer (HAT), a free radical takes a hydrogen atom from an antioxidant, leading the antioxidant itself to become a radical. Conversely, in the single electron transfer (SET) mechanism, antioxidants contribute electrons to free radicals, resulting in the antioxidant adopting a radical cation (Liang & Kitts, 2014). An example illustrating the assessment of antioxidant capability is the ferric ion-reducing power assay, commonly used to measure the electron-donating ability of antioxidants. This characteristic is particularly important for phenolic antioxidants, through the ability to reduce of Fe³⁺ to Fe²⁺ (Irshad *et al.*, 2012). Another instance of SET is seen in the application of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) experiment. In this case, the unpaired electron within DPPH generates a distinctive deep purple colour with an absorption band at 517 nm. However, when this unpaired electron pairs with another electron due to the intervention of an antioxidant, an alteration in colour occurs, resulting in a pale yellow hue (Sadeer *et al.*, 2020).

Prolonged elevation of blood glucose levels significantly contributes to the production of advanced glycation end-products (AGEs). These AGEs are the result of intricate reactions between carbonyl moieties found in reducing sugars and unbound amino groups in proteins, lipids, or nucleic acids. The formation and accumulation of methylglyoxal (MGO), an active dicarbonyl compound, have been intricately associated with the commencement and advancement of type 2 diabetes mellitus (T2DM). The glycolysis pathway generates