



Faculty of Medicine and Health Sciences

Exploring the Potential Effects of *Tinospora crispa* Extracts on RAGE Expression, Cytokine Modulation, and Cell Morphology in Methylglyoxal-Induced Osteoblast Inflammation

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Expression, Cytokine Modulation, and Cell Morphology in Methylglyoxal-
Induced Osteoblast Inflammation

Teknowilie anak Singa

A thesis submitted

In fulfillment of the requirements for the degree of Master of Science

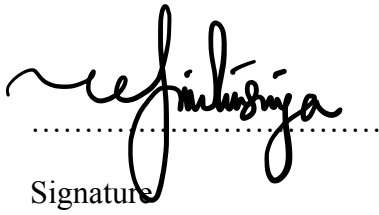
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DECLARATION

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

High blood sugar levels are the primary cause of type 2 diabetes mellitus (T2DM), a complex illness in which bone loss is a major but frequently disregarded consequences. One contributing factor is the formation of advanced glycation end products (AGE), where compounds like methylglyoxal (MGO) react with proteins, impairing their function and causing oxidative stress. This process accelerates tissue damage in uncontrolled diabetes due to prolonged high blood sugar and oxidative stress, thus contributing to diabetic bone loss. Despite *T. crispa*'s established anti-hyperglycaemic properties, its role in mitigating MGO-induced bone disorders is poorly understood. This study explored the antioxidant, anti-inflammatory, and anti-glycation properties of *T. crispa* water (TiW) and methanol (TiM) extracts, alone or combined with metformin, in MGO-stimulated hFOB 1.19 osteoblast cells. Phytochemical screening, GC-MS, FRAP, and BSA-MGO assays evaluated the extracts' active compounds and properties. Cell viability assays identified non-toxic doses (>80%) for TiW (3000 µg/mL) and TiM (476 µg/mL). Luminex assays and ELISAs measured inflammatory markers (IL-1 β , IL-6, M-CSF), RAGE components (fRAGE, sRAGE), and RANKL, while cell morphology was assessed for structural changes. Results showed that TiW (3000 µg/mL) and TiM (476 µg/mL) significantly reduced IL-1 β levels. Additionally, TiM (238 µg/mL and 476 µg/mL) combined with metformin lowered IL-1 β and IL-6 to undetectable levels, indicating synergistic effects. TiW increased sRAGE concentrations, suggesting reduced AGE binding and inflammation, while TiM exhibited varied effects on M-CSF levels. TiW combined with metformin significantly reduced fRAGE levels, supporting a synergistic effect. Morphometric analysis revealed TiM (238 µg/mL) with metformin mitigated MGO-induced cell damage, preserving cell health and structure. In conclusion, *T. crispa* extracts, particularly in combination with metformin, exhibit promising

anti-inflammatory, antioxidative, and anti-glycation properties. These findings highlight the potential of *T. crispa* in preventing T2DM-associated bone loss by modulating inflammatory pathways, reducing AGE-RAGE interactions, and preserving osteoblast integrity.

Keywords: *Tinospora crispa*, osteoblast cells, methylglyoxal, osteoclastogenesis, cell morphology

***Kajian Potensi Kesan Ekstrak Tinospora crispa terhadap Ekspresi RAGE,
Pengubahsuaian Sitokin, dan Morfologi Sel dalam Keradangan Osteoblas yang
Dirangsang oleh Metilglioksal***

ABSTRAK

Tahap gula darah yang tinggi adalah punca utama diabetes mellitus jenis 2 (T2DM), suatu penyakit kompleks di mana kehilangan tulang adalah akibat utama tetapi sering diabaikan. Salah satu punca utama kehilangan tulang ini adalah pembentukan produk akhir glikasi lanjutan (AGE). Salah satu prekursor AGE ialah metilglioksal (MGO), yang bertindak balas dengan protein, menyebabkan gangguan fungsi protein dan mendorong tekanan oksidatif. Dalam keadaan diabetes yang tidak terkawal, paras gula darah tinggi yang berterusan dan tekanan oksidatif memburukkan lagi degradasi tulang, sekali gus menyumbang kepada risiko kehilangan tulang yang lebih tinggi dalam kalangan penghidap T2DM. Walaupun T. crispa diketahui mempunyai sifat anti-hiperglisemik, peranan sediaan T. crispa dalam mencegah gangguan jisim tulang akibat MGO masih kurang difahami. Kajian ini menyelidiki sifat antioksidan, anti-radang, dan anti-glikasi ekstrak air (TiW) dan metanol (TiM) T. crispa, sama ada secara bersendirian atau digabungkan dengan metformin, dalam sel osteoblas hFOB 1.19 yang dirangsang oleh MGO. Saringan fitokimia, analisis GC-MS, ujian FRAP, dan BSA-MGO digunakan untuk menilai sebatian aktif dan sifat ekstrak. Ujian viabiliti sel mendapati TiW (3000 µg/mL) dan TiM (476 µg/mL) adalah dos yang tidak toksik (viabiliti >80%). Luminex dan ELISA mengukur penanda keradangan (IL-1β, IL-6, M-CSF), komponen RAGE (flRAGE, sRAGE), dan RANKL, manakala morfologi sel dinilai untuk perubahan struktur. Hasil menunjukkan TiW (3000 µg/mL) dan TiM (476 µg/mL) secara signifikan mengurangkan paras IL-1β. Selain itu, TiM (238 µg/mL dan 476 µg/mL) yang digabungkan dengan metformin mengurangkan IL-1β dan IL-6 ke paras tidak dapat dikesan, justeru mencadangkan potensi kesan sinergistik. TiW meningkatkan kepekatan sRAGE,

menunjukkan pengurangan pengikatan AGE dan peradangan, manakala TiM menunjukkan kesan berbeza terhadap paras M-CSF. TiW bersama metformin secara signifikan mengurangkan paras fIRAGE, ini turut menyokong potensi kesan sinergistik. Analisis morfometrik menunjukkan TiM (238 µg/mL) dengan metformin mengurangkan kerosakan sel akibat MGO, mengekalkan kesihatan dan struktur sel. Kesimpulannya, ekstrak T. crispa, terutamanya dengan metformin, menunjukkan potensi anti-radang, antioksidan, dan anti-glikasi. Penemuan ini menonjolkan potensi T. crispa dalam mencegah kehilangan tulang berkaitan T2DM dengan memodulasi laluan peradangan, mengurangkan interaksi AGE-RAGE, dan mengekalkan integriti osteoblas.

Kata kunci: Tinospora crispa, sel osteoblas, metilglioksal, osteoklastogenesis, morfologi sel

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Figure 4.9: Determination of non-toxic concentrations of metformin. hFOB 1.19 cells were seeded in 96-well plates at 3.0×10^4 cells/mL and grown to 80% confluency, typically within 24 hours. The cells were then treated with varying concentrations of metformin (mM) for 24 hours. Cytotoxicity was assessed using the Trypan Blue Dye Exclusion Test. Data are presented as mean% $\pm$ SD, $n=3$ /group. Statistical significance compared to the control is indicated by * $p < 0.05$ and ** $p < 0.005$.

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Figure 4.10: Determination of non-toxic concentrations TiM. hFOB 1.19 cells were seeded at 3.0×10^4 cells/mL in 96-well plates and grown to 80% confluency within 24 hours. The cells were then treated with various concentrations of TiM (29.75, 59.50, 119, 238, and 476 $\mu\text{g/mL}$) for 24 hours. Cytotoxicity was assessed using the MTT assay. Data are presented as mean% $\pm$ SD, $n=3$ /group. Statistical significance compared to the control is indicated by * $p < 0.05$ and ** $p < 0.005$. Note: TiM refers to *T. crispa* methanol extract.

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Figure 4.11: Determination of non-toxic concentrations of TiM. hFOB 1.19 cells were seeded in 96-well plates at a density of 3.0×10^4 cells/mL and grown to 80% confluency, typically within 24 hours. The cells were then treated with various concentrations of TiM ($\mu\text{g/mL}$) for 24 hours. Cytotoxicity was assessed using the Trypan Blue Dye Exclusion Test. Data are presented as mean% $\pm$ SD, $n=3$ /group. Statistical significance

compared to the control is indicated by * $p < 0.05$ and ** $p < 0.005$. Note: TiM = *T. crispa* methanol extract.

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Figure 4.12: Determination of non-toxic concentrations of TiW. hFOB 1.19 cells were grown at 3.0×10^4 cells/mL in 96-well plates and grown to 80% confluence within 24 hours. The cells were then treated with TiW at various concentrations (187.5, 375, 750, 1500, and 3000 $\mu\text{g/mL}$) for 24 hours. Cytotoxicity was measured using the MTT assay. Data are shown as mean% $\pm$ SD, $n=3/\text{group}$. Statistical significance compared to the control is marked as * $p < 0.05$ and ** $p < 0.005$. Note: TiW = *T. crispa* hot water extract.

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Figure 4.13: Determination of non-toxic concentrations of TiW. hFOB 1.19 cells were seeded in 96-well plates at 3.0×10^4 cells/mL and grown to 80% confluence, within 24 hours. The cells were then treated with various concentrations of TiW ($\mu\text{g/mL}$) for 24 hours. Cytotoxicity was assessed using the Trypan Blue Dye Exclusion Test. Data are presented as mean% $\pm$ SD, $n=3/\text{group}$. Statistical significance compared to the control is indicated by * $p < 0.05$ and ** $p < 0.005$. Note: TiW = *T. crispa* hot water extract.

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Figure 4.14: Effects of MGO, metformin, *T. crispa* water and methanol extracts on IL-1 β levels in hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and the hot water extract of *T. crispa*, in 6 well plates and incubated at 34°C for 24 hours. All treatments included MGO except for the control. IL-1 β levels in the supernatants were measured with Luminex Assay. Data are presented as mean $\pm$ SD, $n=2/\text{group}$. Significant differences from the MGO only treatment are marked by * $p < 0.05$, ** $p < 0.005$ and from the control by # $p < 0.05$, ## $p < 0.005$. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.15: Effects of *T. crispa* water or methanol extract alone and in combination with metformin on the expression of IL-6 in MGO-stimulated hFOB 1.19. The cells were incubated with MGO, metformin, and *T. crispa* water and methanol extracts for 24 hours. IL-6 concentrations in the supernatants were measured using the Luminex Assay. Data are presented as mean $\pm$ SD, $n=2/\text{group}$. Significant differences compared to MGO alone were marked with * $p < 0.05$, ** $p < 0.005$; compared to the control with # $p < 0.05$, ## $p < 0.005$. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.16: Effects of MGO, metformin, *T. crispa* water and methanol extracts on the concentration of M-CSF in hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and the hot water extract of *T. crispa*, in 6-well plate and incubated at 34°C for 24 hours. All treatments included MGO except for the control. M-CSF in the cell supernatants were measured using Luminex Assay. Data were presented as mean $\pm$

SD, n=2/group. Significant differences from the MGO-only treatment are marked by *p<0.05, **p<0.005, and from the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.17: Effects of MGO, metformin, *T. crispa* water and methanol extracts on the sRAGE levels in hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except for the control. Cell culture supernatants were collected and analysed for sRAGE using ELISA. Data were presented as mean ± SD, n=2/group. Significant differences from the MGO-only treatment are indicated by *p<0.05, **p<0.005, and from the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.18: Effects of MGO, metformin, *T. crispa* water and methanol extracts on fRAGE levels in hFOB 1.19 cells. The cells were treated with MGO, metformin, and *T. crispa* extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except for the control. Cell culture supernatants were collected and analysed for fRAGE levels with ELISA. Data were presented as mean ± SD, n=2/group. Significant differences from the MGO-only treatment are indicated by *p<0.05, **p<0.005, and from the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.19: Effects of MGO, metformin, *T. crispa* water and methanol extracts on RANKL levels in hFOB 1.19 cells. The hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except for the control. Cell culture supernatants were collected and analysed for RANKL concentration using ELISA. Data are presented as mean ± SD, n=2/group. Significant differences from the MGO-only treatment are indicated by *p<0.05, **p<0.005, and from the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.20: Effects of IC₅₀ MGO treatment on hFOB 1.19 cell morphology. hFOB 1.19 cells were treated with MGO (except for control) in 6 well-plates (9.0 × 10⁵ cells/mL) and incubated at 34°C for 24 hours. After incubation, the cells were observed at 20X magnification using inverted microscope in brightfield with images captured by the microscope's software. The figure shows normal, intact apoptotic and disintegrated apoptotic hFOB 1.19 as labelled.

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Figure 4.21: Effects of MGO, metformin, and *T. crispa* extracts on hFOB 1.19 cells area density. hFOB 1.19 cells were treated with MGO, metformin, and

T. crispa water and methanol extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO, except for the control. After incubation, the cells were viewed at 20X magnification using an inverted microscope, and area density was quantified. Data are presented as mean% $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.22: Effects of MGO, metformin, *T. crispa* extracts on hFOB 1.19 volume density. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except the control. After 24 hours, cells were examined at 20X magnification, and volume density was measured. Data are presented as mean% $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.23: Effects of MGO, metformin, and *T. crispa* extracts on Minimum Feret Diameter of hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except the control. Cells were examined at 20X magnification, and minimum Feret diameter was measured. Data are presented as mean $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.24: Effects of MGO, metformin, and *T. crispa* extracts on Maximum Feret Diameter of hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except the control. Cells were examined at 20X magnification, and maximum Feret diameter was measured. Data are presented as mean $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.25: Effects of MGO, metformin, and *T. crispa* extracts on Cell Perimeter of hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in

6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except for the control. Cells were viewed at 20X magnification, and the perimeter was measured. Data are presented as mean $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.26: Effects of MGO, metformin, and *T. crispa* extracts on Ellipticity Index of hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except the control. Cells were examined at 20X magnification, and the ellipticity index was measured. Data are presented as mean $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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Figure 4.27: Effects of MGO, metformin, and *T. crispa* extracts on Circularity Index of hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except the control. Cells were examined at 20X magnification, and the circularity index was measured. Data are presented as mean $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

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LIST OF ABBREVIATIONS

aFGF	Acidic fibroblast growth factor
AP-1	Activator protein 1
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
AGE	Advanced glycation end-products
ALDH	Aldehyde dehydrogenase
AKR	Aldo-keto reductases
ALP	Alkaline phosphatase
ADA	American Diabetes Association
ATCC	American Type Culture Collection
bFGF	Basic fibroblast growth factors
BSC	Biosafety cabinet
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
Ca	Calcium
CO ₂	Carbon dioxide
cells/mL	Cells per millilitre
cm ²	Centimetre square
CLCN7	Chloride channel 7 alpha subunit
cRAGE	Cleaved RAGE
3DG	3-deoxyglucosone
α -DC	α -dicarbonyl compound
DHAP	Dihydroxyacetone phosphate

DMSO	Dimethyl sulfoxide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DPP-4	Dipeptidyl peptidase-4
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
D-PBS	Dulbecco's Phosphate Buffer Saline
ELISA	Enzyme-linked Immunosorbent Assay
EPHB4	Ephrin type-B 4
EGCG	Epigallocatechin-3-gallate
Fe ²⁺	Ferrous ion
Fe ²⁺ -TPTZ	Ferrous tripyridyltriazine
Fe ³⁺	Ferric ion
Fe ³⁺ -TPTZ	Ferric tripyridyltriazine
FeCl ₃	Ferric chloride
FeSO ₄ ·7H ₂ O	Ferrous sulphate heptahydrate
FGF	Fibroblast growth factors
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FDA	Food and Drug Administration
esRAGE	Endogenous secretory RAGE
GC-MS	Gas chromatography-mass spectrophotometry
GLP-1	Glucagon-like peptide 1
G6Pase	Glucose-6-phosphatase
GLUT	Glucose transporters
α-GI	α-glucosidase inhibitor

GSH	Glutathione
G3P	Glyceraldehyde-3-phosphate
GO	Glyoxal
GLO-1	Glyoxalase 1
g	gram
HbA1c	Haemoglobin A1c
IC ₅₀	Half-maximal inhibitory concentration
hPa	hectoPascals
HMGB-1	High mobility group box 1
HPLC-MS	High-performance liquid chromatography-mass spectrometry
HCl	Hydrochloric acid
IGF	Insulin-like growth factors
IL-6	Interleukin 6
IL-1 β	Interleukin 1 beta
JAK/STAT	Janus kinase/signal transducer and activator of transcription
M-CSF	Macrophage colony stimulating factor
Mg	Magnesium
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MGO	Methylglyoxal
μ g	Microgram
μ L	Microlitre
μ m	Micrometre
mg	Milligram
mL	Millilitre

mM	Millimolar
mmol Fe ²⁺ /μg	Millimole ferrous ions per microgram
MAPK	Mitogen-activated protein kinase
nm	Nanometre
NOX4	Nicotinamide adenine dinucleotide phosphate oxidase 4
NAFLD	Non-alcoholic fatty liver disease
NFκB	Nuclear factor kappa-light-chain-enhancer of B cells
NMR	Nuclear magnetic resonance
n	Number of replicates
n/group	Number of replicates per group
OD	Optical density
OAD	Oral antidiabetic drugs
OPG	Osteoprotegerin
PTH	Parathyroid hormone
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PEPCK	Phosphoenolpyruvate carboxy kinase
pg	Picogram
PDGF	Platelet-derived growth factor
ROS	Reactive oxygen species
RANKL	Receptor activator of nuclear factor kappa-B ligand
RAGE	Receptor for advanced glycation end-products
rpm	Revolutions per minute
RNA	Ribonucleic acid
Runx2	Runt-related transcription factor 2
SGLT2	Sodium-glucose co-transporter 2

NaOH	Sodium hydroxide
S1P	Sphingosine 1-phosphate
SD	Standard deviation
H ₂ SO ₄	Sulphuric acid
TCIRG-1	T cell immune regulator 1
TiM	<i>T. crista</i> methanol
TiW	<i>T. crista</i> water
TZD	Thiazolidinedione
TGF- β	Transforming growth factor beta
TPTZ	Tripyridyl-s-triazine
TNF α	Tumour necrosis factor alpha
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
UPS	Ubiquitin-proteasome system
Wnt/ β -catenin	Wingless-related integration site/beta-catenin

CHAPTER 1

INTRODUCTION

1.1 Overview of the study

Harun and Nizam (2024) reported that the National Health and Morbidity Survey (NHMS) 2023 uncovered a significant public health issue in Malaysia, with more than 500,000 adults impacted by four primary chronic diseases: high blood sugar, elevated blood pressure, hypercholesterolemia, and excessive weight gain. Out of all these conditions, diabetes remains particularly concerning, affecting 15.6% of the adult population, equivalent to 3.6 million individuals. The survey also uncovered a substantial proportion of undiagnosed diabetes, especially among younger populations. This is alarming, as delayed diagnosis may lead to the early onset of severe health complications, placing a considerable burden on the healthcare system. These findings emphasise the urgency for improved screening, early intervention, and public health strategies to address the growing diabetes epidemic in Malaysia. Globally, diabetes affected 537 million adults in 2021, with nearly half remaining undiagnosed, predominantly with type 2 diabetes (International Diabetes Federation, 2021). Forecasts suggest that the number of cases will increase to 643 million by the year 2030 and 783 million 15 years later (in the year 2045), illustrating the growing global impact. Underprivilege and moderate-income countries are particularly affected, with more than 75% of cases occurring in these areas, which intensifies the existing healthcare challenges.

Type 2 diabetes mellitus (T2DM), commonly referred to as diabetes, is a multifactorial disorder characterised by high levels of blood glucose. Numerous factors contribute to diabetes and its associated complications, with the non-enzymatic formation of

advanced glycation end products (AGEs) being one of the significant contributors. During AGE formation, active carbonyl compounds such as methylglyoxal (MGO) react with free amino groups of proteins, leading to the impairment or deactivation of protein functions (Lai et al., 2022). These active carbonyl compounds are also a source of oxidative stress, accelerating tissue damage in uncontrolled diabetic patients due to prolonged hyperglycaemia and elevated oxidative stress. Moreover, diabetic complications extend beyond traditional concerns, with emerging research indicating their impact on bone health. While much attention has been given to cardiovascular and renal complications, the skeletal implications of diabetes are equally concerning.

AGE can be formed *in vivo* via lipid peroxidation, the polyol pathway and the enzyme-independent Maillard reaction (Ott et al., 2014). In all three reactions, AGEs are formed through the production of reactive carbonyl compounds, such as glyoxal, methylglyoxal, and 3-deoxyglucosone. AGE will accumulate in the blood, tissues and cells if the detoxification is impaired. Under conditions where detoxification is effective, these carbonyl compounds are eliminated by glyoxalase 1 and 2 enzymes. AGEs that circulate in the bloodstream bind to distinctive cell surface receptors called receptors for AGE (RAGE). RAGE is an immunoglobulin cell surface receptor that interact with multiple ligands including S100 family members, family of β sheet fibrils, amyloid β peptide, amphoterin and AGE themselves (Bucciarelli et al., 2002). When AGEs bind to RAGE, it prompts the stimulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) and the formation of cytokines such as tumour necrosis factor alpha (TNF α), interleukin (IL)-1 β and IL-6 (Ramasamy et al., 2011). Increased activation of cytokines, particularly TNF α , will disrupt bone homeostasis by promoting osteoclastogenesis and inhibiting osteoblast differentiation and proliferation, thereby impairing the synthesis of bone matrix (Ott et al.,

2014). This creates a positive feedback loop which will further activate the signalling pathways involving NF κ B, MAPKs and AP-1, ultimately leading to decreased bone formation. The initial treatment approach for patients who are newly diagnosed with T2DM involves lifestyle changes and medication such as metformin. In a clinical study by Kender and colleagues (2014), metformin demonstrated its effectiveness in reducing MGO levels in T2DM patients while concurrently increasing glyoxalase activity. In addition, animal studies have confirmed that metformin can protect diabetic animals from bone loss (Bahrambeigi et al., 2019).

Plants belonging to the genus *Tinospora* (family: Menispermaceae) are woody climbing shrubs with exstipulate alternate roundish leaves and corky barks on their stems. Among the 34 known species, *T. cordifolia*, *T. crispa* and *T. sinensis* are widely recognised for their therapeutic properties in the treatment of diabetes, jaundice, rheumatoid arthritis, fever, vomiting, anaemia, polyuria, and other ailments (Khan et al., 2016; Sharma et al., 2019). Several clinical studies have shown that the *Tinospora* species exhibit a diverse array of biological activities including anti-diabetic (Lokman et al., 2013), antioxidant (Kannadhasan and Venkataraman, 2013, Jayaprakash et al., 2015), anti-inflammatory (Philip et al., 2018, Birla et al., 2019), anti-tumorigenic (Singh et al., 2004) and anti-osteoporotic (Kapur et al., 2008) properties.

Chronic hyperglycaemia, results in heightened production of advanced glycation end-products (AGEs), which accumulate in the blood and different tissues (Chang et al., 2017). Recent studies have shown an association between AGE accumulation and diabetes-related complications such as diabetic nephropathy, diabetic neuropathy, diabetic

retinopathy, diabetic atherosclerosis, and the lesser known complication of bone fragility (Cavati et al., 2023; Sharma et al., 2012; Singh et al., 2014).

1.2 Problem statement

Diabetes is a chronic inflammatory metabolic disorder that often leads to complications, including the deterioration of bone health. The receptor for advanced glycation end-products (RAGE) plays a key role in mediating inflammation and oxidative stress in diabetes, contributing to bone-related disorders such as osteoporosis and increased fracture risk. While the relationship between Type 1 diabetes mellitus (T1DM) and osteoporosis is well established (Khan & Fraser, 2015), the impact of Type 2 diabetes mellitus (T2DM) on bone fragility remains a subject of ongoing debate. Recent studies have demonstrated a close link between T2DM and an increased risk of bone fractures, highlighting the need for effective interventions to address diabetes-associated bone complications.

Medicinal plants with anti-diabetic and anti-hyperglycaemic properties have gained significant attention for their potential to mitigate diabetes-related complications (Surya et al., 2014). *T. crispa*, in particular, has demonstrated anti-hyperglycaemic effects (Klangjareonchai, 2015), but its ability to prevent methylglyoxal-induced bone-related disorders remains poorly understood. Specifically, there is a lack of research on whether *T. crispa*, alone or in combination with metformin, can modulate RAGE expression, reduce the production of inflammatory and osteoclastogenic cytokines (such as IL-1 β , IL-6, M-CSF, and RANKL), and influence osteoblast morphology in methylglyoxal-induced osteoblast inflammation.

Given the potential anti-inflammatory and antioxidant properties of *T. crispa*, this study seeks to evaluate its efficacy in addressing diabetes-related bone inflammation. Using methylglyoxal-induced osteoblast (hFOB 1.19) cells, this research aims to investigate whether *T. crispa* can modulate RAGE expression, cytokine levels, and osteoblast morphology, thereby providing insights into its potential therapeutic role in mitigating diabetes-associated bone complications.

1.3 Research question

Can water and methanolic extracts of *T. crispa* effectively modulate methylglyoxal-induced bone inflammation associated with diabetes by regulating:

- i. RAGE expression,
- ii. Inflammatory cytokine levels, and
- iii. Osteoblast morphology?

1.4 Hypothesis

Both methanol and hot water extracts of *T. crispa* have the ability to prevent the up-regulation of the RAGE by eliminating AGE or inhibiting the induction of AGE related NFκB signalling. This inhibition subsequently prevents the production of pro-inflammatory cytokines.

1.5 General objective

The purpose of this study was to investigate whether *T. crispa* can prevent MGO-induced cellular inflammation in osteoblasts and inhibit osteoclastogenesis.

1.6 Specific objectives

- i. To determine the effect of MGO on the expression and signalling of RAGE.
- ii. To determine the expression levels of inflammatory and osteoclastogenic cytokines upon independent treatment with *T. crispa* extract and metformin in MGO-stimulated osteoblast cells.
- iii. To determine the potential synergistic effects of *T. crispa* and metformin on the expression of RAGE, inflammatory cytokines and osteoclastogenic cytokines in MGO-stimulated osteoblast cells.

CHAPTER 2

LITERATURE REVIEW

2.1 *Tinospora crispa*

Tinospora crispa, commonly known as “*akar seruntum*” or “*patawali*”, is a medicinal plant that thrives in the tropical and subtropical climate of Southeast Asia and Africa (Pathak et al., 1995). A glabrous, woody climber with succulent, green stem *T. crispa* can grow several meters long. The leaves are shaped like a heart, arranged alternately, and exhibit bright green colour. Small, yellowish-green flowers are produced, which develop into drupe-like fruits. *T. crispa* is a member of the Menispermaceae family and is renowned for its medicinal properties. Numerous species within this genus have been traditionally utilised in different systems of ethnomedicine, particularly in Ayurveda (Thomas et al., 2016) and Chinese traditional medicine (Li et al., 2006). Among the 34 species of the genus *Tinospora* (Haque et al., 2023), *T. crispa* and *T. cordifolia* are most notable for their antioxidant properties. In Malaysia, *T. crispa* has been used as folk remedy for treating fever, jaundice, diabetes, malaria, hypertension, appetite enhancement and as a mosquito's repellent (Rahman et al., 1999; Stephenson, 1931). Its stems are often boiled to make decoctions that are consumed for their health benefits (Ahmad et al., 2016). A substantial number of chemical analyses on various parts of *T. crispa*, including both the whole and its individual parts (leaves, stems and roots), have revealed 167 phytochemicals belonging to 12 different classes of phytochemicals including alkaloids, terpenoids, flavonoids, and phenolic compounds (Haque et al., 2023).

Key bioactive constituents such as columbin, syringin, palmatine and berberine have been identified (Ahmad et al., 2016). These compounds contribute to the plant's therapeutic

effects and are the focus of many pharmacological studies. The diversity of its phytochemicals underpins its wide-ranging medicinal properties. *Tinospora crispa* exhibits significant antioxidant properties, helping to neutralise harmful free-radicals in the body (Rakib et al., 2020). It can potentially show anti-inflammatory effects by lowering the levels of pro-inflammatory cytokines such as IL-1 β and IL-6. The plant has antidiabetic potential, with studies demonstrating its ability to lower blood glucose levels and improve insulin sensitivity (Zuhri et al., 2024). Additionally, its antimicrobial properties have been documented, making it effective against various bacterial and fungal infections (Mohammed et al., 2012). The antioxidant activity of *T. crispa* is primarily due to its high content of phenolic compounds, which scavenge free radicals and mitigate oxidative stress (Zulkefli et al., 2013). Its anti-inflammatory properties are achieved through the inhibition of pro-inflammatory cytokines and the modulation of signalling pathways like NF κ B (Liu et al., 2017). These mechanisms collectively contribute to the plant's broad therapeutic potential in treating chronic diseases and maintaining overall health.

Although *T. crispa* has been extensively studied for its antioxidant and antidiabetic properties, there is limited research on its effects on bone health, particularly in the context of AGE-RAGE signalling and osteoblast inflammation. Previous studies have primarily focused on its hypoglycaemic and antimicrobial effects, with little attention given to its potential role in mitigating AGE-induced bone loss. Furthermore, the mechanisms by which *T. crispa* modulates RAGE expression and cytokine production in osteoblasts remain unclear. This study aims to address these gaps by exploring the effects of *T. crispa* extracts on RAGE expression, cytokine modulation, and cell morphology in methylglyoxal (MGO)-induced osteoblast inflammation. By investigating these aspects, the study seeks to provide

new insights into the potential of *T. crispa* as a therapeutic agent for preventing AGE-related bone complications in diabetic patients.



Figure 2.1: An image of *Tinospora crispa*. The whole plant of *T. crispa* with its woody climber stems and green heart-shaped leaves. *Source:* Mazza (2019).

2.2 Other plant extracts beneficial for bone health

2.2.1 *Orthosiphon stamineus*

Orthosiphon stamineus, widely referred to as *misai kucing* in Malaysia, has been traditionally used for its diuretic and anti-inflammatory properties (Chai et al., 2013). Recent research suggests that its extracts may also have beneficial effects on bone health (Bokhari et al., 2018). *Orthosiphon stamineus* has been found to promote osteoblast proliferation and differentiation while inhibiting osteoclast activity. This dual action is mediated through the

regulation of signalling pathways involved in bone remodelling, including the RANKL/OPG pathway. Animal studies have shown that *Orthosiphon stamineus* extracts inhibit α -amylase and α -glucosidase activities, exhibit antioxidant and anti-inflammatory effects, regulate lipid metabolism, promote insulin secretion and sensitivity, enhance glucose uptake, promote glycolysis, inhibit gluconeogenesis, increase glucagon-like peptide 1 (GLP-1) secretion, and demonstrate antiglycation effects. These properties suggest its potential as a therapeutic agent for various complications of diabetes (Wang et al., 2022b).

2.2.2 *Moringa oleifera*

Known as the drumstick tree, *Moringa oleifera* is rich in vitamins, minerals, and bioactive compounds that support overall health (Islam et al., 2021). Studies have indicated that *Moringa oleifera* leaves and extracts can benefit bone health by promoting osteoblast activity and inhibiting osteoclastogenesis (Liu et al., 2021). The high calcium and phosphorus content in *Moringa*, along with its antioxidant and anti-inflammatory properties, may contribute to its bone-protective effects (Sultana, 2020; Yun et al., 2020). Research in animal models has demonstrated that *Moringa oleifera* can increase bone mineral density and improve bone strength (Hu et al., 2023; Liu et al., 2021). These properties make *Moringa oleifera* a promising natural supplement for enhancing bone health and preventing bone-related diseases.

2.2.3 Curcumin

Derived from turmeric, curcumin exhibits strong anti-inflammatory and antioxidant properties (Nicoliche et al., 2024). It has been shown to inhibit osteoclastogenesis and promote osteoblast activity (Ke et al., 2024), thereby aiding in the maintenance of bone density and strength. Curcumin achieves this by downregulating the expression of pro-

inflammatory cytokines (Gorabi et al., 2021) and transcription factors that are involved in osteoclast differentiation. Furthermore, it enhances the expression of bone morphogenetic proteins (BMPs) and runt-related transcription factor 2 (Runx2), which are critical for osteoblast differentiation and function (Pengjam et al., 2021). Animal studies have demonstrated that curcumin supplementation can prevent bone loss in models of osteoporosis and rheumatoid arthritis (Yang et al., 2023). These findings suggest that curcumin could be a valuable therapeutic agent for promoting bone health and preventing bone-related diseases.

2.2.4 *Epimedium sagittatum*

Epimedium is also known as Horny Goat Weed. Epimedium contains the phytochemical, icariin, which has been demonstrated to stimulate osteoblast proliferation and differentiation while inhibiting osteoclast activity (Long et al., 2022). This dual action makes it a potential therapeutic agent for osteoporosis. Icariin promotes bone formation by upregulating the expression of osteogenic markers such as alkaline phosphatase (ALP), osteopontin, osteocalcin, and collagen type I (Long et al., 2022; Mohammadzadeh et al., 2024). It also boosts the Wnt/ β -catenin signalling pathway, which is essential for bone metabolism (Wang et al., 2016). Research using animal models has demonstrated that icariin can significantly increase bone mineral density and improve bone microarchitecture (Liu et al., 2024). Moreover, its anti-resorptive effects help to maintain the equilibrium between bone resorption and formation, crucial for preventing bone loss in conditions like osteoporosis.

2.2.5 Green tea polyphenols

The polyphenols found in green tea, especially epigallocatechin-3-gallate (EGCG), have been found to enhance bone formation and reduce bone resorption (Chu et al., 2017). EGCG inhibits osteoclastogenesis and promotes osteoblast differentiation through the activation of the Wnt/ β -catenin signalling pathway (Lin et al., 2018). Additionally, EGCG has been shown to suppress the production of pro-inflammatory cytokines, which can otherwise contribute to bone loss (Fan et al., 2023). Animal studies have demonstrated that green tea polyphenols can increase bone mineral density and improve bone strength. Furthermore, EGCG has antioxidant properties that protect bone cells from oxidative stress, which is known to impair bone remodelling. These combined effects suggest that EGCG could be an effective natural compound for maintaining bone health and preventing osteoporosis.

2.2.6 Soy isoflavones

These phytoestrogens mimic the effects of oestrogen in the body, helping to maintain bone density, especially in postmenopausal women (Chen et al., 2015). Soy isoflavones have been shown to promote osteoblast activity and inhibit osteoclast-mediated bone resorption (Hooshier et al., 2022). They achieve this by binding to oestrogen receptors on bone cells, thereby activating signalling pathways that favour bone formation over resorption (Khalid & Krum, 2016). Clinical studies have reported that soy isoflavone supplementation can improve bone mineral density and reduce markers of bone turnover in postmenopausal women (Barańska et al., 2022). Additionally, soy isoflavones have antioxidant properties that protect bone cells from oxidative damage (Kim, 2021). This protection is crucial, as oxidative stress is a significant factor in the development of osteoporosis. These findings

indicate that soy isoflavones could be a beneficial dietary supplement for supporting bone health and reducing the risk of osteoporosis.

2.2.7 Olive oil phenols

Compounds such as oleuropein in olive oil have been reported to enhance bone formation and decrease bone resorption (García-Martínez et al., 2016). Olive oil polyphenols support osteoblast function and inhibit the formation and activity of osteoclasts. These compounds exert their effects by modulating signalling pathways involved in bone metabolism, such as the Wnt/ β -catenin pathway, which is crucial for osteoblast differentiation and activity (Iantomansi et al., 2022). Additionally, olive oil polyphenols have potent antioxidant properties, protecting bone cells from oxidative stress-induced damage (Gorzynik-Debicka et al., 2018; Puel et al., 2008). Animal studies have demonstrated that diets enriched with olive oil polyphenols can improve bone mineral density and bone strength (Chin & Ima-Nirwana, 2016). Furthermore, the anti-inflammatory properties of these polyphenols help to reduce chronic inflammation (Bucciantini et al., 2021), a known risk factor for osteoporosis and other bone-related disorders. These benefits highlight the potential of olive oil polyphenols as a dietary intervention to support bone health and prevent bone loss.

2.3 Methylglyoxal

Methylglyoxal (molecular formula: $C_3H_4O_2$) is a highly reactive organic compound that belongs to the class of α -keto aldehydes (Schalkwijk & Stehouwer, 2020). It is produced as a by-product of various metabolic pathways, particularly during the metabolism of carbohydrates such as glucose and fructose (Aragno & Mastrocola, 2017). Methylglyoxal can also be generated through lipid and protein metabolism (Allaman et al., 2015). It is a

highly reactive molecule due to its α -ketoaldehyde structure (Murata-Kamiya & Kamiya, 2001). It contains two carbonyl groups ($C=O$); a reactive aldehyde group ($-CHO$) and a ketone group ($-C(=O)-$), making it prone to reacting with various biomolecules. It can react with proteins, nucleic acids (DNA and RNA), and lipids, leading to the formation of adducts and modifications that can affect cellular function.

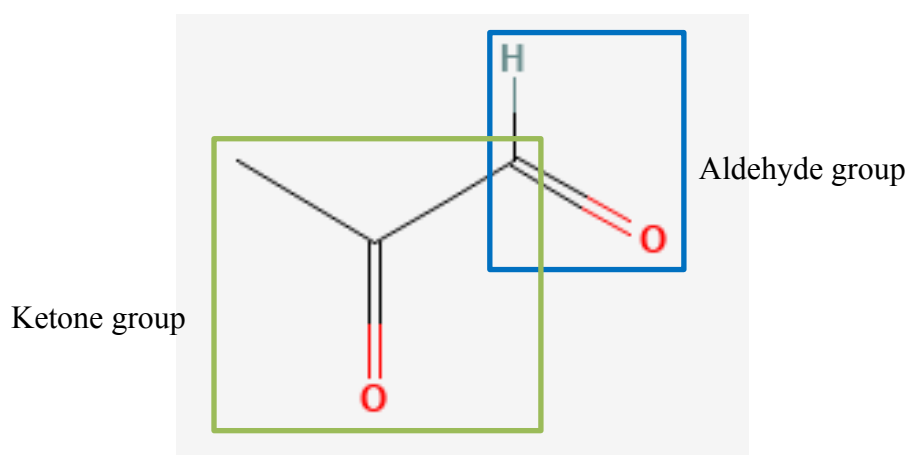


Figure 2.2: Two dimensional structure of methylglyoxal (C₃H₄O₂) illustrating the aldehyde group ($-CHO$) and the ketone group ($-C(=O)-$). The aldehyde group is located on the right side of the molecule, while the ketone group is centrally positioned, both contributing to the compound's reactivity with biomolecules. *Source:* National Center for Biotechnology Information, 2024.

Methylglyoxal can be ingested through dietary sources, particularly in foods and beverages that are high in sugars, such as honey, coffee, and processed foods (Majtan, 2011; Zheng et al., 2021). Once ingested, methylglyoxal can enter bloodstream and potentially contribute to the formation of advanced glycation endproducts (AGEs), which are associated with oxidative stress and inflammation (Twarda-Clapa et al., 2022). The human body can partially detoxify dietary methylglyoxal through the glyoxalase system by converting it into D-lactate using glutathione as a cofactor (Morgenstern et al., 2020). This process relies on the sequential actions of two enzymes, glyoxalase 1 (GLO1) and GLO2, along with reduced glutathione (GSH) (Rabbani et al., 2018). Additional detoxifying mechanisms involve the

enzymes protein deglycase DJ-1, aldehyde dehydrogenases (ALDHs), aldo-keto reductases (AKRs), and those responsible for acetoacetate degradation (Schumacher et al., 2018). Besides detoxification, our body cells also remove harmful compounds, such as carbonylated proteins, through two distinct proteolytic pathways: (1) the ubiquitin-proteasome system (UPS) and (2) the autophagy system (Taylor & Bejarano, 2022). These systems help maintain methylglyoxal levels within a tolerable range, but excessive intake may overwhelm these systems and could lead to increased formation of AGEs (Aragonès et al., 2021). As a consequence, chronic ingestion of high levels of methylglyoxal from food may contribute to the development of metabolic disorders, cardiovascular diseases, and impaired wound healing by exacerbating oxidative stress and inflammatory pathways (Berdowska et al., 2023).

2.3.1 Methylglyoxal and its link to fructose and glucose metabolism

Fructose and glucose are both simple sugars (monosaccharides) that play crucial roles in metabolism (Aragno & Mastrocola, 2017). However, their metabolic pathways differ, and excessive consumption of fructose or glucose can contribute to various metabolic diseases (Hannou et al., 2018; Merino et al., 2019). Glucose is the main energy source for the body's cells and is obtained from carbohydrates in the diet. Glucose is transported into cells via glucose transporters (GLUTs) and undergoes glycolysis, a process in which glucose is broken down into pyruvate (Zhao et al., 2008). Glycolysis occurs in the cytoplasm and generates ATP, which serves as an energy source (Alberts et al., 2002). Pyruvate produced from glycolysis then enters the mitochondria, where it is converted to acetyl-CoA and undergoes aerobic respiration. This process includes the citric acid cycle (Krebs cycle) and oxidative phosphorylation, leading to the production of additional ATP (Melkonian & Schury, 2019). Excess glucose can be converted into glycogen through glycogenesis and

stored in the liver and muscles for later use. However, chronic high intake of glucose, especially from refined sugars and processed foods, can lead to insulin resistance, obesity, type 2 diabetes, and other metabolic disorders (Stanhope, 2016).

Fructose is also absorbed into cells via transporters, primarily GLUT2 and GLUT5, and is metabolised primarily in the liver via fructokinase pathway, through which it is associated with glycolysis, gluconeogenesis, glycogenolysis, and lipid metabolism (Patterson, 2024). In the liver, fructose is converted into fructose-1-phosphate by fructokinase, which can subsequently be transformed into intermediates involved in glycolysis or the pentose phosphate pathway. Unlike glucose, fructose does not trigger insulin release since it is metabolised by the liver and the fructose transporter GLUT5 is not highly expressed in pancreatic β -cells (Khitan & Kim, 2013). High intake of fructose, especially in the form of high-fructose corn syrup (commonly used in sweetened beverages and processed foods), can lead to increased production of triglycerides (Stanhope, 2016), uric acid (Russo et al., 2020), and free radicals in the liver (Bernardes et al., 2017). This can contribute to non-alcoholic fatty liver disease (NAFLD) (Busnatu et al., 2022), insulin resistance (Basciano et al., 2005), metabolic syndrome (Aoun et al., 2022), and cardiovascular diseases (Busnatu et al., 2022).

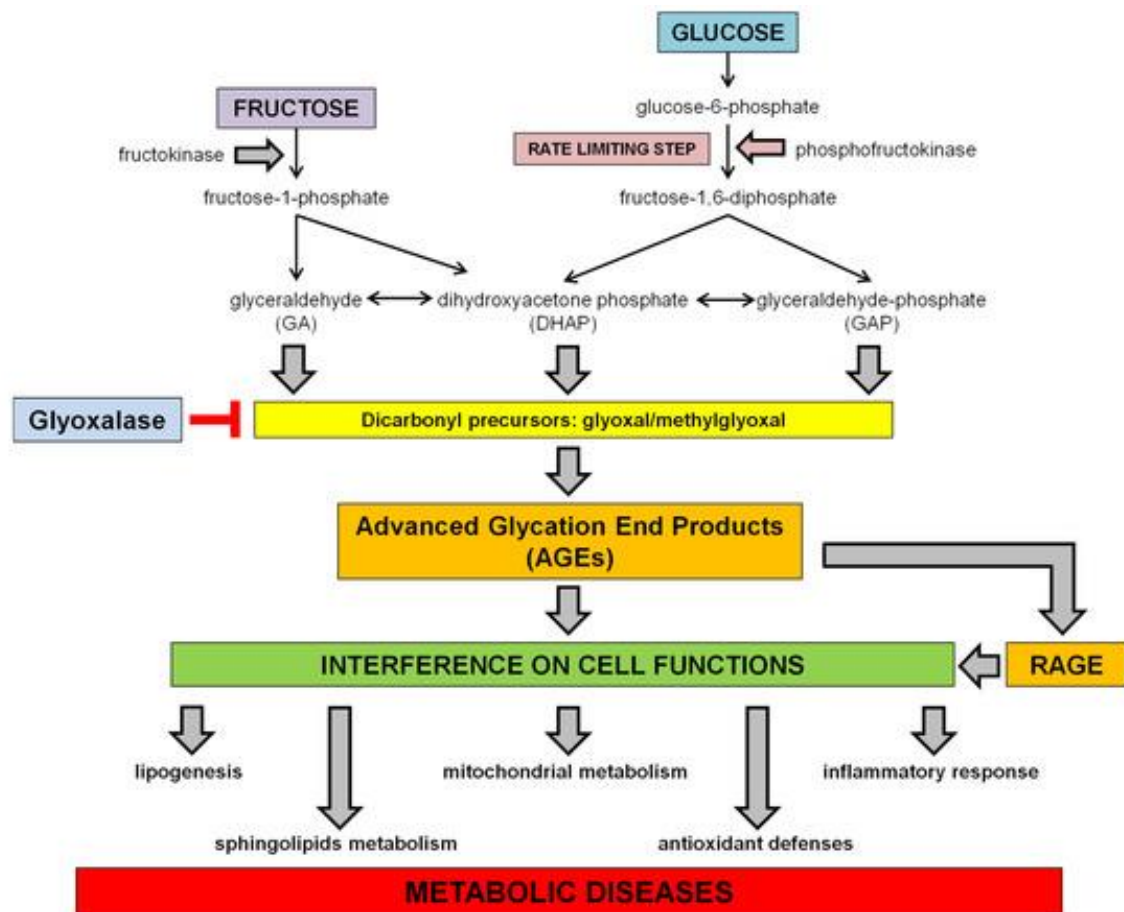


Figure 2.3:Fructose and glucose metabolism that may contribute to AGE accumulation, subsequently the onset of various metabolic diseases. *Source:* Aragno & Mastrocola (2017).

Methylglyoxal is a reactive metabolite that can be formed during glucose and fructose metabolism, particularly through the glycolytic pathway (Allaman et al., 2015). It is produced as a by-product of the degradation of glucose and fructose (Aragno & Mastrocola, 2017), and its accumulation has been linked to several metabolic diseases. This organic compound is formed through the spontaneous degradation of triose phosphates, such as dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP), which are intermediates in the glycolysis (Lai et al., 2022). Under certain conditions, especially in the presence of elevated glucose or fructose levels, the glycolytic pathway can become

overloaded, leading to increased production of triose phosphates and subsequently methylglyoxal (Iacobini et al., 2021).

Methylglyoxal has been implicated in the development and progression of various metabolic diseases, including diabetes, obesity, and cardiovascular diseases. In diabetes, methylglyoxal can contribute to the formation of AGEs, which are associated with diabetic complications such as nephropathy, neuropathy, retinopathy (Sharma et al., 2012) and the lesser-known complication, bone loss (Wu et al., 2022). Additionally, methylglyoxal can induce insulin resistance by impairing insulin signalling pathways and promoting inflammation and oxidative stress (Ng et al., 2022). In an individual with obesity and cardiovascular diseases, methylglyoxal-induced AGE formation and oxidative stress can contribute to endothelial dysfunction, atherosclerosis, and vascular complications (Nigro et al., 2017). The levels of methylglyoxal can be influenced by the metabolism of glucose and fructose (Aragno & Mastrocola, 2017). For instance, high levels of glucose or fructose can increase the production of triose phosphates, leading to elevated methylglyoxal levels (Masterjohn et al., 2013; Yan, 2014). Moreover, fructose metabolism has been specifically linked to increased methylglyoxal production due to its conversion into glyceraldehyde, a precursor of methylglyoxal (White & Xu, 2006).

2.4 Advanced glycation end-products (AGEs)

Advanced glycation end-products (AGEs) are a diverse group of molecules first identified in the early 20th century by French chemist Louis Camille Maillard (Maillard, 1912). They are proteins, nucleic acids or lipids that have been exposed to sugars and became glycated (Goldin *et al.*, 2006). This reaction is called Maillard reaction, which is a natural occurring chemical process that takes place between an ϵ amino group of an exposed amino

acid residue of a protein and an aldehyde group of a free monosaccharide such as glucose and fructose. The reaction between the afore mentioned ϵ amino group of an exposed amino acid of a protein residue such as lysine, and an aldehyde group of a free monosaccharide such as glucose, results in the formation of glucosyl-lysine. This compound then undergoes a supplementary reaction to form a reversible Schiff base (Chaudhuri et al., 2018). The formed Schiff base is an intermediate compound that is unstable and therefore undergoes structural rearrangement into a more stable, covalently bound Amadori product (Yamagishi et al., 2015).

Over the time, the Amadori products undergo a series of complex rearrangement yielding molecules called AGEs. The Amadori products can undergo two pathways: (1) oxidation, resulting in the formation of AGE, or (2) dehydration and deamination (Figure 2.4), leading to the production of highly reactive intermediates known as reactive carbonyl compounds or α -dicarbonyl compounds (α -DC), mainly methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3DG) (Al-Khateeb et al., 2018). Under prolonged hyperglycaemic conditions, the formation of these reactive carbonyl compounds increases as they react with proteins, lipids and DNA thereby generating additional AGEs (Singh et al., 2014). This process may induce intracellular oxidative stress, which is also known as carbonyl stress depending on the number of AGEs within the cells. Anaerobic glycolysis, and to a lesser extent, lipid peroxidation produces α -DCs that react indiscriminately with proteins, lipids, and DNA leading to the generation of more AGEs (Rabbani et al., 2018). MGO, one of the major precursors of AGEs, is produced by the nonenzymatic degradation of the triose phosphates, dihydroxyacetone phosphate and isomers G6P during glycolysis and is primarily due to the β -elimination of a phosphate group from the enediolate phosphate intermediate (Kieffer et al., 2014).

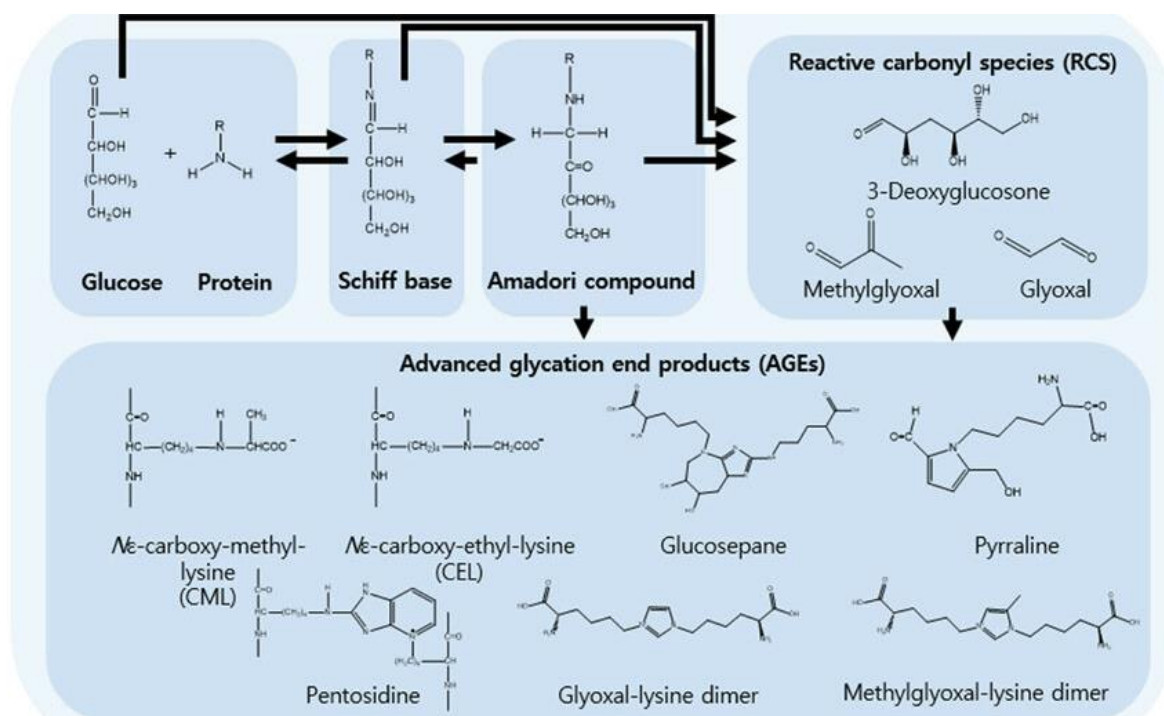


Figure 2.4: The glycation process of glucose and protein. This process lead to the formation of AGEs and reactive carbonyl species (MGO, GO & 3DG). *Source:* Cho et al. (2022).

2.5 Receptor of advanced glycation end-products (RAGE) and its functions

AGEs represent a varied group of compounds resulting from the non-enzymatic glycation of proteins, lipids, and nucleic acids (Twarda-Clapa et al., 2022). The receptor for AGE (RAGE) is a multi-ligand receptor in the immunoglobulin superfamily that is crucial for mediating the biological effects of AGEs across different cellular processes. RAGE, a transmembrane receptor, is found in various cell types, including osteoblasts, endothelial cells, monocytes, macrophages, and neurons (Ramasamy et al., 2008). The full-length RAGE interacts with diverse ligands, including AGEs, S100 proteins, and high mobility group box 1 (HMGB1) (Figure 2.6), initiating downstream signalling cascades (Bongarzone et al., 2017). Activation of RAGE triggers signalling pathways such as NF- κ B, MAPK, and janus kinase/signal transducer and activator of transcription (JAK/STAT), leading to the

transcriptional regulation of pro-inflammatory cytokines, adhesion molecules, and oxidative stress-related genes (Ramasamy et al., 2011; Younessi & Yoonessi, 2011).

2.5.1 Structure and function of RAGE

RAGE consists of an extracellular domain for ligand binding, a transmembrane domain, and an intracellular domain for signal transduction (Bongarzone et al., 2017). The full-length RAGE is a transmembrane receptor with three distinct extracellular domains: a variable (V) domain and two constant (C and C') domains (Figure 2.6) (Lee & Park, 2013). These extracellular domains are essential for binding various ligands, with the majority interacting with the V-type domain. When these ligands attach to the V-type domain, they trigger signal transduction through the intracellular cytosolic domain, leading to various cellular responses.

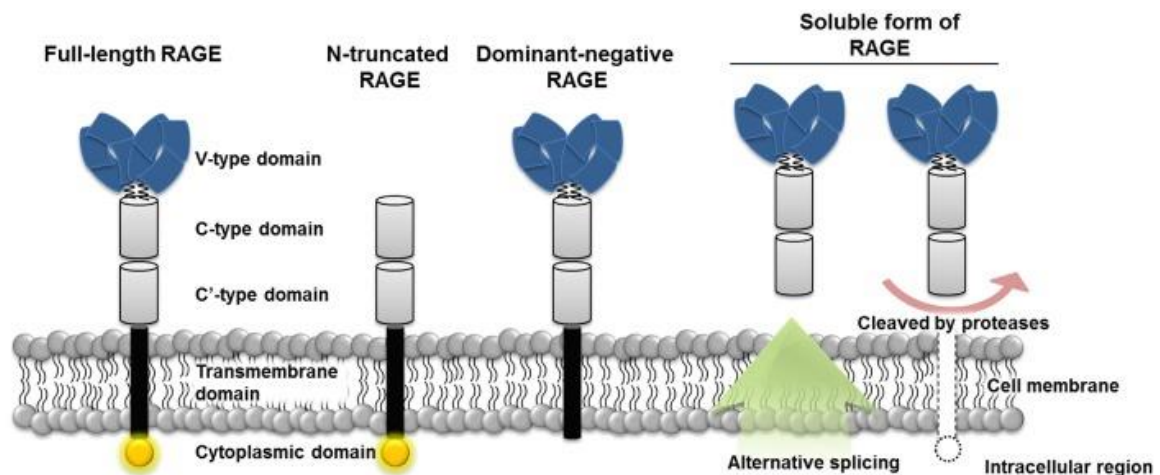


Figure 2.5: Different types of RAGE. Figure above shows the structures of full length RAGE and its three primary isoforms; N-truncated RAGE, dominant-negative RAGE and soluble form of RAGE. *Source:* Lee & Park (2013).

There are several isoforms of RAGE, each with distinct functional characteristics (Figure 2.5). The N-truncated isoform of RAGE lacks the V-type domain, which is essential for receptor-ligand interactions (Lee & Park, 2013). As a result, this isoform is unable to

bind ligands effectively. Another isoform, the dominant-negative form of RAGE, is characterised by the absence of the cytosolic domain. This form can still bind ligands but cannot transduce signals into the cell due to the lack of the intracellular domain, thereby acting as a decoy that inhibits signalling pathways.

Additionally, sRAGE exists in soluble forms and circulates in biological fluids such as plasma and cerebrospinal fluid (Sokół et al., 2017; Raucci et al., 2008). It is formed by two primary mechanisms which are alternative mRNA splicing and proteolytic cleavage of membrane-bound RAGE by protease (Bansal et al., 2023; Schmidt, 2015). Alternative mRNA splicing generates several variants, including the primary soluble isoform of RAGE, referred to as endogenous secretory RAGE (esRAGE) or RAGE_v1 (Hudson et al., 2008; Yonekura et al., 2003). Furthermore, extracellular RAGE can be cleaved from the cell surface by matrix metalloproteinases (MMPs) and A-disintegrin and metalloprotease (ADAM)-10, resulting in a form known as cleaved RAGE (cRAGE) (Raucci et al., 2008; Zhang et al., 2008).

2.5.2 Signalling pathways of RAGE

Upon binding to ligands such as AGEs, S100 proteins, or HMGB1, RAGE activates several key signalling pathways, including NF- κ B, MAPK, and JAK/STAT. These pathways regulate the expression of pro-inflammatory cytokines, adhesion molecules, and oxidative stress-related genes, playing a central role in the inflammatory response and contributing to the pathogenesis of various AGE-related diseases (Ramasamy et al., 2011; Younessi & Yoonessi, 2011).

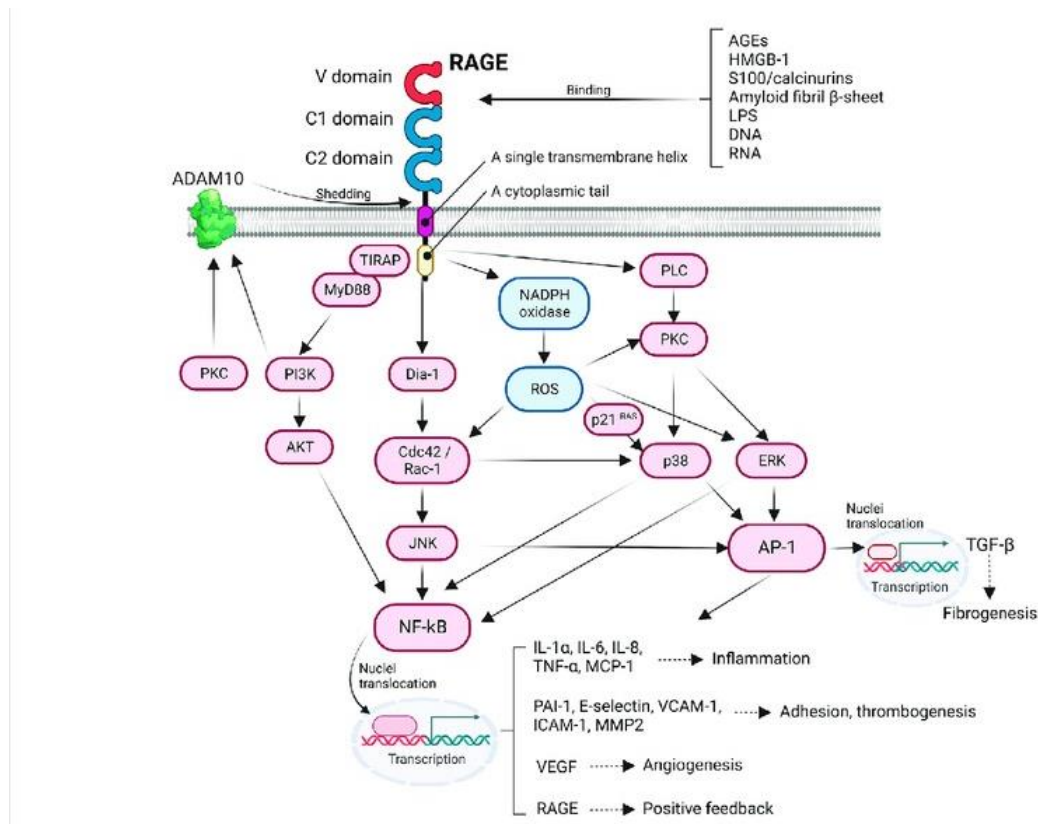


Figure 2.6: Signalling pathways of intracellular RAGE / full length RAGE. RAGE interacts with a variety of ligands, such as AGEs, HMGB-1, S100/calcinurins, and is implicated in angiogenesis, thrombogenesis, cell adhesion, and profibrotic and inflammatory responses. *Source:* Taguchi & Fukami (2023).

The canonical NF- κ B pathway is the most well-studied downstream signalling cascade activated by RAGE. Ligand binding to RAGE recruits adaptor proteins such as MyD88 and TRAF6, which activate the I κ B kinase (IKK) complex. This leads to the phosphorylation and degradation of I κ B α , an inhibitor of NF- κ B, allowing the translocation of the NF- κ B p65/p50 heterodimer into the nucleus. In the nucleus, NF- κ B induces the transcription of pro-inflammatory cytokines (e.g., IL-1 β , IL-6, TNF- α), adhesion molecules, and enzymes involved in oxidative stress (e.g., NADPH oxidase) (Ramasamy et al., 2011). The canonical NF- κ B pathway is central to the inflammatory response and contributes to endothelial dysfunction, oxidative stress, and tissue remodelling processes, which are hallmarks of chronic diseases such as diabetes and atherosclerosis (Dong et al., 2022).

In addition to the canonical pathway, RAGE activation can also trigger the non-canonical NF- κ B pathway, which is typically activated by a subset of stimuli, including certain cytokines and ligands of the TNF receptor superfamily. This pathway involves the activation of NF- κ B-inducing kinase (NIK), which phosphorylates and activates IKK α . IKK α then phosphorylates p100, leading to its proteasomal processing into p52. The p52/RelB heterodimer translocates to the nucleus, where it regulates the expression of genes involved in immune cell development, lymphoid organogenesis, and chronic inflammation (Sun, 2017). While the canonical pathway is primarily associated with acute inflammatory responses, the non-canonical pathway is often linked to chronic inflammation and adaptive immunity (Xiao et al., 2001). In the context of RAGE signalling, the non-canonical pathway may play a role in sustained inflammatory responses and tissue remodelling, particularly in chronic conditions such as diabetes and osteoporosis (Zhang et al., 2017).

Furthermore, RAGE activation stimulates the mitogen-activated protein kinase (MAPK) pathway, which includes ERK, JNK, and p38 MAPK. These kinases regulate critical cellular processes such as proliferation, differentiation, and apoptosis. Dysregulation of the MAPK pathway contributes to chronic inflammation and tissue damage, further exacerbating the pathological effects of AGEs and other RAGE ligands (Dong et al., 2022). For example, sustained activation of p38 MAPK has been linked to increased production of pro-inflammatory cytokines and oxidative stress, which are key drivers of vascular complications in diabetes (Ramasamy et al., 2011).

Another important downstream target of RAGE signalling is the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. This pathway regulates the expression of genes involved in immune responses and inflammation. Upon RAGE

activation, JAK kinases phosphorylate STAT proteins, which then dimerise and translocate to the nucleus to induce the transcription of target genes (Younessi & Yoonessi, 2011). The JAK/STAT pathway plays a critical role in mediating the effects of RAGE in inflammatory conditions, including the upregulation of cytokines and chemokines that promote tissue injury and remodelling (Dong et al., 2022).

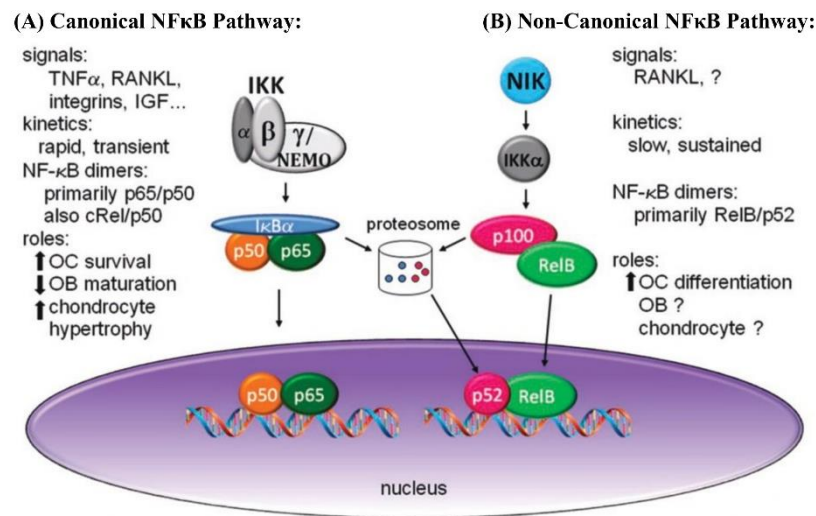


Figure 2.7: Canonical and non-canonical NFκB pathways in skeletal cells. (A) Canonical Pathway: Activated by cytokines like RANKL and TNFα, the canonical pathway involves IKKβ and IKKγ/NEMO phosphorylating IκBα, leading to its degradation. This allows rapid nuclear translocation of p65/p50 dimers, enhancing osteoclast (OC) survival, suppressing osteoblast (OB) function, and promoting chondrocyte hypertrophy. (B) Non-Canonical Pathway: Primarily activated by RANKL, this pathway is regulated by NIK, which activates IKKα to process p100 into p52. The slow, sustained nuclear translocation of RelB/p52 dimers supports osteoclast differentiation and osteoblast activity, promoting bone matrix formation. *Source:* Novack (2011).

2.5.3 Role of RAGE in inflammation and oxidative stress

The activation of RAGE by ligands play a central role in driving inflammation and oxidative stress, two key processes underlying the pathogenesis of chronic diseases. Upon ligand binding, RAGE triggers downstream signalling pathways, including NF-κB, MAPK, and JAK/STAT, which regulate the expression of pro-inflammatory cytokines, adhesion

molecules, and enzymes involved in oxidative stress (Ramasamy et al., 2011; Younessi & Yoonessi, 2011). These pathways create a pro-inflammatory microenvironment that perpetuates tissue damage and contributes to disease progression.

RAGE activation induces the production of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α , which amplify the inflammatory response and recruit immune cells to sites of tissue injury (Dong et al., 2022). For example, in endothelial cells, RAGE signalling upregulates the expression of adhesion molecules (e.g., VCAM-1 and ICAM-1), facilitating the recruitment of monocytes and neutrophils to inflamed tissues (Prasad, 2021). In macrophages, RAGE activation enhances the release of pro-inflammatory mediators, further exacerbating inflammation and tissue damage (Ray et al., 2016). This sustained inflammatory response is a hallmark of chronic diseases such as diabetes, atherosclerosis, and neurodegenerative disorders.

RAGE signaling also contributes to oxidative stress by increasing the production of reactive oxygen species (ROS). Activation of RAGE upregulates the expression of NADPH oxidase, an enzyme complex that generates superoxide anions and other ROS (Yamagishi et al., 2015). These ROS can damage cellular components, including proteins, lipids, and DNA, leading to cellular dysfunction and apoptosis. Additionally, ROS generated by RAGE signalling can further activate NF- κ B, creating a vicious cycle of inflammation and oxidative stress (Ramasamy et al., 2011). This cycle is particularly detrimental in tissues with high metabolic activity, such as the vascular endothelium, neurons, and bone, where oxidative stress contributes to endothelial dysfunction, neuronal damage, and bone loss.

The interplay between inflammation and oxidative stress is a key feature of RAGE-mediated pathology. For example, in diabetes, AGE-RAGE interaction in pancreatic β -cells

induces oxidative stress and inflammation, impairing insulin secretion and exacerbating hyperglycemia (Yamagishi et al., 2015). In atherosclerosis, RAGE activation in endothelial cells and macrophages promotes the formation of ROS and pro-inflammatory cytokines, accelerating plaque formation and instability (Prasad, 2021). Similarly, in neurodegenerative disorders, RAGE signalling in neurons and glial cells drives oxidative stress and neuroinflammation, contributing to neuronal damage and cognitive decline (Ray et al., 2016). These examples highlight the central role of RAGE in linking inflammation and oxidative stress to tissue damage in chronic diseases.

2.5.4 RAGE in osteoblast dysfunction and bone health

RAGE activation has significant implications for bone health, particularly in the context of diabetes and osteoporosis. Osteoblasts, the cells responsible for bone formation, express RAGE, and its activation by AGEs disrupts bone remodelling by promoting inflammation and oxidative stress (Wu et al., 2022). This disruption contributes to decreased bone formation, increased bone resorption, and ultimately, bone loss.

In osteoblasts, AGE-RAGE interaction triggers the activation of NF- κ B and MAPK signalling pathways, leading to the production of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α (Zhou et al., 2019). These cytokines suppress osteoblast differentiation and function by downregulating the expression of key osteogenic markers, including alkaline phosphatase (ALP), osteocalcin, and Runx2 (Wu et al., 2022). Additionally, RAGE signalling in osteoblasts upregulates the expression of RANKL, a key factor in osteoclast differentiation and activation. This promotes osteoclastogenesis and bone resorption, further disrupting bone homeostasis (Boyle et al., 2003).

The role of RAGE in osteoblast dysfunction is particularly relevant in the context of diabetes, where chronic hyperglycemia leads to the accumulation of AGEs in bone tissue. AGE-RAGE interaction in osteoblasts disrupts bone remodelling by suppressing osteoblast activity and promoting osteoclastogenesis, leading to decreased bone density and increased fracture risk (Wu et al., 2022). This process is further exacerbated by the systemic inflammation and oxidative stress associated with diabetes, which contribute to impaired bone healing and increased susceptibility to fractures (Murray & Coleman, 2019).

Targeting RAGE signalling in osteoblasts represents a potential therapeutic strategy for preventing AGE-related bone complications. For example, natural compounds with antioxidant and anti-inflammatory properties, such as those found in *Tinospora crispa*, may inhibit RAGE activation and protect osteoblasts from AGE-induced damage (Haque et al., 2023). Additionally, enhancing the levels of soluble RAGE (sRAGE), which acts as a decoy receptor, could mitigate the harmful effects of AGE-RAGE interaction in bone tissue (Basta et al., 2006). These approaches could help preserve bone health and reduce the risk of fractures in diabetic patients.

2.5.5 sRAGE as a decoy receptor

sRAGE, serves as a key mediator for mitigating the effects of AGEs and other ligands associated with inflammatory and oxidative stress-related diseases (Egaña-Gorroño et al., 2020). Unlike membrane-bound RAGE, sRAGE is secreted into the extracellular space, and serves an important modulatory role. By binding to ligands, sRAGE prevents them from interacting with membrane-bound RAGE, thereby competitively blocking the activation of deleterious effects mediated by cell-bound RAGE (Detzen et al., 2019). This inhibition reduces receptor-mediated cellular responses. sRAGE's ability to act as a decoy receptor

highlights its potential therapeutic value in conditions characterised by excessive RAGE activation.

As a decoy receptor, sRAGE sequesters circulating AGEs, attenuating their interaction with cell surface-bound RAGE (Sparvero et al., 2009). It facilitates the clearance and transport of AGEs by binding circulating AGE-protein complexes and facilitating their uptake and degradation by macrophages and other scavenger cells (Basta et al., 2006). Dysregulation of sRAGE-mediated clearance mechanisms can contribute to AGE accumulation and tissue damage in AGE-related diseases. Furthermore, sRAGE modulates inflammatory and immune responses by regulating the activation of innate immune cells, cytokine production, and leukocyte recruitment (Dong et al., 2022). The sRAGE may act as a natural antagonist, competing with AGEs for cellular receptors and thereby blocking their signalling pathways. By acting as a decoy, sRAGE may play a role in eliminating or neutralising AGEs in circulation (Basta et al., 2006).

2.6 Bone and its functions

Bone is a specialised connective tissue that serves as a dynamic, metabolically active organ with various essential functions for maintaining homeostasis in the body. It is composed of two main components: the bone matrix and cellular elements. The bone matrix consists of both organic and inorganic components. The organic part, which constitutes 20-40% of the matrix, is primarily composed of type I collagen, which provides tensile strength, and proteoglycans that contribute to the compressive strength of bone. Additionally, it includes osteocalcin and osteonectin, proteins crucial for bone mineralisation, as well as growth factors, cytokines, and osteoid that play a role in bone development and repair (Florencio-Silva et al., 2015). The inorganic component, making up 50-70% of the matrix,

consists mainly of calcium hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, which gives the bone its rigidity and hardness. Minor elements such as magnesium, potassium, zinc, and copper also contribute to the bone's structural integrity (Hauschka et al., 1989). Water and lipids, which make up 5-10% of the bone composition, are essential for cellular functions and maintaining structural integrity (Boskey, 2013).

The cellular components of bone include progenitor cells, which are precursor cells that differentiate into osteoblasts, osteoblasts themselves responsible for bone formation, osteocytes that maintain the bone matrix, and osteoclasts, which are involved in bone resorption. These cells work in concert to maintain bone health and remodelling (Clarke, 2008).

Bone serves multiple crucial functions in the body. It provides protection to soft tissues and vital organs, supports body movement by acting as a lever system for muscles, and acts as a reservoir for essential minerals such as calcium, phosphate, magnesium, and sodium. Bone is also a metabolically active tissue, playing a significant role in maintaining the balance of calcium and phosphate, which is closely linked to glucose metabolism (Florencio-Silva et al., 2015). This is particularly important in the context of diabetes, where bone health is influenced by metabolic factors. Osteocalcin, a protein produced by osteoblasts, has been shown to affect insulin secretion and sensitivity, highlighting the importance of studying bone and osteoblast function in relation to diabetes (Ferron & Lacombe, 2014).

Furthermore, bone remodelling is regulated by the balance between two key markers: RANKL and OPG (osteoprotegerin). RANKL is a protein essential for the formation, function, and survival of osteoclasts, promoting bone resorption and leading to bone loss.

OPG acts as a decoy receptor for RANKL, inhibiting its ability to bind to RANK, thereby preventing osteoclast formation and bone resorption. The balance between RANKL and OPG is crucial for maintaining healthy bone remodelling, and disruptions in this balance are associated with various bone disorders (Boyle et al., 2003).

2.7 Bone remodelling

Bone remodelling (Figure 2.7) is a dynamic process essential for maintaining skeletal integrity, regulating calcium homeostasis, and facilitating bone repair and adaptation throughout a person's life (Raggatt & Partridge, 2010). It involves the coordinated action of bone-forming osteoblasts and bone-resorbing osteoclasts, orchestrated by a complex mechanism of molecular signalling pathways, hormonal regulation, and mechanical stimuli (Al-Bari & Al Mamun, 2020). The physiological mechanisms of bone remodelling involve osteoclast-mediated bone resorption, osteoblast-mediated bone formation, and the coupling of bone formation and resorption.

2.7.1 Physiological mechanisms of bone remodelling

The bone remodelling cycle begins with mesenchymal progenitor cells, which, upon activation of the canonical Wnt/ β -catenin signalling pathway, differentiate into osteoblasts. One of the early markers of osteoblast differentiation is Runx2. In addition, osteoblasts also synthesise various extracellular matrix proteins such as ALP, type I collagen, osteocalcin, osteopontin, and osteonectin. Osteoblasts also synthesise and mineralise new bone matrix through the deposition of extracellular matrix proteins, such as collagen fibres and calcium phosphate crystals (Blair et al., 2017). Bone formation is regulated by various growth factors, including BMPs, transforming growth factor β (TGF- β), insulin-like growth factors I and II (IGF-I and IGF-II), platelet-derived growth factor (PDGF), and fibroblast growth factors

(FGFs) including both basic (bFGF) and acidic (aFGF). These factors stimulate osteoblast proliferation, differentiation, and matrix synthesis (Solheim, 1998).

As osteoblasts mature, they take on additional roles, including the secretion of RANKL, which promotes the differentiation of osteoclast precursors into fully developed osteoclasts. Osteoblasts additionally release OPG, a decoy receptor that binds to RANKL, blocking its interaction with the receptor on osteoclast precursors and thereby inhibiting their differentiation into osteoclasts. Moreover, osteoblasts produce M-CSF, which also plays a role in osteoclast differentiation. Osteoclasts, the primary cells responsible for bone resorption, are multinucleated and originate from hematopoietic progenitors. They differentiate into osteoclasts upon exposure to M-CSF and RANKL which is expressed by osteoblasts (Kodama & Kaito, 2020). RANKL binds to its receptor RANK, which is mainly expressed on cells of the monocyte/macrophage lineage, including osteoclast precursors, B and T cells, dendritic cells, and fibroblasts (Takegahara et al., 2022). RANK is also found on the surface of mature osteoclasts. This interaction initiates signalling pathways that facilitate osteoclast differentiation, activation, and survival (Sylvester, 2017). Mature osteoclasts adhere to bone surfaces and secrete acid and proteolytic enzymes, leading to the dissolution of the mineralised bone matrix and the release of calcium and phosphate ions into the extracellular space (Arnett, 2021).

During resorption, osteoclasts release signalling molecules such as TGF- β and IGFs, which influence osteoblast activity. Additionally, osteoclasts express ephrin B2 ligand and sphingosine 1-phosphate (S1P), which interact with the ephrin type-B 4 (EPHB4) receptor on osteoblasts. This interaction promotes osteoblast differentiation and concurrently suppresses osteoclast differentiation. The intricate interplay between osteoclasts and

osteoblasts ensures a coordinated cycle of bone resorption and formation, maintaining bone homeostasis. This complex regulation highlights the close connection between the activities of these two cell types, ensuring the maintenance of healthy bone tissue. The insights into these mechanisms, as detailed by Shyu et al. (2021), provide a comprehensive understanding of the cellular and molecular processes involved in bone remodelling.

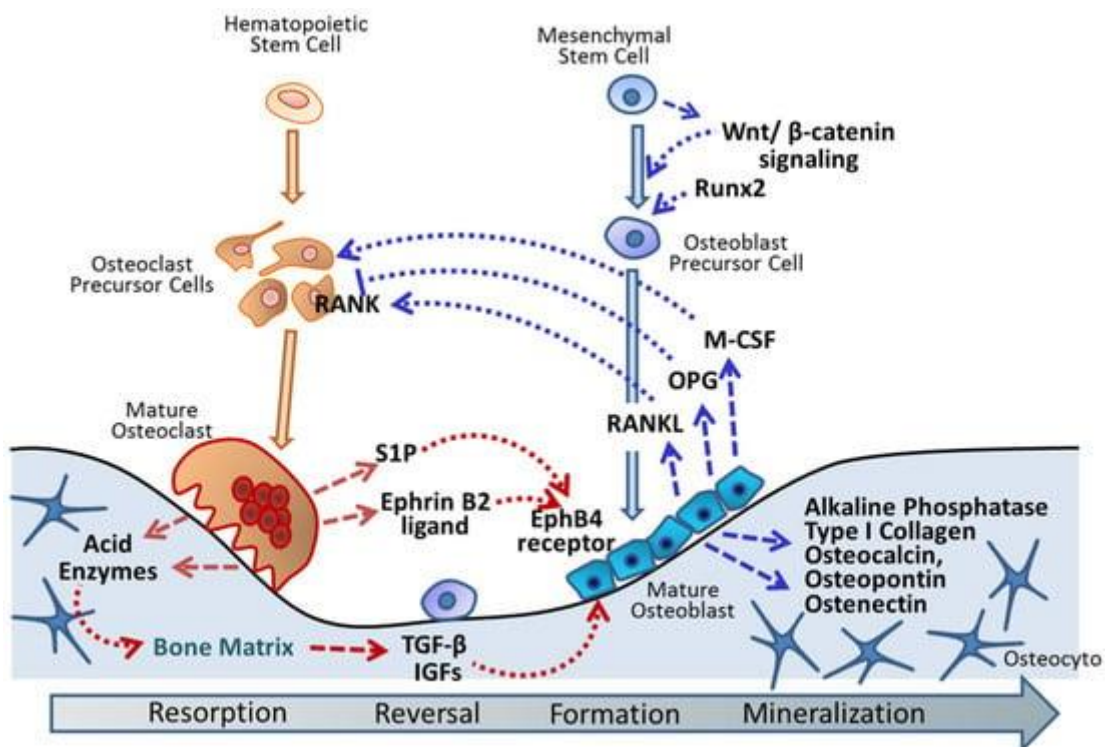


Figure 2.8: Bone remodelling cycle. The bone remodelling cycle aims to maintain normal bone mass through a dynamic equilibrium between resorption and formation. *Source:* Shyu et al. (2021).

2.7.2 Regulation of blood calcium levels

The parathyroid glands secrete parathyroid hormone (PTH), regulates blood calcium levels (Lofrese et al., 2023). In response to low blood calcium levels, PTH is released. PTH targets the kidneys, intestines, and skeleton in an effort to raise calcium levels (Khan et al., 2022). PTH binds to its receptor on osteoblasts in the skeleton, causing these cells to upregulate RANKL. RANKL then binds to RANK on osteoclasts activating signalling

pathways that promote osteoclast differentiation. This process results in bone resorption and the release of calcium into the bloodstream, while simultaneously inhibiting osteoblast activity to reduce calcium deposition in bones (Lofrese et al., 2023). In the kidneys, PTH enhances calcium reabsorption, and it indirectly increases calcium absorption in the intestines by promoting the formation of active calcitriol in the kidneys. Calcitriol, the active form of vitamin D, facilitates dietary calcium uptake. PTH release is suppressed by rising blood calcium levels (Lofrese et al., 2023). The action of PTH is counteracted by calcitonin, where calcitonin inhibits osteoclast function and promotes calcium deposition in bone (Hsiao et al., 2020). Mechanical stimuli, such as weight-bearing exercise and gravitational forces, also exert profound effects on bone remodelling by stimulating osteoblast activity and suppressing osteoclast-mediated bone resorption (Wang et al., 2022a). Mechano-transduction pathways involving Wnt signalling, focal adhesion kinase (FAK), and BMPs mediate the cellular response to mechanical loading and regulate bone remodelling in response to changes in mechanical stress (Robling & Turner, 2009).

2.8 Drugs associated with the treatment of diabetes

2.8.1 Metformin as first line treatment

Metformin is widely regarded as the first-line pharmacological treatment for T2DM due to its well-established efficacy, safety profile, and beneficial effects on glucose metabolism (Andraos et al., 2024). The primary mechanism through which metformin exerts its antidiabetic effects is by reducing hepatic gluconeogenesis, which in turn lowers fasting blood glucose levels. (Zhu et al., 2023) Additionally, metformin improves insulin sensitivity by enhancing peripheral glucose uptake and utilisation, primarily via the activation of AMP-activated protein kinase (AMPK), a crucial enzyme in cellular energy homeostasis (Zhu et al., 2023). The management of diabetes mellitus, particularly type 2 diabetes, often involves

the use of various pharmacological agents to achieve optimal glycaemic control. Although metformin is the cornerstone of T2DM treatment, other classes of medications are also available and may be used either independently or in combination with other medications to help achieve optimal glycaemic control.

2.8.2 Other diabetes-related drugs

2.8.2.1 Sulfonylureas

Sulfonylureas, such as glipizide, glyburide, and glimepiride, have been extensively applied in the intervention of type 2 diabetes (Costello et al., 2023). These agents function by stimulating pancreatic β -cells to secrete insulin. Studies have shown that sulfonylureas can significantly reduce haemoglobin A1c (HbA1c) levels by 1-2% when used as single-drug therapy or in combination with other antidiabetic drugs (Xu & Rajaratnam, 2017). However, the use of sulfonylureas is often correlated with a risk of hypoglycaemia and weight gain (Douros et al., 2018).

2.8.2.2 Thiazolidinediones

Thiazolidinediones (TZDs), including pioglitazone and rosiglitazone, improve insulin sensitivity by activating peroxisome proliferator-activated receptor gamma (PPAR- γ) in adipose tissue, muscle, and the liver (Eggleton & Jialal, 2023). Clinical trials have demonstrated that TZDs can reduce HbA1c levels by approximately 1-1.5% (Xu & Rajaratnam, 2017). Despite their efficacy, TZDs have been associated with negative effects such as fluid retention and weight gain (Ko et al., 2017), and increased risk of bone fractures, particularly in women (Colhoun et al., 2012). In 2010, due to an increased risk of cardiovascular events linked to its use, the European Medicines Agency recommended suspending rosiglitazone, and the US Food and Drug Administration (FDA) significantly restricted its use (Nicholson & Hall, 2011). As a result, pioglitazone remains the only

thiazolidinedione widely used for treating T2DM and is the sole molecule of this class included in the 2012 Position Statement by the American Diabetes Association and the European Association for the Study of Diabetes.

2.8.2.3 Dipeptidyl Peptidase-4 Inhibitors

Dipeptidyl peptidase-4 (DPP-4) inhibitors, such as sitagliptin, saxagliptin, alogliptin and linagliptin are a class of oral antidiabetic drugs (OADs) approved by FDA for the treatment of diabetes mellitus in adults (Kasina & Baradhi, 2023). These agents improve the incretin effect by blocking the enzyme DPP-4, which breaks down incretin hormones (Deacon, 2019). These hormones increase insulin secretion and decrease glucagon levels in a glucose-dependent manner. DPP-4 inhibitors typically reduce HbA1c by 0.74% (Xu & Rajaratnam, 2017). They are generally well-tolerated with good safety profile, with concerns about potential adverse events typically focusing on heart failure (Zannad & Rossignol, 2019), bullous pemphigoid (Liu et al., 2020), pancreatitis (Lee et al., 2019), and severe joint pain (Men et al., 2017).

2.8.2.4 Glucagon-like Peptide-1 Receptor Agonists

Glucagon-like peptide-1 (GLP-1) receptor agonists, including exenatide, liraglutide, and dulaglutide, mimic the action of endogenous GLP-1 to enhance glucose-dependent insulin secretion, inhibit glucagon release, and slow gastric emptying (Mariam & Niazi, 2024). Clinical trials have shown that GLP-1 receptor agonists can reduce HbA1c levels by 1-1.5% and promote weight loss (Zhang et al., 2023). However, they may cause gastrointestinal side effects such as nausea and vomiting (Filippatos et al., 2014), and there are concerns about their potential association with pancreatitis and thyroid tumours (Elashoff et al., 2011).

2.8.2.5 Sodium-Glucose Co-Transporter 2 Inhibitors

Sodium-glucose co-transporter 2 (SGLT2) inhibitors, such as canagliflozin, dapagliflozin, and empagliflozin, lower blood glucose by inhibiting glucose reabsorption in the renal proximal tubules, lead to an increase in glucose excretion in the urine (Ni et al., 2020). SGLT2 inhibitors have been shown to reduce HbA1c by 0.5-1% and are associated with weight loss and a reduction in blood pressure (Ferrannini et al., 2010). Nonetheless, their use can increase the risk of urinary tract infections, genital mycotic infections, and, in rare cases, diabetic ketoacidosis (Gupta et al., 2017; Hazique et al., 2023).

2.8.2.6 α -Glucosidase Inhibitors

α -Glucosidase inhibitors (α -GIs), such as acarbose and miglitol, delay carbohydrate absorption in the intestines, thereby reducing postprandial hyperglycemia (Ueno et al., 2015). These agents typically lower HbA1c by 0.5-0.8% (Wettergreen et al., 2016). Gastrointestinal side effects, including flatulence and diarrhoea, are common with alpha-glucosidase inhibitors, which may limit their use (Dirir et al., 2022).

2.8.2.7 Meglitinides

Meglitinides, such as repaglinide and nateglinide, stimulate rapid, short-term insulin secretion from pancreatic β -cell (Guardado-Mendoza et al., 2013). Both of these agents are valuable for treating T2DM, particularly when used in combination therapy due to their proposed mechanism of action. Similar to sulfonylureas, the insulinotropic effect of repaglinide is mediated through ATP-dependent potassium channels (Guardado-Mendoza et al., 2013). They are particularly effective in reducing postprandial glucose levels and lower HbA1c by 0.5-1% (Inzucchi et al., 2015). The primary side effect of meglitinides is hypoglycaemia, though it is generally less severe compared to sulfonylureas (Inzucchi et al., 2015).

2.8.3 Mechanisms of anti-AGE compounds in inhibiting AGE formation

The build-up of AGEs is closely associated with oxidative stress, inflammation, and the progression of chronic diseases such as diabetes, cardiovascular disease, and neurodegenerative disorders (Byun et al., 2017). Therefore, preventing the formation of AGEs has become a key focus in managing these conditions. Anti-AGE compounds, particularly those derived from natural sources, have shown great potential in reducing AGE formation and its harmful effects. Anti-AGE compounds may act by interfering with the pathways that lead to the formation of AGEs, thereby reducing their accumulation in tissues and preventing the progression of diabetes complications.

One of the key ways in which anti-AGE compounds work is by scavenging reactive carbonyl species (RCS), which are important intermediates in the formation of AGEs. Natural compounds like polyphenols can trap these reactive molecules such as glyoxal (GO), methylglyoxal (MGO), and 3-deoxyglucosone (3-DG), before they have a chance to interact with proteins (Song et al., 2021). For example, curcumin and phloretin are known to form adducts with RCS, effectively blocking AGE formation at an early stage (Alizaedeh & Kheirouri, 2019; Zhou et al., 2019). This approach plays a critical role in preventing the crosslinking of proteins, which is a major contributor to AGE accumulation.

Another important mechanism involves the antioxidant properties of many anti-AGE compounds, which help to reduce oxidative stress. Oxidative stress is a significant factor in glycation, as it promotes the oxidative breakdown of Amadori products, leading to AGEs (Ott et al., 2014). Natural antioxidants such as vitamins C and E, along with polyphenols, are effective in reducing ROS (reactive oxygen species) levels, thereby inhibiting the glycation process (Alizaedeh & Kheirouri, 2019; Grzebyk & Piwowar, 2016). These

antioxidants also help dampen the activity of RAGE (receptor for AGEs), which is known to trigger inflammation and further damage.

In addition, some anti-AGE compounds act by chelating metal ions, which play a role in catalysing glycation reactions. Metal ions like Fe^{2+} and Cu^{2+} can speed up AGE formation by driving oxidative reactions (Song et al., 2021). Natural compounds such as catechins are effective at binding to these metal ions, thereby preventing them from facilitating the oxidation of proteins (Wu et al., 2020). By inhibiting this catalytic activity, these compounds can significantly reduce AGE formation.

Certain natural inhibitors also function by blocking glycation sites on proteins. Compounds like ferulic acid and isoferulic acid compete with reducing sugars for binding sites on proteins (Arfin et al., 2018; Liu et al., 2018), effectively preventing the formation of early glycation products like Schiff bases and Amadori compounds. This mechanism helps maintain protein structure and slows down the overall glycation process.

Lastly, some anti-AGE compounds work by targeting enzymes that play a role in the glycation pathway, such as aldose reductase. Aldose reductase facilitates the conversion of glucose to fructose, a sugar that is far more reactive in glycation (Song et al., 2021). By inhibiting this enzyme, these compounds can reduce the production of fructose, indirectly lowering the rate of AGE formation (Rai et al., 2019). This offers another avenue for reducing the harmful effects of glycation in the body.

Despite the promising effects of these natural compounds, their clinical use is often limited by issues like low bioavailability and rapid metabolism. Nonetheless, ongoing research into their molecular structures provides a valuable basis for developing more effective AGE inhibitors that could have broader therapeutic applications in the future.

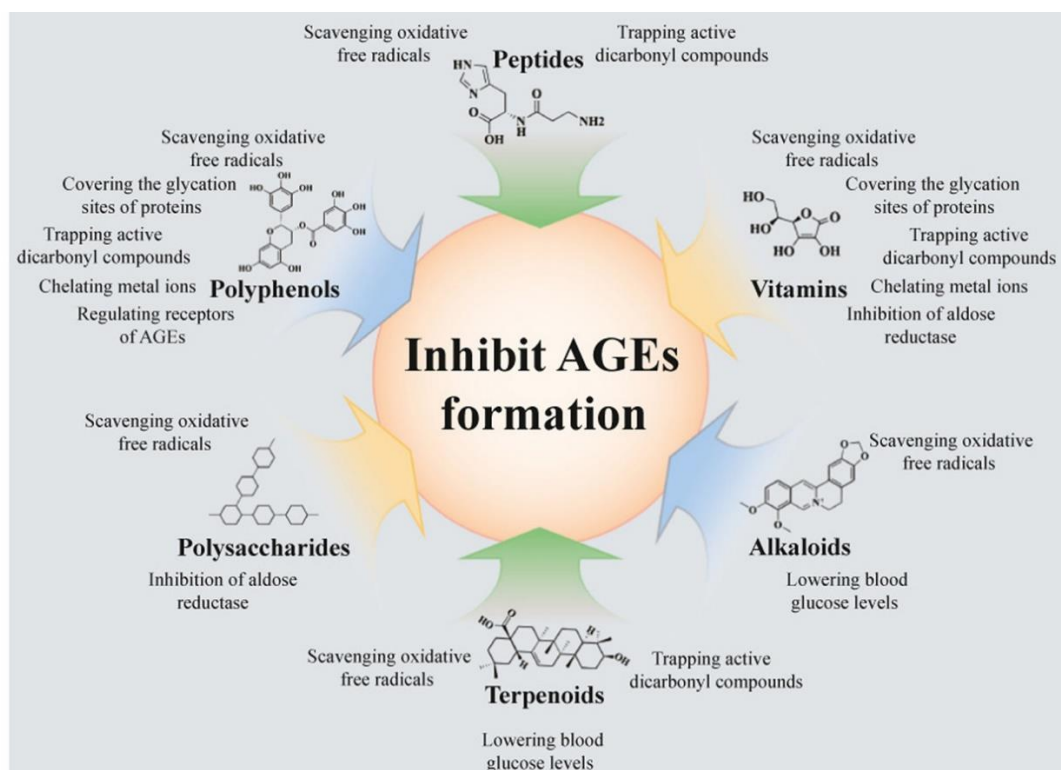


Figure 2.9: Schematic representation of the mechanisms by which various bioactive compounds inhibit the formation of advanced glycation end-products (AGEs). The diagram illustrates the different classes of compounds; peptides, polyphenols, vitamins, polysaccharides, terpenoids, and alkaloids and their key actions. These include scavenging oxidative free radicals, trapping active dicarbonyl compounds, chelating metal ions, covering glycation sites on proteins, and inhibiting enzymes such as aldose reductase, all of which contribute to the prevention of AGE formation and accumulation. *Source:* Song et al. (2021).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemical Reagents

All of the reagents used were of analytical grade. The reagents were diluted using deionised ultra-filtered water, obtained from the ultrapure water system from ELGA Purelab Medica R-7/15 (UK). All of the chemical reagents used for the experiments were listed in Table 3.1 below:

Table 3.1: List of chemical reagents used in the experiments.

No.	Chemical Reagent	Manufacturer
1.	Acetic acid	Merck, Germany
2.	Accutase cell detachment solution	Corning, USA
3.	Aminoguanidine	Sigma-Aldrich, USA
4.	Amphotericin B ($\text{CH}_{47}\text{H}_{73}\text{NO}_{17}$)	Corning, USA
5.	Bovine serum albumin (BSA)	Sigma-Aldrich, USA
6.	Cell culture flasks	Falcon, Corning, USA
7.	Centrifuge tubes	Falcon, Corning, USA
8.	Chloroform (CHCl_3)	Merck, Germany
9.	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) kit	R&D Systems, USA
10.	Dragendorff's reagent	Merck, Germany
11.	Dulbecco's Modification of Eagle's Medium (DMEM)/Ham's F-12 50/50 mix with L-glutamine and 15mM HEPES	Corning, USA
12.	Dulbecco's Phosphate Buffer Saline (D-PBS)	Corning, USA
13.	Ferric chloride (FeCl_3)	Merck, Germany
14.	Ferrous sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	Sigma-Aldrich, USA
15.	Filter paper	Whatman™, UK

Table 3.1: continued

16.	Human ELISA kit for fLRAGE	R&D Systems, USA
17.	Human ELISA kit for RANKL	Abcam, UK
18.	Human ELISA kit for sRAGE	Abcam, UK
19.	Human Magnetic Luminex Assay for IL-1 β , IL-6 and M-CSF	R&D Systems, USA
20.	Metformin hydrochloride	R&D Systems, USA
21.	Methanol (CH ₃ OH)	Merck, Germany
22.	Methylglyoxal (C ₃ H ₄ O ₂)	Sigma-Aldrich, USA
23.	Microcentrifuge tubes	Axygen, USA
24.	Penicillin-streptomycin solution	Corning, USA
25.	Pipette tips	Yiwu, China
26.	Serological pipettes	Trueline, USA
27.	Sodium hydroxide (NaOH)	Merck, Germany
28.	Sulphuric acid (H ₂ SO ₄)	Merck, Germany
29.	<i>T. crista</i> powder	Shine Tech Solutions, Pahang, Malaysia
30.	Transfected foetal osteoblast cells, hFOB 1.19	American Type Culture Collection (ATCC), USA
31.	2,4,6-tripyridyl-s-triazine (TPTZ)	Sigma-Aldrich, USA
32.	Trypan blue solution	Sigma-Aldrich, USA
33.	Well plates	Falcon, Corning, USA

3.1.2 Equipment

All of the equipment used for the experiments were listed in Table 3.2:

Table 3.2: List of equipments used in the experiments.

No.	Equipment	Manufacturer
1.	BPX-5 fused-silica capillary column	Trajan Scientific and Medical, Australia
2.	CellDrop automated cell counter machine	DeNovix, USA
3.	Class II Type 2 Biological Safety Cabinet	ECSO, Japan

Table 3.2: continued

4.	ECLIPSE Ti2 inverted research microscope	Nikon, Japan
5.	Forma Direct Heat CO2 incubator	Thermo Scientific, USA
6.	FreeZone 2.5 freeze dryer	Labconco, USA
7.	GCMS-QP2010 Plus	Shimadzu, Japan
8.	Heraeus Fresco 21 centrifuge machine	Thermo Scientific, USA
9.	Liquid nitrogen storage tank	Thermo Scientific, USA
10.	Micropipette	Eppendorf, Germany
11.	MS104S analytical balance	Mettler Toledo, USA
12.	Multichannel pipette	Gilson, USA
13.	MultiWash microplate washer	Molecular Devices, USA
14.	N-1300 rotary evaporator	Eyela, Japan
15.	Orbital shaker-incubator	Biosan, Latvia
16.	S1 pipet fillers	Thermo Scientific, USA
17.	S911 centrifuge machine	Kubota, Japan
18.	SpectraMax iD3 microplate reader	Molecular Devices, USA
19.	Tomy SS-325 autoclave machine	Tomy, Japan
20.	Ultrapure water purification system	Elga LabWater, UK
21.	Vortex mixer	Velp Scientifica, Italy
22.	Water bath	JeioTech, South Korea

3.2 Flow chart of experimental design

Experimental design for the evaluation of the effects of *T. crispa* extracts on the expression and signalling for the receptors for advanced glycation end-products in methylglyoxal-stimulated osteoblast cells was shown in Figure 3.1 below:

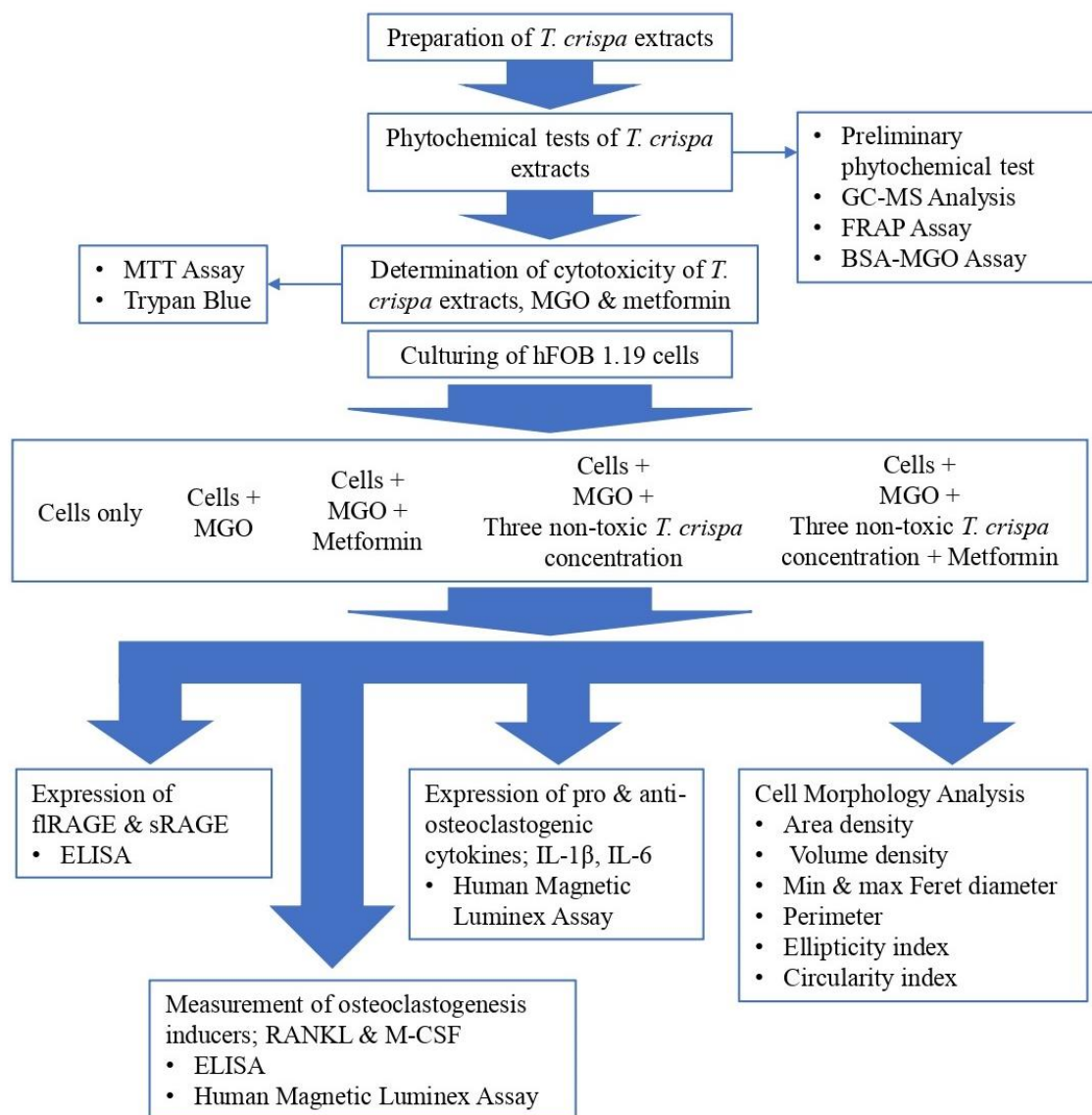


Figure 3.1: Experimental design for the evaluation of the effects of *T. crispa* extracts on the expression and signalling for the receptors for advanced glycation end-products in methylglyoxal-stimulated osteoblast cells.

3.3 Extraction of *T. crispa*

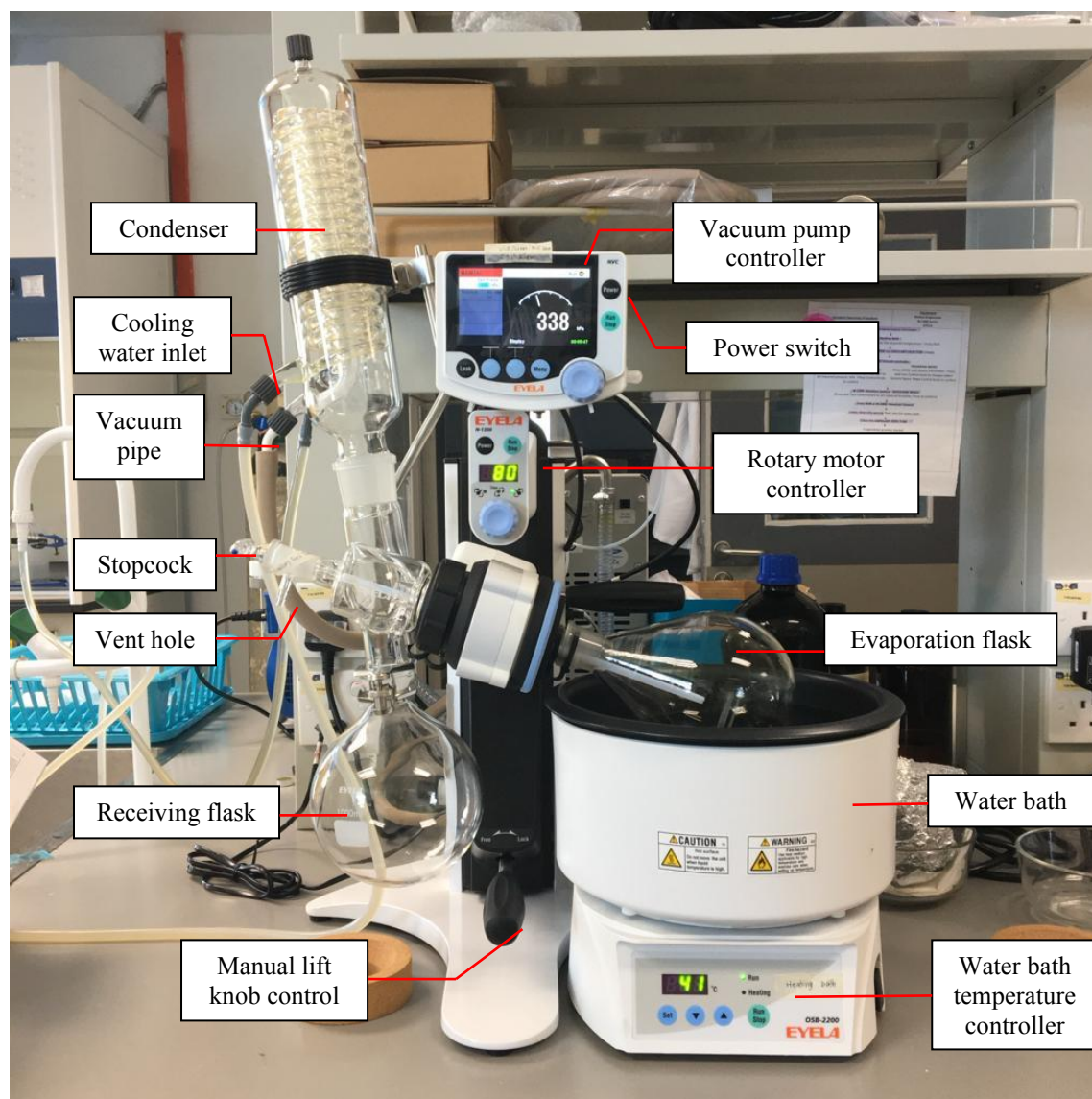


Figure 3.2: *T. crispa* hot water and methanol extracts were concentrated by using N-1300 rotary evaporator (Eyela, Japan).

Commercially available powdered *T. crispa* whole plant (100% pure patawali powder, batch number: 201026) was purchased from Shine Tech Solutions, Pahang, Malaysia. Five grams of *T. crispa* plant powder was macerated in either 100 mL of hot boiled water or 90% methanol for 24 hours at room temperature. To shield the extracts from light exposure during the maceration process, the beakers were covered with aluminium foil and kept in a dark cabinet. The solutions were then filtered using Whatman grade 1 filter paper

(pore size: 11 μm). The filtrate obtained from the filtration was then concentrated by using a rotary evaporator (Figure 3.1). The temperature of the water bath was set at 40°C while the pressure of the rotary evaporator was set at 73 $\times$ hPa for water filtrate and 332 $\times$ hPa for methanol filtrate (pressure was set according to the user manual of the rotary evaporator).

Prior to the freeze-drying process, the concentrated water and methanol filtrates were frozen at -20°C for 24 hours or overnight. The frozen concentrated water and methanol filtrates were then freeze-dried with a freeze dryer (Figure 3.2) at -50°C for a range of 3-7 days until all the moistures were eliminated. The sample tubes or the bottles containing the water and methanol filtrates were kept from direct sunlight throughout the freeze-drying process by wrapping them with aluminium foil to conserve photosensitive compounds. After completed, the freeze-dried extracts were stored at -20°C to -80°C to preserve the extracts for long term usage.

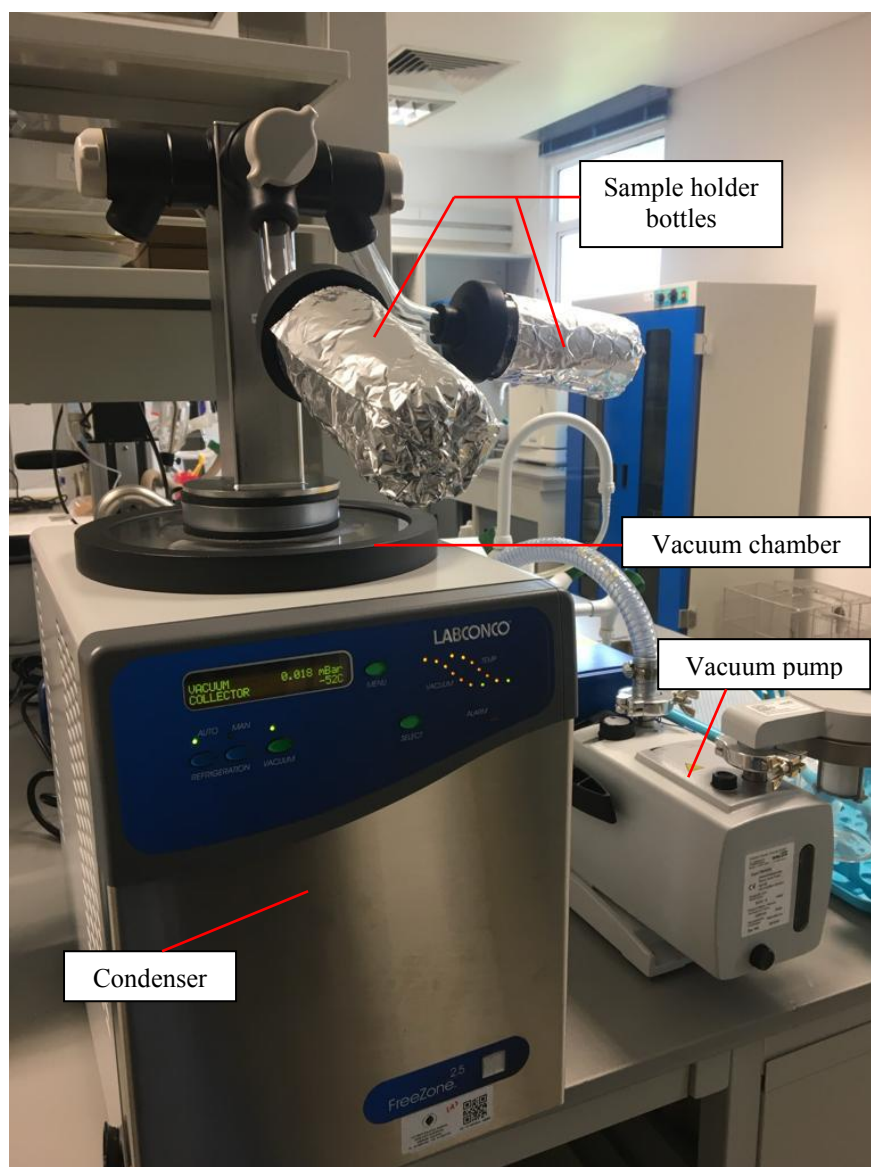


Figure 3.3: The frozen concentrated *T. crisp*a water and methanol extracts were freeze-dried with FreeZone 2.5 freeze dryer (Labconco, USA).

3.4 Culturing of human foetal osteoblast (hFOB) 1.19 cell culture

The hFOB 1.19 cells were purchased from the ATCC (American Type Culture Collection) after material transfer agreement form was signed (Appendix C). The patent number was 5,681,701. According to the product sheet, hFOB 1.19 cells (ATCC CRL-11372) is an osteoblast cell line derived from limb tissue of a spontaneous miscarriage of a human foetus (*Homo sapiens*). To establish this cell line, the temperature-sensitive expression vector pUCSVtsA58 and the neomycin resistance vector pSV2-neo were

introduced into the osteoblasts through transfection. The transfection of these expression vector which contains a mutant of the SV40 large T antigen, contributed to its immortalisation by allowing the cells to proliferate indefinitely. Clones were then selected using 0.6 mg/mL of G418.

3.4.1 Reviving the hFOB 1.19 cells

The protocol to revive the cryopreserved hFOB 1.19 was provided by the ATCC and was slightly modified. The cryovial containing the cryopreserved hFOB 1.19 cells was retrieved from the nitrogen tank. The frozen hFOB 1.19 cells in the cryovial were thawed by gentle agitation in a 37°C-water bath. Once a small chunk of ice was left in the vial, the vial was sprayed with 70% ethanol before it was being transferred to the aseptic ECSO Class II Type 2 Biological Safety Cabinet (BSC). The cell suspension from the cryovial was transferred into a centrifuge tube containing 9 mL of complete culture medium and was spin at 125 x g for 5 to 7 minutes. The complete culture medium consisted of DMEM/Ham's F-12 media Corning, 10% of FBS, 1% of pen-strep, and 0.5% of amphotericin B. The cell pellet was resuspended with 1 mL of complete medium and dispensed into 25 mL Falcon blue vented cell culture flask containing 4 mL complete medium, to make up a total of 5 mL complete medium in 25 mL culture flask. Before adding the contents of the vial and resuspending the cell pellet, the growth medium was placed in the 5% CO₂ incubator (Forma Direct Heat CO₂ Incubator, Thermo Scientific, USA) for at least 15 minutes to allow the medium to reach its optimal pH. The cell culture flask was capped. North, east, south, and west motion were used to evenly distribute the cells. The flask was then placed in the incubator with 5% of CO₂, temperature of 34°C (optimal growth temperature for hFOB1.19 cells) and 95% humidity. The cells were then allowed to grow until there was 70-80% of cell confluency before being passaged.

Although the ATCC's instructions manual did not specify the passage number of the hFOB 1.19 cells after their revival from liquid nitrogen, the cells were generally regarded as passage 2, since the vial they came in with was considered as passage 1. The first subculture of hFOB 1.19 cells would then be regarded as passage 3. The passage number indicates how many times a cell line has been transferred from one vessel to another. Increasing evidence in the literature shows that passage number significantly impacts a cell line's characteristics over time. Higher passage numbers can lead to changes in cell morphology, response to stimuli, expression of protein, growth rates, and transfection efficiency compared to cells at lower passage numbers. Marozin et al. (2021) categorised passage 5-7 as low passages, passage 10-12 as intermediate passages, while passage 18-20 were classified as higher passages. In this study, passage 10 of hFOB 1.19 were selected for various cell analysis, such as the cell morphology analysis, cytotoxicity tests, ELISA, and Luminex assays.

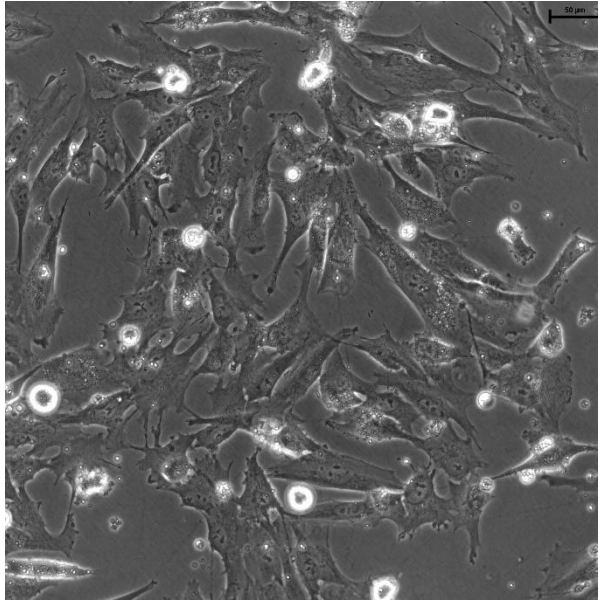


Figure 3.4: hFOB 1.19 cells at 80% confluency viewed at 20X with Nikon Inverted Research Microscope ECLIPSE Ti2.

3.4.2 Passaging the hFOB 1.19 cells (ATCC CRL-11372)

The protocol to passage the hFOB 1.19 (ATCC CRL-11372) cell culture was provided by the ATCC, and was slightly modified. Spent media were removed from the flask and discarded. The cell layers were gently washed with D-PBS (without Ca and Mg) by pipetting the D-PBS into the flask and gently swirling the flask for a few seconds. The D-PBS was then removed and discarded from the flask. Undiluted Accutase cell detachment solution was added to the cell culture flask at 1 mL per 25 cm² of the flask surface area. The cell culture flask was then placed in the CO₂ incubator with a temperature of 34°C for a maximum of 5 minutes. The flask was taken out of the CO₂ incubator and examined under an inverted microscope for the presence of floating, rounded, highly refractile cell bodies. After the cells have been detached from the flask, the Accutase solution containing the hFOB 1.19 cells were transferred into a Falcon centrifuge tube containing 9 mL of complete medium and centrifuged at 250 × g for 5 minutes to pellet the cells. The supernatant was removed and discarded. The cell pellet was resuspended with 1.5 mL media. 100 μL of cell

suspension were removed for cell counting with the Trypan blue exclusion test. The remaining 1.4 mL media were distributed into a few 25 cm² cell culture flasks according to the sub-cultivation ratio recommended by ATCC, which is 1:4. The cell culture flasks were capped. North, east, south, and west motion were used to evenly distribute cells. The flasks were then placed in the incubator with 5% of CO₂, temperature of 34°C and 95% humidity.

3.4.3 Freezing the hFOB 1.19 (ATCC CRL-11372)

Once the cells were detached from the flask, the Accutase solution with the hFOB 1.19 cells was transferred to a centrifuge tube and centrifuged at 250 × g for 5 minutes to form a cell pellet. The supernatant was then removed and discarded. The cell pellet was resuspended in 1.1 mL of cryopreservation reagent; consisted of DMEM/Ham's F-12 media, 7% FBS and 8% dimethyl sulfoxide (DMSO), prepared according to the ATCC's manual. Then, 100 µL of cell suspension were removed for cell counting with the Trypan blue exclusion test. After the cell counting, the number of hFOB 1.19 cells per mL were labelled on the cryovial. The remaining 1 mL of the freezing media was transferred to the cryovial and put in the 4°C fridge for 15 minutes before it was frozen in -20°C freezer for an hour. Subsequently after an hour, the cryovial was moved to a -80°C freezer for overnight or 24 hours before it was relocated into a nitrogen tank for long term storage.

3.5 Trypan blue exclusion test: Cytotoxicity assessment of MGO, metformin and *T. crispata* extracts

The Trypan Blue Exclusion Test was used to assess cell viability by counting the number of viable cells. Live cells, which have an intact membrane, exclude the dye, while dead cells, which lack membrane integrity, take up the dye (Strober, 2015). To determine the cytotoxicity of MGO, the concentrations used in this study were 0.22, 0.44, 0.88, and 1.75 mM. The concentrations of metformin used to determine its cytotoxicity were 3, 6, 12,

and 24 mM. On the other hand, the concentrations of *T. crispa* hot water extract used to test its cytotoxicity were 187.5, 375, 750, 1500, and 3000 µg/mL, while the concentrations of *T. crispa* methanol extract used were 29.7, 59.4, 118.75, 237.5, and 475 µg/mL. These concentrations were chosen in this study as a result of trial runs. Same range of concentrations of MGO, metformin and *T. crispa* extracts were used in the cytotoxicity test with MTT assay (section 3.4). Firstly, 1:1 ratio of cell suspension and Trypan blue dye solution were mixed. The number and percentage of viable and dead cells were automatically counted by the DeNovix CellDrop automated cell counter machine using the formula below (Equation 3.1). The mixture was loaded onto the sample chamber and read three separate times with fresh mixture to obtain triplicate readings (n=3). Percentage viability at 80% and above were considered as healthy (Seegeritz et al., 2017).

Percentage of viability

Equation 3.1

$$= \frac{\text{Total cell count} - \text{Total dead cells}}{\text{Total cell count}} \times 100$$

3.6 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: Cytotoxicity assessment of MGO, metformin and *T. crispa* extracts

The utilisation of the MTT reagent was done by following the user manual provided by the manufacturer. A ratio of 1:10 of MTT reagent solution and D-PBS were added into each well, with triplicate (three wells) for each concentration used (n=3). The plate was then incubated for 2-3 hours. The supernatants were then removed after the incubation, and 100µL of detergent solution was added into each well to solubilise the formazan crystals. The plate was shaken with an orbital shaker for 5 minutes, spin at $250 \times g$ at 4°C for 5 minutes, and then read with SpectraMax iD3 microplate reader (Molecular Devices, USA)

at 570 nm. The percentage of viability for all MGO, metformin and *T. crista* extracts concentrations were calculated with the following formula (Equation 3.2).

$$\text{Percentage of viability} = \frac{\text{Absorbance treated}}{\text{Absorbance control}} \times 100 \quad \text{Equation 3.2}$$

3.7 Preliminary phytochemical analysis of *T. crista* extracts

For the saponins detection test (Das et al., 2014), 5 mL of distilled water was added to 2 mL of extract in a test tube. The tube was then shaken vigorously for about 10 minutes. Froth formation indicates the presence of saponins. For the flavonoid's detection test (Gul et al., 2017), 2 mL of 2% sodium hydroxide (NaOH) was added to the extract. Once the mixture showed a bright colour, 10% sulphuric acid (H₂SO₄) was added to the mixture. If the mixture became colourless after the addition of H₂SO₄, it indicated the presence of flavonoids. For the glycoside's detection test (Gul et al., 2017), 2 mL of acetic acid and 2 mL of chloroform were added to 1 mL of the extract in a test tube. H₂SO₄ was added to the mixture. A green colour indicated the presence of glycosides. For the alkaloid's detection test (Das et al., 2014), 1 mL of Dragendorff's reagent was added to 2 mL of the extract in a test tube. An orange to brown precipitate indicated the presence of alkaloids. For the terpenoids detection test (Shah & Yadav, 2015), 2 mL of chloroform was added to 5 mL of the extract in a test tube. 3 mL of H₂SO₄ was carefully added to the side of the test tube to form a layer. A reddish-brown colour indicated the presence of terpenoids. For the phenol's detection test (Shah & Yadav, 2015), 2 mL of 1% ferric chloride (FeCl₃) was added to 1 mL of the extract in a test tube. A range of colours from red-brown to violet-green indicated the presence of phenols.

3.8 Gas chromatography-mass spectrometry (GC-MS) analysis of *T. crisper* extracts

The GC–MS analysis was conducted using a Shimadzu GCMS-QP2010 Plus (Shimadzu, Japan), following the methods outlined by Samling et al (2021). A BPX-5 fused-silica capillary column (Trajan Scientific and Medical, Australia) with 5% phenyl polysilphenylene-siloxane, 30 m × 0.25 mm i.d., and a film thickness of 0.25 µm was utilized. The initial oven temperature was set at 40°C for 1 minute, then ramped to 220°C at a rate of 5 °C/min and held for 10 minutes. The interface temperature was set at 250°C, and electron impact ionization energy of 70 eV was applied with a scan mass range of 28–400 m/z. The chemical constituents of *T. crisper* water and methanol extracts were identified by comparing their mass spectra with those in the NIST-17 mass spectral library incorporated in the data system to determine their name, molecular weight, and structure. Additionally, experimental retention indices (RIs) were compared with those published in the literature (Adams, 2007).

3.9 Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reduction power for the extracts was measured using FRAP assay, adapted from Samiullah et. al (2017) with slight modifications. The FRAP reagent was prepared by the addition of 20 mL of 300 mM acetate buffer (pH 3.6) with 2 mL of TPTZ (10 mM in 40 mM HCl) and 2 mL of 20 mM FeCl₃ solution. FeSO₄·7H₂O was used as calibration and prepared in different concentrations in mg/mL (0.025, 0.050, 0.100, 0.200 and 0.400). The extracts were prepared according to the following concentrations in mg/mL (0.050, 0.100, 0.200 and 0.400). Three hundred microliters of extracts and 600 mL of distilled water were added to 1200 µL of FRAP reagent. The reaction mixture then was incubated for 10 minutes at 37°C and the absorbance of samples was measured at 593 nm. The same procedure was repeated for FeSO₄·7H₂O to get the values for standard curve. The standard curve for

FeSO₄·7H₂O was plotted and FRAP values were expressed in mg/mL FeSO₄·7H₂O per mg of the extracts.

3.10 Human Magnetic Luminex Multiplex Assay of IL-1 β , IL-6 and M-CSF

3.10.1 Preparation of samples

In this study, hFOB 1.19 cell culture supernatant samples were used to quantify IL-1 β , IL-6 and M-CSF, according to the instructions provided by R&D Systems (USA). The culture supernatant was collected and centrifuged at 1000 x g for 15 minutes at 4 °C. The resulting supernatant was then transferred into a new tube. The sample was assayed immediately or aliquoted and stored at $\leq$ -20 °C. Each tube was sealed with parafilm to prevent sample evaporation or leakage during storage, and repeated freeze-thaw cycles were avoided.

3.10.2 Preparation of reagents

For the preparation of wash buffer, the vial was gently warmed to room temperature and mixed until any crystals in the wash buffer concentrate were fully dissolved. To prepare 500 mL of wash buffer, 20 mL of wash buffer concentrate was added to 480 mL of deionized water.

Each unique Standard Cocktail provided in the kit was reconstituted with Calibrator Diluent RD6-52 and allowed to sit with gentle agitation for at least 15 minutes before making dilutions. Upon reconstitution, each standard cocktail was a 10X concentrate.

To prepare Standard 1, 100 μ L of each reconstituted standard cocktail was combined with Calibrator Diluent RD6-52 in a tube, achieving a final volume of 1000 μ L. Next, 200 μ L of Calibrator Diluent RD6-52 was added to each of five labeled test tubes (2-6). Standard

1 was used to create a 3-fold dilution series, with each tube being thoroughly mixed before the next transfer. Standard 1 acted as the high standard, while Calibrator Diluent RD6-52 was used as the blank.

To prepare the Diluted Microparticle Cocktail, the Microparticle Cocktail vial was centrifuged at 1000 x g for 30 seconds before removing the cap. The vial was then gently vortexed to resuspend the microparticles, being careful not to invert the vial. Subsequently, 500 µL of Microparticle Cocktail was diluted with 5 mL of Diluent RD2-1, providing enough for 96 wells as per the mixing bottle instructions. Following this, 55 µL of the microparticle concentrate was added to 5.5 mL of the diluted microparticles, again for 96 wells, according to the manufacturer's instructions.

For the Biotin-Antibody dilution, the Biotin-Antibody Cocktail vial was centrifuged at 1000 x g for 30 seconds before opening. The vial was then gently vortexed, avoiding inversion. Next, 500 µL of the Biotin-Antibody Cocktail was diluted in 5 mL of Diluent RD2-1, sufficient for 96 wells, as per the provided instructions. The mixture was then gently mixed.

For preparing the Streptavidin-PE, a polypropylene amber bottle or a polypropylene test tube wrapped in aluminium foil was used to protect the Streptavidin-PE from light. The Streptavidin-PE vial was centrifuged at 1000 x g for 30 seconds before removing the cap. The vial was then gently vortexed, taking care not to invert it. Next, 220 µL of Streptavidin-PE concentrate was diluted in 5.35 mL of Wash Buffer, providing enough for 96 wells, in accordance with the manufacturer's guidelines.

3.10.3 Assay procedure

Before use, all reagents and samples were equilibrated to room temperature. It was advised to run all standards and samples in duplicate. Microparticles and Streptavidin-PE were kept protected from light throughout the process. All preparation of reagents, standards, and samples was done according to the specified protocols.

Initially, 50 μ L of each standard or sample was added to the wells, and a plate layout was used to document the standards and samples being tested. The diluted Microparticle Cocktail was gently resuspended by inversion or vortexing, and then 50 μ L of this cocktail was added to each well of the microplate. The plate was then covered with a foil plate sealer and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker with a 0.12" orbit, set to 800 $\pm$ 50 rpm.

For washing, a magnetic device designed for microplates was used. The magnet was placed at the bottom of the microplate for 1 minute to attract the microparticles before the liquid was removed. Each well was then filled with 100 μ L of Wash Buffer, allowed to sit for 1 minute, and the liquid was removed again. Ensuring complete liquid removal was crucial for optimal performance. Note: Blotting was avoided to prevent loss of microparticles. The washing procedure was repeated three times.

3.11 Enzyme-Linked Immunosorbent Assay (ELISA) for full-length RAGE

3.11.1 Preparation of samples

In this study, hFOB 1.19 cell culture lysate samples were obtained to quantify membrane-bound RAGE, following the instructions provided by R&D Systems (USA). The samples were first centrifuged at 16000 x g for 4 minutes to remove the debris, then

aliquoted. After aliquoting, the samples were stored at $\leq -20^{\circ}\text{C}$ to preserve the samples for the ELISA assay, with precautions taken to avoid repeated freeze-thaw cycles.

3.11.2 Preparation of reagents

The Substrate Solution was made by combining equal volumes of Colour Reagents A and B shortly before use, within a 15-minute window. The solution was kept away from light. For each well, 200 μL of the prepared solution was used. Human RAGE Standards were created by dissolving the lyophilised Human RAGE standard in deionised water to produce a stock solution of 50,000 pg/mL. The standard was mixed thoroughly to ensure complete dissolution and was allowed to sit for at least 15 minutes with gentle agitation before proceeding with dilution.

To prepare the dilution series, Calibrator Diluent RD5-5 was added to the 5,000 pg/mL tube first, and then 500 μL of the diluent was pipetted into each of the remaining tubes. The stock solution was used to prepare a dilution series with concentrations of 5,000 pg/mL, 2,500 pg/mL, 1,250 pg/mL, 625 pg/mL, 313 pg/mL, 156 pg/mL, and 78 pg/mL. Each dilution was mixed thoroughly before moving on to the next. The 5,000 pg/mL standard was used as the high standard, and Calibrator Diluent RD5-5 served as the zero standard (0 pg/mL).

3.11.3 Assay procedure

Before use, all reagents and samples were brought to room temperature, as per manufacturer's instructions. Duplicate assays were conducted for all standards, controls, and samples. Following the guidelines outlined in the preceding sections, all necessary reagents and working standards were prepared.

Initially, 100 μ L of Assay Diluent RD1-60 was added to each well. Following this, 50 μ L of the standard, control, or sample was introduced into each well. The plate was then sealed with the provided adhesive strip and incubated for 2 hours at room temperature according to Bio-technne's guidelines. After the incubation period, the liquid was removed from each well, and the wells were washed by adding 400 μ L of Wash Buffer using the automated MultiWash Microplate Washer (Molecular Devices, USA). This washing procedure was performed three times, totaling four washes. It was crucial to completely remove the liquid at each step for optimal performance. After the final wash, any residual Wash Buffer was removed by aspirating or decanting, and the plate was inverted and blotted against clean paper towels.

Next, 200 μ L of Human RAGE conjugate was added to each well. The plate was then covered with a new adhesive strip and incubated for 2 hours at room temperature as instructed by the manufacturer. The aspiration and washing steps described earlier were repeated.

Following this, 200 μ L of Substrate Solution was added to each well, and the plate was incubated for 30 minutes at room temperature, protected from light. Finally, 50 μ L of Stop Solution was added to each well, and the color change from blue to yellow was observed. If any wells showed a green color or uneven color change, the plate was gently tapped to ensure uniform mixing. The absorbance of each well was then measured within 30 minutes using the SpectraMax iD3 microplate reader (Molecular Devices, USA) set to 450 nm.

3.12 ELISA for soluble RAGE

3.12.1 Preparation of samples

In this study, hFOB 1.19 cell culture supernatant samples were obtained to quantify soluble RAGE, according to the instructions provided by Abcam (UK). The cell culture media was centrifuged at 2000 x g for 10 minutes to eliminate debris. Supernatants were collected, and assays were conducted according to the provided guidelines. Undiluted samples were stored at -20°C or below, while repeated freeze-thaw cycles were avoided, as suggested by the manufacturer.

3.12.2 Preparation of reagents

Prior to use, all reagents were allowed to reach room temperature (18-25°C) in accordance with the manufacturer's guidelines. The kit included enough reagents for 96 wells. The recommended sample volumes were adequate for 48 wells (6 x 8-well strips), with adjustments made as necessary based on the number of strips used in the experiment.

The Sample Diluent 50BS might have contained precipitate, which was considered normal according to the manufacturer's guidelines. If gentle mixing did not dissolve the precipitate, it was dissolved by warming and mixing it gently at 37°C for 10 minutes. If any precipitate persisted, it was carefully spun down, and care was taken to avoid pipetting visible precipitates.

Reagents were prepared only as needed on the day of the experiment, adhering to the manufacturer's guidelines. The stability of the Capture and Detector Antibodies was verified solely in their provided 10X concentrations.

A 1X Wash Buffer PT solution was made by diluting 10X Wash Buffer PT with deionised water according to the manufacturer's instructions. To prepare 50 mL of 1X Wash Buffer PT, 5 mL of 10X Wash Buffer PT was combined with 45 mL of deionised water, and the solution was mixed thoroughly and gently.

The Antibody Cocktail was prepared by diluting the capture and detector antibodies in Antibody Diluent 5BI, following Abcam's instructions. For a 3 mL Antibody Cocktail, 300 μ L of 10X Capture Antibody and 300 μ L of 10X Detector Antibody were mixed with 2.4 mL of Antibody Diluent 5BI, and the mixture was stirred gently and thoroughly.

3.12.3 Assay procedure

Reagents, working standards, and samples were prepared according to the guidelines specified by the manufacturer. Excess microplate strips were removed from the plate frame, placed back into the foil pouch with the desiccant pack, resealed to maintain their quality, and stored at 4°C as instructed.

Fifty μ L of each sample or standard was added to the designated wells, followed by 50 μ L of the Antibody Cocktail according to the manufacturer's protocol. The plate was then sealed and incubated for 1 hour at room temperature on a plate shaker set to 400 rpm. Following incubation, each well was washed three times with 350 μ L of 1X Wash Buffer PT. During each wash, the liquid was aspirated or decanted, and 350 μ L of fresh 1X Wash Buffer PT was added to each well. The buffer was left in the wells for at least 10 seconds during each wash to ensure complete removal of residuals, which is crucial for accurate results. After the final wash, the plate was inverted and gently tapped against clean paper towels to remove any remaining liquid.

Next, 100 μ L of TMB Development Solution was added to each well, and the plate was incubated in the dark for 10 minutes on a plate shaker set to 400 rpm, as per the manufacturer's instructions. After the incubation, 100 μ L of Stop Solution was added to each well, and the plate was shaken on a plate shaker for 1 minute to mix thoroughly. The optical density (OD) at 450 nm was then measured as the endpoint of the assay..

3.13 ELISA for RANKL

3.13.1 Preparation of samples

In this study, hFOB 1.19 cell culture supernatant samples were obtained to quantify RANKL, according to the instructions provided by Abcam (UK). Samples designated for assay were stored at 4°C within 24 hours. To prepare samples for assay, particulates were removed through centrifugation at 2000 x g for 10 minutes, and samples were either assayed immediately or aliquoted and stored at -20°C for extended storage.

3.13.2 Preparation of reagents

All reagents were brought to 37°C before use, as recommended by Abcam. Although the assay could be performed at room temperature, conducting it at 37°C was advised for optimal consistency. Additionally, the TMB incubation time estimate (15-25 minutes) was also estimated based on this temperature. The kit was designed for 96 wells, and only the necessary amount of reagent was prepared on the day of the experiment. The RANKL standard solution was prepared no more than two hours before the experiment, in line with the manufacturer's instructions. Each kit included two tubes of RANKL standard (2 x 10 ng), with one tube used for each experiment. To prepare the stock solution, 1 mL of sample diluent buffer was added to one tube creating a 10000 pg/mL Human RANKL stock solution. The tube was kept at room temperature for 10 minutes and mixed thoroughly.

The biotinylated Anti-Human RANKL antibody solution was also prepared no more than 2 hours before the experiment. The total volume was calculated as 100 μ L per well, multiplied by the number of wells, with an additional 100-200 μ L prepared to ensure there was enough for the entire assay. The antibody solution was diluted at a 1:100 ratio with the antibody diluent buffer and mixed thoroughly (e.g., 1 μ L Biotinylated Anti-Human RANKL antibody to 99 μ L antibody diluent buffer).

Before the use, the tubes containing Avidin-Biotin-Peroxidase Complex (ABC) were briefly centrifuged to ensure all contents were not trapped in the lid or sticking to the tube wall. The ABC solution was prepared no more than 1 hour before the experiment with a total volume calculated similarly to the antibody solution. The ABC solution was diluted at a 1:100 ratio with the ABC dilution buffer and mixed thoroughly (e.g., 1 μ L ABC to 99 μ L ABC diluent buffer).

3.13.3 Assay procedure

To ensure accuracy, all assays for standards, controls, and samples were performed in duplicate. The ABC working solution and TMB Colour Developing Agent were pre-warmed to 37°C for 30 minutes before use, following Abcam's guidelines. During dilution, samples and reagents were mixed thoroughly to ensure even distribution. A standard RANKL detection curve was prepared for each experiment, with sample dilution factors based on the estimated RANKL concentration.

For the assay, 100 μ L of Human RANKL standard solutions at concentrations of 5000 pg/mL, 2500 pg/mL, 1250 pg/mL, 625 pg/mL, 312.5 pg/mL, 156.25 pg/mL, and 78.125 pg/mL were added to the pre-coated 96-well plate. In the control well (Zero well), 100 μ L of sample diluent buffer was used. Then, 100 μ L of each diluted hFOB 1.19 cell

culture supernatant was added to the remaining wells. Each Human RANKL standard solution and sample were measured in duplicate. The plate was sealed with a fresh adhesive cover and incubated at 37°C for 90 minutes, or alternatively at room temperature for 120 minutes.

After incubation, the cover was removed, and the contents of the plate were discarded. The plate was then blotted on absorbent material, avoiding complete drying of the wells. Following this, 100 µL of biotinylated anti-Human RANKL antibody working solution was added to each well. The plate was resealed with a new adhesive cover and incubated at 37°C for 60 minutes or at room temperature for 90 minutes.

Subsequently, the plate was washed three times with 0.01 M TBS or 0.01 M PBS using an automated MultiWash Microplate Washer (Molecular Devices, USA). Each wash involved overfilling the wells with buffer, letting it sit for 1 minute before discarding. After washing, the plate was blotted on paper towels or other absorbent material.

Next, 100 µL of the prepared ABC working solution was added to each well. The plate was sealed again and incubated at 37°C for 30 minutes or at room temperature for 40 minutes. Following this, the plate was washed five times with 0.01 M TBS or 0.01 M PBS, with each wash buffer remaining in the wells for 1-2 minutes before being discarded. The plate was blotted once more on paper towels or absorbent material.

Finally, 90 µL of the TMB Colour Developing Agent was added to each well, and the plate was covered and incubated at 37°C in the dark for 15-20 minutes or at room temperature. After this incubation, 100 µL of TMB Stop Solution was added to each well, causing an immediate color change to yellow. The OD absorbance at 450 nm was measured

using a SpectraMax iD3 microplate reader (Molecular Devices, USA) within 30 minutes of adding the TMB Stop Solution, following the manufacturer's instructions.

3.14 Cell morphology analysis of MGO-stimulated hFOB 1.19 cells treated with *T. crispa* water and methanol extracts, and metformin

The hFOB 1.19 cells were cultured according to section 3.2 of this chapter. The cells were then transferred to a 6-well plate and subsequently treated with the IC₅₀ concentration of MGO, non-toxic concentrations of *T. crispa* water and methanol extracts, and the non-toxic concentration of metformin for a period of 24 hours. After the cells were treated for 24 hours, the 6-well plate containing the treated hFOB 1.19 were viewed under a Nikon Inverted Research Microscope ECLIPSE Ti2. The magnification was set at 20X. The image for different treatments of hFOB 1.19 were captured.

The cellular morphometrics of the treated hFOB 1.19 including parameters such as area density of cells, volume density of cells, minimum and maximum Feret diameter of cells and parameter of cells were manually annotated on the captured images of each treated cell. These values were automatically measured using the Nikon NIS-Elements Image Analysis Software (microscope's software). Additionally, the ellipticity index and circularity indices of the cells were calculated using the following formulas described by Chen, (2011):

$$\text{Ellipticity index} = \frac{L}{L_i} \quad \text{Equation 3.3}$$

$$\text{Circularity index} = \frac{4A}{P^2} \quad \text{Equation 3.4}$$

* L = the longest (major) axis, which is the longest line connecting two points on the cell's perimeter, L_i = the shortest (minor) axis, A= area of the cell, P= perimeter of the cell.

3.15 Statistical Analysis

Data from GC-MS analysis, FRAP assay, BSA-MGO assay, cell viability tests, Luminex multiplex assay, ELISA, and cell morphology analysis were analysed, and their means and standard deviations (SD) were calculated using IBM SPSS Statistics Version 24. Using the same software, the data were subjected to one-way analysis of variance (ANOVA), followed by post-hoc tests to identify significant differences between groups. Dunnett's post-hoc test was used to compare each treatment group to a single control group, as it is specifically designed for such comparisons and controls the family-wise error rate. Additionally, Tukey's post-hoc test was performed to assess significant differences between all pairwise combinations of treatment groups. In both tests, a p-value of <0.05 was considered statistically significant. In this study, the Tukey's post-hoc test was additionally performed to explore all possible pairwise differences between treatment groups, ensuring a comprehensive analysis of the data. In addition, linear regression analysis was applied to evaluate three components: (1) the predictive IC_{50} values for cell viability tests, (2) FRAP values, and (3) the quantity of proteins (IL-1 β , IL-6, M-CSF, sRAGE, fRAGE, RANKL) in pg/mL present in the hFOB 1.19 cell supernatant or cell lysate samples treated with MGO, *T. crispa* extracts, metformin, or a combination of *T. crispa* and metformin..

CHAPTER 4

RESULTS

4.1 Preliminary phytochemical analysis of *T. crispa* extracts

The results of the *in vitro* tests for the *T. crispa* water and methanol extracts are presented in detail in Table 4.1 and summarised in Table 4.2. Both extracts were tested for saponins, flavonoids, glycosides, alkaloids, terpenoids and phenols.

The presence of saponins was confirmed in both the water and methanol extracts of *T. crispa* by the formation of froth on the top layer of the reaction mixture. For the flavonoid detection, a two-step chemical addition was done. Initially, a bright yellowish colour was observed when the *T. crispa* water and methanol extracts were mixed with 2% NaOH. Subsequently, the reaction mixture for the methanol extract of *T. crispa* turned colourless after the addition of H₂SO₄ indicating that flavonoids were present. However, the hot water extract only showed slight discolouration suggesting the absence of flavonoids.

A green layer was observed near the bottom of the test tube in both the water and methanol extracts, confirming the presence of glycosides. The formation of a brownish coloured precipitate in the test tube indicated the presence of alkaloids in both extracts. Lastly, a brown colour observed in the hot water extracts of *T. crispa* and a dark green colouration in the methanol extract of *T. crispa* confirmed the presence of phenols.

Table 4.1: The visible colour changes in the water and methanol extracts of *T. crispa* used to test the presence of saponins, flavonoids, glycosides, alkaloids, terpenoids and phenols. Pictures were taken at room temperature in the Biochemistry Lab, Faculty of Medicine and Health Sciences, UNIMAS.

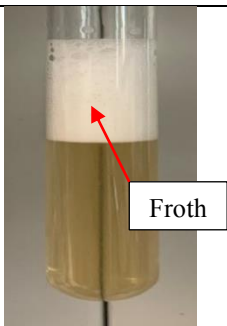
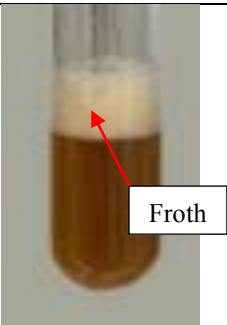
Phytochemical test	Type of <i>T. crispa</i> extracts	Final colour	Remarks
Saponins detection test	Hot water extract		Froth was formed. Saponins were present.
	Methanol extract		Froth was formed. Saponins were present.
Flavonoid's detection test	Hot water extract		The reaction mixture did not turn colourless after H ₂ SO ₄ was added. Flavonoids were absent
	Methanol extract		The reaction mixture turned almost colourless after H ₂ SO ₄ was added. Flavonoids were present

Table 4.1: continued

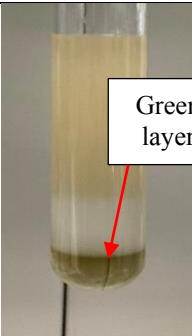
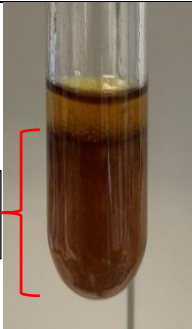
Glycoside's detection test	Hot water extract	 Green layer	Green layer was formed. Glycosides were present.
	Methanol extract	 Green layer	Green layer was formed. Glycosides were present.
Alkaloid's detection test	Hot water extract	 Brown precipitate	Brownish coloured precipitate was formed. Alkaloids were present.
	Methanol extract	 Brown precipitate	Brownish coloured precipitate was formed. Alkaloids were present.

Table 4.1: continued

Terpenoids detection test	Hot water extract		The reaction mixture turned darker brown after the addition of H_2SO_4 . Terpenoids were present.
	Methanol extract		The reaction mixture turned darker brown after the addition of H_2SO_4 . Terpenoids were present.
Phenol's detection test	Hot water extract		Brown colour was observed. Phenols are present
	Methanol extract		Dark green colour was observed. Phenols are present

Table 4.2: The summary of the presence of different types of phytochemicals in *T. crispa* extracts.

Phytochemicals	<i>T. crispa</i> extract	
	Methanol	Hot water
Saponins	+	+
Flavonoids	+	-
Glycosides	+	+
Alkaloids	+	+
Terpenoids	+	+
Phenols	+	+

“+” indicates that the phytochemical was present, while “-” indicates that the phytochemical was absent from the extracts.

4.2 GC-MS Analysis of *T. crispa* extracts

Based on the analysis conducted using gas chromatography with mass spectrophotometry detection, it was determined that there were a total of 13 (methanol extract) and 16 (hot water extract) bioactive compounds of *T. crispa*, respectively (Table 4.3 & Table 4.4). In the methanolic extract of *T. crispa*, oleic acid had the highest recorded concentration at approximately $22.24 \pm 6.29\%$, followed by palmitic acid at $20.01 \pm 6.15\%$ and 3,4-Divanillyltetrahydrofuran at $12.80 \pm 3.70\%$. The remaining phytochemical constituents ranged from 2.15% to 8.40%. Meanwhile, the highest concentration for *T. crispa* hot water extract was observed for santalene ($15.96 \pm 9.60\%$), trans-sinapyl alcohol ($10.64 \pm 1.53\%$), guaiacol ($9.96 \pm 1.71\%$), and columbin ($9.78 \pm 0.31\%$). The remaining phytochemical constituents ranged from 2.06% to 8.14%. Overall, unsaturated fatty acids were detected only in the methanol extract of *T. crispa*, while monosaccharides and aromatic carboxylic acids were found exclusively in the hot water extract of *T. crispa* (Figure 4.1 & Figure 4.2).

Both the methanol and hot water extracts of *T. crispata* shared the presence of the same classes of several phytochemicals, including saturated fatty acids, phenols, lignans, terpenoids, terpenes, and hydrocarbons.

Table 4.3: Detection of phytochemicals through GC-MS Analysis of *T. crispata* methanol extract.

No.	Phytochemical name	Phytochemical class	Molecular formula	(Total % Area) $\pm$ SD
1.	Oleic Acid	Unsaturated fatty acid	C ₁₈ H ₃₄ O ₂	22.24 $\pm$ 6.29
2.	Palmitic acid	Saturated fatty acid	C ₁₆ H ₃₂ O ₂	20.01 $\pm$ 6.15
3.	3,4-Divanillyltetrahydrofuran	Lignan	C ₂₀ H ₂₄ O ₅	12.80 $\pm$ 3.70
4.	Isosativene	Terpenoids	C ₁₅ H ₂₄	8.40 $\pm$ 4.98
5.	Stearic acid	Saturated fatty acid	C ₁₈ H ₃₆ O ₂	6.98 $\pm$ 1.74
6.	Eugenol	Phenol	C ₁₀ H ₁₂ O ₂	6.03 $\pm$ 1.57
7.	Columbin	Terpenes	C ₂₀ H ₂₂ O ₆	4.27 $\pm$ 1.21
8.	Santalene	Terpenoids	C ₁₅ H ₂₄	4.13 $\pm$ 2.72
9.	Dotriacontane	Hydrocarbon	C ₃₂ H ₆₆	3.98 $\pm$ 3.58
10.	Trans-sinapyl alcohol	Phenols	C ₁₁ H ₁₄ O ₄	3.25 $\pm$ 0.90
11.	4-vinylguaiacol	Phenols	C ₉ H ₁₀ O ₂	3.15 $\pm$ 0.97
12.	Syringol	Phenols	C ₈ H ₁₀ O ₃	2.62 $\pm$ 0.67
13.	Vanillin	Phenols	C ₈ H ₈ O ₃	2.15 $\pm$ 0.56

Data were presented as mean% $\pm$ SD, n=2/group.

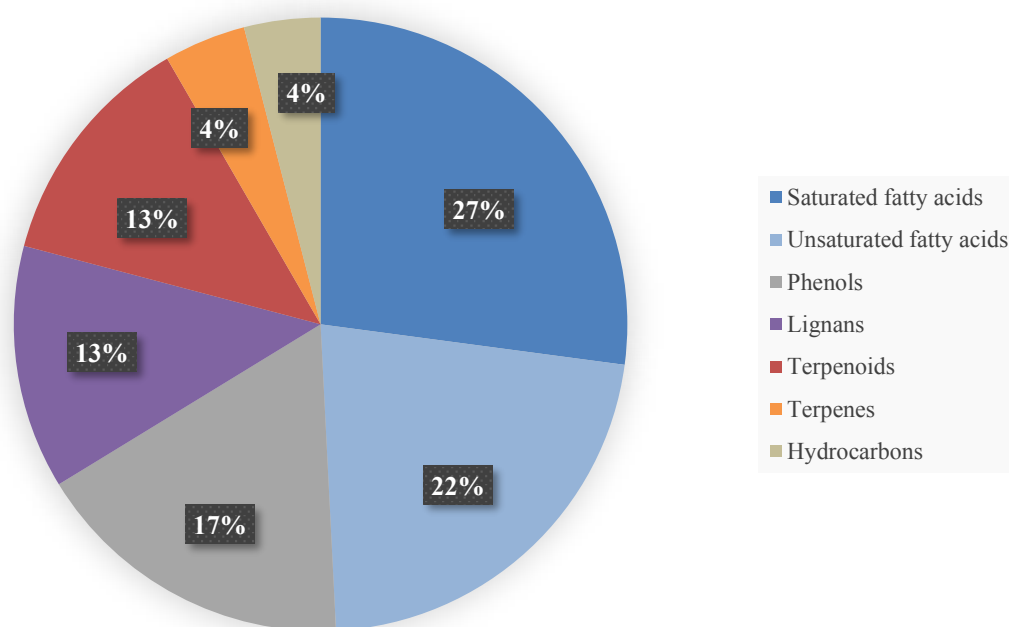


Figure 4.1:Proportion (%) of various phytochemical compounds presence in *T. crista* methanol extract, analysed by the GC-MS.

Table 4.4: Detection of phytochemicals through GC-MS Analysis of *T. crista* hot water extract.

No.	Phytochemical name	Phytochemical class	Molecular formula	(Total % Area) ± SD
1.	Santalene	Terpenoids	C ₁₅ H ₂₄	15.96±9.60
2.	Trans-sinapyl alcohol	Phenols	C ₁₁ H ₁₄ O ₄	10.64±1.53
3.	Guaiacol	Phenols	C ₇ H ₈ O ₂	9.96±1.71
4.	Columbin	Terpenes	C ₂₀ H ₂₂ O ₆	9.78±0.31
5.	2-Methoxy-4-vinylphenol	Phenols	C ₉ H ₁₀ O ₂	8.14±0.27
6.	Eicosane	Hydrocarbon	C ₂₀ H ₄₂	7.03±3.80
7.	2,6,8-Trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol	Terpenoids	C ₁₁ H ₁₈ O ₂	6.64±1.07
8.	2,6-Dimethoxyphenol	Phenol	C ₈ H ₁₀ O ₃	6.09±0.81
9.	Eugenol	Phenols	C ₁₀ H ₁₂ O ₂	4.90±0.40
10.	3,4-Divanillyltetrahydrofuran	Lignan	C ₂₀ H ₂₄ O ₅	4.51±0.62
11.	Vanillin	Phenols	C ₈ H ₈ O ₃	3.18±0.16
12.	Palmitic acid	Saturated fatty acid	C ₁₆ H ₃₂ O ₂	2.95±0.53
13.	Levogluconan	Monosaccharides	C ₆ H ₁₀ O ₅	2.90±1.58
14.	o-Toluic Acid	Aromatic carboxylic acids	C ₈ H ₈ O ₂	2.72±0.32
15.	Syringaldehyde	Phenols	C ₉ H ₁₀ O ₄	2.49±0.53
16.	2,6,10,14-Tetramethyloctadecane	Hydrocarbon	C ₂₂ H ₄₆	2.06±0.36

Data were presented as mean% ± SD, n=2/group.

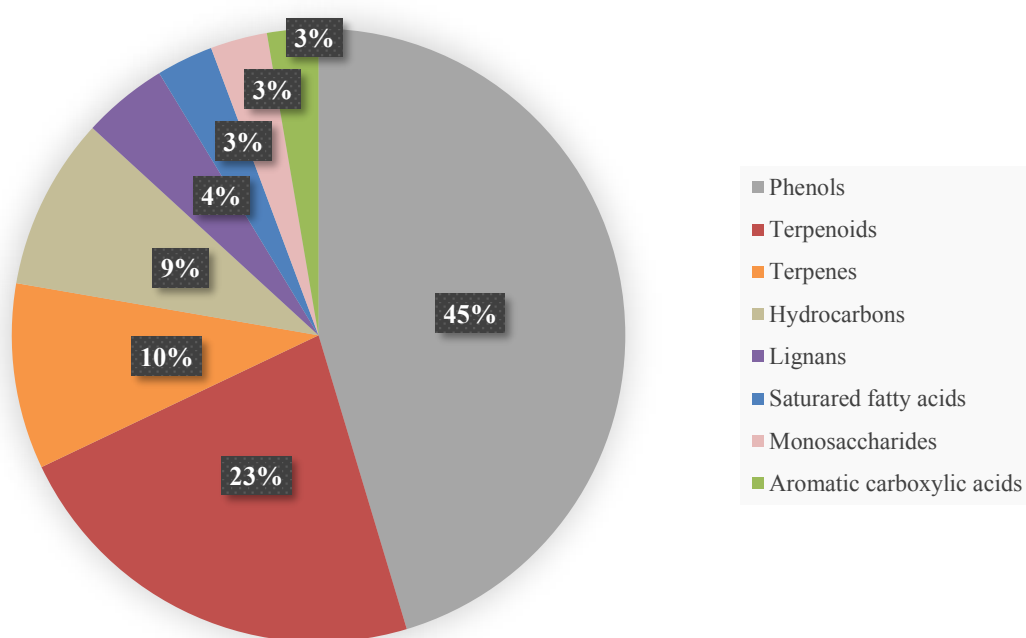


Figure 4.2: Proportion (%) of various phytochemical compounds presence in *T. crispata* hot water extract, analysed by the GC-MS.

4.3 Ferric Reducing Antioxidant Power (FRAP) Assay for *T. crispata* extracts

The FRAP assay measures the ability of an extract to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), reflecting its overall antioxidant potential. Extracts with higher FRAP values are considered to have greater antioxidant levels, better scavenging abilities, and enhanced capacity to counteract oxidative stress in biological systems. In this study, four concentrations (50, 100, 200, and 400 $\mu\text{g/mL}$) of *T. crispata* hot water extract and *T. crispata* methanol extract were selected to assess the impact of solvent type on FRAP values.

Results revealed that as the concentrations of both *T. crispata* water and methanol extracts increased, the FRAP values also increased significantly ($p < 0.05$). At the highest concentration of 400 $\mu\text{g/mL}$, the *T. crispata* methanol extract exhibited a FRAP value of $121.00 \pm 0.82 \text{ mmol Fe}^{2+}/\mu\text{g}$, which was approximately 2.7 times higher ($p < 0.05$) than the FRAP value of the *T. crispata* hot water extract ($45.07 \pm 1.24 \text{ mmol Fe}^{2+}/\mu\text{g}$) at the same concentration (Figure 4.4). Similarly, at the lowest concentration of 50 $\mu\text{g/mL}$, the *T. crispata*

methanol extract showed a FRAP value of 29.55 ± 0.17 mmol $\text{Fe}^{2+}/\mu\text{g}$, which was approximately 3 times higher ($p < 0.05$) than that of the *T. crispata* hot water extract (9.89 ± 0.21 mmol $\text{Fe}^{2+}/\mu\text{g}$) (Figure 4.4).

The significantly higher FRAP values observed in the *T. crispata* methanol extract compared to the hot water extract suggest a higher concentration of potent reducing agents in the methanol extract. These reducing agents likely contribute to its enhanced scavenging abilities, indicating that the methanol extract possesses greater antioxidant activity.

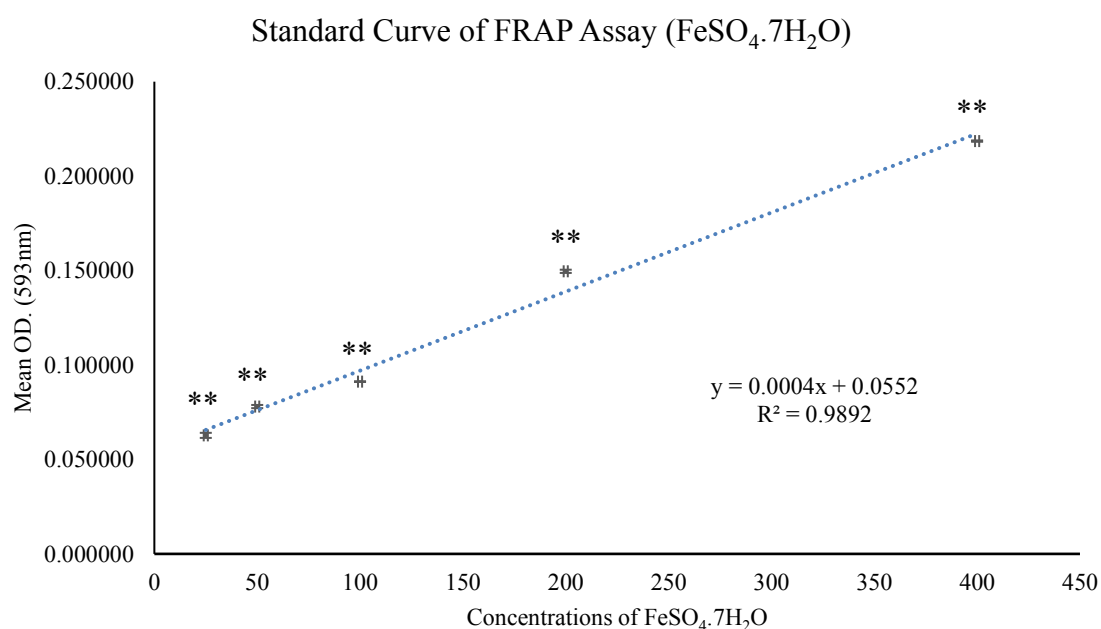


Figure 4.3: Standard curve for FRAP assay. The concentration of the standard was plotted against the optical density. Data were presented as mean $\pm$ SD, ($n=2/\text{group}$). All of the mean OD for different concentrations of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were significantly different from one another at $p < 0.005$.

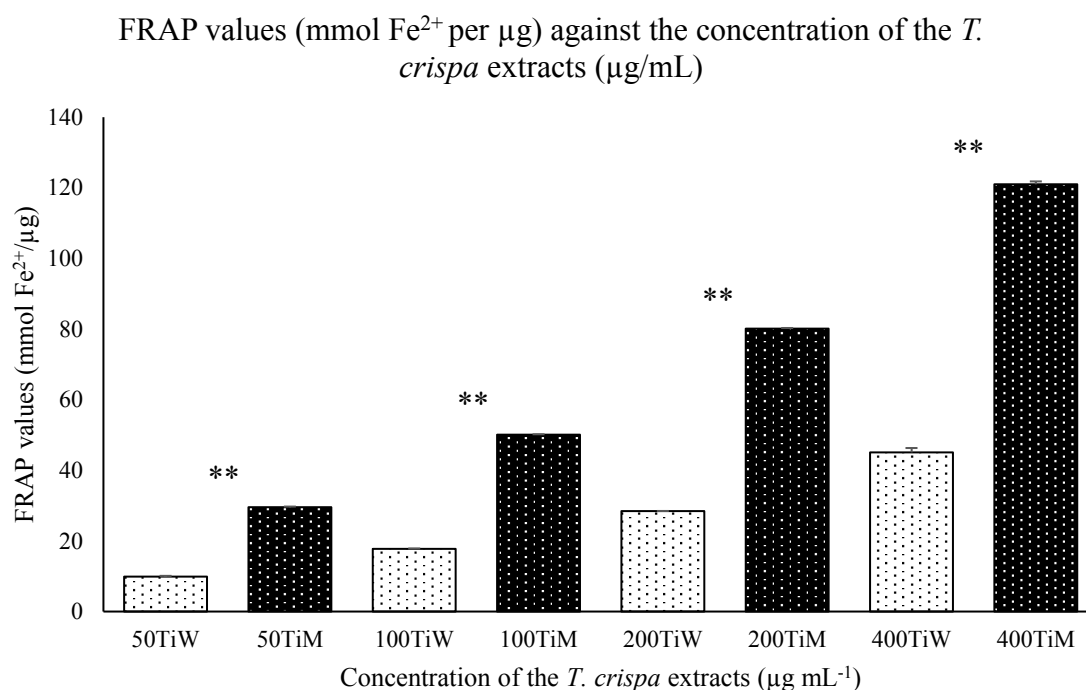


Figure 4.4: FRAP values for *T. crispa* methanol and hot water extracts. Data are presented as mean $\pm$ SD (n=2/group). Statistical significance between extracts at the same concentrations is indicated by *p<0.05 and **p<0.00. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract.

4.4 BSA-MGO preliminary anti-glycation assay

The BSA-MGO assay is a widely used method to evaluate the anti-glycation activity of various compounds. This assay measures the ability of an extract to inhibit the formation of AGEs by inhibiting the reaction between BSA and MGO. AGEs are implicated in the progression of several chronic diseases, including diabetes and its complications, due to their role in promoting oxidative stress and inflammation. Extracts that exhibit high anti-glycation activity can potentially reduce AGE formation, thereby mitigating the associated adverse effects.

In this study, four concentrations (0.05, 0.10, 0.20, and 0.40 mg/mL) of both *T. crispa* hot water extract and *T. crispa* methanol extract were selected to evaluate their efficacy in

inhibiting glycation and to determine if the type of solvent used for extraction affects their anti-glycation properties.

The anti-glycation activity (%) shown in Figure 4.5 increases steadily with the concentration of *T. crispera* extracts. At 0.40 mg/mL, the methanol extract of *T. crispera* showed the highest percentage of glycation inhibition at 48.70% while at its lowest concentration, 0.05 mg/mL, there was 26.44% glycation inhibition. At 0.40 mg/mL, the hot water extract of *T. crispera* showed a lower antiglycation activity than the methanol extract which was at 34.57%. At 0.05 mg/mL the antiglycation activity of the methanol extract was 25.98%. However, both types of *T. crispera* extracts, however, demonstrated significantly lower glycation inhibition compared to aminoguanidine, the positive control, which exhibited 94% glycation inhibition.

These results suggest that while *T. crispera* extracts possess anti-glycation properties, their efficacy is less than that of aminoguanidine. The higher activity observed in the methanol extract compared to the hot water extract could be attributed to the presence of different phytochemicals that are more effective in inhibiting the glycation process. These findings indicate the potential of *T. crispera* extracts in reducing AGE formation, although further optimisation and comparison with more potent inhibitors like aminoguanidine are necessary.

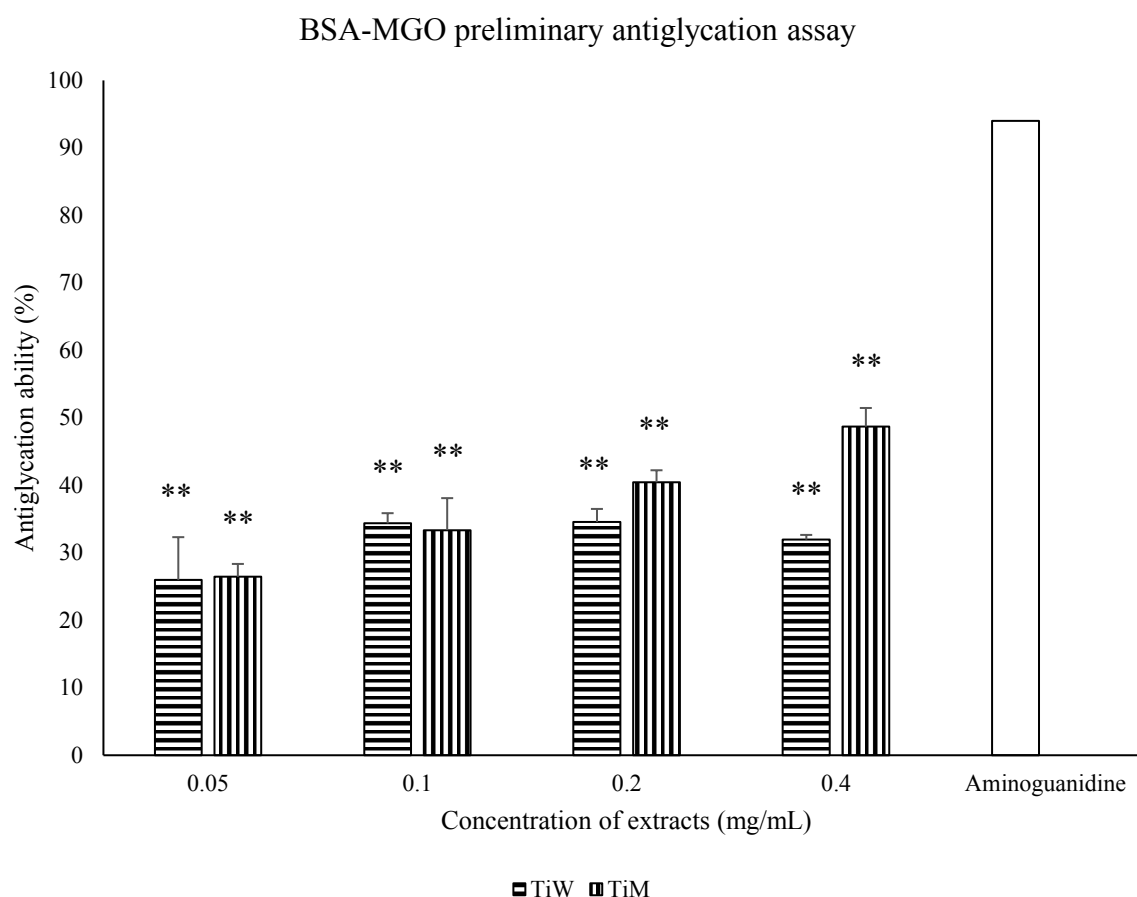


Figure 4.5: Anti-glycation activity of *T. crispa* water and methanolic extracts at varying concentrations (0.05, 0.10, 0.20, 0.40 mg/mL). Data are presented as mean% $\pm$ SD (n=3/group). Significant differences compared to the positive control, aminoguanidine, are indicated by *p<0.05, **p<0.005. Note: TiW= *T. crispa* hot water extract, TiM= *T. crispa* methanol extract.

4.5 Cell Viability Test

4.5.1 Determination of the IC₅₀ of MGO and its effects of MGO on hFOB 1.19 cell viability using the MTT assay and the Trypan Blue Dye Exclusion Test

The MTT assay as shown in Figure 4.6, demonstrated a concentration-dependent decrease in the percentage viability of hFOB 1.19 cells with increasing concentrations of MGO. Treatment with the lowest concentration of MGO (0.2 mM) resulted in 138.90 $\pm$ 8.45% viability of hFOB 1.19 cells, whereas the highest concentration of MGO (1.75 mM) significantly reduced cell viability to 42.21 $\pm$ 1.14%. The IC₅₀ value for MGO, which

represents the concentration MGO at which 50% of the cells are inhibited, was calculated to be 1.69 mM using non-linear regression analysis (Figure 4.6).

Similarly, Figure 4.7 demonstrates a concentration-dependent decrease in hFOB 1.19 cell viability with increasing MGO concentrations, as measured by the Trypan Blue exclusion test. The lowest concentration of MGO (0.2 mM) resulted in $73.30 \pm 1.42\%$ cell viability, while the highest concentration (1.75 mM) reduced viability dramatically to $11.60 \pm 1.20\%$. The IC_{50} value determined from this assay as calculated using non-linear regression analysis was 1.15 mM.

These findings generally highlight the dose-dependent cytotoxic effects of MGO on hFOB 1.19 cells, with the Trypan Blue assay showing a lower IC_{50} value, indicating its greater sensitivity in detecting early cytotoxic effects.

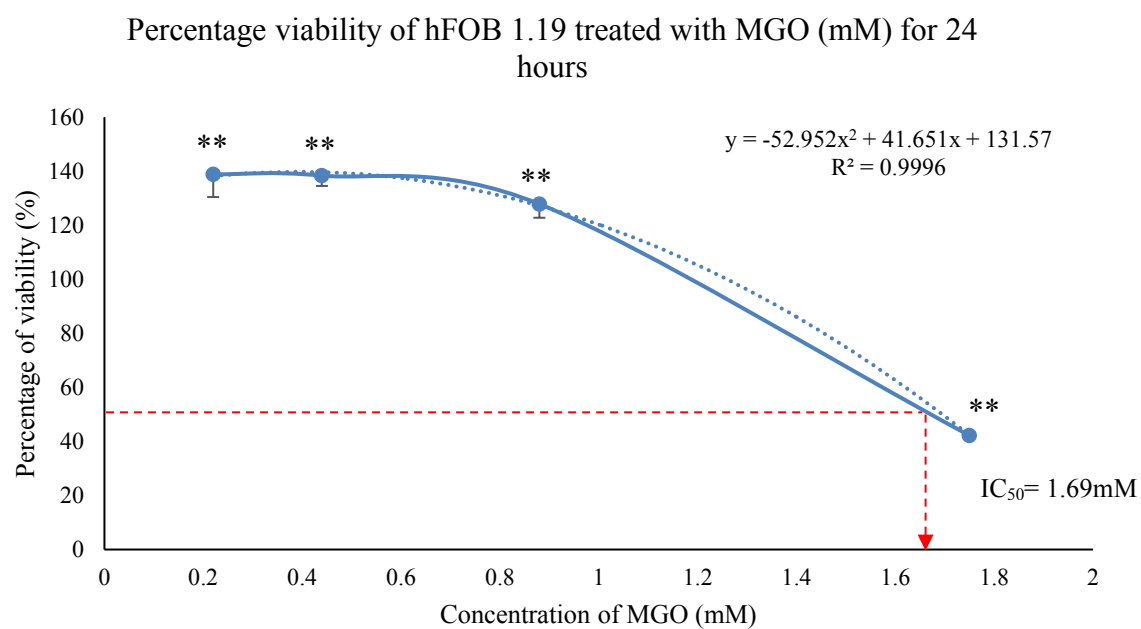


Figure 4.6: hFOB 1.19 cells were seeded at 3.0×10^4 cells/mL in 96-well plates and grown to 80% confluency within 24 hours. Cells were treated with MGO at concentrations of 0.22, 0.44, 0.88, and 1.75 mM for 24 hours. Cytotoxicity was assessed using the MTT assay. Data are presented as mean $\pm$ SD, n=3/group. Statistical significance compared to the control is indicated by *p<0.05 and **p<0.005.

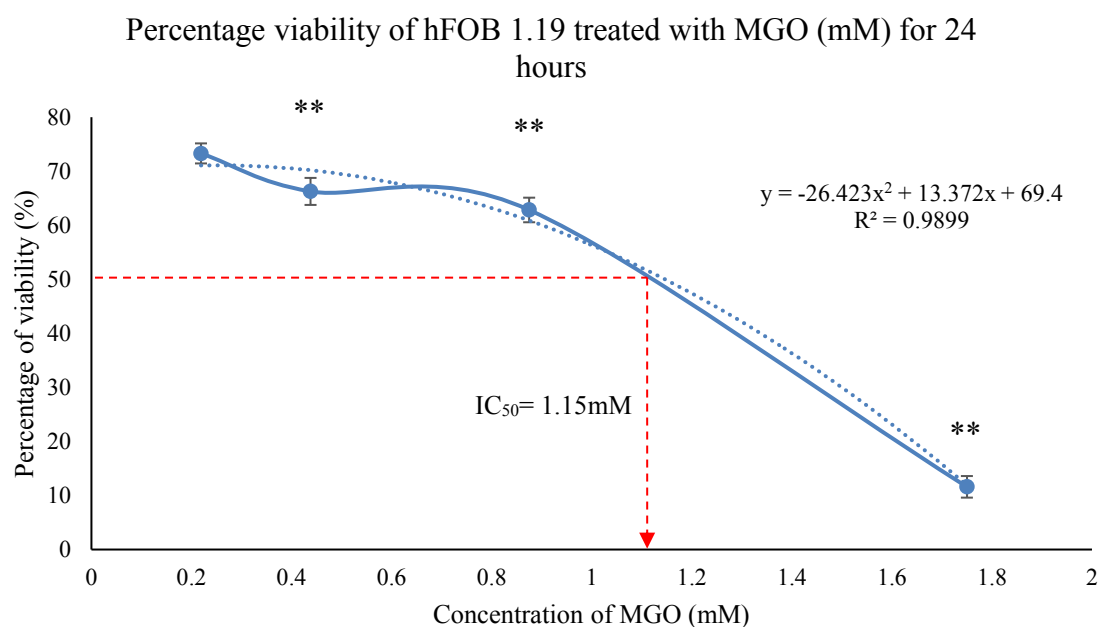


Figure 4.7: hFOB 1.19 cells were grown to 80% confluency in 96-well plates and then treated with various MGO concentrations (0.22, 0.44, 0.88, and 1.75 mM) for 24 hours. Cytotoxicity was assessed using the Trypan Blue Dye Exclusion Test. Data are shown as mean% $\pm$ SD, n=3/group, with an R^2 value of 0.9899. Statistically significant differences from the control are indicated.

4.5.2 Determination of the effects of metformin on hFOB 1.19 cell viability using the MTT assay and the Trypan Blue Dye Exclusion Test

Based on Figure 4.8, the MTT assay shows that the viability of hFOB 1.19 cells decreased as the concentration of metformin increased. The lowest concentration of metformin (3 mM) showed 120.63 ± 12.51 % of cell viability, while the highest concentration (24 mM) of metformin showed 80.68 ± 2.96 % of cell viability.

Similarly, the trypan blue exclusion test (Figure 4.9), shows that the percentage of viability of hFOB 1.19 cells also decreased when treated with metformin. However, in contrast to MTT assay, the decreased in the cell viability between the different concentrations is not significantly different from each other. The lowest concentration of metformin (3 mM) showed 94.30 ± 0.20 % of hFOB 1.19 viability while the highest concentration of metformin (24 mM) showed 92.47 ± 0.25 % of hFOB 1.19 viability.

The percentage of viability of hFOB 1.19 cells treated with 3-24 mM of metformin were still within the non-toxic range ($\geq 80\%$ viability) for both the MTT assay and trypan blue exclusion method. Hence, 24 mM of metformin, which was the highest concentration of metformin used in this study was chosen as the non-toxic concentration of metformin to treat MGO-stimulated hFOB 1.19 cells.

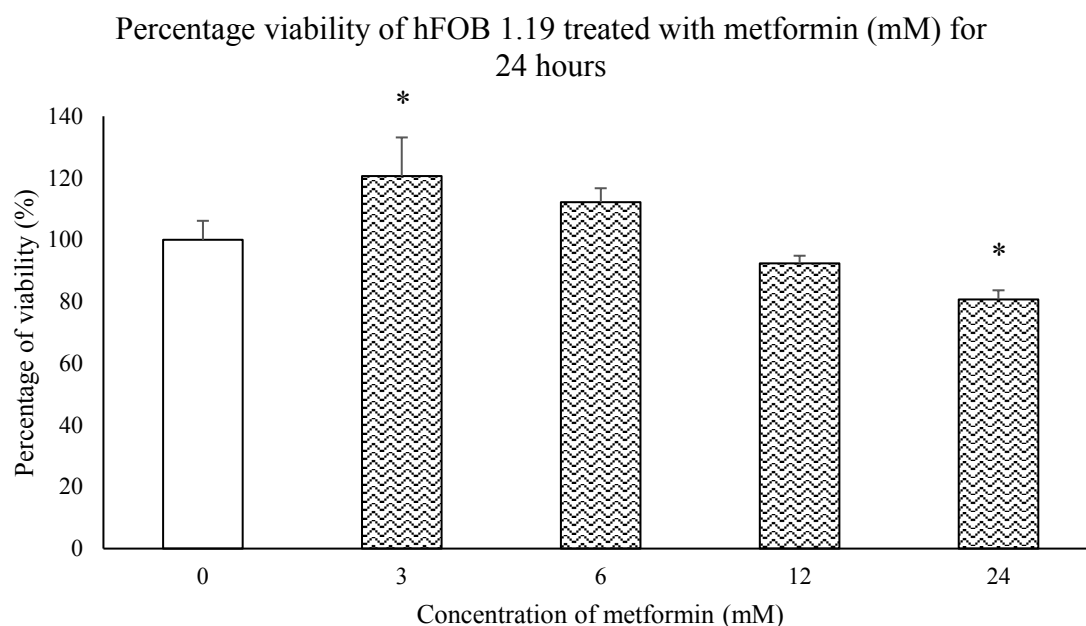


Figure 4.8: Determination of non-toxic concentrations of metformin. hFOB 1.19 cells were seeded in 96-well plates at 3.0×10^4 cells/mL and grown to 80% confluency within 24 hours. The cells were treated with metformin at concentrations of 3, 6, 12, and 24 mM for 24 hours. Cytotoxicity was measured using the MTT assay. Data are shown as mean% $\pm$ SD, n=3/group. Statistical significance compared to the control is marked as *p<0.05 and **p<0.005.

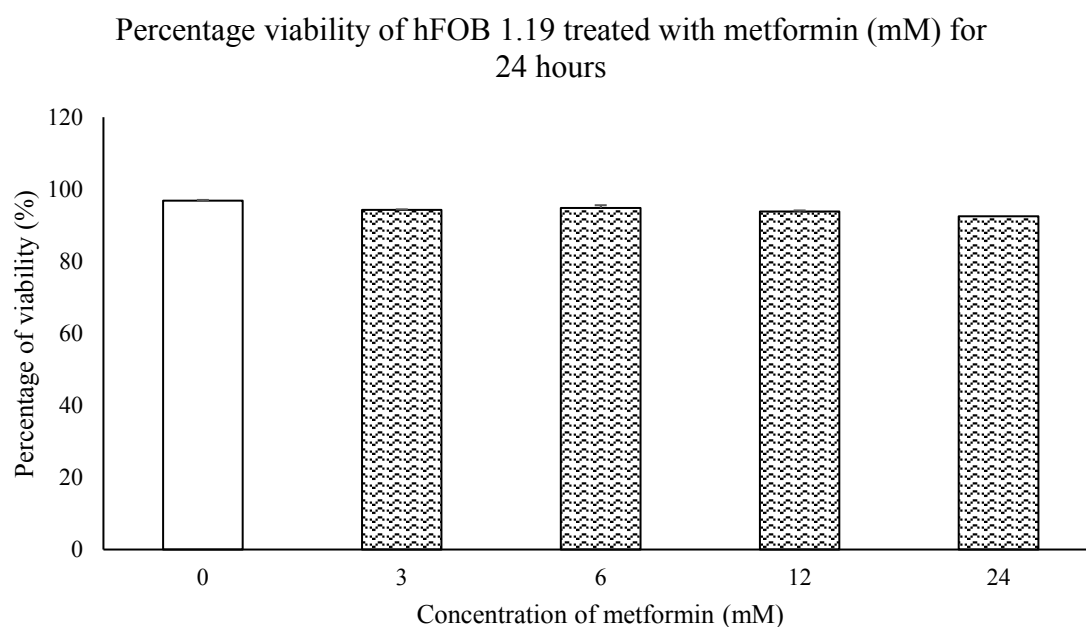


Figure 4.9: Determination of non-toxic concentrations of metformin. hFOB 1.19 cells were seeded in 96-well plates at 3.0×10^4 cells/mL and grown to 80% confluency, typically within 24 hours. The cells were then treated with varying concentrations of metformin (mM) for 24 hours. Cytotoxicity was assessed using the Trypan Blue Dye Exclusion Test. Data are presented as mean% $\pm$ SD, n=3/group. Statistical significance compared to the control is indicated by *p<0.05 and **p<0.005.

4.5.3 Determination of the effects of *T. crista* methanol (TiM) extracts on hFOB 1.19 cell viability using the MTT assay and the Trypan Blue Dye Exclusion Test

Figure 4.10, demonstrates that the MTT assay shows a significant decrease in the percentage of cell viability in hFOB 1.19 cells with increasing concentrations of *T. crista* methanol extract. Cell viability decreased from $134.30 \pm 1.16\%$ at the lowest concentration ($29.75 \mu\text{g/mL}$) to $123.62 \pm 1.91\%$ at the highest concentration ($475 \mu\text{g/mL}$). All the tested concentrations of *T. crista* methanol extract showed statistically significant differences compared to the control ($p < 0.05$). Despite this decline, all tested concentrations yet remained within the non-toxic range ($\geq 80\%$ viability). Consequently, the highest concentrations of 118.75 , 237 , and $475 \mu\text{g/mL}$ were selected for further experiments with MGO-stimulated hFOB 1.19 cells.

Figure 4.11 using the Trypan Blue Exclusion Test, also shows a decrease in cell viability with increasing concentrations of *T. crista* methanol extract, but to a lesser extent. The viability dropped from $95.37 \pm 1.91\%$ at $29.70 \mu\text{g/mL}$ to $94.87 \pm 1.03\%$ at $475 \mu\text{g/mL}$. Although this decrease was less pronounced compared to the MTT assay results in Figure 4.10, all treatments were statistically significant relative to the control. Like the MTT assay results, the trypan blue test also indicated that cell viability remained within the non-toxic range ($\geq 80\%$ viability). Thus, the same highest concentrations of 118.75, 237, and $475 \mu\text{g/mL}$ were selected for treating MGO-stimulated hFOB 1.19 cells.

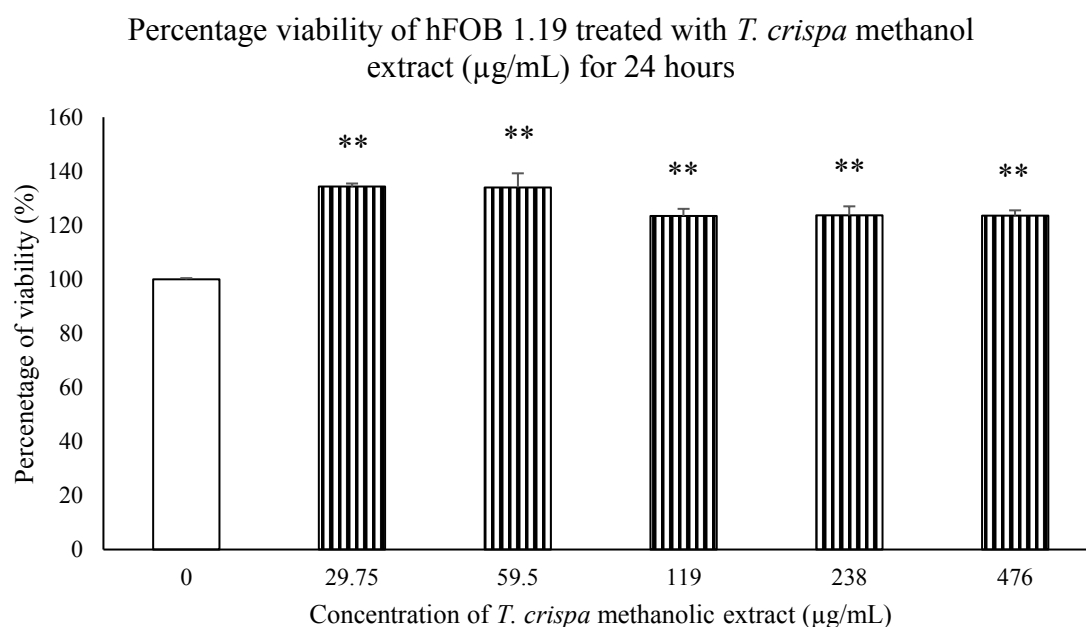


Figure 4.10: Determination of non-toxic concentrations TiM. hFOB 1.19 cells were seeded at 3.0×10^4 cells/mL in 96-well plates and grown to 80% confluency within 24 hours. The cells were then treated with various concentrations of TiM (29.75, 59.50, 119, 238, and 476 µg/mL) for 24 hours. Cytotoxicity was assessed using the MTT assay. Data are presented as mean% $\pm$ SD, n=3/group. Statistical significance compared to the control is indicated by *p<0.05 and **p<0.005. Note: TiM refers to *T. crispata* methanol extract.

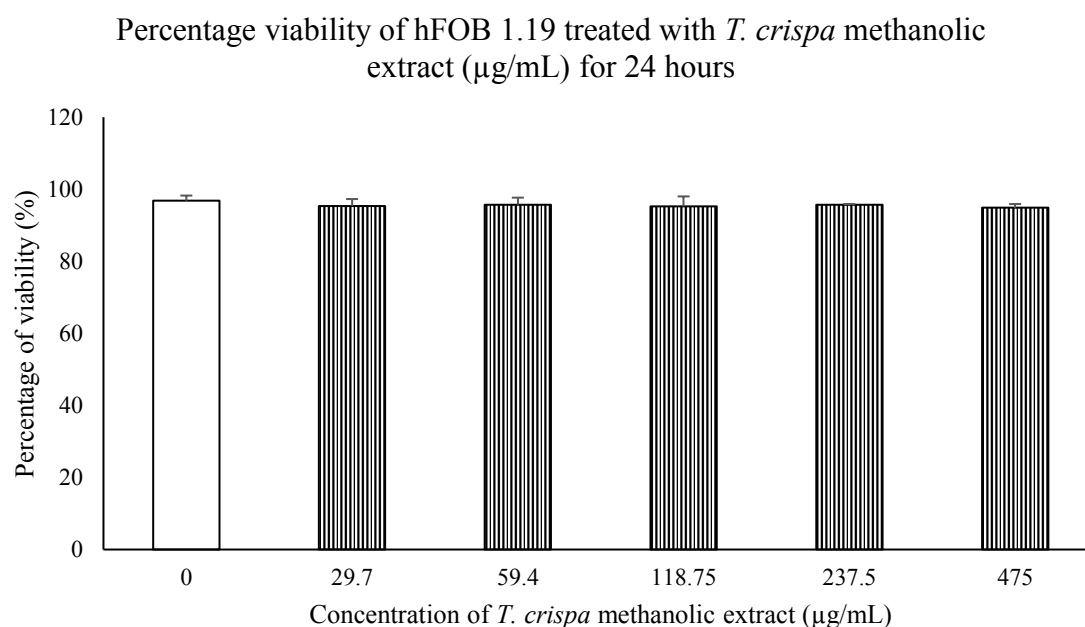


Figure 4.11: Determination of non-toxic concentrations of TiM. hFOB 1.19 cells were seeded in 96-well plates at a density of 3.0×10^4 cells/mL and grown to 80% confluency, typically within 24 hours. The cells were then treated with various concentrations of TiM (µg/mL) for 24 hours. Cytotoxicity was assessed using the Trypan Blue Dye Exclusion Test. Data are presented as mean% $\pm$ SD, n=3/group. Statistical significance compared to the control is indicated by *p<0.05 and **p<0.005. Note: TiM = *T. crispa* methanol extract.

4.5.4 Determination of the effects of *T. crispa* water (TiW) extracts on hFOB 1.19 cell viability using the MTT assay and the Trypan Blue Dye Exclusion Test

The MTT assay (Figure 4.12) shows that the percentage of viability of hFOB 1.19 cells decreased with increasing concentrations of *T. crispa* hot water extract, from 133.51 ± 5.53 % at 187.50 µg/mL to 122.13 ± 0.97 % at 3000 µg/mL. Although this decrease in viability was not statistically significant across the concentration range tested, all concentrations of *T. crispa* hot water extract showed statistically significant differences compared to the control (p<0.05). Despite the observed decrease, the viability remained within the non-toxic range ($\geq 80\%$). Therefore, the highest concentrations of 118.75, 237, and 475 µg/mL were chosen for further treatment of MGO-stimulated hFOB 1.19 cells.

The Trypan Blue Exclusion Test (Figure 4.13) shows a statistically significant ($p < 0.005$) decrease in the percentage of viability of hFOB 1.19 as compared to control. There was a slight increase but not significant in cell viability from the lowest (187.5 $\mu\text{g/mL}$) to the highest (3000 $\mu\text{g/mL}$) concentration of *T. crista* hot water extract, from $85.47 \pm 1.56\%$ to $87.17 \pm 1.68\%$, respectively. The percentage of viability of hFOB 1.19 cell treated with 187.5-3000 $\mu\text{g/mL}$ of *T. crista* hot water extract were still within the non-toxic range ($\geq 80\%$ viability). Therefore, three of the highest concentration of *T. crista* hot water extract from the non-toxic range; 118.75, 237 and 475 $\mu\text{g/mL}$ were selected to treat the MGO-stimulated hFOB 1.19 cells.

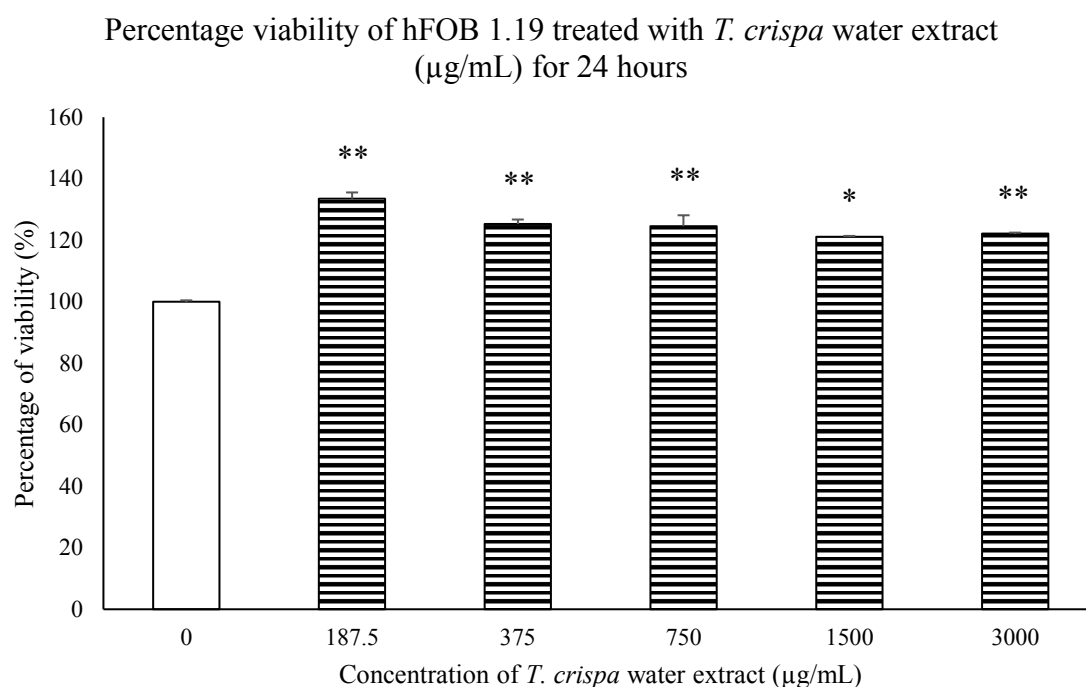


Figure 4.12: Determination of non-toxic concentrations of TiW. hFOB 1.19 cells were grown at 3.0×10^4 cells/mL in 96-well plates and grown to 80% confluence within 24 hours. The cells were then treated with TiW at various concentrations (187.5, 375, 750, 1500, and 3000 $\mu\text{g/mL}$) for 24 hours. Cytotoxicity was measured using the MTT assay. Data are shown as mean% $\pm$ SD, n=3/group. Statistical significance compared to the control is marked as *p<0.05 and **p<0.005. Note: TiW = *T. crispa* hot water extract.

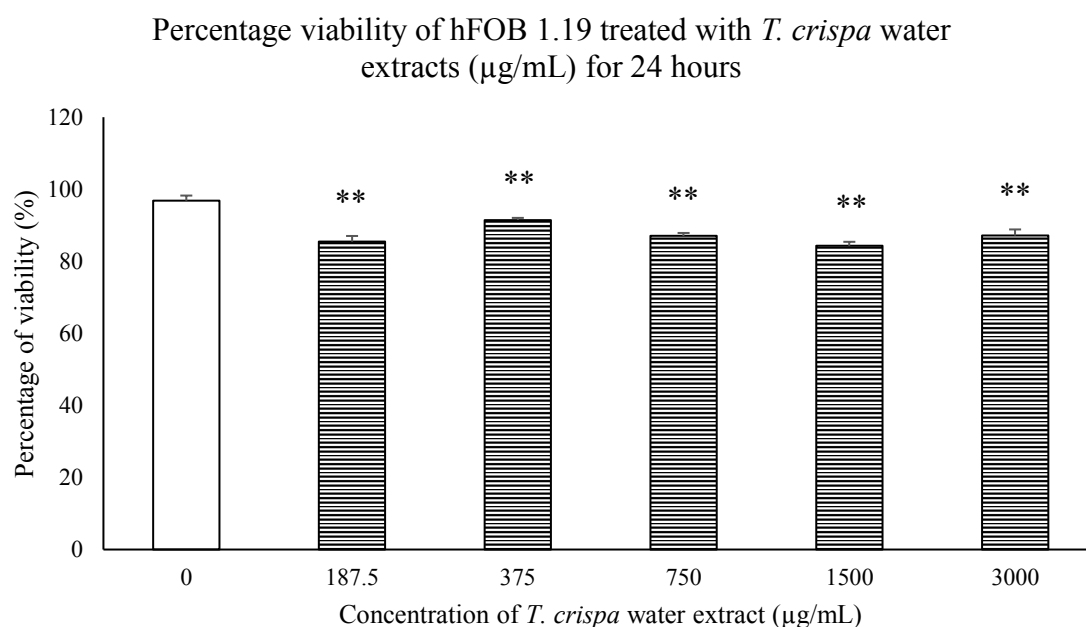


Figure 4.13: Determination of non-toxic concentrations of TiW. hFOB 1.19 cells were seeded in 96-well plates at 3.0×10^4 cells/mL and grown to 80% confluence, within 24 hours. The cells were then treated with various concentrations of TiW (µg/mL) for 24 hours. Cytotoxicity was assessed using the Trypan Blue Dye Exclusion Test. Data are presented as mean% $\pm$ SD, n=3/group. Statistical significance compared to the control is indicated by *p<0.05 and **p<0.005. Note: TiW = *T. crista* hot water extract.

4.6 Human Magnetic Luminex Multiplex Assay of IL-1 β , IL-6 and M-CSF

4.6.1 Effects of *T. crista* water or methanol extract alone and in combination with metformin on the expression of IL-1 β in MGO-stimulated hFOB 1.19

IL-1 β is a key pro-inflammatory cytokine that plays a central role in the inflammatory response to infection or injury. It also promotes the formation and activation of osteoclasts, subsequently stimulates the differentiation of osteoclast precursors into mature osteoclasts, leading to increased bone breakdown.

In this study, the expression of IL-1 β was upregulated in MGO-stimulated osteoblast cells but not significantly reduced by the addition of metformin (p<0.05). When MGO-stimulated hFOB 1.19 cells were treated with 750 µg/mL and 1500 µg/mL *T. crista* hot water extracts, the concentration of IL-1 β were not significantly (p<0.05) decreased

compared to the MGO-stimulated-hFOB 1.19 cells alone. However, treatment with 3000 µg/mL of *T. crispa* hot water extract led to a significant decrease in IL-1β levels ($p < 0.05$).

When combined with metformin, neither 750 µg/mL of *T. crispa* water nor 1500 µg/mL of *T. crispa* hot water extracts resulted in significant reduction in IL-1β ($p < 0.05$). In contrast, the combination of 3000 µg/mL of *T. crispa* hot water extracts with metformin produced a significant decrease in IL-1β levels ($p < 0.05$) was significantly decreased. Figure 4.14 suggesting a potential synergistic effect between the higher concentration of *T. crispa* hot water extract with metformin.

In the case of the methanol extracts, treatment with 119 µg/mL, 238 µg/mL and 476 µg/mL of *T. crispa* methanol extract alone did not result in significant decrease in IL-1β compared to the MGO-stimulated hFOB 1.19 cells. When combined with metformin, 119 µg/mL of *T. crispa* methanol extract still did not significantly decrease the concentration of IL-1β. Notably, the combination of 238 µg/mL or 476 µg/mL of *T. crispa* methanol extract with metformin resulted in IL-1β levels falling below the lowest detection limit (< 18.2305 pg/mL) indicating a potential synergistical effects of *T. crispa* methanol extracts and metformin in reducing the levels of IL-1β. (Figure 4.14). This result could imply that the concentration of IL-1β in the sample is lower than the minimum detectable amount by Luminex assay can reliably detect. In other words, values below the detection limit can indicate that the IL-1β in is either absent or present in extremely low concentrations in the sample.

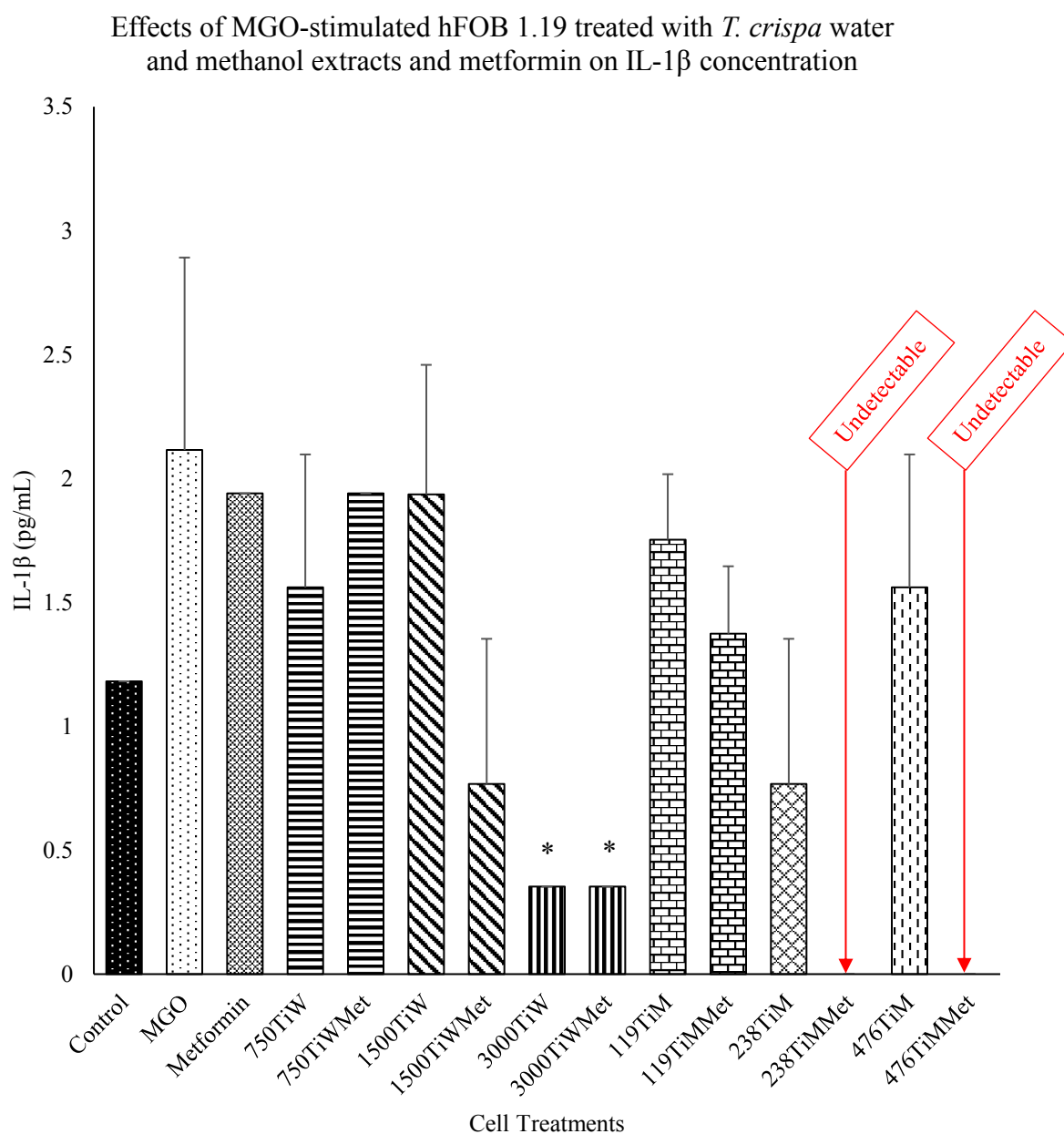


Figure 4.14: Effects of MGO, metformin, *T. crispa* water and methanol extracts on IL-1 β levels in hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and the hot water extract of *T. crispa*, in 6 well plates and incubated at 34°C for 24 hours. All treatments included MGO except for the control. IL-1 β levels in the supernatants were measured with Luminex Assay. Data are presented as mean $\pm$ SD, n=2/group. Significant differences from the MGO only treatment are marked by *p<0.05, **p<0.005 and from the control by #p<0.05, ##p<0.005. Note: TiW= *T. crispa* hot water extract, TiM= *T. crispa* methanol extract, Met= metformin, MGO= methylglyoxal.

4.6.2 Effects of *T. crispa* water or methanol extract alone and in combination with metformin on the IL-6 concentration in MGO-stimulated hFOB 1.19

IL-6 is a cytokine and a key mediator of inflammation and a key regulator of osteoclastogenesis. It stimulates the differentiation of osteoclast precursors into mature osteoclasts, leading to increased bone resorption. Elevated IL-6 levels can contribute to bone loss and conditions such as osteoporosis.

In this study, the effects of MGO, metformin, *T. crispa* water and methanol extracts on the expression level of IL-6 in hFOB 1.19 cells were determined using the Human Magnetic Luminex Multiplex assay. The hFOB 1.19 cells were treated with MGO, metformin, and the hot water extract of *T. crispa*, in 6 well plates and incubated at 34°C for 24 hours. All treatments, except for the control, were treated with MGO. The cell culture supernatants were then collected and analysed for IL-6 concentrations.

In comparison with the control, the expression of IL-6 was significantly upregulated in MGO-stimulated osteoblast cells. Interestingly, the IL-6 levels were not significantly reduced by the addition of metformin ($p < 0.05$). The observed outcomes contrasted from our initial expectations, as it was anticipated that methylglyoxal (MGO) stimulation would lead to a notable increase in IL-6 concentration, whereas the addition of metformin to the MGO-stimulated hFOB 1.19 cells would result in a decrease in IL-6 concentration.

Treatment of MGO-stimulated hFOB 1.19 cells 750 µg/mL *T. crispa* hot water extract alone did not result in reduction of IL-6 levels. However, treatment of MGO-stimulated hFOB 1.19 cells with 750 µg/mL *T. crispa* hot water extract in combination with metformin led to reduction in IL-6 levels as compared to 750 µg/mL *T. crispa* hot water extract alone. Remarkably, treatment with higher concentrations, 1500 µg/mL *T. crispa*

extract and 3000 µg/mL *T. crispa* hot water extract alone or in combination with metformin resulted in a very significant reduction ($p<0.05$) in IL-6 levels when compared to the MGO-stimulated hFOB 1.19 cells alone (Figure 4.15).

Treatment of MGO-stimulated hFOB 1.19 cells with 119 µg/mL and 238 µg/mL *T. crispa* methanol extracts showed a significant reduction in IL-6 levels ($p<0.05$) compared to MGO-stimulated hFOB 1.19 cells alone. The combination of 119 µg/mL *T. crispa* methanol extract with metformin further reduced the IL-6 levels ($p<0.05$). Interestingly, treatment of cells with 238 µg/mL and 476 µg/mL *T. crispa* methanol extracts together with metformin resulted in IL-1 β with values below the lowest detection limit indicating a potential synergistic effects of *T. crispa* methanol extracts and metformin in reducing the levels of IL-6. (Figure 4.15). This could indicate either the near-complete inhibition of IL-6 production or its presence in concentrations so low that they are practically negligible (Figure 4.15).

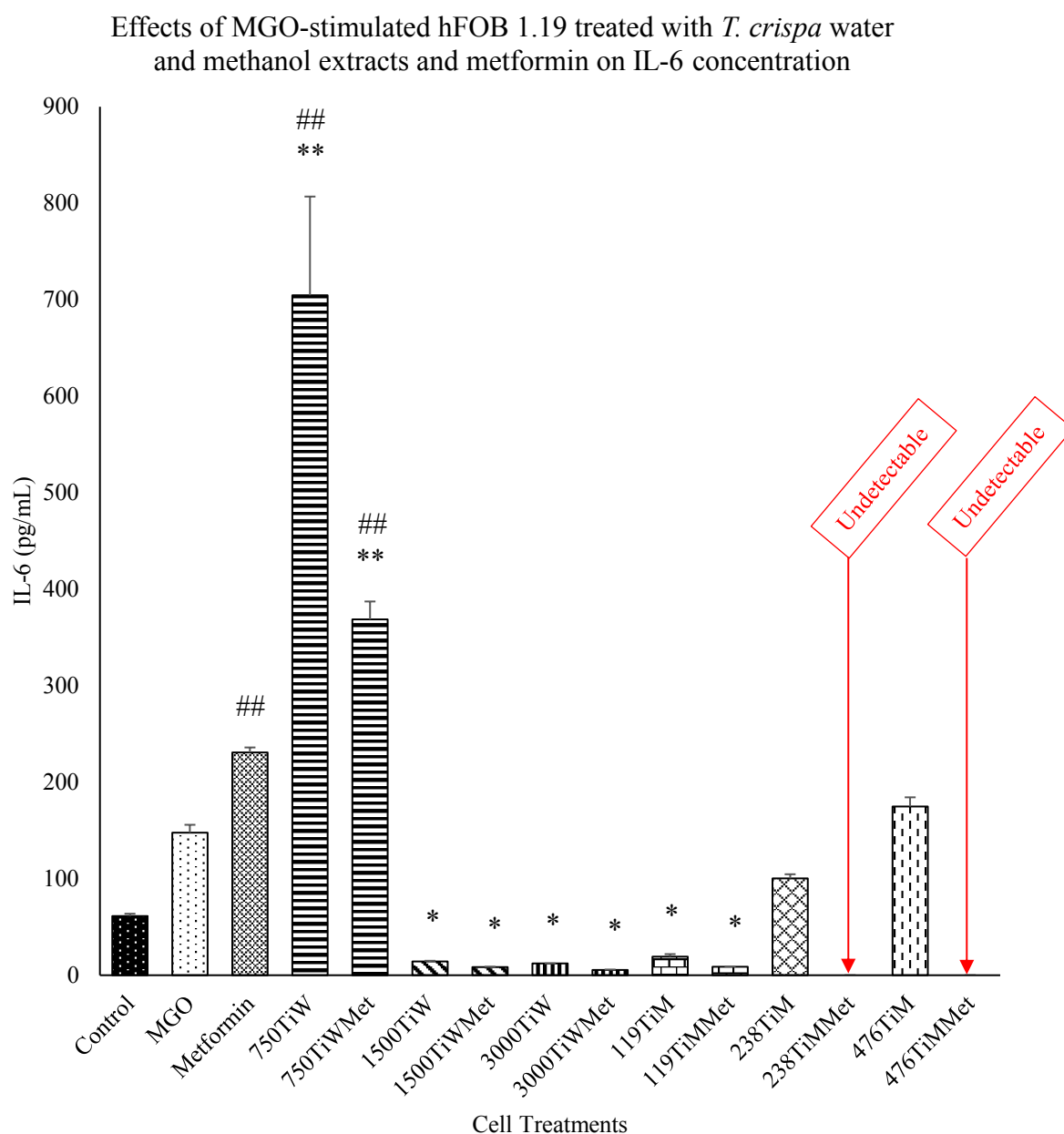


Figure 4.15: Effects of *T. crispa* water or methanol extract alone and in combination with metformin on the expression of IL-6 in MGO-stimulated hFOB 1.19. The cells were incubated with MGO, metformin, and *T. crispa* water and methanol extracts for 24 hours. IL-6 concentrations in the supernatants were measured using the Luminex Assay. Data are presented as mean $\pm$ SD, n=2/group. Significant differences compared to MGO alone were marked with *p<0.05, **p<0.005; compared to the control with #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

4.6.3 Effects of *T. crispa* water or methanol extract alone and in combination with metformin on the M-CSF concentration in MGO-stimulated hFOB 1.19

M-CSF is crucial for the differentiation and survival of osteoclasts. It promotes the proliferation and maturation of osteoclast precursor cells, which are essential for maintaining bone remodelling and homeostasis.

In this study, (Figure 4.16) the treatment of hFOB 1.19 with MGO caused a significant drastic reduction in the levels of M-CSF in the cells compared to the control. However, treatment of MGO-stimulated hFOB 1.19 with metformin alone did not cause increase in M-CSF level. Treatment of MGO-stimulated hFOB 1.19 with 750 µg/mL *T. crispa* hot water extract alone or in combination with metformin did cause increase in M-CSF levels. Notably, treatment with 1500 µg/mL and 3000 µg/mL *T. crispa* hot water extracts significantly increased ($p < 0.05$) the levels of M-CSF. However, there were no significant differences between treatment with *T. crispa* hot water extract alone or in combination with metformin ($p < 0.05$) (Figure 4.16).

For the methanol extract, the concentration of M-CSF in MGO-stimulated hFOB 1.19 cells increased significantly ($p < 0.05$) when treated with 119 µg/mL *T. crispa* methanol extract. However, there was no significance difference in M-CSF when metformin was added together with 119 µg/mL *T. crispa* methanol extract to the MGO-stimulated hFOB 1.19 cells. Interestingly, treatment of MGO-stimulated hFOB 1.19 cells with higher concentrations of methanolic extracts, 238 µg/mL and 476 µg/mL *T. crispa* methanol extract alone or in combination with metformin, did not significantly increase the level of M-CSF as compared to cells treated with MGO alone.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crispa* water and methanol extracts and metformin on M-CSF concentration

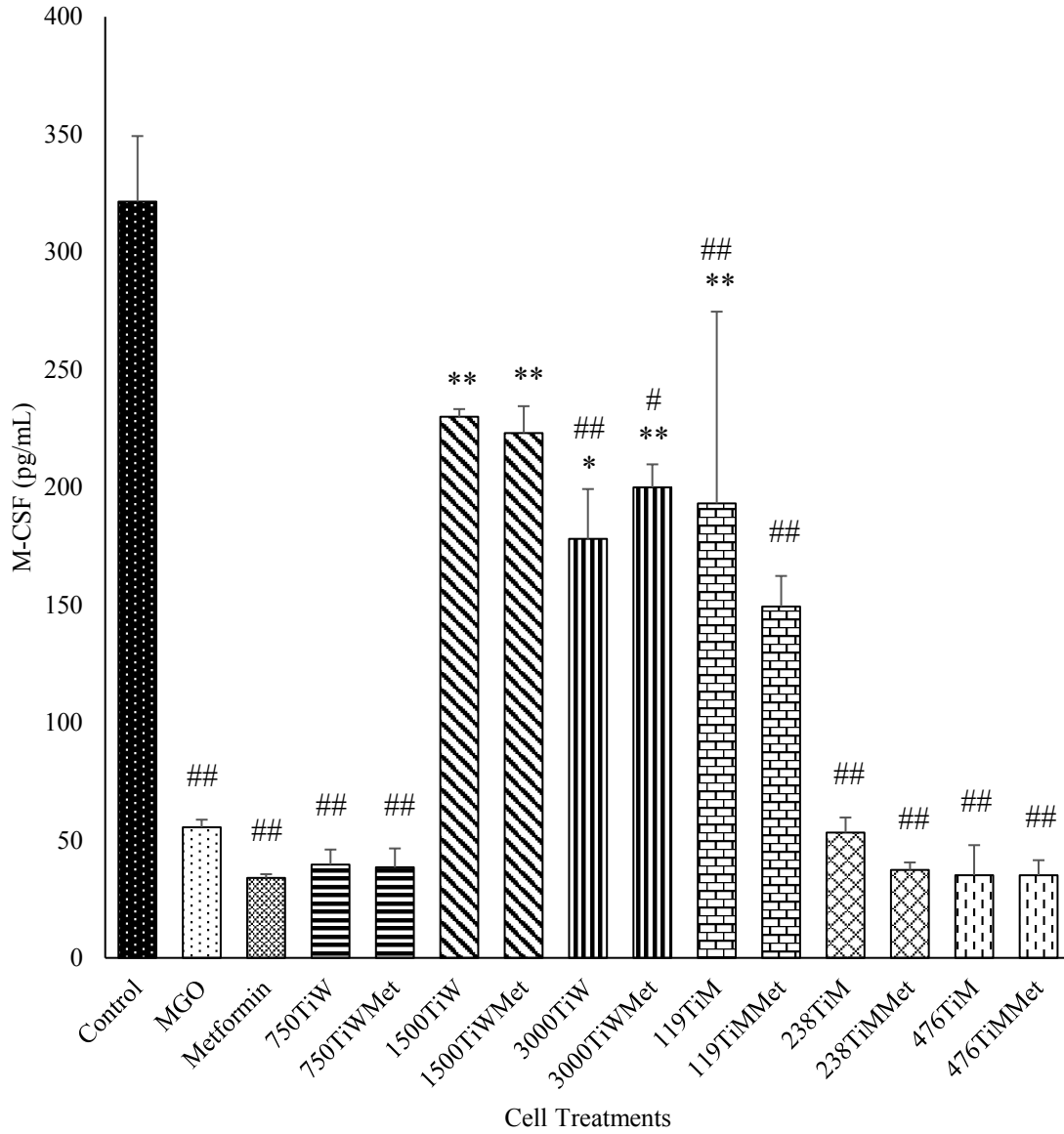


Figure 4.16: Effects of MGO, metformin, *T. crispa* water and methanol extracts on the concentration of M-CSF in hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and the hot water extract of *T. crispa*, in 6-well plate and incubated at 34°C for 24 hours. All treatments included MGO except for the control. M-CSF in the cell supernatants were measured using Luminex Assay. Data were presented as mean $\pm$ SD, n=2/group. Significant differences from the MGO-only treatment are marked by *p<0.05, **p<0.005, and from the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

4.7 Enzyme-linked Immunosorbent Assay (ELISA) for sRAGE, flRAGE and RANKL

4.7.1 Effects of *T. crispa* water or methanol extracts alone and in combination with metformin, on soluble RAGE (sRAGE) concentration in MGO-stimulated hFOB 1.19 cells

The investigation into the effects of *T. crispa* water and methanol extracts, both individually and in combination with metformin, on the soluble receptor for advanced glycation end-products (sRAGE) concentration in MGO-stimulated hFOB 1.19 osteoblast cells was conducted using sRAGE ELISA analysis. Soluble RAGE (sRAGE) is a naturally occurring isoform of the RAGE receptor, which act as a decoy that interact with various signalling pathways. Its role is crucial as it can influence cellular activities involved in bone formation and resorption. Elevated sRAGE can be beneficial, as sRAGE can act as a decoy receptor by binding to advanced glycation end-products (AGEs), preventing reducing their interaction with cell surface bound AGE, hence mitigating inflammation and restoring balance in bone homeostasis.

In the untreated control group, where hFOB 1.19 osteoblasts were not stimulated with MGO, the mean sRAGE concentration was measured at 33.29 ± 1.62 pg/mL. This value serves as a baseline reference for subsequent comparisons. Upon MGO stimulation, the sRAGE concentration modestly increased, but significantly from 33.29 ± 1.62 pg/mL to 47.92 ± 1.68 pg/mL. However, when metformin was introduced to MGO-stimulated hFOB 1.19, the sRAGE concentration increased to 50.24 ± 1.05 pg/mL, but no significant difference. The increase indicates that more decoy RAGE bind with AGEs, thereby reducing AGE interaction with membrane-bound RAGE, which could help in restoring the balance in bone remodelling.

Treatment of MGO-stimulated hFOB 1.19 with non-toxic concentrations of *T. crispa* hot water extracts at 750, 1500 and 3000 $\mu\text{g/mL}$ also led to a significant increase in sRAGE concentration., to 51.58 ± 4.34 pg/mL, 54.45 ± 4.19 pg/mL, and 56.72 ± 2.66 pg/mL respectively (Figure 4.17). The combined treatment of metformin together with *T. crispa* hot water extracts at 750, 1500 and 3000 $\mu\text{g/mL}$ increased the sRAGE concentration significantly to 49.21 ± 0.98 pg/mL, 60.28 ± 6.71 pg/mL, and 63.79 ± 4.54 pg/mL respectively

Similarly, treatment of MGO-stimulated hFOB 1.19 with non-toxic concentrations of *T. crispa* methanol extracts at 119, 238 and 476 $\mu\text{g/mL}$ also led to a significant increase in sRAGE concentration to 54.21 ± 0.77 pg/mL, 57.32 ± 2.24 pg/mL, and 60.24 ± 5.66 pg/mL respectively (Figure 4.17). While the combined treatment of metformin together with TiM extracts at 119, 238 and 476 $\mu\text{g/mL}$ significantly increased the sRAGE concentration to 54.50 ± 0.07 pg/mL, 63.553 ± 4.33 pg/mL, and 59.880 ± 8.67 pg/mL (Figure 4.17).

These results suggest that there are significant differences when *T. crispa* extract alone or together with metformin were incubated together with the MGO-stimulated hFOB 1.19 cells. The increasing trend in sRAGE concentration under these treatment conditions indicates a greater capacity for sRAGE to bind AGEs, thereby reducing their interaction with surface-bound RAGE. that more of these sRAGE will bind to the AGE, hence reducing the binding of AGE to the surface bound RAGE. This effect is likely to mitigate the harmful consequences of MGO-induced stress on bone remodelling.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crispa* water and methanol extracts and metformin on sRAGE concentration

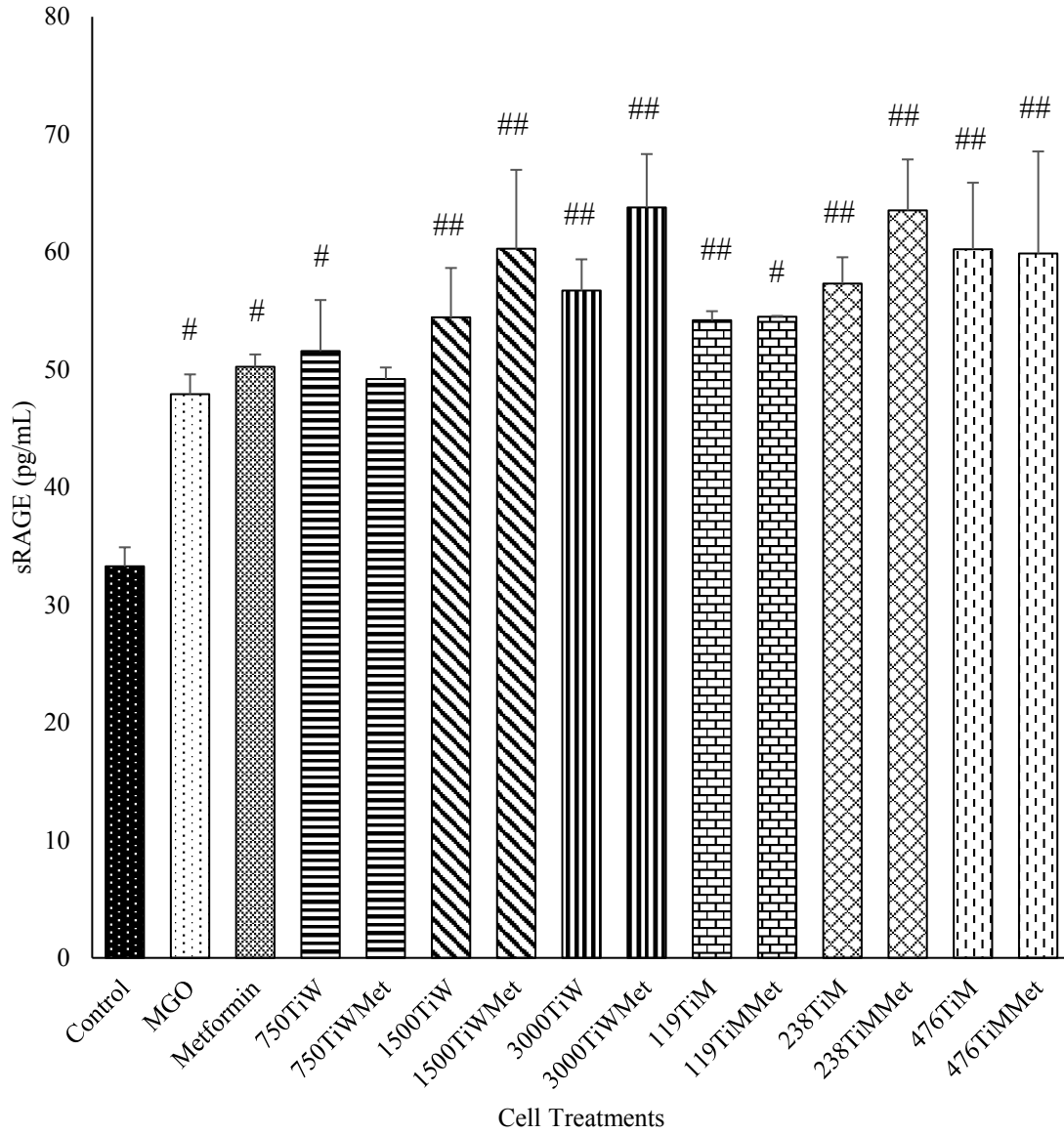


Figure 4.17: Effects of MGO, metformin, *T. crispa* water and methanol extracts on the sRAGE levels in hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except for the control. Cell culture supernatants were collected and analysed for sRAGE using ELISA. Data were presented as mean $\pm$ SD, n=2/group. Significant differences from the MGO-only treatment are indicated by *p<0.05, **p<0.005, and from the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

4.7.2 Effects of *T. crispa* water or methanol extract alone and in combination with metformin on the full-length RAGE (fRAGE) concentration in MGO-stimulated hFOB 1.19

The full-length receptor for advanced glycation end-products (fRAGE) denotes a form of RAGE protein anchored to the cell membrane. This receptor is present on various cell types including osteoblast cells. fRAGE plays a crucial role in bone homeostasis, with its excessive binding to advanced glycation end-products (AGEs) potentially disrupting bone remodelling, promoting osteoclast proliferation, and impairing bone matrix synthesis (Otta et al., 2014). In this study, RAGE ELISA analysis was carried out to evaluate the effects of treatments with *T. crispa* alone and in combination with metformin on the concentration of RAGE in MGO-stimulated hFOB 1.19.

In the untreated control group, where osteoblasts were not exposed to MGO, the mean fRAGE concentration was measured at 34.26 ± 0.81 pg/mL, serving as a baseline for comparison. Upon MGO stimulation, the fRAGE concentration was increased to 35.93 ± 1.52 pg/mL, which is not significant when compared to control. The addition of metformin to MGO-stimulated hFOB 1.19 led to a slight decrease in the membrane-bound fRAGE concentration to 32.79 ± 0.10 pg/mL, but not significant.

Treatment of MGO-stimulated hFOB 1.19 with non-toxic concentrations of *T. crispa* hot water extracts at 750, 1500 and 3000 μ g/mL did not significantly reduce the fRAGE concentration, which measured 30.86 ± 1.01 pg/mL, 31.79 ± 0.30 pg/mL, and 28.79 ± 1.92 pg/mL for *T. crispa* hot water extract respectively, as compared to control and MGO treatment alone. The combined treatment of metformin together with *T. crispa* hot water extracts with concentrations 750, 1500 and 3000 μ g/mL reduced the RAGE concentration

to 30.79 ± 2.93 pg/mL (not significant), 25.43 ± 0.81 pg/mL (significant), and 27.86 ± 2.42 pg/mL (not significant)

Similarly, treatment of MGO-stimulated hFOB 1.19 with non-toxic concentrations of *T. crista* methanol extracts at 119, 238 and 476 μ g/mL resulted in fIRAGE concentrations of 35.14 ± 3.33 pg/mL, 33.50 ± 0.71 pg/mL, and 32.64 ± 0.81 pg/mL respectively (Figure 4.18). When combined with metformin, *T. crista* methanol extract at 476 μ g/mL, significantly reduced fIRAGE to 25.36 ± 3.74 pg/mL if compared to MGO-treated cells only. On the other hand, combined treatment of metformin with *T. crista* methanol extracts at 119 and 238 μ g/mL insignificantly reduced the RAGE concentration to 30.78 ± 2.53 pg/mL and 32.79 ± 0.10 pg/mL (Figure 4.18). These results suggest that there are no significant differences when *T. crista* extract alone or together with metformin were added to the MGO-stimulated hFOB 1.19 cells.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crispa* water and methanol extracts and metformin on fIRAGE concentration

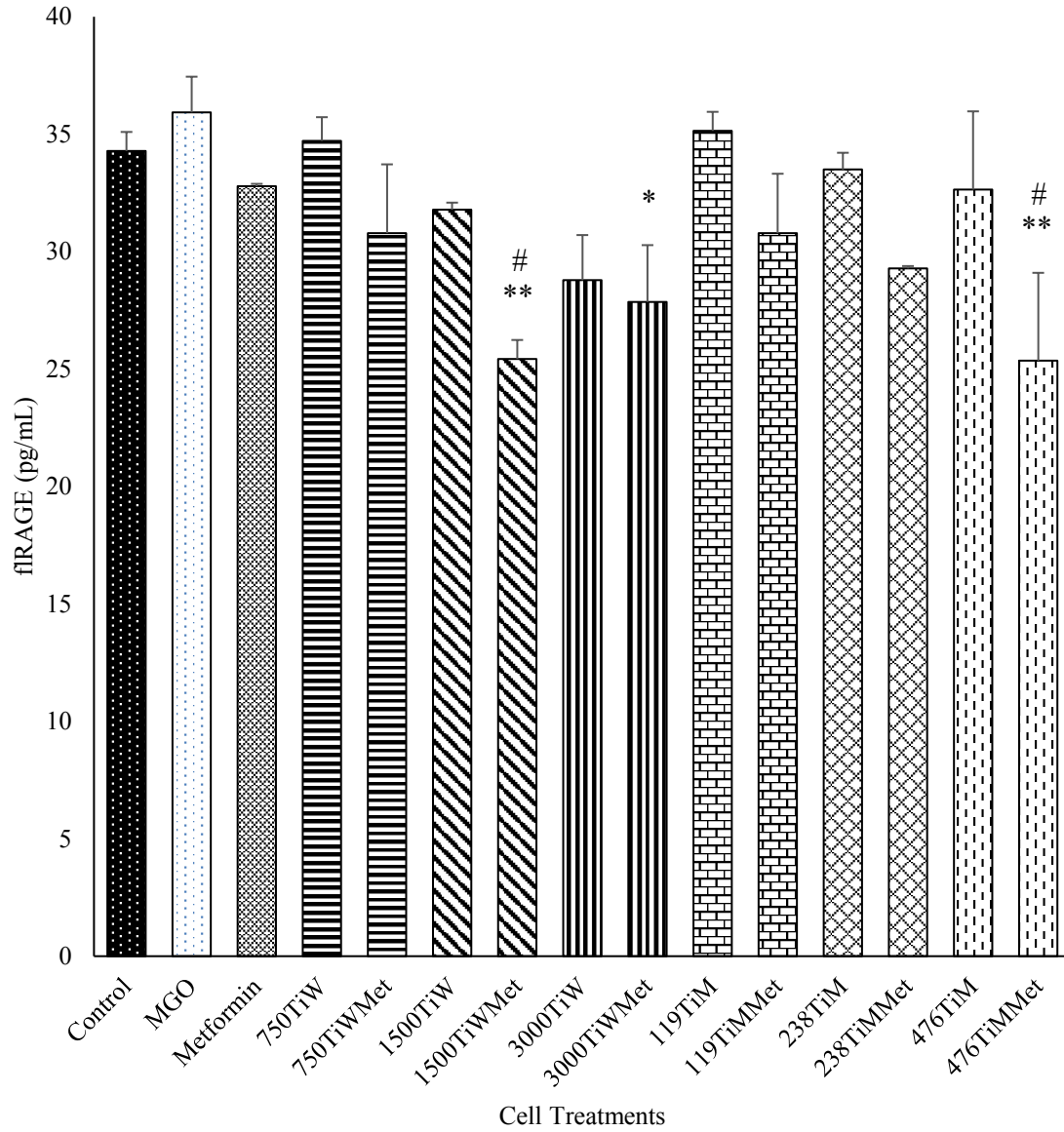


Figure 4.18: Effects of MGO, metformin, *T. crispa* water and methanol extracts on fIRAGE levels in hFOB 1.19 cells. The cells were treated with MGO, metformin, and *T. crispa* extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except for the control. Cell culture supernatants were collected and analysed for fIRAGE levels with ELISA. Data were presented as mean $\pm$ SD, n=2/group. Significant differences from the MGO-only treatment are indicated by *p<0.05, **p<0.005, and from the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

4.7.3 Effects of *T. crispa* water or methanol extract alone and in combination with metformin on the RANKL concentration in MGO-stimulated hFOB 1.19

Receptor Activator of Nuclear Factor κ B Ligand (RANKL) is a protein that belongs to the TNF superfamily, which plays a pivotal role in the regulation of bone remodelling and the immune system function. RANKL is primarily produced by osteoblasts and is a critical cytokine in osteoclastogenesis and bone remodelling, making it a key marker in the study of bone health. This section presents the findings from the RANKL ELISA analysis which aimed to investigate the effects of *T. crispa* extracts alone and in combination with metformin on MGO-stimulated hFOB 1.19.

In the untreated control group, consisting of osteoblasts not subjected to MGO stimulation, the mean RANKL concentration was measured at 82.75 ± 2.72 pg/mL. This value serves as a baseline reference point for subsequent comparisons. Upon MGO stimulation, the RANKL concentration significantly increased to 120.34 ± 0.00 pg/mL, indicating the activation of osteoclastogenic signalling in response to MGO exposure. Conversely, the addition of metformin to MGO-stimulated hFOB 1.19 cells led to a significant reduction in RANKL concentration, which measured at 61.40 ± 0.00 pg/mL, indicating the suppression of osteoclastogenesis.

Meanwhile, the treatment with non-toxic concentrations of *T. crispa* extracts at 750, 1500 and 3000 μ g/mL significantly reduced ($p < 0.05$) RANKL expression in MGO-stimulated hFOB 1.19 cells, resulting in values of 69.39 ± 1.29 pg/mL, 64.63 ± 0.00 pg/mL, and 64.63 ± 1.86 pg/mL respectively. The treatment of MGO-stimulated hFOB 1.19 cells with both *T. crispa* and metformin did not significantly decrease the RANKL concentration if compared to those treated with *T. crispa* extract alone. The resulting RANKL concentration after being treated with *T. crispa* hot water extracts with concentrations 750, 1500 and 3000

µg/mL combined with metformin were measured at 65.64 ± 0.86 pg/mL, 62.41 ± 5.14 pg/mL, and 61.81 ± 4.00 pg/mL respectively.

Similarly, treatment of MGO-stimulated hFOB 1.19 with non-toxic concentrations of *T. crista* methanol extracts at 119, 238 and 476 µg/mL resulted in significant decreased in RANKL levels measured at 1.81 ± 6.44 pg/mL, 67.89 ± 0.60 pg/mL, and 65.950 ± 5.00 pg/mL respectively (Figure 4.19). While the combined treatment of metformin together with *T. crista* methanol extracts at 119, 238 and 476 µg/mL resulted in RANKL concentration measured at 63.83 ± 2.71 pg/mL, 62.01 ± 0.57 pg/mL, and 61.09 ± 1.00 pg/mL respectively (Figure 4.19). These results suggest that *T. crista* and metformin did not work synergistically to reduce RANKL concentration.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crista* water and methanol extracts and metformin on RANKL concentration

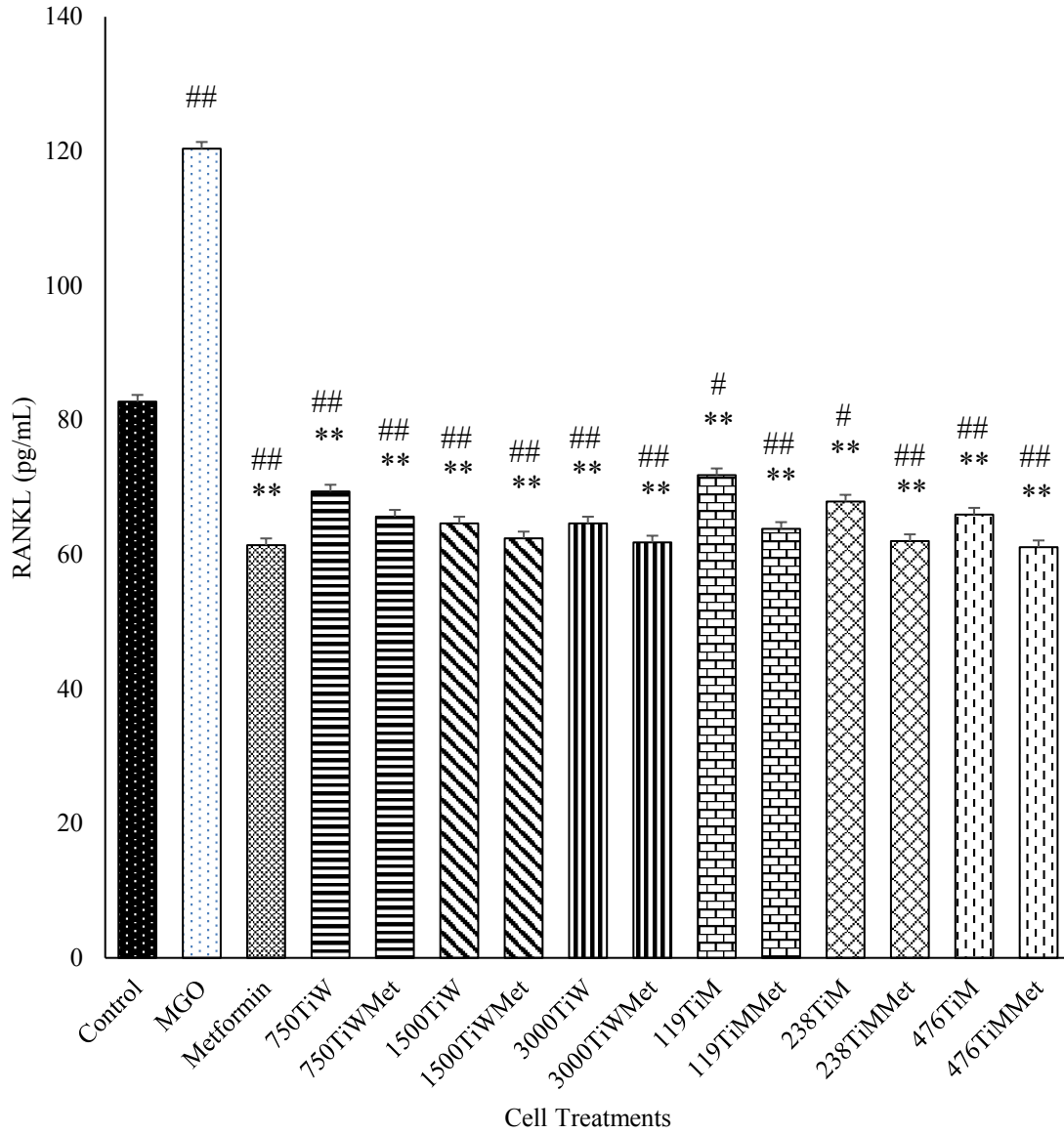


Figure 4.19: Effects of MGO, metformin, *T. crista* water and methanol extracts on RANKL levels in hFOB 1.19 cells. The hFOB 1.19 cells were treated with MGO, metformin, and *T. crista* extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except for the control. Cell culture supernatants were collected and analysed for RANKL concentration using ELISA. Data are presented as mean $\pm$ SD, n=2/group. Significant differences from the MGO-only treatment are indicated by *p<0.05, **p<0.005, and from the control by #p<0.05, ##p<0.005. Note: TiW = *T. crista* hot water extract, TiM = *T. crista* methanol extract, Met = metformin, MGO = methylglyoxal.

4.8 Analysis of hFOB 1.19 Cell Morphology: Quantification of Area Density, Volume Density, Feret Diameters, Perimeter, Ellipticity, and Circularity Indices

The morphological characteristics of hFOB 1.19 cells provide critical insights into their structural integrity, growth patterns, and response to experimental conditions. This section quantifies key morphological parameters to evaluate cellular shape, size, and spatial distribution. The parameters analysed include area density, volume density, Feret diameters, perimeter, ellipticity, and circularity indices, which are defined in the following table:

Table 4.5: Definitions of morphological parameters analysed in hFOB 1.19 cells.

Term	Description
Area Density	The proportion of space occupied by cells on a flat surface.
Volume Density	The proportion of space occupied by cells in a three-dimensional area.
Minimum Feret Diameter	The shortest width of the cell.
Maximum Feret Diameter	The longest length of the cell.
Perimeter	The total length around the edge of the cell.
Ellipticity Index	A measure of how stretched or oval-shaped the cell is.
Circularity Index	A measure of how close the cell is to being a perfect circle (1 = perfect circle).

Figure 4.20 depicts hFOB 1.19 cells treated with IC_{50} of MGO to assess their morphological changes. In the image, three distinct cell morphologies are identified: normal cells, intact apoptotic cells, and disintegrated apoptotic cells. The normal hFOB 1.19 cells, marked within a green rectangle, appear elongated with an intact cell structure. The intact

apoptotic cells show signs of early apoptosis, with condensation of the cytoplasm and membrane blebbing, while the disintegrated apoptotic cells exhibit more advanced apoptosis, with significant fragmentation and breakdown of cell structure. These observations highlight the damaging effects of MGO on hFOB 1.19 cell viability, leading to increased apoptosis, which was visualised under brightfield microscopy at 20X magnification.

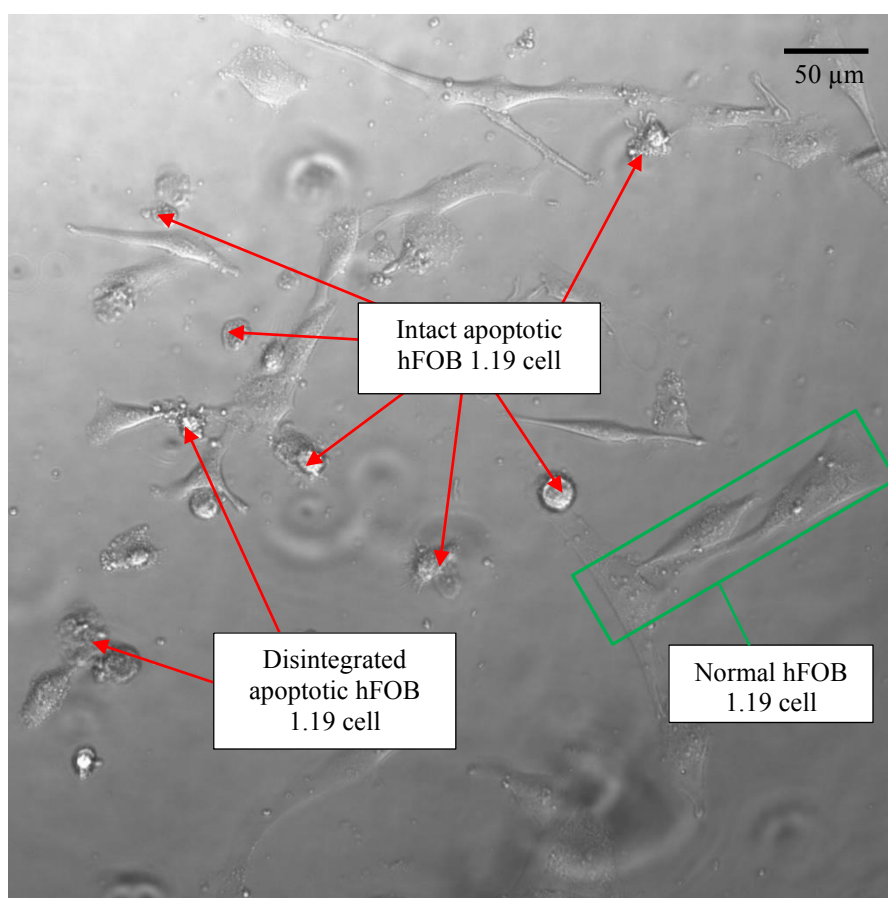


Figure 4.20: Effects of IC₅₀ MGO treatment on hFOB 1.19 cell morphology. hFOB 1.19 cells were treated with MGO (except for control) in 6 well-plates (9.0×10^5 cells/mL) and incubated at 34°C for 24 hours. After incubation, the cells were observed at 20X magnification using inverted microscope in brightfield with images captured by the microscope's software. The figure shows normal, intact apoptotic and disintegrated apoptotic hFOB 1.19 as labelled.

4.8.1 Impact of *T. crista* extracts (water and methanol) and their combination with metformin on the area density of MGO-stimulated hFOB 1.19 cells

In this study, the effect of various treatments on the area density of MGO-stimulated hFOB 1.19 cells was examined. Area density serves as an indicator of cell health, with the higher the area density of the cells reflecting the better cellular conditions of the cells. Compared to the control group (hFOB 1.19 cells without MGO stimulation or any treatment), the MGO-stimulated hFOB 1.19 cells showed a significant reduction in area density dropping from $95.43 \pm 1.35\%$ to $46.74 \pm 1.91\%$. However, when metformin was added to the MGO-stimulated hFOB 1.19, there was a notable improvement in area density, which increased significantly from $46.74 \pm 1.91\%$ to $56.03 \pm 8.57\%$.

When MGO-stimulated hFOB 1.19 cells were treated with hot water extract only with concentrations of 750 $\mu\text{g/mL}$, 1500 $\mu\text{g/mL}$, 3000 $\mu\text{g/mL}$, the area density of hFOB 1.19 increased significantly from $46.74 \pm 1.91\%$ to $74.93 \pm 2.36\%$, $57.69 \pm 8.27\%$, and $62.19 \pm 2.58\%$ respectively. Then metformin was added to each of the extracts to test out their potential synergistic effect with the *T. crista* hot water extracts. When metformin was combined with *T. crista* extracts at concentrations of 750 $\mu\text{g/mL}$, 1500 $\mu\text{g/mL}$, 3000 $\mu\text{g/mL}$, the area densities of the cells were $69.12 \pm 1.05\%$, $74.93 \pm 2.97\%$ and $23.89 \pm 1.39\%$ respectively. Treatment of MGO-stimulated hFOB 1.19 cells with metformin and 750 $\mu\text{g/mL}$ of *T. crista* hot water extract showed synergistic effect as there was increased in area density, from $57.69 \pm 8.27\%$ to $74.93 \pm 2.97\%$.

Similarly, treatment with *T. crista* methanol extract alone with concentrations of 119 $\mu\text{g/mL}$, 238 $\mu\text{g/mL}$ and 476 $\mu\text{g/mL}$ led to significant increase in area density from $46.74 \pm 1.91\%$ to $62.43 \pm 7.33\%$, $56.00 \pm 3.48\%$, and $58.99 \pm 0.46\%$ respectively.

On the other hand, when metformin was added together with 119 $\mu\text{g/mL}$, 238 $\mu\text{g/mL}$, and 476 $\mu\text{g/mL}$ of methanol extracts to MGO-stimulated hFOB 1.19 cells, the area density of the cells increased from $46.74 \pm 1.91\%$ to $70.17 \pm 16.65\%$ (not significant), $86.79 \pm 3.54\%$ (significant), and $52.37 \pm 20.47\%$ (not significant) respectively.

This study showed that *T. crista* water and methanol extracts, when used alone, led to a notable increase in area density. The combination of metformin with *T. crista* extracts generally enhanced area density further, particularly at lower concentrations of the extracts. This synergistic effect was especially evident with the 1500 $\mu\text{g/mL}$ concentrations of the hot water extract and the 119 $\mu\text{g/mL}$ and 238 $\mu\text{g/mL}$ concentrations of the methanol extract. However, higher concentrations of *T. crista* extracts combined with metformin resulted in a decrease in area density, suggesting that the synergistic effect might diminish at higher extract concentrations. Additionally, it is notable that the area density of MGO-stimulated hFOB 1.19 cells treated with the extracts and metformin did not fully return to baseline levels observed in the untreated (negative control) group.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crispa* water and methanol extracts and metformin on the area density of the cells

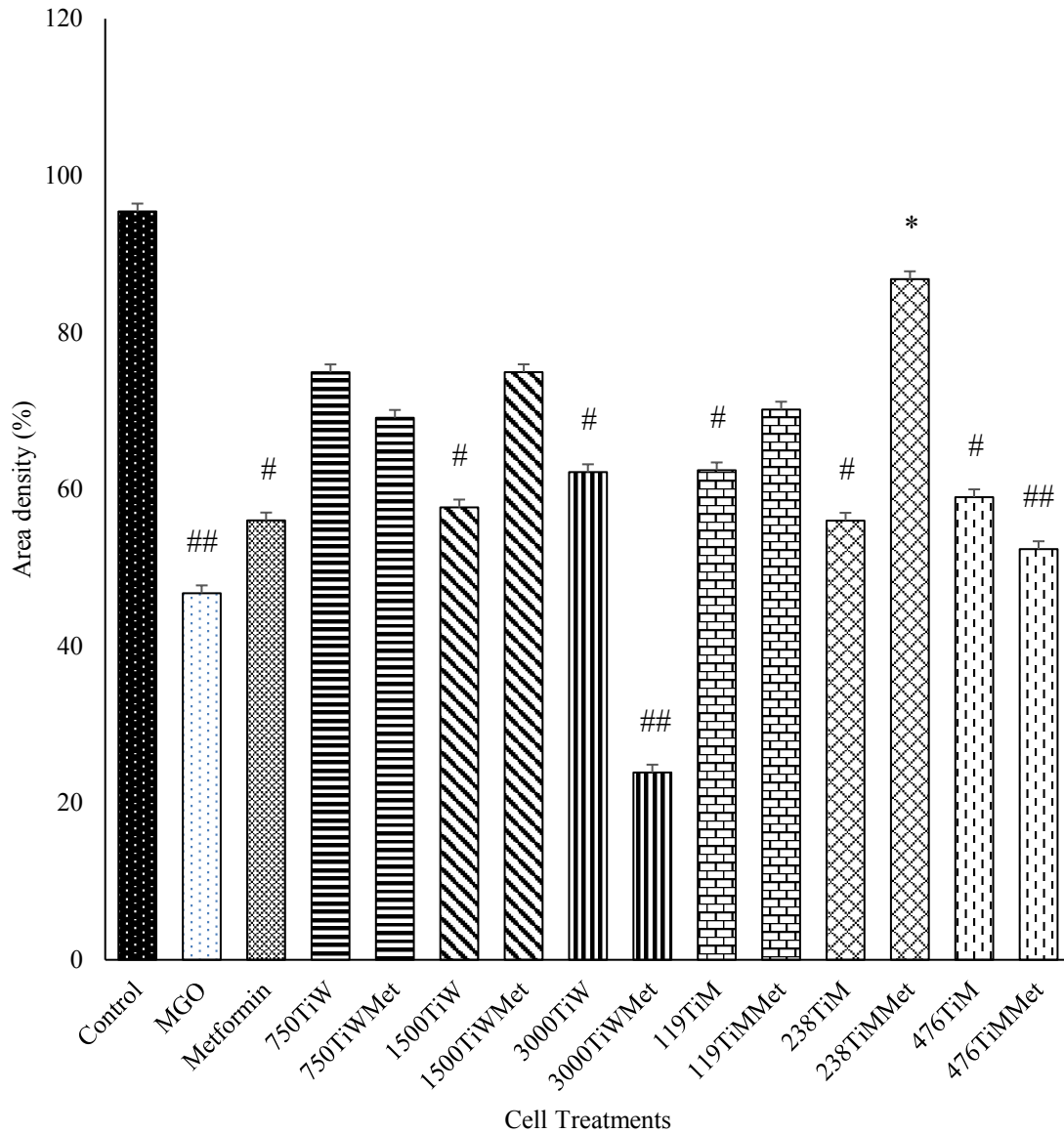


Figure 4.21: Effects of MGO, metformin, and *T. crispa* extracts on hFOB 1.19 cells area density. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water and methanol extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO, except for the control. After incubation, the cells were viewed at 20X magnification using an inverted microscope, and area density was quantified. Data are presented as mean% $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

4.8.2 Impact of *T. crispata* extracts (water and methanol) and their combination with metformin on volume density of hFOB 1.19 cells

In this section, the effects of various treatment on MGO-stimulated hFOB 1.19 cells volume density was investigated. Volume density is an important indicator of cell health, with higher values reflecting better cellular conditions. Compared to the control group (hFOB 1.19 cells without MGO stimulation or any treatment), MGO-stimulated hFOB 1.19 cells showed a significant reduction in volume density dropping from $97.77 \pm 0.47\%$ to $68.89 \pm 6.93\%$. While the addition of metformin to the MGO-stimulated hFOB 1.19 cells did increase volume density of healthy cells to $72.60 \pm 9.04\%$, although this change was not statistically significant.

When MGO-stimulated hFOB 1.19 cells were treated with *T. crispata* hot water extract with concentrations of 750 $\mu\text{g/mL}$, 1500 $\mu\text{g/mL}$, 3000 $\mu\text{g/mL}$ only, the volume density of hFOB 1.19 increased from $68.89 \pm 6.93\%$ to $90.03 \pm 4.01\%$, $77.70 \pm 7.77\%$, and $80.19 \pm 1.86\%$ respectively. This is not significant if compared to the hFOB 1.19 cells stimulated with MGO only. To test out the potential synergistic effect of the *T. crispata* extracts with a drug, metformin was added. When metformin was added together with *T. crispata* hot water extract to MGO-stimulated hFOB 1.19 cells, the volume density of the cells were 84.14 ± 1.49 , $81.46 \pm 3.44\%$ for 750 $\mu\text{g/mL}$ and 1500 $\mu\text{g/mL}$ respectively but decreased to $42.05 \pm 1.07\%$ for 3000 $\mu\text{g/mL}$, all of which were not significant to the area density of MGO-stimulated hFOB 1.19 cells.

When MGO-stimulated hFOB 1.19 cells were treated with *T. crispata* methanol extract alone with concentrations of 119 $\mu\text{g/mL}$, 238 $\mu\text{g/mL}$ and 476 $\mu\text{g/mL}$, there were significant increase in the area density of the cells to $81.93 \pm 3.72\%$, $80.45 \pm 6.77\%$, and $80.89 \pm 1.68\%$ respectively. Interestingly, when metformin was added together with *T. crispata* methanol

extract of 119 $\mu\text{g/mL}$, 238 $\mu\text{g/mL}$ and 476 $\mu\text{g/mL}$ to MGO-stimulated hFOB 1.19 cells, the area density of the cells increased to $88.45 \pm 7.77\%$, $95.55 \pm 3.53\%$, and $76.07 \pm 19.75\%$ respectively. When comparing all treatments, excluding the MGO-treated hFOB 1.19 cells, with the control group, only *T. crista* hot water extract at 3000 $\mu\text{g/mL}$ was unable to mitigate the decrease in volume density of hFOB 1.19 cells caused by exposure to the IC_{50} concentration of MGO.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crispa* water and methanol extracts and metformin on the volume density of the cells

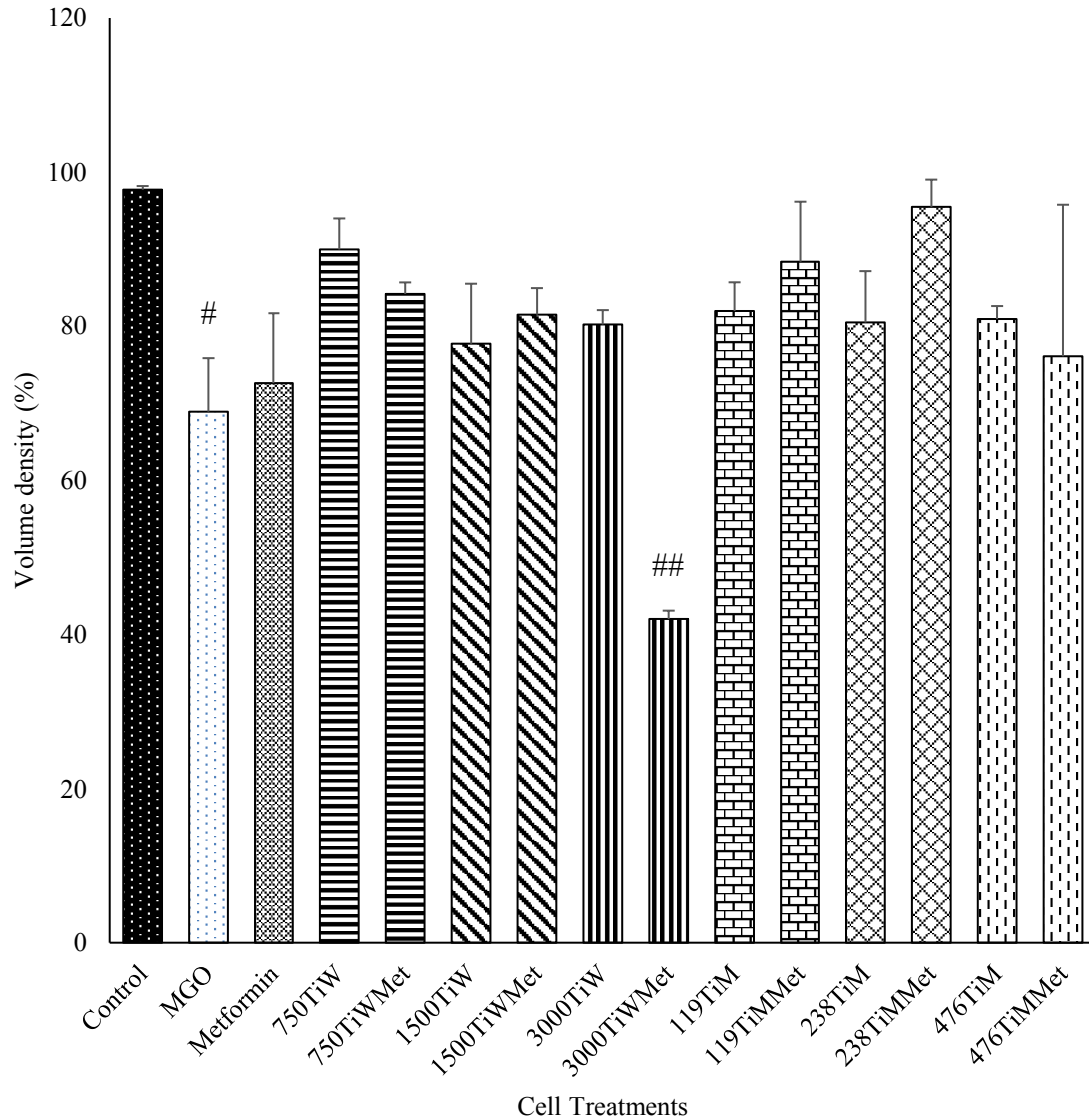


Figure 4.22: Effects of MGO, metformin, *T. crispa* extracts on hFOB 1.19 volume density. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except the control. After 24 hours, cells were examined at 20X magnification, and volume density was measured. Data are presented as mean% $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

4.8.3 Impact of *T. crispa* water or methanol extract alone and in combination with metformin on the minimum and maximum Feret diameter of hFOB 1.19 cells

The longest minimum Feret diameter of hFOB 1.19 cells is 23.26 ± 2.13 μm from the control group while the shortest minimum Feret measured is 15.41 ± 0.97 μm from the treatment with 476 $\mu\text{g/mL}$ of *T. crispa* methanol extract. This measurement represents the smallest distance between two parallel lines that can encompass a healthy cell. It is a crucial parameter for assessing the cell's size, elongation and can be used to calculate the ellipticity index of cells.

On the other hand, the longest maximum Feret diameter of hFOB 1.19 cells is 55.38 ± 10.38 μm from the 1500 $\mu\text{g/mL}$ of *T. crispa* hot water extract in combination with metformin group while the shortest minimum Feret measured is 21.58 ± 1.29 μm from the 3000 $\mu\text{g/mL}$ of *T. crispa* hot water extract in combination with metformin. This measurement represents the longest distance between two parallel lines that can encompass a healthy cell. It is a crucial parameter for assessing the cell's size, elongation and can be used to calculate the ellipticity index of cells.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crispa* water and methanol extracts and metformin on the minimum Feret diameter of the cells

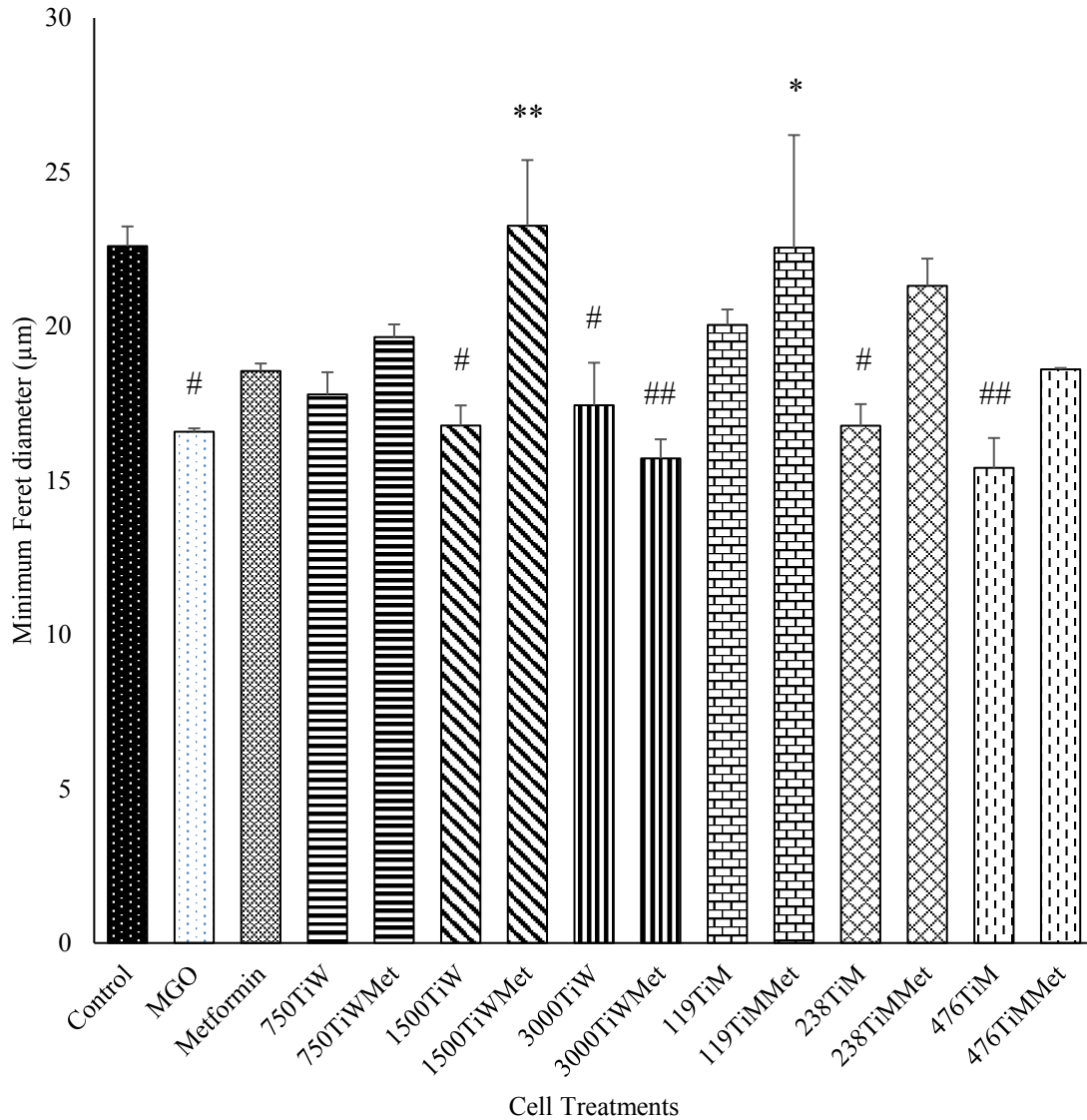


Figure 4.23: Effects of MGO, metformin, and *T. crispa* extracts on Minimum Feret Diameter of hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except the control. Cells were examined at 20X magnification, and minimum Feret diameter was measured. Data are presented as mean $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crispa* water and methanol extracts and metformin on the maximum Feret diameter of the cells

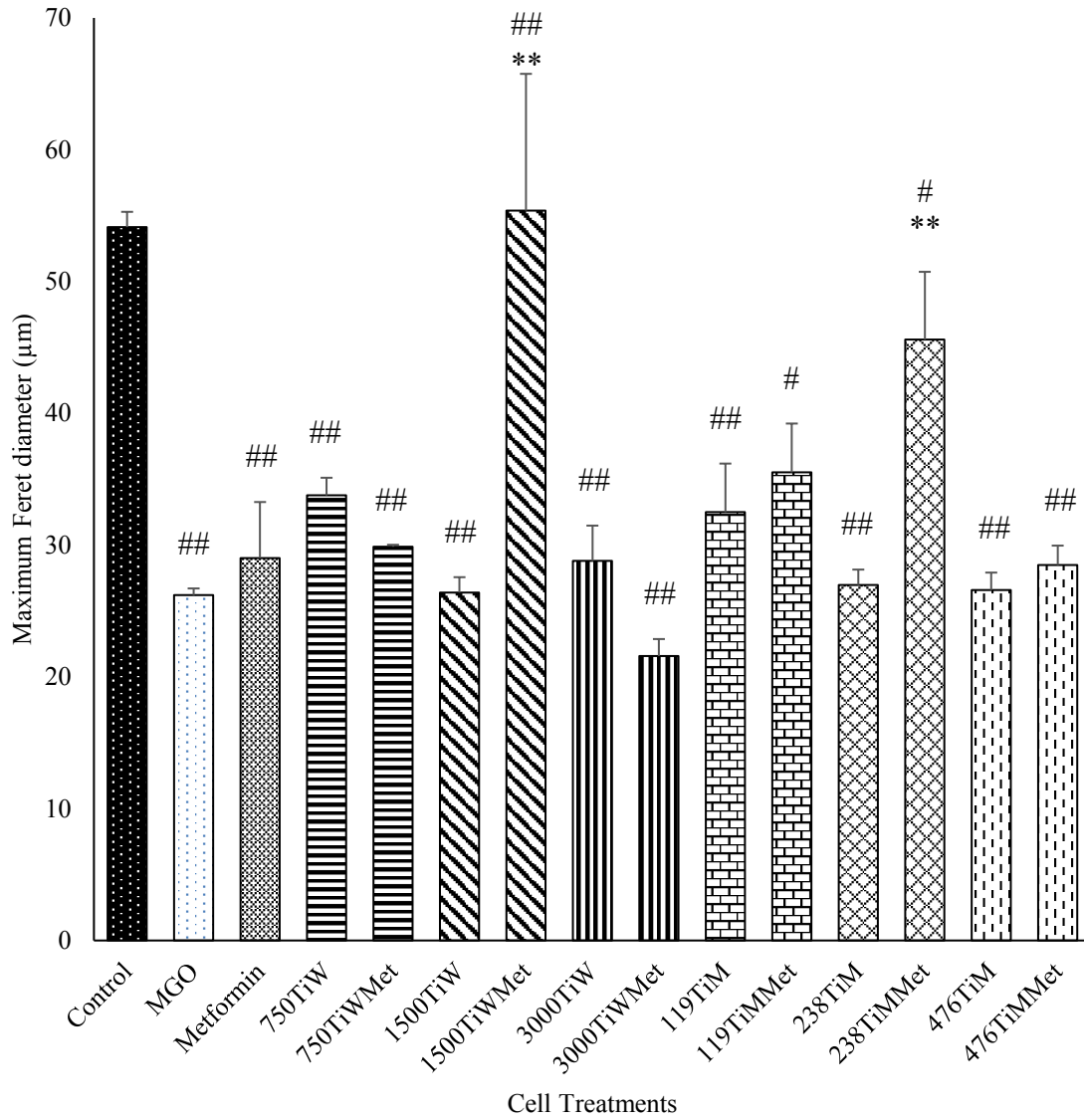


Figure 4.24: Effects of MGO, metformin, and *T. crispa* extracts on Maximum Feret Diameter of hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except the control. Cells were examined at 20X magnification, and maximum Feret diameter was measured. Data are presented as mean $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

4.8.4 Impact of *T. crispa* water or methanol extract alone and in combination with metformin on the perimeter of cells of hFOB 1.19 cells

The perimeter of cells quantifies the total length of the boundary surrounding each cell, one of the most important values to calculate the cell's circularity index. The longest mean perimeter of hFOB 1.19 cells was $130.38 \pm 21.46 \mu\text{m}$ which belongs to the $1500 \mu\text{g/mL}$ of *T. crispa* hot water extract in combination with metformin while the shortest was $68.00 \pm 3.52 \mu\text{m}$ from the treatment $476 \mu\text{g/mL}$ of *T. crispa* methanol extract. In addition, the mean parameter of hFOB 1.19 treated with MGO was measured at $68.78 \pm 1.03 \mu\text{m}$.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crispata* water and methanol extracts and metformin on the perimeter of the cells

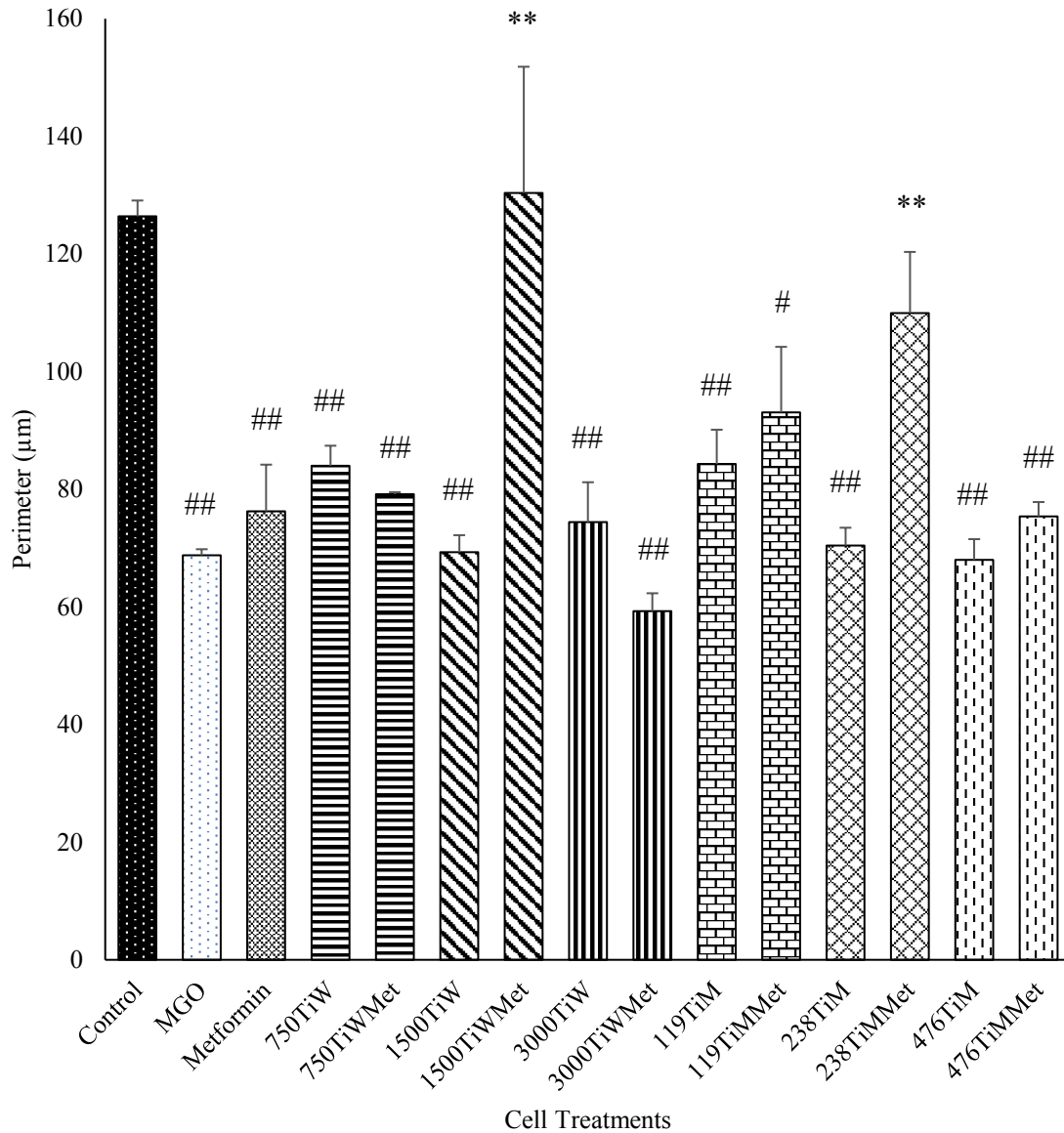


Figure 4.25: Effects of MGO, metformin, and *T. crispata* extracts on Cell Perimeter of hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispata* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except for the control. Cells were viewed at 20X magnification, and the perimeter was measured. Data are presented as mean $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispata* hot water extract, TiM = *T. crispata* methanol extract, Met = metformin, MGO = methylglyoxal.

4.8.5 Impact of *T. crista* water or methanol extract alone and in combination with metformin on the ellipticity index of hFOB 1.19 cells

The ellipticity index quantifies the degree of elongation or the ovality of cells. A higher ellipticity index suggests a more elongated cell shape, while a lower ellipticity index indicates a rounder shape. A value of 0 suggests that the cell shape is closer to a circle, while higher values indicate a more pronounced deviation from circularity, implying a more elongated or elliptical shape. Since osteoblasts are fibroblast-like, a more elongated osteoblast is considered as healthy osteoblast. The ellipticity index of control group is 2.53 ± 0.01 which is higher than most of the treatment groups except for $1500 \mu\text{g/mL}$ *T. crista* hot water extract in combination with metformin which has an ellipticity index of 2.67 ± 0.29 . The ellipticity index for the rest of the treatment group were closer to the value of 0 which might imply that the cells may not have been in optimal shape or condition.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crispa* water and methanol extracts and metformin on the ellipticity index of the cells

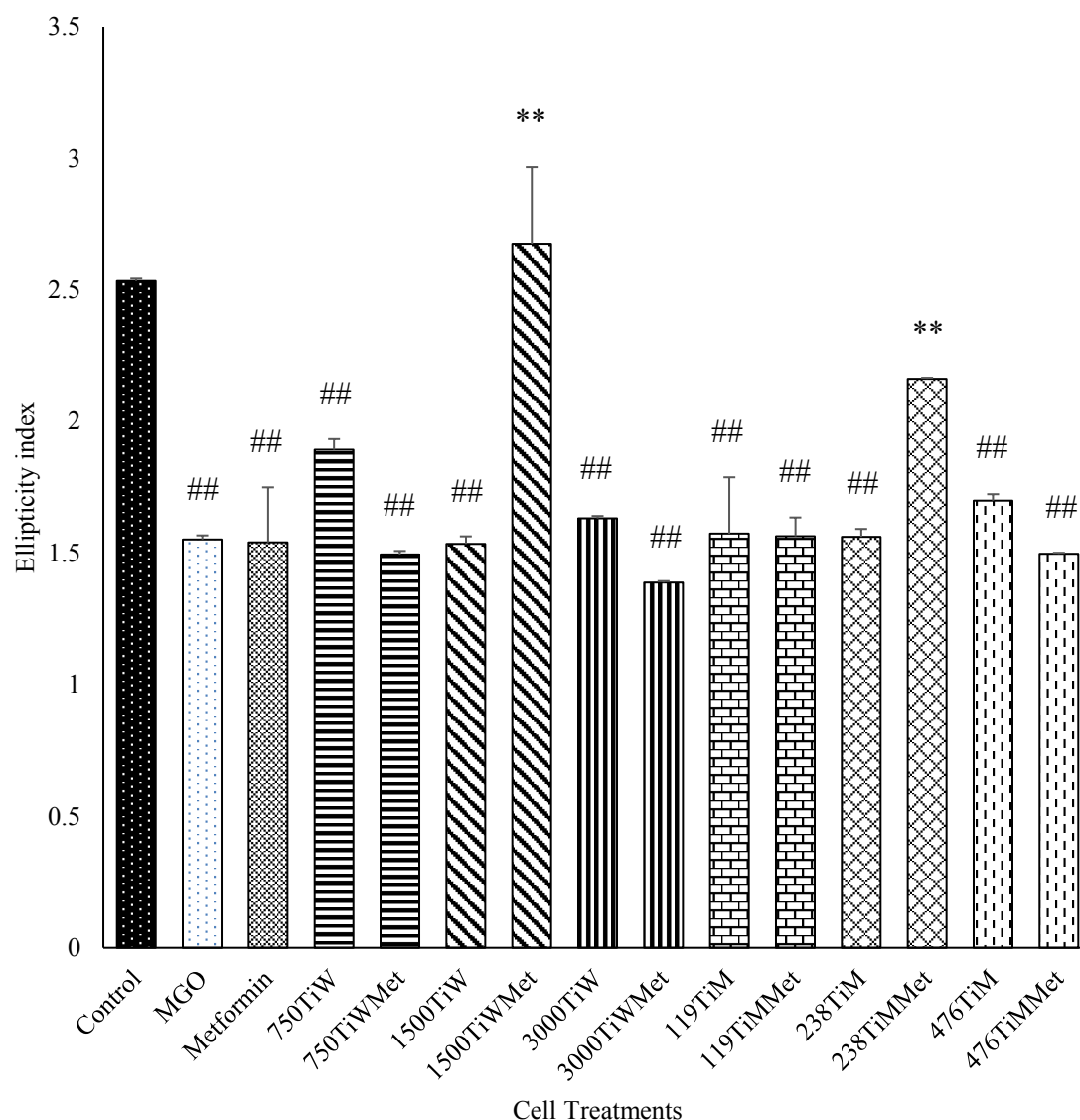


Figure 4.26: Effects of MGO, metformin, and *T. crispa* extracts on Ellipticity Index of hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except the control. Cells were examined at 20X magnification, and the ellipticity index was measured. Data are presented as mean $\pm$ SD, $n=2$ /group. Statistical significance compared to MGO-only treatment is indicated by * $p<0.05$, ** $p<0.005$, and compared to the control by # $p<0.05$, ## $p<0.005$. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

4.8.6 Impact of *T. crispa* water or methanol extract alone and in combination with metformin on the circularity index of hFOB 1.19 cells

The circularity index of a cell is a valuable parameter for assessing the roundness or irregularity of cell shapes. A circularity index value of 1 indicates a perfect circle, while values closer to 0 suggest increasingly irregular shapes. The circularity index of the control group and the 1500 µg/mL *T. crispa* hot water extract were the lowest among all groups, with values of 0.76 ± 0.00 and 0.76 ± 0.05 , respectively. The circularity index for the rest of the treatment groups were very close to the value of 1, which might indicate that the cells may not have been in optimal shape or condition.

Effects of MGO-stimulated hFOB 1.19 treated with *T. crispa* water and methanol extracts and metformin on the circularity index of the cells

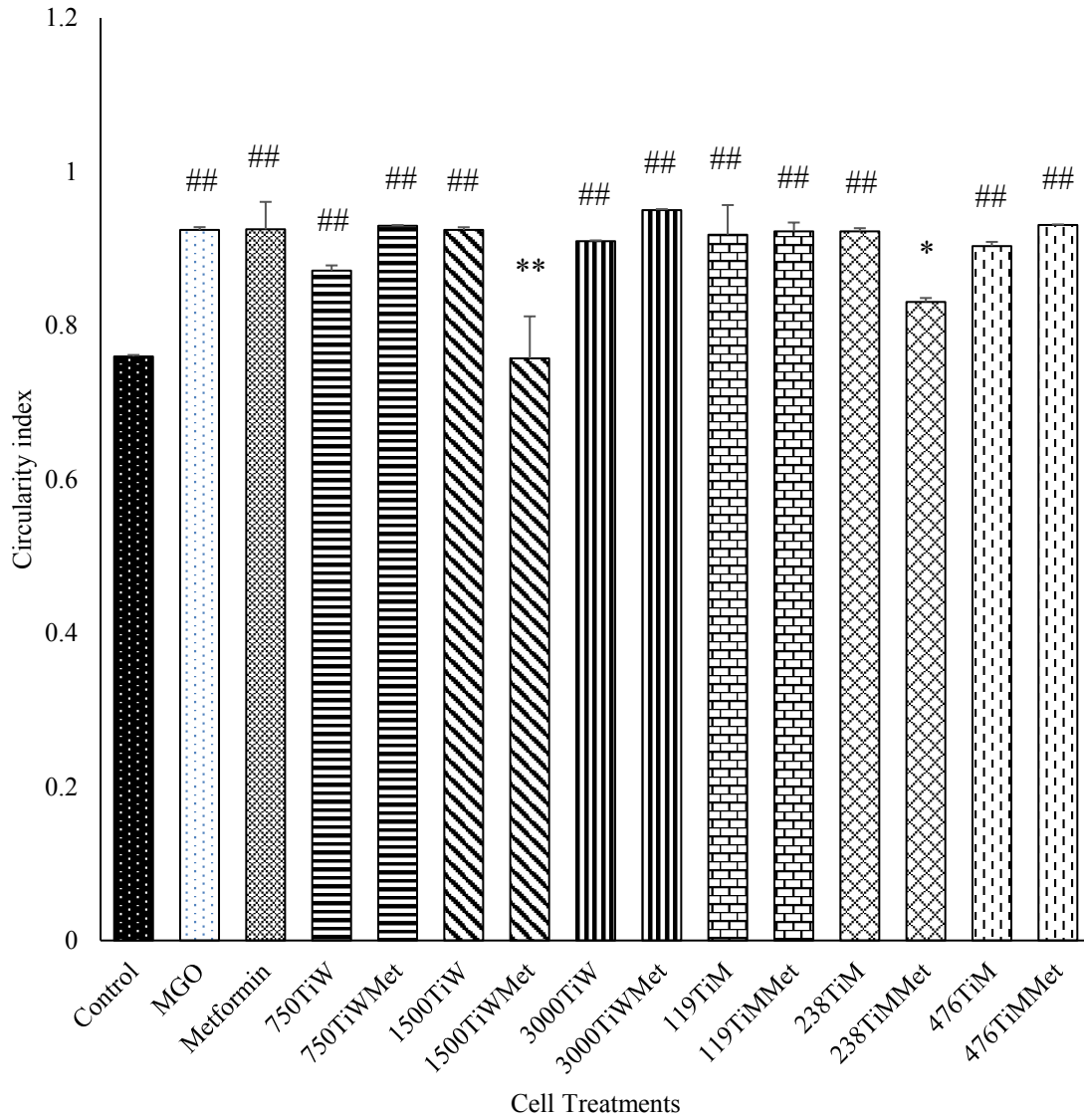


Figure 4.27: Effects of MGO, metformin, and *T. crispa* extracts on Circularity Index of hFOB 1.19 cells. hFOB 1.19 cells were treated with MGO, metformin, and *T. crispa* water (TiW) and methanol (TiM) extracts in 6-well plates and incubated at 34°C for 24 hours. All treatments included MGO except the control. Cells were examined at 20X magnification, and the circularity index was measured. Data are presented as mean $\pm$ SD, n=2/group. Statistical significance compared to MGO-only treatment is indicated by *p<0.05, **p<0.005, and compared to the control by #p<0.05, ##p<0.005. Note: TiW = *T. crispa* hot water extract, TiM = *T. crispa* methanol extract, Met = metformin, MGO = methylglyoxal.

CHAPTER 5

DISCUSSIONS

5.1 Phytochemical components of water and methanol extracts of *T. crispa*

The ethnopharmacological use of plants and other natural sources have given valuable insights into discovery of new potential drugs. A classic example is the isolation of aspirin from the bark of the *Salix alba* species. Therefore, the search for novel drugs derived from natural products is a never-ending quest. Among natural sources, *T. crispa* plants have attracted considerable attention from researchers for their abundant and well-documented phytochemical compounds.

In Malaysia, *T. crispa* is also known by a variety of colloquial names, including "*akar patawali*" and "*akar seruntum*" (Noor & Ashcroft, 1989). According to traditional folklore of South and Southeast Asia, the stem of this plant can be used for a variety of medicinal purposes, including the treatment of diabetes, hypertension, and protection against mosquito bites. Phytochemical analyses of *T. crispa* have shown that it possesses a wide array of beneficial compounds. The phytochemicals include saponins, glycosides, alkaloids, terpenoids, phenols, flavonoids, fatty acids and lignans compounds. These compounds detected through preliminary phytochemical tests and GC-MS analysis, have demonstrated to exhibit potent anti-diabetic, antioxidant, anti-inflammatory, antimicrobial, and anticancer properties, making *T. crispa* a promising candidate for therapeutic applications in various diseases. These findings aligned with previous research on *T. crispa*, which has also reported the presence of similar bioactive compounds (Haque et al., 2023). Similarly, Ahmad et al. (2016) also reported the presence of flavonoids, terpenoids, alkaloids and lignans in *T. crispa* extracts.

The GC-MS analysis revealed the presence of diverse array of bioactive compounds in both the methanol and hot water extracts of *T. crispera*. A total of 13 bioactive compounds were detected in the methanol extract, while 16 bioactive compounds were identified in the hot water extract. The presence of compounds such as santalene, trans-sinapyl alcohol, guaiacol, and columbin in *T. crispera* hot water extract further emphasises the complexity and therapeutic potential of *T. crispera*. Santalene, a terpene, exhibits anti-inflammatory, antioxidant (Toden et al., 2017), and anticancer properties (Bommareddy et al., 2012; Lee et al. 2015; Saraswati et al., 2013). These properties make it beneficial for skin health, immune support, and aromatherapy. Trans-sinapyl alcohol, also known as sinapaldehyde, is a compound found in various plant sources. Further characterisation and quantification of santalene and trans-sinapyl alcohol in *T. crispera* extract could provide valuable perspectives into its pharmacological profile and therapeutic potential. Another significant phenolic compound, guaiacol, offers several beneficial effects, including antioxidant properties that help combat oxidative stress (Gao et al., 2021), antimicrobial activity against various pathogens (Yang et al., 2013), and anti-inflammatory effects that can assist in managing inflammation-related conditions (Murakami et al., 2007).

In the methanol extract, oleic acid emerged as the predominant compound, followed by palmitic acid and 3,4-divanillyltetrahydrofuran. In contrast, the hot water extract showed notable concentrations of santalene, trans-sinapyl alcohol, guaiacol, and columbin. The high concentrations of oleic acid, palmitic acid, and 3,4-divanillyltetrahydrofuran in *T. crispera* methanol extracts indicate the potential bioactivity and pharmacological relevance of *T. crispera*. Oleic acid, known for its anti-inflammatory properties, may contribute to the medicinal value of the plant extract (Carrillo Pérez et al., 2012; Pegoraro et al., 2021). Additionally, oleic acid has been shown to inhibit bone resorption (Cardoso et al., 2017;

Drosatos-Tampakaki et al., 2014), thereby supporting bone health and potentially offering therapeutic benefits for conditions like osteoporosis. On the other hand, palmitic acid, with its various biological activities, could also contribute to therapeutic benefits of *T. crispa* extracts. Contrary to popular belief, moderate consumption of saturated fats like palmitic acid within a balanced diet may not have harmful effects on cardiovascular health and could even provide benefits, such as supporting cell membrane integrity (Carta et al., 2017). However, it is important to note that palmitic acid can also enhance bone resorption (Gunaratnam et al., 2013; Yeh et al., 2014), which may contribute to bone density loss if consumed excessively. Thus, while both oleic acid and palmitic acid are important components of the plant extract, their contrasting effects on bone resorption highlight the need for a balanced approach to their intake for optimal benefits on bone health.

The interplay between these phytochemical constituents emphasised the complexity of the plant extract's bioactivity, suggesting that its medicinal properties are likely influenced by the combined effects of its various phytochemical components. To the best of my knowledge, the compound 3,4-divanillyltetrahydrofuran has been primarily reported in stinging nettle root extracts. As of now, there are no reports indicating its presence in *T. crispa* extract. This compound is known for its potent antioxidant activities (Taheri et al., 2022), anti-inflammatory properties (Hajhashemi & Klooshani, 2013), and its role in supporting testosterone levels which can positively affects muscle growth, libido, and overall vitality (Shan et al., 2016).

Contrary to the findings of Ahmad et al. (2016), borapetol A and borapetol B were not detected in either the methanol or hot water extracts of *T. crispa* extracts during GC-MS analysis, even though these compounds were previously reported to be present in the

methanolic extract of *T. crispa*. This discrepancy suggests that the presence of borapetol A and borapetol B may vary depending on the extraction method used or the specific sample source.

The efficiency of plant extraction is affected by various factors, such as the selection of solvent, as different solvents possess varying polarities which determine their ability to extract specific sets of compounds. For example, methanol, a highly polar solvent, is more polar than ethanol and can extract a broad spectrum of phytochemicals, including non-volatile compounds (Tiwari et al., 2011). In contrast, water, the most polar solvent, surpasses both methanol and ethanol in polarity. This may explain why there were more class of phytochemical compounds detected in *T. crispa* hot water extract.

Additionally, extraction temperature plays a crucial role; higher temperatures can enhance the solubility of certain compounds but may also result in the degradation of heat-sensitive constituents (Putra et al., 2023). This could explain why the hot water extract used in the present studies exhibited lower antioxidant capacity compared to the methanol extracts. The duration of the extraction process also impacts efficiency; while longer extraction times may allow for more thorough extraction. On the other hand, excessively long extractions can degrade sensitive compounds or extract unwanted impurities (Liu et al., 2022). The method of preparing the plant material, such as drying, grinding, and pulverizing, affects the surface area available for solvent interaction, thereby influencing extraction efficiency (Krakowska-Sieprawska et al., 2022).

Variations in the geographical origin, harvesting season, and maturity of the plant can lead to differences in phytochemical content. Environmental factors such as soil quality, climate, and pest exposure impact the concentration of bioactive compounds (Liu et al.,

2018). It is possible that *T. crisper* grown in Pahang, Malaysia, does not produce borapetol A and B in detectable quantities, or at all, compared to *T. crisper* grown in other regions where these compounds have been previously identified. Moreover, the ratio of solvent to plant material is also crucial, as an optimal ratio ensures sufficient solvent interaction with the plant material, maximising extraction efficiency without wasting resources. Additionally, the pH of the extraction medium can influence the solubility and stability of certain compounds, and adjusting the pH can help in selectively extracting specific types of phytochemicals.

GC-MS is a powerful analytical technique widely used for identifying and quantifying compounds in complex mixtures. It merged the separation efficiency of gas chromatography (GC) with the identification precision of mass spectrometry (MS), offering high sensitivity and specificity, which allows for the detection and identification of compounds even at trace levels (Fiehn, 2016). This made the GC-MS particularly useful in environmental analysis, forensic investigations, and food safety testing. Additionally, the GC component enables the efficient separation of volatile and semi-volatile compounds, which was essential for analysing complex mixtures. Extensive mass spectral libraries facilitate the identification of compounds by comparing acquired spectra with reference spectra, enhancing the reliability and speed of identification. Furthermore, GC-MS can provide both qualitative and quantitative data, enabling precise measurement of compound concentrations. However, GC-MS has several limitations. A major limitation is that it can only analyse compounds that are volatile or can be made volatile through derivatization, which exclude many high-molecular-weight or thermally labile compounds from analysis (Garcia & Barbas, 2011). Furthermore, samples frequently require extensive preparation steps, such as extraction, purification, and occasionally derivatisation, which can be time-

consuming and may introduce variability. GC-MS systems are also complex and require regular maintenance and calibration, and they can be expensive to purchase and operate, making them less accessible for some laboratories.

In comparison, high-performance liquid chromatography-mass spectrometry (HPLC-MS) and nuclear magnetic resonance (NMR) are commonly used alternative techniques for compound identification. HPLC-MS can analyse non-volatile and thermally labile compounds, broadening the range of analytes (Holčapek et al., 2008), and allows for the analysis of larger biomolecules such as peptides and proteins. Borapetol A and B, being less volatile, may not be effectively detected by GC-MS. Less volatile or non-volatile compounds are often better suited for detection by HPLC. HPLC separates compounds based on their interactions with the stationary phase and their solubility in the mobile phase, making it more suitable for detecting borapetol A and B. However, HPLC-MS typically has lower resolution compared to GC-MS and may have longer run times due to the liquid chromatography component. On the other hand, NMR provides detailed structural information and can be used to analyse a wide range of compounds without the need for derivatisation, making it particularly powerful for elucidating the structures of complex molecules (Emwas, 2015). Nonetheless, NMR generally may require larger sample quantities and was less sensitive than GC-MS and HPLC-MS, and NMR instruments are expensive and require specialised expertise to operate. While GC-MS offers significant advantages in terms of sensitivity, specificity, and efficiency for volatile compounds, its limitations require the use of complementary techniques like HPLC-MS and NMR for a comprehensive analysis of a wide range of chemical compounds.

5.2 Antioxidant and antiglycation activities of water and methanol extracts of *T. crispera*

In this study, the antioxidant activities of *T. crispera* hot water and methanol extract, was assessed using FRAP assay. The FRAP assay is widely utilised to assess the ability of a substance to reduce a ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex, resulting in the formation of a coloured ferrous tripyridyltriazine (Fe^{2+} -TPTZ). This reaction reflects the overall antioxidant potential of the sample, as it indicates free radical chain breakage through hydrogen atom donation (Rajurkar & Hande, 2011). Extracts with higher FRAP values were indicative of better scavenging abilities and were often associated with higher concentrations of antioxidants, demonstrating their ability to counteract oxidative stress in biological systems (Lalhminghlui & Jagetia, 2018).

The impact of solvent type (hot water vs. methanol) on the FRAP values of *T. crispera* extracts were investigated. The results revealed a significant increase in FRAP values with increasing concentrations for both types of extracts ($p < 0.05$). The higher FRAP values observed in the *T. crispera* methanol extract compared to the hot water extract, suggest a higher concentration of potent reducing agents within the methanol extract. This increased antioxidant capacity suggests that the methanol extract is more effective in neutralising free radicals and mitigating oxidative stress. Such properties are crucial in the context of protecting osteoblast cells from the damaging effects of MGO, a reactive carbonyl compound known to induce oxidative stress and inflammation in cells. The ability of the methanol extract to exhibit stronger antioxidant activity implies its potential to counteract MGO-induced oxidative damage in osteoblasts, thereby preserving cell viability and function. Supporting this, a study by Ghanimi et al. (2022) showed that methanolic extract of *Lavandula mairei* Humbert exhibited significantly higher levels of flavonoids and total

phenolic contents ($p < 0.05$) compared to extracts obtained through Soxhlet or maceration methods. The elevated presence of phenols and flavonoids likely contributed to the plant's superior scavenging abilities and higher antioxidant activities (Aryal et al., 2019). Similarly, the choice of methanol as solvent in this study appears to enhance the extraction of bioactive compounds with strong antioxidant properties from *T. crispa*.

The anti-glycation activity of *T. crispa* extracts, evaluated through the BSA-MGO assay, demonstrated a concentration-dependent inhibition of advanced glycation end-products (AGEs) formation. Both the hot water and methanol extracts of *T. crispa* exhibited significant anti-glycation potential. Notably, the methanol extract consistently demonstrated superior efficacy compared to the hot water extract at all tested concentrations. At the highest concentration (0.40 mg/mL), the methanol extract achieved 47.5% anti-glycation activity, indicating a strong inhibitory effect, although it was still significantly lower than the aminoguanidine positive control. Aminoguanidine is known for its ability to prevent AGE production by capturing intermediates in the initial phases of glycation (Song et al., 2021). The present finding aligns with a similar dose-response inhibition observed in pomegranate extracts, where pomegranate peel, fruit, and leaf extracts also showed anti-AGEs capacity (Amri et al., 2022). Despite their promising activity, the anti-glycation effects of these pomegranate extracts remained below those of aminoguanidine, similar to the results seen in current experiment where aminoguanidine exhibited significantly greater potency than both *T. crispa* water and methanol extracts. These results suggest that the bioactive compounds within *T. crispa*, especially in the methanol extract, may offer protective effects against glycation. Nonetheless, the anti-glycation activity did not reach the level of aminoguanidine, indicating that further optimisation or combination with other agents may be necessary to enhance its anti-glycation potential.

5.3 Effects MGO, metformin and *T. crispa* extract on osteoblast cells viability

The cytotoxicity of MGO, metformin and *T. crispa* extracts on osteoblast cells was assessed using the MTT assay and Trypan Blue Exclusion Tests. The MTT assay measures metabolic activity by converting yellow tetrazolium dye (MTT) to purple formazan crystals via mitochondrial enzymes in living cells. The resulting colour change is quantified by spectrophotometry. In contrast, the Trypan Blue Exclusion Test involving staining cells with Trypan Blue dye, where live cells exclude the dye and dead cells absorb it, allowing for visual differentiation under Cell Drop machine (cell counting machine).

In this study, certain concentrations of MGO, metformin and *T. crispa* extracts resulted in cell viability readings exceeding 100% in the MTT assay. In contrast, using the Trypan Blue Exclusion, cell viability was observed to be around 100% for all the treatments. The observed increase in cell viability in the MTT assay, suggests enhanced metabolic activity of osteoblast cells in response to the treatment. However, this may not necessarily indicate a true increase in cell number but rather an upregulation of mitochondrial dehydrogenase activity. To confirm these findings, appropriate controls such as negative control (hFOB 1.19 cells that are not stimulated or exposed to any treatment), positive control (cell stimulated with MGO), and blank wells without cells were included to rule out any non-specific reduction of MTT by the treatment compound, yet the cell viability still exceeded 100%.

The MTT assay offered several advantages over the Trypan Blue Exclusion Test, including quantitative measurement through absorbance readings, higher sensitivity for detecting subtle cytotoxic effects, suitability for high-throughput screening in multi-well plates, potential for automation, and provision of objective numerical data. While the Trypan

Blue Exclusion Test is simple, quick, and inexpensive, the MTT assay's quantitative, sensitive, and high-throughput capabilities make it a more robust and reliable method for determining the cytotoxic effects of treatments on cells. Thus, despite the merits of the Trypan Blue Exclusion Test, the comprehensive advantages of the MTT assay which made it a more reliable method for determining the cytotoxicity of chemical reagents, particularly MGO, metformin, and *T. crista* extracts, in this study. However, while MTT assay is widely used for assessing cell viability, it has inherent limitations that can affect the interpretation of results. One such limitation is its reliance on measuring mitochondrial metabolic activity, which does not always correlate directly with cell number. Treatments that enhance mitochondrial function or overall metabolic activity can lead to higher MTT reduction, resulting in apparent viability readings greater than 100%. This can be misleading if interpreted as an increase in cell number rather than increased metabolic activity.

5.4 hFOB 1.19 Cell Morphology: Area Density, Volume Density, Feret Diameters, Perimeter, Ellipticity, and Circularity Indices

Morphological analysis has emerged as a robust mathematical tool for examining and addressing cellular informatics (Chen et al., 2012). Automated quantification of cell features by using live imaging microscope stood out as the predominant technique used in this field. Therefore, cellular morphometrics of hFOB 1.19 cells such as area density, volume density, perimeter, minimum and maximum Feret diameter, ellipticity index and circularity index were evaluated quantitatively by adapting the parameters used by Kapitonova et al. (2014), with some modification.

The investigation into the effect of treatments on the cellular parameters of MGO-stimulated hFOB 1.19 cells provided perspectives into cell health and the potential synergistic effects of metformin with *T. crista* extracts. Through the analysis of cell viability

and function using the MTT assay, Trypan Blue Dye Exclusion Test, and cell morphology measurements, some insights into their correlation were obtained. However, not all of the concentrations used in the cytotoxicity test of MGO, metformin, and *T. crispa* were further analysed through cell morphology assessment. Only specific concentrations, such as the IC₅₀ value of MGO and the non-toxic concentrations of metformin and *T. crispa* extracts, were analysed.

The IC₅₀ value of MGO was employed to assess if it could kill half of the cell population (50% lethal dose), with cell morphology analysis confirming significant changes in most parameters compared to the control. Although the cell morphology analysis generally confirmed that the non-toxic concentrations of *T. crispa* extracts and metformin could ameliorate the detrimental effects of MGO, the ellipticity index and circularity index revealed otherwise. Among the treatment groups, the combination of 1500 µg/mL of *T. crispa* hot water extracts with metformin showed a slightly higher ellipticity index, suggesting a positive effect on maintaining or promoting a normal cell shape. However, it was noteworthy that the ellipticity index for most of the treatment groups were closer to the value of 0, indicating a more circular or less elongated cell shape.

On the other hand, the circularity index was used to assess the effects of different treatments on cell morphology. The control group and the 1500 µg/mL of *T. crispa* hot water extracts with metformin exhibited the lowest circularity index values, suggested less rounded and potentially more fibroblast-like cell shapes. Conversely, the circularity index values for the other treatment groups were closer to 1, indicating that these cells might have more rounded shapes, which could be associated with cellular stress or changes in cell function. These findings suggested that certain treatments, such as 1500 µg/mL of *T. crispa* hot water

extracts with metformin, may positively impact in maintaining or restoring normal cell morphology. However, some of these treatment conditions did not effectively maintain the fibroblast-like morphology characteristic of normal osteoblasts, suggesting potential cellular stress or altered cellular dynamics under these treatments.

The observed differences in cell morphology, particularly the variations in ellipticity and circularity indices, could be attributed to several factors. The slightly higher ellipticity index in the 1500 µg/mL of *T. crista* hot water extracts with metformin suggested that this combination might enhance cytoskeletal stability or promote healthier cell shape adaptations in response to stress. This could be due to metformin's known effects on improving mitochondrial function and reducing oxidative stress (Apostolova et al., 2020), thereby supporting better cell health and structure. Conversely, the lower circularity index in the control group and the 1500 µg/mL of *T. crista* hot water extracts with metformin, indicated that less rounded and potentially healthier cell shapes, might result from these treatments' ability to maintain normal cell adhesion and spreading properties. This analysis highlighted the potential of specific treatments, particularly the combination of 1500 µg/mL of *T. crista* hot water extracts with metformin, to mitigate adverse morphological changes and preserve cell viability under stress conditions. The closer-to-1 circularity index values in other treatment groups could reflect cellular stress responses, such as cytoskeletal reorganisation or membrane blebbing, which often lead to more rounded or irregular shapes (Chikina et al., 2019). Additionally, the irregular shapes observed under other treatment conditions might be due to the higher levels of reactive oxygen species (ROS) or inflammatory cytokines induced by MGO, which can disrupt normal cell morphology and function (Forrester et al., 2018). High ROS levels can cause oxidative damage to cellular components such as lipids, proteins, and DNA, compromising cell integrity and viability (Mittal et al., 2014). The

inflammatory cytokines, such as IL-1 β and IL-6, can further exacerbate cellular stress by promoting inflammation and apoptosis (Kany et al., 2019). This inflammatory microenvironment not only altered cell morphology but also impaired the cells' ability to proliferate and maintain normal metabolic functions. Thus, the observed morphological changes were likely a manifestation of the cumulative stress and damage inflicted by these biochemical alterations.

5.5 Biological markers involved in the osteoclastogenesis and inflammation in MGO-stimulated osteoblast cells

To study the biological markers involved in the osteoclastogenesis and inflammation in MGO-stimulated osteoblast cells, protein quantification assays such as Human Magnetic Luminex Assay and ELISA were employed. Osteoclastogenesis, the formation and activation of osteoclasts, is a critical process in bone remodelling and is tightly regulated by a network of cytokines and signalling molecules (Kitaura et al., 2020). Among these, IL-1 β , IL-6, M-CSF, RANKL, and RAGE (both full-length and soluble form) played crucial roles. Understanding how these markers interact and influence osteoblast function was essential to explain the mechanisms underlying bone diseases characterised by excessive bone resorption. Cytokines such as IL-1 β and IL-6 were central to the inflammatory response and significantly impacted osteoclastogenesis through their effects on osteoblasts (Zhou et al., 2022). Both IL-1 β and IL-6 were known to upregulate the expression of RANKL, a critical osteoclastogenic factor produced by osteoblasts (Xu et al., 2023).

The study observed that increased levels of IL-1 β and IL-6 (not significant to control group, $p>0.05$) in osteoblasts correlated with increased RANKL production (not significant to control group, $p>0.05$), thereby potentially promoting osteoclast differentiation and activation. The correlation between increased IL-1 β and IL-6 levels demonstrated their

combined role in enhancing osteoclastogenesis and bone resorption. The treatments of 1500 µg/mL of *T. crispa* hot water extracts with and without metformin reduced the IL-1β concentration while the treatments of 3000 µg/mL of *T. crispa* hot water extracts with and without metformin, 119 µg/mL of *T. crispa* methanol extracts with and without metformin reduced the IL-6 concentration (but not significant, $p < 0.05$) if compared to the concentration of these two cytokines in MGO-stimulated hFOB 1.19 cells. Interestingly, the treatments of 238 µg/mL and 476 µg/mL of *T. crispa* hot water extracts combined with metformin resulted in IL-1β and IL-6 concentrations below the lowest detection limit. To put it simply, the concentrations of IL-1β and IL-6 fell below the detection limit, which was lower than the lowest concentration of IL-6 in the standards, indicating that the levels were undetectable within the sensitivity range of the Human Magnetic Luminex Assay. This observation highlighted the potential for these treatments to profoundly suppress the inflammatory response induced by MGO in hFOB 1.19 cells. Another possibility, IL-1β and IL-6 cytokines were absent from the cell culture supernatant samples for 238 µg/mL and 476 µg/mL *T. crispa* methanol with metformin. Meanwhile, there was a significant increase in RANKL concentration upon MGO stimulation, indicating activation of osteoclastogenic signalling in response to MGO exposure. All of the treatments which reduced the levels of IL-1β and IL-6 in osteoblasts were shown to effectively diminish RANKL expression, highlighted the potential of targeting these cytokines to control excessive osteoclast activity.

Generally, M-CSF synergised with RANKL to drive the proliferation and survival of osteoclast precursors (Xu et al., 2023). The interrelation between M-CSF and RANKL was critical for osteoclastogenesis, as M-CSF primed osteoclast precursors, making them more responsive to RANKL signalling. The reduction in M-CSF concentration upon the stimulation of MGO in osteoblast cells can be linked to the oxidative stress mechanisms

involved. A study by Werner et al. (2020) discussed the absence of M-CSF expression in osteocytes and its association with increased oxidative stress/ROS produced by nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4). Given that MGO downregulated M-CSF in osteoblast cells in this study, it was plausible that the function of M-CSF in this context was related to the oxidative stress induced by MGO. Rather than promoting osteoclastogenesis alongside RANKL, M-CSF's role appeared to be more focused on enhancing the oxidative stress caused by MGO. Moreover, another potential mechanism involved an autocrine effect of M-CSF on RANKL produced by osteoblasts (Wittrant et al., 2009). Although Wittrant et al. (2009) indicated that M-CSF is produced by various cell types, including osteoblasts, it is possible that in the presence of MGO, the expression of these cytokines is differentially regulated. Specifically, the reduction of M-CSF produced by osteoblasts could decrease the proliferation of osteoclast precursors, while the concurrent increase in RANKL expression might still promote the differentiation of any existing precursors into mature osteoclasts. This differential regulation suggests that while M-CSF and RANKL are typically co-regulated, MGO stimulation might uncouple their expression, leading to complex effects on osteoclastogenesis. Consequently, osteoclast formation driven by RANKL and M-CSF may still occur through M-CSF produced by other cell types, such as immune cells, epithelial cells, and osteocytes. Furthermore, the current study showed that treatments with 1500 µg/mL of *T. crispa* hot water extracts with and without metformin, 3000 µg/mL of *T. crispa* hot water extracts with and without metformin, and 119 µg/mL of *T. crispa* methanol extract caused a significant increase in M-CSF concentration compared to the osteoblast cells treated with MGO alone. This suggested that these treatments may counteract the MGO-induced downregulation of M-CSF, potentially mitigating the oxidative stress and promoting a cellular environment more conducive to osteoclastogenesis. The

increased M-CSF levels with these treatments showcased their potential to influence the bone remodelling process by modulating M-CSF availability and, consequently, osteoclast precursor proliferation and survival. Currently, there is a lack of research specifically investigating the interaction between hFOB1.19 cells, MGO, and M-CSF. Therefore, the hypotheses presented above, which suggested that MGO downregulated M-CSF and that this downregulation contributed to oxidative stress rather than osteoclastogenesis, remain speculative. Further studies are necessary to confirm these theories and to understand the accurate mechanisms by which MGO influences M-CSF expression and its subsequent effects on osteoblast function and bone homeostasis.

The role of the RAGE added another layer of complexity to the regulation of osteoclastogenesis. flRAGE on osteoblasts interacted with AGEs, contributing to inflammatory responses and increased RANKL production (Asadipooya & Uy, 2019). In the present study, the stimulation of MGO on osteoblast increased the concentration of flRAGE. Only a few treatments, such as the 1500 µg/mL, 3000 µg/mL of *T. crispa* hot water extracts with metformin, and 476 µg/mL of *T. crispa* methanol extract with metformin, were able to significantly decrease the concentration of full-length RAGE in MGO-stimulated osteoblast cells. On the other hand, sRAGE referred to a form of the RAGE, which was generated by the cleavage of flRAGE or through alternative splicing during the production of RAGE mRNA (Schmidt, 2015). It acted as a decoy receptor, competing with membrane-bound RAGE, or flRAGE for binding to AGEs and other ligands (Sparvero et al., 2009). In MGO-stimulated osteoblast cells, sRAGE production increased, but this increase was not significant compared to the untreated cells (control). All of the treatments showed elevated sRAGE concentrations, although these increases were not significant compared to the cells treated with MGO only. However, almost all of the treatments demonstrated significantly

increased sRAGE concentrations when compared to the untreated osteoblasts. The significantly elevated sRAGE levels compared to the control might indicate that more sRAGE was able to bind to the AGEs released by osteoblast cells due to the action of MGO. In this context, elevated levels of sRAGE were associated with reduced RANKL production and decreased osteoclastogenesis, suggesting that enhancing sRAGE levels may offer a therapeutic strategy to counteract bone loss induced by chronic inflammation and AGEs.

Since the hFOB 1.19 cells were incubated with MGO, a reactive dicarbonyl compound, they are known to readily enter cells through various pathways. Once inside, MGO can interact with intracellular components, including proteins and lipids, leading to the formation of AGEs. These intracellular AGEs can disrupt normal cellular functions by cross-linking proteins, impairing enzyme activities, and altering cellular signalling pathways. In addition to its intracellular effects, MGO in the incubation media can also react with extracellular proteins and lipids to form AGEs. This extracellular glycation can further compound the problem by increasing the pool of AGEs available to interact with cell surface receptors such as RAGE. The binding of AGEs to membrane-bound RAGE triggers a cascade of pro-inflammatory and pro-oxidative signals, exacerbating cellular damage and promoting inflammatory responses. In parallel, BSA-MGO assay demonstrated that both methanol and hot water extracts of *T. crispa* possess anti-glycation activity, with the methanol extract showing higher glycation inhibition. This preliminary finding suggests that the methanol extract is more effective in preventing the formation of AGE, which play a significant role in the pathogenesis of various chronic diseases, including diabetes and its complications. The higher anti-glycation activity of the methanol extract compared to the hot water extract could be attributed to the distinct phytochemical profiles extracted by the solvents. Despite these promising results, the glycation inhibition capabilities of *T. crispa*

extracts are notably lower than that of aminoguanidine, the positive control. The combination of anti-glycation properties and the ability to elevate sRAGE levels positions *T. crista* extracts as promising candidates for mitigating AGE-related damage. While the methanol extract showed higher glycation inhibition in the BSA-MGO assay, both methanol and hot water extracts demonstrated potential in elevating sRAGE levels in the osteoblast cell model. This dual action of reducing AGE formation and increasing sRAGE levels suggests that *T. crista* extracts offer a comprehensive approach to managing AGE-related complications.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The goal of this study sought to investigate the potential of *T. crispa* extracts in mitigating bone loss associated with T2DM, specifically focusing on their effects on receptor for advanced glycation end-products (RAGE) expression and associated inflammatory pathways in methylglyoxal (MGO)-stimulated osteoblast cells. The findings of this study revealed several key findings.

Preliminary phytochemical screening, GC-MS analysis, FRAP assay, and BSA-MGO assay confirmed that *T. crispa* extracts, both water and methanol, possess antioxidant and anti-glycation properties. These properties are attributed to the bioactive compounds present in the extracts. Cell cytotoxicity assays demonstrated that MGO, metformin and *T. crispa* extracts of water and methanol extracts decreased cell viability in a dose-dependent manner, allowing the identification of their IC₅₀ and non-toxic concentrations for further experimentation. The results showed that 3000 µg/mL *T. crispa* hot water extract and 476 µg/mL *T. crispa* methanol extract significantly reduced IL-1β levels. Notably, the combination of 238 µg/mL and 476 µg/mL of *T. crispa* methanol extract with metformin reduced IL-1β to undetectable levels, indicating a potential synergistic effect. Additionally, the extracts altered IL-6 concentrations, with significant reductions observed when combined with metformin, highlighting their impact on inflammatory pathways. The RAGE ELISA analysis indicated that while *T. crispa* extracts alone did not significantly affect fRAGE levels, the combination of TiW extracts with metformin significantly reduced RAGE levels at certain concentrations. This suggests a potential synergistic effect between

T. crispa water and metformin in reducing RAGE expression in MGO-stimulated cells. Moreover, the sRAGE analysis revealed significant increases in sRAGE concentrations with *T. crispa* extracts, suggesting a role in reducing AGE binding and inflammation, although the impact on fRAGE levels was less pronounced. Cell morphology analysis showed significant changes in cell shape and structure in response to treatments, with the 238 µg/mL of *T. crispa* methanol extract with metformin mitigating the detrimental effects caused by MGO. This highlights the potential of *T. crispa* extracts and metformin in preserving cellular health in the context of diabetic bone loss.

6.2 Limitations and Recommendations

Despite these promising results, the study has limitations. The use of a single cell line model and the need for further validation in *in vivo* systems are notable constraints. Further research should involve *in vivo* studies to substantiate the efficacy of *T. crispa* extracts in mitigating bone loss and to comprehensively evaluate their potential adverse effects. *In vivo* models will offer a deeper understanding of the systemic impacts and safety profile of these extracts.

Additionally, future studies should focus on investigating the specific molecular mechanisms through which *T. crispa* extracts modulate RAGE expression and inflammatory pathways. For example, incorporating OPG ELISA in future research is recommended to assess how *T. crispa* extracts influence the OPG-RANKL-RANK interaction, which is crucial for regulating osteoclastogenesis and bone resorption, thereby providing a more comprehensive understanding of their impact on bone health. Next, osteocalcin measurement in future research is crucial to evaluate the effects of *T. crispa* extracts, or the combination of both on bone formation, as osteocalcin is a key marker of osteoblast activity

and bone matrix synthesis. Moreover, it is recommended to measure the concentrations of TNF α because it is one of the most important cytokines that involved in bone homeostasis and osteoclastogenesis. The understanding of these bone markers and its mechanisms could offer some insights into the extracts' potential to support bone health and counteract bone loss.

The exploration of combinations of different concentrations of *T. crispa* extracts with other therapeutic agents is also recommended. This approach could optimise the therapeutic potential of *T. crispa* and aid in creating effective treatment regimens for managing diabetic bone loss. Furthermore, clinical trials should be designed and conducted to assess the effectiveness and safety of *T. crispa* extracts in human subjects with T2DM. Such trials are essential for translating preclinical findings into practical treatments and confirming the benefits observed in cell-based studies. Long-term studies are also necessary to evaluate the chronic effects of *T. crispa* extracts on bone health and overall metabolic function, providing crucial data on the sustainability and safety of these extracts as therapeutic interventions. Lastly, employing diverse cell models and tissue types in research will help assess the broader applicability of *T. crispa* extracts, determining whether the effects observed in osteoblast cells are consistent across different cell types and biological contexts.

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APPENDICES

Appendix A: New Account Form for Biosafety Cabinet Level 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@spdscientific.com	
New Account Application BSL 2			
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 ☐ 3 rd 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		Room Number Admin office, level 2	
Building FMHS		City Kota Samarahan	
Street Address/P.O. Box -		Country Malaysia	
State/Province Sarawak		Zip/Postal Code 94300	
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		Room Number Admin office level 2	
Building FMHS		City Kota Samarahan	
Street Address (PO Boxes cannot be accepted)		Country Malaysia	
State/Province Sarawak		Zip/Postal Code 94300	
Telephone (including Country Code) +60 133835613		Fax (including Country Code) +6082665152	
		E-mail ratgabriele@unimas.my	
End User Information (Primary End User)			

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) +60135693721	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

☒ 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

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. crispa 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 B: Biosafety Forms

NBB/N/CU/22/FORM E

NBB REF.NO : JBK(S) 600-3/1/
(For Office Use)

BIOSAFETY ACT 2007

BIOSAFETY REGULATIONS 2010

NBB/N/CU/22/FORM E

NOTIFICATION FOR CONTAINED USE AND IMPORT FOR CONTAINED USE ACTIVITIES INVOLVING LIVING MODIFIED ORGANISM (LMO) FOR BIOSAFETY LEVELS 1, 2, 3 AND 4

Please refer to the Explanatory Notes of NBB/N/CU/22/FORM E (at the end of the form) before filling out this form.

PROJECT TITLE: Investigating the preventive effects of *Tinospora crispa* and *T. cordifolia* on methylglyoxal-induced cytotoxicity and metabolic dysregulation in osteoblast cells

NOTIFICATION CHECK LIST

1. Form NBB/N/CU/22/FORM E is complete with the relevant signatures.	☐
2. Cover letter from Applicant's institute provided.	☐
3. Notification has been assessed and sent through the IBC (if activity involves modern biotechnology research and development).	☐
4. Any information to be treated as confidential business information (CBI) has been clearly marked "CBI" in the notification.	☐
5. One (1) original Form E and six (6) hardcopies of the completed Form E are submitted, including all supporting documents (Standard Operating Procedures, Inspection Reports, approval from other relevant authorities, training records etc.). The original Form E and hardcopies submitted must be identical.	☐
6. One (1) original and six (6) hardcopies of the IBC Assessment report (IBC/AP/20/ANNEX 2) are submitted.	☐
7. A soft copy of the Form E that is identical to the hardcopies is submitted. The softcopy should include Form E, IBC Assessment Report (IBC/AP/20/ANNEX 2) and all supporting documents (Standard Operating Procedures, Inspection Reports, approval from other relevant authorities, training records etc.).	☐

(Information claimed to be CBI must be excluded from the softcopy submitted).	
8. All supporting documents/attachments required are submitted, including: i) Most recent Inspection report(s) of the premises used (inspection done not more than two years ago from date of submission) ii) SOP for LMO transportation (if activity involves movement of LMO between premises stated in Table 3) ii) SOP for LMO treatment and disposal iii) SOP for solid and liquid waste treatment and disposal iv) SOP for waste water treatment and disposal v) SOP for decontamination (All SOPs submitted must be endorsed by the IBC prior to submission and dated not more than two years from the date of submission).	☐
9. A copy of clearance documents (e.g. import permit, etc.) from the relevant Government agencies (if applicable).	☒
10. A letter of authorization from the IBC of the collaborating agency/institution/organization must be provided (If any premises outside the Applicant's organization is used for LMO work).	☐
11. A copy of the completed notification retained for records	☐

PRELIMINARY INFORMATION

1. Organization:	UNIMAS, Faculty of Medicine and Health Science
2. Name of Applicant (Principal Investigator):	Gabriele Ruth Anisah Fromming
3. Position in Organization:	Professor
Telephone (office):	082510000ext 7310
Telephone (mobile):	0133835613
Fax number:	082665152
E-mail address:	rafgabriele@unimas.my
Postal address:	Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak

Project Title:	Investigating the preventive effects of <i>Tinospora crispa</i> and <i>T. cordifolia</i> on methylglyoxal-induced cytotoxicity and metabolic dysregulation in osteoblast cells
IBC Project Identification No:	Click here to enter text.
Is this the first time the activity is being notified?	☒ Yes ☐ No
If this is not the first time the activity is notified, please answer the following: i) Please provide the NBB reference number of your previous notification. ii) How is this notification different from the previous notification submitted for this activity? (describe the differences or if this activity is using LMO produced through a previous notification)	Click here to enter text. Click here to enter text.

DETAILS OF IMPORTER

Importer refers to the Applicant as importer, or person or business importing/bringing the LMO on behalf of the Applicant. This section to be filled out only if the LMO is imported.

1. Importing person/ company/ organization:	Faculty of Medicine and Health Science, UNIMAS via SPD Scientific
2. Contact Person:	Gabriele Ruth Anisah Fromming
3. Designation:	Professor
Telephone (office):	082581000 ext 7310
Telephone (mobile):	0133835613
Fax number:	082665152
E-mail address:	rafgabriele@unimas.my
Postal address:	Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak

SUMMARY OF IBC ASSESSMENT (Refer to IBC/AP/20/ANNEX 2)

This section to be completed by the registered IBC of the Applicant's organization. Please mark [X] in the appropriate box.

1	Name of Principal Investigator:	Gabriele Ruth Anisah Fromming	
2	Project Title:	Investigating the preventive effects of <i>Tinospora crispa</i> and <i>T. cordifolia</i> on methylglyoxal-induced cytotoxicity and metabolic dysregulation in osteoblast cells I	
3	Date of the IBC Assessment:	Click here to enter text.	
4	Does the IBC consider that the Principal Investigator and every other person authorized to be involved in the contained use of the LMO have adequate training and experience for the task?	☒ Yes ☐ No	
5	The following information related to this project has been checked and approved		
	a) The description of project activities	☒ Yes ☐ No	
	b) The description and genetics of the LMO	☒ Yes ☐ No	
	c) The emergency response plan and the specific measures to be taken in relation to a contained use activity involving LMO.	☒ Yes ☐ No	
	d) All persons involved are appropriately trained:	☒ Yes ☐ No	
6	Has the information provided in Form NBB/N/CU/22/FORM E been checked by the IBC and found to be complete?	☒ Yes ☐ No	
7	Has the IBC assessed the biosafety of the proposed project? <i>The risks that the IBC is required to assess are:</i> a) <i>risks to the health and safety of human (occupational exposure) from the activities associated with genetic modification</i> b) <i>risks to the health and safety of human and animals from an unintentional release of the LMO; and</i> c) <i>risks to the environment from an unintentional release of the LMO</i>	☒ Yes ☐ No	

NBB/N/CU/22/FORM E

A template of the IBC Assessment report (IBC/AP/20/ANNEX2) can be obtained at https://www.biosafety.gov.my/wp-content/uploads/2021/08/IBC-ANNEX-2_revised-9.1.2020.pdf	
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SIGNATURES AND STATUTORY DECLARATION

Please mark [X] in the appropriate box

- ☐ This application is for contained use activity involving LMO and has been assessed as above and endorsed by the IBC.
- ☒ This application is for commercial production and does not involve modern biotechnology research and development.

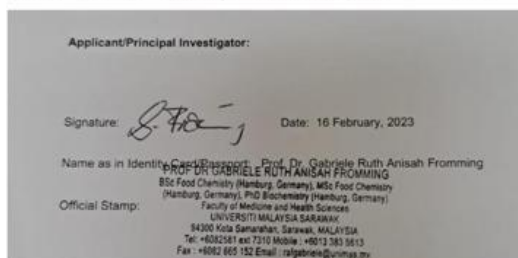
We declare that all information and documents herein are true and correct. We understand that providing misleading information to the NBB, deliberately or otherwise, is an offence under the Biosafety Act 2007.

Applicant/Principal Investigator:

Signature:

Date: 16 February, 2023

Name as in Identity Card/Passport: Prof. Dr. Gabriele Ruth Anisah Fromming



Official Stamp:

IBC Chairperson:

This section is applicable to organizations involved in modern biotechnology research and development.

NBB/N/CU/22/FORM E

Signature:

Date: Click or tap to enter a date.

Name as in Identity Card/Passport: Click here to enter text.

Official Stamp:

Head of Organization/Authorized representative:

Signature:

Date: Click or tap to enter a date.

Name as in Identity Card/Passport: Click here to enter text.

Official Stamp:

PART A: DETAILS OF TEAM MEMBERS

1. Project team members' details.

Note 1: Information required is for ALL persons involved in the project and IBC should have their records.

Note 2: All persons listed here should be assessed and approved by IBC.

Table 1: Description of team members' details

Name	Address, contact number & e-mail	Qualifications & Relevant Experience	Designation in Organisation
Gabriele Ruth Anisah Fromming	Faculty of Medicine and Health Science, UNIMAS rafgabriele@unimas.my	BSc Food Chemistry (Hamburg, Germany), MSc Food Chemistry (Hamburg, Germany), PhD Biochemistry (Hamburg, Germany)	Professor
Luqman bin Jaya	Faculty of Medicine and Health Science, UNIMAS	SARJANA MUDA, Biokesihatan	Masters student
Teknowilie anak Singa	Faculty of Medicine and Health Science, UNIMAS	Bachelor of Science, Resource Biotechnology	Master student, research assistant
Zunika binti Amit	Faculty of Medicine and Health Science, UNIMAS	PhD in Biochemistry	Associate Professor, Co-PI

PART B: PROJECT DESCRIPTION

Applicant is required to describe the proposed activities with the LMO within the context of the project.

2. General Objective:

The overall objective of this study is to determine the beneficial effects of *T. crispa* and *T. cordifolia* on methylglyoxal (MGO)-induced cytotoxicity and dysregulation of insulin signaling in human osteoblast cells.

..

Specific Objective(s): (if any)

The specific objectives are:

1. To determine the mode of cell death (apoptosis and/or autophagy) and oxidative stress induced by MGO and its reduction/prevention by *T. crispa*, *T. cordifolia* and metformin.
2. To determine MGO-induced NFκB expression and consequently upregulated expression of pro-inflammatory cytokines (IL-6, IL1β, TNFα and IL-18) and its attenuation by *T. crispa*, *T. cordifolia* and metformin.
3. To determine the effect of *T. crispa*, *T. cordifolia* and metformin on MGO-induced downregulation of the insulin receptors in osteoblast cells and downregulated RUNX2 expression and therefore decreased expression of osteocalcin and GLUT4.

3. Description of project activities

Note 1: Please provide a brief description that clearly shows the involvement of handling LMO materials and a flow chart of the activities that accurately reflects the narrative provided.

Note 2: In the flowchart, state all premises where each activity is conducted.

Note 3: A separate attachment may be provided for the flowchart or incorporated into the text box below:

The overall experimental design is outlined in the Flow Chart attached below (Appendix 1)

Briefly, human osteoblast (HOB) cells will be grown in tissue culture flasks and then stimulated with 30 μM methylglyoxal(MGO) for 24h in the absence or presence of metformin (Reference), *T. crispa* or *T. cordifolia* (3 best, non-toxic concentrations). The measurement of the effects of *T. crispa* and *T. cordifolia* on MGO-induced cell death will be investigated using MTT assay and fluorescence labelling of Annexin V and LC3B (differentiation between apoptosis and autophagy). Then the effects of the *T. crispa* and *T. cordifolia* on MGO-induced dysregulation of insulin signalling in the stressed HOB cells will be evaluated by measuring the expression of the insulin receptor, GLUT4, TWIST2 and RUNX 2 expression as well as osteocalcin (carboxylated and undercarboxylated forms). Additionally, we will measure the expression of NFκB and pro-inflammatory cytokines (IL-1β, IL6, TNFα and IL18).

a) Objective 1 (mode of cell death (apoptosis and/or autophagy) and oxidative stress induced by MGO)

The stimulated cells will be examined for changes in cell viability and death pathway (apoptosis or autophagy). The cell viability will be determined using Trypan Blue and MTT assay, while apoptosis and autophagy will be differentiated by measuring Annexin V and LC3B fluorescence. The gene expression of BAX, BCL2 and caspase 3 will support the data on apoptosis as mode of cell death

For the determination of Oxidative stress caused by MGO incubation a) Reactive Oxygen species (ROS) and AGE will be measured. Intracellular ROS production will be measured using a fluorescence kit from Cell Biolabs. The assay measures hydroxyl, peroxy, or other reactive oxygen species activity within a cell by incubating cells with the cell-permeable fluorogenic probe 2', 7'- Dichlorodihydrofluorescein diacetate (DCFH-DA. B) Advanced glycation endproducts will be measured using an ELISA kit from Cell Biolabs.

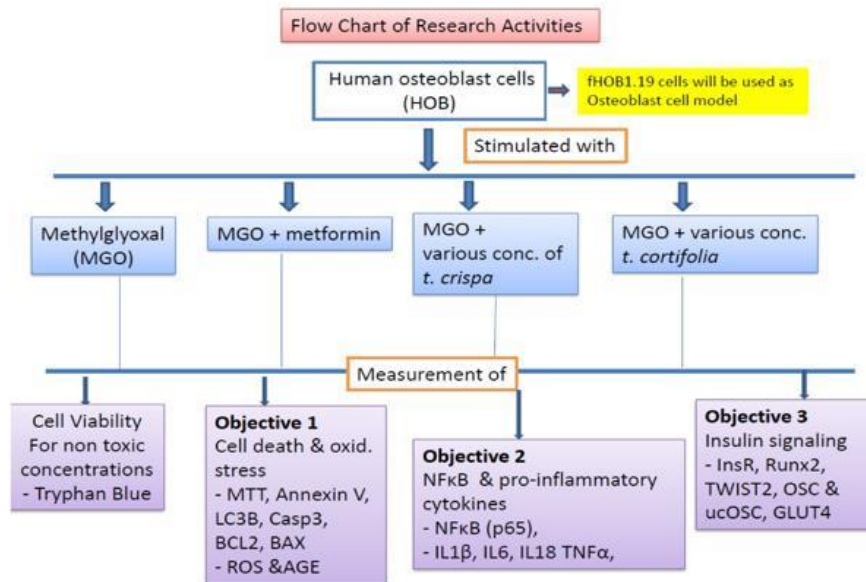
Objective 2 (expression of NFκB, and pro-inflammatory cytokines (IL-6, IL1β, TNFα and IL-18)

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The cell supernatant and/or cell extract will be tested for IL-6, TNF α , IL1 β and IL18 proteins using commercially available ELISA kits according to the supplier's instruction.

Objective 3 (insulin receptor (IR), RUNX2, osteocalcin, undercarboxylated osteocalcin and GLUT4)

The expression of the insulin receptor, GLUT4, TWIST 2, RUNX2, osteocalcin and undercarboxylated osteocalcin will be measured using a commercially available ELISA kit or by Western blotting (if an ELISA kit can't be found). The expression of osteocalcin and undercarboxylated osteocalcin will be measured using colourimetric Sandwich ELISA kits from NOVOS Biologicals.



4. Biosafety Level (BSL) of the proposed activity:

Note: The biosafety containment level is determined by the risk assessment of the activity.

BSL 1 ☐ BSL 2 ☒ BSL 3 ☐ BSL 4 ☐

5. Estimated duration of activity

Note 1: Please indicate the duration of this activity

Note 2: Please provide a Gantt chart that itemizes the sequence of the activity within the time period stipulated.

Note 3: Separate attachment may be provided for the Gantt chart or incorporated into the text box below:

November 2022 to July 2023

6. Intended Date of Commencement:

Note: Please provide an estimate of a feasible date that takes into account the documents processing time period of IBC (if relevant) and documents processing time period of the Department of Biosafety.

January 2023

7. Expected Date of Completion:

Note: Please ensure that the information provided here is consistent with what is provided in the Gantt Chart.

The work for the FRGS grant has been delayed due to various reasons e.g. MCO and problems with growing primary osteoblast cells. Therefore, we have decided to use an immortalised cell line. Hopefully the labwork will be done by the end of July 2023

8. Date of importation or expected importation of LMO (if relevant)

Note: Import of LMO is not allowed until Applicant receives the Letter of Acknowledgement from the Director General of the Department of Biosafety.

We not planning to import an LMO. Part its DNA has been used to conditionally immortalized fetal osteoblast cells. Attaching the NIH guidelines, which classifies the SV40 as not harmful as mentioned in **Appendix B-V. Animal Viral Etiologic Agents in Common Use**

9. If the experiments are successful, are there plans for an application for field experiment / large scale production /commercialization?

☐ Yes ☒ No

PART C: DESCRIPTION OF THE LMO

10. Please refer to the Explanatory Notes of NBB/N/CU/22/FORM E (at the end of the form) on part C before filling in the specific information in a tabulated form as shown below.

Table 2: Description of the LMO for contained use activities

<i>LMO</i>	Common and scientific name(s) of recipient organism	Common and scientific name(s) of donor organism	Vector(s) and method of genetic modification	Class of modified trait (Refer to Box 1 of the Explanatory Notes)	Modified trait	Number of genes involved (Please provide the gene construct(s) map)	Identity and function of the gene(s) involved and protein(s) expressed

1.	hFOB 1.19; Osteoblast; Human (Homo sapiens)	osteoblast; SV40 large T antigen transfected	This line was established by transfection of limb tissue obtained from a spontaneous miscarriage with the temperature sensitive expression vector pUCSVtsA58 and the neomycin resistance expression vector pSV2-neo. Clones were selected in the presence of 0.6 mg/mL G418. Not harmful according to Appendix B-V. Animal Viral Etiologic Agents in Common Use, NIH guidelines	19	The cells provide a homogenous, rapidly proliferating model system for studying normal human osteoblast differentiation, osteoblast physiology, and hormonal, growth factor, and other cytokine effects on osteoblast function and differentiation conditionally immortalized fetal osteoblast cells	SV40 large T antigen  SV40 T helicase domain hexamer, Simian virus <table><thead><tr><th colspan="2">Identifiers</th></tr></thead><tbody><tr><td>Organism</td><td>Simian virus 40 [2]</td></tr><tr><td>Symbol</td><td>?</td></tr><tr><td>UniProt</td><td>P03070 [2]</td></tr></tbody></table> The SV40 large T-antigen has been used as a model protein to study nuclear localization signals (NLSs). It is imported into the nucleus by its interaction with importin α. The NLS sequence is PKKKRKV. Copied from Wikipedia: https://en.wikipedia.org/wiki/SV40_large_T_antigen	Identifiers		Organism	Simian virus 40 [2]	Symbol	?	UniProt	P03070 [2]	osteoblast; SV40 large T antigen transfected
Identifiers															
Organism	Simian virus 40 [2]														
Symbol	?														
UniProt	P03070 [2]														

PART D: THE PREMISES

11. Please provide information for **all of the premises** being used for the contained use activities in the table below. This includes premises for production/ handling of LMO materials, treatment of LMO materials and storage of LMO materials.

Note 1: For notifications with more than one premises, use additional columns provided.

Note 2: For a Research and Development collaboration involving more than one organization (premises is under the oversight of a different IBC), please provide proof of collaboration (such as letter of authorization) to use the premises.

Table 3: Details of premises

Information required	Premises 1	Premises 2*	Premises 3*	Premises 4*
1. Name of premises:	Faculty of Medicine and Health Science, UNIMAS	Click here to enter text.	Click here to enter text.	Click here to enter text.
2. Premises type: <i>Note 1:</i> Example of premises type includes, animal containment premises, laboratory, insect containment premises, greenhouse, etc.) <i>Note 2:</i> Please specify if it is a large scale facility involving culture volume greater than or equal to 10L of culture of any LMO.	Biochemistry and Medical Genetics Research Laboratory	Click here to enter text.	Click here to enter text.	Click here to enter text.
3. Biosafety level (BSL):	2	Click here to enter text.	Click here to enter text.	Click here to enter text.

Information required	Premises 1	Premises 2*	Premises 3*	Premises 4*
4. Purpose of using the premises <i>Note:</i> Please indicate which step(s) of the activity the premises is used for, e.g. genetic manipulation or the organism, feeding study on mice, treatment of LMO materials, or for storage.	Investigating the effect of T crispa and T cordifolia on methyl glyoxal incubated osteoblast cells. This is a cell culture study FMHS/Genetics lab/Biochemistry lab have a laminar flow/BSC class2, CO2 incubator and the required analytical instruments e.g. ELISA plate reader, fluorescence microscope etc	Click here to enter text.	Click here to enter text.	Click here to enter text.
5. Who undertook the most recent inspection of the premises? <i>Note:</i> Please indicate which IBC, if more than one IBC is involved in the premises used or if it the most recent inspection was done by the Department of Biosafety enforcement.	The cell culture-related instruments are serviced on a regular basis. For more information refer to the science officer in charge	Click here to enter text.	Click here to enter text.	Click here to enter text.

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Information required	Premises 1	Premises 2*	Premises 3*	Premises 4*
6. Date of the most recent inspection: <i>Note: Provide the most recent inspection report which is NOT MORE than two years from the commencement date of the activity and include a record of any corrective actions as recommended by the report.</i>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
7. Fill the following if premises is BSL 3 or BSL 4: <i>Note: Provide the latest inspection report and a record of any corrective actions as recommended by the report.</i> a) Date of certification by competent authority b) Certificate reference no:	Click here to enter text. Click here to enter text.	Click here to enter text. Click here to enter text.	Click here to enter text. Click here to enter text.	Click here to enter text. Click here to enter text.
8. Full address of premises: <i>Note: Includes floor level, if relevant, and name of building.</i>	Level 1, Research wing, Faculty of Medicine and Health Science	Click here to enter text.	Click here to enter text.	Click here to enter text.
9. Name of contact person in charge of premises/	Safarudin Bin Pa'ie Prof Chew Keng Sheng	Click here to enter text.	Click here to enter text.	Click here to enter text.

10

NBB/N/CU/22/FORM E

Information required	Premises 1	Premises 2*	Premises 3*	Premises 4*
<i>Note: This could be the Biosafety Officer or Laboratory Manager or any other person in charge of the premises</i>				
10. Office/ Premises phone number: <i>Note: Please provide a number that the person in charge will be accessible to take the call.</i>	+6082-267446	Click here to enter text.	Click here to enter text.	Click here to enter text.
11. Mobile phone number:	0138464934	Click here to enter text.	Click here to enter text.	Click here to enter text.
12. Fax number:	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
13. E-mail address:	psafarudin@unimas.my kschew@unimas.my	Click here to enter text.	Click here to enter text.	Click here to enter text.

PART E: RISK ASSESSMENT AND MANAGEMENT

E1 Risk Assessment (Basic information)

12. You are required to fill in the matrix below. Consider what are the possible hazard(s), and the likelihood and consequences of the hazard(s) occurring (i.e. the risk) from the genetic modification(s) and proposed activity to the health and safety of human, plant and animals and the environment (including unintentional release).
Please refer to Chapter 4 of Biosafety Guidelines: Contained use activity of Living Modified Organism (<https://www.biosafety.gov.my/wp-content/uploads/2021/08/Garis-Panduan-Aktiviti-Kegunaan-Terkawal-LMO.pdf>).

RISK ASSESSMENT MATRIX

11

Assessment category	Identification of Potential hazard	Comments on risk	Risk Management by Applicant
Potential risk from the science of genetic modification <i>Points for consideration</i> a) Hazards from gene(s), protein(s), change in virulence /pathogenicity of LMO b) Risk from vector used (e.g. retroviral vector, etc) ...	Nil Refer to <i>NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules p 49</i> 	Click here to enter text.	Click here to enter text.
	nil	Click here to enter text.	Click here to enter text.
	nil	Click here to enter text.	Click here to enter text.
	nil	Click here to enter text.	Click here to enter text.
Risk to human health (occupational exposure) <i>Points for consideration</i> a) Activities with LMO (e.g. large volume, handling sharps, competency of personnel, use of PPE, compliance to SOPs) b) Equipment used (e.g. BSC)	nil	Click here to enter text.	Click here to enter text.
	nil	Click here to enter text.	Click here to enter text.
	nil	Click here to enter text.	Click here to enter text.
Containment integrity (risk of unintentional release of the LMO to the environment)	nil	Click here to enter text.	Click here to enter text.

Assessment category	Identification of Potential hazard	Comments on risk	Risk Management by Applicant
<i>Points for consideration</i> a) Maintenance of the facility and equipment b) Transfer of LMO between premises c) Waste decontamination	nil	Click here to enter text.	Click here to enter text.
	nil	Click here to enter text.	Click here to enter text.
	nil	Click here to enter text.	Click here to enter text.
Special risks unique to notification <i>Points for consideration</i> a) Long term duration b) Use of animals/ arthropods/ exotic species c) Immunocompromised personnel d) Techniques used (e.g. synthetic biology, genome editing) e) Large scale volume of LMO	nil	Click here to enter text.	Click here to enter text.
	nil	Click here to enter text.	Click here to enter text.

E2 Risk Management

For questions 13-17, an activity specific SOP endorsed by the IBC must be provided. The date that the SOP has been developed/approved or reviewed/revised should not exceed more than two years from the date of submission to this form.

13. Do you propose to transfer/transport the LMO between premises?

Note 1: Please ensure all the premises used are included in Part D of this form and are stated in the activity flowchart provided.

Note 2: If yes, please give statement(s) and identify/ state the relevant SOP provided for this.

No

14. What is the treatment method and how will the LMO and related wastes be disposed of?

Note 1: If the activity involves LMO at various growth stages (seedlings, trees), the SOP should cover the disposal of LMO at each growth stage.

Note 2: Please give statement(s) and identify/ state the relevant SOP provided for this.

Note 3: Please refer to Chapter 12 and 13 of Biosafety Guidelines: Contained use activity of Living Modified Organism (<https://www.biosafety.gov.my/wp-content/uploads/2021/08/Garis-Panduan-Aktiviti-Kegunaan-Terkawal-LMO.pdf>)

We will follow standard biosafety procedures for cell culture material e.g. autoclaving and using chlorox. Below are examples of treatment of cells, media and tissue culture consumables copied from MERCK

Tissue culture waste (culture medium) – inactivate for at least 2 hours in a solution of hypochlorite (10,000ppm) prior to disposal to drain with an excess of water Contaminated pipettes should be placed in hypochlorite solution (2500ppm) overnight before disposal by incineration Solid waste such as flasks, centrifuge tubes, contaminated gloves, tissues, etc., should be placed inside heavy-duty sacks for contaminated waste and incinerated If at all possible waste should be incinerated rather than autoclaved copied from <https://www.sigmaaldrich.com/MY/en/technical-documents/technical-article/cell-culture-and-cell-culture-analysis/mammalian-cell-culture/safety-aspects-of>

15. How will the solid and liquid wastes from the activities be treated/decontaminated and disposed of?

Note 1: Examples of wastes include media, disposable gloves, planting materials, plant parts, etc.

Note 2: Please give statement(s) and identify/ state the relevant SOP provided for this.

We will follow standard biosafety procedures for cell cultures, for example: Alcohol (e.g. Ethanol, Isopropanol) Effective concentrations: 70% for ethanol, 60-70% for isopropanol Their mode of action is dehydration and fixation Effective against bacteria. Ethanol is effective against most viruses but not nonenveloped viruses Isopropanol is not effective against viruses Aldehydes

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(e.g. Formaldehyde) Aldehydes are irritants, and their use should be limited due to problems of sensitization. Should only be used in well ventilated areas. Formaldehyde is used to fumigate laboratories. The formaldehyde is heated in a device so it will vaporize and all exposed surfaces are coated with the disinfectant. Copied from Merck

<https://www.sigmaaldrich.com/MY/en/technical-documents/technical-article/cell-culture-and-cell-culture-analysis/mammalian-cell-culture/safety-aspects-of>

16. How will the wastewater from the activities be disposed of?

Note 1: Examples of wastewater include water used for cleaning equipment, watering the plants, keeping fish, etc.

Note 2: Please give statement(s) and identify/ state the relevant SOP provided for this.

Normal biosafety procedures for culture cells

17. How will the equipment/tools/surfaces used during the activities be decontaminated?

Note 1: Examples of equipment/tools/surfaces include sharps, pipette, decontaminated glassware, etc.

Note 2: Please give statement(s) and identify/ state the relevant SOP provided for this.

Normal biosafety procedures for culture cells

E3 Emergency Response Plan

For questions 18-22, form [IBC/IR/10/ANNEX3](#) or [IBCOD/10/ANNEX4](#) shall to be used to report all incidents or occupational exposure to LMO materials to IBC and Department of Biosafety in addition to any internal administrative procedures in place. This requirement must be included in the SOP provided.

18. Provide plans for protecting human and animal health and the environment in case of the occurrence of an adverse effect observed during contained use activities.

Note 1: Examples include medical management which includes first aid and hospitalization, line of communication both within and outside the organization.

Note 2: Please give statement(s) and identify/ state the relevant SOP provided for this.

Normal biosafety procedures for culture cells

19. Provide plans for removal of the LMO in the affected areas in the case of an unintentional release

Note 1: Examples include to contain and treat spillage.

Note 2: Please give statement(s) and identify/ state the relevant SOP provided for this.

Normal biosafety procedures for culture cells

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20. Provide plans for disposal of plants, animals and any other organisms exposed during the unintentional release.

Note: Please give statement(s) and identify/ state the relevant SOP provided for this.

Normal biosafety procedures for culture cells

21. Provide plans for isolation of the area affected by the unintentional release

Note 1: Examples include evacuation and quarantine.

Note 2: Please give statement(s) and identify/ state the relevant SOP provided for this.

Normal biosafety procedures for culture cells

22. Provide details of any other contingency measure that will be in place to rectify any unintended consequences if an adverse effect becomes evident during the contained use activities or when an unintentional release occurs.

Note: Please give statement(s) and identify/ state the relevant SOP provided for this.

Normal biosafety procedures for culture cells

PART F: CONFIDENTIAL BUSINESS INFORMATION

Enter in this section any information required in Parts A - E for which confidentiality is claimed together with full justification for that claim.

Criteria for confidentiality are as follows (section 59 of Biosafety Act 2007):

- a) that the information is not known generally among, or readily accessible to, any person within the circle that normally deals with the kind of information sought to be made confidential
- b) that the information has commercial value because it is secret
- c) that reasonable steps have been taken to keep the information secret.

[Click here to enter text.](#)

PART G: REFERENCES

Please include references mentioned in the Project Description, supporting documents for any statements in the Risk Assessment matrix or any other relevant references associated with this activity.

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1. NIH GUIDELINES FOR RESEARCH INVOLVING RECOMBINANT OR SYNTHETIC NUCLEIC ACID MOLECULES (NIH GUIDELINES) APRIL 2019 (PDF file is attached)
2. Alyaa R. AL-Khateeb, Nurhafizah Md. Shaari, Suhaila Abd. Muid, Gabriele RA Froemming, A Critical Link Between Advanced Glycation End Products, Osteoporosis And Type 2 Diabetes Mellitus, Regenerative Research 6(2) 2018 1-9
3. General guidelines for cell immortalization from ABM
4. Subramaniam M, Jalal SM, Rickard DJ, Harris SA, Bolander ME, Spelsberg TC. Further characterization of human fetal osteoblastic hFOB 1.19 and hFOB/ER alpha cells: bone formation in vivo and karyotype analysis using multicolor fluorescent in situ hybridization. J Cell Biochem. 2002;87(1):9-15. doi: 10.1002/jcb.10259. PMID: 12210717

EXPLANATORY NOTES FOR FORM E

**NOTIFICATION FOR CONTAINED USE AND IMPORT FOR CONTAINED USE ACTIVITIES
INVOLVING LIVING MODIFIED ORGANISM (LMO) FOR BIOSAFETY LEVELS 1, 2, 3 AND 4**

NBB/N/CU/20/FORM E shall be submitted to the Director General as a notification for contained use and import for contained use [not involving release into the environment of Living Modified Organism (LMO) as specified in Second Schedule of the Biosafety Act 2007]. Any organization undertaking modern biotechnology research and development shall submit the notification through its Institutional Biosafety Committee (IBC) that is registered with the National Biosafety Board (NBB). The IBC should do an assessment prior to submission and submit the result of the assessment via the [IBC Assessment Form \(IBC/AP/20/ANNEX 2\)](#). Not all parts in this form will apply to every case. Therefore, Applicants will only address the specific questions/parameters that are appropriate to individual applications.

In each case where it is not technically possible or it does not appear necessary to give the information, the reasons shall be stated. If there are other related documents (example If a comprehensive description of the activity is provided in the Research Project proposal, please provide a summary in in Form E but you may provide a reference to the proposal document for more details). The risk assessment, risk management plan, emergency response plan and the fulfillment of any other requirements under the Biosafety Act 2007 will be the basis of the decision by the NBB.

The Applicant shall submit 1 original and 6 copies of the notification to the Director General. The six copies submitted should be identical to the original form. Please ensure that the information provided can be clearly read/seen. This submission should be accompanied by a cover letter from the Applicant's institution. A soft copy of the submitted notification **(including all supporting documents/attachments, if any)** shall also be provided by the Applicant. However, **all information that has been declared as Confidential Business Information (CBI) should be omitted from the softcopy**. You may collate documents related to one notification into one document/ softcopy (do not combine with any other notification that you may submit concurrently).

Providing information

The information provided in this notification will be used to evaluate the emergency response plan as specified in section 37 of the Biosafety Act 2007 and specific measures to be taken in relation to a contained use activity involving LMO. Therefore it is important to provide accurate and timely information that is as comprehensive as existing scientific knowledge would permit, and supported by whatever data available.

If the LMO is imported, details of importer, date of intended importation and approval from relevant authorities like Department of Agriculture (DOA), Ministry of Health, Malaysia, etc. should be

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provided.

If the activity involves work with animals (example of feeding studies involving LMO products), please provide the status of approval from the Institutional Animal Care and Use.

The NBB may require additional information, and the applicant will be notified should this be the case. If the applicant fails to provide the additional information requested, the notification shall be deemed to have been withdrawn but it shall not affect the right of the applicant to make a fresh notification.

Description of LMO – Table 2 (Part C)

- a) 'Recipient organism' refers to the final recipient of the intended genetic modification.
- b) 'Donor organism' refers to the source of the genetic sequences used for modification. If more than one gene is used and the source is different for each gene, ensure that the donor organism for each gene used is stated.
- c) 'Vector' should include all vectors and method (s) used.
- d) 'Modified trait' can be stated as "unknown" if for example building a genomic library.
- e) Identity and function of gene(s) of donor organism responsible for the modified trait can be stated as "unknown" if for example building a genomic library.

Class of modified trait, please refer box below.

If the LMO has more than one modified trait please list all. If the modified trait is not listed in the Box 1, please list it as "other" and provide details of the modified traits

Box 1: Class of modified trait

NO	Class (type) of trait
1	Abiotic stress resistance
2	Altered agronomic characteristics
3	Altered nutritional characteristics
4	Altered pharmaceutical characteristics
5	Altered physical product characteristics
6	Antibiotic resistance
7	Foreign antigen expression
8	Attenuation
9	Bacterial resistance
10	Disease resistance
11	Flower colour
12	Fungal resistance
13	Herbicide tolerance
14	Immuno-modulatory protein expression

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NO	Class (type) of trait
15	Pest resistance e.g. insect resistance
16	Protein expression (please specify)
17	Reporter/marker gene expression
18	Virus resistance
19	Others (please specify)

Accuracy of information

The notification should also be carefully checked before submission to ensure that all the information is accurate. If the information provided is incorrect, incomplete or misleading, the NBB may issue a withdrawal of the acknowledgement of receipt of notification without prejudice to the submission of a fresh notification

Confidentiality

Any information within this notification which is to be treated as Confidential Business Information (CBI), as described in section 59(3) of the Biosafety Act 2007 should be clearly marked "CBI" in the relevant parts of the notification by providing the justification for the request for CBI. The following information shall not be considered confidential:

- a) The name and address of the applicant
- b) A general description of the LMO
- c) A summary of the risk assessment of the effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health; and
- d) Any methods and plans for emergency response

Authorization

Please ensure that if this notification is being completed on behalf of the proposed user, that the person completing this notification holds proper authority to submit this notification for the proposed user. Please provide written proof of authorization.

For further information or any queries related to filling up this form, please contact jbksnp@biosafety.gov.my

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The completed form, cover letter and relevant documents shall be submitted to:

The Director General
Department of Biosafety
Level 4, Block F11, Complex F
Lebuh Perdana Timur, Precinct 1
Federal Government Administrative Centre
62000 Putrajaya, Malaysia

Acknowledgement of Receipt

Upon receipt of the notification, the Director General of the Department of Biosafety shall send to the applicant a Letter of Acknowledgement of receipt with an assigned reference number. The reference number should be used in all correspondence with respect to the notification. The activity (contained use activity and import of any LMO) can start only after the Letter of Acknowledgement is issued. The Principal Investigator is still required to be complaint to any decisions made by the NBB (as described in section 30(3) of the Biosafety Act 2007 and is required to comply with other written laws governing LMO.

Exemption

[The First Schedule of the Biosafety \(Approval and Notification\) Regulations 2010](#) allows exemptions for some types of techniques and contained use activities in relation to LMO posing a very low risk (i.e. contained research activities involving very well understood organisms and processes for creating and studying LMO). Exempted activities should be carried out under conditions of standard laboratory practice. Appropriate biosafety levels as according to Second Schedule of the Biosafety (Approval and Notification) Regulations 2010 should be used for the exempted activities and personnel should have appropriate training. Principal Investigators who believe that the work falls into any of the exemptions should nevertheless notify their IBC of the proposed project. The IBC shall review all submitted research projects to determine their exemption or non-exemption status. The IBC will provide oversight of the exempted activities and report to the Department of Biosafety through the IBC Annual Report.

Please retain a copy of your completed notification.

Appendix C: Material Transfer Agreement Form



Material Transfer Agreement

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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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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

Address: FMHS, Universiti Malaysia Sarawak (UNIMAS), 94300 Kota Samarahan,
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

Appendix D: Tables for Chapter 4- Results

Table 6.1: Percentage viability of hFOB 1.19 cells when treated with different concentration of MGO (mM) for 24 hours. There are significant differences ($p<0.05$) between all MGO concentrations used and the control group, $n=3$.

Concentrations of MGO (mM)	Percentage of viability (%)			Mean $\pm$ SD
	Replicate 1	Replicate 2	Replicate 3	
0.00	97.10	99.04	103.86	100.00 $\pm$ 3.4775
0.22	129.58	146.06	141.05	138.90 $\pm$ 8.4510
0.44	136.65	135.74	142.76	138.38 $\pm$ 3.8192
0.88	131.94	129.45	122.18	127.86 $\pm$ 5.0711
1.75	41.56	41.54	43.52	42.21 $\pm$ 1.1397

Table 6.2: Percentage viability of hFOB 1.19 cells when treated with different concentration of metformin (mM) for 24 hours, $n=3$.

Concentrations of metformin (mM)	Percentage of viability (%)			Mean $\pm$ SD
	Replicate 1	Replicate 2	Replicate 3	
0	94.55	106.65	98.79	100.00 $\pm$ 6.14161
3	129.88	125.62	106.40	120.63 $\pm$ 12.5077
6	117.08	111.25	108.21	112.18 $\pm$ 4.5049
12	91.91	90.16	95.03	92.37 $\pm$ 2.4672
24	77.70	83.63	80.71	80.68 $\pm$ 2.9635

Table 6.3: Percentage viability of hFOB 1.19 when treated with different concentration of *T. crista* methanol for 24 hours. There are significant differences ($p<0.05$) between all different *T. crista* methanol extract concentrations and the control group, $n=3$.

Concentrations of TiM ($\mu\text{g mL}^{-1}$)	Percentage of viability (%)			Mean $\pm$ SD
	Replicate 1	Replicate 2	Replicate 3	
0	100.14	99.51	100.36	100.00 $\pm$ 0.4417
29.70	135.55	134.11	133.26	134.30 $\pm$ 1.1605
59.40	139.79	132.64	129.68	134.04 $\pm$ 5.1963
118.75	126.20	123.22	121.02	123.48 $\pm$ 2.6015
237.50	122.70	120.87	127.39	123.65 $\pm$ 3.3606
475.00	125.74	123.07	122.04	123.62 $\pm$ 1.9101

Table 6.4: Percentage viability of hFOB 1.19 when treated with different concentration of *T. crista* water for 24 hours. There are significant differences ($p<0.05$) between all of the different *T. crista* water extract concentrations and the control group, $n=3$.

Concentrations of TiW ($\mu\text{g mL}^{-1}$)	Percentage of viability (%)			Mean $\pm$ SD
	Replicate 1	Replicate 2	Replicate 3	
.00	100.14	99.51	100.36	100.00 $\pm$ 0.4417
187.50	136.29	137.11	127.15	133.51 $\pm$ 5.5302
375.00	122.07	133.38	120.35	125.27 $\pm$ 7.0801
750.00	125.07	120.81	127.83	124.57 $\pm$ 3.5348
1500.00	127.46	127.00	108.95	121.14 $\pm$ 10.5573
3000.00	122.70	122.68	121.00	122.13 $\pm$ 0.9780

Table 6.5: Background-subtracted O.D raw data with the mean O.D $\pm$ SD for different concentrations of ferrous sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), the standard of FRAP assay.

Standard Curve Measurements			
Concentrations of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ($\mu\text{g/mL}$)	O.D 450 nm		Mean O.D $\pm$ SD
	Replicate 1	Replicate 2	
25	0.0618	0.0637	0.0628 $\pm$ 0.0013
50	0.0786	0.0774	0.0780 $\pm$ 0.0008
100	0.0912	0.0912	0.0912 $\pm$ 0.0000
200	0.1502	0.1490	0.1496 $\pm$ 0.0008
400	0.2188	0.2183	0.2186 $\pm$ 0.0004

Table 6.6: Table below show FRAP values (mmol Fe^{2+} per μg of extract) presented as mean $\pm$ standard deviation (n=3). Asterisk (*) symbol indicates that the value of the mean is significantly different ($p < 0.05$) when compared to their respective standards with the same concentration.

Concentration of the extracts ($\mu\text{g mL}^{-1}$)	FRAP (mmol Fe^{2+} per μg of extract)	
	<i>T. crispa</i> water extract	<i>T. crispa</i> methanol extract
50	9.8932 $\pm$ 0.2131	29.5539 $\pm$ 0.1776
100	17.7524 $\pm$ 0.1065	50.0935 $\pm$ 0.1065
200	28.4491 $\pm$ 0.0355	80.1245 $\pm$ 0.1776
400	45.0716 $\pm$ 1.2429	121.0028 $\pm$ 0.8167

Table 6.7: Net RFI raw data with the mean concentrations of IL-1 β $\pm$ SD (pg/mL) for different treatment combinations of MGO-stimulated hFOB1.19 cells. ALL of the results presented in this table are extrapolated from the available data.

MG-stimulated hFOB 1.19 treatments					
Combination of treatments	Concentrations of IL-1 β				Mean IL-1 β $\pm$ SD (pg/mL)
	Net RFI		(pg/mL)		
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	
Control	1.75	1.75	1.1815	1.1815	1.1815 $\pm$ 0.0000
MG only	2.25	3.75	1.5662	2.6641	2.1151 $\pm$ 0.7764
MG + Met	2.75	2.75	1.9399	1.9399	1.9399 $\pm$ 0.0000
MG + TiW-1	2.75	1.75	1.9399	1.1815	1.5607 $\pm$ 0.5363
MG + TiW-2	3.25	2.25	2.3054	1.5662	1.9358 $\pm$ 0.5227
MG + TiW-3	0.75	0.75	0.3533	0.3533	0.3533 $\pm$ 0.0000
MG + TiM-1	2.75	2.25	1.9399	1.5662	1.7530 $\pm$ 0.2643
MG + TiM-2	0.75	1.75	0.3533	1.1815	0.7674 $\pm$ 0.5856
MG + TiM-3	2.75	1.75	1.9399	1.1815	1.5607 $\pm$ 0.5363
MG + TiW-1 + Met	2.75	2.75	1.9399	1.9399	1.9399 $\pm$ 0.0000
MG + TiW-2 + Met	1.75	0.75	1.1815	0.3533	0.7674 $\pm$ 0.5856
MG + TiW-3 + Met	0.75	0.75	0.3533	0.3533	0.3533 $\pm$ 0.0000
MG + TiM-1 + Met	2.25	1.75	1.5662	1.1815	1.3738 $\pm$ 0.2720
MG + TiM-2 + Met	-0.25	-0.25	<18.2305	<18.2305	<18.2305
MG + TiM-3 + Met	0.25	-0.75	<18.2305	<18.2305	<18.2305

Table 6.8: Net RFI raw data with the mean concentrations of IL-6 $\pm$ SD (pg/mL) for different treatment combinations of MGO-stimulated hFOB1.19 cells. The results presented in this table labelled with an asterisk (*) are extrapolated from the available data.

MG-stimulated hFOB 1.19 treatments					
Combination of treatments	Net RFI		Concentrations of IL-6		Mean IL-6 ± SD (pg/mL)
			(pg/mL)		
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	
Control	248.000	234.000	63.0126	59.6015	61.3070±2.4120
MG only	628.000	579.000	153.4963	141.9501	147.7232±8.1644
MG + Met	937.000	967.000	227.1529	234.5170	230.8350±5.2072
*MG + TiW-1	1800.500	1765.000	776.9610	632.2832	704.6221±102.3026
MG + TiW-2	51.000	55.000	13.7344	14.7805	14.2575±0.7397
MG + TiW-3	45.500	45.000	12.2897	12.1580	12.2239±0.0932
MG + TiM-1	65.000	79.500	17.3804	21.1176	19.2490±2.6426
MG + TiM-2	416.000	390.500	103.3860	97.3113	100.3486±4.2955
MG + TiM-3	690.000	747.000	168.1117	181.5812	174.8464±9.5244
*MG + TiW-1 + Met	1466.000	1466.000	381.8751	355.6507	368.7629±18.5435
MG + TiW-2 + Met	30.000	32.000	8.1692	8.7057	8.4374±0.3794
MG + TiW-3 + Met	19.000	21.000	5.1834	5.7314	5.4574±0.3875
MG + TiM-1 + Met	31.500	33.000	8.5717	8.9733	8.7725±0.2008
*MG + TiM-2 + Met	0	0	<1.5226	<1.5226	<1.5226
*MG + TiM-3 + Met	0	-1.000	<1.5226	<1.5226	<1.5226

Table 6.9: Net RFI raw data with the mean concentrations of M-CSF $\pm$ SD (pg/mL) for different treatment combinations of MGO-stimulated hFOB1.19 cells. The results presented in this table labelled with an asterisk (*) are extrapolated from the available data.

MG-stimulated hFOB 1.19 treatments					
Combination of treatments	Concentrations of				Mean M-CSF ± SD (pg/mL)
	Net RFI		M-CSF (pg/mL)		
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	
Control	60.5	69.0	301.7244	341.1436	321.4340±27.8736
*MG only	6.5	7.5	53.2545	57.8006	55.5275±3.2146
*MG + Met	4.5	2.5	32.8579	35.1184	33.9882±1.5984
*MG + TiW-1	2.5	4.5	35.1184	44.1758	39.6471±6.4046
MG + TiW-2	44.5	45.5	227.6890	232.3090	229.9990±3.2668
MG + TiW-3	30.5	37.0	163.1316	193.0744	178.1030±21.1727
MG + TiM-1	24.5	49.5	135.5474	250.7993	193.1734±81.4954
*MG + TiM-2	7.5	5.5	57.8006	48.7127	53.2567±6.4261
*MG + TiM-3	0.5	4.5	26.0877	44.1758	35.1317±12.7902
*MG + TiW-1 + Met	2.0	4.5	32.8579	44.1758	38.5169±8.0029
MG + TiW-2 + Met	43.5	47.0	223.0701	239.2410	231.1555±11.4346
MG + TiW-3 + Met	37.0	40.0	193.0744	206.9124	199.9934±9.7850
MG + TiM-1 + Met	29.5	25.5	158.5303	140.1407	149.3355±13.0034
*MG + TiM-2 + Met	2.5	3.5	35.1184	39.6442	37.3813±3.2002
*MG + TiM-3 + Met	3.5	1.5	39.6442	30.5993	35.1217±6.3957

Table 6.10: Background-subtracted O.D raw data with the mean O.D $\pm$ SD for different concentrations of sRAGE (pg/mL) standards.

Standard Curve Measurements			
Concentrations of sRAGE (pg/mL)	O.D 450 nm		Mean O.D $\pm$ SD
	Replicate 1	Replicate 2	
23.43	0.00275	0.01515	0.00895 $\pm$ 0.00895
46.87	0.02865	0.02765	0.02815 $\pm$ 0.00071
93.75	0.06355	0.05035	0.05695 $\pm$ 0.00933
187.50	0.17545	0.16795	0.17170 $\pm$ 0.00530
375.00	0.39075	0.34185	0.36630 $\pm$ 0.03458
750.00	0.76175	0.79795	0.77985 $\pm$ 0.02560
1500.00	1.78765	1.68485	1.73625 $\pm$ 0.07269

Table 6.11: Background-subtracted O.D raw data with the mean concentrations of sRAGE $\pm$ SD (pg/mL) for different treatment combinations of MGO-stimulated hFOB1.19 cells.

MGO-stimulated hFOB 1.19 treatments					
Combination of treatments	O.D 450 nm		Concentrations of RAGE (pg/mL)		Mean sRAGE $\pm$ SD (pg/mL)
			Replicate 1	Replicate 2	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	
Control	0.01325	0.01485	32.1467	34.4314	33.2891 $\pm$ 1.6155
MG only	0.02725	0.02965	46.7316	49.1088	47.9202 $\pm$ 1.6809
MG + Met	0.03005	0.03155	49.5049	50.9900	50.2475 $\pm$ 1.0501
MG + TiW-1	0.02905	0.03525	48.5146	54.6513	51.5830 $\pm$ 4.3393
MG + TiW-2	0.03205	0.03805	51.4849	57.4203	54.4526 $\pm$ 4.1970
MG + TiW-3	0.03545	0.03925	54.8492	58.6065	56.7279 $\pm$ 2.6568
MG + TiM-1	0.03535	0.03425	54.7502	53.6620	54.2061 $\pm$ 0.7696

Table 6.11: continued

MG + TiM-2	0.03635	0.03955	55.7393	58.9030	57.3212±2.2371
MG + TiM-3	0.03685	0.04495	56.2338	64.2374	60.2356±5.6594
MG + TiW-1 + Met	0.03045	0.02905	49.9010	48.5146	49.2078±0.9803
MG + TiW-2 + Met	0.04575	0.03615	65.0271	55.5415	60.2843±6.7073
MG + TiW-3 + Met	0.04125	0.04775	60.5830	67.0011	63.7921±4.5383
MG + TiM-1 + Met	0.03515	0.03505	54.5524	54.4535	54.5030±0.0699
MG + TiM-2 + Met	0.04115	0.04735	60.4842	66.6064	63.5453±4.3291
MG + TiM-3 + Met	0.04675	0.04445	66.0142	53.7437	59.8780±8.6766

Table 6.12: Background-subtracted O.D raw data with the mean O.D ± SD for different concentrations of RAGE (pg/mL) standards.

Standard Curve Measurements			
Concentrations of RAGE (pg/mL)	O.D 450 nm		Mean O.D ± SD
	Replicate 1	Replicate 2	
78.13	0.1192	0.1257	0.1225±0.0046
156.25	0.2047	0.2073	0.2060±0.0018
312.50	0.3192	0.3081	0.3137±0.0078
625.00	0.5779	0.6056	0.5918±0.1959
1250.00	0.9367	1.0647	1.0007±0.0640
2500.00	1.9030	1.9637	1.9334±0.0429
5000.00	3.3870	3.5051	3.4461±0.0835

Table 6.13: Background-subtracted O.D raw data with the mean concentrations of RAGE $\pm$ SD (pg/mL) for different treatment combinations of MGO-stimulated hFOB1.19 cells.

MGO-stimulated hFOB 1.19 treatments					
Combination of treatments	O.D 450 nm		Concentrations of RAGE (pg/mL)		Mean RAGE±SD (pg/mL)
			Replicate	Replicate	
	1	2	1	2	
Control	0.1534	0.1526	34.8571	33.7143	34.2857±0.8081
MG only	0.1534	0.1549	34.8571	37.0000	35.9286±1.5153
MG + Met	0.1520	0.1519	32.8571	32.7143	32.7857±0.1010
MG + TiW-1	0.1501	0.1511	30.1429	31.5714	30.8572±1.0101
MG + TiW-2	0.1514	0.1511	32.0000	31.5714	31.7857±0.3031
MG + TiW-3	0.1501	0.1482	30.1429	27.4286	28.7857±1.9193
MG + TiM-1	0.1540	0.1532	35.7143	34.5714	35.1429±3.3335
MG + TiM-2	0.1528	34.0000	0.1521	33.0000	33.5000±0.7071
MG + TiM-3	0.1502	0.1535	30.2857	35.0000	32.6429±0.8082
MG + TiW-1 + Met	0.1520	0.1491	32.8571	28.7143	30.7857±2.9294
MG + TiW-2 + Met	0.1472	0.1464	26.0000	24.8571	25.4286±0.8082
MG + TiW-3 + Met	0.1497	0.1473	29.5714	26.1429	27.8571±2.4243
MG + TiM-1 + Met	0.1493	0.1518	29.0000	32.5714	30.7857±2.5254
MG + TiM-2 + Met	0.1520	0.1470	32.8571	32.7143	32.7857±0.1010
MG + TiM-3 + Met	0.1486	0.1449	28.0000	22.7143	25.3571±3.7376

Table 6.14: Background-subtracted O.D raw data with the mean O.D $\pm$ SD for different concentrations of RANKL (pg/mL) standards.

Standard Curve Measurements			
Concentrations of RANKL (pg/mL)	O.D 450 nm		Mean O.D $\pm$ SD
	Replicate 1	Replicate 2	
78.13	0.0154	0.0218	0.01860 $\pm$ 0.00453
156.25	0.0566	0.0426	0.04960 $\pm$ 0.00990
312.50	0.1099	0.1383	0.12410 $\pm$ 0.02008
625.00	0.2285	0.1795	0.20400 $\pm$ 0.03465
1250.00	0.4281	0.4589	0.44350 $\pm$ 0.02178
2500.00	0.7062	1.0567	0.88145 $\pm$ 0.24784
5000.00	1.0935	1.355	1.22425 $\pm$ 0.18491

Table 6.15: Background-subtracted O.D raw data with the mean concentrations of RANKL $\pm$ SD (pg/mL) for different treatment combinations of MG-stimulated hFOB1.19 cells.

MG-stimulated hFOB 1.19 treatments					
Combination of treatments	O.D 450 nm		Concentrations of RANKL (pg/mL)		Mean RANKL±SD (pg/mL)
			Replicate 1	Replicate 2	
		Replicate 1	Replicate 2	Replicate 1	Replicate 2
Control	0.01305	0.01115	84.6736	80.8226	82.7481±2.72
MG only	0.03835	0.02285	136.184	104.575	120.3795±0.00
MG + Met	0.00155	0.00155	61.4016	61.4016	61.4016±0.00
MG + TiW-1	0.00505	0.00595	68.4751	70.2953	69.3852±1.29
MG + TiW-2	0.00315	0.00315	64.6342	64.6342	64.6342±0.00
MG + TiW-3	0.00295	0.00425	64.23	66.8576	64.6342±1.86
MG + TiM-1	0.00445	0.00895	67.2619	76.3665	71.8142±6.44

Table 6.15: continued

MG + TiM-2	0.00455	0.00497	67.4641	68.3133	67.8887±0.60
MG + TiM-3	0.00555	0.00205	69.4863	62.4116	65.9490±5.00
MG + TiW-1 + Met	0.00335	0.00395	65.0384	66.2511	65.6448±0.86
MG + TiW-2 + Met	0.00025	0.00385	58.7764	66.049	62.4127±5.14
MG + TiW-3 + Met	0.00035	0.00315	58.9783	64.6342	61.8063±4.00
MG + TiM-1 + Met	0.00365	0.00185	65.6447	62.0076	63.8262±2.71
MG + TiM-2 + Met	0.00205	0.00165	62.4116	61.6036	62.0076±0.57
MG + TiM-3 + Met	0.00175	0.00105	61.8056	60.3918	61.0987±1.00

Table 6.16: Area density of healthy and dead (%) MGO-stimulated hFOB 1.19 cells when treated with *T. crista* water and methanol extracts alone, and in combination with metformin.

MGO-stimulated hFOB 1.19 treatments						
Combination of treatments	Area density of healthy cells (%)			Area density of dead cells (%)		
	Replicate 1	Replicate 2	Mean±SD	Replicate 1	Replicate 2	Mean±SD
Control	94.4713	96.3819	95.4266±1.3510	5.5287	3.6181	4.5734±1.3510
MG only	45.3966	48.0922	46.7444±1.9061	54.5034	51.9078	53.2056±1.9061
MG + Met	64.6041	47.4608	56.0325±12.1221	35.3959	52.5393	43.9676±8.5717
MG + TiW-1	76.5932	73.2567	74.9250±2.3593	23.4069	26.7434	25.0752±1.6683
MG + TiW-2	51.8418	63.5399	57.6909±8.2718	48.1582	36.4601	42.3092±8.2718
MG + TiW-3	64.0165	60.3621	62.1893±2.5841	35.9835	39.6379	37.8107±2.5841

Table 6.16: continued

MG + TiM-1	67.6110	57.2400	62.4255±7.3334	32.3890	42.7600	37.5745±7.3334
MG + TiM-2	58.4641	53.5411	56.0026±3.4811	41.5359	46.4589	43.9974±3.4811
MG + TiM-3	59.3146	58.6683	58.9915±0.4570	40.6854	41.3318	41.0086±0.4570
MG + TiW-1 + Met	68.3782	69.8575	69.1179±1.0460	31.6218	30.1425	30.8822±1.0460
MG + TiW-2 + Met	72.8304	77.0363	74.9333±2.9740	27.1697	22.9637	25.0667±2.9740
MG + TiW-3 + Met	24.8703	22.9029	23.8866±1.3912	75.1298	77.0971	76.1135±1.3912
MG + TiM-1 + Met	81.9394	58.3922	70.1658±16.6504	18.0606	41.6079	29.8343±16.6504
MG + TiM-2 + Met	84.2907	89.2981	86.7944±3.5408	15.7093	10.7019	13.2056±3.5408
MG + TiM-3 + Met	66.8451	37.8979	52.3715±20.4688	33.1549	62.1021	47.6285±20.4688

Table 6.17: Volume density of healthy and dead (%) MGO-stimulated hFOB 1.19 cells when treated with *T. crispa* water and methanol extracts alone, and in combination with metformin.

MGO-stimulated hFOB 1.19 treatments						
Combination of treatments	Volume density of healthy cells (%)			Volume density of dead cells (%)		
	Replicate 1	Replicate 2	Mean±SD	Replicate 1	Replicate 2	Mean±SD
Control	97.4399	98.1043	97.7721±0.4698	2.5601	1.8957	2.2279±0.4698
MG only	63.9890	73.7951	68.8921±6.9340	36.0110	26.2049	31.1080±6.9340
MG + Met	78.9915	66.1991	72.5953±9.0456	21.0085	38.8010	29.9048±9.0456
MG + TiW-1	92.8687	87.1986	90.0337±4.0094	7.1314	12.8014	9.9664±4.0094
MG + TiW-2	72.2058	83.1906	77.6982±7.7674	27.7942	16.8095	22.3019±7.7674
MG + TiW-3	81.5008	78.8724	80.1866±1.8586	18.4992	21.1276	19.8134±1.8586
MG + TiM-1	84.5481	79.2926	81.9304±3.7162	15.4519	20.7074	18.0797±3.7162
MG + TiM-2	85.2390	75.6614	80.4502±6.7724	14.7610	24.3386	19.5498±6.7724
MG + TiM-3	79.7023	82.0791	80.8907±1.6807	20.2978	17.9209	19.1094±1.6807

Table 6.17: continued

MG + TiW-1 + Met	83.0851	85.1961	84.1406±1.4927	16.9149	14.8039	15.8594±1.4927
MG + TiW-2 + Met	83.8874	79.0278	81.4576±3.4362	16.1126	20.9722	18.5424±3.4362
MG + TiW-3 + Met	41.2919	42.8112	42.0516±1.0743	58.7081	57.1888	57.9485±1.0743
MG + TiM-1 + Met	93.9376	82.9525	88.4451±7.7676	6.0625	17.0475	11.5550±7.7676
MG + TiM-2 + Met	93.0527	98.0403	95.5465±3.5268	6.9473	1.9597	4.4535±3.5268
MG + TiM-3 + Met	90.0339	62.1021	76.0680±19.7508	9.9661	37.2219	23.5940±19.2728

Table 6.18: Minimum and maximum Feret (µm) of MGO-stimulated hFOB 1.19 cells when treated with *T. crispa* water and methanol extracts alone, and in combination with metformin.

MGO-stimulated hFOB 1.19 treatments						
Combination of treatments	Minimum Feret of cells (µm)			Maximum Feret of cells (µm)		
	Replicate	Replicate	Mean±SD	Replicate	Replicate	Mean±SD
	1	2		1	2	
Control	22.1499	23.0521	22.6010±0.6380	53.3045	54.9481	54.1263±1.1622
MG only	16.5031	16.6582	16.5807±0.1097	25.8524	26.5658	26.2091±0.5045
MG + Met	18.7218	18.3761	18.5490±0.2444	32.0224	26.0068	29.0146±4.2537
MG + TiW-1	18.2996	17.2794	17.7895±0.7214	34.7198	32.8389	33.7794±1.3300
MG + TiW-2	16.3192	17.2430	16.7811±0.6532	25.5711	27.2238	26.3975±1.1686
MG + TiW-3	16.4644	18.4152	17.4398±1.3794	26.9113	30.6958	28.8036±2.6760
MG + TiM-1	19.6913	20.4000	20.0457±0.5011	35.1081	29.9025	32.5053±3.6809
MG + TiM-2	17.2709	16.2807	16.7758±0.7002	27.8009	26.1451	26.9730±1.1708
MG + TiM-3	16.0939	14.7249	15.4094±0.9680	27.5274	25.6532	26.5903±1.3253
MG + TiW-1 + Met	19.3604	19.9421	19.6513±0.4113	29.9817	29.7712	29.8765±0.1488
MG + TiW-2 + Met	21.7553	24.7676	23.2615±2.1300	48.0440	62.7180	55.3810±10.3761
MG + TiW-3 + Met	16.1535	15.2766	15.7151±0.6201	22.4957	20.6740	21.5849±1.2881
MG + TiM-1 + Met	25.1326	19.9703	22.5515±3.6503	38.1398	32.9053	35.5226±3.7014

Table 6.18: continued

MG + TiM-2 + Met	20.6885	21.9366	21.3126±0.8825	41.9646	49.2300	45.5973±5.1374
MG + TiM-3 + Met	18.5731	18.6349	18.6040±0.0437	29.5313	27.4466	28.4890±1.4741

Table 6.19: Perimeter (µm) of MGO-stimulated hFOB 1.19 cells when treated with *T. crispa* water and methanol extracts alone, and in combination with metformin.

MGO-stimulated hFOB 1.19 treatments			
Combination of treatments	Perimeter of cells (µm)		
	Replicate 1	Replicate 2	Mean±SD
Control	124.5127	128.3094	126.4111±2.6847
MG only	68.0494	69.5095	68.7795±1.0324
MG + Met	81.8651	70.6400	76.2526±7.9373
MG + TiW-1	86.4114	81.5861	83.9988±3.4120
MG + TiW-2	67.2615	71.3511	69.3063±2.8918
MG + TiW-3	69.6276	79.2124	74.4200±6.7775
MG + TiM-1	88.4213	80.1889	84.3051±5.8212
MG + TiM-2	72.5770	68.2920	70.4345±3.0300
MG + TiM-3	70.4885	65.5073	67.9979±3.5222
MG + TiW-1 + Met	78.9313	79.4073	79.1693±0.3366
MG + TiW-2 + Met	115.2037	145.5551	130.3794±21.4617
MG + TiW-3 + Met	61.4294	57.1318	59.2806±3.0389
MG + TiM-1 + Met	100.9530	85.1913	93.0722±11.1452
MG + TiM-2 + Met	102.5994	117.3098	109.9546±10.4018
MG + TiM-3 + Met	77.1131	73.6626	75.3879±2.4399

Table 6.20: Ellipticity index of MGO-stimulated hFOB 1.19 cells when treated with *T. crispa* water and methanol extracts alone, and in combination with metformin.

MGO-stimulated hFOB 1.19 treatments			
Combination of treatments	Ellipticity index of cells		
	Replicate 1	Replicate 2	Mean±SD
Control	2.5406	2.5279	2.5343±0.0090
MG only	1.5409	1.5629	1.5519±0.0156
MG + Met	1.6888	1.3930	1.5409±0.2092
MG + TiW-1	1.8644	1.9215	1.8930±0.0404
MG + TiW-2	1.5554	1.5148	1.5351±0.0287
MG + TiW-3	1.6260	1.6385	1.6323±0.0088
MG + TiM-1	1.7256	1.4230	1.5743±0.2140
MG + TiM-2	1.5406	1.5831	1.5619±0.0301
MG + TiM-3	1.7169	1.6822	1.6996±0.0245
MG + TiW-1 + Met	1.5044	1.4855	1.4950±0.0134
MG + TiW-2 + Met	2.4644	2.8809	2.6727±0.2945
MG + TiW-3 + Met	1.3839	1.3920	1.3880±0.0057
MG + TiM-1 + Met	1.5139	1.6147	1.5643±0.0713
MG + TiM-2 + Met	2.1596	2.1653	2.1625±0.0040
MG + TiM-3 + Met	1.5002	1.4947	1.4975±0.0039

Table 6.21: Circularity index of MGO-stimulated hFOB 1.19 cells when treated with *T. crispa* water and methanol extracts alone, and in combination with metformin.

MGO-stimulated hFOB 1.19 treatments			
Combination of treatments	Circularity index of cells		
	Replicate 1	Replicate 2	Mean±SD
Control	0.7610	0.7583	0.7597±0.0019
MG only	0.9268	0.9217	0.9243±0.0036

Table 6.21: continued

MG + Met	0.8999	0.9503	0.9251±0.0356
MG + TiW-1	0.8761	0.8665	0.8713±0.0068
MG + TiW-2	0.9218	0.9267	0.9243±0.0035
MG + TiW-3	0.9089	0.9103	0.9096±0.0010
MG + TiM-1	0.8907	0.9453	0.9180±0.0386
MG + TiM-2	0.9253	0.9194	0.9224±0.0042
MG + TiM-3	0.8993	0.9071	0.9032±0.0055
MG + TiW-1 + Met	0.9290	0.9303	0.9297±0.0009
MG + TiW-2 + Met	0.7958	0.7185	0.7572±0.0547
MG + TiW-3 + Met	0.9509	0.9491	0.9500±0.0013
MG + TiM-1 + Met	0.9305	0.9142	0.9224±0.0115
MG + TiM-2 + Met	0.8342	0.8269	0.8306±0.0052
MG + TiM-3 + Met	0.9302	0.9312	0.9307±0.0007

Appendix E: Tabulated treatments of hFOB 1.19 cells with MGO, metformin, *T. crista* water and methanol extracts.

Table 6.22: Experimental design for cell morphology analysis, cell culture supernatant and cell lysate collection for Luminex assay and ELISA.

No.	Treatment combinations	MGO volume	TiW volume	TiM volume	Metformin volume	Final volume of media
1.	Cells only					4500 μ L
2.	Cells +MGO	468.6 μ L				4031.4 μ L
3.	Cells +MGO +Metformin	468.6 μ L			894 μ L	3137.4 μ L
4.	Cells +MGO +TiW-1	468.6 μ L	112.5 μ L			3918.9 μ L
5.	Cells +MGO +TiW-2	468.6 μ L	225 μ L			3806.4 μ L
6.	Cells +MGO +TiW-3	468.6 μ L	450 μ L			3581.4 μ L
7.	Cells +MGO +TiM-1	468.6 μ L		106.8 μ L		3924.6 μ L
8.	Cells +MGO +TiM-2	468.6 μ L		213.8 μ L		3817.6 μ L
9.	Cells +MGO +TiM-3	468.6 μ L		427.5 μ L		3603.9 μ L
10.	Cells +MGO +TiW-1 + Met	468.6 μ L	112.5 μ L		894 μ L	3024.9 μ L
11.	Cells +MGO +TiW-2 + Met	468.6 μ L	225 μ L		894 μ L	2912.4 μ L
12.	Cells +MGO +TiW-3 + Met	468.6 μ L	450 μ L		894 μ L	2687.4 μ L
13.	Cells +MGO +TiM-1 + Met	468.6 μ L		106.8 μ L	894 μ L	3030.6 μ L
14.	Cells +MGO +TiM-2 + Met	468.6 μ L		213.8 μ L	894 μ L	2923.6 μ L
15.	Cells +MGO +TiM-3 + Met	468.6 μ L		427.5 μ L	894 μ L	2709.9 μ L

Appendix F: Images of hFOB 1.19 cells treated with different treatments combination

Table 6.23: Images of hFOB 1.19 cells under the 20X inverted microscope after being treated with their respective treatments for 24 hours.

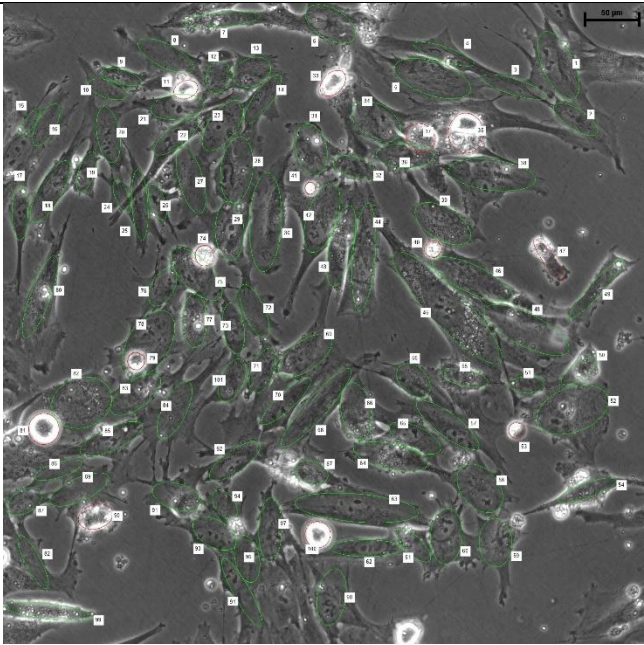
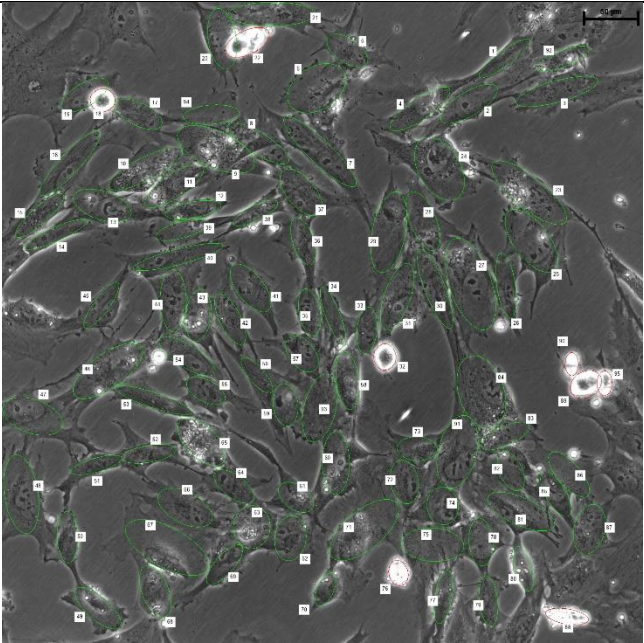
Treatments	hFOB 1.19 images
Cells only (R1)	
Cells only (R2)	

Table 6.23: continued

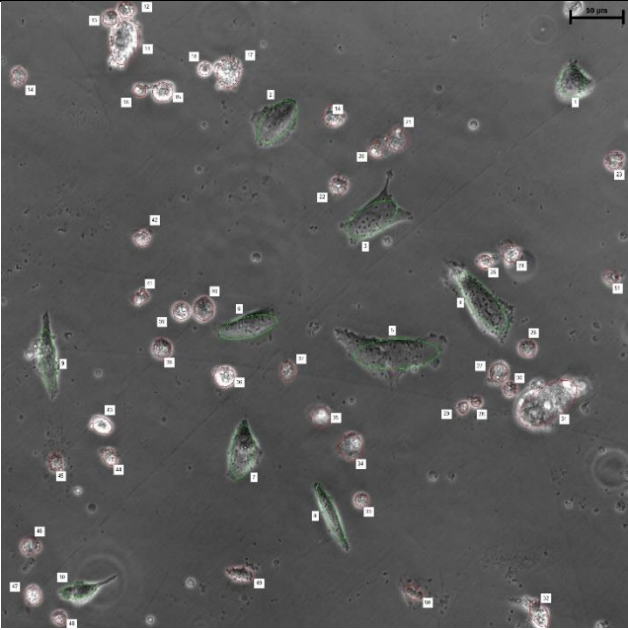
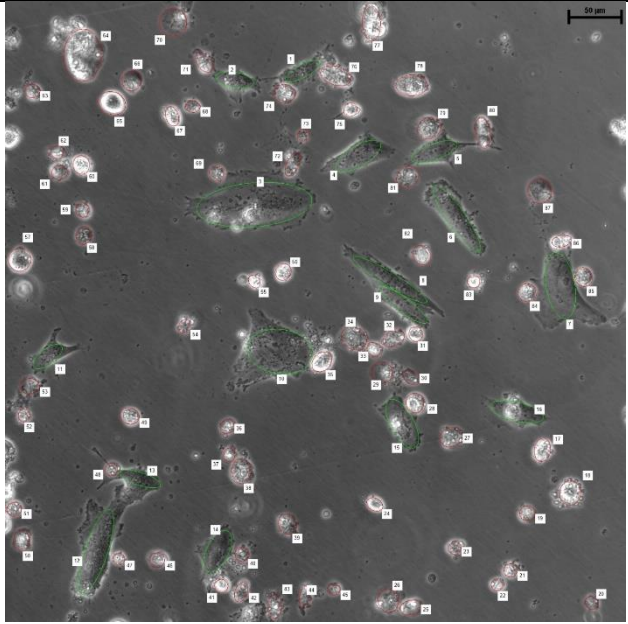
Cells +MGO (R1)	
Cells +MGO (R2)	

Table 6.23: continued

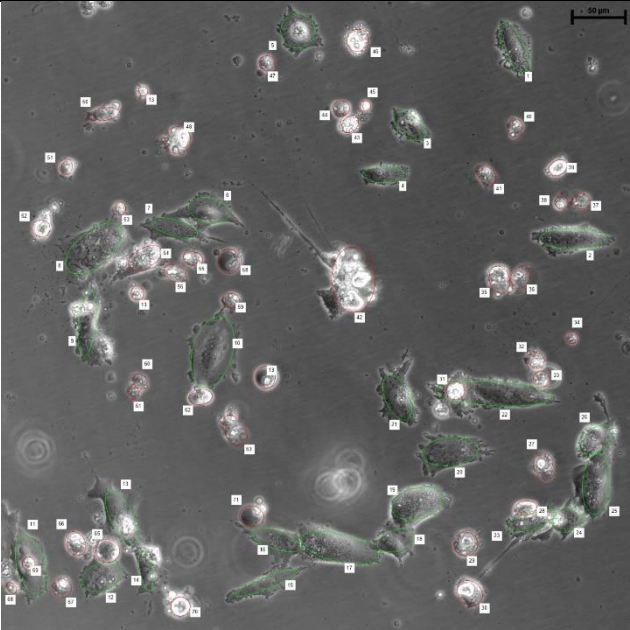
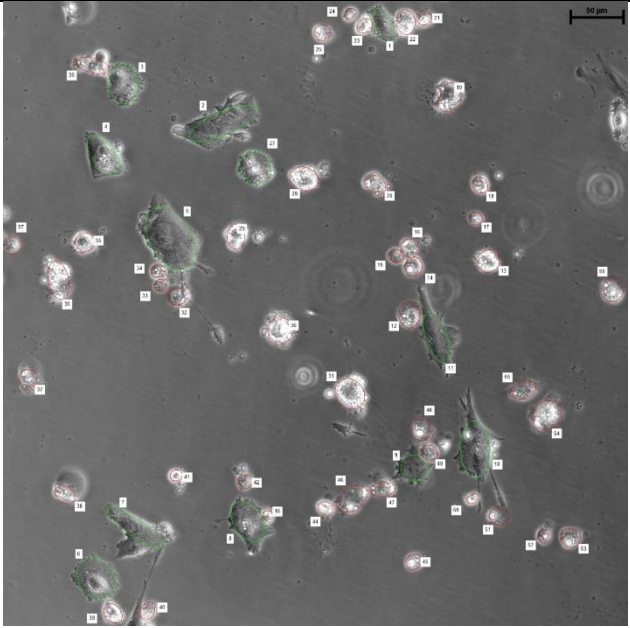
Cells	+MGO	+Met	
(R1)			
Cells	+MGO	+Met	
(R2)			

Table 6.23: continued

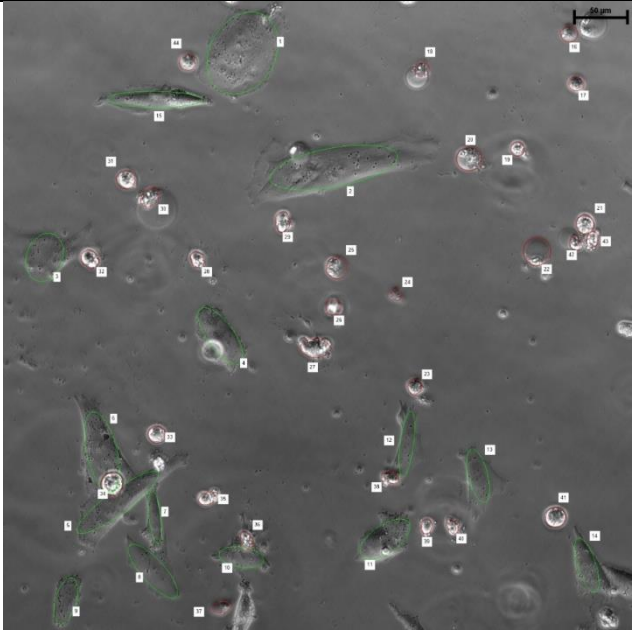
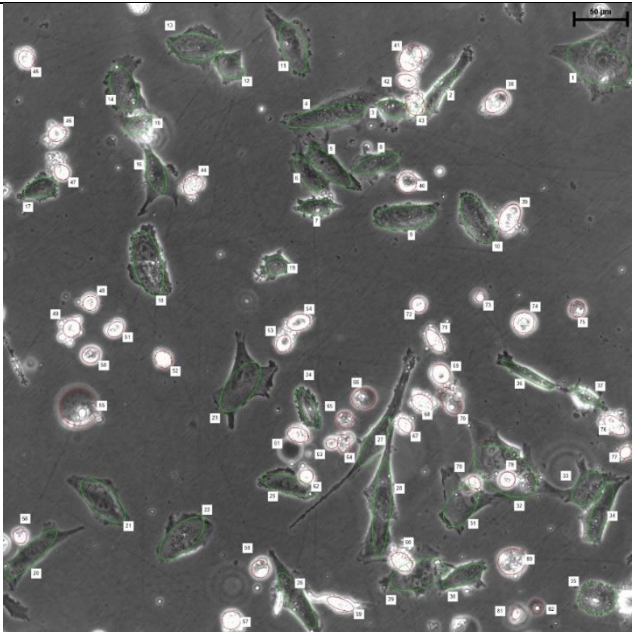
Cells +MGO +TiW-1 (R1)	
Cells +MGO +TiW-1 (R2)	

Table 6.23: continued

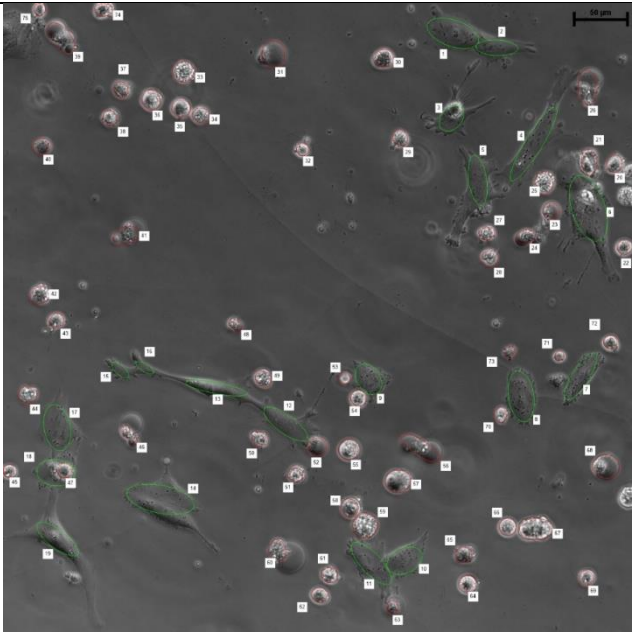
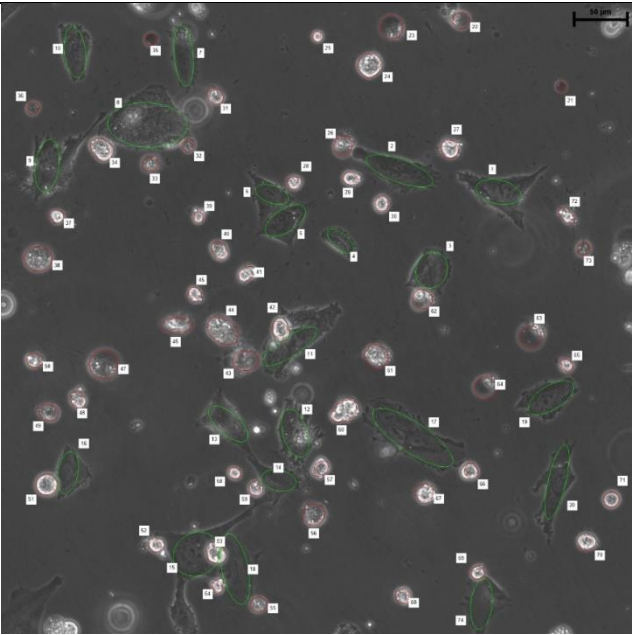
Cells +MGO +TiW-2 (R1)	
Cells +MGO +TiW-2 (R2)	

Table 6.23: continued

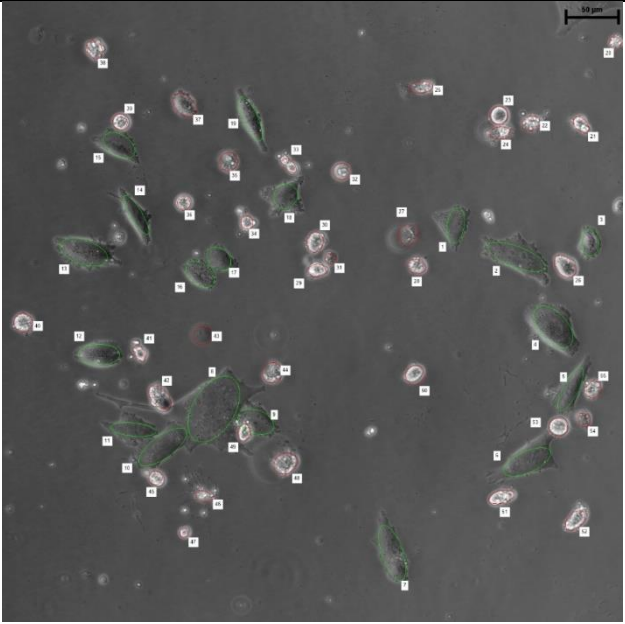
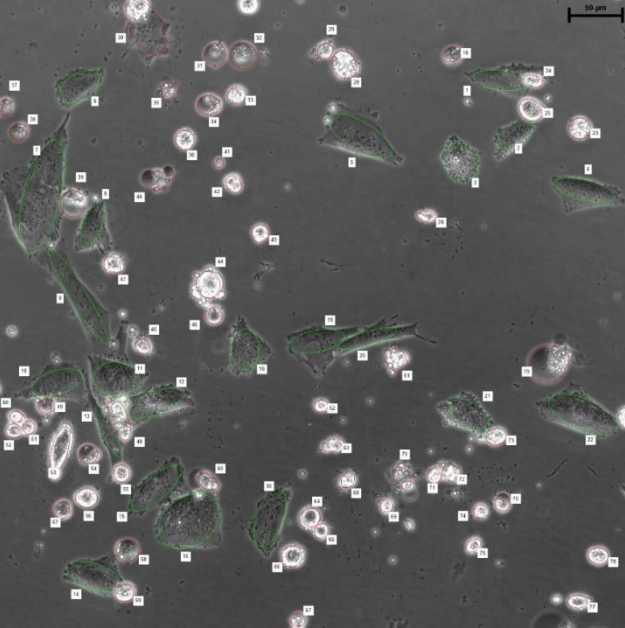
Cells +MGO +TiW-3 (R1)	
Cells +MGO +TiW-3 (R2)	

Table 6.23: continued

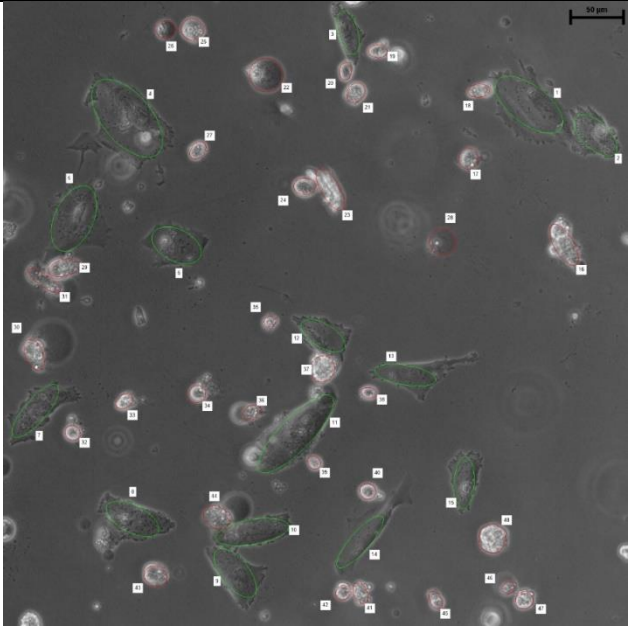
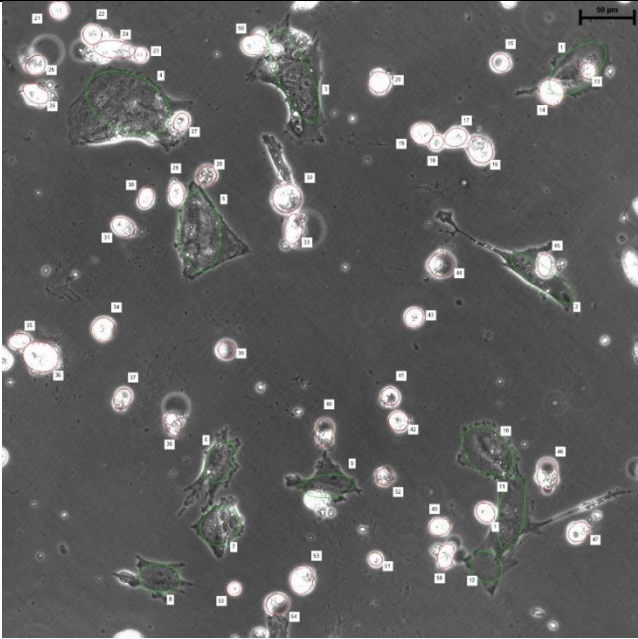
Cells +MGO +TiM-1 (R1)	
Cells +MGO +TiM-1 (R2)	

Table 6.23: continued

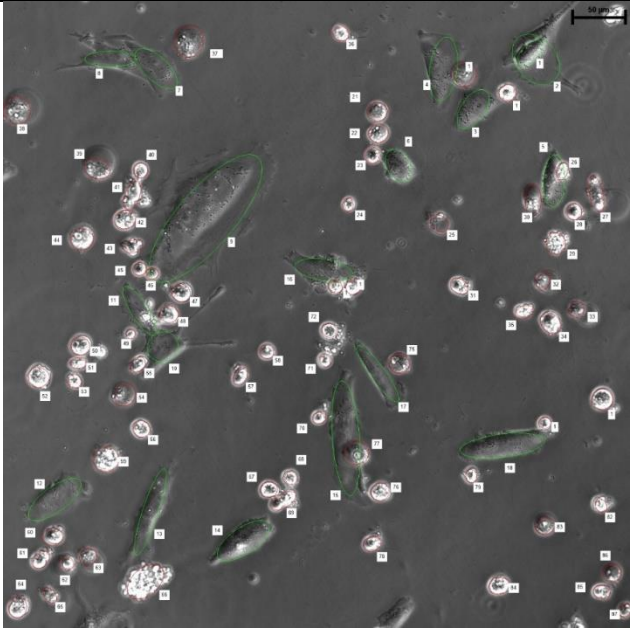
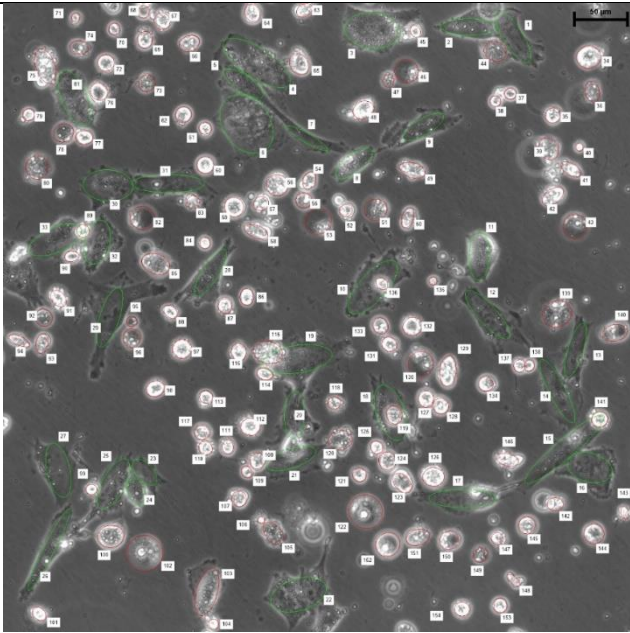
Cells +MGO +TiM-2 (R1)	
Cells +MGO +TiM-2 (R2)	

Table 6.23: continued

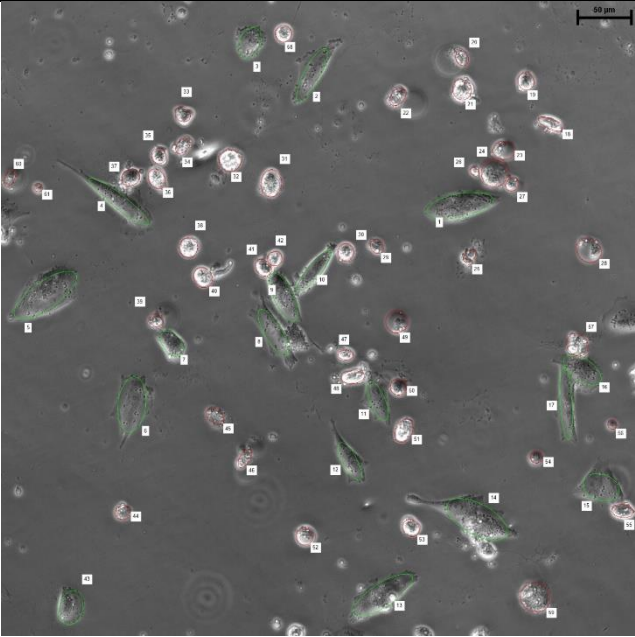
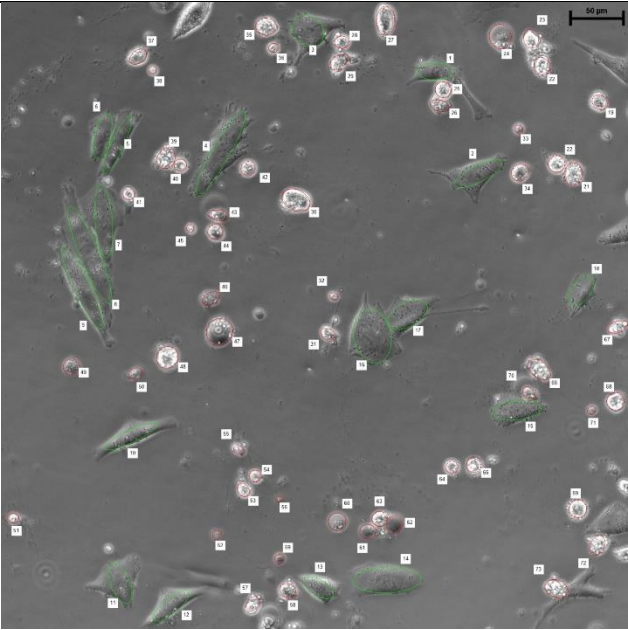
Cells +MGO +TiM-3 (R1)	
Cells +MGO +TiM-3 (R2)	

Table 6.23: continued

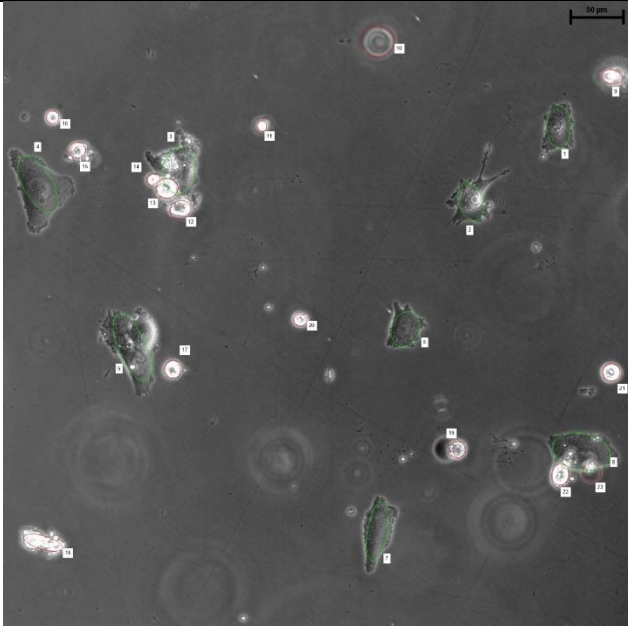
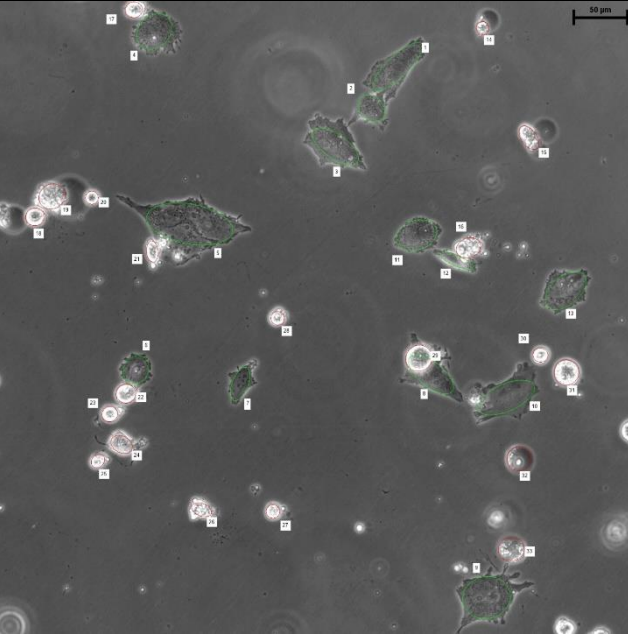
Cells +MGO +TiW-1 + Met (R1)	
Cells +MGO +TiW-1 + Met (R2)	

Table 6.23: continued

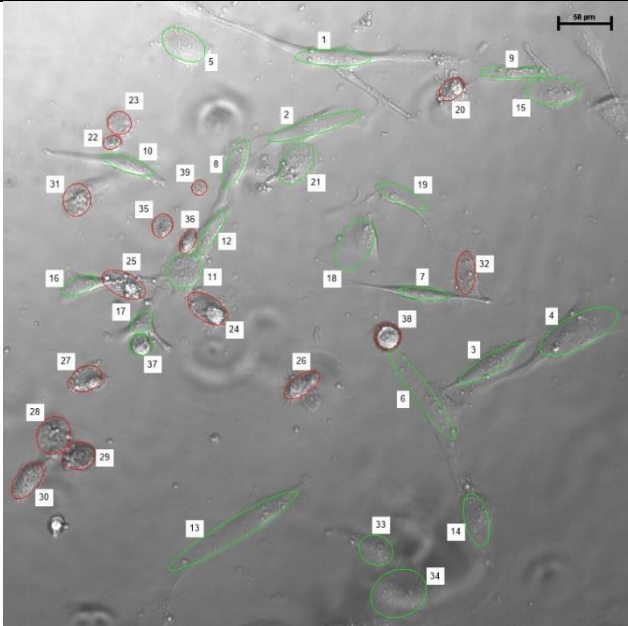
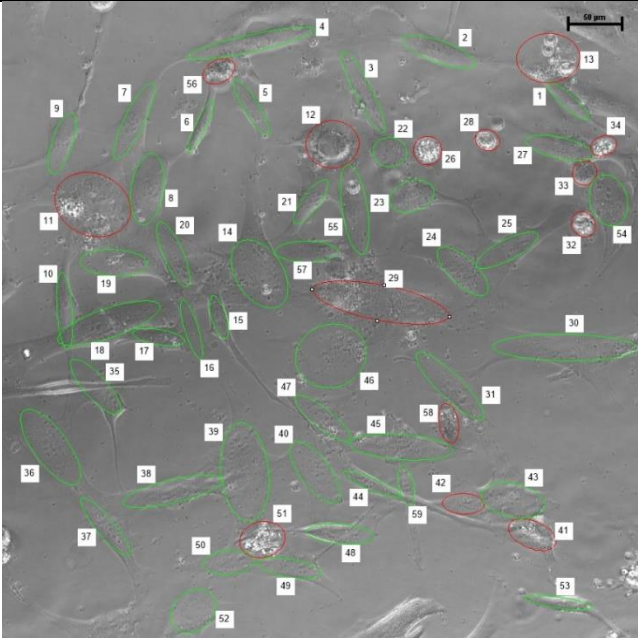
Cells +MGO +TiW-2 + Met (R1)	
Cells +MGO +TiW-2 + Met (R2)	

Table 6.23: continued

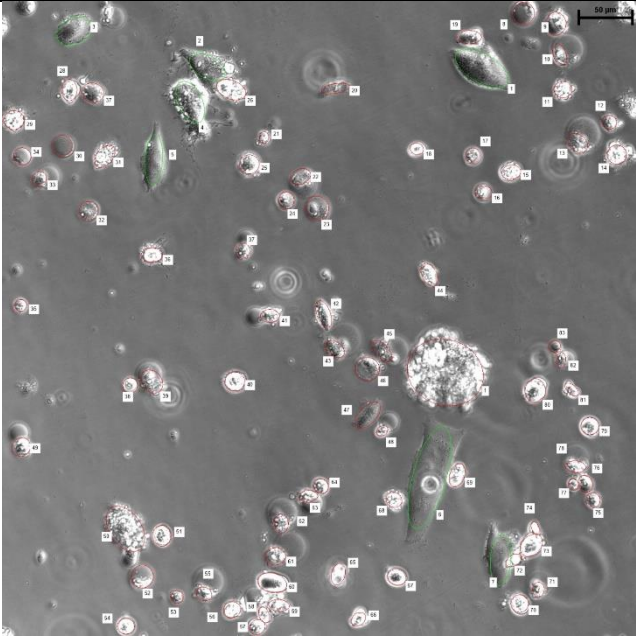
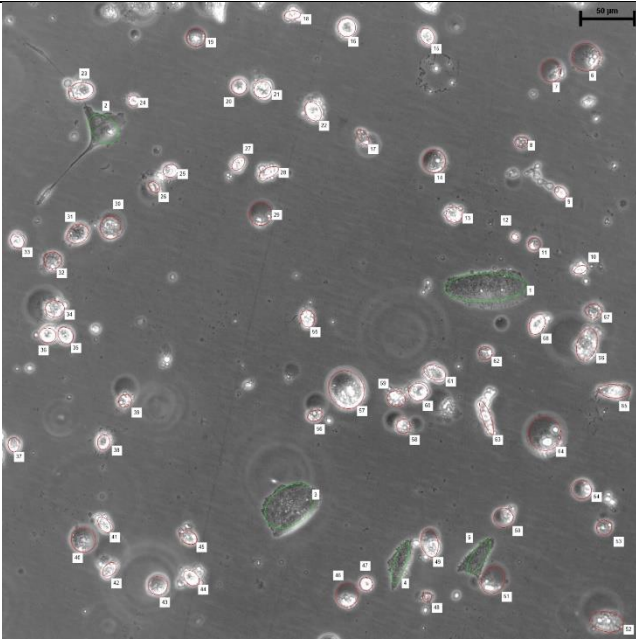
Cells +MGO +TiW-3 + Met (R1)	
Cells +MGO +TiW-3 + Met (R2)	

Table 6.23: continued

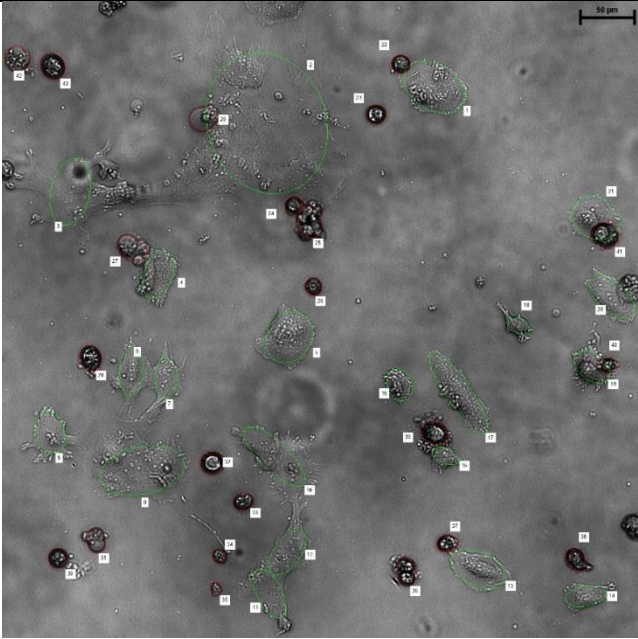
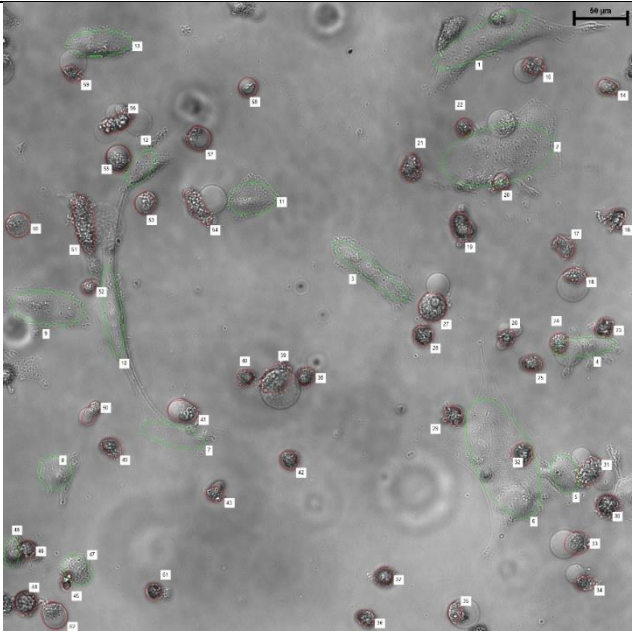
Cells +MGO +TiM-1 + Met (R1)	
Cells +MGO +TiM-1 + Met (R2)	

Table 6.23: continued

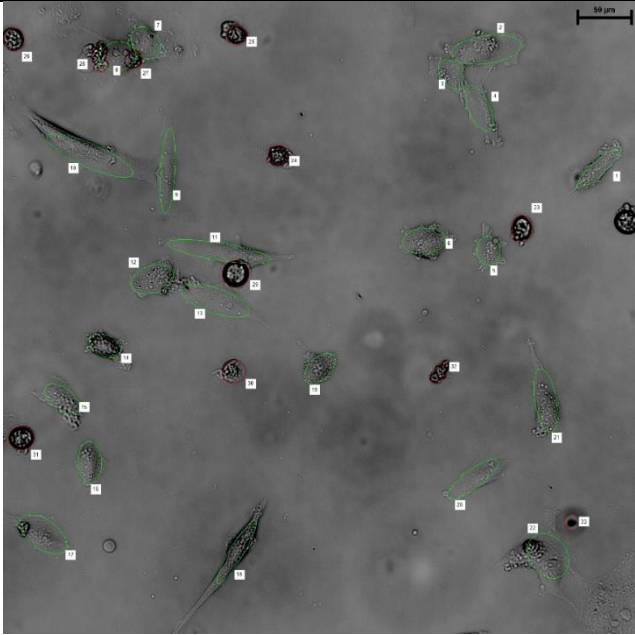
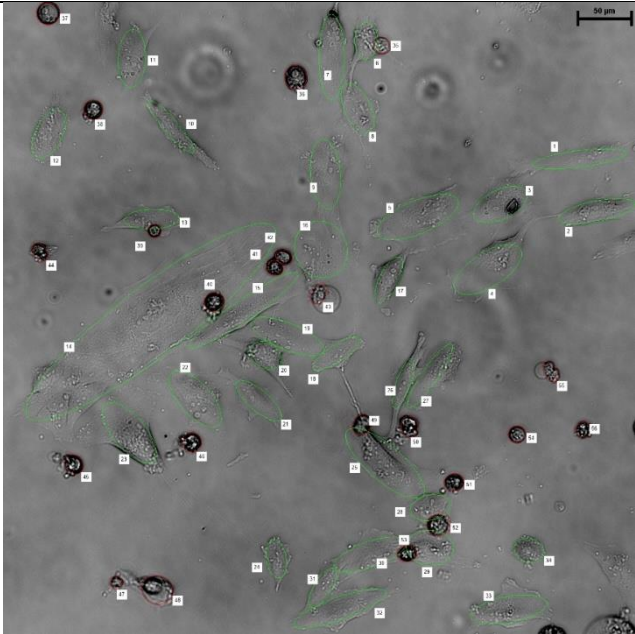
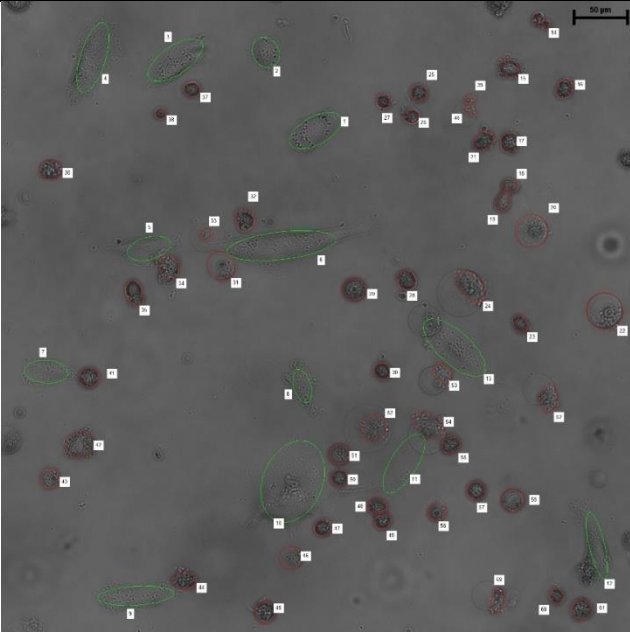
Cells +MGO +TiM-2 + Met (R1)	
Cells +MGO +TiM-2 + Met (R2)	

Table 6.23: continued

Cells +MGO +TiM-3 + Met (R1)	
Cells +MGO +TiM-3 + Met (R2)	