IN VITRO ANTI-PROLIFERATIVE MECHANISM OF COMBINATION THERAPY WITH TANNIC ACID AND CISPLATIN AGAINST HUMAN OSTEOSARCOMA CELL LINE U2OS

MOHAMAD ZAHID BIN MOHD KASIRAM

UNIVERSITI SAINS MALAYSIA

2024

IN VITRO ANTI-PROLIFERATIVE MECHANISM OF COMBINATION THERAPY WITH TANNIC ACID AND CISPLATIN AGAINST HUMAN OSTEOSARCOMA CELL LINE U2OS

by

MOHAMAD ZAHID BIN MOHD KASIRAM

Thesis submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy

JUNE 2024

ACKNOWLEDGEMENT

Alhamdulillah, thanks to Allah for giving me His strength and blessing in completing this thesis. I would like to express my special appreciation to my supervisor, Assoc. Prof. Dr. Hermizi Hapidin, for her invaluable guidance and unwavering support from the beginning to the end of my journey in pursuing knowledge. My deepest gratitude for all the knowledge and wisdom provided that fostered me with intellectual growth throughout this PhD journey. Her expertise, commitment and insightful feedback have instrumentally contributed in determining the direction of this study. My special thanks also to my co-supervisor, Assoc. Prof. Dr. Hasmah Abdullah and Assoc. Prof. Dr. Azlina Ahmad, for the persistent guidance and support. I would like to acknowledge the Universiti Sains Malaysia (USM) for providing the funds under the Research University Grant (RUI) (1001/PPSK/8012318). Sincere acknowledgement also to the Craniofacial Laboratory, School of Dental Sciences, and Biomedicine Laboratory, School of Health Sciences USM for providing the research facility to complete this study. Finally, I would like to express special thanks of gratitude to my beloved parents, Mohd Kasiram bin Che Hussin and Rozipah binti Hamzah, for their unconditional support and understanding along the journey. Thank you for all comforting words given which indeed helped me to continuously walk and traverse the passageway until the terminal tunnel is met. Thanks also to all my friends that helped me during this study.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF SYMBOLS

α	Alpha
*	Asterisk
β	Beta
cm^2	Centimetre square
°C	Degree Celsius
δ	Delta
=	Equal
F	Freedom
γ	Gamma
g	Gram
x g	Gravitational force
kV	Kilo volts
<	Less than
>	More than
mL	Millilitre
µg/mL	Microgram per millilitre
µM	Micromolar
mg/mL	Milligram per millilitre
µL	Microlitre
nM	Nanomolar
%	Percentage
+	Plus
±	Plus-minus

p	Probability value
n	Sample size
Δ	Symmetric difference
:	Ratio
v/v	Volume to volume
v:v	Volume ratio volume

LIST OF ABBREVIATION

AP-1	Activator protein-1
Apaf-1	Apoptotic protease activator factor 1
APS	Ammonium persulfate
AJCC	American Joint Committee on Cancer
ASK	Apoptosis Signal-Regulating Kinase
ATF-2	Activating Transcription Factor 2
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-Related
BAX	BCL2 associated X
BCL2	B-cell lymphoma 2
Ca^{2+}	Calcium ions
CI	Combination Index
CO_2	Carbon Dioxide
Cells/mL	Cells per millilitre
DHT	Dihydrotestosterone
DMEM F12	Dulbecco's Modified Eagle Medium F12
DMSO	Dimethyl Sulphoxide
DISC	Death-Inducing Signalling Complex
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
FADD	Fas-Associated Protein with Death Domain
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate

EC_{50}	Half-Maximal Effective Concentration
ECL	Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular- Signal-Regulated Kinases
FACS	Fluorescence-Activated Cell Sorting
FBS	Foetal Bovine Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GCK	Germinal Center Kinase
GCCP	Good Cell Culture Practice
hFOB 1.19	Human Foetal Osteoblast Cells
HMDS	Hexamethyldisilazane
HMG	High Mobility Group
HRP	Horseradish Peroxidase
IC_{50}	Half-Maximal Inhibitory Concentration
JAK/STAT	Janus Kinase/Signal Transducers and Activators of Transcription
JNK	C-Jun N-Terminal Kinase
MAPK	Mitogen-Activated Protein Kinase
MAPKK	MAPK Kinase
MAPKKK	MAPKK Kinase
MAPK2	MAPK-Activated Kinase 2
MAP	Doxorubicin, Cisplatin, and Methotrexate
MDM2	Mouse Double Minute 2 Homolog
MEK	MAPK ERK Kinase
MMP	Matrix Metalloproteinases
MKK4	MAPK Kinase 4
MLK	Mixed-Linage Kinases

MRI	Magnetic Resonance Imaging
MRI	Mean Relative Intensity
MSK1	Mitogen- and Stress-Activated Protein Kinase 1
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MTX	Metrotrexate
NCCN	National Comprehensive Cancer Network
MSC	Mesenchymal Stem Cells
OD	Optical Density
OPG	Osteoprotegerin
PC	Polycarbonate
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffer Saline
PET	Positron Emission Tomography
PI	Propidium Iodide
PUMA	p53 Up-Regulated Modulator of Apoptosis
PVDF	Polyvinylidene Difluoride
RANK	Receptor Activator of Nfκb
RANKL	Receptor Activator of Nfκb Ligand
RB1	Rb Transcriptional Corepressor 1
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
ROS	Reactive Oxygen Species
Saos-2	Sarcoma Osteogenic-2
SDS	Sodium Dodecyl Sulfate Solution
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscope
SEER	Surveillance, Epidemiology, and End Results

SNV	Single Nucleotide Variants
TA	Tannic Acid
TAE	Tris Acetic EDTA
TBST	Tris-Buffer Saline Tween-20
TP53	Tumour Suppressor 53
TNFR1	Tumour Necrosis Factor Type 1
TRADD	Tumour Necrosis Factor Receptor Type 1-Associated DEATH Domain Protein
TRAIL	TNF-Related Apoptosis-Inducing Ligand Receptors
U2OS	Human Osteosarcoma Cells
UV	Ultraviolet

LIST OF APPENDICES

MEKANISME ANTI-PROLIFERATIF *IN VITRO* BAGI GABUNGAN TERAPI ANTARA ASID TANIK DAN CISPLATIN TERHADAP MODEL SEL OSTEOSARKOMA MANUSIA U2OS

ABSTRAK

Osteosarcoma adalah kanser tulang yang menyumbang kepada punca kedua tertinggi kematian berkaitan kanser dalam kalangan kanak-kanak dan remaja. Kadar kelangsungan kehidupan masih tidak memuaskan terutamanya untuk kes metastasis. Asid tannik (TA) adalah sebatian fenol yang terdapat dalam tumbuhan ubatan dan telah berjaya meningkatkan keberkesanan ubat kemoterapi terhadap pelbagai jenis kanser. Oleh itu, kajian ini bertujuan untuk menyiasat keberkesanan rawatan gabungan TA dengan cisplatin ke atas titisan sel osteosarkoma manusia (U2OS). Kepekatan perencatan separuh maksima (IC_{50}) bagi TA dan gabungan dengan cisplatin terhadap sel U2OS ditentukan menggunakan ujian MTT. Ujian MTT juga menilai ketoksikan sel TA terhadap titisan sel normal osteoblas janin manusia (hFOB 1.19). Interaksi farmakologi antara TA dan cisplatin dinilai menggunakan perisian *CompuSyn*. Aktiviti anti-proliferasi dinilai menggunakan ujian pengecualian biru trypan. Perubahan morfologi dan ultramorfologi sel U2OS diperiksa menggunakan mikroskop terbalik fasa kontras dan mikroskop elektron pengimbasan (SEM). Perubahan morfologi nukleus telah dinilai menggunakan pewarnaan nuklear *Hoechst 33258* dan diperhatikan di bawah mikroskop pendarfluor. Peratusan sel apoptosis dikira menggunakan *flow cytometer* selepas melakukan ujian *annexin V/propidium iodide* (PI). Penilaian mekanistik dijalankan melalui penilaian ekspresi mRNA untuk laluan pengaktifan mitogen protein kinase (MAPK) dan apoptosis mengunakan *reverse transcriptase quantitative polymerase chain reaction* (RT-qPCR). Nilai IC_{50} untuk TA

dan cisplatin masing-masing dikenalpasti sebanyak 4.47 µg/mL dan 16.25 µg/mL. TA tidak menyebabkan kesan perencatan yang ketara terhadap sel hFOB 1.19 tetapi menunjukan kesan proliferasi yang memberangsangkan. Gabungan antara TA dan cisplatin pada nisbah peratusan 90:10 dan 85:15 telah menyebabkan interaksi sinergi. Manakala gabungan pada nisbah 75:25 dan 50:50 menyebabkan interaksi antagonis. Gabungan pada 90:10 menunjukkan potensi tertinggi dengan nilai IC_{50} terendah iaitu sebanyak 3.56 µg/mL. Kesan anti-proliferasi dengan perubahan morfologi dan ultramorfologi yang ketara telah dikesan pada sel rawatan gabungan. Sel-sel kelihatan mengecut, membran berbonjol serta penampakan struktur *lamellipodia* dan *filopodia* yang lebih rendah. Di samping itu, perubahan morfologi nukleus yang menonjol dan peratusan tertinggi sel apoptosis yang tinggi telah dicatatkan pada sel rawatan gabungan. Analisis RT-qPCR menunjukkan peningkatan ketara ERK2, BAX, caspase-9, dan caspase-3 dan penurunan regulasi ekspresi mRNA JNK1 dan BCL2 dalam TA dan sel yang dirawat gabungan. Secara mekanismanya, kesan anti-proliferasi dan apoptosis oleh TA dan gabungan dengan cisplatin terhadap cel U2OS mungkin dimodulasi melalui laluan perencatan JNK1, pengaktifan ERK2 dan apoptosis intrinsik. Hasil penemuan kajian ini mencadangkan potensi kemasukan TA ke dalam rejimen kemoterapi berasaskan cisplatin untuk rawatan osteosarcoma dan kajian selanjutnya perlu dilakukan.

IN VITRO ANTI-PROLIFERATIVE MECHANISM OF COMBINATION THERAPY WITH TANNIC ACID AND CISPLATIN AGAINST HUMAN OSTEOSARCOMA CELL LINE U2OS

ABSTRACT

Osteosarcoma is most common primary bone malignancy that affects children and young adults. The survival rate remains low, especially in metastatic cases. Tannic acid (TA), a phenolic compound found in medicinal plants, has shown promising efficacy in enhancing chemotherapeutic drugs against various cancers. Hence, this study aimed to investigate the effect of TA treatment combined with cisplatin on human osteosarcoma cell line (U2OS). The half-maximal inhibitory concentration (IC_{50}) values of TA and its combination with cisplatin against U2OS cells were determined using MTT assay. MTT assay also assessed TA cytotoxicity on normal human foetal osteoblast (hFOB 1.19) cell line. The pharmacological interaction between TA and cisplatin was evaluated using CompuSyn software. The anti-proliferative activity was evaluated by using trypan blue exclusion assay. The morphological and ultramorphological alteration of the U2OS cells were examined using phase contrast inverted microscope and scanning electron microscope (SEM), respectively. The nuclear morphological changes were evaluated by Hoechst 33258 nuclear staining and observed under fluorescence microscope. The percentage of apoptotic cells was measured using flow cytometer after conducting annexin V/propidium iodide (PI) assay. A mechanistic study was conducted by evaluating mRNA expression of mitogen-activated protein kinase (MAPK) and apoptotic pathway using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). The IC_{50} values of TA and cisplatin were determined at 4.47 and 16.25 µg/mL,

respectively. TA induced no significant inhibitory effect on hFOB 1.19 cells, but potent proliferative effect was indicated. The combination of TA with cisplatin at percentage ratios of 90:10 and 85:15 induced synergistic interaction, while the combination at 75:25 and 50:50 induced antagonistic interaction. The combination at 90:10 showed the highest potency with the lowest IC_{50} value at 3.56 µg/mL. A significant anti-proliferative effect with prominent morphological and ultramorphological alteration was detected in the combination-treated cells. The cells appeared shrunk, with blebbed membrane and reduced appearance of lamellipodia and filopodia. Additionally, prominent nuclear morphological alteration and highest percentage of apoptotic cells were noted in the combination-treated cells. RT-qPCR analysis indicated the significant upregulation of ERK2, BAX, caspase-9, and caspase-3 and downregulation of JNK1 and BCL2 mRNA expression in the TA- and combination-treated cells. Mechanistically, anti-proliferative and apoptotic effect of TA and its combination with cisplatin on U2OS cells were suggested to be modulated via JNK1 inhibition, ERK2 activation, and intrinsic apoptotic pathways. The findings suggest the potential inclusion of TA in cisplatin-based chemotherapy for osteosarcoma and warrant further investigation.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Osteosarcoma is a bone cancer due to the uncontrolled production of osteoid (Liao *et al.*, 2013). It is a primary malignant bone tumour originating from the primitive mesenchymal stem cells and characterised by the immature deposition of osteoid matrix by the spindle cells (Isakoff *et al.*, 2015). Osteosarcoma is the most common primary malignant bone tumour with annual incidence from 8 to 11 million cases (de Azevedo *et al.,* 2019; Beird *et al.,* 2022). The worldwide prevalence of osteosarcoma is bimodally distributed, affecting two age groups. The prominent peak is among children and adolescents at the pubertal age, and another smaller peak is among the elderlies of more than 60 years old (Nie & Peng, 2018; Rojas *et al.*, 2021; Beird *et al.,* 2022). Rapid bone development during the pubertal growth period contributes to the higher number of cases among children and adolescents (Sadykova *et al.,* 2020).

In Malaysia, osteosarcoma has been determined as the fifth most common cancer among the age group of 0 to 19 years old with highest cases detected in male (National Cancer Institute, 2020). For osteosarcoma, men possess higher risk to be diagnosed as they have a longer duration of skeletal growth compared to women (Rojas *et al.*, 2021). In humans, skeletal growth takes place in the metaphyseal region of the long bone. Due to that, osteosarcoma commonly develops around this region. Osteosarcoma has been mostly detected in long bones like the femur, tibia, and humerus (Zhao *et al.*, 2021). Like other cancers, osteosarcoma can be classified into localised and metastatic cases. Localised osteosarcoma affects the local area where the tumour initially develops and accounts for 80% of osteosarcoma cases

(Papakonstantinou *et al.*, 2020). Meanwhile, the key indication for the advanced stage of osteosarcoma is metastasis, which usually has a poorer prognosis. Advanced osteosarcoma is indicated when the local tumour progressively spreads to other distant organs. Metastatic osteosarcoma accounts for 20% of cases, and the lung is the most common organ affected. Approximately 90% of osteosarcoma patients are detected with lung metastasis (Marko *et al.*, 2016).

The exact aetiology of osteosarcoma is not clear. Nevertheless, genetic aberration is recognised as a key factor that leads to the neoplastic transformation and mitotic error in osteosarcoma (Ottaviani & Jaffe, 2009b; de-Azevedo *et al.*, 2019). Any genetic aberrations in patients might alter signal transduction that consequently dysregulates cell proliferation, causing cells to divide uncontrollably (Dey *et al.*, 2023). Mitogen-activated protein kinase (MAPK) pathways are one of the signal transductions that regulate cell growth and death. MAPK pathways comprise three signalling pathways: JNK, p38, and ERK (Braicu *et al.*, 2019). MAPK pathways have been reported to regulate osteosarcoma cell proliferation and progression, and these pathways have emerged as a promising therapeutic target (Chandhanayingyong *et al.*, 2012).

The defect of programmed cell death is a crucial hallmark of cancer development. Apoptosis is one of the programmed cell deaths that prevent excess cell growth. Apoptosis is regulated by intrinsic and extrinsic apoptotic pathways (D'Arcy, 2019a). A balanced coordination between cell proliferation and cell death is pivotal in avoiding uncontrolled cell growth, which can become pathologically harmful to the body system (Morana *et al.*, 2022). Thus, apoptosis is an essential barrier in preventing cancer development by restricting the cancer cells from surviving and disseminating (Su *et al.*, 2015). Several studies have reported that the MAPK pathways also

contribute a critical role in regulating cytotoxic drug-induced apoptosis in osteosarcoma (You & Park, 2011; Chen *et al.*, 2009). Cross interaction between MAPK and apoptosis signalling pathways was previously described to modulate cell proliferation and cell death (Yeh *et al.*, 2019; Sun *et al.*, 2015). Several new chemotherapeutic agent developments have focused on various aspects of MAPK and apoptosis signalling pathway as these seem effective in eradicating cancer cells (Jensen *et al.*, 2008; Braicu *et al.*, 2019; Jan & Chaudhry, 2019).

Currently, chemotherapy is considered a standard treatment component for osteosarcoma, along with surgery and radiotherapy. In chemotherapy, cisplatin is actively employed as the standard chemotherapeutic drug for osteosarcoma (Li *et al.*, 2018). Cisplatin (cis-diamminedichloroplatinum) is a cytotoxic drug that inhibits cancer growth by interfering with DNA transcription and replication (Li *et al.*, 2018; Fuertes *et al.*, 2003). The chemotherapy for osteosarcoma consists of several regimens that composed of several combinations of chemotherapeutic drugs used at the defined dosage, frequency, and duration. Cisplatin has a high efficacy and is usually placed in the first-line regimen (Mohanty *et al.*, 2019).

To date, the 5-year survival rate for localised osteosarcoma patients is between 65% to 70% (Xu *et al.*, 2021; Moukengue *et al.*, 2022). Meanwhile, the survival rate for advanced osteosarcoma patients is lower at 30% (Beird *et al.*, 2022; Wang *et al.*, 2017). The survival rate of osteosarcoma is still unsatisfactory as it appeared to plateau over the past three decades despite therapeutic advancement and multiple options for chemotherapeutic regimens available (Camuzard *et al.*, 2019; Harrison *et al.*, 2018). In addition, approximately 20% of osteosarcoma patients relapsed within 5 years of treatment (Cheng *et al.*, 2016; Gazouli *et al.*, 2021). Currently, the efficacy of chemotherapy for osteosarcoma is transient and mostly absent in the advanced stage

(Luetke *et al.*, 2014; Lilienthal & Herold, 2020; Verma *et al.*, 2021; Tsuda *et al.*, 2020). Furthermore, the treatment regimen for osteosarcoma has not changed over the last three decades (Rastogi *et al.*, 2018; Jaffe *et al.*, 2013).

Moreover, concerns have been raised regarding the chemoresistance of osteosarcoma (Prudowsky & Yustein, 2020). Sequential and long-term exposure to the same drug components induces DNA mutation which ultimately alters the biological target of the drug. This condition consequently limits chemotherapy effectiveness for osteosarcoma (Mansoori *et al.*, 2017; Wang *et al.*, 2015). Other than that, excessive side effects of the current chemotherapeutic drugs are also one of the problems that need to be resolved. Therefore, searching for a novel agent is urgently required to overcome these problems.

Current new drug discovery studies for cancer have mostly shifted towards phytochemical compounds, which are naturally occurring chemical compounds found in plants. They are relatively safe and are easily found in various plants (Dewi *et al.*, 2022; Elkordy *et al.*, 2021). Certain phytochemical compounds such as curcumin, quercetin, and resveratrol have demonstrated the ability to inhibit the proliferation of many types of cancer cells (Li *et al.*, 2023). Besides that, some phytochemical compounds like curcumin, gallic acid and oleandrin have also been reported to ameliorate the total chemotherapeutic effect and reduce the toxicity of chemotherapeutic drugs (Abdel-Daim *et al.*, 2016). Combining phytochemical compounds with currently used chemotherapeutic drugs might be a novel strategy to improve the survival rate, address the chemoresistance problem, and reduce adverse side effects of the chemotherapeutic agents.

Phenols are the largest category of phytochemical compounds. They are a major secondary metabolite and ubiquitously found in most medicinal plants (King &

Young, 1999; Albulescu, 2015). Tannic acid (TA) is one of the phenolic compounds under the group of nonflavanoid-hydrolisable tannins. TA can be isolated from the leaves, fruit, and roots of various medicinal plants like *Phyllanthus emblica*, *Quercus infectoria*, and *Punica granatum*. A previous study demonstrated that TA possesses a potent anti-proliferative effect against many types of cancer cells (Jing *et al.*, 2022). Nagesh *et al.* (2020) reported that treating prostate cancer cells with TA resulted in an upregulation of the associated apoptotic markers. In another study conducted by Liu *et al.* (2013), the highest growth inhibition of the human osteosarcoma cell line (Saos-2) was recorded when it was treated with condensed tannin extracted from *Caulis spatholobi* (Chinese medicinal plant). The application of phenolic compounds like curcumin and quercetin in combination with cisplatin has been found to enhance the drug's killing effect on osteosarcoma cells (Zhang *et al.*, 2015; Jiang & Huang, 2020). Until now, the effect of TA in combination with cisplatin on osteosarcoma cells has not yet been evaluated. Furthermore, the mechanism of TA and its combination with cisplatin in mediating the anti-proliferative effect on osteosarcoma is also necessary to be elucidated. Therefore, this study was conducted to investigate the anti-proliferative and apoptotic effect of TA and its combination with cisplatin against the human osteosarcoma cell line (U2OS).

1.2 The rationale of the study

Cancer is one of the most diagnosed non-communicable diseases in humans (Budreviciute *et al.*, 2020; World Health Organisation, 2022). Even though childhood cancer is not common, it is still among the leading causes of death in children and adolescents (World Health Organisation, 2021). About 4.7 million children and adolescents between 0 and 19 years old are diagnosed with cancer annually (Yan & Xiang, 2021). Osteosarcoma is a primary malignancy of bone that commonly affects growing children. It is highly aggressive type of tumour with high propensity to invade local areas and spread early to the lungs or other bone (Rothzerg *et al.*, 2023). The survival rate of osteosarcoma patients has not improved over the last few decades, especially for those diagnosed with the advanced stage (Smeland *et al.*, 2019).

Chemotherapy is a standard treatment component for osteosarcoma and is applied along with surgery and radiotherapy. Cisplatin is one of the chemotherapeutic drugs that are primarily included in the chemotherapeutic regimens for osteosarcoma (Li *et al.*, 2018). Chemotherapeutic regimen is structured treatment plan that consist of several combination of chemotherapeutic agents administered over a specific period at the predetermined schedule. The chemotherapeutic regimen for osteosarcoma has not undergone significant modification over 30 years (Rothzerg *et al.*, 2022). Osteosarcoma is still currently known as a pathological condition with unchanged standard therapy (Hu *et al.*, 2022). The currently used chemotherapeutic regimen has narrow therapeutic indices (Belayneh *et al.*, 2021). Cisplatin usage has also raised disputes over its excessive side effects (Fanelli *et al.*, 2020). Cisplatin has low specificity and it affects both the osteosarcoma cells and the surrounding normal osteoblasts, resulting in deterioration of the local bone formation (Münz *et al.*, 2021; Young *et al.*, 1997). Therefore, there is an urgent need to search for a new potential

agent that not only can elevate therapeutic efficacy but also overcome the problem of chemoresistance and adverse side effects.

Plant is a vital source of active compounds and is harnessed due to its diverse range of medicinal properties. The naturally isolated compounds, also known as phytochemical compounds, have gained wide attention in new drug discovery studies nowadays as they have relatively low toxicity, are cost-effective, and are easily found (De-Luca *et al.,* 2022). Phenols are one of the phytochemical groups commonly studied for anti-cancer activity (Briguglio *et al.*, 2020). The phenolic compound like curcumin, quercetin and resveratrol have been reported to have a potent anti-osteosarcoma property (Zahedipour *et al.,* 2021; Maleki-Dana *et al.,* 2021; De-Luca *et al.,* 2022). TA is one of the phenolic compounds under the group of hydrolysable tannins (Doughari, 2012). It has been reported to have anti-cancer activity against various cancer types like prostate cancer (Nagesh *et al.*, 2020), lung cancer (Hatami *et al.*, 2022), ovarian cancer (Sun *et al.*, 2012), breast cancer (Darvin *et al.,* 2017), colon adenocarcinoma (Cosan *et al.*, 2009), and gingival cancer (Darvin *et al.*, 2015). In a study conducted by Liu *et al.* (2013), condensed tannin extracted from Chinese medical plant (*Caulis spatholobi*) effectively inhibited osteosarcoma cell line (Saos-2). To date, no study has evaluated the specific effect of TA on human osteosarcoma (U2OS) cell line. Hence, there is an opportunity to harness the possible anti-osteosarcoma activity of TA through detailed *in vitro* investigation on human osteosarcoma cells. This evaluation could also provide a way to enhance the efficacy of the currently available treatment regimen for osteosarcoma.

Chemotherapy for osteosarcoma utilised the combination of several chemotherapeutic drugs to make up several treatment regimens. This method has been proven to enhance the therapeutic efficacy of osteosarcoma treatment (Mokhtari *et al.*,

2017). Nevertheless, long-term exposure to the same drugs is associated with the risk of chemoresistance. Furthermore, the chemotherapeutic efficacy of these drugs is transient, especially in metastatic cases. The amalgamation of phytochemical compounds with the current chemotherapeutic drugs may improve their therapeutic effect on osteosarcoma. This method has also been proven to overcome the chemoresistance problem and adverse side effects. The stability and diverse structural components of phytochemical compounds enable them to act effectively which ultimately induce numerous medicinal benefits. When used in combination with chemotherapeutic drugs, they may enhance the effectiveness of the treatments as well as overcoming the ineffective responses of current drugs (Kumar *et al.,* 2023). Additionally, this combination strategy allows for dose reduction of drugs like cisplatin, which are known to induce adverse side effect.

The effectiveness of the combination of phenolic compounds with cisplatin against several cancer types has been widely reported. Cisplatin combined with gallic acid and methyl gallate induced a killing effect on cervical cancer cells (Norlida *et al.*, 2021). Meanwhile, the application of TA in combination with cisplatin has been shown to enhance anti-proliferative effect on liver cancer cells (Geng *et al.*, 2019) and human ovarian carcinoma cells (Sun *et al.,* 2012). However, the effect of TA and cisplatin combination against human osteosarcoma cell line has not yet been reported. Thus, this study was conducted to evaluate the effect of TA and cisplatin combination as an effort to elevate the current chemotherapeutic efficacy, overcome the chemoresistance problem, and reduce the adverse side effects of chemotherapy in osteosarcoma.

Comprehensive understanding of the drug's mechanism of action is a fundamental aspect of developing an effective cancer treatment. An effective therapeutic agent can interfere with and interrupt specific signalling pathways that

regulate tumour development. Phytochemical compounds exhibit inhibitory effects by dysregulating diverse key pathways that control the vital functions of cells (Petric *et al.*, 2015). Mitogen-activated protein kinase (MAPK) signalling pathways (ERK, JNK, and p38) are among the vital regulators of cellular proliferation and apoptosis (Ravingerová *et al.*, 2003; Braicu *et al.*, 2019). Apoptosis is also explicitly regulated by intrinsic and extrinsic pathways. Both MAPK and apoptosis pathways are among the most targeted pathways in the therapeutic evaluation of new cancer treatment (Braicu *et al.*, 2019; Pfeffer & Singh, 2018).

In osteosarcoma, the genetic mutation in key components of these signalling pathways has dysregulate the signalling, contributing to the development and progression of osteosarcoma. TA has been previously reported to effectively target MAPK and apoptosis signalling markers, ultimately inhibiting cell proliferation and inducing cell death of several cancer cells (Youness *et al.*, 2021; Jing *et al.*, 2022; Anjum *et al.*, 2022). At this time, the effect of TA and its combination with cisplatin on the regulation of MAPK and apoptosis pathways has not yet been elucidated. The mechanism of action of TA and its combination with cisplatin in mediating the anti-proliferative and apoptotic effect on osteosarcoma cells need to be investigated. Understanding its mechanism of action is crucial for the development of effective therapy as it can provide comprehensive findings of the drug's dynamic action as well as monitor the treatment response, safety and tolerability (Swinney, 2015). Thus, targeting these pathways might be advantageous for controlling the development of osteosarcoma by specifically targeting its key regulatory pathways (MAPK and apoptosis pathway). Hence, this study was undertaken to investigate the role of TA and its combination with cisplatin in inducing anti-proliferative and apoptotic effects on the human osteosarcoma cell line (U2OS).

1.3 Research objectives

General objective

To study the anti-cancer effect of TA and its combination with cisplatin on human osteosarcoma (U2OS) cells.

Specific objectives

1) To determine the half-maximal inhibitory concentration (IC_{50}) of TA and its combination with cisplatin against U2OS cell lines by MTT assay.
2) To determine the pharmacological interaction in the combination treatment between TA and cisplatin on U2OS cell lines through the calculation of combination index (CI) number.
3) To investigate the anti-proliferative activity of U2OS cells in response to the treatment with TA and its combination with cisplatin using trypan blue exclusion assay.
4) To determine the occurrence of apoptotic cell death by evaluating morphological changes in U2OS cells in response to treatment with TA and its combination with cisplatin using phase contrast inverted microscopy, scanning electron microscopy (SEM), and fluorescence microscopy.
5) To quantify the apoptotic U2OS cells in response to the treatment with TA and its combination with cisplatin using flow cytometer.
6) To elucidate the mechanism of anti-proliferative and apoptotic cell death by evaluating the mRNA expression of MAPK (ERK2, JNK1, and p38) and apoptosis (BAX, BCL2, caspases 3, 8, and 9) pathway markers in U2OS cells after treatment with TA and its combination with cisplatin using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).

1.4 Hypotheses

Null hypothesis (Ho):

The TA and its combination with cisplatin do not possess anti-cancer effect against human osteosarcoma (U2OS) cells.

Alternative hypotheses (H_1):

1) The combination of TA and cisplatin is expected to be more potent compared to the single agent of cisplatin and TA based on the IC_{50} values identified.
2) The combination of TA with cisplatin at the lowest dose of cisplatin is expected to have lower CI value, indicating favourable synergistic interaction.
3) The combination treatment with TA and cisplatin is expected to induce potent anti-proliferative effect with lowest number of viable U2OS cells compared to the single agent treatment with cisplatin and TA.
4) Treatment with TA and its combination with cisplatin are expected induce morphology of apoptosis in U2OS cells.
5) The combination treatment with TA and cisplatin is expected to elevate the percentage apoptotic U2OS cells compared to the single agent treatment with cisplatin and TA.
6) Treatment with TA and its combination with cisplatin are expected induce anti-proliferative and apoptotic cells death mechanism via MAPK and apoptosis pathways by downregulating the mRNA expression of JNK1, p38, and BAX and upregulating the expression of ERK2, BCL2, caspase 3, caspase 8, and caspase 9.

1.5 Conceptual framework

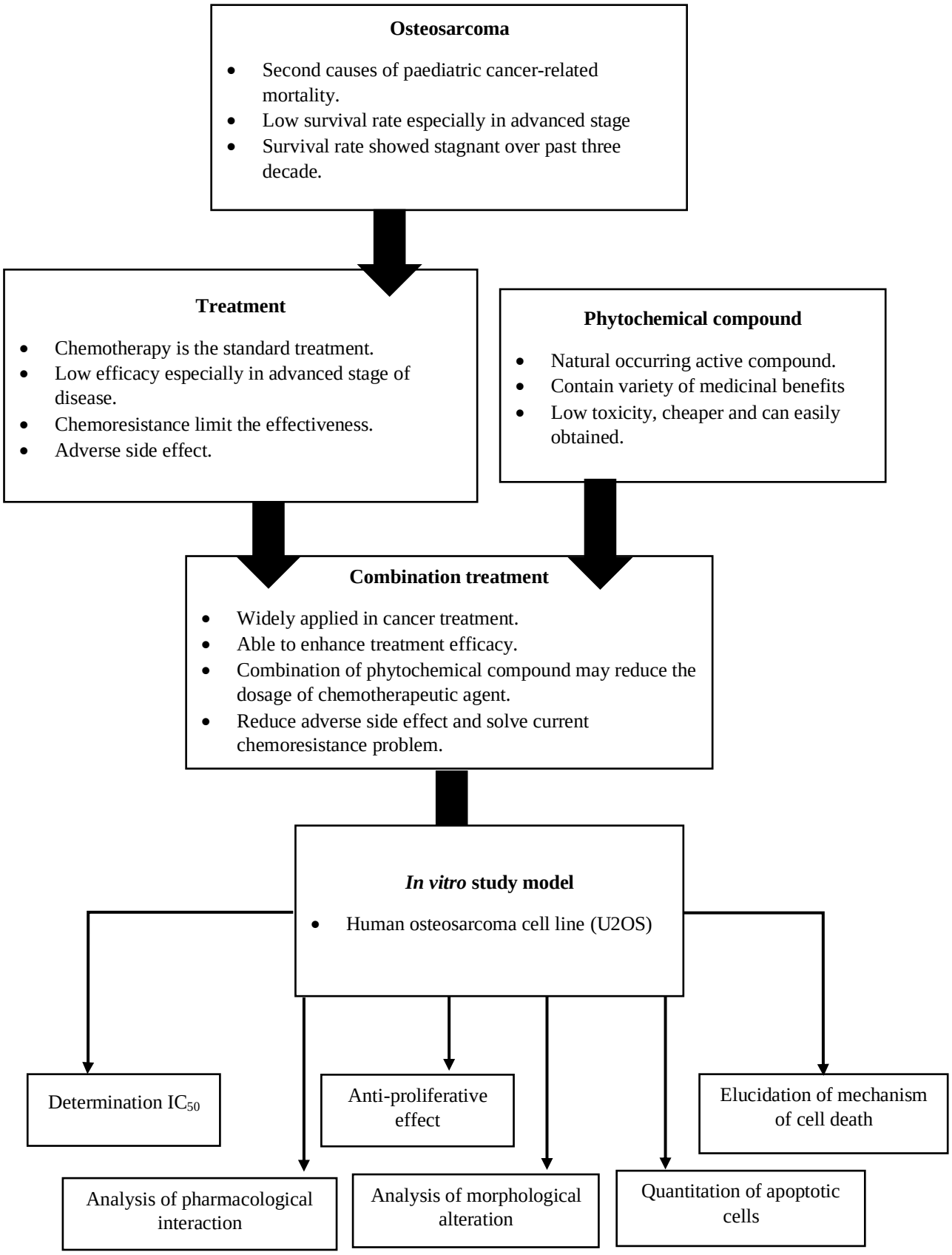


Figure 1.1 Conceptual framework of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer in children

Cancer is a disease with noxious pathological conditions with a wide range of clinical features. Each year, millions of fatalities are recorded due to cancer. In 2020 alone, there were approximately 18.1 million new cancer cases detected (World Cancer Research Fund International, 2022). Cancer in children is considered rare, but it is the leading cause of death for children under age 18 worldwide. Globally, approximately 175,000 new cases of cancer in children are diagnosed each year (Ren *et al.*, 2022). The burden of cancer in children is expected to continue increasing in the future, especially in low- and middle-income countries. (Gadepalli *et al.*, 2021). In Malaysia, 77.4 per million children aged lower than 15 years have been diagnosed with cancer. The incidence of childhood cancer in Malaysia was reported to increase from 2012 to 2016. The number of cancer cases and deaths is projected to swiftly grow as the population continues growing (National Cancer Institute, 2020).

Cancer in children behaves differently than in adults and this has propelled oncologists to study cancer in children separately. Even though childhood cancer is classified at ages 0 to 14 years, certain types of cancer in children peak at the age group of 15 to 19 years. Childhood cancer is a major cause of morbidity in children (C.-E. Tan et al., 2022). Looking at long-term outcomes, childhood cancer survivors are likely to encounter late effects of health related outcome like impaired growth and maturation and health issues between the age of 20 and 39 years (Erdmann *et al.*, 2021). These late effects are usually due to the side effect of the treatment which appear years after the completion of cancer therapy. Studies have demonstrated that adults who received treatment for cancer during childhood possess 60% to 90% risk

for chronic health conditions and 20% to 80% risk of developing life-threatening complications (Salloum *et al.*, 2019; Hudson *et al.*, 2013).

Continuous cell proliferation is a fundamental abnormality in cancer development. Cancer cells inappropriately respond to the transduced signal for cell proliferation that causes the cells to uncontrollably proliferate, subsequently invading the surrounding healthy tissues and eventually spreading throughout the body (Cullen & Breen, 2016). Cancer development is a complex pathophysiology resulting from the net accumulation of multiple abnormalities in the cell's genetic and regulatory system which induce distinguished behaviour from its normal counterparts (Carbone *et al.*, 2020). The transformation of normal cells into malignant tumours involves a multistage process that generally progresses from uncontrolled cell proliferation to the precancerous lesion until the formation of a malignant tumour. These changes result from the interaction of several causative factors that induce cancer development (Curtius *et al.*, 2017).

Lifestyle-related risk factor is not the main factor contribute to the development of childhood cancer because this factor usually requires several years to influence cancer growth. Cancer in children is mainly due genetic alteration either inherited or developed during infancy and childhood time. Genetic alterations in osteosarcoma are influenced by a combination of intrinsic factors, such as inherited genetic predispositions, spontaneous mutations, and epigenetic changes, as well as extrinsic factors, including environmental exposures and viral infections. The genetic alteration influenced the activation of oncogene as well as prevent the normal regulation of tumour suppressor gene which consequently induced carcinogenesis (Kontomanolis *et al.*, 2020).

2.2 Bone cancer

Bone cancer refers to malignant tumours that develop in the bone tissue. There are more than 200 bones in the body that vitally support the body, protect internal organs, and act as levers for muscles so that humans can stand and move rigidly. Bone cancer can arise at any bone in the body (Keil, 2020). Bone cancer can be classified into primary and secondary types. Primary bone cancer, also called bone sarcoma, develops initially in the bone. The most common forms of primary bone cancer are osteosarcoma, chondrosarcoma, and Ewing sarcoma, constituting about 35%, 30%, and 16% of occurrences, respectively. Chondrosarcoma usually affects middle-aged and older adults. In contrast, osteosarcoma and Ewing sarcoma mainly affect children and young adults. There are also several other primary bone cancers that are rarely detected, such as malignant fibrous histiocytoma, fibrosarcoma, chordoma, and giant cell tumour of the bone (GCTB), constituting about 1% to 5% of all primary bone cancers (Lam, 2020). Meanwhile, secondary bone cancer results from other cancers that develop elsewhere in the body and spread to the bones. The skeletal system is determined to be the third most common site for cancer metastases after the lung and liver (Wood, 2020). Each of these types of cancer varies in biological behaviour, imaging appearance, and demographics (Pullan & Lotfollahzadeh, 2024).

2.3 Osteosarcoma

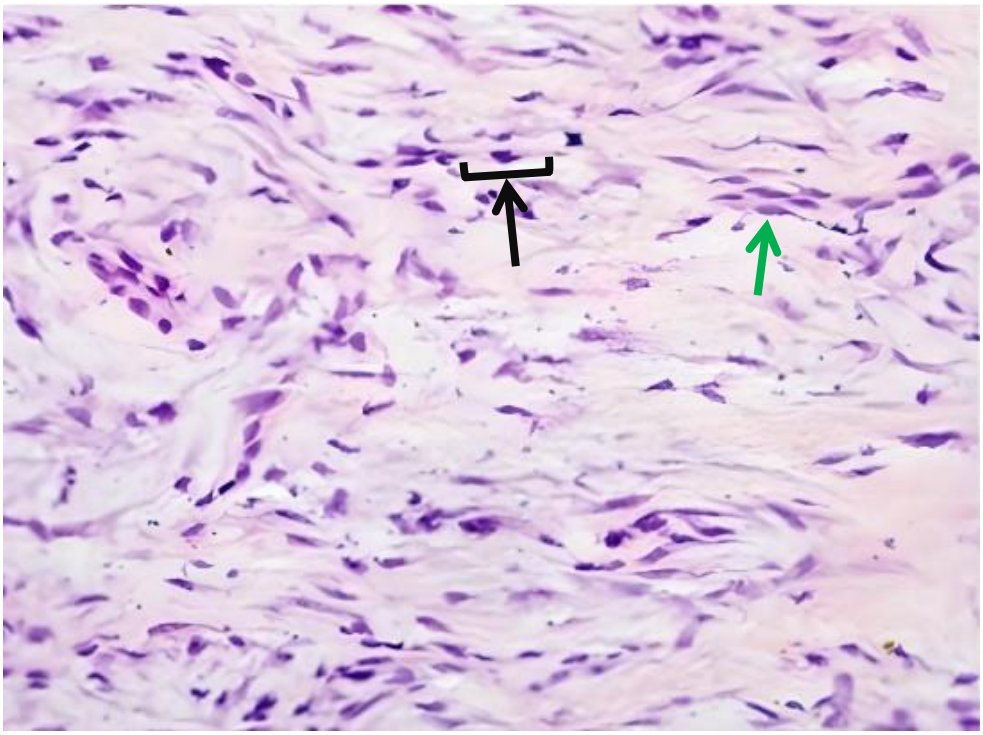
Osteosarcoma is a typical childhood cancer and has been recognised leading cause of death among bone cancer in children under age 18 (Bielack *et al.*, 2022). Osteosarcoma is a primary malignant tumour due to the uncontrolled production of immature bone (Beird *et al.*, 2022). It is developed from primitive mesenchymal stem cells that are responsible for the development of bone, cartilage, or fibrous tissue

(Isakoff *et al.,* 2015). Osteosarcoma is the most common primary malignant bone tumour (Zhao *et al.,* 2021).

2.3.1 Morphology of osteosarcoma

Osteosarcoma is described as spindle cell neoplasm and may be presented with different degrees of cytological atypia and pleomorphism (Doyle, 2014). Commonly, osteosarcoma cells appear in triangle, elongated, spindle to polyhedral shape (Huang *et al.,* 2013; Broadhead *et al.,* 2011; Pautke *et al.,* 2004). The cell's cytoplasm is commonly moderate to abundant. The appearance of the nuclear structure varies, although it is typically observed to be hyperchromatic with prominent nucleoli and large rounded with central to eccentric position. The atypical mitotic cells undergoing mitosis are easily identified with multinucleated giant cells (Gupta *et al.,* 2021). The spindled-shaped cells were usually associated with osteoid deposition (Doyle, 2014). Osteoid is an unmineralised or organic component that undergoes calcification to become a key structure of bone matrix (Trammell & Kroman, 2013). The osteoid can be easily recognised as it is stained pink to magenta-coloured. The osteoid is helpful in cytological analysis for diagnosing osteosarcoma based on cytomorphologic features (Gupta *et al.,* 2021). In cell culture, osteosarcoma is normally attached to the dish and grows in monolayer (Chim *et al.,* 2023; Pautke *et al.,* 2004). Compared to osteoblasts, osteosarcoma cells possess different morphological structures. The morphology of osteoblast is described to be in cuboidal single-layer cell shape (Rutkovskiy *et al.,* 2016). The average comparison of osteosarcoma cell size was approximately 1/6 to osteoblasts. Unlike osteoblasts, osteosarcoma cell lines have consistent cell size regardless of cell density (Pautke *et al.,* 2004) (Figure 2.1).

(A)



(B)

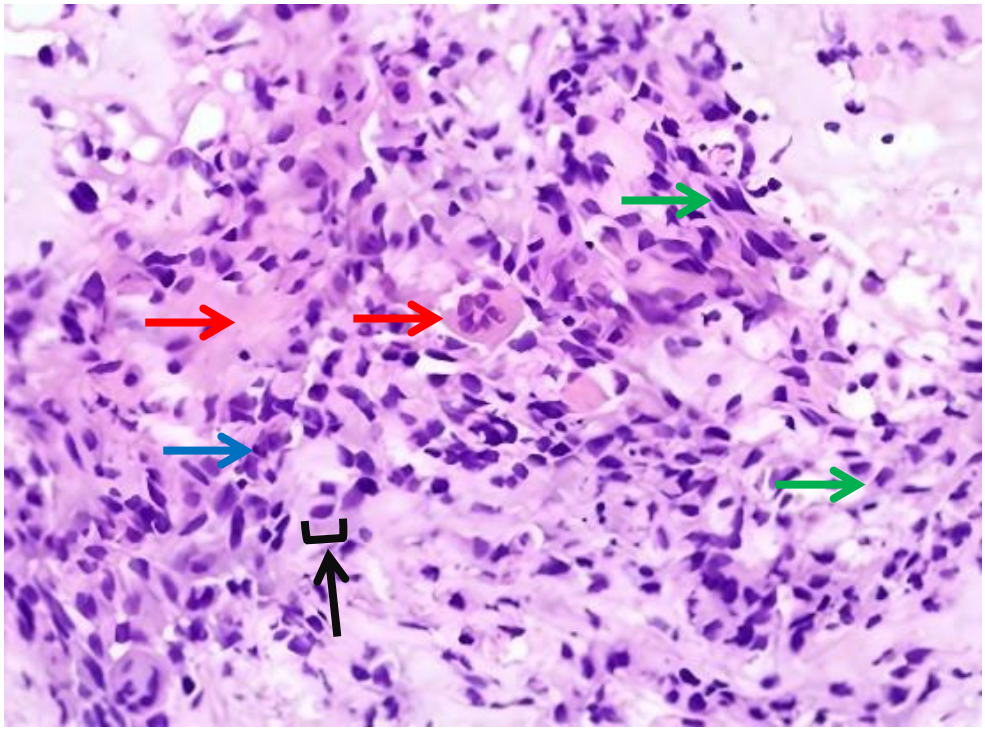


Figure 2.1 The histology of (A) adjacent normal bone and (B) osteosarcoma tissue in response to haematoxylin and eosin (H&E) staining observed under phase contrast microscope at 100X magnification. Osteoblast cells appeared with uniform spindle shape while osteosarcoma cells also presented in spindle shape but at different degrees of cytological atypia and pleomorphism (→). The osteoblast mostly appeared longer in size compared to osteosarcoma cells (→). The osteoid can be recognised with pink to magenta-coloured stained (→). Prominent nucleoli of osteosarcoma cells can be easily detected (→). Adapted from Liu *et al.* (2014)

The usage of osteosarcoma cell lines is widespread and provides an excellent model system for *in vitro* evaluation of malignancy, drug discovery, and general cell biology. In new cancer drug discovery studies, the application of cell lines is of foremost importance as an initial assessment before further testing in animal models, which facilitates clinical studies (Mohseny *et al.*, 2011). Most *in vitro* evaluations use a wide range of human osteosarcoma cell lines, such as MG-63, U2OS, HOS, Saos-2, and OSA 1777. Among all osteosarcoma cell lines, MG-63 and U2OS are the most frequently used in *in vitro* evaluations (Kasiram *et al.,* 2022). Both, MG-63 and U2OS cell lines have been shown to have similar proliferation rates (Jiang *et al.,* 2020).

The U2OS cell line is the first derived osteosarcoma cell line and has been used for studying mitosis, apoptosis, and cellular mechanisms. U2OS is a cell line with moderately differentiated morphology that was derived in 1964 from 15-year-old girl that has sarcoma of the tibia (Yu *et al.,* 2021). U2OS cells are relatively stable genetically, making them suitable for long-term culture and experiments requiring consistent genetic backgrounds. This stability facilitates the reproducibility and reliability of experimental results. U2OS cells are easy to culture and maintain in the laboratory compared to some other osteosarcoma cell lines. They grow adherently under standard cell culture conditions, allowing for the scalability of experiments. U2OS cells exhibit a rapid proliferation rate, allowing for quicker experimentation and easier maintenance in culture. This is a significant benefit for studies requiring large numbers of cells (Burmester *et al.*, 2014; Musa, 2013).

2.3.2 Classification, staging and diagnosis of osteosarcoma

Osteosarcoma is a bone cancer due to atypical excess production of osteoid. Histologically, different degrees of differentiation resulting variable histological patterns of osteosarcoma according to its grade (Wadhwa, 2014). Osteosarcoma can be classified into two types which are primary and secondary. Primary osteosarcoma mainly originates from the bone cells and is not associated with other diseases (Pullan & Lotfollahzadeh, 2024). Primary osteosarcoma can be further classified according to the tumour location within the bone like central (medullary) and peripheral (surface). There are several subtypes within each group. The osteosarcoma subtype for central (medullary) are conventional, low-grade, telangiectatic, and small-cell, whereas for surface (peripheral) subtypes are periosteal, parosteal, and high-grade surface (Rothzerg *et al.,* 2023).

Meanwhile, secondary osteosarcoma is due to association with an underlying osseous disorder or other cancer. Commonly, around 90% of primary osteosarcoma were detected at the metaphyseal region of the long bone (Bertoni *et al.,* 2020) [Figure 2.2 (A)]. Nevertheless, tumour location for secondary osteosarcoma possesses wider distribution and varies according to the nature of the underlying predisposed condition. Adult populations are mostly diagnosed with secondary osteosarcoma. Flat bones, especially the pelvis, are commonly affected by Paget's disease and notably exhibit a higher incidence of secondary osteosarcoma (Prater & McKeon, 2023).

Like other cancers, osteosarcoma can either be localised or advanced stage. Localised osteosarcoma only affects the bone where the tumour initially developed and its surrounding tissues, such as muscle and tendon. Localised osteosarcoma accounts for 80% of all osteosarcoma cases (Cable & Randall, 2019). About 45% to 75% of localised osteosarcoma has been found to affect the distal femur, 19% to 80%

affect the proximal tibia (Figure 2.3), 10% to 90% affect the proximal humerus, 8% affect the pelvis, skull, and jaw (Menendez et al., 2022; Lamplot *et al.,* 2013) [Figure 2.2 (B)]. Osteosarcoma of long bone vastly affects children and adolescents. Meanwhile, osteosarcoma at the craniofacial and axial bone is usually detected in elderly patients. The primary osteosarcoma of axial was recorded in 41.3% of elderly patients compared to only 12% in patients under 24 years old. Axial osteosarcoma commonly develops in the mandible, skull, ribs, pelvis, and spine (Durfee *et al.,* 2016) [Figure 2.2 (B)].

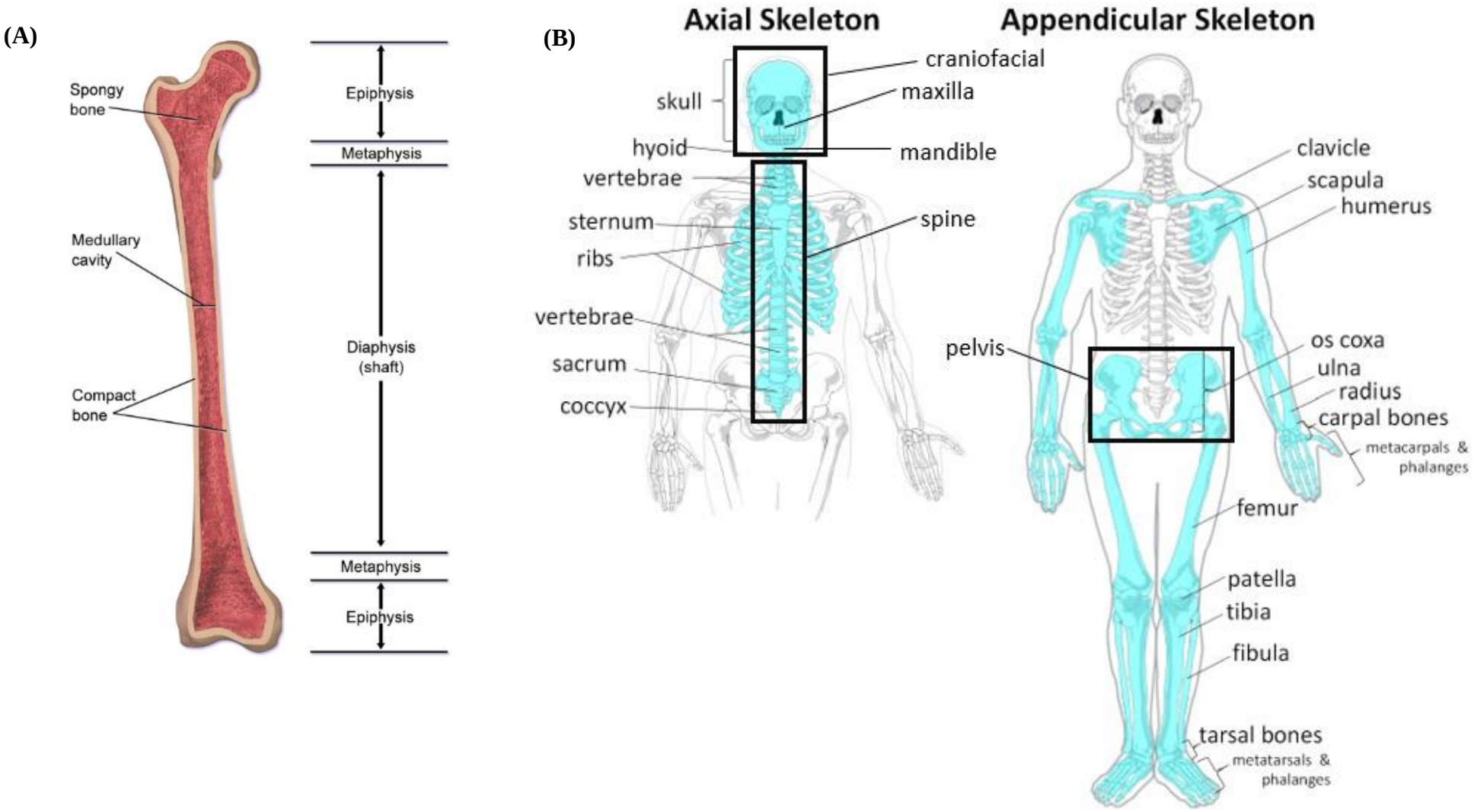


Figure 2.2 (A) Structure of the long bone. Adapted from *Features of a Typical Long Bone* (2018). (B) Human skeletal system. Modified from Aquino (2019)

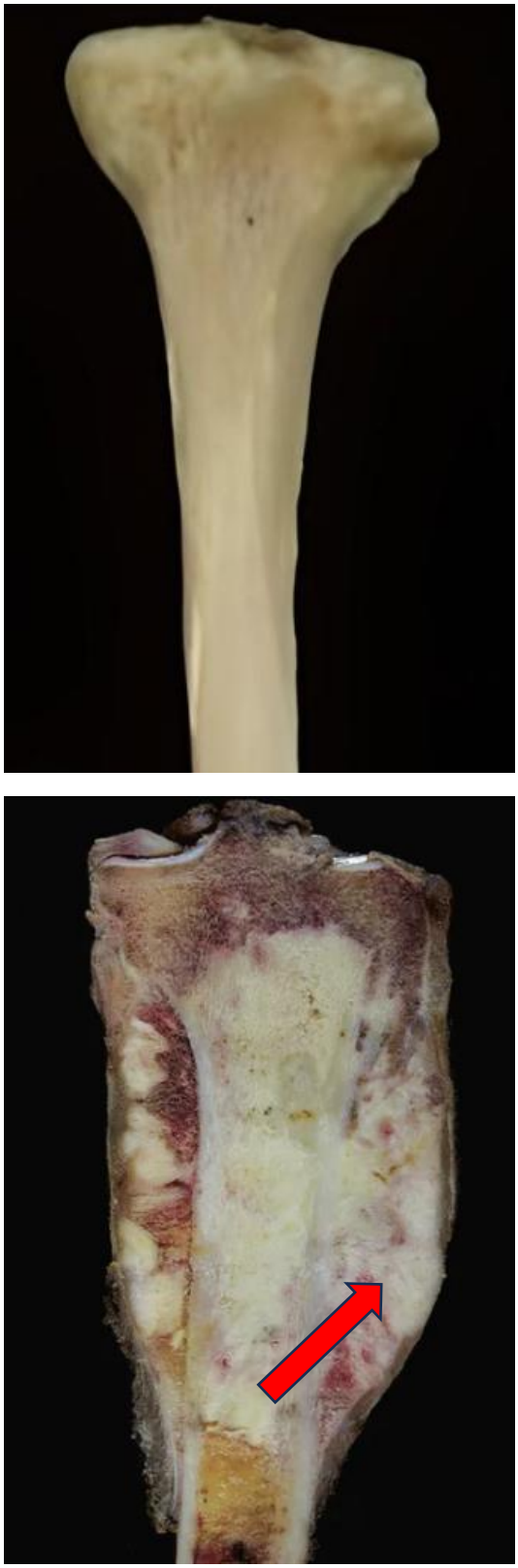


Figure 2.3 (A) The posterior normal tibia of human. Adapted from *OsteoID Bone Identification* (2024). (B) Gross pathology of conventional intramedullary osteosarcoma at proximal tibia. The tumour extensively affects the metaphyseal region of the proximal tibia (Red arrow). Adapted from *Osteosarcoma* (2017)

Advanced osteosarcoma is developed once the tumour begins to progress and invades other organs. Disseminated or metastatic osteosarcoma constituted 20% of all osteosarcoma cases (Cable & Randall, 2019). It has been found that the lung is the most common distant metastasis of osteosarcoma, comprising about 83% to 90% of all metastatic cases (Durfee *et al.*, 2016). The second most common site for metastasis in osteosarcoma is another type of bone and collectively makes up around 8% to 10% of all metastatic cases. Other organs like the brain account for 1.8% to 21.9% of metastatic cases of osteosarcoma (Xu *et al.*, 2022). Patients with brain metastasis from osteosarcoma are rare and usually associated with lower survival rate (Martin *et al.*, 2019).

The pathological diagnosis of osteosarcoma is mainly based on the interpretation of cell morphology and osteoid matrix produced (Nguyen *et al.*, 2022). It is recognised as a hallmark diagnosis of osteosarcoma which either be determined through the histologic analysis from the biopsy or through the radiograph (Abarrategi *et al.*, 2016). Conventional plain radiographs like X-ray, MRI, and PET are considered the best tools used for diagnosing osteosarcoma. These radiographs are capable of accurately showing the atypical bone features of periosteal reaction like the appearance of sunburst, onion skin, and Codman's triangle, as well as the new bone formation in the soft tissue with the permeative pattern that indicates the born destruction (Figure 2.4). The radiograph method can also be used to detect metastasis, especially in the lung. As for biopsy, definitive surgery is performed to take a tissue sample for histological analysis in the laboratory. Histological analysis is essential to confirm and determine the grade of tumours. Other than that, biomarker analysis for makers like alkaline phosphatase and lactate dehydrogenase are applied for making a prognosis and evaluation of the treatment response (Hirayama *et al.*, 2020; Basoli *et al.*, 2023).

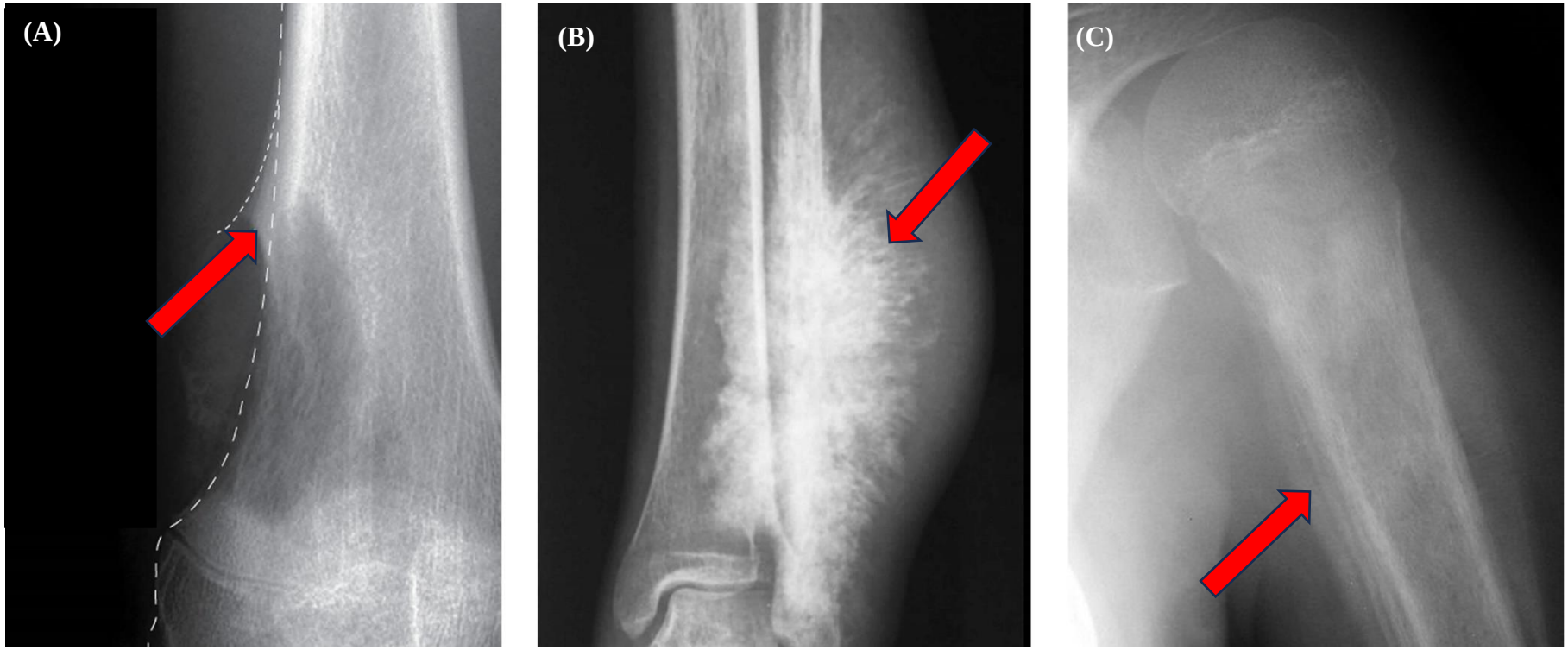


Figure 2.4 The radiograph of atypical bone features due to periosteal reaction of bone tumour. (A) Codman's triangle appearance. Adapted from *Periosteal Reaction* (2019) (B) Sunburst appearance. Adapted from *Periosteal Reaction* (2019) and (C) Onion skin appearance. Adapted from Plant & Cannon (2016). Red arrow shows the bone deformation due to tumour development.