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Cytotoxicity study and sicroscopy evaluation on the effects of *Quercus infectoria* Gall extract on Human Osteosarcoma Cell Line (U2OS)

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The necessity for a new treatment plan has arisen due to the increased occurrence of cisplatin chemoresistance cases and other adverse outcomes from osteosarcoma therapy. *Quercus infectoria* (QI) gall extract possesses an antiproliferative effect against various cancer cells. Therefore, this study investigates the effects of QI gall extract on cell viability and morphological changes in the U2OS human osteosarcoma cell line. Rotary evaporator and freeze dryer were used to prepare QI gall extract. MTT assay was used to determine the half-maximal inhibitory concentration (IC_{50}) for QI gall extract, while the cytotoxicity effect of the cisplatin and QI gall extract treatment was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue exclusion assay. The morphological changes of cells were observed under a phase-contrast inverted microscope. QI gall extract had cytotoxic effects on U2OS cells, with an IC_{50} value of 27.19 µg/mL. Cisplatin-treated cells exhibited the highest level of growth inhibition, accompanied by distinct morphological alterations resembling the hallmark features of apoptotic cells like distinct chromosomal condensation, nuclear fragmentation, reduction of nuclear volume, and notable apoptotic bodies. No significant differences were seen between cisplatin and QI gall extract treatment groups at 24, 48, and 72 h when comparing the groups for the same treatment durations. QI gall extract treatment had a cytotoxic effect on U2OS cells and induced prominent morphological changes on the cells. Further investigation and comprehensive studies are required to draw a conclusion regarding the potential of QI gall extract as a chemotherapeutic regimen for osteosarcoma treatment.

Keywords: Cytotoxicity effect; Cisplatin; Human osteosarcoma cell line; Morphology; *Quercus infectoria*

INTRODUCTION

The American Cancer Society reported that less than 1% of all malignancies are primary bone cancers, which are rare cancers that begin in the bones. Adults are far more likely to develop secondary bone cancers (often referred to as bone metastases) than primary bone cancers. Bone cancer is a form of cancer that originates in the bones and primarily affects the long bones and the pelvis (Pylostomous et al. 2023). The most common type of bone cancer is bone osteosarcoma (Ferguson & Turner, 2018; American Cancer Society, 2023), followed by chondrosarcoma and Ewing sarcoma. Osteosarcoma is a highly malignant bone tumour that develops from mesenchymal cells, which are spindle-shaped stromal cells that can create tissues that mimic bones (Raymond et al. 2010). These cells carry cancer stem cells, which are responsible for the tumour's growth and resistance to chemotherapy (Martins-Neves et al. 2023).

Chemotherapy and radiation therapy are two frequently used methods in modern osteosarcoma cancer therapy and are associated with numerous health risks. Chemotherapy is the gold standard for cancer treatment, but its efficacy is limited by cytotoxic side effects, tumour-targeting consistency, and chemoresistance development (Lopes et al. 2023; Liu et al. 2022). Furthermore, the high levels of medication resistance linked to chemotherapy and radiation therapy have a significant impact on the quality of life and treatment outcomes for patients (Fang et al. 2023; Imran et al. 2022).

One of the most important obstacles to improving osteosarcoma therapy efficacy is overcoming chemoresistance, which is frequently linked to the body's defence mechanism against oxidative stress. For instance, chemoresistance development significantly reduces the therapeutic efficacy of cisplatin, a frequently used first-line treatment for osteosarcoma (Zhang et al.

2021). Although the prognosis for osteosarcoma patients has improved over the past few decades, bone sarcomas remain an urgent disorder requiring an interdisciplinary approach to both diagnosis and treatment (Bläsius et al. 2022).

As conventional cancer treatments may have detrimental side effects on healthy cells and tissues, research is still being done to find cancer therapy agents with fewer side effects (Tajudin et al. 2012; Khalili et al. 2017; Ismail et al. 2018). One promising approach to lessen these negative effects is the use of medicinal plants in cancer treatment (Desai et al. 2008; Ismail et al. 2021). Medicinal plants have been recognised for their therapeutic and pharmaceutical benefits throughout history. The consumption of natural plant-based products for medical purposes has become progressively more common in recent years. Ismail et al. (2021) reported that some phytochemicals derived from plants possess anticancer effects. According to the World Health Organization (WHO), between 70% and 95% of people in developing countries use medicinal plants as their main source of healthcare (Singh et al. 2010). Because of their unique properties, which include their nontoxicity to healthy cells and their cytotoxicity to malignant cells, therapeutic plants are becoming ever more prominent (Greenwell & Rahman, 2015).

Quercus infectoria (QI) is a small oak belonging to the Fagaceae family and is indigenous to Greece, Asia Minor, and Iran. It grows to a modest height of 4 to 6 feet and has a characteristic crooked stem. This oak species, which has shiny, colourful leaves and tall, slender, scaly, fluffy acorns, has drawn notice for its potential medicinal uses (Ismail et al. 2021; Shrestha et al. 2014). In Ayurvedic medicine, QI is highly valued for its astringent powers, antidiabetic, antitremor, antipyretic, and anti-Parkinson effects (Aroonrerk et al. 2009). QI galls also possess several biological activities such as anticancer (Yusof & Abdullah, 2020; Kasiram et al. 2022; Tofigh et al. 2024), antimicrobial (Tayel et al. 2018), and anti-inflammatory properties addressing on female genital disorders (Mahboubi et al. 2020).

Furthermore, QI gall extracts have been shown to contain a variety of tannins, including hexamethyl ether, isocryptomerin, syringic acid, ellagic acid, gallic acid, β-sitosterol, amentoflavone, methyl betulate, methyl oleanate, and hexagalloyl glucose (Ismail et al. 2021). A previous study by Yusof and Abdullah (2020) concluded that out of all the extracts examined, QI ethyl acetate extract showed the strongest cytotoxic effect against HeLa cervical cancer cells, with the lowest half-maximal inhibitory (IC_{50}) value. Apoptotic cell death shown by DNA fragmentation and chromatin condensation in the treated cells is the basis for the cytotoxic activity of QI ethyl acetate.

The presence of a distinct variety of phytochemicals in the QI gall extract, such as tannins, alkaloids, flavonoids, glucosides, saponins, terpenoids, and phenolic compounds, may have contributed to the cytotoxic action and cell death event. Therefore, this study was conducted to investigate the cytotoxicity and morphological changes caused by QI gall extract on the human osteosarcoma cell line U2OS upon treatment.

MATERIALS AND METHODS

Preparation of *Quercus infectoria* (QI) Gall Extract

Quercus infectoria (QI) galls were obtained from Syarikat Jaffar Rawas Sdn. Bhd., Kota Bharu, Kelantan. The QI galls were crushed and ground into powder form. The QI gall powder was then added to distilled water and extracted by refluxing in a water bath at 50 °C for 24 h. The extract was filtered by using a filter paper and concentrated by using a rotary evaporator (Heidolph Rotavac, Germany). The aqueous QI gall extract was lyophilised in a freeze dryer until a powder was obtained; finally, it was stored at −20 °C until use.

Cell Culture

Cell revival and subculture

The human osteosarcoma cell line U2OS (HTB-96™) used in this study was purchased from the American Type Culture Collection (ATCC®) (Manassas, VA, USA). It was maintained in McCoy's 5A modified medium (Invitrogen, Massachusetts, USA) supplemented with 10% (v/v) foetal bovine serum (Invitrogen, Massachusetts, USA) and 1% (v/v) penicillin–streptomycin (Gibco, Gaithersburg, USA). Cells were incubated in a humidified atmosphere of 5% CO2 incubator at 37 °C and closely monitored every 24 h. All cell culture-related work was conducted in a biosafety cabinet Class II to maintain sterility.

Preparation for cell treatment

Cisplatin was used as the positive control while untreated cells served as the negative control. QI gall extract and cisplatin (TargetMol, Boston, USA) were dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque, Japan) to make stock solutions of 10 mg/mL. The stock solutions of each agent were serially diluted to produce a working solution at several concentrations (10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.02, & 0.01 mg/mL).

Cells were initially seeded at 5×10^4 cells/mL in 96-well plates and incubated overnight at 37 °C in 5% CO_2 incubator for cellular adherence. Then, the cells were treated with the serially diluted agents. DMSO was used in the control group. The cells were incubated in a 5% CO_2 incubator at 37 °C for 72 h. The tests were conducted in three independent experiments in triplicate to ensure that the results were reliable and accepted.

Determination of Half-Maximal Inhibitory Concentration (IC_{50}) by MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Nacalai Tesque, Japan) was performed to obtain the value of half-maximal inhibitory concentration (IC_{50}) of QI gall extract. The MTT working solution was prepared at 5 mg/mL in phosphate-buffered saline. MTT solution was pipetted into each well of the control and treatment groups. The 96-well plate was then wrapped with aluminium foil and incubated in a 5% CO_2 incubator at 37 ℃ for 4 h. After the incubation, the supernatant was aspirated from the wells, and DMSO was added to solubilise the purple formazan crystal. The 96-well plate was shaken for 3 min to ensure complete solubilisation. The absorbance (OD) was measured at 570 nm by using an ELISA microplate reader (Tecan, Switzerland). DMSO was used as blank. The percentage of viable cells was calculated by using the following formula:

$$\text{Percentage of viable cells (\%)} = \frac{(\text{OD value of treated cells} - \text{OD value of blank})}{(\text{OD value of untreated cells} - \text{OD value of blank})} \times 100$$

The dose-response curve of cell viability (%) against the final concentration was plotted, and the IC_{50} values were determined using GraphPad Prism software, version 9.5.1.

Cell Morphology Observation by Phase-contrast Inverted Microscope

U2OS cells were initially seeded at 5 × 10^4 cells/mL in a 6-well plate and incubated overnight at 37 °C in a 5% CO_2 incubator. The cells were treated with cisplatin at IC_{50} =16.25 µg/mL based on previous data from Kasiram et al. (2022) and QI gall extract at IC_{50} = 27.19 µg/mL as determined in this study. DMSO was used in the control group. The morphology of the U2OS cells in each control, cisplatin, and QI extract treatment group was observed after 24, 48, and 72 h of incubation at 37 °C in 5% CO_2 incubator. The cell morphological changes were observed under a phase-contrast inverted microscope (Carl Zeiss, Germany). Images of cells in each treatment and control group were captured at 40× magnification.

Trypan Blue Exclusion Assay

The cytotoxicity activity was evaluated through the trypan blue exclusion assay. U2OS cells at 5 × 10^4 cells/mL were initially seeded and incubated overnight at 37 ℃ in 5% CO_2 incubator. After that, the cells were treated with cisplatin and QI at the IC_{50} determined earlier. After 24, 48, and 72 h, the cells were trypsinised with 0.25% trypsin/EDTA (Invitrogen, Massachusetts, USA). The cells were then stained with trypan blue for cell counting. Countess™ Automated Cell Counter (Invitrogen, Massachusetts, USA) was utilised to count the number of cells in each treatment and control group. A graph of viable cells versus the time for each treatment and control group was plotted and analysed.

Statistical Analysis

Data obtained were expressed as mean ± SEM (standard error mean) from three independent experiments (n = 3). Half-maximal inhibitory concentration (IC_{50}) was determined by using GraphPad Prism, version 9.5.1. The obtained data were initially tested for normality and homogeneity of variance through the Shapiro–Wilk test. Then, a statistical comparison was performed through one-way ANOVA, followed by Tukey's honest significant difference (HSD) post hoc test. The result was considered statistically significant if $p < 0.05$. Each analysis was conducted using IBM SPSS Statistics version 20.

RESULTS

Half-Maximal Inhibitory Concentration (IC_{50}) of *Quercus infectoria* (QI) Gall Extract and Cisplatin for U2OS Cells

After 72 h of treatment, cisplatin and QI gall extract showed different cytotoxicity effects on U2OS cells. QI gall extract demonstrated an IC_{50} value of 27.19 µg/mL, which is lower than that of cisplatin as obtained in previous study at 16.25 µg/mL Kasiram et al. (2022). The lower IC_{50} value for cisplatin indicates greater cytotoxicity effect against U2OS cells compared to QI gall extract, which is expected since it is a well-known chemotherapy drug.

To visualise the cytotoxicity pattern of the U2OS cells, the percentage of cell viability (%) was plotted against the log_{10} concentration (µg/mL) of the treatment (Figure 1). The percentage of U2OS cell viability consistently decreased with increasing concentrations of the treatment agents, suggesting dose-dependent effects of both QI gall extract and cisplatin. Cisplatin and QI gall extract were then used to treat U2OS cells in the subsequent cytotoxicity evaluation at the obtained IC_{50} values to observe the number of viable cells and effect on cell morphology.

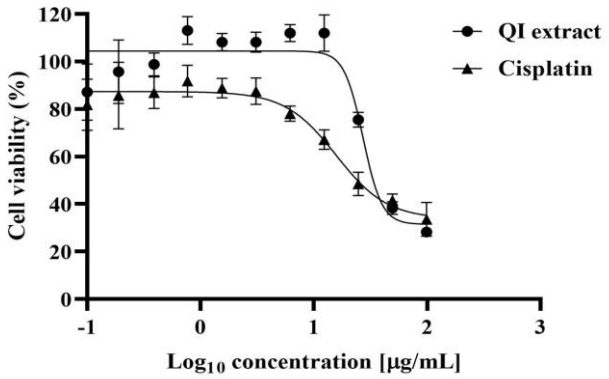

Figure 1: Graph of percentage of cell viability (%) against log_{10} concentration of cisplatin [data from Kasiram et al. (2022) and *Quercus infectoria* (QI] gall extract.

Half-maximal inhibitory concentration (IC_{50}) values

were calculated by using dose-response stimulation curve in GraphPad Prism version 9.5.1. The IC_{50} for QI gall extract was 27.19 µg/mL, higher than IC_{50} for cisplatin, 16.25 µg/mL.

Morphological Changes on U2OS after Treatment with *Quercus infectoria* (QI) Gall Extract and Cisplatin

U2OS cells after seeding appeared to be rounded in shape and were spread freely in the flask as they were suspended in the medium and not yet attached to the surface. Following overnight incubation, the cells adhered to the flask surface, displaying an elongated and spindle-shaped form characteristic of epithelial and adherent morphology.

The experimental treatment was then administered to all groups except the control group. Morphological changes in U2OS cells were assessed at 24, 48, and 72 h across the three groups: control group (untreated), cisplatin treatment (positive control), and QI gall extract treatment [Figure 2 (A–H)]. After 24 h of treatment, cells in both the cisplatin and QI gall extract treatment groups transitioned to a rounded shape with increased nuclear chromatin density [Figure 2 (B, C)]. At 48 h of treatment, the cisplatin treatment induced cell blebbing and chromatin condensation in U2OS cells. Meanwhile, the QI gall extract treatment primarily resulted in cell shrinkage and chromatin condensation.

After 72 h of treatment, cells in all treatment groups exhibited a sparsely distributed pattern with deteriorated shapes [Figure 2 (H, I)]. The QI gall extract caused cells to adopt an oval and round shape, displaying a blebbing and shrinkage-like appearance along with chromatin condensation in most cells [Figure 2 (I)]. In contrast, the cisplatin treatment group exhibited more pronounced morphological alterations, including apoptotic hallmarks such as chromatin condensation, cell shrinkage, cell blebbing, and the presence of apoptotic bodies [Figure 2 (H)]. Most cells treated with cisplatin were dead, evident through the extensive deterioration of cell shape and the presence of apoptotic bodies.

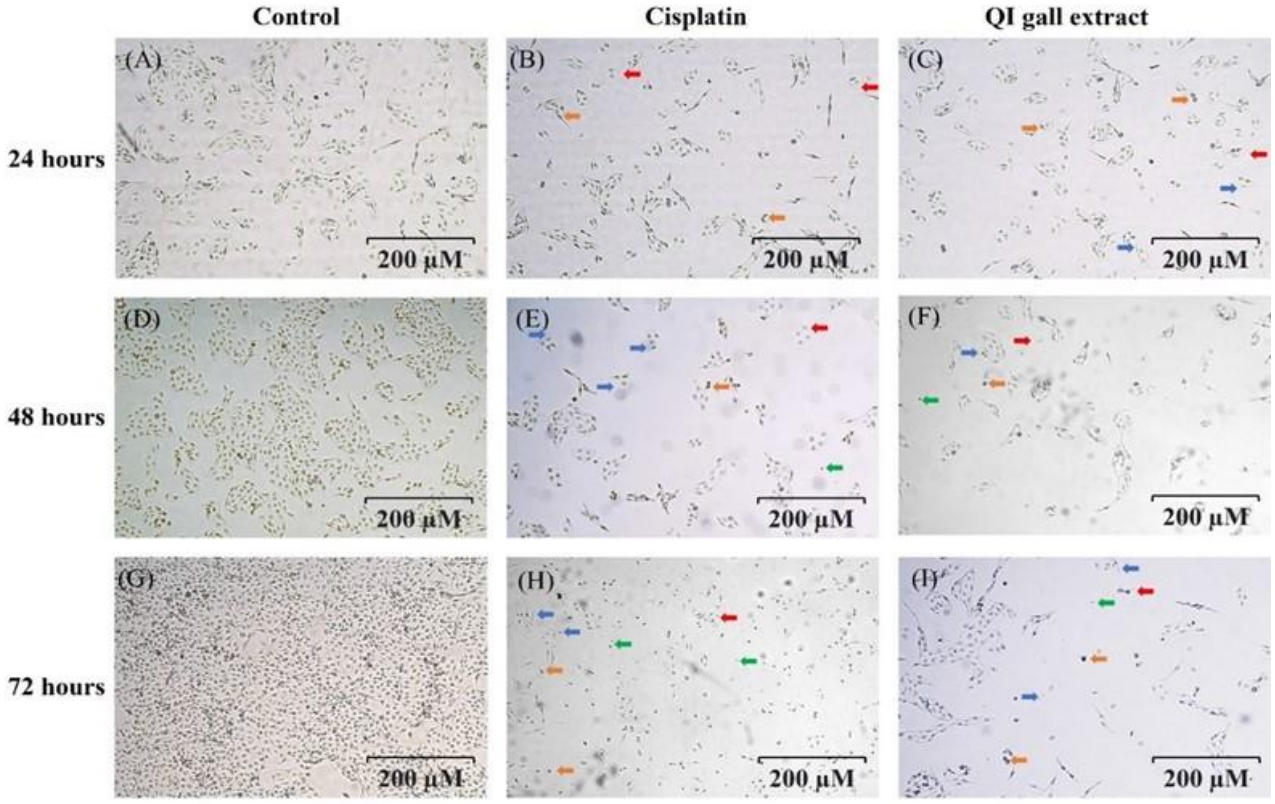

Figure 2: Morphological changes of control and treated U2OS cells with cisplatin and QI gall extract. (A, B, C) Morphology of U2OS after 24 h of treatment. (D, E, F) Morphology of U2OS after 48 h of treatment. (G, H, I) Morphology of U2OS after 72 h of treatment.

Cell morphologies were observed and captured by using a phase-contrast inverted microscope (Carl Zeiss, Germany) at 40× magnification. Red arrows indicate cell shrinkage, blue arrows represent cell blebbing, green arrows indicate apoptotic body, and orange arrows represent chromatin condensation.

Cisplatin and *Quercus infectoria* (QI) Gall Extract Cytotoxicity Activity on U2OS Cells

In this study, the cytotoxic activity of QI gall extract and cisplatin on U2OS cells was assessed using trypan blue exclusion assay. The number of viable U2OS cells upon exposure to QI gall extract and cisplatin adjusted to their respective IC_{50} values was determined. As depicted in Figure 3, the number of viable cells decreased at each time point across all treatment groups.

Notably, both the positive control (cisplatin) and QI gall extract caused an approximately similar reduction in viable cells. At 48 and 72 h, both cisplatin and QI gall groups showed significantly lower cell viability as compared to the control (untreated group).

Statistical analysis using a one-way ANOVA revealed a significant effect within similar groups at different treatment times for both cisplatin (p = 0.005) and QI gall extract (p = 0.023). However, no significant differences were found between cisplatin and QI gall extract treatment groups at 24 h (p = 1.00), 48 h (p = 0.972), and 72 h (p = 0.761) when comparing groups with similar treatment durations

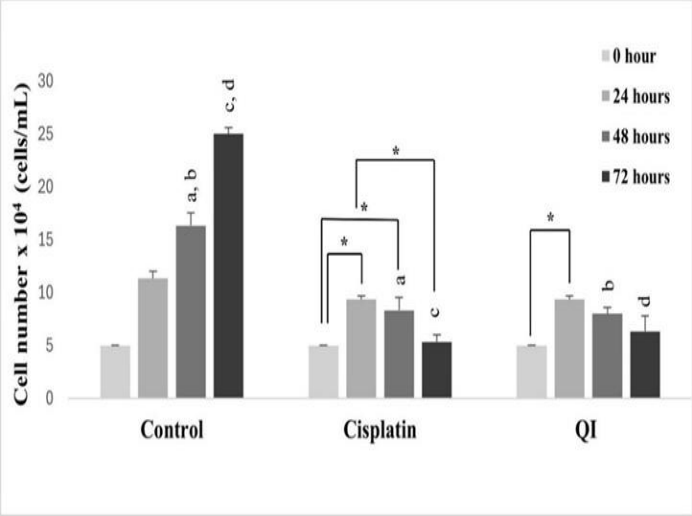

Figure 3: **The number of viable U2OS cells (×10^4) in response to treatment for 0, 24, 48, and 72 h in three groups (control, cisplatin, and QI gall extract treatment groups) determined by trypan blue exclusion assay.**

The bar represents mean ± standard error mean (SEM). The asterisk (*) indicates a significant difference (p < 0.05) within a similar group in different treatment hours. Groups that share the same letter showed significant differences among the different groups in the similar treatment hours. The statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test

DISCUSSION

This study evaluated the cytotoxicity and morphological effect of *Quercus infectoria* (QI) gall extract on the human osteosarcoma cell line U2OS model. Initially, the half-maximal inhibitory concentration (IC_{50}) value of QI gall extract against U2OS cells was determined. The viability of U2OS cells decreased in a dose- and time-dependent manner after treatment with QI gall extract and the chemotherapy drug cisplatin.

The IC_{50} value of the QI gall extract was 27.19 µg/mL, whereas cisplatin exhibited an IC_{50} value of 16.25 µg/mL. This higher IC_{50} value indicates that the concentration of QI gall extract required to induce cytotoxicity in U2OS cells is higher compared to that of cisplatin. Hence, this finding suggests that the cytotoxic effect on U2OS cells is more potent when the cells were treated with cisplatin compared to QI gall extract. As highlighted by Zhang et al. (2021), established chemotherapy drugs like cisplatin, carboplatin, and oxaliplatin are widely utilised in medical practice due to their proven therapeutic efficacy and well-understood mechanisms of action. Moreover, cisplatin is an extensively used platinum-based chemotherapeutic drug that plays a crucial role in the treatment of various tumour types (Zhang & Lu, 2021). Thus, our findings align with existing knowledge, showing that cisplatin elicits a stronger cytotoxicity effect compared to QI gall extract.

However, being a first-generation drug, cisplatin lacks selectivity to cancer cells, which can potentially lead to systemic side effects (Zhang et al. 2021; Wong & Giandomenico, 1999). Furthermore, it is also capable of inhibiting the growth of normal cells (Yusof & Abdullah, 2020). The lack of selective cytotoxic action by cisplatin has intensified the search for potential natural compounds to solve this issue. Moreover, certain natural compounds such as flavonoids, polyphenolics, steroids, and tannins have been associated with a range of health benefits (Yoo et al. 2018). For instance, the gallotannin-enriched fraction from QI galls has been identified as an antioxidant and inhibitory agent against human glioblastoma multiforme (Kamarudin et al. 2021). Due to the abundance of active compounds present in QI gall extract, it remains unclear which specific phytochemicals exert the most potent cytotoxic effects on U2OS cells. Therefore, conducting further phytochemical screening is necessary to identify the compound and elucidate its anticancer activities.

Yusof and Abdullah (2020) reported IC_{50} values for QI methanol extract against cervical cancer cell HeLa, breast cancer cell line MDA-MB-231, and hepatoma cell line Hep G2 to be 23.8 ± 0.91, 90.2 ± 0.89, and 85.1 ± 0.34 µg/mL, respectively. The QI methanol extract exhibited minimal toxicity against MCF-7 breast cancer cell line, with an IC_{50} value below 99 µg/mL. On the other hand, the QI ethyl acetate extract displayed significant cytotoxicity, boasting an IC_{50} value of 6.33 ± 0.33 µg/mL, highlighting its potent efficacy, particularly against HeLa cells. Hence, the IC_{50} value currently obtained for QI gall extract against U2OS cells (27.19 µg/mL) is consistent with the range of 6.33 to 99 µg/mL reported for various cancer cell lines.

Nevertheless, when comparing groups with similar treatment times, no significant differences in viable cells were identified between cisplatin and QI gall extract at 24 h (p = 1.00), 48 h (p = 0.972), and 72 h (p = 0.761). It implies that the effects of cisplatin and QI gall extract are similar across those specific time intervals, indicating a comparable outcome at those specific time intervals.

To support the results from the cytotoxicity study, a phase-contrast inverted microscope was used to observe the morphological changes of the U2OS cells before and after treatment. Microscopic examination unveiled notable alterations indicative of apoptosis, including cell shrinkage and chromatin condensation (Elmore et al. 2013; Power et al. 2002). Apoptosis, a vital defence mechanism against cellular damage, stress, and external stimuli, plays a pivotal role in preventing the accumulation of non-functional cells in tissues (Kiraz et al. 2016). However, cancer cells have a malfunctioning death machinery that causes them to proliferate uncontrollably (Labi & Erlacher, 2015). It is essential to consistently induce apoptosis to stop cancer cells from proliferating uncontrollably. Yet, for a comprehensive understanding, it is crucial to thoroughly evaluate other complex nuclear alterations associated with apoptosis including chromatin condensation, DNA fragmentation, and nuclear fragmentation using both light and fluorescence microscopy. Hence, further investigation will strengthen the trustworthiness of the results and provide a comprehensive understanding of the apoptotic process.

The nuclear and cellular morphology of QI gall extract-treated cells consistently exhibited similar alterations when compared to the cells treated with cisplatin. Notably, the cisplatin-treated group displayed prominent morphological alterations with numerous apoptotic bodies after 72 h of treatment. Although the morphological alterations induced by QI gall extract were not as prominent as those by cisplatin, the results still suggest that the QI extract is capable of inducing apoptosis in U2OS cells. This is based on the cellular and nuclear morphological changes observed in the cells for both treatments, which resemble the morphological features of apoptotic cells. Several common morphological characteristics of apoptosis include cell shrinking, fragmentation into membrane-bound apoptotic bodies, and quick phagocytosis by nearby cells (Elmore et al. 2013; Power et al. 2002; Kerr et al. 1972). Nevertheless, further apoptosis evaluation is required for confirmation.

CONCLUSIONS

In conclusion, a cytotoxicity effect with prominent morphological changes was detected in the U2OS cells upon treatment with QI gall extract. Cisplatin-treated cells exhibited more remarkable alterations in their overall morphology, characterised by the presence of numerous apoptotic bodies, cell shrinkage, chromatin condensation, and cell blebbing compared to the QI gall extract-treated cells after 72 h of treatment. Even so, no significant variations in viable cell counts were identified between cisplatin-treated and QI gall extract-treated groups at 24, 48, and 72 h of treatment. This implies that the effects of cisplatin and QI gall extract are similar across those specific time intervals, indicating a comparable outcome at those specific time intervals. These pilot findings warrant further preclinical investigations into the potential of QI gall extract as an alternative chemotherapeutic regimen for osteosarcoma.

Supplementary materials

Not applicable.

Author contributions

HH conceived and designed the project and drafted the manuscript. RSSRS and MZK performed experiments, and data analysis. HH, RSSRS and MZK revised the article, gave final approval of the version, and agreed to all aspects of the work.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

All materials are available via the corresponding authors.

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Conflict of interest

The authors declare that they have no competing interests.

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