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Research Article

6,3'-dimethoxy flavonol: Evidence-based insights into anti-proliferative and apoptotic effect on osteosarcoma cells

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Abstract: Osteosarcoma, a cancer predominantly affecting children and teenagers, has shown limited improvements in long-term survival rates despite advances in treatment, necessitating new therapeutic approaches. Natural compounds, particularly flavonoids, are being investigated for their anti-cancer properties due to their ability to modulate signaling pathways and induce apoptosis in cancer cells. This study evaluates the anti-carcinogenic effects of 6,3'-dimethoxy flavonol on MG-63 osteosarcoma cells, a *p53*-null model suitable for testing novel therapies, using various assays. Cell viability and proliferation were measured via MTT assay, showing dose dependent inhibition with an IC_{50} of 221.017 µg/ml at 48 hours. Phase-contrast microscopy revealed morphological changes consistent with apoptosis, including cell shrinkage, reduced density and cytoplasmic blebbing. Wound healing assays demonstrated significant inhibition of cell migration at 100 µg/ml and 234.12 µg/ml, highlighting its anti-metastatic potential. Acridine orange/ethidium bromide (AO/EB) staining confirmed apoptotic cell death. Real-time PCR analysis revealed an increased (Bax/Bcl-2) ratio and upregulation of *p53* expression, indicating activation of the intrinsic apoptotic pathway. These findings demonstrate that 6,3'-dimethoxy flavonol effectively induces apoptosis and inhibits migration in MG-63 cells by modulating apoptotic markers and signaling pathways. The results suggest its potential as a therapeutic agent for osteosarcoma. Future studies should explore its*invivo* efficacy, possible synergistic effects in combination therapies, and its mechanisms in *p53*-positive cell lines. This evidence underscores the promise of flavonoid based interventions in cancer treatment.

Özet: Çoğunlukla çocukları ve gençleri etkileyen bir kanser türü olan osteosarkom, tedavi alanındaki gelişmelere rağmen uzun süreli sağkalım oranlarında sınırlı iyileşmeler göstermiş, bu durum da yeni tedavi yaklaşımlarını gerekli kılmıştır. Doğal bileşikler, özellikle de flavonoidler, kanser hücrelerinde sinyal yollarını düzenleme ve apoptozu başlatma kabiliyetlerine bağlı olarak kanser karşıtı özellikleri açısından araştırılmaktadır. Bu çalışmada, 6,3'-dimetoksi flavonolün yeni tedavilerin test edilmesine uygun bir model olan MG-63 osteosarkom hücreleri üzerindeki antikarsinojenik etkileri çeşitli analizler kullanılarak değerlendirilmiştir. MTT testi ile hücre canlılığı ve çoğalması ölçülmüş ve 48 saatte 221.017 µg/ml'lik bir IC_{50} ile doza bağlı bir inhibisyon tespit edilmiştir. Faz-kontrast mikroskopisi incelemesi, hücre büzülmesi, yoğunluğun azalması ve sitoplazmik kabarcıklanma gibi apoptozla uyumlu morfolojik değişiklikleri ortaya koymuştur. Yara iyileşme deneyleri, 100 µg/ml ve 234,12 µg/ml'de hücre göçünün önemli ölçüde inhibe edildiğini göstererek 6,3'-dimethoxy flavonolün anti-metastatik potansiyelini öne çıkarmıştır. Uygulanan Akridin turuncusu/etidyum bromür (AT/EB) boyaması apoptotik hücre ölümünü doğrulamıştır. Bax/Bcl-2 oranında artış ve *p53* ekspresyonunda artış olduğunu ortaya koyan gerçek zamanlı PCR analizi, intrinsik apoptotik yolağın aktive olduğunu göstermiştir. Bu bulgular, 6,3'-dimetoksi flavonolün apoptotik belirteçleri ve sinyal yollarını düzenleyerek MG-63 hücrelerinde apoptozu etkili bir şekilde indüklediğini ve hücre göçünü engellediğini göstermektedir. Sonuçlar, 6,3'-dimetoksi flavonolün osteosarkom için tedavi edici bir ajan olarak potansiyeli olduğunu da göstermektedir. Gelecek çalışmalarda 6,3'-dimetoksi flavonolün *in vivo* etkinliği, kombinasyon tedavilerindeki olası sinerjik etkileri ve *p53* pozitif hücre hatlarındaki mekanizmaları araştırılmalıdır. Çalışmada sunulan kanıtlar, kanser tedavisinde flavonoid bazlı müdahalelerin umut verici olduğunun altını çizmektedir.

Introduction

Cancer remains a major global health concern due to its high rate of morbidity and mortality (Singh *et al.* 2024, Bushi *et al.* 2025). Osteosarcoma, also known as osteogenic sarcoma (Joshy *et al.* 2023, Gavarraju *et al.* 2024), is a type of cancer that arises in the bones and originates from primitive bone-forming cells (Kannan *et al.* 2024, Zuvairiya *et al.* 2024, Nautiyal *et al.* 2024). Osteosarcoma, sometimes called osteogenic sarcoma that arises in the bones (Joshy *et al.* 2023, Gavarraju *et al.* 2024). It is considered as a rare malignant disease, and mainly affects children and teenagers (Gavarraju *et al.* 2024). It is an uncommon and dangerous form of bone cancer that mostly affects the body's long bones, such the arms and legs (Prithiksha & Priyadharshini *et al.* 2024, Sekar *et al.* 2025). Treatment for osteosarcoma, includingradiation, chemotherapy and possibly surgery, has advanced significantly in the modern period. Individuals with osteosarcoma are currently evaluated to have a 5-year survival rate of 60 to 70%. Among the chemotherapeutic medications used, methotrexate and ifosfamide are the most common ones, but a number of combinations and additional cytotoxic compounds, such as etoposide, have also been reported in related literature (Kansara *et al.* 2014). However, using these drugs may result in a number of problems and side effects including emesis, nausea, mouth fissures, fatigue, severe diarrhea and neutropenia. Among the acute reactions, anthracyclines may be the most notorious perpetrators, causing shortness of breath and chest pain, highlighting how these serious side effects constitute a major drawback of chemotherapy (Broder *et al.* 2008). Another problem with modern treatments is chemoresistance (Broder *et al.* 2008). In order to uncover innovative treatments for a range of malignancies, including osteosarcoma, researchers have been motivated by these therapeutic limits to start exploring new directions, such as identifying novel targets and comprehending their mechanisms. The most widely used cell lines for osteosarcoma are MG-63, which were produced from the fibroblastic or epithelial origins of young, white patients. MG-63 cells exhibit a phenotype characterized by high proliferation, rapid growth rate, and moderate differentiation, making them a widely used in vitro model for studying osteosarcoma biology and anticancer drug screening (Czekanska *et al.* 2012). This particular cell line was chosen because it is accessible, reasonably priced and useful for experimental study. Due to their aggressive proliferative nature, MG-63 cells serve as an ideal model to evaluate the anticancer potential of various therapeutic agents, including natural compounds like flavonoids, which have shown promising cytotoxic and apoptotic effects against osteosarcoma cells, which have interesting properties such as the activation of epigenetic changes and the Ubiquitin-proteasome pathway (Nabavi *et al.* 2018, Barreca *et al.* 2021, Khan *et al.* 2021, Gervasi *et al.* 2022). Because flavonols selectively engage with specific intercellular signaling pathways to promote different activities inside the cells, they have shown anti-cancer properties in numerous *invitro* and *invivo* studies. 6,3'-dimethoxy flavonol is a naturally occurring flavonoid derivative that belongs to the larger class of flavonols, which have a number of pharmacological characteristics, including anti-inflammatory, anti-cancer, and antioxidant effects. The structure of flavonols is characterized by a hydroxyl group on the 3rd carbon of the flavone backbone, while 6,3'-dimethoxy flavonol features methoxy groups at the 6 and 3' positions, which may influence its biological activity by affecting its solubility, cell permeability, and interaction with biomolecules (Fig. 1).

Like other flavonoids, flavonols are known to have anticancer properties through a number of ways, including apoptosis induction, cell growth suppression and disruption of cell signaling pathways implicated in the development of cancer. Specifically, compounds with methoxy substitutions often show enhanced bioactivity, as methylation can improve cell uptake and metabolic stability compared to their hydroxylated counterparts. The study suggests that flavonoids with methoxy groups, such as 6,3'-dimethoxy flavonol, have the potential to modulate cellular oxidative stress, induce cell cycle arrest, and inhibit key enzymes involved in tumor progression. Flavonoids with methoxy groups, such as 6,3'-dimethoxy flavonol, have the potential to modulate cellular oxidative stress, induce cell cycle arrest, and inhibit key enzymes involved in tumor progression. These actions make them promising candidates for cancer therapy (López-Lázaro *et. al.* 2009). Also, Studies showed its antioxidant activities, and role in neuropathy pain and inflammation (Nadipelly *et al.* 2018, Sayeli *et al.* 2019).

This study aims to investigate the anticancer effects of 6,3'-dimethoxy flavonol on human cancer cell lines, with a particular emphasis on how it affects gene expression, migration, and cell viability. To evaluate cytotoxicity, the MTT assay was employed, providing insights into 6,3'-dimethoxy flavonol's dose-dependent effects on cell survival. The scratch migration assay was conducted to assess the influence of 6,3'-dimethoxy flavonol (DMF) on cellular movement, a critical factor in cancer metastasis. We also analyzed gene expression changes in key regulatory genes to understand the molecular mechanisms underlying 6,3'-dimethoxy flavonol's anticancer activity.

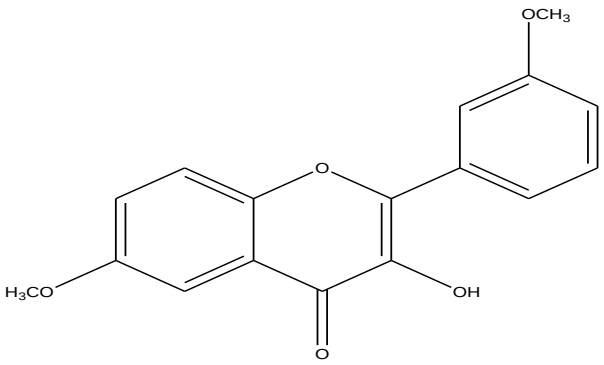

Fig. 1. The chemical structure of 6,3'-dimethoxy flavonol.

By exploring the effects of 6,3'-dimethoxy flavonol on cancer cells through multiple assays, this study sheds light on its potential as a therapeutic candidate, advancing our understanding of naturally derived compounds in cancer treatment.

Materials and Methods

Chemicals

The 6,3'-dimethoxy flavonol used in the study was procured from Research Organics, Chennai, India.

Cell line maintenance

We obtained MG-63 Cell lines from the NCCS in Pune. The cells were cultivated in DMEM and RPMI in T25 culture flasks supplemented with 10% FBS and 1% antibiotics. The cells were kept in a humidified environment with 5% CO_2 at 37°C, and were trypsinised and passaged once they had reached confluency.

Cell viability (MTT) assay

A common colorimetric method for assessing a drug's detrimental effects is the MTT assay, which measures mitochondrial activity in living cells (Fig. 2). NAD(P)H-dependent oxidoreductase enzymes, the basis of this assay, are only active in metabolically active cells. Spectrophotometry is used to assay the colour intensity, when these enzymes convert the yellow MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into soluble insoluble purple formazan crystals (Ahn *et al.* 2010). This reduction mechanism only takes place in living cells since it is dependent on continuous mitochondrial activity. The MTT assay is a trustworthy technique for determining cell viability and the cytotoxic effect of a test item since the absorbance is directly proportional to the number of viable cells. The MTT assay (Van Meerloo *et al.* 2011) was used to evaluate the cell viability of the MG-63 Cell line treated with 6,3'-dimethoxy flavonol. The assay relies on metabolically active cells reducing soluble yellow tetrazolium salt to insoluble purple formazan crystals. The osteosarcoma cell line was plated at a density of $5x10^3$ cells/well in 96-well plates, and then cultured for 3 h at 37ºC in serum-free medium to starve them. Then, two washes were performed using 100µl of serum-free media. Following a 24 h fast, cells were exposed to varying doses of 6,3'-dimethoxy flavonol (50-350 µg/ml). Following the treatment, 100µl of MTT containing DMEM (0.5 mg/ml) was added to each well after the media from the control and Rutin-treated cells had been removed.

Morphology study

We identified the ideal dosages for additional research based on the MTT experiment (IC_{50}: 234.12 µg/ml for osteosarcoma cell line and 100 and 234.12 µg/ml for MG-63 cell system) using a phase contrast microscope to evaluate changes in cell morphology. After being grown on 6 well plates, 2×10^5 cells were exposed to 6,3'-dimethoxy flavonol for a full day. Cells were given a single wash with phosphate buffer saline (PBS pH 7.4) following the removal of the medium at the end of the incubation period. A phase contrast microscope was used to view the plates (Perumal *et.al.* 2023).

Scratch wound healing assay

In six-well culture plates, osteosarcoma cells (2×10^5 cells/well) were cultivated. After making a wound with a 200µl tip, the cell monolayer was cleaned with PBS and captured on camera using an inverted microscope. Following a 24 h treatment with the IC_{50} dose and the administration of serum-free growth media to control cells, images of the damaged area were captured using the same microscope. The experiments were carried out three times for each treatment group (Felice *et al.* 2015).

Using acridine orange (AO)/ethidium bromide (EtBr) dual labelling to identify the mode of cell demise

By using the previously published AO/EtBr dual staining, the effects of 6,3'-dimethoxy flavonol (100 & 234.12 µg/ml) on osteosarcoma cell mortality were ascertained. Following a 24 h treatment with 6,3'-dimethoxy flavonol, after being collected, Ice-cold PBS was used to wash the cells. 5 µl of acridine orange (1 mg/ml) and 5 µl of EtBr (1 mg/ml) were used to re-suspend the pellets. A fluorescent microscope was then used to view the apoptotic alterations in the designated cells (Ezhilarasan *et al.* 2019).

Real Time PCR

Real-time PCR was used to measure the gene expression of apoptotic signaling molecules. Trizol Reagent (Sigma) was used in the standardized procedure to isolate total RNA. A Prime Script first strand cDNA synthesis kit (TakaRa, Japan) was used to synthesize reverse transcription cDNA from two µg of RNA. Certain primers were used to amplify the pertinent genes (Table 1). The GoTaq® qPCR Master Mix (Promega), which includes SYBR green dye and all the PCR components, was used to conduct the PCR reaction. The CFX96 PCR system (Biorad) was used to perform the real-time PCR. The comparative CT method was used to assess the results and Schmittgen & Livak's $2^{-\Delta\Delta CT}$ method was used to compute the fold change (Morrison *et al.* 1998).

Table 1. List of the primers used. Tm corresponds to Melting Temperature

Gene	Forward Primer	Reverse Primer	Tm
p53	AGGCCTTGGAACTCAAGGAT	TGAGTCAGGCCCTTCTGTCT	60
Bax	TACCTCTTCCCTTCCTTTCTCC	TCCTGGATGAAACTAGAGTGGG	58
Bcl-2	CATGTGTGTGGAGAGCGTCAAC	CAGATAGGCACCCAGGGTGAT	58
Bad	GCTGGACATTGGACTTCCTC	CTCAGCCCATCTTCTTCCAG	60
Caspase-3	GCTATTGTAGGCGGTTGT	TGTTTCCCTGAGGTTTGC	55
GAPDH	CGACCACTTTGTCAAGCTCA	CCCCTCTTCAAGGGGTCTAC	58

Statistical analysis

The collected data were analyzed using SPSS software, employing One-Way ANOVA and Student's t-test, and compared between the control and treated groups at various concentrations (100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml, and 350 µg/ml). A threshold of $p < 0.05$ was set for statistical significance, and the results were presented in triplicate as mean ± standard deviation (SD).

Abbreviations

AO/Et Br - Acridine Orange / Ethidium Bromide
Bad - Bcl-2-associated death promoter
Bax - Bcl-2-Associated X Protein
DMEM - Dulbecco's Modified Eagle Medium
FBS - Fetal Bovine Serum
IC - Inhibitory Concentration
MTT - 3-(4,3–Dimethylthizaol-2-yl)–2,5–diphenyl tetrazolium bromide Assay
MOMP - Mitochondrial Outer Membrane Permeabilization
NCCS - National Centre for Cell Sciences
PBS - Phosphate Buffered Saline
p53 - Tumor Protein
PCR - Polymerase Chain Reaction
SAR - Structural Activity Relationship

Results

6,3'-dimethoxy flavonol significantly reduces MG-63 cell viability

The cytotoxic effects of 6,3'-dimethoxy flavonol were evaluated at varying concentrations (50-350 µg/ml) using the MTT assay, with absorbance values used to calculate percentage cell viability. Following a 24 h incubation period, the outcomes were contrasted with untreated control cells (Fig. 2). In Table 2, the cytotoxicity was expressed as mean ± SD, and indicate a dose-dependent cytotoxic effect of 6,3'-dimethoxy flavonol. The control showed 100% viability, while at 50 µg/ml, viability was 95.3% ± 2.9 with minimal variability. As the concentration increased, viability decreased: 80.5% ± 2.8 (100 µg/ml), 73.9% ± 5 (150 µg/ml), 56.7% ± 9.8 (200 µg/ml), 36.1% ± 5.4 (250 µg/ml), 21.3% ± 4.5 (300 µg/ml), and 15.7% ± 3.8 (350 µg/ml). The increasing SD at higher concentrations suggests greater variability in cellular response.

Table 2. Dose-dependent cytotoxic effect of 6,3'-dimethoxy flavonol.

Test Compound	Percentage Viability
Control	100
6,3'-dimethoxy flavonol (50 µg/ml)	95.3 ± 2.9
6,3'-dimethoxy flavonol (100 µg/ml)	80.5 ± 2.8
6,3'-dimethoxy flavonol (150 µg/ml)	73.9 ± 5
6,3'-dimethoxy flavonol (200 µg/ml)	56.7 ± 9.8
6,3'-dimethoxy flavonol (250 µg/ml)	36.1 ± 5.4
6,3'-dimethoxy flavonol (300 µg/ml)	21.3 ± 4.5
6,3'-dimethoxy flavonol (350 µg/ml)	15.7 ± 3.8

The data shown as mean ± SD ($n = 3$).

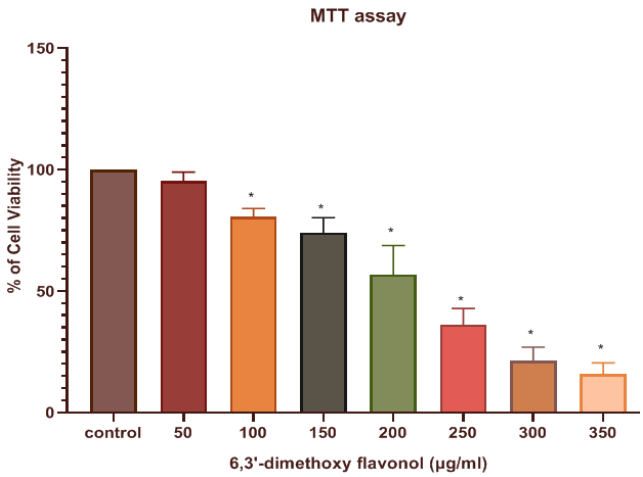

Fig. 2. The cytotoxic effects of 6,3'-dimethoxy flavonol on osteosarcoma (MG-63) cells. Cells were treated with 6,3'-dimethoxy flavonol (50 -350 µg/ml) for 24 hours.

Morphological study of 6,3'-dimethoxy flavonol

Cell were treated with 6,3'-dimethoxy flavonol (100 µg/ml, 234.12 µg/ml & 300 µg/ml) for 24 hours. The cell population significantly decreased after treatment, indicating strong cytotoxic effects. The cells showed signs of apoptosis, including cell shrinkage and blebbing of the cytoplasmic membrane. These alterations indicate cellular damage and stress. An inverted phase-contrast microscope set to ×10 magnification was used to monitor and record these changes (Fig. 3).

6,3'-dimethoxy flavonol decreases wound closure in MG-63 cells

Using the scratch wound healing assay, the effect of 6,3'-dimethoxy flavonol on migration were assessed. Following injury to a cell, migration experiment was conducted 24 h later with and without the administration of 6,3'-dimethoxy flavonol (100 and 234.12 µg/ml).

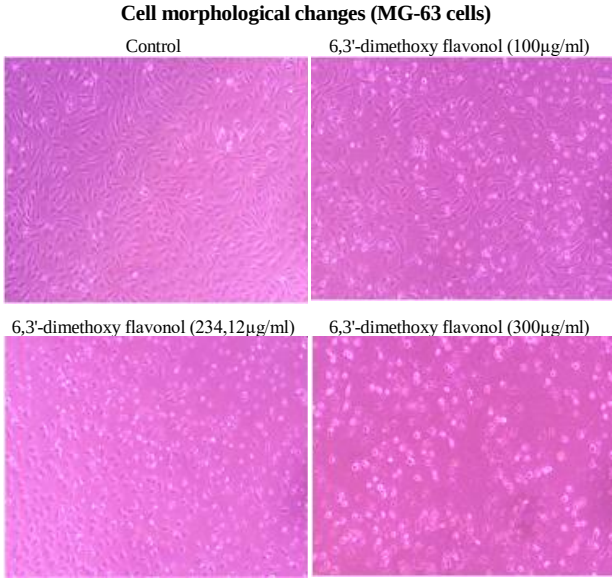

Fig. 3. Effect of 6,3'-dimethoxy flavonol (100 µg/ml, 234.12 µg/ml & 300 µg/ml) on cell morphology.

A uniform scratch was introduced into a confluent cell monolayer, and the wound closure was monitored after treatment. In comparison to the untreated control, both doses markedly reduced cell migration, and a dose-dependent delay in wound closure was noted. These results indicate that 6,3'-dimethoxy flavonol effectively impairs the migratory ability of MG-63 cells, highlighting its potential to suppress metastatic behavior in osteosarcoma (Felice *et al.* 2015) (Fig. 4).

Acridine Orange/Ethidium bromide (AO/EB) staining

Ethidium Bromide (EB) only stains dead cells with compromised membrane integrity, whereas Acridine Orange (AO) stains both living and dead cells. When using this technique to identify apoptosis, the nuclei of live cells show consistent green fluorescence, whereas the nuclei of early apoptotic cells display bright green patches due to chromatin condensation. On the other hand, late apoptotic cells with broken or constricted nuclei and lost membrane integrity appear orange due to EB binding. Different stages of cell death can be easily distinguished. Red-stained necrotic cells have a nucleus structure similar to that of living cells, but they lack condensed chromatin. Following treatment with 6,3'-dimethoxy flavonol (100 and 234.12 µg/ml), the quantity of green-fluorescent cells, which indicates a reduction in viable cells, decreased in a dose-dependent manner. The presence of early apoptotic cells with fragmented DNA was indicated by the emergence of high-intensity green spots on nuclei. Compared to the control group, the percentage of orange-stained necrotic or late apoptotic cells was significantly higher (X% ± SD, $p < 0.05$) in the 6,3'-dimethoxy flavonol-treated group (Fig. 5).

Gene expression profiles induced by 6,3'-dimethoxy flavonol

Gene expression analysis of MG-63 cells treated with 6,3'-dimethoxy flavonol at 100 µg/ml and 234.12 µg/ml revealed significant modulation of key apoptotic markers. The treatment downregulated Bcl-2, an anti-apoptotic gene, while upregulating Bax, a pro-apoptotic gene, thereby increasing the Bax/Bcl-2 ratio and promoting apoptosis. Additionally, the expression of *p53*, a tumor suppressor, was enhanced, indicating its role in regulating apoptotic pathways.

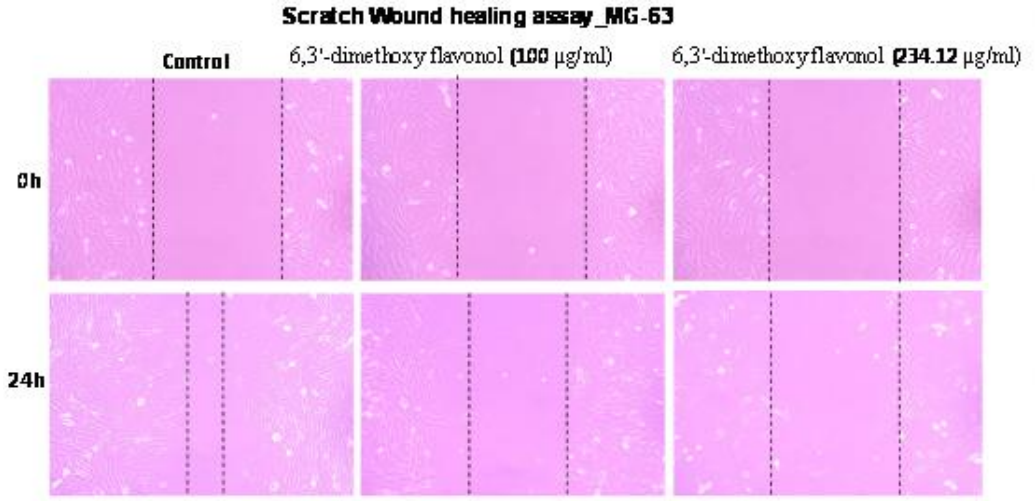

Fig. 4. MG-63 Cells were injured and cell migration assay with and without treatment of 6,3'-dimethoxy flavonol (100 and 234.12 µg/ml) was performed at 24 h. Images were obtained using an inverted phase contrast microscope.

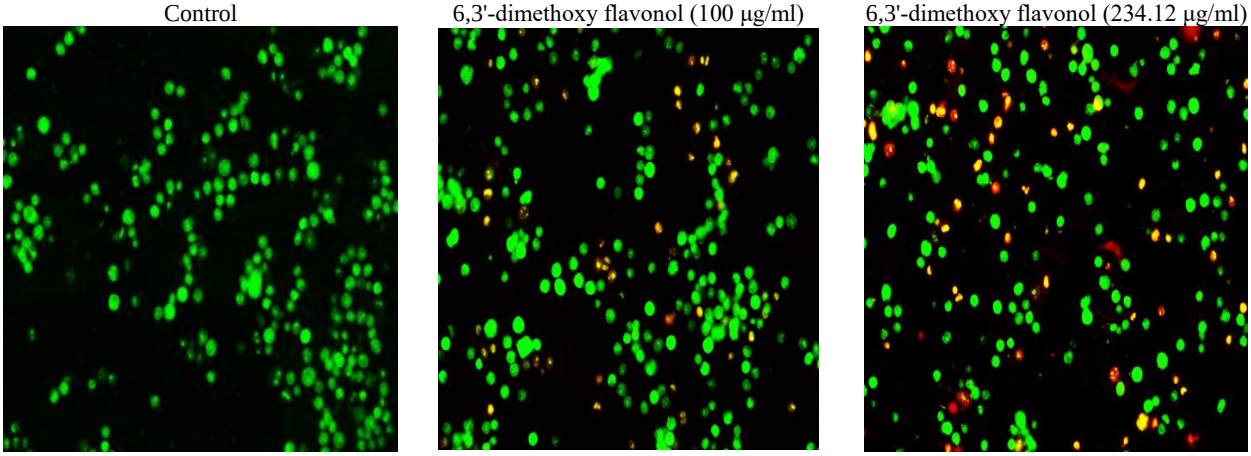

Fig. 5. Effect of 6,3'-dimethoxy flavonol (100µg/ml & 234.12 µg/ml) on nuclear morphology of MG-63 Cell line.

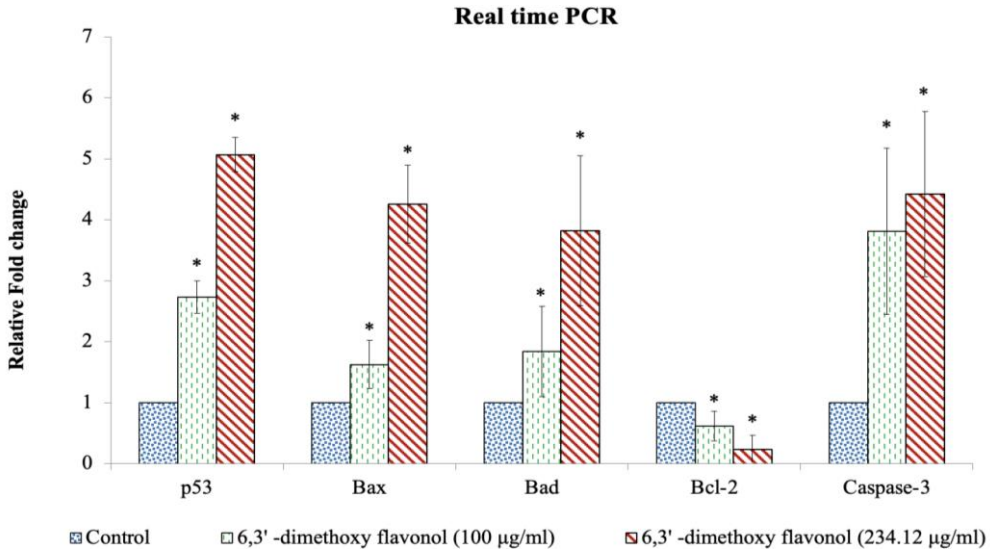

Fig. 6. Effect of 6,3'-dimethoxy flavonol (100 and 234.12 µg/ml) on pro-apoptotic gene (Bax, Bad, Bcl-2, and Caspase-3) expressions in osteosarcoma cell line. Target gene expression is normalized to GAPDH mRNA expression and the results are expressed as fold change from the control.

These findings align with the growing body of evidence that flavonoids, including flavonols, exhibit anti-cancer properties by targeting key signaling pathways involved in cell survival and apoptosis. The specificity of 6,3'-dimethoxy flavonol in modulating these pathways at effective concentrations (100 µg/ml and 234.12 µg/ml) highlights its potential therapeutic application. Furthermore, the dose-dependent response observed suggests that higher concentrations mayelicit a stronger apoptotic response, providing insights into its pharmacological activity (Fig. 6). Table 3 illustrates apoptotic marker expression (*p53*, Bax, Bad, Bcl-2, Caspase-3) under different conditions. In the experimental setup, *p53* increased (2.728 to 5.068), accompanied by elevated Bax, Bad, and Caspase-3 levels, while Bcl-2 decreased (0.6182 to 0.2377), indicating apoptosis. The control showed minimal marker expression, with slight increases under experimental conditions. Overall, *p53* activation enhanced pro-apoptotic markers and suppressed Bcl-2, reinforcing an apoptosis-driven response.

Table 3. Apoptotic marker expressions.

Gene	6,3'-dimethoxy flavonol (100 µg/ml)	6,3'-dimethoxy flavonol (234.12 µg/ml)
p53	2.728 ± 0.26	5.06 ± 0.27
Bax	1.62 ± 0.39	4.25 ± 0.63
Bad	1.84 ± 0.74	3.82 ± 1.23
Bcl-2	0.61 ± 0.24	0.237 ± 0.232
Caspase-3	3.80 ± 1.36	4.41 ± 1.35

Discussion

Osteosarcoma, a rare cancer worldwide, primarily affects children and teenagers. Even though new treatment approaches for osteosarcoma have improved the prognosis, the long-term survival rate for the disease has remained stagnant. The development of new, creative treatments is necessary to improve the long-term prognosis for patients with osteosarcoma. Common cancer therapies include chemotherapy, surgery, and radiation therapy, each of which has a number of disadvantages. Nowadays, the majority of anticancer medications come from natural sources. Natural compounds inhibit cell signaling pathways and trigger cellular apoptosis (Baskar *et al.* 2012) to produce their anticancer effects. In this study, we examined the anti-carcinogenic properties of 6,3'-dimethoxy flavonol on human osteosarcoma cell line. MG-63 cells make a great model cell line for developing novel therapeutic treatments for patients with osteosarcoma (Hashem *et al.* 2022). The current study's findings are consistent with other research showing flavonol has a variety of biological effects, like anti-inflammatory, anti-oxidative, anti-proliferative, and anticoagulative properties (Lee *et al.* 2019, Kim *et al.* 2008). No research has so far evaluated for 6,3'-dimethoxy flavonol's *in vitro* anti-cancer activities in osteosarcoma. Thus, the current study's goal was to look into 6,3'-dimethoxy flavonol's anti-cancer effects in osteosarcoma. In order to do this, the impact of 6,3'-dimethoxy flavonol on osteosarcoma cell viability and proliferation was assessed, and the signaling pathways that mediate these effects were examined.

6,3'-dimethoxy flavonol decreased cell proliferation in the MG-63 cell line in a dose-dependent way in our experiments. After 24 and 48 h of treatment, the IC_{50} was reported to be 221.017 µg/ml (Fig. 2). This finding suggests that 6,3'-dimethoxy flavonol is primarily cytotoxic to cancerous cells. Following the cytotoxicity assessment of 6,3'-dimethoxy flavonol, its effect on MG-63 cell morphology was analyzed using an inverted phase-contrast microscope. The impact of 6,3'-dimethoxy flavonol on cell morphology is thoroughly analysed by using different concentrations (100 µg/ml, 234.12 µg/ml,

and 300 µg/ml). The findings showed that cells treated with 6,3'-dimethoxy flavonol showed significant morphological changes in comparison to untreated cells. These alterations included shrinkage, reduced cell density, and dose-dependent cytoplasmic blebbing all of which are traits of apoptotic cells (Fig. 3).

The observed inhibition of MG-63 cell migration at both concentrations (100 and 234.12 µg/ml) suggests that this compound significantly impairs the migratory ability of cancer cells, which is a critical step in the metastatic cascade (Van *et al.* 2011). Cell migration plays a pivotal role in cancer progression and metastasis, enabling tumor cells to invade surrounding tissues and establish secondary tumors. The dose-dependent inhibition of wound closure indicates that 6,3'-dimethoxy flavonol interferes with cellular processes essential for migration. The delayed wound closure observed in treated groups further underscores the compound's potential to suppress osteosarcoma metastasis. The dose-dependent nature of inhibition indicates that the higher concentration of 6,3'-dimethoxy flavonol (234.12 µg/ml) exerts a stronger inhibitory effect, aligning with the compound's bioactivity profile. These results are particularly significant as osteosarcoma is known for its aggressive nature and high propensity for metastasis, especially to the bone cells. By targeting the migratory capacity of MG-63 cells, 6,3'-dimethoxy flavonol could serve as a valuable therapeutic agent in reducing metastatic risk. 6,3'-dimethoxy flavonol demonstrates a strong anti-migratory effect on MG-63 cells, supporting its potential role in managing metastatic osteosarcoma (Fig. 4).

Apoptotic cell death is characterized by nuclear fragmentation and cell shrinkage. Using dyes that bind fluorescent DNA, like AO/EB, morphological alterations and apoptosis-induced cell death were detected. Acridine orange is a necessary dye that can be used to stain both living and dead cells. The fluorescent intercalating chemical ethidium bromide, which creates linkages between DNA bases, only stains cells with damaged cytoplasmic membranes, giving their nucleus a red hue when exposed to UV light.

While late apoptotic cells contain contracted and broken orange chromatin (Renvoize *et al.* 1988, Liu *et al.* 2015), early apoptotic cells have condensed or fragmented chromatin with a bright green nucleus. In our study, green cells were uniformly distributed throughout the control group or untreated cells. According to the applied stainings, cells treated with 6,3'-dimethoxy flavonol exhibited more orange to red nuclei and perinuclear bright green patches than the control group. This suggests that 6,3'-dimethoxy flavonol increases both early and late apoptotic cells at doses of 100 and 234.12 µg/ml. Furthermore, the treatment enhances apoptosis by increasing the levels of active cleaved caspase-3 and cleaved poly-ADP ribose polymerase (PARP), both of which are key markers of programmed cell death. Additionally, it elevates the Bax/Bcl-2 ratio, promoting a pro-apoptotic balance that favors cell death in cancerous cells. (Fig. 5)

The gene expression of pro-apoptotic and anti-apoptotic cells were evaluated with the help of real-time PCR. The results showed that 6,3'-dimethoxy flavonol can cause MG-63 osteosarcoma cells to undergo apoptosis by altering important apoptotic indicators. A disruption of mitochondrial membrane integrity is suggested by the observed downregulation of the anti-apoptotic protein Bcl-2 and the overexpression of the pro-apoptotic protein Bax, a characteristic of the intrinsic apoptotic pathway (Fig. 6). The elevated Bax/Bcl-2 ratio, which is generally acknowledged as a crucial element of mitochondrial outer MOMP, which results in the release of cytochrome c and the subsequent activation of caspases, lends additional credence to this. The intrinsic pathway's involvement is further shown by the overexpression of *p53*, a tumor suppressor gene that is essential for controlling cell cycle arrest and apoptosis. It is known that *p53* represses anti-apoptotic genes like Bcl-2 while transcriptionally activating pro-apoptotic genes like Bax. It may function, at least in part, by activating *p53*-dependent apoptotic signaling, as evidenced by its enhanced expression in response to therapy. The enhanced expression of *p53* may also indicate the ability of 6,3'-dimethoxy flavonol to induce DNA damage or stress responses, triggering apoptosis as a protective mechanism against malignant transformation. These results are consistent with the increasing amount of data showing that flavonoids, notably flavonols, have anti-cancer effects by focusing on important signaling pathways that are involved in apoptosis and cell survival. The specificity of 6,3'-dimethoxy flavonol in modulating these pathways at effective concentrations (100 µg/ml and 234.12 µg/ml) highlights its potential therapeutic application. Furthermore, the dose-dependent response observed suggests that higher concentrations may elicit a stronger apoptotic response, providing insights into its pharmacological activity. Further exploration of its therapeutic applications could provide new avenues for combating this aggressive cancer.

Future research should expand the investigation of 6,3'-dimethoxy flavonol to other osteosarcoma cell lines, such as SaOS-2 and U2OS, to validate its broad anticancer efficacy. To evaluate its pharmacokinetics, biodistribution, and capacity to impede tumor growth and metastasis in a physiological setting, *in vivo* research employing animal models is crucial. Exploring combination therapy with standard chemotherapeutic agents like doxorubicin could enhance efficacy while minimizing toxicity. Elucidating molecular mechanisms beyond *p53*-dependent pathways, particularly in *p53*-positive cell lines, will provide a deeper understanding of its anticancer action. Structure-activity relationship (SAR) studies could optimize its bioactivity, while developing nanoparticle-based delivery systems may improve its bioavailability and reduce systemic toxicity. Additionally, the exploration of its immunomodulatory

effects on the tumor microenvironment, such as modulating immune cell infiltration and cytokine production, may reveal synergistic therapeutic potential. Clinical translational studies, including early-phase clinical trials, will be crucial to establish its safety and efficacy in osteosarcoma patients. High-throughput genomic and proteomic profiling could further identify biomarkers for patient stratification, enabling personalized treatment approaches. These future directions could position 6,3'-dimethoxy flavonol as a promising candidate in the development of innovative osteosarcoma therapies.

Conclusion

This study highlights the therapeutic potential of 6,3'-dimethoxy flavonol, a bioactive molecule with strong antiproliferative effects on MG-63 osteosarcoma cells. Morphological analysis post-treatment revealed hallmark signs of apoptosis, such as decreased density and cell shrinkage, indicating a successful interference with cancer cell survival. In a wound-healing study, the molecule also demonstrated anti-migratory qualities, suggesting that it may prevent metastasis. The Bax/Bcl-2 ratio shifted towards apoptosis at the molecular level, as demonstrated by RT-PCR analysis, which revealed elevated (anti-apoptotic) expression. Its potential as a selective anticancer drug is highlighted by this dual mechanism. The study does, however, admit many limitations, such as its exclusive emphasis on a particular cell line. To confirm efficacy and safety, further osteosarcoma models and *in vivo* investigations are essential for future study. In summary, 6,3'-dimethoxy flavonol exhibits strong anticancer properties, providing a potentially effective therapy option for osteosarcoma.

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