Saudi Journal of Biological Sciences 28 (2021) 3641–3649

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Rhaponticin suppresses osteosarcoma through the inhibition of PI3K-Akt-mTOR pathway

Suresh Mickymaray [a], Faiz Abdulaziz Alfaiz [a], Anand Paramasivam [b], Vishnu Priya Veeraraghavan [c], Nanthini Devi Periadurai [d], Krishna Mohan Surapaneni [e], Guangfeng Niu [f,g,*]

[a] Department of Biology, College of Science, Al-Zulfi, Majmaah University, Riyadh Region, Majmaah 11952, Saudi Arabia
[b] Department of Basic Medical Sciences, College of Dentistry, Al-Zulfi, Majmaah University, Riyadh Region, Majmaah 11952, Saudi Arabia
[c] Department of Biochemistry, Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai 600 077, India
[d] Departments of Microbiology, Molecular Virology and Hospital Infection Control, Panimalar Medical College Hospital & Research Institute, Varadharajapuram, Poonamallee, Chennai 600 123, India
[e] Department of Biochemistry, Panimalar Medical College Hospital & Research Institute, Varadharajapuram, Poonamallee, Chennai 600 123, India
[f] Department of Orthopaedics, Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University, No. 324 Jingwu Weiqi Road, Jinan, Shandong 250021, China
[g] Department of Orthopaedics, Shandong Provincial Hospital Affiliated to Shandong First Medical University, No. 324 Jingwu Weiqi Road, Jinan, Shandong 250021, China

ARTICLE INFO

Article history:
Received 19 April 2021
Revised 1 May 2021
Accepted 2 May 2021
Available online 8 May 2021

Keywords:
Rhaponticin
Osteosarcoma
Apoptosis
PI3K-Akt-mTOR pathway
MG-63 cells

ABSTRACT

Osteosarcoma is the frequent pediatric bone cancer where pediatric osteosarcoma incidences are more than 10% within the population. Most of the patients with osteosarcoma fall within the age of 15–30 years. Therefore, in this research, we examined the anticancer effect of Rhaponticin against the human osteosarcoma (MG-63) cells. The cytotoxicity of Rhaponticin on the MC3T3-E1 and MG-63 cells was examined through the MTT assay. The intracellular ROS accumulation, cell nuclear morphological alterations, apoptotic cell death and nuclear damages, and MMP status of Rhaponticin administered MG-63 cells were inspected by fluorescent staining techniques. The cell migration was assessed through scratch assay. The mRNA expressions of PI3K-Akt-mTOR signaling proteins were studied by RT-PCR analysis. Rhaponticin showed potent cytotoxicity, substantially inhibited the MG-63 cell growth, and displayed morphological alterations. However, rhaponticin did not affect the MC3T3-E1 cell viability. Rhaponticin administered MG-63 cells demonstrated augmented intracellular ROS accretion, weakened MMP, increased nuclear damages, and increased apoptosis. Rhaponticin effectively down-regulated the PI3K-Akt-mTOR signaling cascade in the MG-63 cells. These outcomes proved that the Rhaponticin can be a hopeful chemotherapeutic agent in the future to treat human osteosarcoma.

1. Introduction

Osteosarcoma or bone cancer is one of the frequent types of cancers worldwide that mainly affects children and adolescents (Meazza and Scanagatta, 2016). Metastasis of bone cancer leads to the augmented death rate, joint pains, elevated calcium depositions, joint disabilities, spinal cord compression, and other relevant pathological complications, which results in the deprived life quality of patients. These tumors possess sturdy local intrusiveness, and they can undoubtedly metastasize at the early phase and have a deprived prognosis (Yu et al., 2019). Osteosarcoma primarily targets metaphyseal sites of the long bones like a proximal tibia and distal femur attributes for nearly half of total osteosarcoma incidences. Most osteosarcoma patients are noted between 15 and 30 years (Chou and Gorlick, 2006; Longhi et al., 2006). Although numerous cancer treatment advancements have improved the survival rate of patients, this type of cancer is still regarded as one of the deadliest today. In such cases, the metastasis pattern shows it is highly complicated to treat with the currently existing treatment approaches

* Corresponding authors at: Department of Orthopaedics, Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University, No. 324 Jingwu Weiqi Road, Jinan, Shandong 250021, China (G. Niu).
E-mail address: niu_guangfeng@sina.com (G. Niu).

Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.05.006
1319-562X/

are often linked with many side effects (Fornetti et al., 2018; Ke et al., 2019).

Clinical advancements like chemotherapy and surgery lead to the augmented 5-year survival rate of osteosarcoma victims, but nearly 50% of patients have still experienced localized reappearance (Harrison et al., 2018; Alsagaby et al., 2020). There is no successful treatment for metastatic and recurring osteosarcoma (Kager et al., 2017). Usually, the administration of chemotherapy drugs like cyclophosphamide, methotrexate, cisplatin, ifosfamide, and methotrexate is an extensively utilized approach to treat osteosarcoma. A high dose of chemotherapy drugs is currently utilized for osteosarcoma treatment, but it is linked to higher side effects, restricting their usage (Ferrari et al., 2018). Ionizing radiation therapy is also an imperative approach to treat osteosarcoma, and it demonstrates marginal damages to the nearby normal cells. However, radiotherapy as a local treatment is often restricted because of the radioresistance (Feng et al., 2016). The chemotherapeutic approach may result in drug resistance and numerous deleterious outcomes like drug-induced cytotoxicity to the normal cells (Friebele et al., 2015; Wang et al., 2017; Mickymaray, 2019a,b; Chen et al., 2021). Hence, the need for substitute approaches to treat osteosarcoma has highly emerged.

The tumor microenvironment is generally regarded as an essential and imperative factor of all cancers, and the cells penetrating such microenvironment are the sources of pro-inflammatory regulators. Interleukin (IL)-1β has pleiotropic upshots on several cancer cells, especially mediating pro-oncogenic transcription factors like NF-κB (Dmitrieva et al., 2016). The PI3K/Akt cascade is a vital pathway that controls numerous cellular mechanisms like metabolism and cell proliferation. The deregulation of the PI3K/Akt cascade is generally interconnected with malignancy and the progression of cancers (Yu and Cui, 2016; Chen et al., 2021).

Akt (protein kinase-B) is a crucial mediator situated downstream of the PI3K cascade. Mammalian target of rapamycin (mTOR), a type of threonine/serine protein kinase, from the phosphatidylinositol 3-kinase family (Hu et al., 2016). The PI3K-Akt-mTOR cascade performs critical functions in the cells, mediating numerous cellular mechanisms like multiplication, survival, angiogenesis, autophagy, and apoptosis. Several ailments like autoimmune disease, neuropathy, and cancers are caused by the deregulated PI3K-Akt-mTOR cascade (Zhang et al., 2015). PI3K-Akt-mTOR cascade is also associated with many essential processes of cell growth. It was reported that the PI3K/Akt cascade is recurrently hyper-activated in osteosarcoma and donates to tumorigenesis, multiplication, invasion, and progression of tumor cells. Therefore, the PI3K/Akt cascade suppression could avert cancer development (Xu et al., 2014). So an explicit recognition of the PI3K-Akt-mTOR cascade serves to the cancer drug discovery (Wang et al., 2016).

Rhaponticin is a primary bioactive compound present in the *Rheum rhaponticum* that is known for its numerous pharmacological activities. Rhaponticin possessed potent anti-diabetic (Chen et al., 2009; Choi et al., 2006), anti-tumor (Sun and Zhao, 2012), estrogenic (Wober et al., 2007), anti-angiogenic (Kim and Ma, 2018), antioxidant (Zhang et al., 2007), and anti-inflammatory (Kolodziejczyk-Czepas and Czepas, 2019) activities. Rhaponticin demonstrated potential anticancer activity (Kim and Ma, 2018) and ameliorated retinal oxidative stress and inflammation (Shi et al., 2020). Nonetheless, the anticancer activity of Rhaponticin against osteosarcoma was not explored yet. Therefore, in this investigation, we planned to scrutinize the anti-proliferative and apoptosis-triggering potentials of Rhaponticin against the MG-63 cells via the suppression of the PI3K-Akt-mTOR pathway.

2. Materials and methods

2.1. Chemicals

Rhaponticin, 2, 7-diacetyl dichlorofluorescein (DCFH-DA), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), 4′6-diamidino-2-phenylin dole (DAPI), propidium iodide (PI), and Rhodamine-124 (Rh-123) were procured from Sigma Aldrich, USA. All test kits were obtained from Promega, Wisconsin, USA, and Origene, Maryland, USA.

2.2. Collection and maintenance of MG-63 cells

The MG-63 cells and mouse-derived normal osteoblast (MC3T3-E1) cells were acquired from American type culture collection (ATCC, USA) and sustained in the DMEM medium, which consisting of FBS (10%) and antimycotic (1%) mixture. Cells were sustained at 37 °C with 5% CO_2 in an incubator.

2.3. MTT cytotoxicity assay on osteosarcoma and normal osteoblast cells

The cytotoxicity of Rhaponticin on the MG-63 cells and normal osteoblast (MC3T3-E1) cells was examined through the MTT assay (Mosmann, 1983). Briefly, 2×10^4 cells were loaded onto the 96-well plate with DMEM medium and sustained in 5% of CO_2 at 37 °C for 24 h. Rhaponticin was prepared in DMSO, and diverse concentrations of Rhaponticin (5–100 μM) were administered to each well. The same volume of DMSO was utilized for control cells. After 48 h, the medium was replaced with fresh medium in each well along with 50 μl of MTT solution. After 4 h of incubation, the medium was reinstated with 100 μl of DMSO to solubilize formazan crystals. UV absorbance was taken at 570 nm on a microplate reader to determine the percentages of cell viability and IC_{50} value calculation.

For the morphological examination, MG-63 cells were loaded in a 96 – well plate and then supplemented with 25 μM and 50 μM of Rhaponticin for 24 h. Followed by the incubation, cells were investigated using an optical microscope to distinguish morphological alterations.

2.4. Measurement of ROS production via DCFH-DA staining

MG-63 cells were cultured in 6-well plates at 1×10^6 cells/well and sustained for 24 h at 37 °C. Then medium substituted with a fresh one supplemented with 25 μM and 50 μM of Rhaponticin and again incubated at 37 °C for 24 h. Cells were then cleansed with buffered saline, 10 μg of DCHF-DA stain was added to each well and maintained for 30mins in the dark. In the end, cells were cleaned with PBS and examined using a fluorescent microscope. The fluorescent strength of captured images was analyzed with ImageJ software.

2.5. Measurement of mitochondrial membrane potential (MMP)

The MMP of Rhaponticin administered MG-63 cells were studied through Rh-123 staining. MG-63 cells grown in a 6-well plate then supplemented with 25 μM and 50 μM of Rhaponticin for 24 h. Then cells were cleansed with buffered saline then stained with Rh-123 (10 μg/ml) for 30 min at 37 °C. Then the MMP status of Rhaponticin administered MG-63 cells were examined using a fluorescent microscope.

2.6. Acridine orange/ethidium bromide (AO/EtBr) dual staining assay

The apoptosis stimulation by Rhaponticin on MG-63 cells were examined through dual staining. Briefly, MG-63 cells were grown on a 6-well plate with DMEM medium in a moistened CO_2 (5%) incubator at 37 °C for 24hrs. Cells were then cleansed with buffered saline, and cell nuclei were counterstained with AO/EB mixture (1:1, 100 μg/ml) for 15 min. Lastly, cells were cleaned with saline, inspected, and photographed using a fluorescent microscope. The captured images were analyzed using ImageJ software.

2.7. PI staining assay

The apoptotic cell death in the Rhaponticin administered MG-63 cells were studied using the PI staining. Cells were cultured in a 6-well plate and administered with 25 μM and 50 μM of Rhaponticin for 24 h. The cells were pelleted, washed with buffered saline, and stained with the 10 μg/ml of PI for 2 h in the dark. Lastly, the cells were examined using a fluorescent microscope for apoptosis detection.

2.8. DAPI staining assay

The cell nuclear morphological alterations stimulated by Rhaponticin on MG-63 cells were examined through DAPI staining. For this, MG-63 cells were grown in a 6-well plate along with the 25 μM and 50 μM of Rhaponticin for 24 h at 37 °C in a 5% CO_2 incubator. Then cells were cleansed with buffered saline and stained with 200 μg/mL of DAPI. After that, cells were examined using a fluorescent microscope to detect the chromatin remodeling or nucleus condensation induced by Rhaponticin. Then images were analyzed using ImageJ software.

2.9. Cell adhesion study

The osteosarcoma MG-63 cells were cultured on a gelatin-coated culture dish and then administered with 15 μM and 20 μM of Rhaponticin and maintained for 24 h in a 5% CO_2 incubator at 37 °C. The cells were then cleansed with saline and stained with trypan blue to detect and distinguish the viable and dead cells. The dead cells get stained with trypan blue, and viable cells remain unstained. Cells were monitored under the microscope, and viable and dead cell numbers were noted.

2.10. Scratch assay

MG-63 cells were grown in a 6-well plate, and then scratch was made after attaining 80% confluency with the aid of 200 μl micro tip in each well. After scratching, the detached cells were eliminated with saline. The undetached cells were then supplemented with 25 μM and 50 μM of Rhaponticin and incubated for 24 h. Then these cells were cleansed with PBS with gentle shaking for 30 s. The scratch closure was investigated under the microscope, and the image of 0hr and 24 h was observed through a microscope.

2.11. Real-time polymerase chain reaction analysis (RT-PCR)

Total RNA from both control and Rhaponticin administered MG-63 cells were extracted using a commercial kit as per the manufacturer's protocol (Promega, Wisconsin, USA). The cDNA was constructed using extracted RNA from MG-63 cells using a commercial kit following the protocols of the manufacturer (Origene, Maryland, USA). RT-PCR was then executed through a commercial PCR kit following the manufacturer's guidelines (Origene, Maryland, USA). The primer sequences for the PI3K, mTOR, Akt, and internal control β-actin were illustrated in Table 1.

Table 1
Primer sequences for RT-PCR.

PI3K	Upstream	5′-ACACCACGGTTTGGACTATGG-3′
	Downstream	5′-GGCTACAGTAGTGGGCTTGG-3′
Akt	Upstream	5′-TGGGTCAAGGAACAGAAGCA-3′
	Downstream	5′-TCACACTGACCACTGACACA-3′
mTOR	Upstream	5′-CGGGACTCTTTACACTGCG-3′
	Downstream	5′-CCTTCAGGCTCAACCAACA-3′
β-actin	Upstream	5′-AACAAGAGGCCACACAAATAGG-3′
	Downstream	5′-CAGATGTACAGGAATAGCCTCCG-3′

All tests were executed in triplicate, and the outcomes were displayed as relative mRNA expressions (Fold change).

2.12. Statistical analysis

Data were inspected using SPSS software (ver.18). The results are portrayed as mean ± SD of three measurements. One-way ANOVA and, successively, the DMRT test was performed to investigate the statistical differences between groups. *P*-value of less than 0.05 was significant.

3. Results

3.1. Effect of Rhaponticin on the cell morphology and viability of MG-63 cells

The cytotoxic potential of Rhaponticin against the MG-63 cells were scrutinized through the MTT assay. Fig. 1A revealed that the Rhaponticin repressed the growth of MG-63 cells where the highest dosage of Rhaponticin demonstrated increased cytotoxicity. Rhaponticin was tested in diverse doses, i.e., 5–100 μM; among them, 25 μM of Rhaponticin exhibited 50% growth inhibition on MG-63 cells. Thus 25 μM of Rhaponticin was chosen as IC_{50}; eventually, 25 μM and 50 μM of Rhaponticin opted for further experiments.

Morphological investigations revealed drastic alterations in the morphology of MG-63 cells. Notable variations were detected in the morphology of Rhaponticin administered MG-63 cells when compared with control cells. Rhaponticin (25 μM and 50 μM) treatment possessed remarkable morphological modifications like cell shrinkage, rounding, unequal shape, and cell detachment (Fig. 1B).

3.2. Effect of Rhaponticin on the viability of normal osteoblast MC3T3-E1 cells

The effect of Rhaponticin on the viability of normal osteoblast MC3T3-E1 cells were investigated via an MTT assay and the

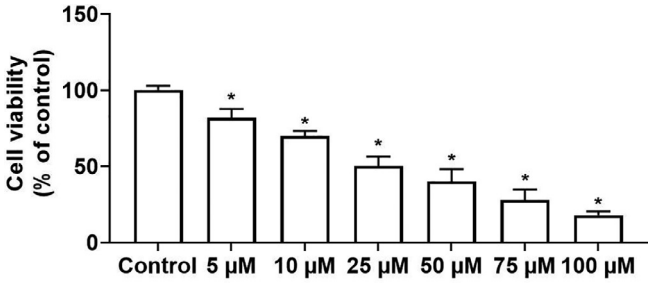

Fig. 1. (A&B): Effect of rhaponticin on the cell viability of MG-63 cells. This figure illustrates the cytotoxicity of rhaponticin on the MG-63 cells. Data were described as mean ± SD of triplicate. Significance was calculated by ANOVA successively DMRT assay; note: '*' $p < 0.05$ is significant when compared with control.

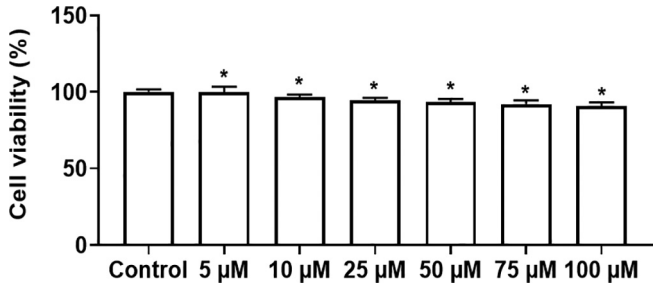

Fig. 2. Effect of rhaponticin on the cell viability of normal osteoblast (MC3T3-E1) cells. This figure depicts that rhaponticin treatment did not show any toxicity to the MC3T3-E1 cells. Data were described as mean ± SD of triplicate. Significance was calculated by ANOVA successively DMRT assay; note: '*' $p < 0.05$ is significant when compared with control.

outcomes were illustrated in Fig. 2. The Rhaponticin treatment at different doses 5–100 μM did not possess any toxicity to the normal osteoblast MC3T3-E1 cells. The MC3T3-E1 cell viability was not affected by the rhaponticin treatment. This outcome was proved that the rhaponticin was toxic only against the osteosarcoma (MG-63) cells.

3.3. Effect of Rhaponticin on the ROS accumulation

The upshot of Rhaponticin on the stimulation of intracellular ROS accretion in the MG-63 cells was examined via DCFH-DA staining. Fig. 3 proved that the Rhaponticin improved the ROS accumulation in MG-63 cells. A bright green fluorescence revealed the elevated ROS accretion, in that way, promoting the oxidative stress-mediated cell damage in the MG-63 cells. Control cells showed no fluorescence, which is in contrast to the Rhaponticin administered MG-63 cells. This outcome was evident due to the stimulation of excess ROS generation in MG-63 cells by the Rhaponticin.

3.4. Effect of Rhaponticin on the MMP status of MG-63 cells

Rhaponticin stimulated alterations in the MMP of MG-63 cells were inspected via Rh-123 staining. An elevated level of MMP in the cells exhibited green fluorescence and weakened MMP indicated disaggregated cells that display reduced or dull green fluorescence. Fig. 4 displayed that the control cells possess a high green fluorescence; alternatively, the MG-63 cells administered with Rhaponticin demonstrated the dull green fluorescence that proves the declined MMP. In that order, it was clear that the Rhaponticin can weaken the MMP of MG-63 cells.

3.5. Effect of Rhaponticin on the apoptotic induction in MG-63 cells

The apoptosis stimulating capacity of Rhaponticin in the MG-63 cells were investigated by dual (AO/EB) staining assay. AO/EB staining method was utilized to detect nuclear damages during apoptotic cell death. Fig. 5 illustrates that the control cells demonstrated AO stained green fluorescence, whereas the Rhaponticin administered MG-63 cells displayed intense EB stained orange fluorescence indicating apoptotic cell death. Hence, this outcome proved that Rhaponticin could stimulate apoptosis in MG-63 cells. Both concentrations (25 μM and 50 μM) of the Rhaponticin have induced nuclear damage, as proven by the intense orange fluorescence cells (Fig. 5).

3.6. Effect of Rhaponticin on the cell necrosis in MG-63 cells

The PI is a fluorescent stain that is widely employed to differentiate the necrotic cells from viable cells. PI cannot enter the viable cell membrane, so only apoptotic or necrotic cells can take up the PI. Fig. 6 demonstrates strong red fluorescence in the Rhaponticin supplemented MG-63 cells, proving necrotic cells. The amount of red fluorescence indicated the number of necrotic cells. Hence it was clear that the Rhaponticin could stimulate cell necrosis in MG-63 cells.

3.7. Effect of Rhaponticin on the apoptotic cell death in MG-63 cells

The effect of Rhaponticin on the apoptotic cell death in MG-63 cells were inspected via DAPI staining, and the result is displayed in Fig. 7. It thus proved that Rhaponticin noticeably altered the nuclear morphology, which demonstrates the apoptotic condition of MG-63 cells. A bright blue fluorescence was noticed in MG-63 cells that was supplemented with Rhaponticin. Fig. 7 confirmed that the Rhaponticin efficiently altered the nuclear content, promoting apoptotic cell death in MG-63 cells.

3.8. Effect of Rhaponticin on the cell adhesion of MG-63 cells

Fig. 8 demonstrates that Rhaponticin treatment reduced the cell adhesion of MG-63 cells. The control cells possessed increased cell adhesion, whereas the 25 μM and 50 μM of Rhaponticin administered MG-63 cells revealed diminished cell adhesion. These outcomes proved that the Rhaponticin could disaggregate the cells and inhibit the adhesiveness of MG-63 cells (Fig. 8).

3.9. Effect of Rhaponticin on the cell migration of MG-63 cells

The inhibitory properties of Rhaponticin on the cell migration of MG-63 cells were investigated by scratch assay. Fig. 9 revealed that the control cells demonstrated increased cell migration, evidenced

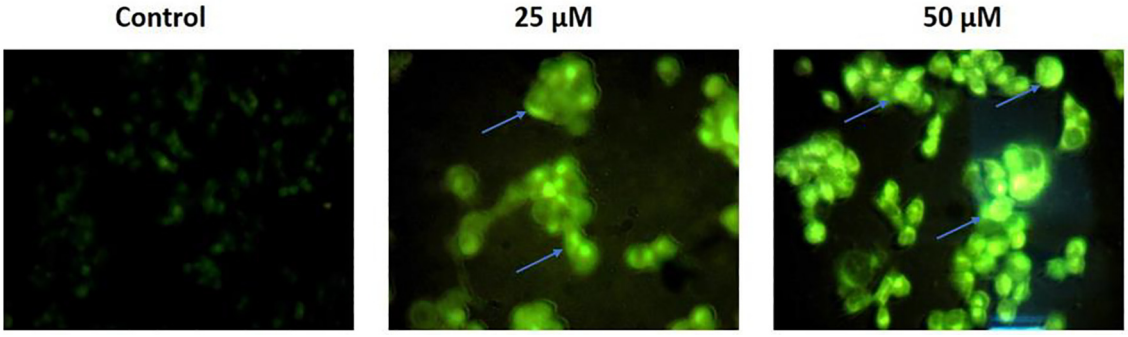

Fig. 3. Effect of rhaponticin on the ROS accumulation in MG-63 cells. A bright green fluorescence in the 25 and 50 μM of rhaponticin administered MG-63 cells (blue arrows) illustrates the augmented accumulation of ROS.

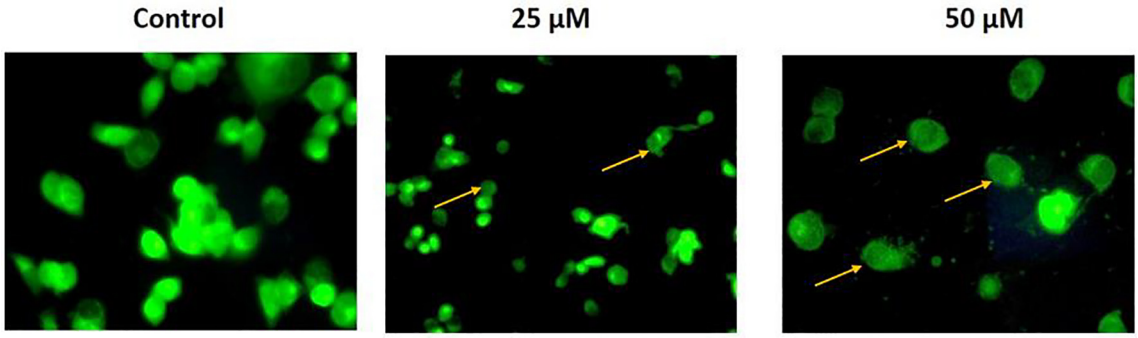

Fig. 4. Effect of rhaponticin on the MMP status of MG-63 cells. The untreated control cells possessing an intense green fluorescence, alternatively, the 25 and 50 µM of rhaponticin administered MG-63 cells demonstrating the dull green fluorescence (yellow arrows) that confirming the declined MMP status.

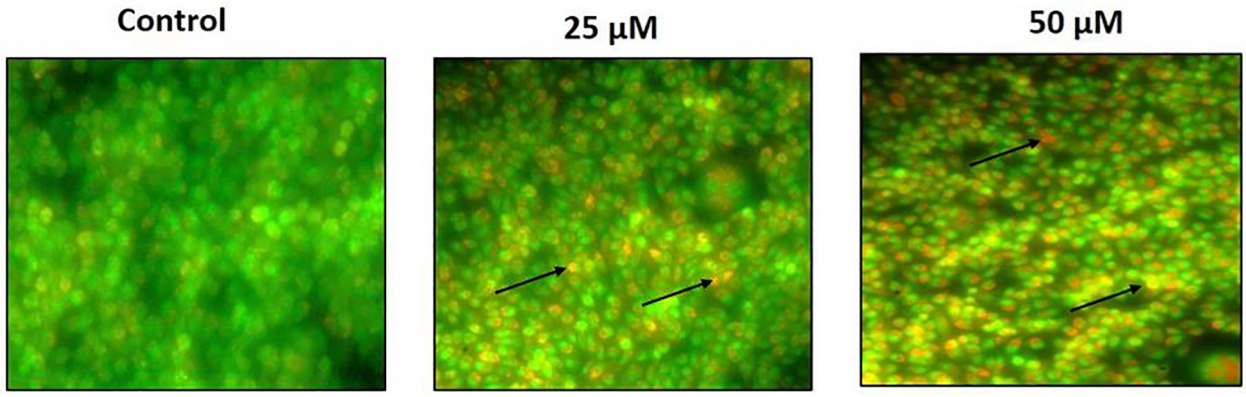

Fig. 5. Effect of rhaponticin on the apoptotic induction in MG-63 cells. The control cells demonstrating the AO stained green fluorescence, whereas the 25 and 50 µM of rhaponticin administered MG-63 cells illustrating the intense EB stained orange fluorescence (black arrows) that indicates the apoptotic cell death.

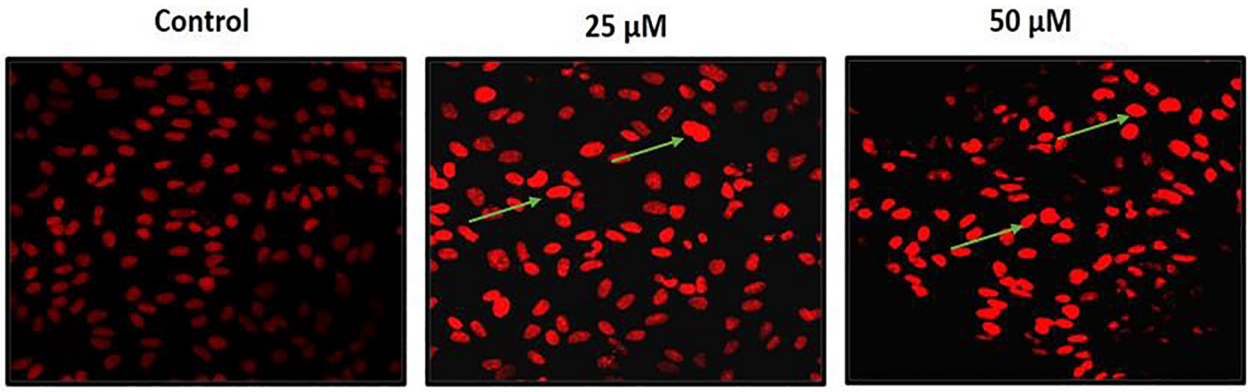

Fig. 6. Effect of rhaponticin on the cell necrosis in MG-63 cells. This figure demonstrating the strong red fluorescence in the morphology of 25 and 50 µM of rhaponticin supplemented MG-63 cells (green arrows), which proves the occurrence of necrotic cells.

by the augmented wound closure rate, whereas the wound closure rate of 25 µM and 50 µM of Rhaponticin administered MG-63 cells was drastically diminished, indicating the inhibition of cell migration. Hence, this outcome revealed that Rhaponticin could inhibit cell migration in MG-63 cells (Fig. 9).

3.10. Effect of Rhaponticin on the PI3K-Akt-mTOR signaling pathway in MG-63 cells

The mRNA expression status of PI3K, Akt, and mTOR proteins was investigated through the RT-PCR analysis. The outcomes proved that the Rhaponticin administration had suppressed the PI3K-Akt-mTOR protein expressions (Fig. 10). The Rhaponticin appreciably prevented the PI3K, Akt, and mTOR expressions in the MG-63 cells. This upshot confirmed that Rhaponticin inhibited the PI3K-Akt-mTOR pathway, thereby inhibiting cellular functions in the MG-63 cells. The antiproliferative and apoptotic inducing capacities of Rhaponticin could be because of the hindering of the PI3K-Akt-mTOR cascade.

4. Discussion

Osteosarcoma is a frequent type of pediatric bone cancer, allegedly said that the incidences of pediatric osteosarcoma may be

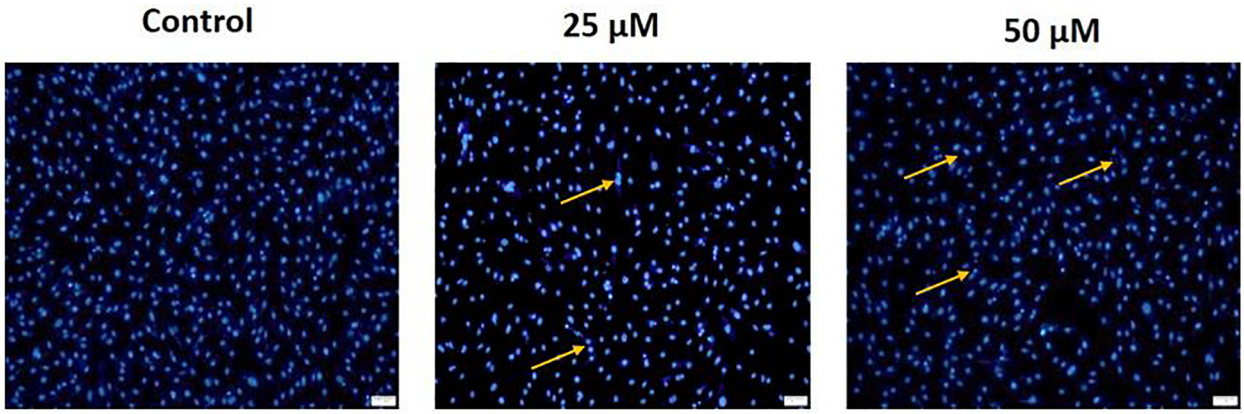

Fig. 7. Effect of rhaponticin on the apoptotic cell death in MG-63 cells. This figure proves that the 25 and 50 µM of rhaponticin noticeably altered the nuclear morphology (yellow arrows), which demonstrating the apoptotic condition in MG-63 cells.

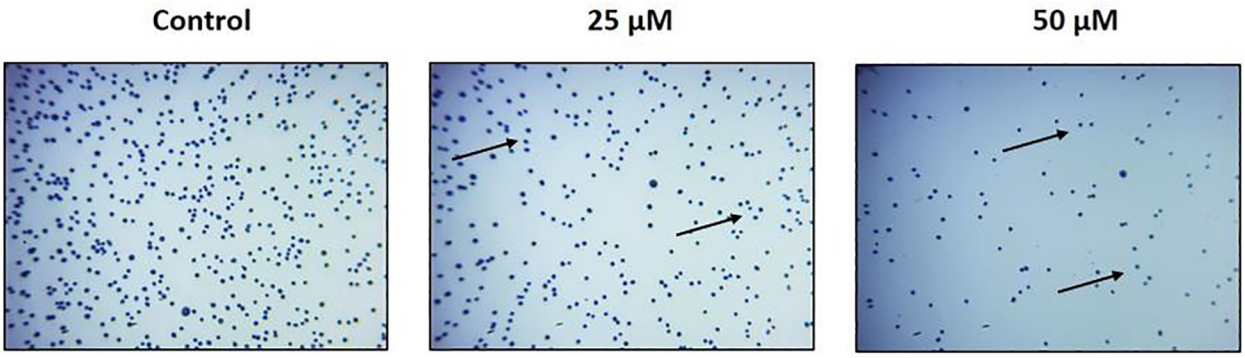

Fig. 8. Effect of rhaponticin on the cell adhesion in MG-63 cells. This figure demonstrates the effects of rhaponticin on cell adhesion. The decreased number of adhesive cells (black arrows) was noted in the 25 and 50 µM of rhaponticin treatment.

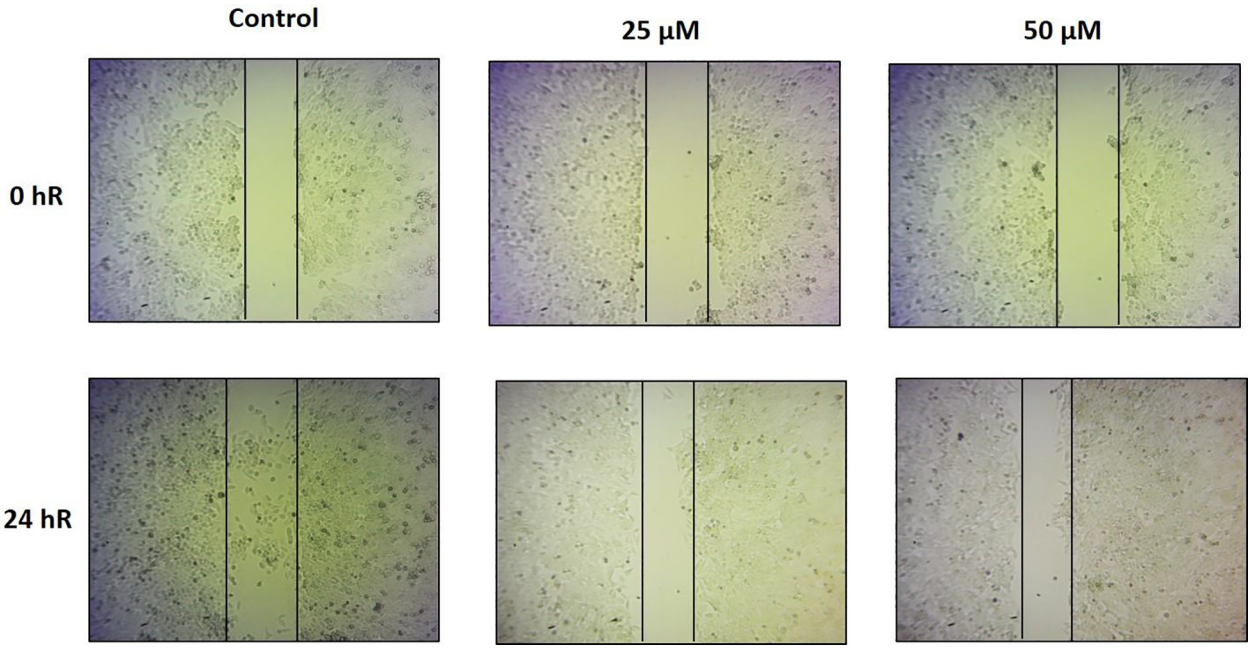

Fig. 9. Effect of rhaponticin on the cell migration in MG-63 cells. This figure revealed the inhibitory potential of rhaponticin on cell migration. The control cells revealed the increased wound closure, whereas the 25 and 50 µM of rhaponticin administered cells possessed the decreased wound closure that proves the inhibition of MG-63 cell migration.

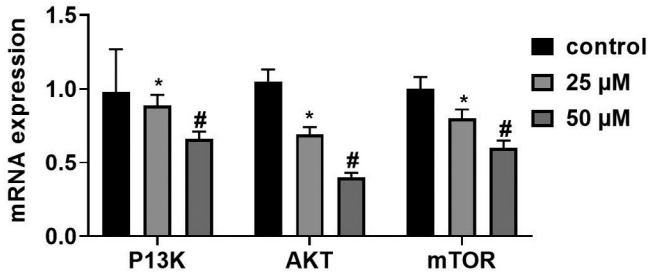

Fig. 10. Effect of rhaponticin on the PI3K-Akt-mTOR signaling pathway in MG-63 cells. Data were described as mean ± SD of triplicate. Significance was calculated by ANOVA successively DMRT assay; note: '*' $p < 0.05$ is significant when compared with control and '#' $p < 0.05$ is significant when compared with 25 μM rhaponticin treatment.

more than 10%. There have been many advancements in diagnostic technology, but researchers and doctors have not been able to find an efficient approach to treat osteosarcoma because of the high metastasis rate and drug resistance (Ward et al., 2014). The administration of chemotherapeutic drugs noticeably improved the survival rate of osteosarcoma patients and diminished the recurrence rate and metastasis. However, these drugs have deprived chemotherapeutic upshots in some victims and often experienced the many side effects that lead to cancer recurrence and metastasis (Altaf et al., 2013). While the osteosarcoma prognosis has been augmented due to the novel treatment approaches, osteosarcoma treatment's success rate is minimal (Li et al., 2015). Administration of chemotherapeutic drugs on or before the surgical procedures may enhance patient survival rates to nearly 60% higher than the surgery alone (Bernthal et al., 2012; Aboody and Mickymaray, 2020). Though surgical procedures and chemotherapy have been widely used, 30% of osteosarcoma victims experienced metastasis to other organs with less prognosis. The actual survival period of metastatic cancer victims is less than one year (Luetke et al., 2014). Hence, the exploration of novel agents with efficient therapeutic outcomes is the primary need to accelerate osteosarcoma patients' successful recovery with improved life quality. Therefore, in this research work, we explored the anticancer effects of Rhaponticin against the MG-63 cells via triggering apoptosis and suppression of PI3K-Akt-mTOR cascade.

The two imperative events that trigger cancer cells' progression are tumor development and metastasis, resulting in augmented cell proliferation and reduced apoptosis. Accordingly, the instigation of apoptosis in tumor cells may improve the therapeutic possibilities to inhibit tumor cell growth (Zhu et al., 2017). Apoptotic cell death is regulated via extrinsic or intrinsic cascades, and it is distinguished by mitochondrial membrane depolarization with membrane potential diminution; such imbalances lead to the augmented ROS generation (Kurokawa and Caspases, 2009; Huang et al., 2017; Mickymaray and Alturaiki, 2018). Augmented intracellular ROS generation leads to the elevated apoptosis rate of cancer cells (Yoon et al., 2018). Accumulation of intracellular ROS performs a crucial function in pathological mechanisms like cell multiplication and oxidative damage (He et al., 2017). Cancer cells are incredibly susceptible to augmented ROS accumulation, improving the apoptotic rate (Gallego et al., 2008). The altered or reduced antioxidant mechanisms increase the ROS accretion and injures cellular macromolecules like lipids, proteins, and DNA (Mickymaray and Al Aboody, 2019; Mickymaray, 2019). Hence, the target agent that augments the intracellular ROS accumulation could improve mitochondrial injury and finally lead to the escalated apoptotic processes in tumor cells (Pramanik et al., 2011).

ROS and the mitochondrial oxidative mechanisms perform a crucial task in triggering apoptosis under pathological and physiological circumstances. ROS can trigger various pathological conditions like the stimulation of apoptotic pathways and oxidative alterations of proteins, lipids, or DNA (Circu and Aw, 2010; Suresh et al., 2020). Flaws in apoptosis play a vital function in tumor progression and cytotoxic outcomes of numerous chemotherapeutic agents are primarily conveyed by augmented apoptosis (Hassan et al., 2014). So, the efficient approach to treat cancers is primarily through developing drugs that improve intracellular ROS production and ensure apoptosis. The level of intracellular ROS accretion can be inspected through the fluorescent dye DCFH-DA, and the MMP level of the cells can be visualized through the Rh-123 staining technique (Zhou et al., 2018). In this investigation, the DCFH-DA staining assay outcomes proved that the Rhaponticin had improved the ROS accumulation in the osteosarcoma MG-63 cells (Fig. 2). Rh-123 staining result confirmed that the Rhaponticin administered MG-63 cells had diminished MMP (Fig. 3).

The dual (AO/EB) staining technique was extensively used to distinguish the nuclear damages mediated by apoptosis. The apoptosis stimulating capacity of sample agents is usually examined via AO/EB staining assay. This technique can distinctly differentiate the various stages of apoptosis (Gherghi et al., 2003). DAPI staining is commonly used to detect the DNA fragmentation and nuclear condensation directly linked to the apoptotic process (Vandghanooni et al., 2013). In this work, we noticed that the Rhaponticin administered MG-63 cells were possessed increased apoptotic cell death and nuclear damages, which evidenced by the AO/EB (Fig. 4) and DAPI staining (Fig. 6). The Rhaponticin treatment also revealed reduced cell adhesion (Fig. 7) and cell migration (Fig. 8) of osteosarcoma cells.

Akt protein mediates the activities of numerous substrates implicated in cell multiplication, the advancement of the cell cycle, and growth. It was stated in recent decades that PI3K/Akt signaling cascade is modified in several human cancers. Inhibition of the Akt signaling cascade may cause cancer cell necrosis (Zhang et al., 2017). PI3K/AKT/mTOR cascade is reported as an imperative cascade in cancer progression implicated with cell multiplication, cell cycle, and tumor development (Dey et al., 2017). In some ailments like cancer, this cascade's stimulation could diminish the apoptosis and speed up the metastasis (Pierobon et al., 2017). PI3K/AKT/mTOR pathway is mainly concerned with cell multiplication, invasion, improves cell cycle progression, and mediates autophagy and apoptosis (Liu et al., 2019). The activation of PI3K possesses consistent biological effects (Gao et al., 2018; Liu et al., 2018). It was already identified that the disordered PI3K/AKT/mTOR cascade tightly allied with the cell growth in osteosarcoma (Keremu et al., 2017). Interestingly, in this investigation, we found that the PI3K/AKT/mTOR signaling cascade was appreciably downregulated in the Rhaponticin administered MG-63 cells (Fig. 9). The potent anti-proliferative capacity and triggering of apoptosis in MG-63 cells via Rhaponticin could be because of the hindering of PI3K-Akt-mTOR signaling cascade in the MG-63 cells.

5. Conclusion

Our outcomes from this research work revealed that the Rhaponticin possessed cytotoxic and apoptotic effects against the MG-63 cells. The outcomes proved that the Rhaponticin diminished the cell viability, elevated the intercellular ROS accumulation, and reduced the MMP, thereby promoting apoptotic cell death in MG-63 cells. Rhaponticin effectively inhibited the PI3K/AKT/mTOR cascade in the MG-63 cells. These outcomes proved that the Rhaponticin can be a hopeful chemotherapeutic agent in the future for osteosarcoma treatment. However, further investigations are still required in the future to understand the exact curative role of Rhaponticin.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Aboody, M.S.A., Mickymaray, S., 2020. Anti-fungal efficacy and mechanisms of flavonoids. Antibiotics (Basel) 9 (2), 45.

Alsagaby, S.A., Vijayakumar, R., Premanathan, M., Mickymaray, S., Alturaiki, W., Al-Baradie, R.S., AlGhamdi, S., Aziz, M.A., Alhumaydhi, F.A., Alzahrani, F.A., Alwashmi, A.S., Al Abdulmonem, W., Alharbi, N.K., Pepper, C., 2020. Transcriptomics-based characterization of the toxicity of ZnO nanoparticles against chronic myeloid leukemia cells. Int. J. Nanomed. 15, 7901–7921.

Altaf, S., Enders, F., Jeavons, E., Krailo, M., Barkauskas, D.A., Meyers, P., Arndt, C., 2013. High–BMI at diagnosis is associated with inferior survival in patients with osteosarcoma: A report from the children's oncology group. Pediatr. Blood Cancer 60, 2042–2046.

Bernthal, N.M., Federman, N., Eilber, F.R., Nelson, S.D., Eckardt, J.J., Eilber, F.C., Tap, W.D., 2012. Long-term results (>25 years) of a randomized, prospective clinical trial evaluating chemotherapy in patients with high-grade, operable osteosarcoma. Cancer 118 (23), 5888–5893.

Chen, H., Feng, X., Gao, L., Mickymaray, S., Paramasivam, A., Abdulaziz Alfaiz, F., Almasmoum, H.A., Ghaith, M.M., Almaimani, R.A., Aziz Ibrahim, I.A., 2021. Inhibiting the PI3K/AKT/mTOR signalling pathway with copper oxide nanoparticles from *Houttuynia cordata* plant: attenuating the proliferation of cervical cancer cells. Artif. Cells Nanomed. Biotechnol. 49 (1), 240–249.

Chen, J., Ma, M., Lu, Y., Wang, L., Wu, C., Duan, H., 2009. Rhaponticin from rhubarb rhizomes alleviates liver steatosis and improves blood glucose and lipid profiles in KK/Ay diabetic mice. Planta Med. 75 (5), 472–477.

Choi, S.B., Ko, B.S., Park, S.K., Jang, J.S., Park, S., 2006. Insulin sensitizing and alpha-glucoamylase inhibitory action of sennosides, rheins and rhaponticin in Rhei Rhizoma. Life Sci. 78 (9), 934–942.

Chou, A.J., Gorlick, R., 2006. Chemotherapy resistance in osteosarcoma: Current challenges and future directions. Expert Rev. Anticancer There. 6, 1075–1085.

Circu, M.L., Aw, T.Y., 2010. Reactive oxygen species, cellular redox systems and apoptosis. Free Radic. Biol. Med. 48, 749–762.

Dey, N., De, P., Leyland-Jones, B., 2017. PI3K-AKT-mTOR inhibitors in breast cancers: from tumor cell signaling to clinical trials. Pharmacol. Ther. 175, 91–106.

Dmitrieva, O.S., Shilovskiy, I.P., Khaitov, M.R., Grivennikov, S.I., 2016. Interleukins 1 and 6 as main mediators of inflammation and cancer. Biochem 81, 80–90.

Feng, H., Wang, J., Chen, W., Shan, B., Guo, Y., Xu, J., Wang, L., Guo, P., Zhang, Y., 2016. Hypoxia-induced autophagy as an additional mechanism in human osteosarcoma radioresistance. J Bone Oncol. 5, 67–73.

Ferrari, S., Bielack, S.S., Smeland, S., et al., 2018. EURO-B.O.S.S.: A European study on chemotherapy in bone-sarcoma patients aged over 40: outcome in primary high-grade osteosarcoma. Tumori 104 (1), 30–36.

Fornetti, J., Welm, A.L., Stewart, S.A., 2018. Understanding the bone in cancer metastasis. J. Bone Miner. Res. 33, 2099–2113.

Friebele, J.C., Peck, J., Pan, X., Abdel-Rasoul, M., Mayerson, J.L., 2015. Osteosarcoma: a meta-analysis and review of the literature. Am. J. Orthoped. 44 (12), 547–553.

Gallego, M.A., Ballot, C., Kluza, J., Hajji, N., Martoriati, A., Castera, L., Cuevas, C., Formstecher, P., Joseph, B., Kroemer, G., Bailly, C., Marchetti, P., 2008. Overcoming chemoresistance of non-small cell lung carcinoma through restoration of an AIF-dependent apoptotic pathway. Oncogene 27 (14), 1981–1992.

Gao, W., Wu, X.L., Li, D.Z., Liu, H.D., 2018. HOTTIP participates in mammary cancer by promoting cell proliferation via PI3K/AKT pathway. Eur. Rev. Med. Pharmacol. Sci. 22, 4181–4187.

Gherghi, I.C., Girousi, S.T., Voulgaropoulos, A.N., Tzimou-Tsitouridou, R., 2003. Study of interactions between DNA-ethidium bromide (EB) and DNA-acridine orange (AO), in solution, using hanging mercury drop electrode (HMDE). Talanta 61, 103–112.

Harrison, D.J., Geller, D.S., Gill, J.D., Lewis, V.O., Gorlick, R., 2018. Current and future therapeutic approaches for osteosarcoma. Expert Rev. Anticancer Ther. 18 (1), 39–50.

Hassan, M., Watari, H., AbuAlmaaty, A., Ohba, Y., Sakuragi, N., 2014. Apoptosis and molecular targeting therapy in cancer. Biomed Res. Int., 150845

He, L., He, T., Farrar, S., Ji, L., Liu, T., Ma, X., 2017. Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. Cell. Physiol. Biochem. 44 (2), 532–553.

Hu, K., Dai, H.B., Qiu, Z.L., 2016. mTOR signaling in osteosarcoma: Oncogenesis and therapeutic aspects (Review). Oncol. Rep. 36, 1219–1225.

Huang, H., Chen, J., Lu, H., Zhou, M., Chai, Z., Hu, Y., 2017. Two mTOR inhibitors, rapamycin and Torin 1, differentially regulate iron-induced generation of mitochondrial ROS. Biometals 30 (6), 975–980.

Kager, L., Tamamyan, G., Bielack, S., 2017. Novel insights and therapeutic interventions for pediatric osteosarcoma. Future Oncol. 13 (4), 357–368.

Ke, Y., Al Aboody, M.S., Alturaiki, W., Alsagaby, S.A., Alfaiz, F.A., Veeraraghavan, V.P., Mickymaray, S., 2019. Photosynthesized gold nanoparticles from Catharanthus roseus induces caspase-mediated apoptosis in cervical cancer cells (HeLa). Artif. Cells Nanomed. Biotechnol. 47 (1), 1938–1946.

Keremu, A., Maimaiti, X., Aimaiti, A., Yushan, M., Alike, Y., Yilihamu, Y., Yusufu, A., 2017. NRSN2 promotes osteosarcoma cell proliferation and growth through PI3K/Akt/mTOR and Wnt/beta-catenin signaling. Am. J. Cancer Res. 7 (3), 565–573.

Kim, A., Ma, J.Y., 2018. Rhaponticin decreases the metastatic and angiogenic abilities of cancer cells via suppression of the HIF-1α pathway. Int. J. Oncol. 53 (3), 1160–1170.

Kolodziejczyk-Czepas, J., Czepas, J., 2019. Rhaponticin as an anti-inflammatory component of rhubarb: a mini review of the current state of the art and prospects for future research. Phytochem. Rev. 18, 1375–1386.

Kurokawa, M., Kornbluth, S., 2009. Caspases and kinases in a death grip. Cell 138 (5), 838–854.

Li, H.Y., Zhang, J., Sun, L.L., Li, B.H., Gao, H.L., Xie, T., Zhang, N., Ye, Z.M., 2015. Celastrol induces apoptosis and autophagy via the ROS/JNK signaling pathway in human osteosarcoma cells: An in vitro and in vivo study. Cell Death Dis. 6, e1604.

Liu, H.W., Bi, W.T., Huang, H.T., Li, R.X., Xi, Q., Feng, L., Bo, W., Hu, M., Wen, W.S., 2018. Satb1 promotes Schwann cell viability and migration via activation of PI3K/AKT pathway. Eur. Rev. Med. Pharmacol. Sci. 22, 4268–4277.

Liu, J.Z., Hu, Y.L., Feng, Y., Guo, Y.B., Liu, Y.F., Yang, J.L., Mao, Q.S., Xue, W.J., 2019. Rafoxanide promotes apoptosis and autophagy of gastric cancer cells by suppressing PI3K/Akt/mTOR pathway. Exp. Cell Res. 385, 111691.

Longhi, A., Errani, C., De Paolis, M., Mercuri, M., Bacci, G., 2006. Primary bone osteosarcoma in the pediatric age: State of the art. Cancer Treat. Rev. 32, 423–436.

Luetke, A., Meyers, P.A., Lewis, I., Juergens, H., 2014. Osteosarcoma treatment—where do we stand? A state of the art review. Cancer Treatment Rev. 40 (4), 523–532.

Meazza, C., Scanagatta, P., 2016. Metastatic osteosarcoma: a challenging multidisciplinary treatment. Expert Rev. Anticancer Ther. 16 (5), 543–556.

Mickymaray, S., Al Aboody, M.S., 2019. In Vitro Antioxidant and Bactericidal Efficacy of 15 Common Spices: Novel Therapeutics for Urinary Tract Infections?. Medicina (Kaunas) 55 (6), 289.

Mickymaray, S., Alturaiki, W., 2018. Antifungal Efficacy of Marine Macroalgae against Fungal Isolates from Bronchial Asthmatic Cases. Molecules 23 (11), 3032.

Mickymaray, S., 2019a. Efficacy and Mechanism of Traditional Medicinal Plants and Bioactive Compounds against Clinically Important Pathogens. Antibiotics (Basel) 8 (4), 257.

Mickymaray, S., 2019. One-step Synthesis of Silver Nanoparticles Using Saudi Arabian Desert Seasonal Plant *Sisymbrium irio* and Antibacterial Activity Against Multidrug-Resistant Bacterial Strains. Biomolecules 9 (11), 662.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Pierobon, M., Ramos, C., Wong, S., et al., 2017. Enrichment of PI3K-AKTmTOR pathway activation in hepatic metastases from breast cancer. Clin. Cancer Res. 23 (16), 4919–4928.

Pramanik, K.C., Boreddy, S.R., Srivastava, S.K., 2011. Role of mitochondrial electron transport chain complexes in capsaicin mediated oxidative stress leading to apoptosis in pancreatic cancer cells. PLoS ONE 6, (5) e20151.

Shi, Q., Cheng, Y., Dong, X., Zhang, M., Pei, C., Zhang, M., 2020. Effects of rhaponticin on retinal oxidative stress and inflammation in diabetes through NRF2/HO-1/NF-kB signaling. J. Biochem. Mol. Toxicol. 34, (11) e22568.

Sun, Y., Zhao, Y., 2012. Enhanced pharmacokinetics and anti-tumor efficacy of PEGylated liposomal rhaponticin and plasma protein binding ability of rhaponticin. J. Nanosci. Nanotechnol. 12 (10), 7677–7684.

Suresh, M., Alfonisan, M., Alturaiki, W., Al Aboody, M.S., Alfaiz, F.A., Premanathan, M., Vijayakumar, R., Umamagheswari, K., Al Ghamdi, S., Alsagaby, S.A., 2020. Investigations of Bioactivity of Acalypha indica (L.), Centella asiatica (L.) and Croton bonplandianus (Baill) against Multidrug Resistant Bacteria and Cancer Cells. J Herbal Med., 100359 https://doi.org/10.1016/j.hermed.2020.100359.

Vandghanooni, S., Forouharmehr, A., Eskandani, M., Barzegari, A., Kafil, V., Kashanian, S., Dolatabadi, E.N., 2013. Cytotoxicity and DNA fragmentation properties of butylated hydroxyanisole. DNA Cell Biol. 32 (3), 98–103.

Wang, W., Yang, J., Wang, Y., Wang, D., Han, G., Jia, J., Xu, M., Bi, W., 2017. Survival and prognostic factors in Chinese patients with osteosarcoma: 13-year experience in 365 patients treated at a single institution. Pathol. Res. Pract. 213 (2), 119–125.

Wang, Y., Sun, Y., Wu, Y., Zhang, J., 2016. Cucurbitacin E inhibits osteosarcoma cells proliferation and invasion through attenuation of PI3K/AKT/mTOR signaling. Biosci Rep. BSR20160165.

Ward, E., Desantis, C., Robbins, A., Kohler, B., Jemal, A., 2014. Childhood and adolescent cancer statistics, 2014. CA: Cancer J. Clin. 64 (2), 83–103.

Wober, J., Möller, F., Richter, T., Unger, C., Weigt, C., Jandausch, A., Zierau, O., Rettenberger, R., Kaszkin-Bettag, M., Vollmer, G., 2007. Activation of estrogen receptor-beta by a special extract of *Rheum rhaponticum* (ERr 731), its aglycones and structurally related compounds. J. Steroid Biochem. Mol. Biol. 107, 191–201.

Xu, Y., Li, N., Xiang, R., Sun, P., 2014. Emerging roles of the p38 MAPK and PI3K/AKT/mTOR pathways in oncogene-induced senescence. Trends Biochem. Sci. 39, 268–276.

Yoon, D.S., Lee, M.H., Cha, D.S., 2018. Measurement of intracellular ROS in Caenorhabditis elegans using 2′, 7′-dichlorodihydrofluorescein diacetate. Bio Protoc. 8 (6), e2774.

Yu, J.S., Cui, W., 2016. Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. Development 143, 3050–3060.

Yu, W., Wang, Y., Zhu, J., Jin, L., Liu, B., Xia, K., Wang, J., Gao, J., Liang, C., Tao, H., 2019. Autophagy inhibitor enhance ZnPc/BSA nanoparticle induced photodynamic therapy by suppressing PD- L1 expression in osteosarcoma immunotherapy. Biomaterials 192, 128–139.

Zhang, J., Yu, X.H., Yan, Y.G., Wang, C., Wang, W.J., 2015. PI3K/Akt signaling in osteosarcoma. Clin. Chim. Acta 444, 182–192.

Zhang, R., Kang, K.A., Piao, M.J., Lee, K.H., Jang, H.S., Park, M.J., Kim, B.J., Kim, J.S., Kim, Y.S., Ryu, S.Y., Hyun, J.W., 2007. Rhapontigenin from Rheum undulatum protects against oxidative-stress- induced cell damage through antioxidant activity. J. Toxicol. Environ. Health 70 (13), 1155–1166.

Zhang, S., Deng, Z., Yao, C., Huang, P., Zhang, Y., Cao, S., Li, X., 2017. AT7867 Inhibits human colorectal cancer cells via AKT-Dependent and AKT-Independent mechanisms. PLoS ONE 12 (1), e0169585.

Zhou, G.Z., Shi, Y.Y., Cui, L.S., Li, A.F., Wang, Q.Q., Liu, M., 2018. Oxymatrine induces A549 human non-small lung cancer cell apoptosis via extrinsic and intrinsic pathways. Mol. Med. Rep. 17 (1), 1071–1076.

Zhu, J., Liu, M., Liu, Y., Zhang, Y., Yang, B., Zhang, W., 2017. Zoledronic Acid Regulates Autophagy and Induces Apoptosis in Colon Cancer Cell Line CT26. Biomed Res. Int. 2017, 6.