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ORIGINAL ARTICLE

CONTENTS

Osteoblastic MG63 CELLS promoted by ATP shows apoptotic LDH release under guggulsterone exposure

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ABSTRACT

Aim: To identify the anticancer potential of guggulsterone on Mg63 cells. ATP was administered to promotes growth was given to MG-63 human osteosarcoma cells that have osteoblastic features.

Materials and Methods: Different amounts of guggulsterone were added to MG 63 cells, and their shape and rate of growth were studied. The amount of LDH in cells was measured to evaluate cell toxicity. The findings show that guggulsterone caused more changes in the shape of MG63 cells after they were exposed to it.

Results: With higher amounts of guggulsterone, cell growth slowed down significantly. The results that were seen in this study were similar to what had been seen in other studies on different cell types.

Conclusions: Based on the results, we conclude that guggulsterone plays an important part in the changes that happen to the shape of cancer cells when they grow faster.

KEY WORDS: MG63, cell lines, osteoblasts, guggulesterone, ATP, LDH, apoptosis

Wiad Lek. 2026;79(4):712-719. doi: 10.36740/WLek/215497 DOI

INTRODUCTION

OSTEOSARCOMA AND CELLULAR RESEARCH

Osteosarcoma is the most common type of cancerous bone growth, and it tends to grow rapidly. Even though it's rare, "osteosarcoma" makes up 1% of all cancers [1]. It mostly affects kids and young people, and 5–10% of their joints are affected [2]. It's not likely that osteosarcoma will go away. Even though surgery and treatment have come a long way, only 60 to 70% of people with locally advanced disease will still be alive after 5 years. For those with a spread, that number drops to 20 to 30 percent [3]. Osteosarcoma is the second most common type of cancer in kids and teens. The outlook is not good because chemotherapy and radiation treatment did not work. To come up with successful treatment plans, it is important to understand how tumors grow and spread. Xenograft models and cancer cell lines are useful for studying these processes and testing out new ways to treat cancer. Herbal drugs have become popular recently as a way to treat a wide range of illnesses. Researchers are now interested in getting these plant parts out and figuring out how these chemicals work to treat different types of cancer and other diseases. Guggulsterone (pregna-4,17-diene-3,16-dione; $C21H28O2$) is a strong plant sterol that comes from the roots of many guggul trees and the myrrh tree. Scientists have found that guggulsterone can bind to and block the farnesoid X receptor (FXR), which stops the FXR gene from being expressed when an activator is present [4].

GUGGULSTERONE AS ANTICANCER THERAPY

Continouse research is carried out to look into how guggulsterone can help fight cancer. Aside from that, not much is known about how guggulsterone can be used in early and clinical studies. It will be clearer how guggulsterone can be used in humans and animals, and how well it works to treat different types of cancer, after a thorough review and meta-analysis. As of now, there hasn't been any thorough or organized study done to show how guggulsterone affects the growth and spread of cancer cells [5]. For cancer to grow, attack, and spread, the cancer cells and surrounding cells must be able to communicate with each other. Tumor cells produce growth-promoting factors, chemokines, and cytokines during cancer growth. These chemicals attract stromal

cells and let immune cells and nerves get inside. Tumor growth, spread, and metastasis rely on tumor cells and their surroundings being able to talk to each other and share information. When cancer grows, tumor cells make chemokines, cytokines, and growth-promoting factors that bring in stromal cells, immune cells, and nerves [6-8]. So, these cells also release some ECM proteins, growth factors, proteases, and basement membrane parts [9].

The TME is mostly made up of immune and inflammatory cells, fibroblasts and myofibroblasts, the extracellular matrix (ECM), and blood vessels. The molecular and cellular parts of the TME are crucial for stopping the growth, spreading, and invasion of cancer [10, 11]. The TME provides tumor cells with nutrients like glucose, glutamine, and vital amino acids, enabling them to continue growing unhindered [12, 13]. It is interesting to note that the TME had a lot more ATP than the other samples. A new study by Patrizia Pellegatti et al. uses bioluminescence imaging and pme LUC (plasma membrane luciferase) to find out how much ATP is in the body. They found that the amount of ATP in tumor tissue was hundreds of micromoles, which was more than the amount of ATP in healthy tissue, which was less than 100 nanomoles per liter [14].

There are many different sources and processes that cause eATP to build up inside the TME. The TME is made up of different levels of oxygenation caused by cellular stress, hypoxia, damage, and death, all of which produce ATP. ATP can also be released into the extracellular area by autophagy, damage to the cell membrane, and the effects of cancer drugs [15, 16]. ATP builds up in the TME with the help of cytolytic ATP release and the rapid release of ATP from cells that are activating or dying through vesicle exocytosis, transporters, and membrane-bound pathways. Some cells, like defense cells (like T lymphocytes), platelets, lymphocytes, and vascular cells, have been shown to go through exocytosis, which is the process of ATP being released from inside cells into the area outside of cells [17, 18]. ABC receptors, such as the sulfonylurea receptor (SUR), the multidrug resistance (MDR) gene product (also called P-glycoprotein), and the cystic fibrosis transmembrane conductance regulator (CFTR), move ATP from cells and do other things besides vesicle release. Purinergic messaging functions in both directions [19].

Finally, intercellular channels help release ATP. These include maximal anion channels and pore-forming channels such as connexins, pannexins, and P2X purinergic receptor 7 (P2X7R). For example, it is thought that Pannexin 1 (Panx1) and other specific pore-forming plasma proteins are the major way that ATP is released in the TME in many cells. Reports say that Panx1 creates a six-part system that lets ATP move into the space around cells in reaction to different triggers, such as low oxygen or cell death [20, 21]. Basically, Panx1 stops the negative buildup of ATP outside of cells by managing the release of ATP through a negative P2X7R-mediated route. As it turns out, Panx1 is highly linked to P2X7R, and Panx1 has a smaller affinity for ATP than P2X7R. So, as eATP rises, ATP starts to bind to P2X7R and stops Panx1 from working. For the record, ATP can be released in the TME by both stressed or dead cells and live cells in different ways [22, 23].

AIM

This study was made to look into how guggulsterone can help treat different types of cancer by using different types of cancer cell lines. The aim of this study is to identify the anticancer potential of guggulsterone on Mg63 cells and morphological changes that are induced to Mg63 cell upon exposure.

MATERIALS AND METHODS

For this study, ATCC CRL-1427TM human osteosarcoma cells MG-63 were chosen because they have osteoblastic features [24]. The cells were grown in a special medium that helped them grow bones. It had Dulbecco-Vogt's Modified Eagle's (DME) medium and Ham's F-12 (H) medium mixed together 3:1 (v/v). It also had 24.3 µg/mL adenine, 10 µg/mL human epidermal growth factor, 0.4 µg/mL hydrocortisone, 5 µg/mL bovine insulin, 5 µg/mL human transferrin, 2×10^{-9} M 3, 3′, 5′-triiodo-L-thyronine, 100 µg/mL penicillin, 25 µg/mL gentamicin, and 10% fetal calf serum. To keep the cell growth going, the culturing was done at 37ºC in a wet 5% CO_2 environment. The culture medium was changed every 24 hours until the confluence level of 80 to 90 percent was reached.

PREPARATION OF GUGGULSTERONE AND ATP

Z-Guggulsterone was bought from Steraloids, Inc. in Newport, RI. It was mixed with dimethyl sulfoxide (DMSO) to make a 10 mM stock solution and kept at -20°C. Consecutive concentrations were made to use in the study (0, 1, 5,10,25,50, 75 µM). Adenosine triphosphate purchased from Sigma Aldrich / UK was used in a concentration of 100 µM throughout the experiment as a promoter for cellular growth.

GUGGULSTERONE EFFECT ON OSTEOBLASTS GROWTH AND MORPHOLOGY

Three times as many osteoblastic cells were put into each well of a 6-well plate. The growth medium was

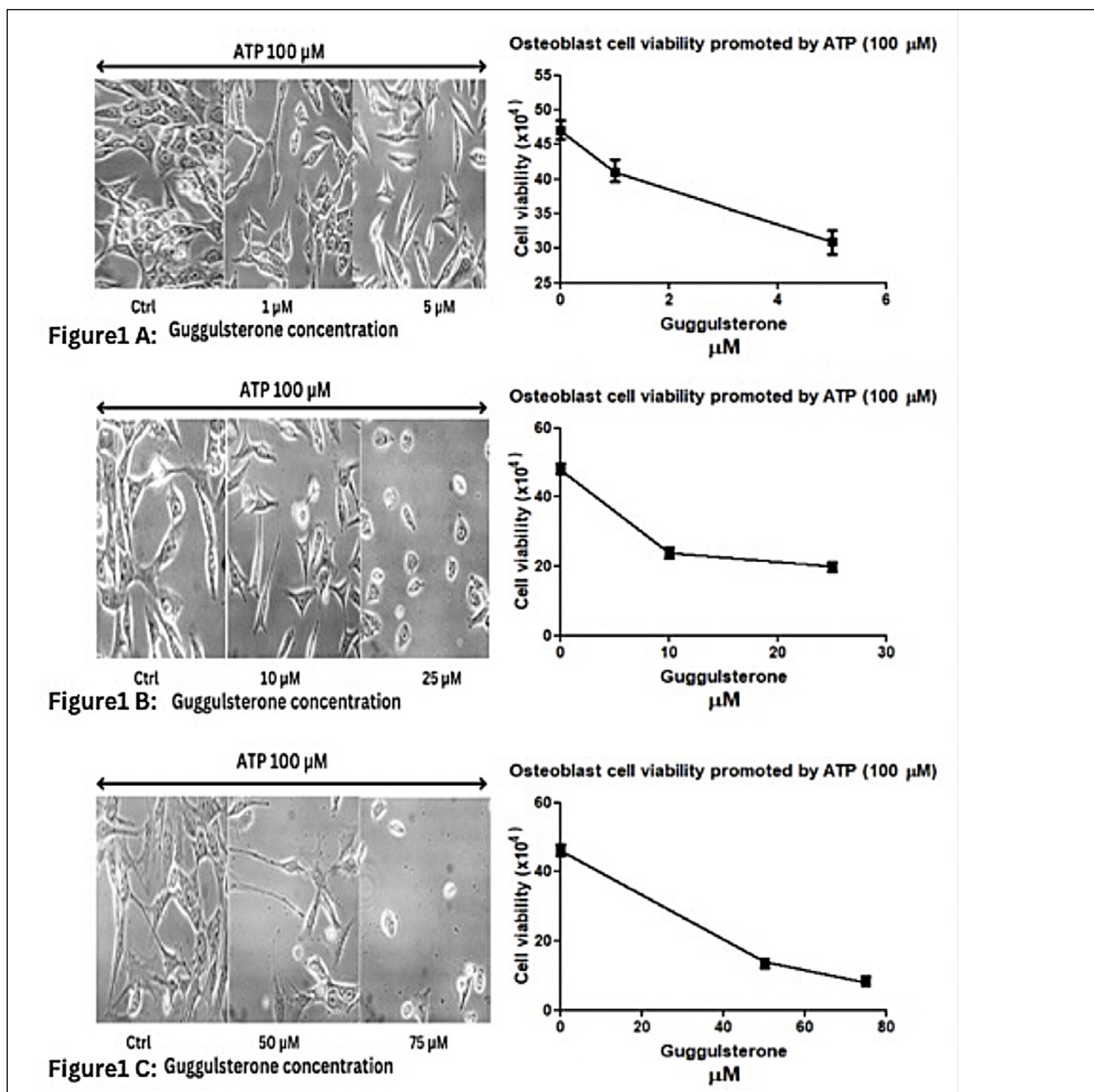

Fig. 1. Osteoblasts promoted by ATP and exposed to increased guggulsterone concentration shows round cells with reduced cell contact compared to control cells (A). Cell viability also decreases with an increase in guggulsterone concentration (A).
Osteoblasts promoted by ATP with increased guggulsterone concentration shows round cells with apoptotic signs and reduced cell contact compared to control cells (B). Cell viability also decreases with an increase in guggulsterone concertation.
Increased guggulsterone concentration shows round cells with apoptotic signs and reduced cell contact compared to control cells (C). Cell viability also decreases with an increase in guggulsterone concentration.
Source: Own materials

treated with guggulsterone at different amounts (0, 1, 5, 25 μM), and 2 ml was added to each well. The cells were kept at 37°C with 5% CO_2 for 24 hours. To sum up, trypsin was used to separate the cells from each culture well. The cells were then washed twice with culture water and used to test the survival of the cells. Each cell pellet was mixed with 1 mL of culture medium. Twenty microliters of each cell solution were mixed with twenty microliters of TB and left to sit on ice for five minutes. The mix of cells was put into a hemacytometer. An inverted optical microscope (Leica) was used to count the number of living cells (not colored blue) and dead cells (colored blue). The tests were done at least three times, each time with a copy of the original.

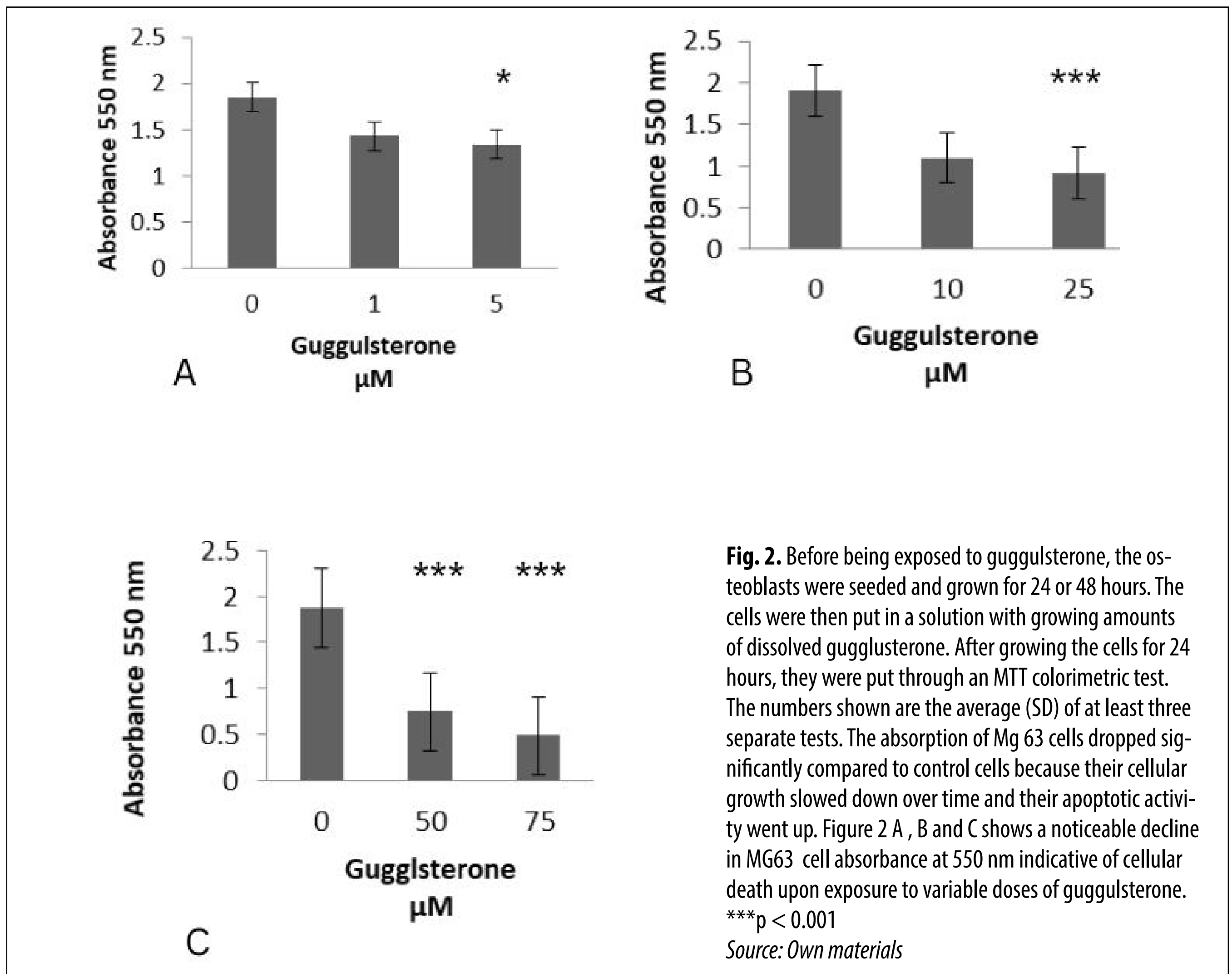

Fig. 2. Before being exposed to guggulsterone, the osteoblasts were seeded and grown for 24 or 48 hours. The cells were then put in a solution with growing amounts of dissolved gugglusterone. After growing the cells for 24 hours, they were put through an MTT colorimetric test. The numbers shown are the average (SD) of at least three separate tests. The absorption of Mg 63 cells dropped significantly compared to control cells because their cellular growth slowed down over time and their apoptotic activity went up. Figure 2 A , B and C shows a noticeable decline in MG63 cell absorbance at 550 nm indicative of cellular death upon exposure to variable doses of guggulsterone.
***$p < 0.001$
Source: Own materials

GUGGULSTERONE EFFECT ON CELLULAR LACTATE DEHYDROGENASE (LDH)

Mg63 cells were seeded into each well of a 6-well plate. The cells were then introduced to e-liquid solutions at 0%, 1%, and 5% amounts for 24 hours. After the time was up, the cell growth medium was taken out and the LDH cytotoxicity test (abcam, UK) was used to find out how harmful the cells were. In short, 50 µL of supernatant was put into a 96-well flat-bottom plate along with 50 µL of restored substrate mix. The plate was then left to sit at room temperature for 30 minutes in the dark. Lactate dehydrogenase (LDH), an enzyme that is steady and soluble and is found in all live cells, is released into the area outside of cells. 50 µL of an acid solution was added to each well to stop the process. After that, 100 µL of each reaction solution was added four times to a new 96-well flat-bottom plate, and the absorbance was measured at 490nm using an Agilent microplate spectrophotometer (Agilent, USA). Making a positive control, which means letting the cells sit in 1% Triton x 100, was done to get the most LDH release. To get the least amount of LDH production, a negative control was used, which meant that the cells were incubated without Guggulsterone. The experiment was done at least three times, each time exactly the same.

BIOSTATISTICAL ANALYSIS

The results were given as means with a standard deviation (SD). At least three times of each experiment were done. The differences between the sets of data were compared. The Shapiro-Wilk test and Levene's test of variance were used to check if the data were normal. At $p \leq 0.001$, the P value was thought to be important. A one-way analysis of variance (ANOVA) parametric test was used to find out if there was a statistically significant difference between the numbers that came from a normal distribution. A non-parametric Kruskal Wallis test was done on the data that did not have a normal distribution. Tukey and Bonferroni's change of p-value was used to compare differences between groups within and between groups after post hoc analysis. SPSS

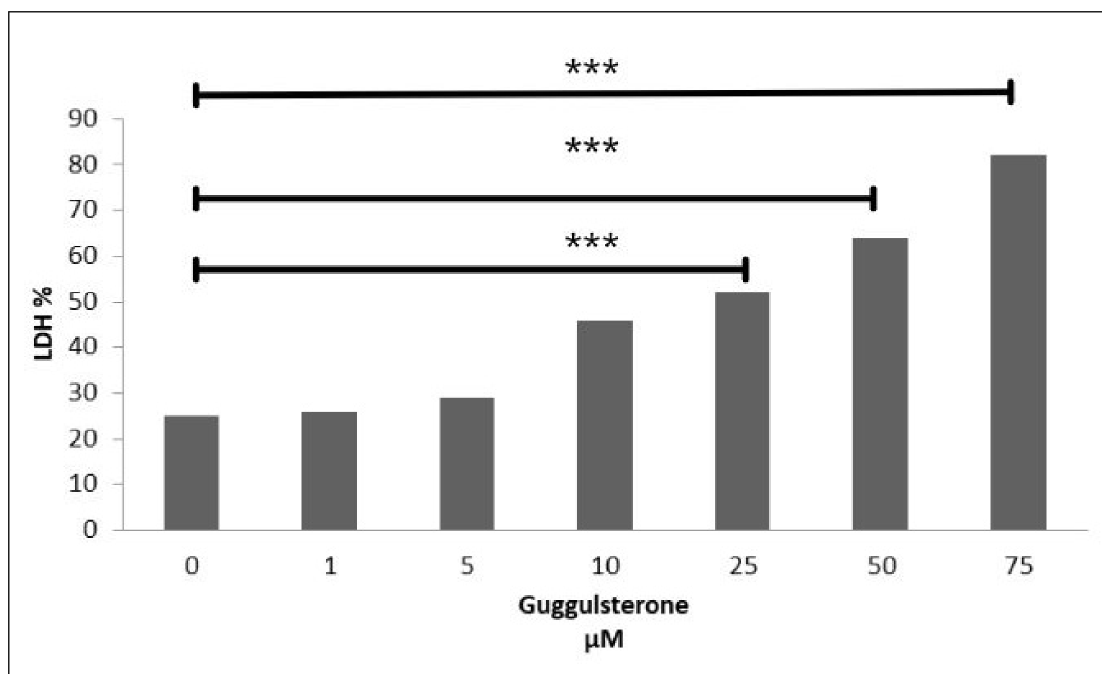

Fig. 3. The osteoblasts were seeded and cultured for 24 hrs, prior to exposure to guggulsterone. LDH levels were measured to evaluate the apoptotic activity of osteoblast cells. . The presented results are LDH level percentage after 24hrs of guggulsterone exposure. Mg 63 cells showed a significant LDH level increase *** $p < 0.01$ under 25, 50 and 75 µM of guggulsterone.
Source: Own materials

version 21.0 (IBM Corp., Armonk, NY, USA) was used for all of those statistical studies.

RESULTS AND DISCUSSION

OSTEOBLAST CELL MORPHOLOGY AND VIABILITY UPON EXPOSURE TO GUGGULSTERONE

This study showed the increasing effect of guggulsterone on actively promoted osteoblast cells. Each concentration was compared to a control medium free from gugglusterone to establish its anticancer potential. Cellular morphology was analyzed using inverted microscopy (Fig. 1). Cellular contact was decreased gradually and apoptotic changes were seen compared to control conditions. Each bar represents the mean ± SD of at least three independent experiments ***$p \leq 0.001$ when comparing the control to the other conditions. The exposure to an increasing concentration of guggulsterone showed an increased in cell roundness and overall reduction in cellular contact (Fig. 1). Osteoblast cells began losing their cell contact and change to round cell morphology after exposure to 1 µM of guggulsterone and this has increased at 5 um (Fig. 1) their viability response was also seen to decrease to 31 ± 1.75 x10^4 compared to control cells 47 ± 1.35 x10^4. Increasing the concentration of guggulsterone inflicted as shown in figure 1. Cells exposed to 10 and 50 µM of guggulsterone showed reduced cellular contact and apoptotic changes which suggests a cytotoxic effect. This is further confirmed through viability study which showed a reduction in cell viability at 10 µM of guggulsterone. Cell viability was reduced from 48 ± 1.45 x104 in control conditions to 24 ± 1.5 x10^4 at 10 µM and 20 ± 1.35 x10^4 at 25 µM of guggulsterone.

Under increased exposure of guggulsterone, osteoblast cells showed increased cellular roundness and decreased cellular adhesion and contact compared to normally grown cells. To validate this further viability test showed a significant decrease in cellular viability. Osteoblast cells exposed to a 50 µM of guggulsterone had a lower viability 14 ± 1.45 x104 compared to control cells 46 ± 1.7 x104 while osteoblast cells were less significantly less viable 8 ± 1.45 x104 at 75 µM compared to control conditions. Overall increased osteoblast cells that have been exposed to increasing guggulsterone concentration showed a reduction in dendrites and increased floating which suggests that cellular adhesion has been lost due to weak cellular growth as well as increased apoptotic changes.

OSTEOBLAST GROWTH FOLLOWING GUGGULSTERONE EXPOSURE

An MTT assay was preformed to confirm the cellular viability and proliferative capacity in each group of osteoblast cells. The cells showed significant decreases upon

exposure to guggulsterone (Fig. 2). Guggulsterone supplemented at 1 μM resulted in a decrease in cellular absorbance 1.43 ± 0.34 in and a significant decrease (p 0.001) 1.34 ± 0.21 at 5 μM when compared to control cells in the same group 1.85± 0.34 (Fig. 2A). A significant reduction in MTT metabolism in osteoblast cells is noticeably lower 1.1 ± 0.2 ($p \leq 0.001$) in cells exposed to 10 μM of guggulsterone and further stronger significant reduction (p 0.001) 0.91 ± 0.18 is seen in 25 μM of guggulsterone indicating compromised cellular metabolism in comparison to control cells which had an absorbance of 1.91 ± 0.4 (Fig. 2B). Cellular proliferation and growth is confirmed by significant reduction ($p \leq 0.001$) in MTT activity through reduced cellular absorbance when cells are exposed to 50 of μM 0.75 ± 0.15. When exposed to 75 μM of guggulsterone the cells showed a very low absorbance 0.49 ± 0.11 which is significantly lower than control cells in the same group which had an absorbance of 1.87 ± 0.34 (Fig. 2C). Moreover Chen and colleagues [25] indicated that guggulsterone induces apoptosis and inhibits lysosomal-dependent migration in human bladder cancer cells. Similarly Wu et al. [26] demonstrated that Z-guggulsterone induces cell cycle arrest and apoptosis by targeting the p53/CCNB1/PLK1 pathway in triple-negative breast cancer.

EFFECT OF GUGGULSTERONE ON CELLULAR RELEASE OF LDH APOPTOTIC MARKER

Evidence from the previous experiment in the current study demonstrated the strong effect of guggulsterone on osteoblast cells. This further confirmed by LDH apoptotic assay to determine the apoptotic behavior and release of cellular LDH. The amount of LDH was measured over a period of 24 hrs post exposure to different concentrations of guggulsterone. The results in figure 3 shows the percentage of LDH released from osteoblast cells under different concentrations of guggulsterone compared to control conditions. A noticeable significant ($P \leq 0.01$) increase in LDH activity is seen at 25 μM of guggulsterone reaching up to 46% when compared to normal conditions while reaching 64% at 50 μM and 82% at 75 μM which were both significant in comparison to control conditions. There was no statistical significant in LDH release under 1, 5 and 10 μM compared to control conditions as shown in figure 3. The significant rise in LDH release indicated the potent cytotoxic activity of guggulsterone on osteoblast cells through morphological changes and growth rate reduction. A study by Wang et al. [27] demonstrated the protective effect of guggulsterone against cardiomyocyte injury induced by doxorubicin in vitro using LDH as a marker for cellular damage.

CONCLUSIONS

In conclusion, this study points out the potential cytotoxic effect of guggulsterone on osteoblast. The noted effects seen in the current study were concurrent with previous evidence shown by previous studies on various other cell lines. The effects provided an insight and solid evidence on the active role of guggulsterone in cellular morphological changes that takes place in cancerous cells during promoted growth. Guggulsterone exposure leads to a decrease in cellular contact and an increase in cell roundness in osteoblast cells. The study suggests that guggulsterone has anticancer potential by inducing apoptotic changes and reducing cellular adhesion and viability in osteoblast cells.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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RECEIVED: 28.09.2025
ACCEPTED: 11.12.2025