Gallic acid Inhibiting TGF-beta and modifying Intrinsic Apoptotic Signalling Pathways to suppress Osteosarcoma Cell Migration, Proliferation and to induce Apoptosis

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Abstract

The most prevalent primary cancer in bone tissues is osteosarcoma (OS). For thousands of years, traditional Chinese medicines (TCM) have been employed and their use offers valuable insights into the treatment of OS. Natural phenolic acid found in a variety of foods and herbs is called gallic acid (GA). GA has a number of well-established pharmacological properties including anti-oxidation and anti-inflammatory properties. Its biological role in OS is still not entirely known, nevertheless. GA's putative anti-cancer qualities were assessed in MG63 cells. In these OS cells, its effects on migration, apoptosis, cell cycle and proliferation were investigated.

The JAK-STAT signalling pathway was identified using experiments using flow cytometry and RT-PCR. Molecular docking was used to examine the in silico impact of GA on proteins. GA was discovered in this work to prevent invasion and metastasis in addition to causing cell cycle arrest and death in OS cells which in turn suppresses tumour growth in vitro. The binding affinity of GA with the JAK-STAT proteins was determined using the Autodock program. When viewed as a whole, our findings showed that GA impeded tumour growth in OS cells by regulating the JAK-STAT, TGF and SMAD signalling pathways.

Keywords: Osteosarcoma, gallic acid, apoptosis, migration, MG-63.

Introduction

Osteosarcoma is the third leading cause of cancer-related mortality in children and adolescents, the most prevalent primary malignant bone tumor (median age of diagnosis: 18 years). Unfortunately, throughout the past few decades, the survival rate, which reaches between 50 and 70 percent after five years, depending on the series, has stayed the same[13]. The typical course of treatment for osteosarcoma involves total surgical excision combined with adjuvant and neoadjuvant chemotherapy which may include doxorubicin, cisplatin, methotrexate, or ifosfamide. Because this kind of tumor is radiation resistant, radiotherapy is rarely performed. Metastasis disorders claim the lives of many individuals; the most prevalent metastatic site for osteosarcoma is the lung[12].

Estimates for bone and joint cancer in 2023 indicate that there will be 2,140 fatal cases and 3,970 new cases; data from the Surveillance, Epidemiology and End Results Programme (SEER) and the American Cancer Society (ACS) show that 67% of children and adolescents with osteosarcoma have received a diagnosis[15]. Gallic acid is a type of phenolic acid that is widely distributed in many foods and herbs. Naturally occurring plants that contain large amounts of gallic acid (3,4,5-trihydroxybenzoic acid) include gallnuts, grapes, sumach, oak bark, peels from green tea apples, grapes, strawberries, pineapples, bananas, lemons and red and white wine. Biological actions of gallic acid include antibacterial, antiviral, anti-inflammatory, antioxidant and anticancer properties.

Additional advantages of gallic acid include its anti-diabetic and anti-angiogenic properties as well as its modulation of MMP-2/9 levels and the cytoskeletal remodelling signal pathway in gastric cancer cells, which enhances nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity[7]. Research indicates that gallic acid can be used to treat multiple malignancies by either inducing apoptosis, suppressing cell viability, or blocking migration. It has also been shown to prevent osteosarcoma cell invasion and migration[4]. The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is thought to be one of the primary pathways for cell function communication.

The JAK/STAT signaling system has been linked to the identification of over 50 cytokines and growth factors including hormones, interferons (IFN), interleukins (ILs) and colony-stimulating factors[5]. A variety of downstream processes are mediated by JAK/STAT including immunological fitness, apoptosis, adipogenesis, tissue repair and hematopoiesis. Many diseases in humans are linked to loss or mutation of JAK/STAT components. Tyrosine phosphorylation of receptors, recruitment of one or more STAT proteins and noncovalent association of cytokine receptors are the functions of JAKs[11]. Tyrosine-phosphorylated STATs regulate particular genes by dimerizing and entering the nucleus through the nuclear membrane. Different STATs have distinct biological effects,

even though they can be activated by partially overlapping cytokines[18].

One of the most prevalent growth factors in the bone matrix is the transforming growth factor (TGF-β). Through stimulating tumor development, triggering the epithelial-mesenchymal transition (EMT), impeding antitumor immune responses, elevating tumor-associated fibrosis and augmenting angiogenesis, TGF-β acts as a promoter of metastasis. Significant interest has been created by numerous studies regarding TGF-β signaling pathway targeting as a potential osteosarcoma therapy[3]. SMADs the Drosophila Mad and C. elegans Sma genes, which are located downstream of the BMP-analogous ligand-receptor systems in these organisms, are the source of the substrates for TβRI kinases, which are shown to have a signaling role.

Within cells, TGF-β signaling is mediated by SMAD proteins its plays a significant role in osteosarcoma cell line. The present investigation explores the potential of gallic acid as a therapeutic agent in managing osteosarcoma by examining its inhibitory effects on transforming growth factor-beta (TGF-beta) signaling, a pathway known to promote tumor growth and metastasis in osteosarcoma. Additionally, the research looks at how gallic acid modulates intrinsic apoptotic signaling pathways, thereby suppressing cell migration and proliferation while inducing apoptosis in osteosarcoma cells.

Material and Methods

Cell line maintenance: Human osteosarcoma cell lines (MG-63) were obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM and RPMI supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37◦C in a humidified atmosphere containing 5% CO_2. Upon reaching confluency, the cells were trypsinized and passaged.

Cell viability (MTT) assay: The cell viability of gallic acid treated osteosarcoma cell line was assessed by MTT assay. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Osteosarcoma cell line was plated in 96 well plates at a concentration of 5x103 cells. Cells were washed twice with 100µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, cells were treated with different concentrations of gallic acid (25- 250µM/ml) for 24 hours. At the end of treatment, the medium from control and gallic acid treated cells were discarded and 100µl of MTT containing DMEM (0.5 mg/ml) was added to each well.

The cells were then incubated for 4h at 37ºC in the CO_2 incubator. The MTT containing medium was then discarded and the cells were washed with 1x PBS. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100µl) and incubated in dark for an hour. The intensity of the color developed was assayed using a Micro Elisa plate reader at 570 nm. The number of viable cells was expressed as percentage of control cells cultured in serum-free medium. Cell viability in control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells]×100.

Morphology study: Based on MTT assay, we selected the optimal doses (IC-50: 150µM/ml for osteosarcoma cell line and 100 and 150µM/ml for MG-63 cell line) for further studies. Analysis of cell morphology changes by a phase contrast microscope. 2×105 cells was seeded in 6 well plates and treated with gallic acid for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope.

Determination of mode of cell death by acridine orange (AO)/ethidium bromide (EtBr) dual staining: The effects of gallic acid on osteosarcoma cell death were also determined by AO/EtBr dual staining as described previously. The cells were treated with gallic acid for 24 h and then the cells were harvested and washed with ice-cold PBS. The pellets were resuspended in 5 µl of acridine orange (1 mg/mL) and 5 µl of EtBr (1 mg/mL). The apoptotic changes of the stained cells were then observed by using a fluorescence microscope.

Real Time PCR: The gene expression of apoptosis signaling molecules was analysed using real-time PCR. The total RNA was isolated by the standardized protocol using trizol reagent (Sigma). 2µg of RNA were used for cDNA synthesis using reverse transcription using a PrimeScript 1st strand cDNA synthesis kit (TakaRa, Japan). The targeted genes were amplified using specific primers. PCR reaction was performed with GoTaq® qPCR Master Mix (Promega). It contains SYBR green dye and all the PCR components. Real time-PCR was performed in a CFX96 PCR system (Biorad). The results were analyzed by comparative CT method and $2^{-\Delta\Delta CT}$ method was used for fold change calculation.

Using a flow cytometer to evaluate cell cycles: A total of 1 × 106 MG-63 cells were grown on 100-mm culture plates supplemented with growth medium. After a period of fasting, the cells were subjected to a 24-hour treatment with 60 M/ml gallic acid, collected using 0.25% trypsin and centrifuged at 3000xg for five minutes. The cells were then washed with PBS.

Following centrifugation, the cells were frozen in 70% ice-cold ethanol at -20°C for a whole night. Subsequently, the cells were cultured in PBS with 50 g/ml propidium iodide and 1 mg/ml ribonuclease for 30 minute. BD FACSCantoTMII (Becton and Dickinson Biosciences, Mountain View, CA, USA) was used for cell cycle investigations and BD FACSC anto clinical software was used for data analysis.

Real-Time PCR: Real-time PCR was used to analyse the apoptotic signalling molecules' gene expression. Using the trizol reagent (Sigma), a standardised process was followed to extract the total RNA. PrimeScript first strand cDNA synthesis kit (TakaRa, Japan) and 2µg of RNA were used for reverse transcription-based cDNA synthesis. Using particular primers, targeted gene amplification was achieved. The GoTaq® qPCR Master Mix (Promega), which included SYBR green dye and other necessary PCR components was used to conduct the PCR reaction. PCR was carried out in real time using a Biorad CFX96 PCR device. The comparative CT approach was utilised to analyse the results and Schmittgen and Livak's 2−ΔΔCT method was employed to calculate the fold change.

Assay for scratch wound healing: Six-well culture plates were seeded with MG-63 cells (2×105 cells/well). Using a 200µl tip, the cell monolayer was scratched to produce a wound and then it was cleaned with PBS and captured on camera using an inverted microscope. After receiving gallic acid for 24 hours and control cells receiving DMSO (0.01%) in culture medium in lieu of gallic acid, the injured area was captured on camera using the same microscope. Furthermore, for every treatment group, the studies were conducted three times.

Molecular docking: The NMR and crystal structures of the target protein, namely JAK 2 and STAT3 (PDB ID – 6AAJ and 6NJS), were obtained from the Protein Data Bank (PDB). The protein structures were pre-processed and minimized using Pymol and Swiss PDBViewer. In AutoDock, the processed macromolecules were imported, Hydrogen atoms were appended to the polar residues. Atom types were assigned to AD4 classification and Kollman charges were applied to the atoms and the net charge was distributed accordingly. The ligand Mahanine (PubChem CID: 10281) structure was obtained from PubChem in SDF format and subsequently converted into PDBQT format. This ligand was then incorporated into AutoDock, where the torsion roots were identified.

The grid box was created based on the amino acid residues that interact with the co-crystal ligand in the above structures. The molecular docking simulations were conducted using the AutoDock algorithm, which involved running 20 genetic search algorithm iterations for each protein target. Subsequently, the docked complex with the highest binding energy was retrieved and subjected to interaction analysis.

Statistical analysis: All of the data were examined using SPSS and were expressed as mean ± SD for triplicate. A one-way ANOVA was followed by a student's-t-test. The level of statistical significance was set at $p<0.05$.

Results and Discussion

Cell Viability Assay: After 24 hours of treatment, the GA-treated cells were subjected to the cell viability assay. In a dose-dependent manner, GA slowed the proliferation of MG -63 osteosarcoma cancer cells. With an IC50 of between 20 and 40 µg/ml, the cell growth was markedly suppressed (Figure 1). According to Otçu et al[10], the GA and Dox treated group exhibited the highest amount of cytotoxicity as measured by MTT. After IC50 calculations, it was discovered that Dox had an IC50 of 124.6 for 48 hours while GA had an IC50 of 242.4. The A549 cell line, which represents 85% of lung cancer cases, was found to be significantly inhibited by GA or CDDP in terms of cell viability by Ko et al[8]. They also discovered that the viability of human lung cancer cells was impacted by GA in a dose-dependent manner. In A549 cells, the IC50 value of GA was 74.15 µM.

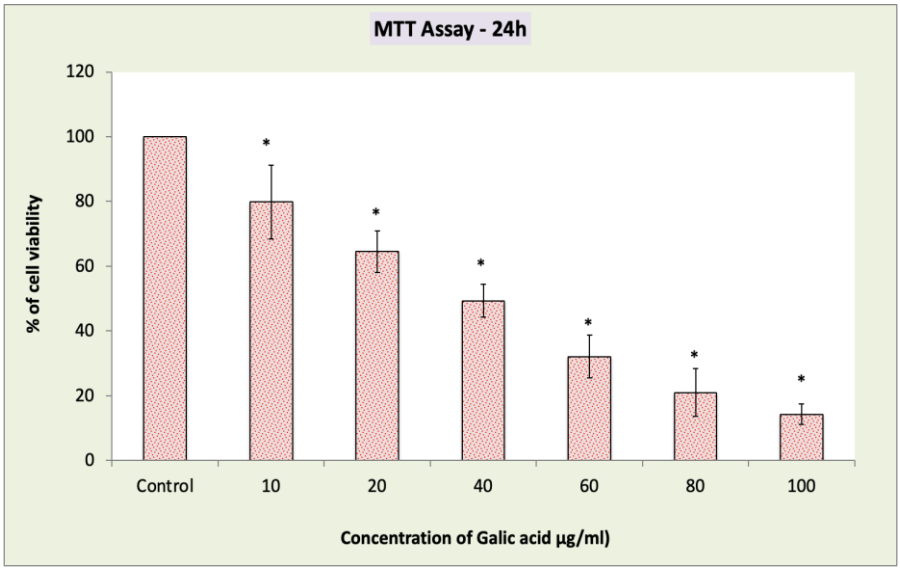

Fig. 1: The cytotoxic effects of Gallic acid on osteosarcoma cancer cells. Cells were treated with Gallic acid (10 - 100µg/ml) for 24h and cell viability was evaluated by MTT assay. Data are shown as means ± SD (n = 3). * compared with the control blank group, p < 0.05.

Cell morphological changes (MG-63 cells): The GA-treated cells exhibit typical alterations, such as shrinkage and membrane blebbing, when compared to the untreated cells. These modifications include the induction of apoptosis upon treatment with GA. In order to obtain high-resolution morphological images of the MG-63 cells, an inverted phase contrast microscope was used in addition to the digital microscopic image analysis. Figure 2 shows pictures of MG-63 cells that were not treated and MG-63 cells that were treated with GA for 24 hours. The pictures clearly demonstrate that the GA-treated cells exhibited standard apoptotic symptoms, such as shrinkage and membrane blebbing. Normal cells, however, did not exhibit any discernible shrinking. Thus, the signals of apoptosis caused by GA were further supported by the microscopic images.

Additionally, the microscopic inspection indicated rough, shrinking, irregularly shaped cells and Aye et al[1] observed that declines in cell density were associated with cell death. Notably, they employed greater doses of GA and Pira due to evident alterations in the morphology of the cells. The microscopic analysis of the cytotoxicity showed that 30 µg/ml and 60 µg/mL of boldine treatment respectively, resulted in changed morphology and less HCT-116 and Saos-2 cells[2].

AO/EtBr (Acridine orange/Ethidium Bromide dual staining)–MG-63 cell: Green nuclei are shown in live cells with normal DNA when stained with AO/EB double-staining, but orange and red nuclei are seen in necrotic and apoptotic cells with fragmented DNA. The number of apoptotic cells was substantially higher in the GA (40 µg/ml) than in the GA (20 µg/ml)-treated group, according to our results (Fig. 3).

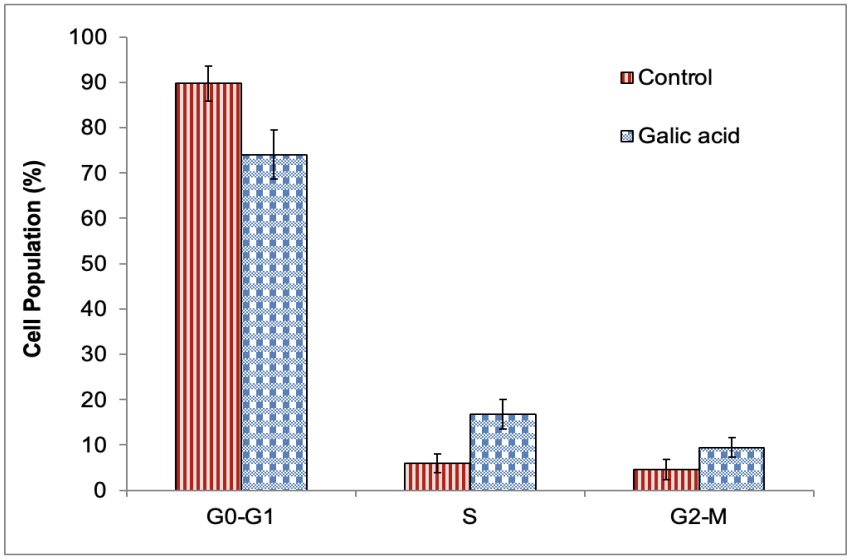

Fig. 2: Effect of Gallic acid on cell morphology of human osteosarcoma cells (MG-63). Cells were treated with Gallic acid (20 and 40µg/ml) for 24 h and cells were observed under an inverted phase contrast microscope. The number of cells decreased after Gallic acid treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.

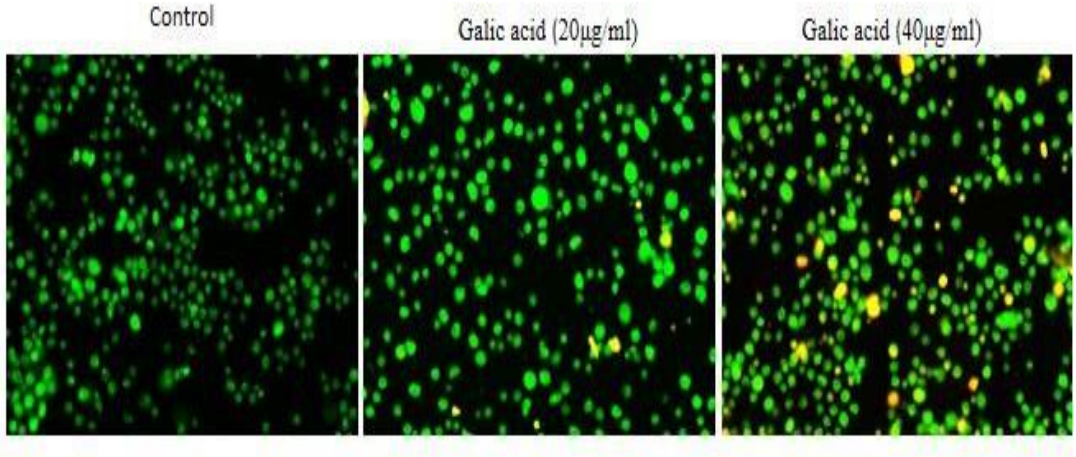

Fig. 3: AO/EtBr (Acridine orange/Ethidium Bromide dual staining) – MG-63 cell Human osteosarcoma cells were treated with Gallic acid (20 and 40 µg/ml) for 24h along with the control group. After the treatment, the cells were incubated with AO/EtBr dual staining. Images were obtained using an Inverted Fluorescence Phase contrast microscope.

According to Shruthi et al[14], EA cells had condensed nuclei and looked bright green, while LA cells had shattered nuclei and were tinted orange. Untreated control cells were found to have intact nuclei and plasma membranes; they also appeared green when stained with AO. The presence of N cells in CPT-treated MCF-7 cells was demonstrated by EtBr penetrating dead cell membranes and staining their nuclei red. When MCF-7 cells were treated with GA (5 µg/mL)+CPT (10 µg/mL), the EA and LA cells were seen. AO/EB staining was used to ascertain whether the cytotoxicity was caused by cell apoptosis.

Green, yellow and orange dyes were used to indicate early apoptotic, late apoptotic and normal live cells respectively. The number of apoptotic cells rose and the number of viable cells were dramatically lowered by the boldine treatment. Consequently, boldine caused HCT-116 and Saos-2 cells to undergo apoptosis[2].

Realtime- PCR: Additionally, the apoptotic genes' gene expression was examined. The expression of the proteins Bax, p53 and caspase-3 was dramatically up-regulated in comparison to the control group, but the expression of the protein Bcl-2 was significantly down-regulated.

Similarly, the gene expression of gallic acid was performed for the apoptotic genes. Gene expression analysis was also done. The Bcl-2 protein was dramatically down-regulated in comparison to the control group, whereas the expression of the proteins Bax, p53 and caspase-3 was significantly up-regulated[19].

Flow cytometry: Apoptosis and cell cycle inhibition are directly connected. Therefore, gallic acid (200µM/ml)-induced apoptosis was investigated further using a flow cytometry assay. The propidium iodide double staining technique was used to analyse apoptosis. The percentage of apoptosis was G0-G1:90.10%, S: 8.56% and G2/M: 6.67% for the control group following a 24-hour treatment with different dosages of gallic acid. In contrast, the MG-63 cells treated with gallic acid showed G0-G1: 74.61%, S: 9.12% and G2/M: 6.28%, as shown in figure 5. These findings demonstrate that the percentages of S phase and G2/M phase cells in MG-63 treated with gallic acid were higher than in the control group. These results showed that by interfering with proteins linked to the cell cycle and proliferation, gallic acid inhibited proliferation and caused cell cycle blockage.

The effects of GA on the cycle of HCC1806 cells were evaluated by Lin et al using flow cytometry. In the control group, the percentage of HCC1806 cells was 50.47% ± 1.63% in the G0/G1 phase, 23.32% ± 2.25% in the S phase and 25.88% ± 2.13% in the G2/M phase. In comparison with the control group, the percentage of G0/G1 phase in GA treatment groups (200, 250, 300 µM) was 39.18% ± 0.30%, 37.27% ± 0.22%, 36.87% ± 0.14% respectively. The percentage of G0/G1 phase in GA treatment groups was significantly decreased. In comparison with the control group, the percentage of S phase in GA treatment groups (200, 250, 300 µM) was 31.37% ± 1.07%, 34.06% ± 1.27% and 34.16% ± 1.27% respectively. The percentage of S phase in GA treatment groups was significantly increased. According to their findings, GA prevented the S phase of the HCC1806 cell cycle.

RT-PCR: Numerous physiological and pathological processes, including body homeostasis, cell elimination and embryonic development, are influenced by programmed cell death or apoptosis. In an effort to enhance patient prognosis, a number of research seek to comprehend the connection between apoptosis and the development of cancer. The goal of this study was to employ gallic acid intrinsic apoptotic mechanism to induce apoptosis in osteosarcoma cells.

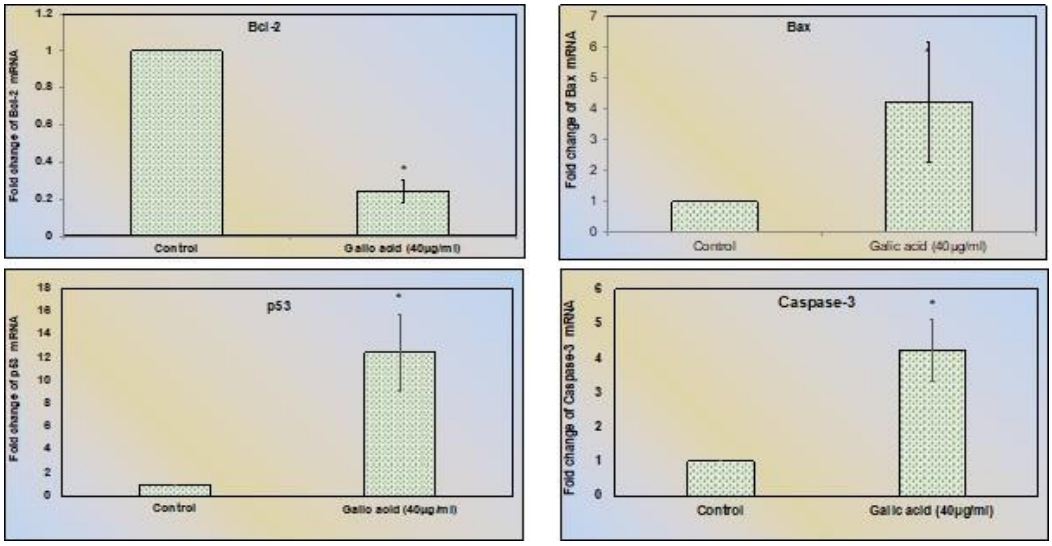

Fig. 4: Effect of Gallic acid (40µM/ml) on pro-apoptotic genes (p53, Bcl-2, Bax and Caspase-3) expression in osteosarcoma cell line. Target gene expression is normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents mean + SEM of three independent observations. '*'represents statistical significance between control versus drug treatment groups at p<0.05level.

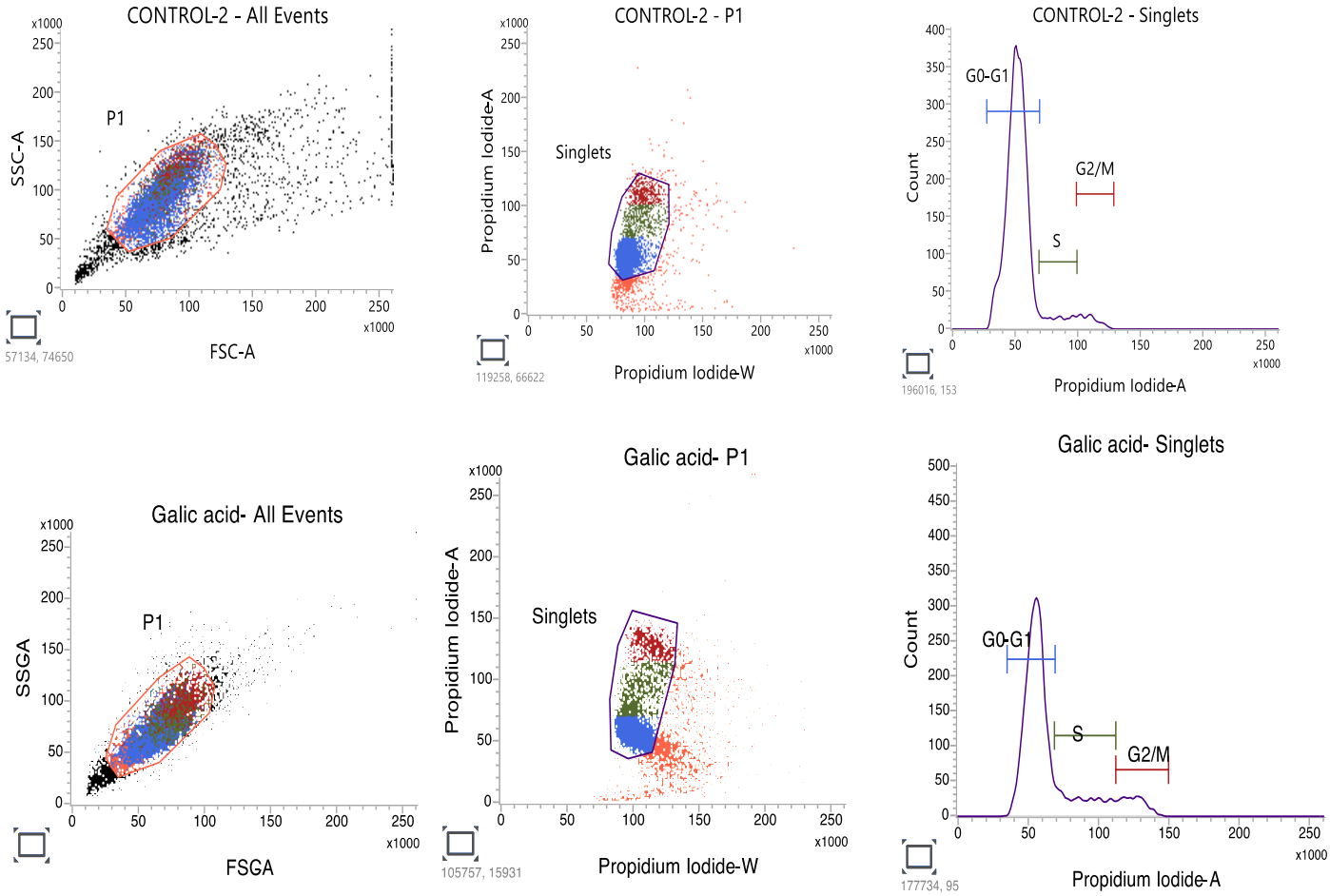

Fig. 5: Effect of gallic (40µM/ml) on cell cycle analysis in osteosarcoma cells. The cell cycle analysis was evaluated using propidium iodine (PI) staining followed by flow cytometric analysis. A. Representative plots of showing PI staining of osteosarcoma cells treated with the thymoquinone. B. Results of 3 independent experiments. Data represent the mean±S.D. of three independent experiments.

The YGF and SMAD -2 apoptotic genes in the MG-63 cells were measured to assess the impact of gallic acid on these genes.

Figure 6 demonstrates that gallic acid (40 µM/ml) dramatically increased the mRNA expression of apoptotic genes in the MG-63 cell line as compared to the untreated control cells. Simultaneously, in this MG-63 osteosarcoma cell line, gallic acid lowered the expression of the anti-apoptotic gene TGF and raised the expression of pro-apoptotic genes like SMAD-2.

The potential of gallic acid in cell cycle analysis was assessed by Sourani et al[16] using the lymphoblastic leukaemia cell line (C121). The results showed that the percentages of apoptotic cells increased significantly in the GA treated cells when compared to the control group (without treatment). At concentrations of 10, 30, 50 and 80 µM, the mean apoptosis was 25%, 35%, 50% and 86% respectively while it was 3.80% in the control group. Lin and colleagues[9] have investigated the expression of associated genes and proteins. The expression of Bcl-2 protein was significantly reduced while the expression of Bax and P53 proteins was significantly increased in the GA treatment groups when compared to the control group.

Additionally, there was a significant increase in the ratio of cleaved-Caspase-3/Caspase-3 and cleaved-Caspase-9/Caspase-9. In the meantime, the expression of Bcl-2 mRNA was dramatically reduced in the GA treatment groups compared to the control group while the expression of Bax, Caspase-3, Caspase-9 and P53 mRNA was significantly elevated. The conclusion was that the mitochondrial apoptosis pathway was connected to the apoptosis induction of HCC1806 cells by GA.

Scratch wound healing assay: In this experiment, MG-63 cells were moved and an *in vitro* wound healing assay was used to evaluate the impact of gallic acid. Following the initiation of medicines, the number of cells that had migrated into the area that had been scratched, were counted and converted into a percentage of migration using pictures. MG-63 cells were tested for their ability to migrate using a wound-healing experiment is shown in figure 3.

Following a 24-hour incubation period for injured cells, the groups treated with 40 µM/ml gallic acid respectively, showed an increase in the percentage wound width. The

study's findings showed that this dramatically and independently decreased MG-63 cells' migration.

The experiment for wound healing demonstrated by Truzzi et al[17] varying amounts of the two phenolic acids might either promote or inhibit the healing of wounds in intestinal tissue. Over the course of 48 hours, commencing 4 hours after the stimulus was added and ending 48 hours later, cell migration was observed. The effects that were most intriguing were shown 24 hours following the therapy. A dose of 40 mg/L decreased wound healing, while ferulic acid shown a substantial stimulating effect at 5, 10 and 20 mg/L in comparison to the control. In reference to gallic acid, fibroblast migration was enhanced at 2.5 and 5 mg/L, but this action was blocked at 40 mg/L.

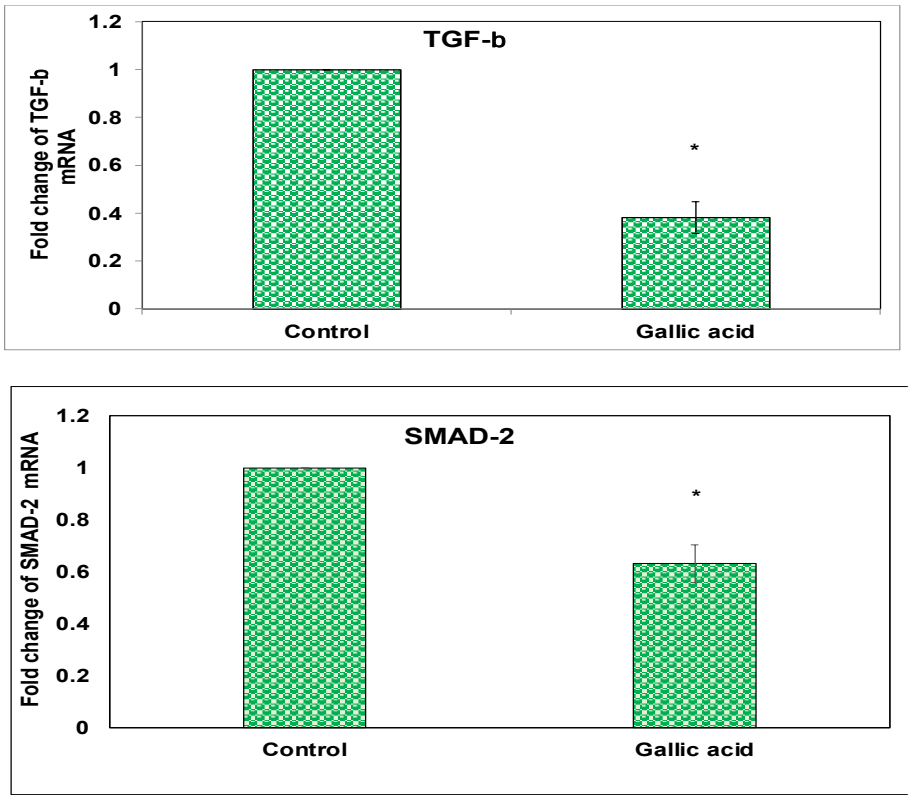

Figure 6: Effect of Gallic acid (40µM/ml) on TGF/SMAD -2 genes expression in the osteosarcoma cell line. Target gene expression is normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents mean + SEM of three independent observations. '*' represents statistical significance between control versus drug treatment groups at p<0.05 level.

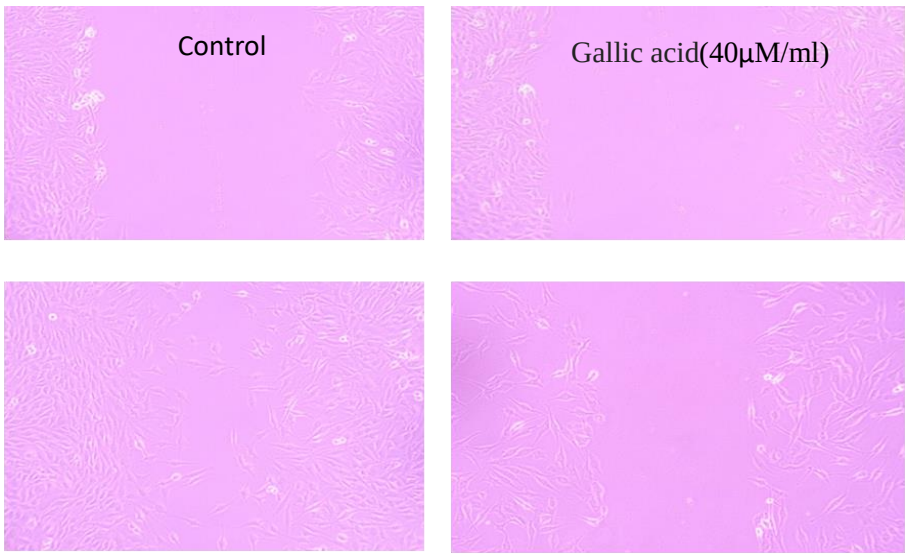

Figure 7: *In vitro* scratch wound healing assay. Human osteosarcoma cells were injured and cell migration assay with and without treatment of gallic (40 µM/ml) was performed at 24h. Images were obtained using an inverted Phase contrast microscope

Table 1
Molecular docking of JAK, TYK2 and STAT-3 with gallic acid.

S.N.	Target Name	Complex	Binding Energy	Interacting Amino acids
1	JAK 1	3	-0.80	-
2	TYK 2	5	-5.35	Hydrogen Bonding: VAL 981
3	STAT 3	6	-4.46	Alkyl bonding: MET 648, ILE 653

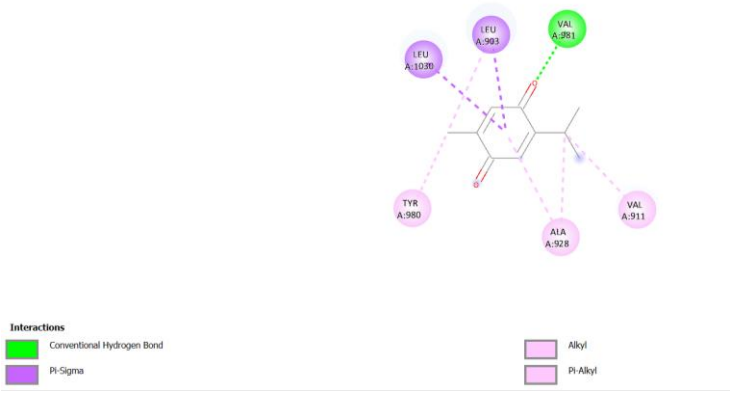

Figure 8: 2D of gallic acid with TYK2

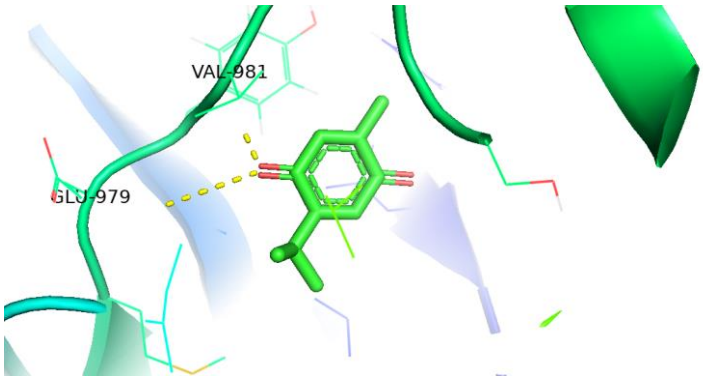

Figure 9: 3D of gallic acid with TYK2

Molecular docking: From the molecular docking analysis, the compound thymoquinone binds to TYK2 and STAT3 of JAK-STAT pathway and it could act as a potential inhibitor and modulate JAK-STAT pathway. When comparing thymoquinone, it binds to TYK2 with high binding affinity than JAK 1. This binding affinity changes because of binding of the compound to different pockects. On further studies, this compound will reveal the lung cancer migration with unique perspective targeting TYK2 protein.

Conclusion

According to the current research, gallic acid therapy for osteosarcoma may be a beneficial course of action. Based on all findings, we deduced that GA inhibited metastasis induced apoptosis and cell cycle arrest and suppressed cell proliferation in OS cells in order to perform its anti-cancer effect. It was shown that GA dramatically reduced Bcl-2, which in turn caused the disruption of Jak-STAT signalling in OS cells. These findings offer new perspectives on the treatment of cancer by combining traditional pharmacotherapy with molecular target therapies that works synergistically.

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