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# Research Article
# Transcriptome Analysis and Characterized Differentially Regulated Genes Between Treated and Untreated SaOS-2 Cells with Baicalein

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## Abstract

**Background and Objective:** Baicalein is an effective anti-cancer treatment of several cancer types. Although numerous studies on baicalein efficacy have been published, the specific mechanism of its cancer inhibition remains unclear. Present study was aimed to provide detailed molecular targets and signaling pathways that baicalein regulated, which may contribute to better application of baicalein in osteosarcoma cure. **Materials and Methods:** In this study, after treatment of human osteosarcoma cells lines SaOS-2 with baicalein, the CCK-8 assay was used to determine cell viability. The RNA sequencing was used to examine whole transcriptome variation and analyzed by Gene ontology, Kyoto Encyclopedia of Genes and Genomes and Hierarchical clustering. Apoptosis was studied by flow cytometry. Wound healing, adhesion, ATP assays followed by evaluating the expression of mRNA and protein of COL1A2, COL6A1, LAMA5, ITGA4, ITGB1, MMP2 and BINP3 via quantitative real-time PCR and western blot respectively. **Results:** The dose of 25 μg $mL^{-1}$ of baicalein inhibited the cells proliferation in time dependent manner. The extracellular matrix receptors including the integrin (ITGA4 and ITGB1), collagen (COL1A2, COL6A1) and laminin (LAMA5) were significantly repressed in challenged cells groups at messenger RNA and protein expression level. Furthermore, the migration, adhesion as well as ATP level were statically reduced, whereas cell apoptosis was stimulated. **Conclusion:** The result supported that baicalein promotes the SaOS-2 apoptosis process. This study suggests that baicalein may be used alone or with other anti-cancer treatments to reverse ECM receptor-associated malignant phenotype of human cancers.

Key words: Baicalein, RNA sequencing, bone cancer, apoptosis, extracellular matrix receptors

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Osteosarcoma (OS) is a primary malignant bone tumor that mainly presents in children and teenagers[1]. OS develops through a series of genetic and epigenetic aberrations causing dysregulation of core cell signaling pathways involved in malignant behavior, growth and therapy-resistance[2].

Baicalein is a Chinese herbal medicine that is a bioactive flavonoid compound which can be isolated from the *Scutellaria baicalensis* Georgi roots[3]. Recent studies have demonstrated that baicalein has cytoprotective effects, anti-inflammatory agent and acts as an antioxidant[4-7]. Moreover, baicalein prompted cellular irregularities in different human cancer cell lines by altering multiple signaling pathways, including: cell apoptosis and caspase activation, cell proliferation, death receptor mitochondrial, protein kinase and tumor suppressor pathways[8,9]. Baicalein was confirmed to show anti-metastatic and anti-proliferative properties in osteosarcoma, but, the mechanism of its effects remains unclear[10]. Recently, more studies have focused on searching for natural chemopreventive combinations able to inhibit or improve the multistep carcinogenesis with minimum toxicity. In recent decades, more than 70% of cancer drugs are either natural or based on natural products[11].

Here, RNA-seq was applied to gain a global vision of baicalein-induced whole transcriptome adaptation in human osteosarcoma (SaOS-2) cell lines. After a systemic analysis of baicalein-induced whole transcriptome variation, the current study provided detailed molecular targets and signaling pathways effected by baicalein, which contributes to better application of baicalein in bone cancer treatment.

## MATERIALS AND METHODS

This study was carried out in the Beijing key laboratory of traditional Chinese veterinary medicine, Beijing University of Agriculture, during the period from January, 2017 to June, 2019.

**Reagents and cell culture:** Baicalein (purity: 98%) was purchased from the National Institutes for Food and Drug Control (Beijing, China). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Baicalein was dissolved in 100% DMSO to prepare a 10 mg stock solution, stored at -20°C.

The SaOS-2 osteosarcoma cell line in this study was obtained from Peking Union Medical College Hospital. The cells were grown in McCoy's 5A medium with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) supplemented with penicillin-streptomycin. The cell line was routinely cultured in 25 $cm^2$ culture flasks, 96-well or 6-well plates and maintained at 37°C in a humidified 5% $CO_2$ atmosphere. Culture medium was refreshed every 3 days.

**Cell assays:** All assays were done as previously described with minor modifications[12,13]. Briefly, SaOS-2 Osteosarcoma cells were cultivated in 6-well plates and incubated to approximately 75% confluence, at which point the cells were treated with concentration of 25 μg $mL^{-1}$ baicalein in either McCoy's 5A or control media, unless otherwise stated.

**CCK-8 and ATP assessment:** The cell viability after baicalein treatment was measured with Cell Counting Kit-8 (CCK8) assay. Briefly, after confluence, the cells were treated with different concentrations of baicalein. After incubation for 24, 48 or 72 h, the medium in each well was replaced with free FBS culture medium containing 10 μL CCK8 (Dojindo, Japan)[14]. The plates were incubated at 37°C for an additional 3 h. The absorbance was then recorded at OD450 nm using a MK3 Microplate Reader (Thermo, Waltham, MA, USA). Additionally, intracellular ATP was extracted from SaOS-2 cells and the concentration was determined by the ATP colorimetric kit (A095, Jiancheng Bioengineering, Nanjing, China)[15]. All tests were performed with three biological replicates.

**RNA sequencing:** SaOS-2 cells were lysed using Trizol method (Invitrogen) and the RNA was extracted in the chosen time points. The RNA-seq process and data analysis were employed according to Song *et al.*[16]. And quantitative real time (qRT) PCR were done as previously described[17-20].

**Flow cytometry analysis:** After treatment with baicalein for 48 or 72 h, SaOS-2 cells were harvested. Untreated cells were used as a control. Apoptosis assay was performed with an Annexin V FITC-A/PI apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's guidelines. Briefly, cells were washed twice with cold PBS buffer and resuspended in the 1×binding buffer at a concentration of ~$1 \times 10^6$ cells $mL^{-1}$, 5 μL of Annexin V FITC-A conjugate and 5 μL of PI solution were added to each 500 μL cell suspension. Cells were stained by Annexin V FITC-A/PI for 15 min at room temperature in darkness. Stained samples were analyzed using BD LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) and the apoptosis rate was determined with FACS Diva 6.0 software[21].

**Adhesion assay:** SaOS-2 cells were pre-challenged with baicalein for 48 or 72 h, trypsinized, harvested, plated and incubated for 2 h on 96-well microplates that were previously layered with 100 μg $mL^{-1}$ Matrigel™ (Sigma-Aldrich, St Louis, MO, USA). Next, each group was washed for 0.5-1 h to discard any free cells. The CCK-8 test was used to evaluate the cell adhesion capability through absorbance measurements at OD450 nm with an MK3 Microplate Reader (Thermo, MA, USA).

**Wound healing assay:** The cell monolayer was wounded with a sterile 100 μL pipette tip to make a narrow wound-like gap as previously described[17]. Briefly, medium was discarded and rinsed twice with PBS to remove cell debris and free cells, the cells were treated with baicalein in either McCoy's 5A or control media. After 48 or 72 h, the wounds were assessed under the microscope.

**Western blot assay:** After confluence, cells were treated with baicalein in either McCoy's 5A or control media. Procedures followed Fan *et al.*[13].

**Statistical analysis:** All statistical analysis were carried out using SPSS 20.0 software. Data are listed as mean value±SD. Student's t-test was used when the variance between samples are similar. The $p \leq 0.05$ was considered statistically significant.

## RESULTS

**SaOS-2 cells proliferation assay:** Before studying the transcriptome of SaOS-2 cells treated by baicalein, CCK8 assay was employed in order to identify any anti-proliferation effect. As shown in Fig. 1, baicalein significantly decreased the number of SaOS-2 cells in a time-dependent manner. Among the concentrations tested, 25 μg $mL^{-1}$ was most effective at both 48 and 72 h following treatment, for this reason, this dosage level was applied with additional assays.

**RNA-seq analysis:** The transcriptome analysis of SaOS-2 cells assessed baicalein treatment after 48 and 72 h. The fragment statistical information was listed in Table S1. The RNA-seq data was reported from both baicalein-treated and control SaOS-2 cells with Q30 >90% (Table S2). The transcriptome of baicalein-treated SaOS-2 cells showed significantly different from control cells (Fig. S1). Pair-wise comparisons, detected the significantly different genes between groups (Table 1).

Gene ontology (GO) pathway analysis identified many terms, the 10 most significantly enriched GO terms were listed in Fig. S2. The three most significant biological terms that were downregulated after treatment in a time-dependent manner were: extracellular matrix organization, extracellular structure organization and anatomical structure morphogenesis

Table S1: Statistical summary of the mapping results (Median)

| Sample name | Raw pairs | Trimmed | mtRNAs (%) | rRNAs (%) | Mapped (%) | Unmapped (%) |
|---|---|---|---|---|---|---|
| C48 | 16471205 | 16469164 | 9.42 | 2.43 | 90.27 | 9.73 |
| C72 | 20882810 | 20879089 | 10.93 | 2.80 | 89.76 | 10.24 |
| M48 | 17862831 | 17859354 | 10.10 | 4.99 | 89.18 | 10.82 |
| M72 | 18561224 | 18557342 | 10.72 | 5.04 | 89.85 | 10.15 |

mtRNA, rRNA: Fragments aligned to rRNA, mtRNA, Raw Pairs: Raw sequencing fragments number (read pairs), Trimmed: Fragments number (read pairs) after 5', 3'-adaptor trimmed and ltered ≤16 bp reads. mtRNAs (%): Proportion of mtRNA fragments (read pairs) in Trimmed pairs, rRNA (%): Proportion of rRNA fragments (read pairs) in Trimmed pairs, Mapped (%): Proportion of fragments number (read pairs) aligning to reference genome in Trimmed pairs, Unmapped (%): Proportion of not aligned fragments number (read pairs) in Trimmed pairs, C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h

Table S2: Quality score data, the Q30 (%) (sequencing error rate <0.1%)

| Sample name | Reads count | Bases number | Bases number (Q≥30) | Q30 (%) |
|---|---|---|---|---|
| C48 | 32942410 | 4941361500 | 4442350310 | 90.34 |
| C72 | 41765620 | 6264843000 | 5647035324 | 90.14 |
| M48 | 35725662 | 5358849300 | 4873594715 | 90.09 |
| M72 | 37122448 | 5568367200 | 5024674834 | 90.37 |

C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h

Table 1: Number of the different expression genes (DEGs) in the comparisons of treated to untreated cells

| | Down \|log2 (FC)\|<-1 | | | Up \|log2 (FC)\|>1 | | |
|---|---|---|---|---|---|---|
| Express gene number | All | p≤0.05 | p≤0.01 | All | p≤0.05 | p≤0.01 |
| 48 h | 586 | 566 | 548 | 777 | 758 | 722 |
| 72 h | 904 | 885 | 832 | 960 | 941 | 851 |

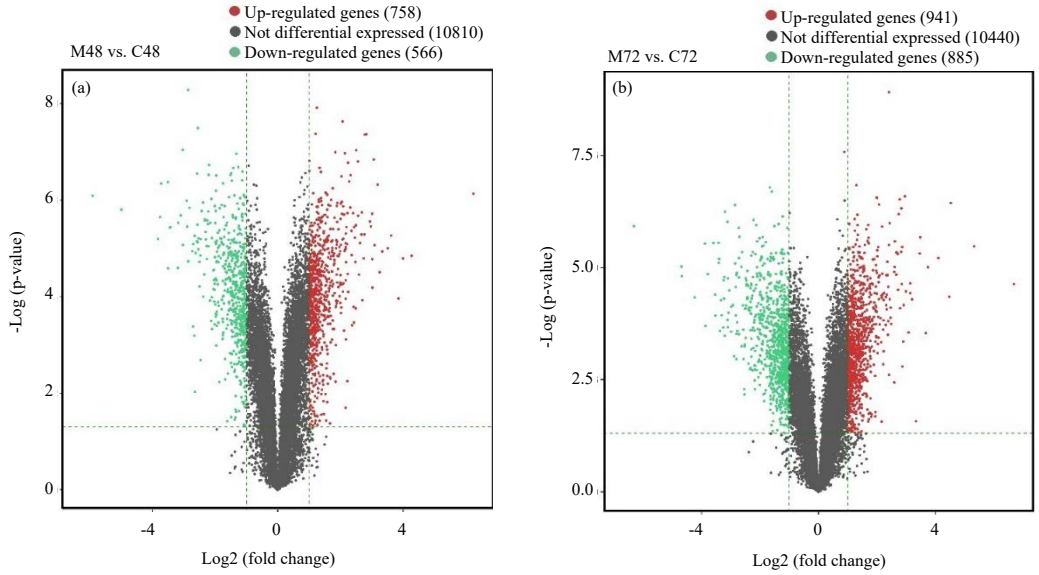


Fig. S1: Values of X and Y axes in the volcano plot are (a) Log2 transformed fold change and (b) Log10 transformed p-values between the 2 groups, respectively

Red/Green circles indicate statistically significant differentially expressed genes with fold change no less than 2 and p-value = 0.05, (Red: up-regulated, Green: Down-regulated), Gray circles indicate non-differentially expressed genes, with FC and/or p-value are not meeting the cutoff thresholds, C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h

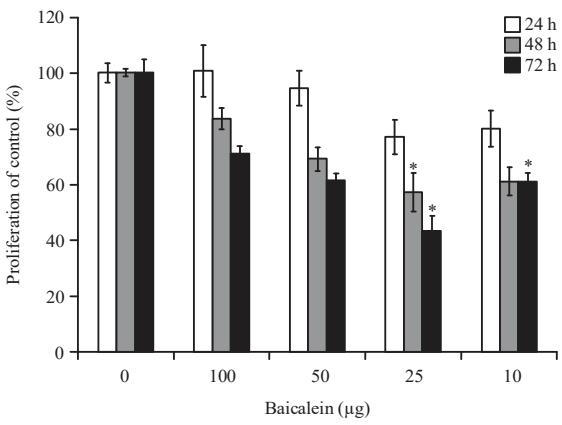


Fig. 1: Cells were incubated with different concentrations of baicalein for 24, 48, or 72 h and cell proliferation was analyzed using the CCK-8 cell proliferation assay kit

Data are represented as the Mean±SD of 3 determinations, p = 0.05 vs. the control group, *p = 0.01 compared to control

(p≤0.01). While the three most significant biological terms for upregulation were: regulation of nucleic acid, regulation of RNA biosynthetic process and regulation of nitrogen compound metabolic process (p≤0.01).

Pathway enrichment analyses were based on latest Kyoto encyclopedia of genes and genomes (KEGG) database. 28 pathways were significantly down regulated (p<0.05) while 43 and 44 pathways were significantly up regulated in 48 and 72 h, respectively. The top 10 upregulated and down regulated pathways were listed in Fig. S3. The most significantly down regulated pathways were: ECM-receptor, focal adhesion, PI3K/Akt signaling and proteoglycans in cancer (p≤0.01). The most significantly upregulated pathways were: homologous recombination, autophagy-animal, mTOR signaling and FoxO signaling (p≤0.01). These results were matched with observed phenotypes of the baicalein-treated SaOS-2 cells, including the induction of cell death and suppression of cell proliferation (osteoblast differentiation, cell adhesion and PI3K-Akt signaling pathways). Also, the ECM-receptor interaction pathway was suppressed after baicalein treatment, suggesting that baicalein may lead to repress SaOS-2 cell adhesion.

**Hierarchical clustering of differentially expressed genes:** A clustering method was employed to analyze gene expression. For the analysis of gene expression samples was arranged into various groups based on their expression level (FPKM values), which is allowed to assess the relationships among different samples. The dendrogram showed the relationships among mRNA expression patterns among different samples. Two main clusters of genes were observed: Fig. 2 shown down regulated genes (ECM-receptor, focal adhesion, PI3K/Akt,

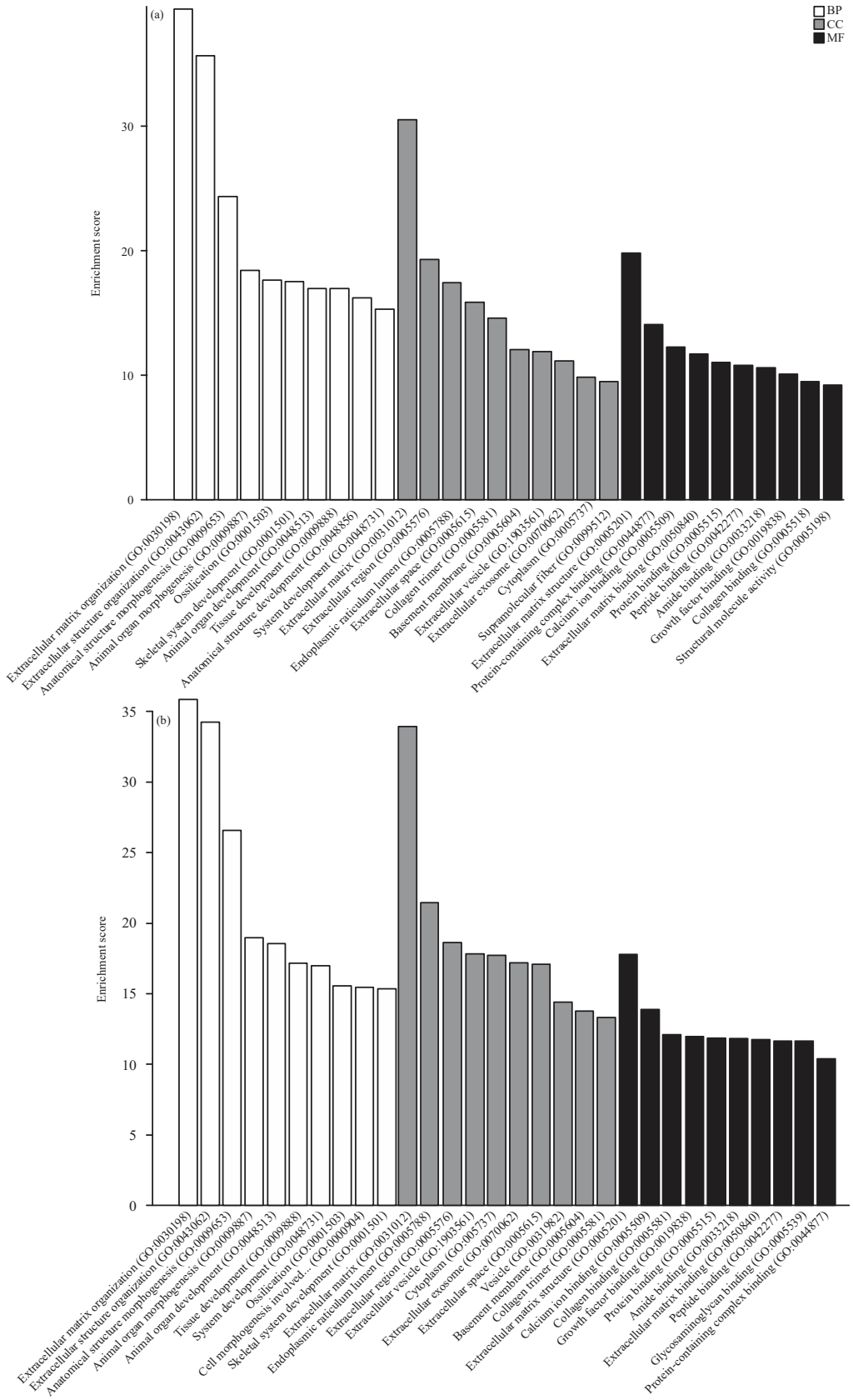


Fig. S2(a-d): Continue

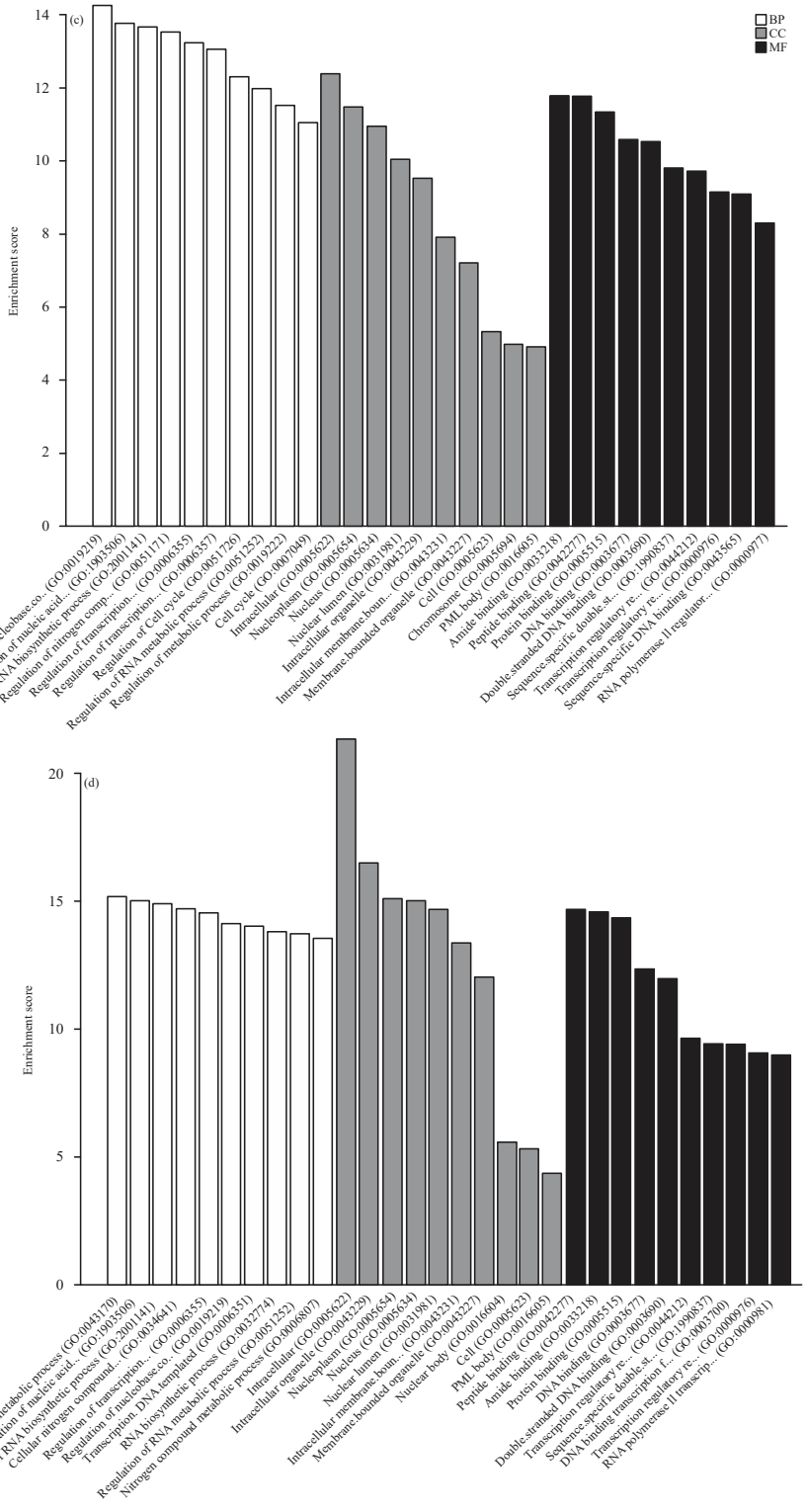


Fig. S2(a-d): Results of GO analysis of the gene products (a, b) Top ten enrichment Score values of the significantly down-regulated terms for biological process, cellular components and molecular function and (c, d) Top ten enrichment score values of the significantly up-regulated terms for biological process, cellular components and molecular function

BP: Biological process, CC: Cellular components, MF: Molecular function, C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h

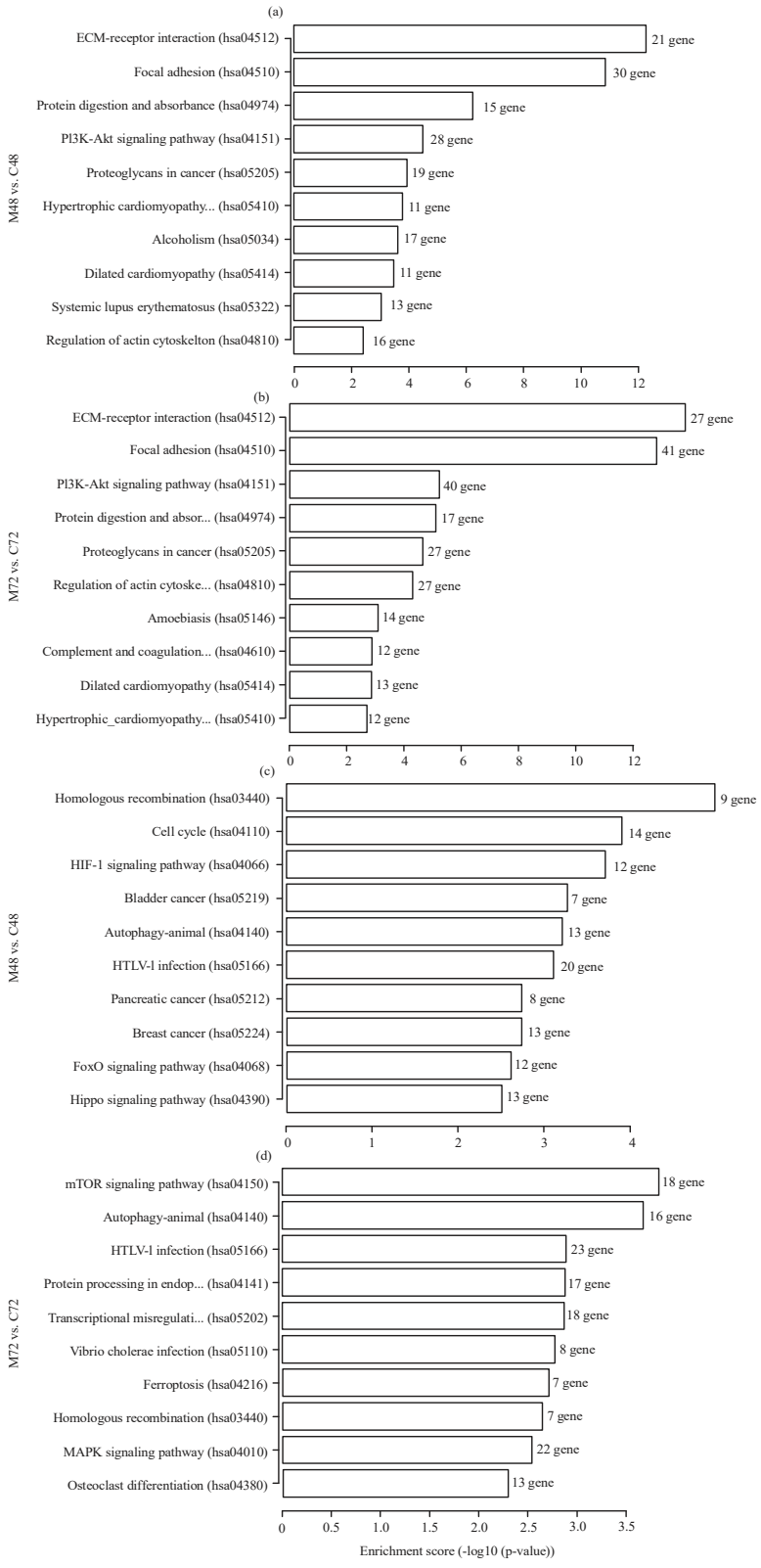


Fig. S3(a-d): Enrichment score (-log10 (p-value)) values of all (a, b) Significantly down-regulated (c, d) and up-regulated pathways of DE gene

C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h

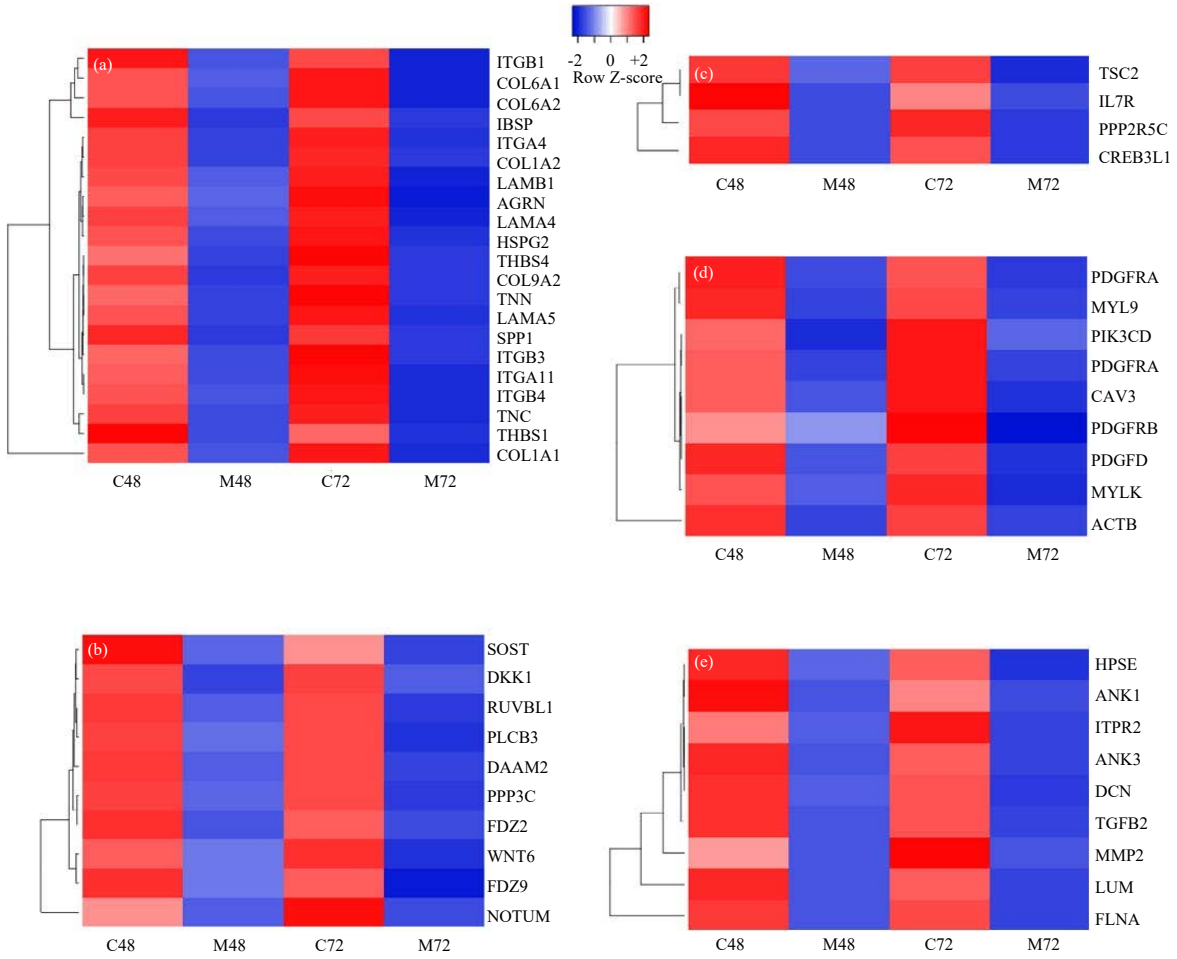


Fig. 2(a-e): Heat maps of different down-regulated pathways, (a) ECM-receptor interaction, (b) Wnt signaling pathway, (c) P13K-Akt signaling pathway, (d) Focal adhesion and (e) Proteoglycans in cancer

Relative mean log2 expressions of genes in several pathways were calculated and visualized over time using excel, C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h

proteoglycans in cancer and Wnt signaling), while Fig. S4 shown the upregulated genes (homologous recombination, autophagy-animal, mTOR signaling pathway, MAPK signaling pathway, HIF-1 signaling and FOXO signaling) and Fig. 3a has the ATP-regulated genes. The result showed that ATP levels decreased significantly after challenging SaOS-2 with baicalein during the period of the study (Fig. 3b).

**Apoptosis analysis:** In order to determine whether baicalein could affect the regulation of apoptosis in SaOS-2 cells, flow cytometry analysis was conducted. Apoptotic cells were assessed as the percentage of early and late apoptosis in order to quantify its induction. After exposure to baicalein the apoptosis rate increased significantly when compared to the control group (Fig. 4a, b). Furthermore, the rate of apoptosis of SaOS-2 cells induced by baicalein increases in a time-dependent manner. To further confirm that baicalein induces apoptosis, different protein ratios (caspase-3, BNIP3 and BAX/Bcl-2) were detected with western blot. Baicalein causedan upregulation of caspase-3, BAX and BNIP3, while it downregulated Bcl-2 levels in SaOS-2 cells (Fig. 5a). To assess the involvement of the Akt pathway in apoptosis, Akt levels by determining the phosphorylated Akt proteins were assayed using a western blot. As a result, the level of phosphorylated Akt was significantly reduced after treatment but that no changes were detected in the total Akt expression levels (Fig. 5a, b).

**Baicalein reduces cell adhesion and migration of SaOS-2 cells:** Generally, the metastatic properties of cancer are

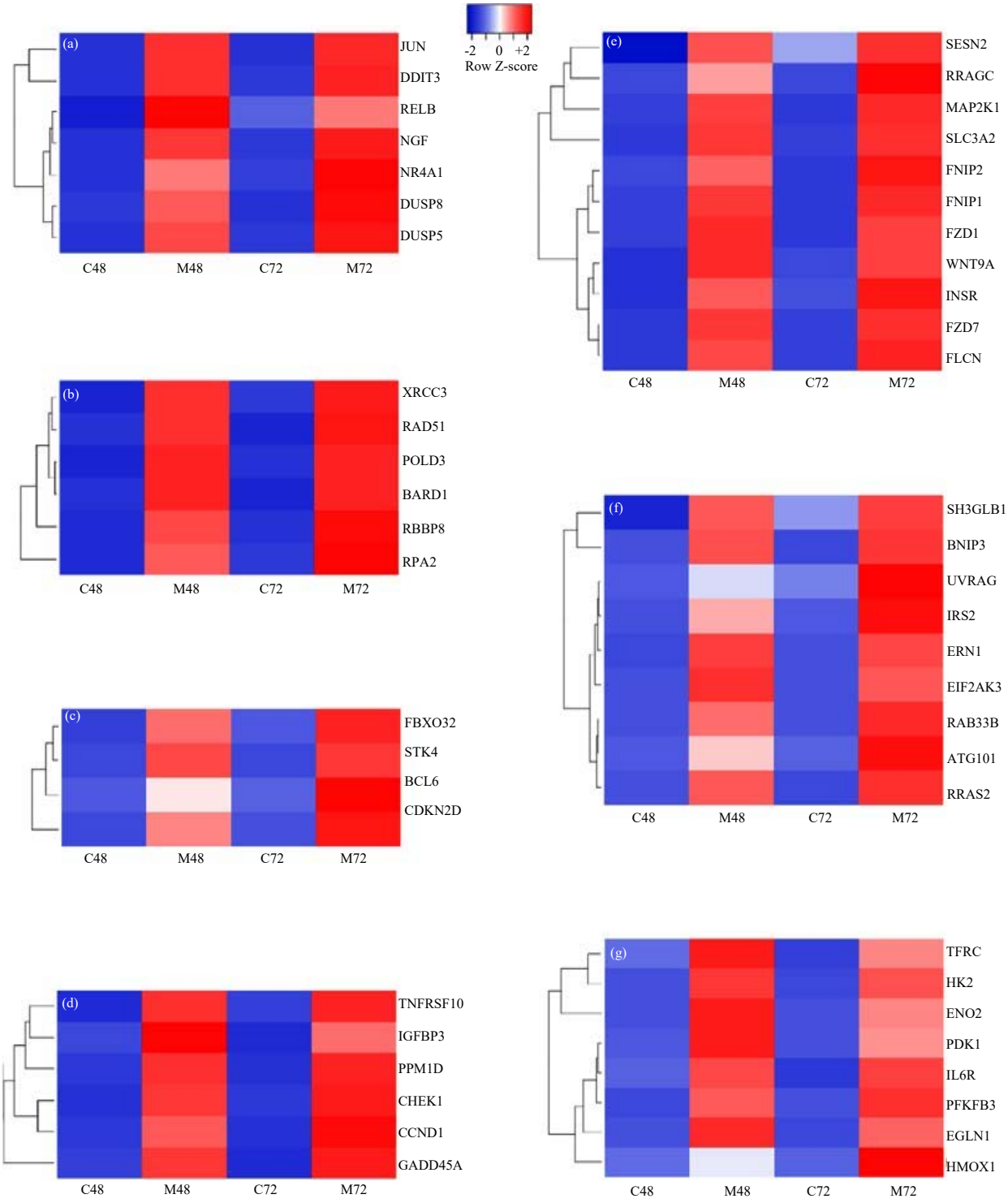


Fig. S4(a-g): Heat maps of different up-regulated pathways, (a) MAPK signaling pathway, (b) Homologous recombination, (c) FoxO signaling pathway, (d) p53 signaling pathway, (e) mTOR signaling pathway, (f) Autophagy animal and (g) HIF 1 signaling pathway

Relative mean log2 expressions of genes in several pathways were calculated and visualized over time using excel, C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h

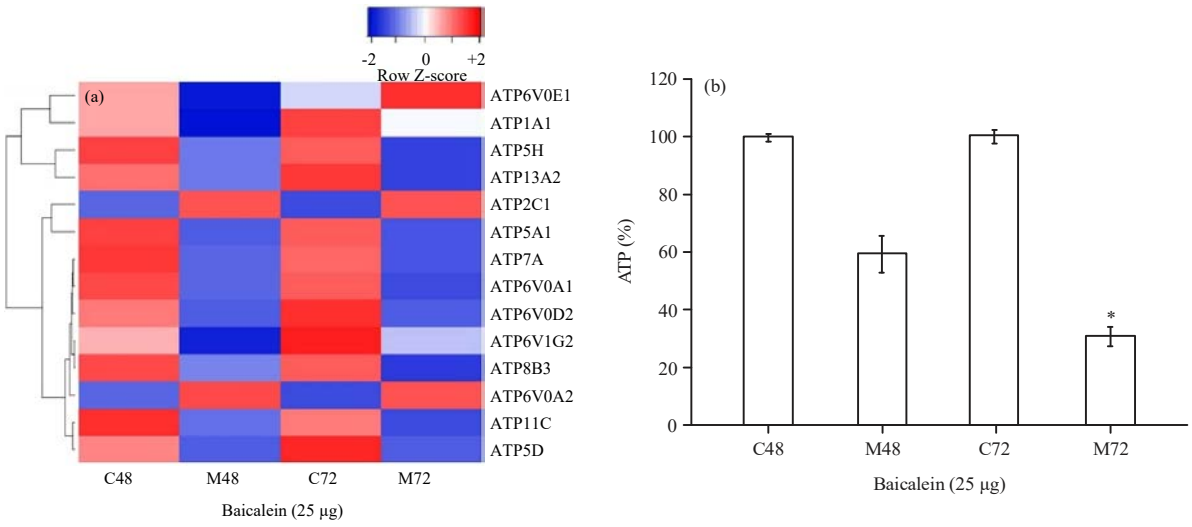


Fig. 3(a-b): (a) Heat map of ATP synthesis pathway and (b) Intracellular ATP levels of SaOS-2 after treating with Baicalein

Relative mean log2 expressions of different genes were calculated and visualized over time using excel, Baicalein treatment significantly reduced the levels of ATP as compared to control cells ($p<0.05$), values are given as Mean±SD of 3 experiments in each group, C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h, *$p \leq 0.01$ compared to control

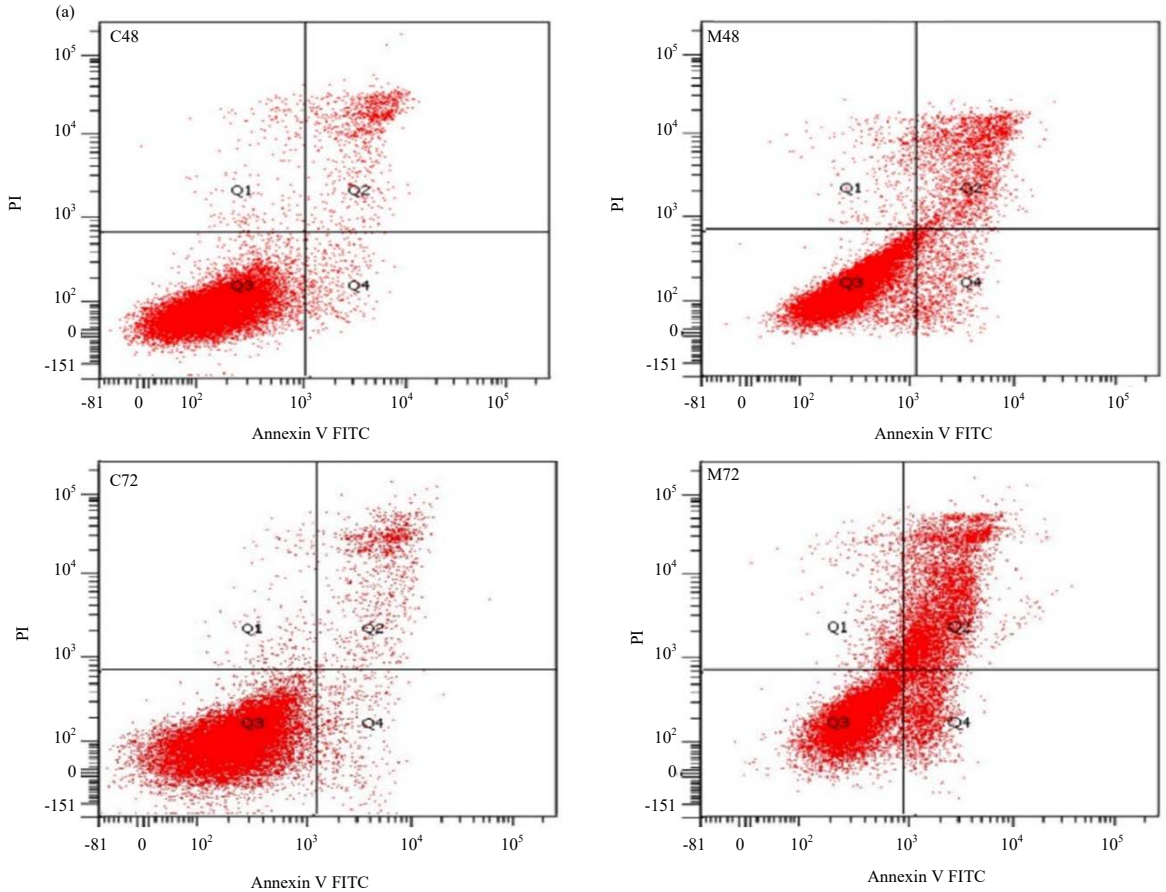


Fig. 4(a-b): Continue

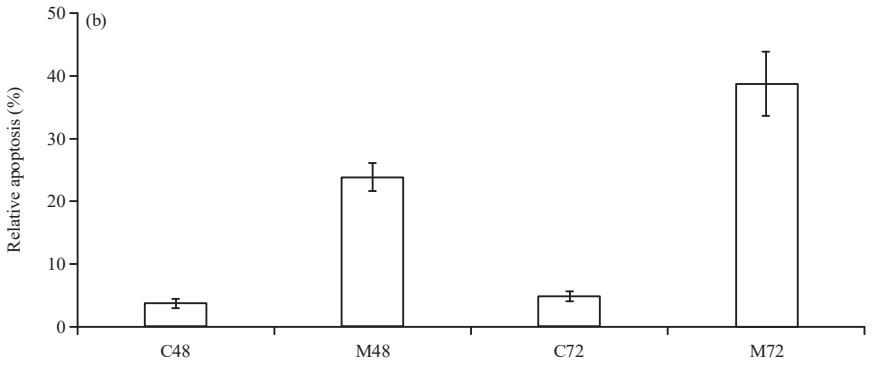


Fig. 4(a-b): (a) Flow cytometry analysis of apoptotic ratio in SaOS-2 cells which were treated with or without baicalein for 48 and 72 h and (b) Quantitative analysis of apoptosis of SaOS-2 cells

Data is presented from 3 independent experiments (C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h), data was expressed as mean±SEM, $p \leq 0.05$ vs. the control group

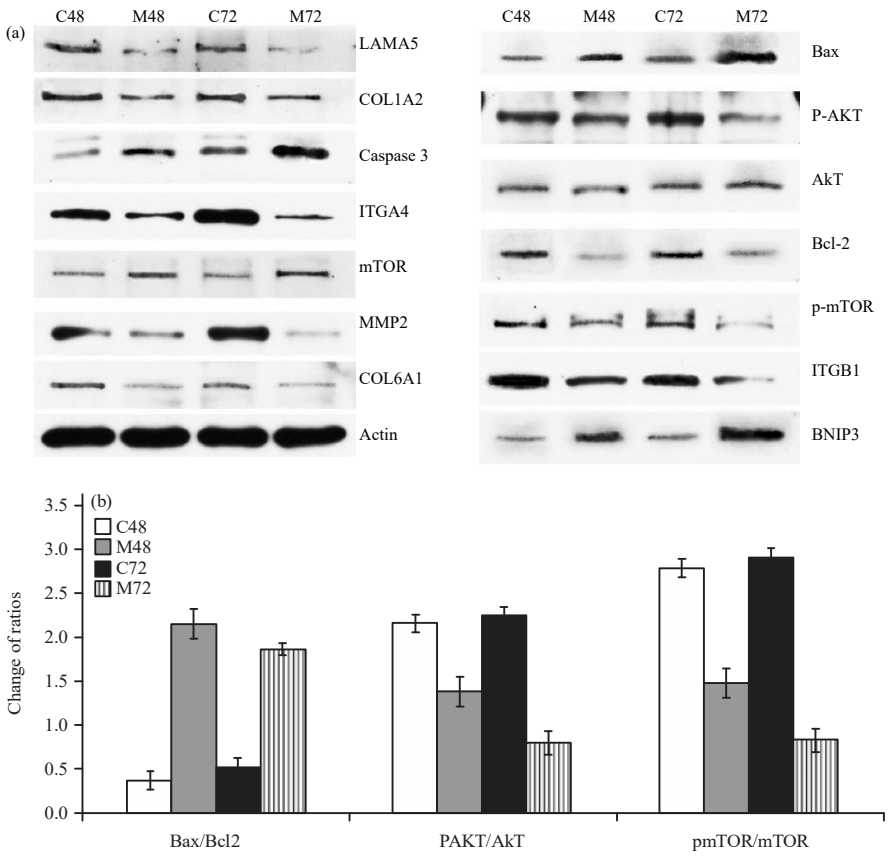


Fig. 5(a-b): (a) Confirmation of the baicalein effect on the SaOS-2 cells on the protein level assayed by western blot method and (b) Bax/Bcl2, p-AKT/AkT and p-mTOR/mTOR ratios were quantified by densitometry analysis

Data are represented as the Mean±SD of 3 determinations, $p \leq 0.05$ vs. the control group, C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h

strongly linked to its ability to migrate and adhere to other tissues. The cell adhesion test was achieved to evaluate the effects of baicalein on adhesion of SaOS-2 cells. Current results indicate that the adhesion of osteosarcoma cells to Matrigel was decreased after 48 and 72 h of baicalein treatment respectively compared to control cells. Next, wound healing was assessed to examine SaOS-2 cell migration. Figure 6 demonstrated that migration of treated cells was significantly

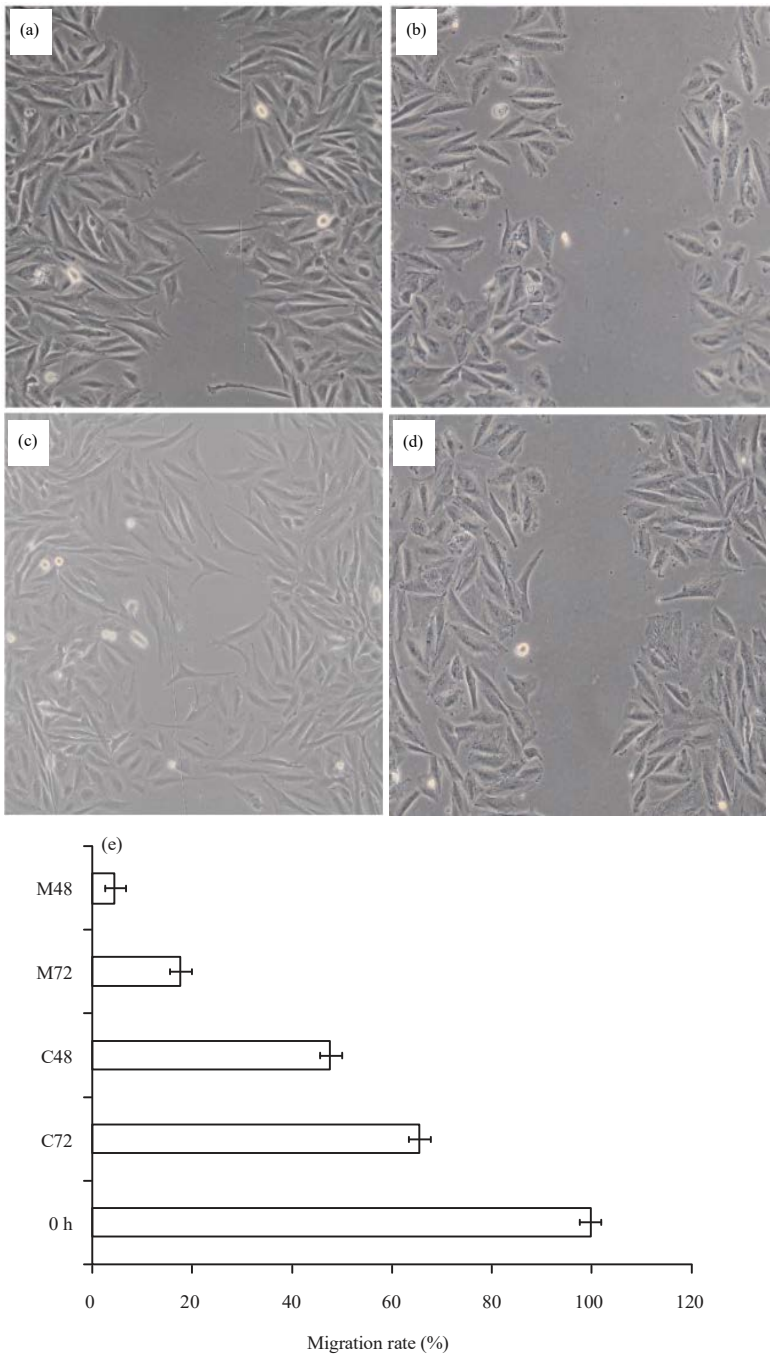


Fig. 6(a-e): Cell line wound developing during the treatment time points, (a, b) Untreated cells after 48 and 72 h respectively, (c, d) Baicalein treated cells after 48 and 72 h, respectively and (e) Percent migration rate is expressed as a percentage of the 0 h control

C48 and C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h

inhibited in a time-dependent manner. To confirm this result on a protein level, western blot assay was performed on phospho-mTOR, mTOR, phospho-Akt, Akt and MMP-2 proteins. After treatment with baicalein for 48 or 72 h, the levels of phospho-mTOR, phospho-Akt and MMP-2 proteins were significantly decreased (Fig. 5a) as were phospho-Akt/Akt and phospho-mTOR/mTOR protein ratios in a time-dependent manner (Fig. 5b).

To confirm RNA-seq accuracy of the ECM pathway, potentially targeted proteins were selected and analyzed

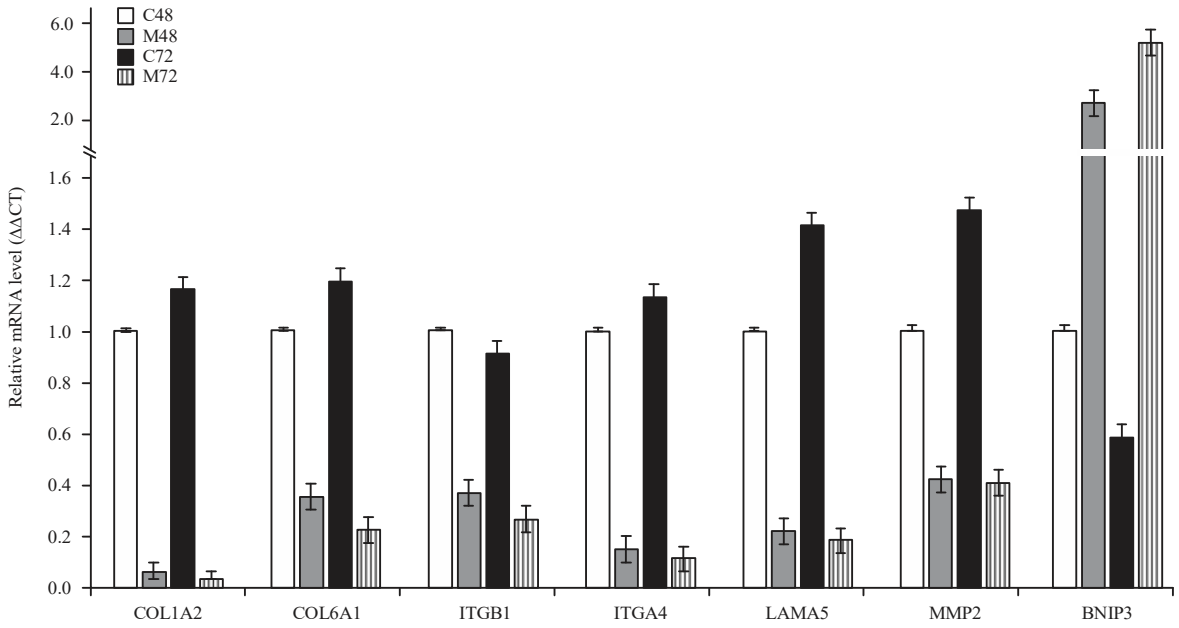


Fig. 7: Differentially expressed genes detected by RNA sequencing confirmed by qRT-PCR
qRT-PCR was performed for 7 genes that were identified to be differentially expressed between treated and control cells (C48, C72: Control groups for 48 and 72 h, M48, M72: Baicalein treated groups for 48 and 72 h)

by western blot. As a result, the ECM receptors ITGB1, ITGA4, COL1A2, COL6A1 and LAMA5 were clearly inhibited following baicalein treatment compared to the controls in a time-dependent manner (Fig. 5a).

**Real-time RT-PCR validation of RNA-seq results:** Seven potential target genes (COL1A2, ITGB1, ITGA4COL6A1, LAMA5, MMP-2 and BNIP3) were assayed with qRT-PCR. When SaOS-2 cells were treated with baicalein, the mRNA levels of ECM receptors were significantly reduced, which is consistent with the RNA-seq results (Fig. 7). From these observations, this study suggested that baicalein may not only affect SaOS-2 cell motility and proliferation but also induce cancer cell death by down regulating ECM receptors and focal adhesion-related genes.

## DISCUSSION

Osteosarcoma is a malignant cancer that mostly affects children and juveniles[1]. Recent developments in diagnoses and treatment methods have led to an increase in the survival rate for these patients, however, the rate remains[2]. Numerous TCM components have been documented to have benefits and low toxicity (e.g., curcumin, wogonin and baicalein[22,23].

Baicalein is formed from one of the flavones found in the root of *Scutellaria baicalensis* Georgi. It can be used to improve several conditions including microbial infections, inflammation and cardiovascular disease. Recent studies investigating several types of human cancer cell lines suggest baicalein has antitumor activity while possessing a low cytotoxicity to healthy cells[17,24-26]. In recent decades, numerous studies have confirmed that baicalein is capable of suppressing tumor cell growth and survival, inducing cell cycle arrest and apoptosis, in different types of human cancers (e.g., pancreatic, liver, breast, gallbladder, bone, colon, prostate and gastric cancers[27-30]. These observations may be due to induced cell cycle arrest or to apoptosis of bone cancer cells through a mitochondrial-mediated pathway[17].

Here, baicalein-altered signaling pathways at the whole transcriptome level with RNA-seq in SaOS-2 cell lines were studied. Many signaling pathways were altered in baicalein-treated cells, including cell proliferation, adhesion, migration and apoptosis. The current RNA-seq data indicated that baicalein has significant inhibitory effects on the expression of ECM receptors (laminin (LAMA5), integrin (ITGB1 and ITGA4) and collagen (COL1A2 and COL6A1)) in SaOS-2 cells. Baicalein leaded to downregulation of the expression of ECM receptors and MMP2, while BNIP3 displayed upregulation at both RNA and protein levels.

The ECM receptors are transmembrane proteins that are necessary for cell-cell or cell-extracellular binding. Collagen (COL1A2 and COL6A1), integrin (ITGA4 and ITGB1) and laminin (LAMA5) in SaOS-2 cells were significantly inhibited after treatment compared to control cells in a time-dependent

manner. This result has been confirmed through proliferation, adhesion, migration and apoptosis assays.

The atypical activation of the ECM-receptor pathway is linked to survival, migration, polarity, proliferation and differentiation of cells[31,32]. When cancer cells adhere to the ECM, through integrin and collagen, the activity of several pro-survival pathways are increased (e.g., PI3K/Akt and p53/MAPK)[33-35]. Integrins (ITGA4 and ITGB1) are bioactive molecules necessary for cell-adhesion and expression changes have been linked with tumor development, invasion, metastasis and migration[36,37]. Integrin promotes the growth, migration, cell signaling and tumor metastasis of many types of cancer cells. The promotion of cell signaling is especially critical for the migration and regulation of osteoblast proliferation[38,39]. A previous study suggested that ITGB1 could be applied as a therapeutic target for the treatment of cancers[40].

The ECM contains several protein fibrils and growth factors including collagens and laminins, these components provide the supporting scaffold for a developing tumor. The ECM additionally facilitates the formation of blood vessels, which is imperative for nutrient acquisition. Type I collagen is the major element in both bone and ECM tumors, which is key to promoting metastasis and proliferation of tumor cells[41-43]. Previous reports, showed that COL1A2 interacts with the matrix metalloprotease (MMP) family, including MMP2 and plays an important role in the tumorigenesis of osteosarcoma. Because osteosarcoma causes an upregulation of the COL gene family, it may be possible to use this signature as a diagnostic marker for in osteosarcoma patients, while restoring type I collagen levels, may be a promising therapeutic agent[42].

COL6A1 is one of the major subunits of collagen VI, it is a key protein of ECM that provides support[44,45]. This subunit regulates apoptosis and proliferation through many different signaling pathways[46-49].

The present results of RNA and protein levels indicated that COL1A2 and COL6A1 were significantly decreased after baicalein treatment compared to untreated cells in a time-dependent manner. LAMA5 has a main role in cancer cell migration[50]. The results showed that LAMA5 expression was significantly down regulated in treated cells in a time-dependent manner, also the expression of phospho-Akt and phospho-mTOR were reduced which might be due to the down regulation of LAMA5 (Fig. 5).

Cell adhesion and migration are known to play key roles in tumor metastases[51]. Adhesion and wound-healing were assayed to test the ability of cell adhesion and migration after baicalein challenge. The results indicated that baicalein could inhibit motility of SaOS-2 cells in a concentration-dependent manner (Fig. 6).

Tumor cells stick to the basic membrane and ECM by secreting several protease enzymes, such as MMPs, to degrade the ECM and migrate via biological barriers, this phenotype is critical for metastasis[52]. Consequently, MMP-2 levels are elevated in tumors compared with the healthy tissues. The present results displayed significant decrease of MMP-2 after treatment with baicalein, suggesting that baicalein has the ability to inhibit the cell migration process.

ATP levels decline when cells suffer apoptosis, necrosis or under some toxic conditions. Glucose stimulation may upregulate intracellular ATP levels. Baicalein treatment exhibits mitochondrial toxicity and affects mitochondrial energy. Different reports showed that baicalein has a slight inhibition of cell development and proliferation in normal human osteoblasts[10,17]. The decline in ATP was observed in the studied baicalein-treated cells, suggesting the toxic role of baicalein in cancer cells[53].

Apoptosis is known as a tumor-suppressor mechanism, which is organized by cell-suicide machineries prompted by specific signals. Presently, several pathways related with the apoptosis induction are recognized (e.g., Bcl-2 family, mitochondrial and PIK3/Akt signaling pathways[54]). However, this study was investigated the effect of baicalein on the Bcl-2 family (BAX and Bcl-2). The balance between BAX and Bcl-2 were previously suggested to have a critical role in conferring cell susceptibility to apoptosis[55]. After treatment with baicalein, the expression levels of BAX and Bcl-2 were elevated, suggesting that baicalein-induced apoptosis was associated with alterations in BAX and Bcl-2 expression, consistent with previous studies[10,25].

The PIK3/Akt signaling pathway controls cellular responses and play a serious role in keeping the balance between cell survival and apoptosis. Current reports showed that stimulation of the PIK3/Akt pathway by translocation, mutation and amplification occurs on a more frequent basis than in other pathways in cancer patients[56]. Therefore, inhibition of the PIK3 pathway may offer a suitable target for therapeutics due to the fact that phospho-mTOR has been observed to promote proliferation, migration and survival in cancer cells[57]. The results indicated that baicalein challenge on SaOS-2 cells reduced mTOR phosphorylation and AKT activity. These results proposed that baicalein may induce its anticancer effect by inhibiting the Akt/mTOR pathway.

Furthermore, Akt has been described to support cell survival via the direct regulation of Bcl-2 family members.

Previous reports suggested that reduction of Akt activity can induce the upregulation of BAX and downregulation of Bcl-2 expression prompted by baicalein. Indicating that Akt may be upstream of the baicalein-induced apoptosis in prostate cancer cells[24]. The present study found that phospho-mTOR/mTOR and phospho-Akt/Akt ratios decreased in treated cells in a time-dependent manner.

Additionally, early studies indicated that BNIP3 interacts with mitochondrial dynamic-related proteins, after which, the mitochondria degrade followed by cell apoptosis mediated by BNIP3 regulation of Bax and Bcl-2 expression[10]. The results showed that BNIP3 was significantly upregulated protein level and gene expression.

## CONCLUSION

In conclusion, all evidence supports that baicalein promotes the SaOS-2 apoptosis process. Present study is the first to describe that ECM receptors are the main target of baicalein. This suggests that baicalein may be used alone or with other anti-cancer treatments to reverse ECM receptor-associated malignant phenotype of human cancers. However, outcomes are only based on *in vitro* study (SaOS-2 cell line) and additional investigations on different cell lines as well as *in vivo* studies are needed to estimate the application of baicalein in future studies

## SIGNIFICANCE STATEMENT

This study discovers the possible inhibitory effects of baicalein on tumor cells development that could be beneficial for cancer treatment in particularly osteosarcoma. This study will help the researcher to uncover the critical areas osteosarcoma curing and baicalein applications that many researchers were not able to explore. Thus, a new theory on the therapeutic effect of baicalein though repressing ECM receptors activity may be arrived at.

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