Induction of Apoptosis in Ehrlich ascites carcinoma cells through an intrinsic pathway by Co(II)-benzoin Thiosemicarbazone Complex [$Co(BTSC)_2$]

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Abstract

A group of cells exhibit uncontrolled proliferation, invasion, and occasionally metastasis in cancer, a category of disorders. According to statistics, cancer is the second largest cause of mortality in the world. As a result, current cancer research places a strong priority on the discovery and development of novel, effective, and selective anticancer medications. The purpose of this work was to determine the method by which Ehrlich ascites cancer (EAC) cells are inhibited by Co(II)-benzoin thiosemicarbazone complex in Swiss albino mice. DNA fragmentation assays and nuclear morphology observations both supported the induction of apoptosis in EAC cells. The mRNA expression levels of many tumor-related antiapoptotic genes, including B-cell lymphoma 2 (bcl-2), B-cell lymphoma extra-large (bcl-xL), and caspase-8, as well as proapoptotic genes, including p53 or tumor protein, bcl-2 associated X protein (bax), caspase-9, caspase-3, and poly-ADP ribose polymerase (PARP-1) and in vitro effect of caspase inhibitors on EAC cells. Using 2', 7'- dichlorodihydrofluorescein diacetate (DCFH-DA) staining, reactive oxygen species (ROS) production following $Co(BTSC)_2$ treatment were quantified. The findings of this investigation revealed that the induction of apoptosis by $Co(BTSC)_2$ occurred via an intrinsic mitochondria-mediated ROS-dependent mechanism as opposed to an extrinsic one, and that this intrinsic pathway was controlled by the bcl-2 protein family. As a result, this study offers support for conducting more research to develop novel anticancer drugs.

Keywords

EAC cells, Cobalt benzoin thiosemicarbazone complex, intrinsic pathway, ROS, Caspase inhibitor.

Introduction

Hugo Schiff described Schiff bases for the first time in 1864. Schiff bases are the condensation products of aldehydes and ketones with primary amines. These are substances with imine or azomethine (-HC=N-) functional groups, and it has been discovered that they are flexible pharmacophores for the synthesis of numerous bioactive lead compounds. In many different domains, including analytical, biological, and inorganic chemistry, Schiff bases and Schiff base complexes with transition metals are among the most frequently utilized classes of organic and organometallic molecules. Due to a wide range of biological activities, including anti-inflammatory, analgesic, antimicrobial, anticonvulsant, antitubercular, antioxidant, anthelmintic, anti-HIV, anti-leprosy, anti-leukemia, and anticancer properties, Schiff bases and Schiff base metal complexes have recently attracted the interest of many researchers in the medical and pharmaceutical fields. On the effective application of this family of chemicals as anticancer agents, several papers have been published [1-4]. However, further research is required to fully comprehend their anticancer mechanism (s).

Cellular signaling pathways control the process of programmed cell death known as apoptosis, which is morphologically characterized by cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA degradation. One of two pathways—the intrinsic pathway or the extrinsic pathway—can cause apoptosis to begin [5]. Cellular stress, infection, and DNA damage trigger the intrinsic (mitochondrial) pathway, which activates pro-apoptotic BCL-2 associated X (BAX) or BCL-2 homologous killer (BAK) proteins. These proteins oligomerize to form pores on the mitochondrial outer membrane and release cytochrome c into the cytosol [6]. A death ligand such tumor necrosis factor (TNF) or TNF-related apoptosis inducing ligand (TRAIL) interacting to cell surface receptors to activate caspase-8, which then directly activates downstream caspase-3, which is crucial for programmed cell death, starts the extrinsic (death receptor) pathway [7]. These two processes are mostly carried out by endoproteases known as caspases. Caspases 8 and 9 connect the extrinsic and intrinsic pathways, respectively, and are important components of cell regulatory networks that regulate inflammation and cell death [8–9]. Reactive oxygen species (ROS), a group of byproducts of oxygen metabolism, also play a part in cell signaling, including the activation of cell signaling cascades, gene expression, and death [10]. In this study, we describe the activation of the intrinsic signaling cascade leading to apoptosis and the anticancer effects of the Cobalt (II) benzoin thiosemicarbazone complex against EAC cells in Swiss albino mice.

Materials and Methods

Chemicals

All chemicals and reagents used to carry out the research work were of reagent grade.

Experimental animal model

Male adult Swiss albino mice were obtained from the animal resource division of the International Centre for Diarrheal Disease Research (ICDDR'B), Mohakhali, Dhaka, Bangladesh. The mice were 5-7 weeks old and weighed (25 ± 4 g). Animals were kept in iron cages with bedding made of sawdust and sterile rice husk under sanitary circumstances, with a maximum of six animals per cage. They were kept in a controlled environment with 12:12 h of light and dark and a temperature of 22 ± 5 °C). To ensure that the mice had enough nourishment and liquids, the ICDDR'B prescribed and prepared a standard mouse meal, along with water.

Ethical clearance

The Institute of Biological Sciences, University of Rajshahi, Bangladesh (225/320-IAMEBBC/IBSc), gained institutional animal, medical ethics, biosafety, and biosecurity committee permission for experiments on animals, humans, microorganisms, and live natural sources. The University Animal Ethical Committee gave its approval to the study's protocol for using mice as research subjects (27/08/RUBCMB).

Synthesis of the compounds

Synthesis of Co(II)-benzoin thiosemicarbazone complex

The compound was synthesized using the procedure outlined in the literature [11–12]. Benzoin and thiosemicarbazide were combined in a 1:1 molar ratio, refluxed for three to four hours, and then distilled to get benzoin thiosemicarbazone (BTSC). The condensed solution was mixed with a saturated solution of metal (II) acetate [Co(II)] in ethanol. Crystals of Co(II)-benzoin thiosemicarbazone were produced in a short period of time. The crystal was dried in an oven at 50°C, twice recrystallized, washed with ethanol, and then placed in a desiccator.

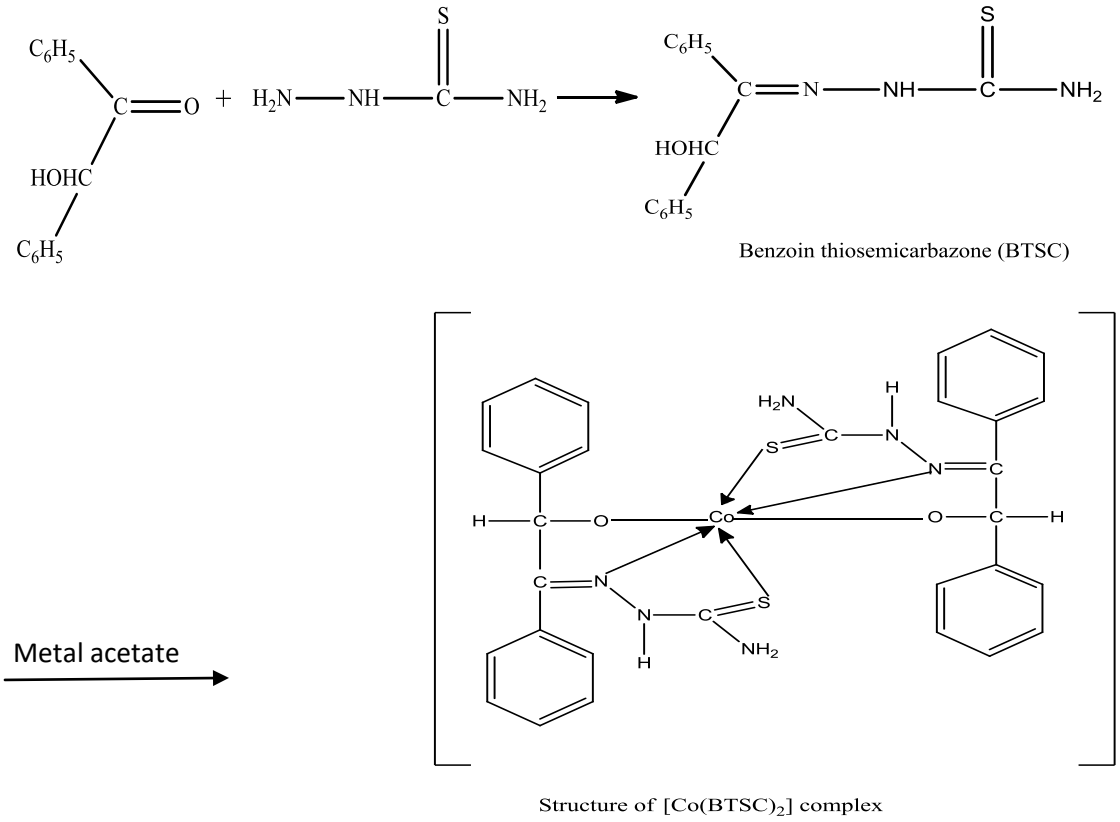

Figure- 1: Synthesis of Co(II)-benzoin thiosemicarbazone complex

Cell lines

We were able to collect Ehrlich ascites carcinoma (EAC) cells thanks to the Indian Institute of Chemical Biology (IICB), Kolkata, India. Swiss albino mice were given 2×10^6 cells/mouse intraperitoneally (biweekly) to maintain the cells as mammary gland cancer cells in ascites. EAC cells were grown in RPMI-1640 media that had been adjusted to include glucose, 2 mM L-glutamine, 10% fetal calf serum (FCS), and 1% penicillin-streptomycin (v/v) for the in vitro investigation. At 37°C, cells were incubated in a humidified environment with 5% CO_2.

In vitro cell growth inhibition

By using the MTT colorimetric method, the in vitro impact of Co(BTSC)2 against EAC cell growth suppression was evaluated. Under specific circumstances, NAD(P)H-dependent cellular oxidoreductase enzymes may serve as an indicator of the quantity of alive cells. The tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] may be reduced by these enzymes to its insoluble formazan, which has a purple hue. On this experiment, 4×10^6 EAC cells were plated in a 96-well flat bottom culture plate with 200 µLof RPMI-1640 medium and six different doses of $Co(BTSC)_2$ (5 µg, 10 µg, 20 µg, 40 µg, 80 µg and 120 µg/mL). The cells were then incubated for 24 hours at 37 °C in a CO_2 incubator. Dimethyl sulfoxide (DMSO), employed as a control, was applied to EAC cells. To eliminate experimental bias, the experiments were carried out in triplicate.

Dimethyl sulfoxide (DMSO), was applied to EAC cells, employed as a control. To prevent experimental mistakes, the experiments were run in triplicate. After the incubation time, the aliquots were carefully removed, and each well received 180 µL of phosphate buffered saline (PBS) and 20 µL of MTT, followed by another 8 h of incubation at 37 °C. The aliquot was once again withdrawn, 200 µL of acidic isopropanol was added, and the mixture was once more incubated at 37 °C for 1 hour. Subsequently, absorbance was taken at 570 nm in microtiter plate reader (Optica Microplate Reader, Mikura Ltd., Horsham, UK).The following equation was used to calculate the cell proliferation inhibition ratio:

$$\text{Proliferation inhibition ratio (\%)} = \frac{(A - B) \times 100}{A}$$

Where A is the $OD_{570\ nm}$ of the cellular homogenate (control) without experimental compounds and B is the $OD_{570\ nm}$ of the cellular homogenate with experimental compounds.

DNA fragmentation assay

The procedure outlined in the preceding paragraph [13] was used to determine the DNA fragmentation test by agarose gel electrophoresis. EAC cells were taken from mice given the experimental substance $Co(BTSC)_2$ for five days in a row at a concentration of 8 mg/kg/mice, as well as from control mice. The cells were cleaned and put back together in PBS. A DNA extraction kit from Promega, Madison, WI, USA, was used to separate the genomic DNA. This DNA was then electrophoresed on a 1.5% agarose gel containing 0.1 g/mL ethidium bromide and examined using a conventional UV trans-illuminator.

Effect of caspase inhibitors on EAC cells

To validate the participation of caspases [14] in the reaction to experimental compounds that produced cell death, untreated EAC cells were collected and washed with PBS on day six following tumor implantation. The cells were then treated with Z-DEVD-FMK (caspase-3 inhibition, 2 mol/mL) and Z-IETD-FMK (caspase-8 inhibitor, 2 mol/mL) for 2 hours in a CO_2 incubator using RPMI-1064 medium. An further 24 hours were spent incubating the $Co(BTSC)_2$-treated cells at 370C in a CO_2 incubator. In order to assess the impact of experimental drugs on

cell growth inhibition in the presence or absence of certain inhibitors, the cells were last counted using a hemocytometer.

Measurement of Reactive Oxygen Species (ROS) generation

Using 2', 7'-dichlorodihydrofluorescein diacetate, the cells' ROS level was assessed (DCFH-DA; Invitrogen, USA). On the sixth day following tumor injection, cells were harvested from control and treated mice and were then washed in PBS at a speed of 1200 rpm for two minutes. The cells were then exposed to DCFH-DA 10µL (50µM final concentration) for 30 min at 37 ^{0}C. The cells were once again maintained in 1mL of culture media after being washed twice with PBS. A fluorescent microscope was used to measure ROS production at 485nm and 530nm for excitation and emission, respectively [15].

Reverse transcription polymerase chain reaction

On the sixth day after tumor implantation, total RNA was extracted using the TRIzol technique from mice receiving $Co(BTSC)_2$ at a dosage of 8 mg/kg/day and control animals carrying EAC. The following procedure was used to reverse-transcribe total RNA: each 20µL reaction contained 3µg of total RNA, 50 units of MuLV reverse transcriptase from New England Biolab in Ipawich, Massachusetts, USA, 125M deoxynucleotide (dNTP), 100 pmol random hexamer, 100 pmol oligo dT18 primer, and diethylpyrocarbonate (DEPC)-treated water. Briefly, after 15 minutes of incubation at 70 degrees Celsius with the oligo dT18 primer, the RNA and other components were added and incubated for 1 hour at 42 degrees Celsius before being moved to 70 degrees Celsius for 15 minutes.
Using this cDNA as a template for PCR, the expression of one housekeeping gene (gapdh) and eight growth regulating genes—namely, bcl-2, bax, p53, bcl-xl, parp 1 and caspase-3,-8,-9—were investigated. 25 µL of nuclease-free water, 2 µL of cDNA, 1 x of Taq polymerase, 25 pmol of forward and reverse primers, 2.5 mM of each dNTP, and 0.25 U of platinum Tag polymerase (Tiangen, Beijing, China) were used to produce the reaction mixture. Table 8 contains a collection of primer sequences. The amplifications were done using a gradient thermal cycler by BioRad (Hercules, California, USA). A 1.5% agarose gel was used to evaluate all of the PCR results, and a Gene Ruler 1 kb DNA ladder from Fermentas in Pittsburgh, Pennsylvania, USA, was employed as a marker. 35 cycles of 95 °C/1 min were followed by 3 min of 95 °C for the first PCR activation phase. The annealing temperatures for the genes p53, Bcl-2, and caspase-3, -8, and -9 were 55 °C/1 min, 72 °C/1 min, and 72 °C/1 min for the final extension. The annealing temperature for Bax, GAPDH, and Bcl-xL was 54 °C.

Statistical analysis

The mean S.E.M. of the experimental data has been used as an expression. One-way analysis of variance (ANOVA) was used to compute the parametric values, and the Dunnett "t" test was used to determine the statistical significance (version 20). When $p < 0.05$, the difference was deemed statistically significant.

Results

In vitro EAC cell growth inhibition with $Co(BTSC)_2$

Using the MTT test, the in vitro effects of $Co(BTSC)_2$ on the suppression of EAC cell proliferation have been studied. EAC cell death caused by $Co(BTSC)_2$ occurs in a dose-dependent manner (Figure 2). At doses of 5 10 20 40 80 and 120 µg/mL, the $Co(BTSC)_2$ inhibited cell growth by 16.2%, 32.3%, 45.7%, 61.62%, 73.9%, and 89.72%, respectively. The impact diminished when $Co(BTSC)_2$ concentration was lowered.

DNA fragmentation assay

The most recognizable biochemical indicator of apoptosis is the activation of the endogenous Ca^{2+}/Mg^{2+} dependent endonuclease. By cleaving internucleosomes, this active endonuclease produces oligonucleotide fragments that are 180–200 base pairs long or their polymers. When genomic DNA from the experimental chemical $Co(BTSC)_2$ treated EAC cells was subjected to PCR amplification and then agarose gel electrophoresis, a DNA band was produced from the gel. This DNA had the defining trait of apoptotic induction. Conversely, the control group had DNA degradation that resembled a smear. Figure 3 displays the outcome.

Effect of caspases on $Co(BTSC)_2$-induced cytotoxicity in EAC cells

Z-DEVD-FMK (a caspase-3 inhibitor) and Z-IETD-FMK (a caspase-8 inhibitor) were employed to identify the role of particular caspases in the apoptotic cell death that was brought on in EAC cells as a result of exposure to the experimental substance $Co(BTSC)_2$. Growth inhibition of EAC cells was 78.89% when $Co(BTSC)_2$ was present, and it was decreased to 3.88 and 75.01% when Z-DEVD-FMK (a caspase-3 inhibitor) and Z-IETD-FMK (a caspase-8 inhibitor) were present, respectively, in the cell culture media (figure 4).

Altered expressions of cancer-related genes in $Co(BTSC)_2$ treated EAC cells

Reverse transcription In control and $Co(BTSC)_2$-treated EAC cells, PCR was used to examine the mRNA expression levels of numerous tumor-related antiapoptotic genes (bcl-2, bcl-xl, and caspase 8) and proapoptotic genes (p53, bax, parp-1, caspase-3, and caspase-9) (Figure 5). Gapdh primers were used to check that the isolated RNAs were appropriate for PCR. Bcl-2, Bcl-xl, and caspase 8 genes were highly expressed in control cells, but they were expressed at lower levels in EAC cells that had been exposed to the experimental chemicals. The expression of the genes p53, bax, parp-1, caspase-3, and caspase-9 was also elevated in the treated EAC cells. However, in control mice, there was no evidence of these genes being expressed.

Determination of intracellular reactive oxygen system in EAC cells

A popular fluorescent probe called 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) may be used to find various ROS in biological media. The intracellular esterase first hydrolyzes the DCFH-DA to DCFH, which is subsequently oxidized by reactive species to produce the fluorescent chemical 2, 7- dichlorofluorescein (DCF), whose fluorescence intensity is inversely correlated with the ROS concentrations. The impact of $Co(BTSC)_2$ on ROS production in EAC cells after 24 h of treatment was evaluated here using DCFH-DA. In contrast to increasing doses

of the experimental drug, which resulted in significantly stronger signals, with 8 mg/kg (i. p.) of $Co(BTSC)_2$ yielding the greatest intensity, the untreated control cells showed minimal green fluorescence, as seen in figure 6.

Conclusion

In view of the aforementioned findings, the experimental drug may be regarded as an efficient anticancer agent and is at least as effective as bleomycin in terms of inhibiting cell proliferation, lowering the average tumor weight, and lengthening the survival time of tumor-bearing mice. Therefore, this substance may be regarded as one of the most effective anticancer drugs. Rather than an extrinsic pathway, ROS dependent mitochondria-mediated intrinsic pathway, which is controlled by the BCL-2 protein family, may underlie its anticancer activity. It is important to do more research against additional cancer cell lines as well as higher tests in animal models utilizing cutting-edge techniques in order to determine whether these compounds are a unique potential anticancer medication. These results unquestionably provide strong justification for conducting more investigation to develop brand-new anticancer medications.

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Competing Interests

Authors declare that they do not have any conflict of interest.

Authors' Contributions

This work was done in collaboration among all the five authors. Author Hossain Mohammad Zakir designed the study, performed the analysis and wrote the first draft of the manuscript. Author Md. Murshed Hasan Sarker and Sha Md. Shahan Shariar were analysed the data. Author Mele Jesmin and Shaikh M Mohsin Ali were supervised. All the authors managed the literature search writing of the final manuscript. All authors read and approved the final manuscript.

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Table- 1: Primer sequences and PCR protocol used for the RT-PCR assay

Sl. No.	Target genes	Primers	Amplification (pb)
1	*bcl-2* F *bcl-2* R	5´-GTGGAGGAGCTCTTCAGGGA-3´ 5´-AGGCACCCAGGGTGATGCAA-3´	0.200
2	*bax* F *bax* R	5´-CGCCCACCAGCTCTGAGCAGA-3´ 5´-GCCACGTGGGCGTCCCAAAGT-3´	0.500
3	p^{53} F p^{53} R	5´-GCGTCTTAGAGACAGTTGACT-3´ 5´-GGATAGGTCGGCGGTTCATGC-3´	0.550
4	*bcl-x* F *bcl-x* R	5´-TTGGACAATGGACTGGTTGA-3´ 5´-GTAGAGTGGATGGTCAGTG-3´	0.700
5	*caspase-3* F *caspase-3* R	5´-TTAATAAAGGTATCCATGGAGAACACT-3´ 5´-TTAGTGATAAAAATAGAGTTCTTTTGT-3´	0.300
6	*caspase-8* F *caspase-8* R	5´-CTGCTGGGGATGGCCACTGTG-3´ 5´-TCGCCTCGAGGACATCGCTCTC-3´	0.450
7	*caspase-9* F *caspase-9* R	5´-ATGGACGAAGCGGATCGG-3´ 5´-CCCTGGCCTTATGATGTT-3´	0.400
8	*parp-1* F *parp-1* R	5´-AGGCCCTAAAGGCTCAGAAT-3´ 5´-CTAGGTTTCTGTGTCTTGAC-3´	0.270
9	*gapdh* F *gapdh* R	5´- GTGGAAGGACTCATGACCACAG-3´ 5´- CTGGTGCTCAGTGTAGCCCAG-3´	0.350

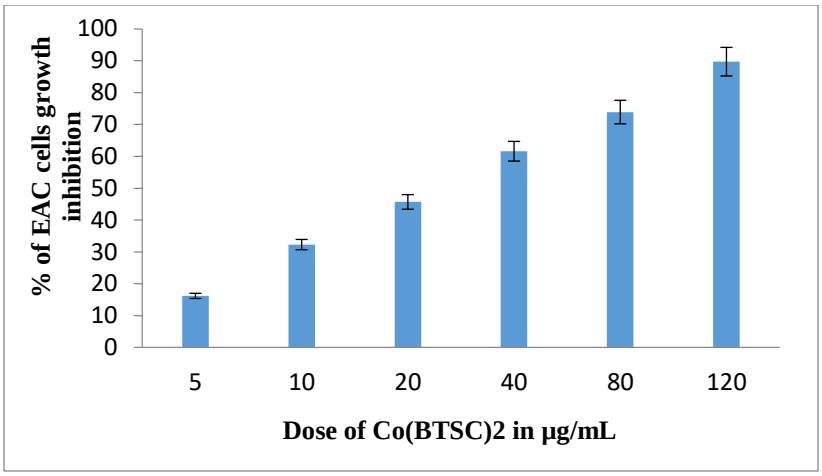


Figure-2. *In vitro* effect of $Co(BTSC)_2$ on EAC cell growth inhibition. Data are expressed as the mean of results in 3 repeated experiments. Error bar represent standard error the of mean.

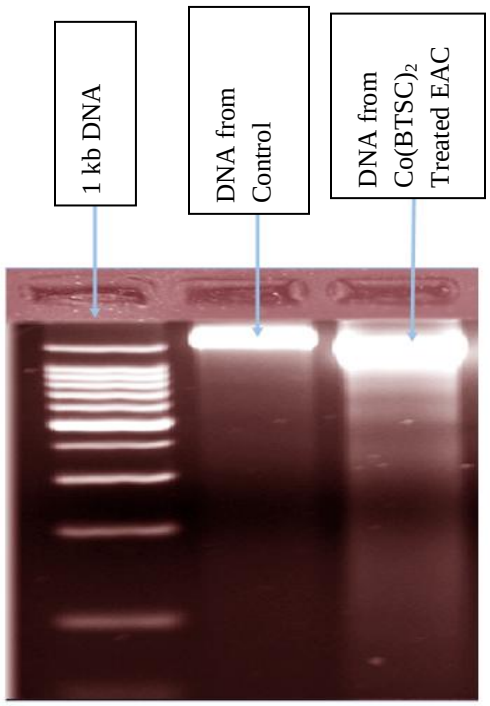


Figure-3: DNA fragmentation assay in $Co(BTSC)_2$-treated mice

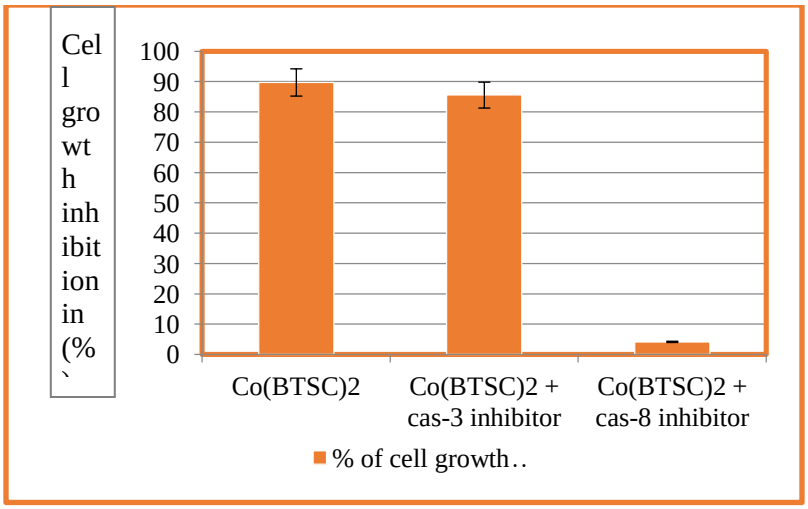


Figure- 4: Effect of caspases on $Co(BTSC)_2$- induced cytotoxicity in EAC cells. The number of mice in each experiment was 6. The results are shown as mean±SEM. Significant value is, **p<0.01.

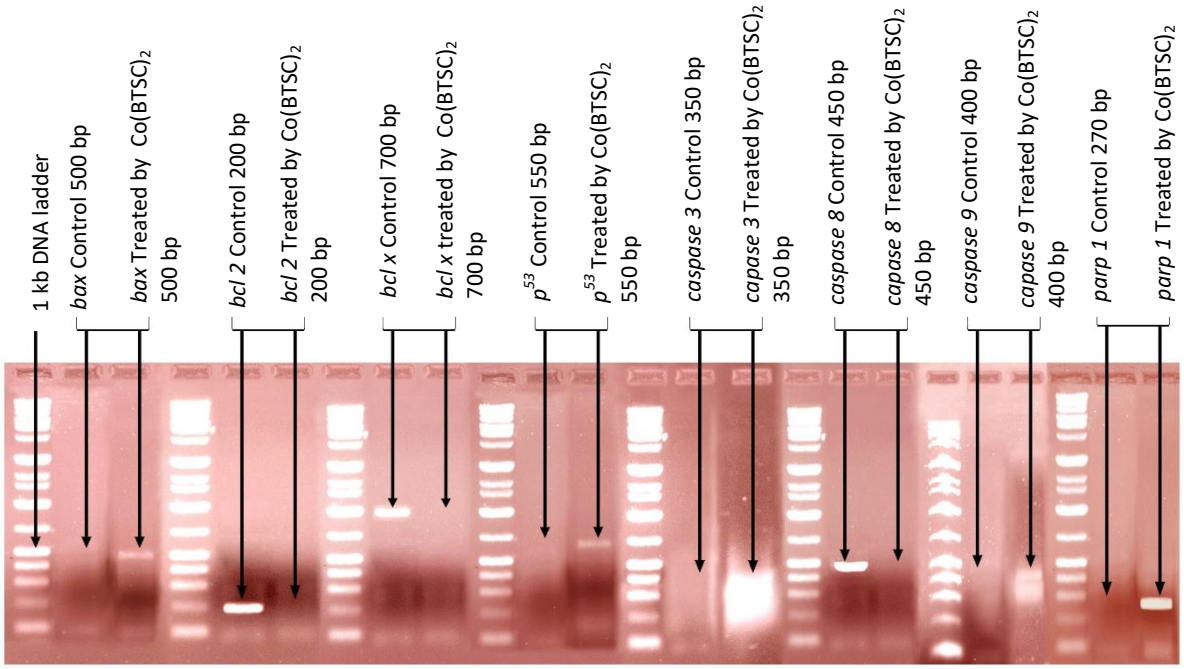
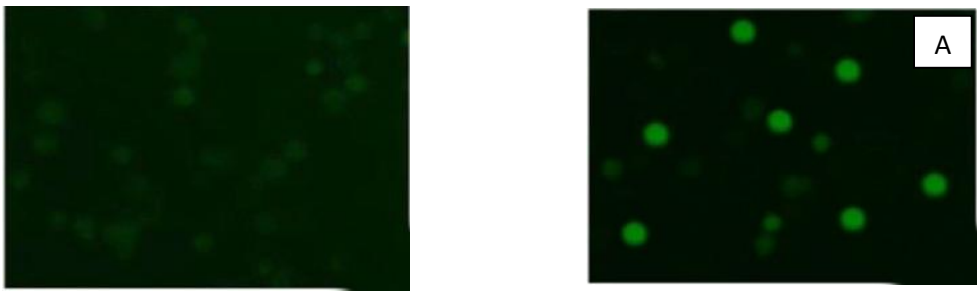


Figure-5: Expressions of different cancer-related genes in $Co(BTSC)_2$-treated EAC cells. RNA was extracted from the experimental mice on day 6 and level of mRNA expression of antiapoptotic genes (Bcl-2, Bcl-xL and caspase-8) and proapoptotic genes (p53, Bax, Caspase-3, -9 and PARP-1) were studied. In $Co(BTSC)_2$ treated mice.



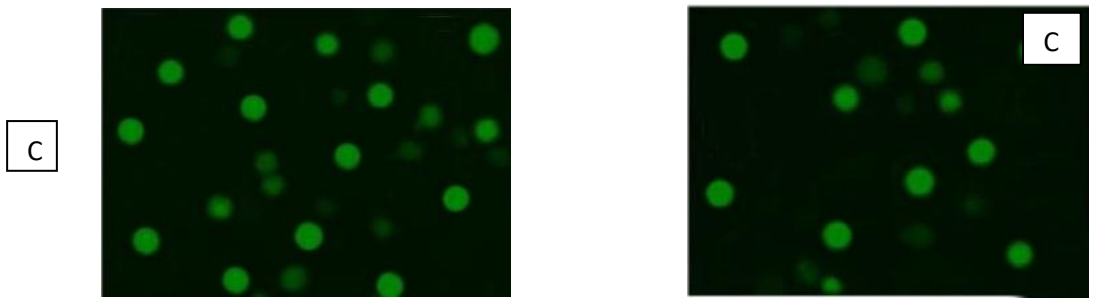


Figure-6: Effect of $Co(BTSC)_2$on the generation of ROS in EAC cells after 24 h treatment. Fluorescent microscopy observations (200× magnification) of cells treated with DCFH-DA. (A) Control cells, (B) 2 mg/kg (*i.p*), (C) 4 mg/kg (*i.p*.) and (D) 8 mg/kg (*i.p.*).