Original Research Article

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

Abstract

Cancer is a class of diseases in which a group of cells display uncontrolled growth, invasion and sometimes metastasis. Cancer is recognized as the second leading cause of death globally. Therefore, the discovery and development of new potent and selective anticancer drugs is are of high importance in modern cancer research. The objective of this study was to find out the mechanism through which Co(II)-benzoin thiosemicarbazone complex exerts its antitumor activity against Eehrlich ascites carcinoma (EAC) cells in bearing Ssswiss albino mice. Induction of apoptosis in EAC cells was confirmed by observation of nuclear morphology and DNA fragmentation assay. The intrinsic apoptotic pathway induced by $Co(BTSC)_2$ was evidenced by the mRNA expression levels of several tumor related anti-apoptotic genes B-cell lymphoma 2 (bcl-2), B-cell lymphoma extra-large (bcl-xL) and caspase-8, and pro-apoptotic genes 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. Reactive oxygen species (ROS) generation after $Co(BTSC)_2$ treatment was determined by 2´, 7´-dicholorodihydrofluorescein diacetate (DCFH-DA) staining. The results found during this study, confirmed that the induction of apoptosis by $Co(BTSC)_2$ occurred through an ROS-dependent mitochondria-mediated intrinsic pathway rather than an extrinsic pathway, and was regulated by the bcl-2 protein family. Thus, this study provides evidence to carry out further researches in a way to formulate novel anticancer drugs.

Keywords

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

Introduction

Schiff bases are condensation products of aldehyde and ketones with primary amines and were first reported by Hugo Schiff in 1864. These are the compounds containing imine or azomethine (-HC=N-) functional group and are found to be a versatile pharmacophore for design and development of various bioactive lead compounds. Schiff bases as well as schiff Schiff base

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complexes with transition metals form an important class of the most widely used organic and organometallic compounds and have a wide variety of applications in many fields including analytical, biological and inorganic chemistry. In recent times, Sschiff bases and Sschiff base metal complexes have drawn the attention of many researchers in medicinal and pharmaceutical fields due to a broad spectrum of biological activities, like anti-inflammatory, analgesic, antimicrobial, anticonvulsant, antitubercular, antioxidant, anthelmintic, anti HIV, antileprosy, antileukemia and, anticancer and so on. Several reports have been published as on the successful use of such class of compounds as anticancer agents [1-5]. However, further investigation is necessary for biological studies in order to understand more about their antitumor mechanism(s).

Apoptosis is a process of programmed cell death regulated by cellular signaling pathways and characterized morphologically by cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation and global mRNA decay. Apoptosis can be initiated through one of two pathways, one is the intrinsic pathway and other is the extrinsic pathway [6]. The intrinsic (mitochondrial) pathway is induced by cellular stress, infection and DNA damage and results in the activation of pro-apoptotic BCL-2 associated X (BAX) or BCL-2 homologous killer (BAKak) proteins that oligomerize to create holes or pores on the mitochondrial outer membrane and release cytochrome c, to the cytosol [7]. The another pathway is extrinsic (death receptor) pathway is initiated by a death ligand such as tumor necrosis factor α (TNF α) or TNF-related apoptosis inducing ligand (TRAIL) binding to cell surface receptors to and activate caspase-8, which then activates downstream caspase-3 directly, which plays an essential role in programmed cell death [8]. These two pathways are executed mainly by caspases a family of endoproteases that provide critical links in cell regulatory networks controlling inflammation and cell death with caspase-8 and -9 connectingengaging in the extrinsic and intrinsic pathways, respectively [9-10]. In addition, reactive oxygen species (ROS), a series of oxygen metabolism byproducts, have a role in cell signaling including; apoptosis; gene expression; and the activation of cell signaling cascades [11]. In theis present paper we have reported anticancer activities of Cobalt (II) benzoin thiosemicarbazone complex against EAC cells in Sswiss albino mice and the activation of the intrinsic signaling pathway leading to the mechanism of apoptosis.

Materials and Methods

Chemicals

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

Experimental animal model

Adult male Swiss albino mice, 5–7 weeks old (25 ± 4 g body weight), were collected from animal resource branch of the International Centre for Diarrheal Disease Research (ICDDR'B), Mohakhali, Dhaka, Bangladesh. Animals were housed in iron cages containing sterile paddy husk and saw dust as bedding material under hygienic conditions with a maximum of six animals ~~in a~~per cage. They were maintained under controlled conditions (12:12 h light–dark with a temperature of 22 ± 5 °C). Standard mouse diet (recommended and prepared by ICDDR'B~~International Centre for Diarrheal Disease Research, Bangladesh Mohakhali, Dhaka~~) and water were given to provide adequate nutrition and fluids~~in adequate~~.

Ethical approval was obtained from institutional animal, medical ethics, biosafety and biosecurity committee for experimentation~~s~~ on animal, human, microbes, and living natural sources from the Institute of Biological Sciences, University of Rajshahi, Bangladesh (225/320-IAMEBBC/IBSc).

Synthesis of the compounds

Synthesis of Co(II)-benzoin thiosemicarbazone complex

The compound was synthesized according to the method ~~as~~ described in the literature [12-13] For benzoin thiosemicarbazone (BTSC), benzoin and thiosemicarbazide (1:1 molar ratio) were mixed together and refluxed for a period of 3-4 hours and then distilled to half of the total volume. A saturated solution of metal (II) acetate [Co(II)] in ethanol was added to the condensed solution. Within a few minutes crystals of Co(II)-benzoin thiosemicarbazone were~~as~~ obtained. The crystals were~~as~~ washed with ethanol and recrystallized twice, dried in an oven at 50°C and stored in a desiccator.

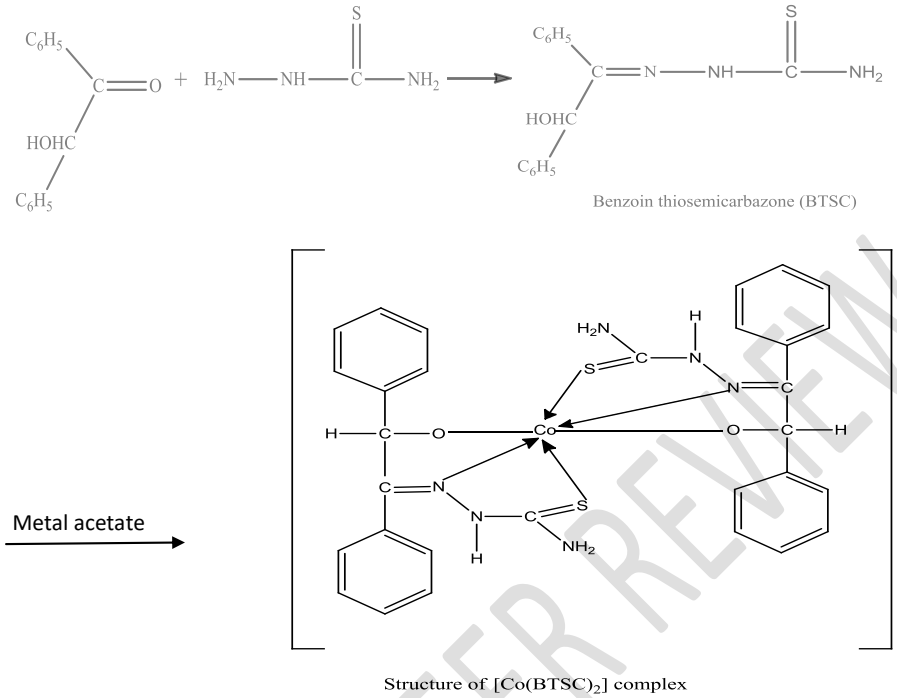

Figure- 1: Synthesis of Co(II)-benzoin thiosemicarbazone complex

Cell lines

Ehrlich ascites carcinoma (EAC) cells were obtained by the courtesy of Indian Institute of Chemical Biology (IICB), Kolkata, India. The cells were maintained as mammary gland cancer cells in ascites in Swiss albino mice by intraperitoneal inoculation (biweekly) of 2×10^6 cells/mouse. For *in vitro* study, EAC cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium, modified to contain glucose, 2 mM L-glutamine, in presence of 10% fetal calf serum (FCS), and 1% (v/v) penicillin-streptomycin (v/v) Cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C. in a humidified atmosphere of 5% CO_2 at 37 °C.

***In vitro* cell growth inhibition**

In vitro effect of $Co(BTSC)_2$ against EAC cell growth inhibition was assayed by MTT (3-(4,5-Dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide) colorimetric technique.

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NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to its insoluble formazan, which has a purple color. For this experiment, 4×10^6 EAC cells in 200 µL RPMI-1640 media were plated in the 96-well flat bottom culture plate in the presence of six different concentrations (5 µg, 10 µg, 20 µg, 40 µg, 80 µg and 120 µg/mL) of $Co(BTSC)_2$ and incubated for 24 h at 37 °C in a CO_2 tissue culture incubator. EAC cells treated with dimethyl sulfoxide (DMSO), were used as control. The assays were performed in triplicates to avoid experimental errors. After the incubation period, aliquot were removed carefully, and 180 µL of phosphate buffered saline (PBS) and 20 µL of MTT were added to each well and further incubated for 8 h at 37 °C. The aliquot was removed again and 200 µL of acidic isopropanol was added and incubated again at 37 °C for 1 h. Subsequently, absorbance was taken at 570 nm in a microtiter plate reader (Optical 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

DNA fragmentation assay by agarose gel electrophoresis was determined by the method described previously [14]. EAC cells were collected from mice treated with the experimental compounds $Co(BTSC)_2$ at the dose of 8mg/kg/mice (five consecutive days) and from control mice. The cells were washed with PBS and resuspended again in PBS. The genomic DNA was isolated by using a DNA extraction kit (Promega, Madison, WI, USA) and analyzed by electrophoresis on 1.5% agarose gel containing 0.1µg/mL ethidium bromide and visualized underon a standard Ultraviolet UV trans-illuminator.

Effect of caspase inhibitors on EAC cells

In order to confirm the involvement of caspases [15] in the response to experimental compounds that induced cell death, the untreated EAC cells were collected on day six of tumor inoculation

and washed with PBS. Then these cells were incubated in a CO_2 tissue culture incubator (RPMI-1064 media) with Z-DEVD-FMK (caspase-3 inhibition, 2µmol/mL) and Z-IETD-FMK (caspase-8 inhibitor, 2µmol/mL) for 2h. ~~Then~~ T~~t~~he cells ~~were~~ treated with $Co(BTSC)_2$ ~~and~~ were incubated for another 24 hours at 37^0C in a CO_2 tissue culture incubator. Finally, the cells were counted using hemocytometer ~~and~~ in order to determine~~d~~ the effect~~s~~ of experimental compounds on cell growth inhibition in presence or absence of specific inhibitors.

Measurement of Reactive Oxygen Species (ROS) generation

The ROS level of the cells was examined using 2´, 7´-dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen, USA). The cells were collected from control mice and treated mice on day six of tumor inoculation and washed with PBS at 1200rpm for 2minutes. Then the cells were incubated with DCFH-DA 10µL (50µM final concentration) at 37^0C for 30minutes in the dark. Again the cells were washed twice with PBS and maintained in 1mL culture medium. ROS generation was assessed using a fluorescence microscope at excitation and emission wavelengths of 485nm and 530nm, respectively [16].

Reverse transcription polymerase chain reaction

Total RNA was extracted using TRIzol method from mice receiving $Co(BTSC)_2$ at the dose of 8mg/kg/day and control EAC bearing mice on day six of tumor implantation. Total RNA was used as a template for reverse transcription using the following protocol: each 20µL reaction contained 125µM deoxynucleotide (dNTP), 100 pmol random hexamer, 100 pmol oligo dT_{18} primer, 50 units of MuLV reverse transcriptase (~~-~~New England Bio lab, Ipawich, MA, USA), diethylpyrocarbonate (DEPC) treated water and 3µg of total RNA. Briefly, RNA and oligo dT_{18} primer where incubated at 70°C for 15 minutes then immediately placed on ice, ~~after which~~ the other components were added and incubated at 42°C for 1h and then at 70°C for 15 minutes. Expression of one housekeeping gene (*gapgh*) and eight growth regulatory genes, namely, *bcl-2, bax, p53, bcl-xl, parp 1* and Caspase-3,-8,-9 were examined using these cDNA as template for PCR. Reaction mixture were prepared containing 1x~~X~~ ~~of~~ Taq polymerase, 25pmol each of forward and reverse primer, 2.5mM of each dNTP and 0.25U of platinum Tag polymerase (Tiangen, Beijing, China), 2µL cDNA and nuclease-free water to a total volume of 25µL. Primer sequences are listed in Table 8. BioRad (Hercules CA, USA) gradient thermal cycler was used for amplifications. All the PCR products were analyzed in 1.5% agarose gel and the Gene Rule~~a~~r 1kb DNA ladder (Fermenta~~u~~s, Pittsburgh, PA, USA) was used as marker. The cycling condition

for initial PCR activation step ~~of~~ was 3 min at 95 ˚C, followed by 35 cycles of 95 ˚C/1 min. For p53, Bcl-2 and Caspase-3, -8, -9 genes the annealing temperature was 55 ˚C/1 min, followed by 72 ˚C/1 min and a final extension of 72 ˚C/1 min. In the case of Bax, GAPDH and Bcl-xL, the annealing temperature was 54 ^{0}C~~e~~.

Statistical analysis

The experimental results have been expressed as the mean ± S.E.M. The parametric values were calculated by one-way analysis of variance ~~Data have been calculated by o~~ (ANOVA) followed by Dunnett "t" test for the determination of statistical significance using SPSS software ~~of 20~~ (version 20). The difference was considered to be statistically significant when $p < 0.05$.

Results and Discussion

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

In vitro effect of Co(BTSC)$_2$ on EAC cell growth inhibition have been investigated using the ~~by~~ MTT assay. The Co(BTSC)$_2$ induced EAC cell ~~-~~death is a dose dependent manner (Figure 2). The Co(BTSC)$_2$ showed 16.2%, 32.3%, 45.7%, 61.62%, 73.9% and 89.72% cell growth inhibition at the concentrations of 5, 10, 20, 40, 80 and 120 µg/mL respectively. The effect decreased with the reduction in concentration of Co(BTSC)$_2$.

DNA fragmentation assay

The activation of the endogenous Ca^{2+}/Mg^{2+} dependent endonuclease is the most distinctive biochemical hallmark of apoptosis. This activated endonuclease mediates~~d~~ the cleavage of internucleosomes and generates oligonucleotide fragments of about 180-200 base pairs length or their polymers. A DNA~~-~~ band was obtained from ~~-~~agarose gel~~–~~ when genomic DNA from the experimental compound Co(BTSC)$_2$ treated EAC cells was undergone PCR amplification followed by agarose gel electrophoresis. This DNA showed the characteristic feature of apoptosis induction. On the other hand, control group showed smear-like DNA degradation. Result ~~was~~ is shown in figure 3.

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

Caspase inhibitors Z-DEVD-FMK (caspase-3 inhibitor) and Z-IETD-FMK (caspase-8 inhibitor) were used to detect the involvement of~~-~~ specific caspases in the apoptotic cell death of EAC cells induced by the treatment with the ~~of~~ experimental compound Co(BTSC)$_2$. In the presence of Co(BTSC)$_2$ growth inhibition of EAC cells was of 78.89% and it decreased to 3.88 and 75.01%

in the presence of Z-DEVD-FMK (caspase-3 inhibitor) and Z-IETD-FMK (caspase-8 inhibitor) respectively in the cell culture medium (figure 4).

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

Reverse transcription PCR was used to study the mRNA expression levels of several tumor-related anti-apoptotic genes (*bcl-2, bcl-xl* and caspase 8) and proapoptotic genes (*p53, bax, parp-1*, caspase-3 and caspase-9) in control and Co(BTSC)$_2$-treated EAC cells (Figure 5). *gapdh* primers were used ~~for an amplification reaction~~ to confirm the suitability of the purified RNAs ~~and the samples were found to be suitable~~ for PCR. The control cells showed high expression~~s~~ of *bcl-2, bcl-xl* and caspase 8 genes, whereas EAC cells treated with experimental compounds showed reduced expression of *bcl-2, bcl-xl* and caspase 8 mRNA~~s~~ ~~expressions~~. ~~-~~In addition,~~–~~ the *p53, bax, parp-1*, caspase-3 and caspase-9 genes showed~~—~~ increased expressions in treated EAC cells. On the other hand, no expression of these genes was found in control mice.

Determination of intracellular reactive oxygen system in EAC cells

2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) is a common fluorescent probe that can be used for detecting several ROS in biological media. The DCFH-DA is firstly hydrolyzed to DCFH by intracellular esterase, which is then oxidized by reactive species and originates a fluorescent compound 2, 7- dichlorofluorescein (DCF), whose fluorescence intensity is proportional to the levels of ROS. Here, DCFH-DA was used to assess the effect of Co(BTSC)$_2$ on ~~-~~ROS generation in ~~-~~EAC~~-~~ cells ~~–~~after 24 h~~-~~ treatment. As shown in figure. 6,~~-~~ the~~–~~ untreated control cells~~–~~ displayed little green fluorescence, while increasing concentrations of the experimental compound resulted in much stronger signals, with 8 mg/kg (*i. p.*) of ~~the~~ Co(BTSC)$_2$~~compounds~~ producing the ~~-~~strongest intensity.

Conclusion

In the light of above observations the experimental compound~~s~~ can be considered as effective antitumor agent~~s~~ and are comparable in efficiency with that of *bleomycin* at least in cell growth inhibition, reduction in average tumor weight and enhancement of survival time of tumor bearing mice. ~~These~~ This compound~~s~~ may therefore be considered primarily as potent anticancer agents and the possible mechanism for ~~the~~ its anticancer effect is ROS dependent mitochondria-mediated intrinsic pathway rather than an extrinsic pathway and ~~was~~ it is regulated by the BCL-2 protein family. In order to ascertain these compound~~s~~ as a novel potential anticancer drug~~s~~, it is necessary to carry out further experiments against other cancer cell lines along with ~~higher~~ more

test in animal models ~~and with~~ using advanced techniques. These findings definitely give positive support to carry out further research~~es~~ in a way to formulate novel anticancer drugs.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

References

[1]. Zakir HM, Mondol MM, Shahriar SM, Hossain MA, Rahim MA, Dey AK, Amin R, Rahman MZ, Chowdhury AN, Moniruzzaman M, Khanam J, et al. Benzoin Thiosemicarbazone Inhibits Growth and Triggers Apoptosis in Earlich Ascites Carcinoma Cells through an Intrinsic Pathway. American Journal of Cancer. 2019. 7(1):1-9.

[2]. Ali SM, Zakir HM, Shahriar SM, Sarkar MH, Dey AK, Nur HP, Jesmin M, et al. In vivo anticancer activities of Ni (II)-Benzoin thiosemicarbazone complex [Ni (BTSC) 2] against ehrlich ascites carcinoma cells. Journal of Bio-Science. 2015. 23:77-88.

[3]. Zakir HM, Mondol MM, Shahriar SM, Hossain MA, Rahim MA, Dey AK, Amin R, Rahman MZ, Chowdhury AN, Moniruzzaman M, Khanam J, et al. Benzoin Thiosemicarbazone Inhibits Growth and Triggers Apoptosis in Earlich Ascites Carcinoma Cells through an Intrinsic Pathway. American Journal of Cancer. 2019. 7(1):1-9.

[4]. Zakir HM, Ali MM, Jesmin M, et al. Antibacterial activities of benzoin thiosemicarbazone and its complexes with Co (II) and Ni (II), et al. Asian Journal of Medical and Pharmaceutical Researches. 2016. 6(4):32-40.

[5]. Zakir HM, Islam AM, Nurtaj K, Ali MM, Jesmin M, et al. Pesticidal activities of benzoin thiosemicarbazone and its complexes with Co (II) and Ni (II). International Journal of Biosciences. 2016. 9(6):92-100.

[6]. Bai L, Wang S, et al. Targeting apoptosis pathways for new cancer therapeutics. Annu Rev Med. 2014. 65:139-155.

[7]. Tait SW, Green DR, et al. Mitochondria and cell death; outer membrane permeabilization and beyond. NRMCB. 2010. 11:621-632.

[8]. Wallach D, Kang TB, Kovalenko A, et al. The extrinsic cell death pathway and the elanmortel. Cell death Differ. 2008. 15:1533-1541.

[9]. Ola MS, Nawaz M, Ahsan H, et al. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Mol Cell Biochem. 2011. 351:41-58.

[10]. Mcllwain DR, Berger T, Mak WT, et al. Caspase functions in cell death and disease. Cold spring harb perspect biol. 2013.1; 5(4): a008656.

[11]. Hancock JT, Desikan R, Neill SJ, et al. Role of reactive oxygen species in cell signaling pathways. Biochem Soc Trans. 2001.29:345-350.

[12]. Longman, Elementary Practical Organic Chemistry Part-2: Qualitative Organic Analysis. 2nd ed. London. 1974; 118.

[13]. El-Shahawi MS, Al-Jahdali MS, Bashammakh AS, Al-Sibaai AA, Nassef HM, et al. Spectroscopic and electrochemical characterization of some Schiff base metal complexes containing benzoin moiety.Acta Part A: Mol and Biom Spectroscopy. 2013. 113:459–465.

[14]. Islam F, Khatun H, Khatun M, Ali MM, Khanam JA, et al. Growth inhibition and apoptosis of Ehrlich ascites carcinoma cells by the methanol extract of Eucalyptus camaldulensis. Pharm Biol. 2014. 52:281–290.

[15]. Yinyuan W, Dianjun W, Xiaodong W, Yinyin W, Fangli R, Donald C, Zhijie C, Baoqing J, et al. Caspase-3 is activated through caspase-8 instead of caspase-9 during H_2O_2-induced apoptosis in HeLa Cells. Cell Physical Biochemistry. 2011. 27:539-546.

[16]. Xiao-Dong W, Chen-Yang L, Miao-Miao J, Dong L, Ping W, Xun S, Jun-Da C, Li-Xuan G, Xiao-Peng H, Guo-Qiang L, Jian Z, Chun-Hua W, Zhen-Dan H, et al. Induction of apoptosis in human leukemia cells through an intrinsic pathway by cathachunine, a unique alkaloid isolation from Catharanthusroseus. Phytomedicine. 2016. 23:641-653.

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

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

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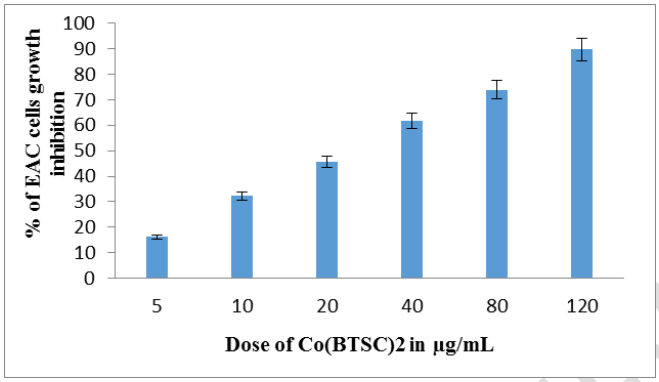

Figure-2. *In vitro* effect of $Co(BTSC)_2$ on EAC cell growth inhibition. Data are expressed as the mean of results in 3 repeated experiments. Error bars represent standard error of the mean.

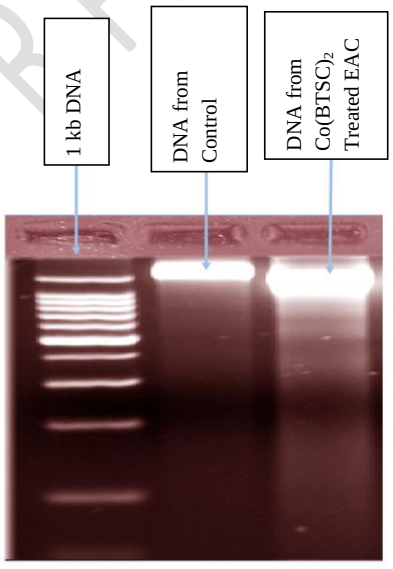

Figure-3: DNA fragmentation assay in $Co(BTSC)_2$-treated mice

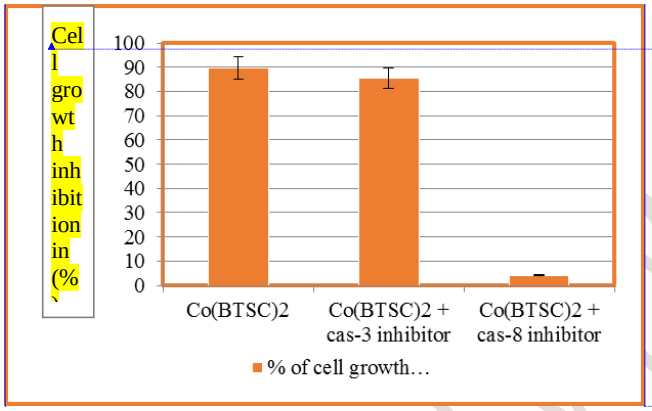

Figure- 4: Effect of caspases on $Co(BTSC)_2$- induced cytotoxicity in EAC cells. The n~~N~~umber~~s~~ of mice in each experiment was~~ere~~ 6. The results are shown ~~in~~ as mean±SEM. Significant value is, **p<0.01.

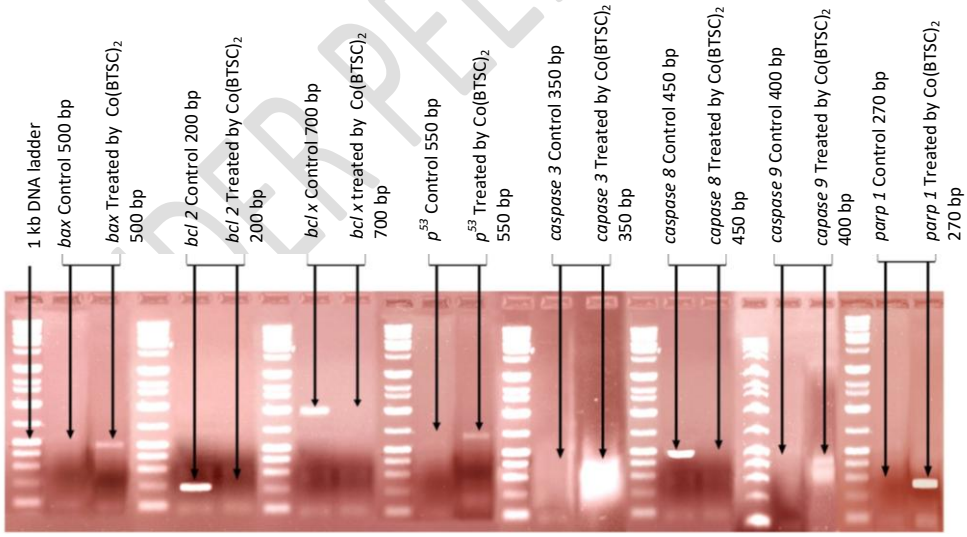

Figure-5: Expressions of different cancer-related genes in $Co(BTSC)_2$-treated EAC cells. RNA was extracted from the experimental mice on day 6 and level of mRNA expression of anti-apoptotic genes (Bcl-2, Bcl-xL and caspase-8) and proapoptotic genes (p53, Bax, Caspase-3, -9 and PARP-1) were studied. In $Ni(BTSC)_2$ treated mice, ~~all the proapoptotic genes expression increased remarkably, and the expression of antiapoptotic genes were not observed. In control group, opposite results were observed.~~

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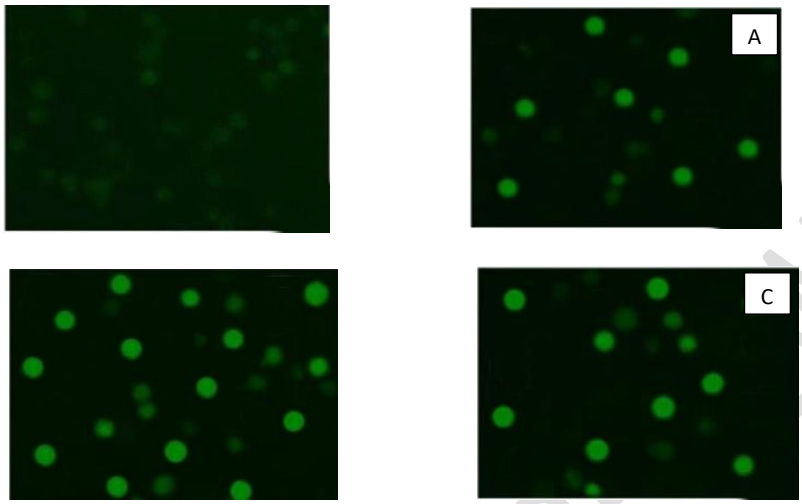

Figure-6: Effect of $Co(BTSC)_2$ on intracellular ~~-~~ROS generation. Effect~~s~~ of $Co(BTSC)_2$ on the generation of ROS in EAC cells after 24 h treatment. Fluorescent microscopy observations ~~Observation of cells by fluorescence microscopy~~ (200× magnification) ~~after incubation with~~of cells 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*.).

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