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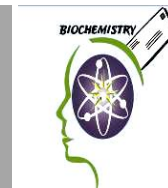

Antimicrobial, cytotoxic and genotoxic assessment of p-Cresol on different organism systems

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ABSTRACT

Background and Aim: The antimicrobial activity of p-cresol against different bacterial, fungal and yeast strains which have medical and pharmacological importance, also the effects of this pollutant on Murine fibroblast-like L929 cells and leukemia HL-60 cell line were evaluated in this study. **Materials and methods:** Agar diffusion test was used to reveal the antimicrobial action of p-cresol, Cytotoxicity was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5 - diphenyltetrazolium bromide (MTT) assay. Propidium iodide flow cytometry was used to analyze cell cycle. Genotoxicity and characterization of DNA damage on L929 cells was evaluated by alkaline comet assay, and then the change of expressions of the BCL-2 gene was detected by RT-PCR. **Results:** We found a broad spectrum of activity against clinically important bacterial, fungal and yeast strains. P-cresol also decreased the immortality of L929 cells with respect to concentration gradient. The apoptosis is a possible cause of the morphological alterations observed. The repression of growth of L292 and HL-60 cells by p-cresol was associated to stimulation of arresting S-phase cell cycle. There was higher % Tail DNA after treatment of p-cresol in a dose dependent manner leading to apoptosis, also the expression of BCL-2 mRNA decreased significantly. **Conclusion:** Knowledge the cytotoxic and genotexic effect of p-cresol may be useful in discovering treatment for patients with p-cresol intoxication.

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INTRODUCTION

Pollution generated by petroleum compounds elevated in recent years all over the world. Para-Cresol $CH_3C_6H_4$ is a phenolic organic compound used in antiseptics and disinfectants, in the production of artificial resins, in photographic plates and in explosives [1]. When human exposed to P-cresol by inhalation or oral administration this may lead to poisoning, and liver injury possibly

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due to blood clots and hepatic circulation disorder [2]. Furthermore being immensely toxic, the p-cresol will cause hurtful effects on the cardiovascular system, CNS, blood, lungs, kidneys, and liver [3]. Environmental Protection Agency categorized p-cresol as a toxin of group C [4].

P-cresol is a natural metabolism product, produced from tyrosine with anaerobic bacteria and stays in the small intestine then accomplish the last part of large intestine [5]. It is absorbed by the epithelial cells of large intestine, passed on the blood circulation, processed in the liver, finally excreted into urine [6]. Moreover, p-cresol is identified as a uremic noxious waste, and in patients with CKD, its deposition or that of p-cresyl-sulfate, causes to the progress of renal and cardiovascular diseases [7, 8].

Antimicrobial resistance is one of the most important health problems worldwide that efficiently inhibit many infections caused by bacteria, viruses and fungi that are not yet liable to the ordinary drugs used to treat them [9]. Also, the problem caused by high cost and increasing toxic adverse effects of these manufacturing drugs as well as their inadequacy in the treatment of diseases that are especially found in developing countries cannot be overcome [10]. Therefore, the discovery of new alternatives is necessary for treatment of infections involving resistant microorganisms.

The objectives of the present study are to investigate the antimicrobial potential, also, unveiled on the cytotoxicity and genotoxicity of p-cresol on other organisms, so we may use these beneficial properties as treatment mechanisms against different pathogenic organisms.

Materials and methods

P-cresol with purity 99% was attained from Sigma (St Louis, MO). The p-cresol dissolved in DMSO and diluted in different concentrations by sterile distilled water.

Cell lines were attained from the American Type Culture Collection (ATCC: PtK2) and the German Collection of Microorganisms and Cell Cultures (L-929, HL-60). Murine fibroblast-like L929 cells (ATCC CCL 1, NCTC clone 929 of strain L) spreaded and grownup on DME medium supplemented with 10% newborn calf serum (NCS). Human myeloid leukemic cell line HL-60 were purchased from ATCC and they grownup in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% NCS. Culture media and Fetal Bovine Serum (FBS) were from Gibco-BRL (Eggenstein, Germany, plastic wares and glass slides from Nunc (Roskilde, Denmark. Cells were cultured and maintained for three days at 37 ^{o}C in humidified atmosphere of 5% CO2.

Test Microorganisms

Bacterial strains: *Escherichia coli* and *Staphylococcus aureus* (ATCC 6538). Also, fungal strains *Botrytis cinerea and Pythium debaryanum* (ATCC 9998) as well as the yeasts *Hansenula anomala and Candida albicans* (DSM 1577). All strains were chosen based on their clinical and pharmacological importance.

Microorganism's growth condition

Escherichia coli: cells were cultivated aerobically in rich medium Luria-Bertani broth (LB) or in LB medium containing 1.5% (w/v) LB agar at 37˚C .

Staphylococcus aureus: was cultivated overnight in a flask containing 80 ml Tryptone Soya Broth (TSB; Oxoid)

Botrytis cinerea: were cultivated in Petri dishes with synthetic minimal medium (MM). The strains were incubated at 20˚C using incubator equipped with cool white light fluorescent tubes.

Pythium debaryanum: was cultivated on potato dextrose containing 2% agar (PDA) at 23 ˚C in the dark, as stated by the guidance for growth media given by DSMZ, for inoculation experiments.

Hansenula anomala (*Pichia anomala*): were grown in yeast/malt (YM) agar.

Candida albicans: were cultivated on Yeast Extract-Peptone-Dextrose (YPD) Agar at 30°C.

Antimicrobial activity of p-cresol:

Agar disc diffusion method by Perez et al. [11] was followed to determine the antimicrobial activity. Sterile paper discs were impregnated with 10 µl of test substance. The diameter inhibition clear zone was measured with a highly accurate ruler. After incubation period 24 and 48 h, plates were removed and the diameters of zone of inhibition (ZOI) were recorded. All the tested microorganisms were conducted in triplicate times and averages were taken.

Antiproliferative activity of p-cresol:

p-cresol effect on L929 cell proliferation was measured in 96-well plates. p-cresol dissolved in dimethylsulphoxide (DMSO), diluted with water (conc. ranging from 0.001-250 µmol) was added to suspended L929 mouse fibroblast cells (120 ml, 50,000 cells/ mL ; two replicates). Five days later, the metabolic activity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

cell morphology

The L929 fibroblast cells were grown to near confluency on glass coverslips (13 mm in diameter) at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's minimal essential medium supplemented with 2 mM glutamine, 0.1 mM non-essential amino acids, 10 % FBS (GibcoBRL). After 48 h, the indicated concentrations of p- cresol were added and the cells were incubated for an additional 24 h. The cells were fixed with Azur B-Eosin in methanol 1:15 (v:v) before the staining with Azur B-Eosin solution .

cell cycle distribution:

The influence of p-Cresol on apportionment of HL-60 cell cycle carried out by flow-cytometric study. Where 10^6 HL-60 cells (treated with p-cresol for 72 h) were collected by centrifugation and renovated with cold 80% methyl alcohol. 30 min later incubation at 0 °C, the restored cells were washed with PBS and then with saponin containing PBS (0.1 %, w/v). Finally 500 mL propidium iodide (20 µg/mL), and RNase (1mg/mL) were added and the cells were incubated at 37°C for 30 min. Samples were analyzed by a FacScan.

Quantification of DNA damage (comet) assay

L-929 cells were usually used for characterization of DNA damage. This was accomplished as explained previously [12]. All steps were done in faint light to reduce the UV damage. 10 µl cell suspension (mixed with 120 µl agarose (0.5% in PBS), put in glass slides load beforehand with a layer of 1.5% agarose. The slides were covered with coverslips and left for 10 minutes at 4 ºC. Coverslips were removed and a second layer of agarose only without cells was added, the slides were covered again then left at 4Cº for 5 minutes. At last the slides were submerged for 1 h in the dark at 4°C in the lysis buffer to eliminate proteins of cells. Following lysis, Slides were treated with a time of alkali denaturation and electrophoresis (0.86 V/cm) of 25 min each. Finally, slides were stained with 50 AL of ethidium bromide (2 mg/ml), covered up by a cover slip and examined at 400 magnification. Slides were observed using OptikaAxioscope fluorescence microscope. For each sample 100 randomly selected cells, respectively, were photographed and scanned. The mean tail moment (percentage of DNA in the tail × tail length) of the solitary cells was founded along with the image examination software (Loats Associates Inc., USA).

RT-PCR gene expression

After treatment of L-929 cells with distinctive concentrations of p-cresol for 24 hours, about 3.2 × 106 cells were collected for RT-PCR study. The cellular mRNA was isolated using the Dynabeads mRNA Direct Kit (Life Science). Contaminating genomic DNA was separated from quantified RNA samples to exclude false positive findings in RT-PCR

by DNaseI enzymatic digestion using DNaseI. Total mRNA was then reverse transcribed for 1 hr at 42°C. 1 mg of cDNA mixed with SYBR green PCR reagent. Real-time PCR was acheived using ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using the fluorescent dye Sybr® Green Master Mix.

The real time PCR conditions were fixed along with PCR parameters for primer optimization, and each sample of cDNA was studied with β-actin which act as home-management genes using applied biological systems (Biosystems® Life Technologies Power SYBR® Green PCR Master Mix). The PCR requirement was as follows: purification at 50°C for 2 min, denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 20 sec and at hybridization 60°C for 40 sec. Specific primers were designed for gel-based RT-PCR using the sequences obtained in Gene Bank :Bcl-2 sense primer: 5'-ACGGGGTGAACTGGGGGAGGA-3' antisense primer: 5'-TGTTTGGGGCAGGCATGTTGACTT-3' and β-actin sense primer: 5'-TGCGGCAGTGGCCATCTCTTGCTCG AAGTC-3', antisense primer: 5'-AGCAGCCGTGGCCATCTCTTGCTCG AACTG-3'. Relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ method, where CT is the threshold cycle [13].

Results

Effect of p-cresol on different microbial activities:

Antimicrobial activity of p-cresol was assessed opposed to a group of microorganisms as their intensity were evaluated both with qualitative and quantitative methods. Fig. (1) represents the antimicrobial activity of p-cresol established by the disc diffusion technique. It was found that the p-cresol inhibit the growth of a wide spectrum of microorganisms. It was found that the mean values of inhibition zones for E. coli, S. aureus, H. anomala, C. albicans, B. cinerea and P. debaryanum were 20, 12, 16, 17, 16. 14 mm diameter respectively as clear zones (C. zones). These values were calculated for 100 μmol as a mean of 5 values. It represents also 27 and 32 mm diameter of unclear diffusion zones (D. zones) with the fungal strains; B. cinerea and P. debaryanum respectively.

P-cresol effect on proliferative activity of L929 cells

Determination of cells proliferation was carried out using MTT technique. Viability and growth of cell was measured by absorbance at 490 nm of the purple formazan that was formed from MTT mitochondrial LDH, that is only active in vital cells.

Fig (2) showed that treatment of L929 cells with different concentrations of p-cresol resulted in growth inhibition in a concentration-dependent manner. Overall, it can be concluded that p-cresol has cytotoxic activities against animal cells.

P-cresol effect on morphology of L-929 cells:

Microscopic examination of gross morphology of L929 cells is shown in Figure 3.The characteristic of cell growth in the presence of p-cresol (200μmol) provided a consideration of how treated L929 fibroblast cells dramatically differ from aligned and closely packed in network fashion controls (Figure 3 A&B). The treated cells have grown chaotic, lost their alignment and adherence to the dish so that they appear almost round. The apoptosis may be a possible cause of these morphological alterations. Some cells appeared to be 'shrinking', and apoptotic 'spikes' were also observed .

Fluorescence microscopy study of contaminated DAPI cells was performed to study nuclear changes and the composition of the programmed cell death body, as apoptosis markers. Cells given p-cresol exhibited apoptotic morphology. The detected alterations in cell Morphology included cytoplasmic and nuclear contraction, chromatin compression and formation of programmed cell death bodies were revealed after treatment with

p-cresol. In the untreated or DMSO treated L929 fibroblast cells, the stained nuclei were rounded and equivalently stained with DAPI, whereas cells dealt with p-cresol exhibited a changed nuclear DNA staining configuration with condensed chromatin nuclear fragmentation that indicated apoptosis (Figure 3 C&D). Overall, it can be concluded that p-Cresol hindered the growth of L-929 fibroblast cells also in a time- contingent way.

P-cresol affects cell cycle

The influence of p-Cresol on the distribution of cell cycle (HL-60 cell line was used) achieved by flow cytometry assessment. HL-60 cells are myeloid leukemia cells of human developed from white blood cells of a Caucasian female with acute vascular leukemia. The influence of p-Cresol on the distribution of HL-60 cell cycle give rise to 29.7 % of the cells accumulated in S phase on day later the addition of the p-Cresol and 80.9 % in S phase after 48 h (Fig 4 B&C). Almost all of the cells (98%) were arrested in the S phase after 72 h (Fig 4 D). On the other hand, only 33% of the cells were in the S phase in untreated control cells (Fig 4 A).

Genotoxicity of p-cresol (comet assay)

With the intention of additional prove the relationship between p-Cresol and DNA damage in L-929 fibroblast cell line we carried out solitary cell gel-electrophoresis (alkaline comet assay), a high effective and direct test utilized to determine DNA damages. The tail moment level, that is an outcome of tail length and DNA content in the tail, was positively linked with the amount of DNA damage in a cell. We found greater evidence of DNA breaks, in terms of % tail DNA. There was higher % Tail DNA after treatment of p-cresol (depending on dose) leading to apoptosis (Fig 5).

P-cresol treatment and BCl-2 gene expression

BCL-2 is well-known apoptosis marker gene which acts as important factor in regulation of apoptosis. In the present study the change of expressions of the BCL-2 was detected by quantitative real-time PCR. It was decreased significantly (fig 6).The expression of BCL-2 mRNA decreased by about only 7 % after treatment with 50 mmol p- cresol and reached about 34% and 50% with higher concentration (100 and 150 mmol respectively).

Discussion

P-cresol, low-molecular-weight compound, is a member of the large family of the phenols is also used as an antiseptic and disinfectant because of its bactericidal and fungicidal properties, but there are still insufficient data to support its safety for use in cosmetics [14]. P-Cresol has many applications in the fragrance and dye industries.

The present study revealed a broad spectrum of activity against clinically important bacterial, fungal and yeast strains. The agar disc diffusion method showed anti-microbial activities of p-cresol against the tested microorganisms. Its high efficacy against E. coli, S. aureus, *H. anomala*, *C.albicans, B. cinerea* and *P. debaryanum* hence para-cresol is used as a disinfectant and in the manufacture of herbicides. The obtained results confirm that p-cresol is bactericide and fungicide, and is in agreement with that obtained by Höferl *et al.* [15]. The p-cresol is added to soaps as disinfectants and mixtures of m- and p-cresol often serve as disinfectants and preservatives [16].

P-cresol represses the function of lining the normal blood vessels, such as proliferation, wound healing and restraint to cytokines [17, 18]; it additionally prevents the liberation of platelet activators by macrophages in rat that represent a vital function of platelet [19]. Also, many lethal poisoning cases with cresol were recently reported [20].

Cell proliferation represents one of the most important characteristic of growth and maturity of cell. The current research revealed the inhibition curve depending on the concentration of L929 mouse fibroblasts by p-cresol. Markedly it

inhibited the viability of L-929 fibroblast cell determined using MTT assay, and reduction of the metabolic action that might be shown as cytotoxic effects. This study agrees with chang *et al.* [21] and Andriamihaja et al. [22] who proved the cytotoxic influence of p cresol on different cells.

In the current research the p-cresol induced cell morphological changes and reduced the percentage of viable cells. The treated cells have grown chaotic, lost their alignment and adherence to the dish so that they appear almost dense and round. The nuclei of the cells stained with DAPI were increased in size and some appeared fragmented. These results indicate that inhibition of cell development and maturity using p-cresol was due to initiation of cell apoptosis. Our results match with de Carvalho *et al.* [23].

For further understanding of whether the growth-inhibition outcome of p-cresol is associated with cell apoptosis flow cytometric analysis was utilized to analyze the Cell cycle of HL-60 cells. HL-60 cell line is a long-term suspension culture of human myeloid leukemia. It was obtained from white blood cells of Caucasian female with acute vascular leukemia [24].

P-cresol may stimulate G2/M cell cycle stop of endothelial progenitor cells [25]. The initial and main HL-60 cell line had numerous features of malignant cells and expressed several oncogenes. This study showed that p-cresol drives the cells to be cumulative in the S phase of the cell cycle in a time-dependent manner. On the other hand, the large proportion of the untreated control cells was distributed in G_0/G_1 phase. Our finding matched with that of chang *et al.* [21] who demonstrated that p-cresol reduced the growth and viability and number of EAHY and U937 cells, showed also that the toxicity of p-cresol was correlated to stimulation of S-phase programmed cell death and stimulation of ROS production. The study presented in this article clearly demonstrated that p-cresol clearly prevented the growth and viability of different cancer and non-cancer cell lines. L-929 cells were utilized as a typical cell line that was commonly used to identify genotoxic ability in various genotoxicity assays, and characterization of DNA damage. Our results indicate a limited potential genotoxic effects on L-929 cells. This agrees with Andriamihaja *et al.* [22].

We next examined whether the different doses of p-cresol affected mRNA expression of genes involved in cell apoptosis. RT-PCR exhibited that the expression of Bcl-2 was down-regulated. This gene is involved in cancer cell anti-apoptosis mechanism. The results demonstrated decreases in demonstration of intrinsic anti-apoptotic pathway Bcl-2 mRNAs. This suggests the participation of intrinsic pathway in programmed cell death initiation by p-cresol in L929 cells. The decreased expression of Bcl-2 could be candidate mechanism underlies induction of apoptosis in L-929 by p-cresol.

In conclusion, p-cresol has wide range antimicrobial activity against different bacterial, fungal and yeast strains. This proved its use as disinfectant and in fragrance industry. Also, p-cresol repressed cell maturation and development by initiating programmed cell death in fibroblast L-929 cells and HL-60 cells; exert also the cytotoxic effect by triggering the intrinsic apoptotic pathway.

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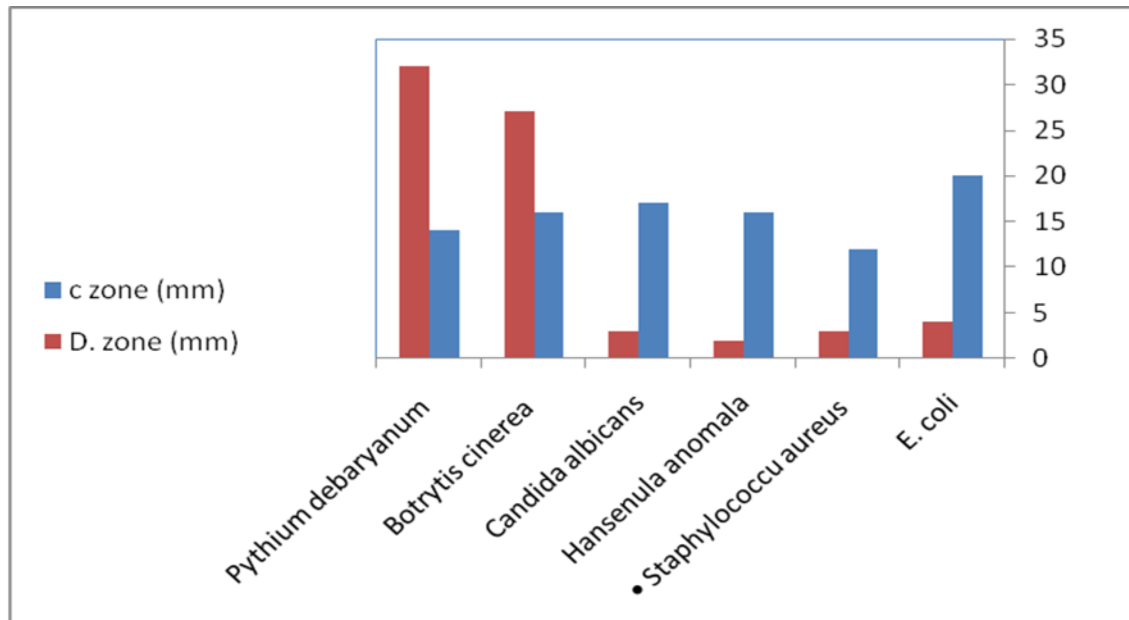

Figure 1: antimicrobial activity (Disk diffusion method) of p-cresol (100μmol)

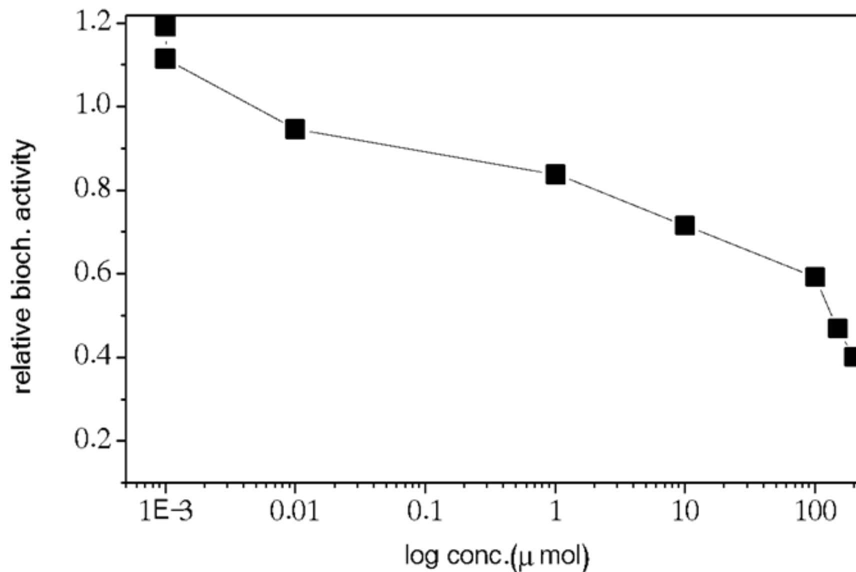

Figure 2: Cells proliferation was determined using MTT assay. After 48 h the value of OD was measured by absorbance at 490 nm.

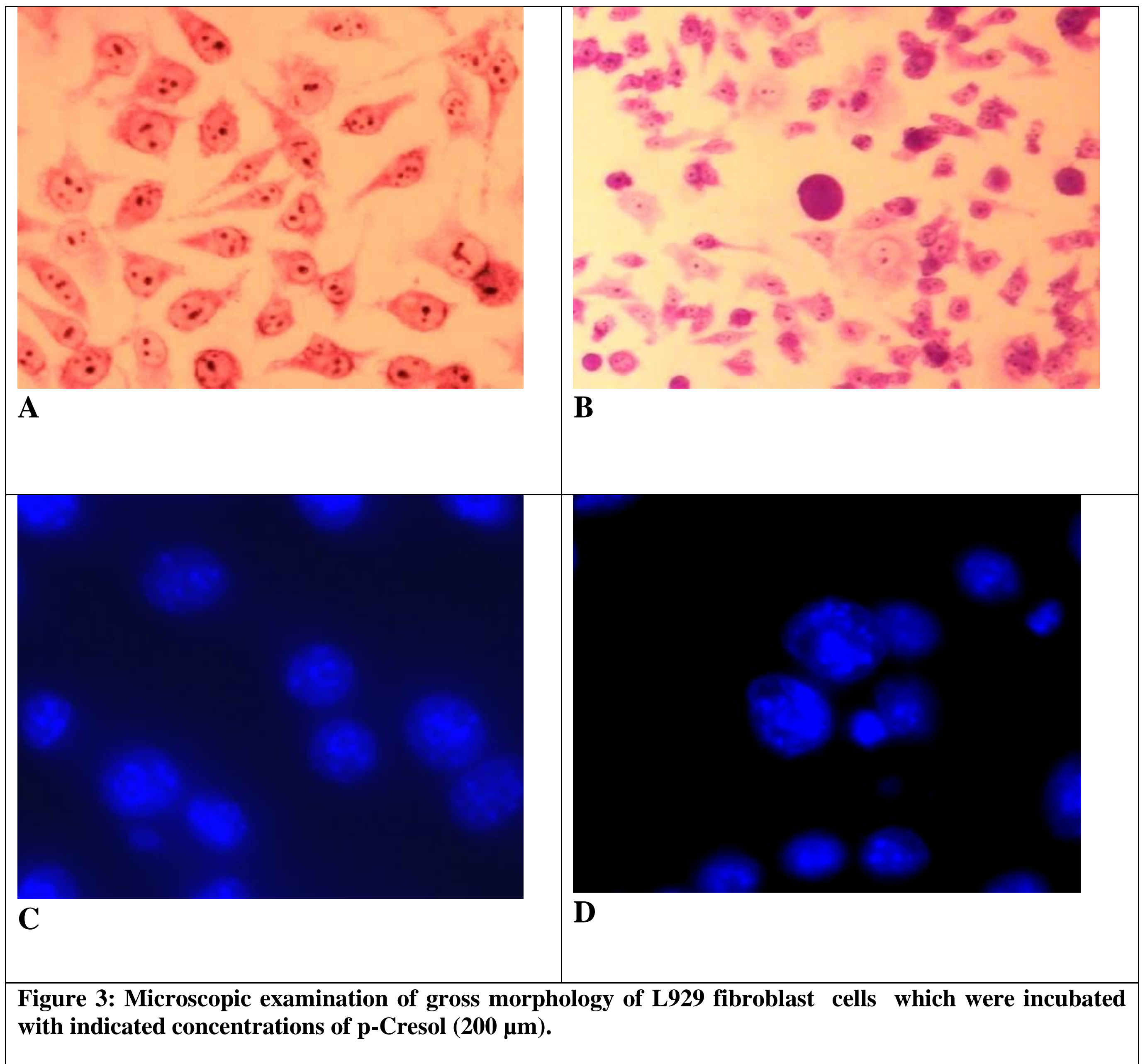

Figure 3: Microscopic examination of gross morphology of L929 fibroblast cells which were incubated with indicated concentrations of p-Cresol (200 µm).

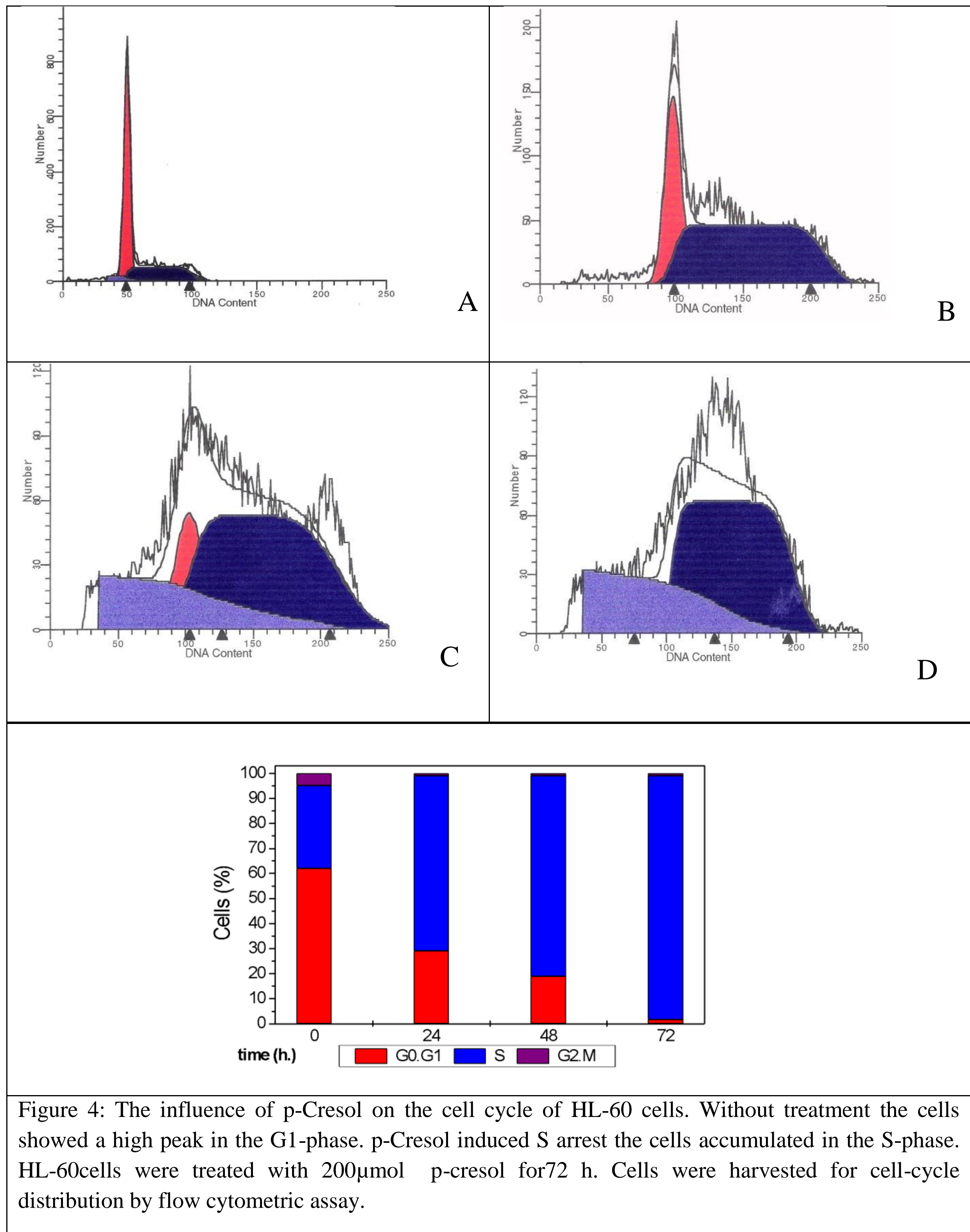

Figure 4: The influence of p-Cresol on the cell cycle of HL-60 cells. Without treatment the cells showed a high peak in the G1-phase. p-Cresol induced S arrest the cells accumulated in the S-phase. HL-60cells were treated with 200µmol p-cresol for72 h. Cells were harvested for cell-cycle distribution by flow cytometric assay.

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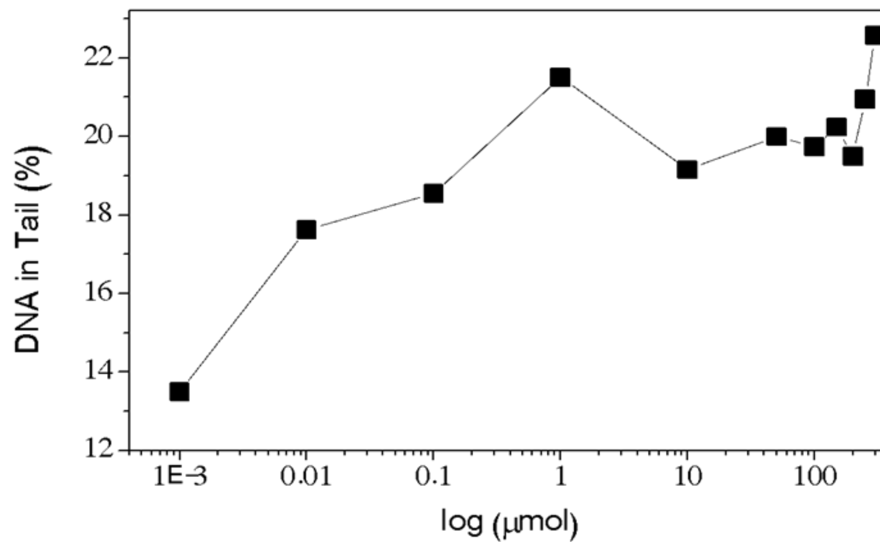

Figure 5: Genotoxicity (DNA damage in L-929 fibroblast) of p-Cresol Using Comet assay. P-cresol in the concentrations of 0.01 to 250 μM increased the DNA damage relative to control.

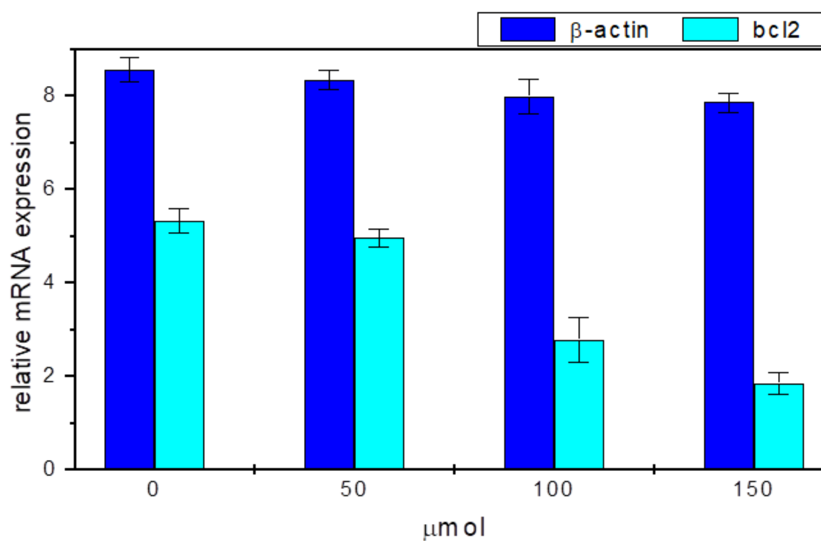

Figure 6: The expression of apoptotic BCL-2 to β-actin genes in L-929 fibroblast cell. The expression levels were determined by real- time PCR. In cell treated with p- cresol for 24 hours against untreated control cell. β Actin was used as a loading control respectively