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CYTOTOXIC EFFECTS OF THEOBROMINE DERIVED COPPER NANOPARTICLES ORAL CANCER CELLS VIA P53 ACTIVATION

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ABSTRACT

Background: Oral cancer remains a major global concern , with limited success from conventional therapies due to chemoresistance and systemic toxicity.Phytochemical assisted nanoparticles due to chemoresistance and systemic toxicity.Phytochemical assisted nanoparticles reflect a biocompatible alternative with multi potency.This study investigates the cytotoxicity and molecular effect of Theobromine derived copper nanoparticles (Theobro-CuONPs) in KB oral cancer cells

Objective: To investigate the cytotoxicity of Theobro-CuONPs by emphasizing p53 mediated apoptosis and inflammatory signalling in a gene expression profile in KB cells

Methods: Theobro-CuONPs have been synthesized employing theobromine , a natural and green reducing agent. The synthesized nanoparticles were characterized by standard techniques. The cytotoxic effect was determined by performing an MTT assay , whereas the expression of p53 , Bax , Bcl-2 , Caspase-3 , TNF - α , IL-6 and IL-10 genes was assessed by quantitative real time PCR in treated vs control KB cells.

Results: Theobro-CuONPs decreased cell viability more than theobromine or CuO alone in a dose-dependent manner. Gene expression profiling indicated that while the expressions of p53 , Bax and Caspase-3 were upregulated , those of Bcl-2 , TNF-α , IL-6 and IL-10 were downregulated . Thus , the intrinsic apoptotic pathway got activated while suppressing inflammatory responses.

Conclusion: Theobro- CuONPs exhibit major anticancer effects by altering the expression of key apoptotic and inflammatory genes , thus proving their immense potential as a green , biocompatible candidate for oral cancer therapy

Keywords: Theobromine , Copper nanoparticles , Oral cancer , p53 activation , apoptosis , inflammation , green synthesis

1.Introduction

Oral cancer is a global health concern , being among the world's leading ten most prevalent cancers. Despite the improvement in diagnostic and therapeutic modalities , the outlook of advanced stage Oral squamous cell carcinoma patients is bleak due to recurrence , metastasis , and drug resistance. (Sung *et al.*, 2021)The inadequacy of current therapy forms is the basis for the design of new therapeutic approaches with potential alternatives , particularly through the synthesis of metal based nanoparticles with enhanced cellular uptake , targeted efficacy and low systemic toxicity. (Aditya *et al.*, 2021)

Among various metal-based nanomaterials , Copper nanoparticles are well known effective candidates for cancer treatment. Copper is a trace metal involved in fundamental physiological functions like the maintenance of oxidative stress, mitochondrial respiration, and enzyme catalysis . (Cobine and Brady, 2022)In incorporation into the niche of nanoscale materials , copper has the ability to selectively induce cancer cell oxidative damage , trigger apoptosis , and suppress pro-survival signals.CuNPs were also seen to exhibit significant cytotoxicity against a broad range of human cancer cell lines , and hence hem is a drug that can be investigated to cure oral cancer.(Eissa *et al.*, 2023)

Nanoparticles synthesized through green methods using naturally occurring or plant widely promoted as green and safer alternatives over chemical synthesis , conventionally pursued .(Sreenivasagan *et al.*, 2023) One of the target phytochemicals is theobromine , which is a methylxanthine alkaloid amply found in cacao beans and possesses antioxidant , anti-inflammatory , and mild cytotoxic activities.(Khaldari, Naghavi and Motamedi, 2021) With its low molecular weight configuration that is similar to those of caffeine and theophylline, theobromine exhibits modulatory activity toward cell cycle progression , oxidative stress response , and immune signalling. Based on its typical molecular structure , it may be used as a reducing and stabilizing agent in nanomaterial synthesis . In green synthesis of copper nanoparticles, theobromine imparts not only biocompatibility but also some additional bioactivity that may enhance the therapeutic potential of the final preparation.(Siddiqui *et al.*, 2013)

Tumor suppressor protein p53 is a significant protein involved in the maintenance of genomic stability and regulation of cellular response to stress. It is referred to as the “guardian of the genome” for its tumor suppressive role in maintaining control over a wide array of downstream events such as DNA repair , cell cycle arrest , and apoptosis.(Pandiar *et al.*, 2022) p53 is frequently mutant or nonfunctional in plants and animals , such as oral cancers , and is highly correlated with disease outcome and chemoresistance. (Ghasemi *et al.*, 2023)Thus p53 upregulation is considered to be a promising therapeutic strategy to restore apoptotic sensitivity in cancer cells. Importantly , p53 also controls the transcriptional regulation of essential apoptotic genes such as Bax and Bcl-2 , and caspases that are essential effectors of programmed cell death.

Bax (Bcl-2 associated X protein) is a pro-apoptotic Bcl-2 family protein that is accountable for induction of mitochondrial membrane permeabilization and cytochrome c release and activation of the intrinsic apoptotic pathway. Bcl-2 on the other hand , is an anti apoptotic protein that is involved in maintaining the integrity of the mitochondria and preventing apoptosis.(Mahmood *et al.*, 2022) The balance between Bax expression and Bcl-2 expression is a molecular switch determining cell fate.In cancer cells , this balance is disturbed in the direction of survival. Restoration of the balance by employing specific therapeutic drugs is one of the most important objectives of apoptosis based-cancer treatment.(Mulay *et al.*, 2019)

Caspase-3 , a key executioner caspase , is activated downstream of mitochondrial signalling and is involved in the cleavage of structural and regulatory proteins to promote apoptotic cell death. Activation of this caspase-3 is a hallmark of effective pro-apoptotic intervention, and upregulation of the same has been utilized as an effective indicator of intrinsic apoptosis for decades.(AlOthman, 2020) In addition to the regulation of cell death, cancer treatment also needs to address tumor-induced inflammation, which subsequently places itself in a double role of aiding in cancer growth and immune system regulation of cell death , cancer treatment also needs to address tumor-induced inflammation, which subsequently places itself in a double role of aiding in cancer growth and immune system regulation.(Rehana *et al.*, 2017) Cytokines such as TNF-α(tumor necrosis factor-alpha) and IL-6(interleukin-6) have been documented to play a mediating role in chronic inflammation in the tumor

micro environment toward proliferation, angiogenesis and metastasis. These cytokines are normally associated with poor prognosis and metastasis. These cytokines are generally associated with poor prognosis in oral cancers. Alternatively , anti-inflammatory cytokine IL-10 plays a multifaceted role with its ability to suppress pro-inflammatory responses and limit immune mediated tissue injury .(Landskron *et al.*, 2014) In cancer, nonetheless, IL-10 is able to promote tumor tolerance and immune evasion.

This study was designed to explore the cytotoxicity and molecular mechanism of Theobromine originated copper nanoparticles (Theobro-CuONPs) towards oral cancer cells with specific focus on p53 activation and further pro-apoptotic and anti-inflammatory Metrygene expression. Synthesis of Theobro the-CuONPs followed green, theobromine mediated approach and was assessed in vitro using reference biological assays.(Liu *et al.*, 2022)Cytotoxicity dose dependent,y of Theobro-CuONPs was quantitated by MTT assay on oral cancer cells. MTT assay is a colorimetric assay that provides quantitative information regarding cellular viability and metabolic activity and thus a useful screen test for first-line anticancer drugs.(Qian *et al.*, 2022) In parallel , qRT-PCR-based analysis of gene expression was also performed to examine the transcriptional response of the important genes p53 , Bax , Bcl-2 , Caspase-3 , TNF-α , Il-6 and Il-10. These genes have been chosen so as to know the coordination between apoptosis induction, reactivation of tumor suppressor function, and modulation of inflammation after Theobro-CuONP exposure. (Paramasivam, George and Priyadharsini, 2021)

With the application of green nanotechnology in molecular oncology, this study attempts to gather together the increasing evidence favoring the application of photochemical stabilized metal nanoparticles as targeted , multifunctional anticancer agents. The cross-talk between cytotoxicity assays and gene-expression analysis provides mechanistic insights into how Theobro-CuONPs regulate the key pathways involved in cell death and immune modulation and oral cancer models. Overall , this study proposes Theobro-CuONPs as a candidate compound for construction of new oral cancer therapy.(Kanchi and Ahmed, 2018) Through p53 upregulation and subsequent downstream induction of apoptosis markers and control of inflammatory mediators , Theobro-CuONPs potentially offers a dual mechanism approach of inhibiting tumor growth and control of pro-tumorigenic inflammation. The outcomes of this research may inform future preclinical development towards novel nanoparticle-based therapeutic agents for the treatment of oral cancer.(Naikoo *et al.*, 2021)

2. Materials and Methods

2.1 Chemicals and Reagents

Theobromine (≥98%) as a reducing and stabilizing agent in nanoparticle synthesis was obtained from Sigma-Aldrich(St. louis, MO , USA) . Copper sulfate pentahydrate (CuSO4.5H20) as a precursor for copper and sodium hydroxide (NaOH) as a pH adjuster were obtained from Merck (Darmstadt, Germany). Absolute ethanol was utilized for washing nanoparticles and was supplied by Himedia Laboratories (Mumbai, India). Distilled water was utilized in washing and synthesis for preventing contamination. Human oral cancer cell line was obtained from National Centre for Cell Science (NCCS) , Pune , India , for cell based research study. The cells were grown in Dulbecco's Modified Eagle Medium(DMEM; Gibco , USA) containing 10% fetal bovine serum(FBS; Gibco) and 1% Penicillin-Streptomycin solution at standard conditions (37°C, 5% CO2). To determine cytotoxicity, MTT(3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyltetrazolium bromide)was provided by Thermo Fisher Scientific (USA). For gene expression analysis, TRIzol reagent (Invitrogen, USA) was used to isolate total RNA and reverse transcription of cDNA was performed using a high capacity cDNA Reverse Transcription Kit (_signature of Applied Biosystems, USA). SYBR Green-based real time PCR

amplification was conducted using Takara SYBR Premix Ex Taqll (Takara Bio Inc , Canada) with a compactable qPCR system.

2.2 Green synthesis of Theobro-CuONPs

Theobro-CuoNPs were synthesized via green chemistry using theobromine as reducing and stabilizing agent. In room temperature magnetic stirring , 10 mg of theobromine was dissolved in 50 mL deionized water to form a clear solution. 50 mL of a 1mm solution of copper sulphate pentahydrate(CuSO4.5H2O) was also prepared and was added dropwise with stirring to the solution of theobromine. Reaction mixture was left at room temperature for 3-4 hours and, within this time frame, was observed to be transformed from light blue to dark brown color as a result of the formation of copper nanoparticles. The pH level of the solution was increased to about 9 using 1M NaOH in order to facilitate complete reduction of Cu2+ ions . The obtained Theobro-CuONPs were isolated following the completion of the reaction by centrifugation at 12,000 rpm for 20 min and sequentially washed three times with absolute ethanol and deionized water to eliminate by-products and unreacted residues. The final pellet was dried in a vacuum oven at 40°C overnight and sealed away in containers for further characterization and biological investigation.

2.3 Methods of characterization of nanoparticles

The synthesized Theobro-CuONPs were then identified through a set of routine physicochemical techniques. Proof of the nanoparticle formation was confirmed through UV-Visible spectrophotometry by scanning the absorbance between 200-800 nm with Shimadzu UV-2600 spectrophotometer. For detection of the reduction and stabilizing entities, Fourier Transform Infrared Spectroscopy (FTIR) was analyzed by recording PerkinElmer FTIR spectrometer with range of 400-4000 cm^{-1}. To visualize particle size and surface morphology, Scanning Electron Microsscopy(SEM) was employed. The X-ray Diffraction (XRD) technique employing Rigaku Miniflex diffractometer(Japan) with operating voltage 40kv and current 30mA using Cu-Kα (λ = 1.5406 Å) radiation was employed to identify crystalline structure and phase characterization of the synthesized Theobro-CuONPs. Diffraction pattern was recorded in 2θ range 10° to 80° at scan rate 2°/min. Average crystallite size was determined by applying the Debye-Scherrer equation.

2.4 In vitro cytotoxicity study with MTT assay

MTT assay was employed to approximate Theobro-CuONPs cytotoxicity towards KB cells. Human oral cancer KB cells were kindly gifted by National Centre for Cell Science (NCCS) , Pune , India and these cells were plated in Dulbecco's Modified Eagle Medium (DMEM; Gibco , USA) with 10% fetal bovine serum (FBS; Gibco) and 1% Pencillin-Streptomycin. The cells were grown at 37°C under a humidified 5% CO2 atmosphere. KB cells were plated in 96 well plates at 1×104 cells/well for assay and left overnight to get adhered. The cells were treated with various concentrations of Theobro-CuONPs (5,10,25,50 and 100 µg/ml) for 24 hours. The negative control was untreated cells. 20µL of MTT solution (5 µg/mL in PBS) was added to each well after treatment and the plates incubated again for 4 hours at 37°C. Formazan crystals formed were solubilized in 100µL of DMSO, and absorbance was read at 570 nm in a microplate reader (Bio-Rad, USA). Cell viability was calculated as a percentage compared to the control group.

2.5 Gene expression analysis through Quantitative Real Time PCR

Comparative relative gene expression of apoptosis and inflammation-related genes was calculated by quantitative real-time PCR(qRT-PCR) for investigating molecular mechanisms of cytotoxic activity. Theobro-CuONps (50µg/mL) were incubated with KB cells for 24 hours. Total RNA isolation was performed on treated and untreated cells by TRIzol reagent (Invitogen,USA) as per the vendor protocol. The integrity and the amount of isolated RNA were analyzed spectrophotometrically , and

1µg of RNA was reverse-transcribed using complementary DNA (cDNA) by a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). SYBR Premix Ex TaqTM II (Takara Biosystems SteponePlus, USA) was applied for qRT-PCR. Gene-specific primers targeted were for p53, BAX, Bcl-2, Caspase-3, TNF-α, IL-6, and IL-10, using GAPDH as internal housekeeping control. All three reactions were carried out in triplicate in a volume of 20µL that comprised 10 µL SYBR Green master mix, 1µL each of one primer, 2 µL cDNA, and 6 µL nuclease free water. The thermal cycling condition used was a pre-diffusion step at 95°C for 5 minutes, followed by 40 denatures of 15 seconds at 95°C and 40 annealing/extension of 30 seconds at 60°C. Relative levels of gene expression were obtained from the 2^–ΔΔCt method and normalized to GAPDH expression

Table 1: Primers used for gene expression

Gene	**Forward Primer (5'→3')**	**Reverse Primer (5'→3')**	**Amplicon size (bp)**
P53	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC	150
Bax	TTGCTTCAGGGTTTCATCCA	GATCAGCTCGGGCACTTTAG	120
Bcl-2	GTGGAGGAGCTCTTCAGGGA	AGGCACCCAGGGTGATGCA	130
Caspase-3	CATGGAAGCGAATCAATGGACT	CTGTACCAGACCGAGATGTCA	140
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG	110
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG	108
IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG	122
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	121

Results:

3.1 Physicochemical characterization of Theobro-CuONPs

Several physicochemical analyses were conducted on the synthesized Theobro-CuONPs in order to confirm their formation , structural integrity , and morphological features. UV-Vis spectroscopy was conducted 200-800 nm for studying the optical properties and surface plasmon resonance (SPR) features of the nanoparticles. Appearance of a single absorption band between 570-590 nm confirmed successful synthesis of CuNPs. FTIR spectrum in the 4000-400 cm^{-1} range identified functional groups involved in nanoparticle stabilization. Absorbance peaks indicating O-H , N-H , C=O stretching conformed with the presence of theobromine capping and copper ion reduction. Surface topography and size of nanoparticle were recorded by SEM in order to reveal dominance of spherical or quasispheroidal nanoparticles with negligible agglomeration and average particle size 40-80 nm. Crystalline nature of the nanoparticles was established through XRD analysis, and typical diffraction peaks were seen similar to face centred cubic (FCC) structure of copper. The size of the crystallite was approximately 45-50 nm from the use of the Debye-Scherrer formula. All these detailed analyses confirmed that green synthesis using theobromine effectively produced

stable, crystalline copper nanoparticles with predefined physicochemical characteristics suitable for biological purposes (Figure 1).

Figure 1 : Physicochemical characterization of Theobro-CuONPs whereas ,
a) UV-Visible spectrum demonstrating the surface plasmon resonance and validation of successful synthesis of copper nanoparticles b) FTIR spectrum with peaks corresponding to O-H , N-H , and C=O functional groups, demonstrating theobromine in reduction and stabilization of CuNPs. c) SEM micrograph demonstrating spherical to quasi-spherical nanoparticles. d) XRD pattern with sharp peaks which indicates successful copper nanoparticle synthesis.

3.2 Cytotoxicity assessment of Theobro-CuONPs using MTT assay

Cytotoxicity of Theobro-CuONPs against KB oral cancer cells was determined using MTT assay with concentrations ranging from 5-100 µg/mL. Results revealed a clear dose-dependent inhibition of cellular viability following 24 hours of exposure to the cells. Low concentrations (5 and 10 µg/mL) resulted in moderate inhibition of viability, whereas with higher concentrations (50, 75, and 100 µg/mL), there was excessive cytotoxicity against the control (Figure 2).

At 100 µg/mL, Theobro-CuONPs exhibited maximum cytotoxicity, reducing cell viability to below 20%, exhibiting drastic anticancer activity. The value of IC_{50} obtained using nonlinear regression analysis was in the mid-dose category, which again validated the efficacy of the nanoparticle treatment. These results suggest that Theobro-CuONPs exhibit drastic inhibitory activity towards KB cell metabolic activity through cell membrane disruption and apoptosis-inducing mechanisms. The superior cytotoxicity of Theobro-CuONPs over theobromine or individual copper ions is due to the cooperative interaction of bioactive theobromine with oxidative stress-inducing ability of nanoscale copper resulting in greater cellular uptake and target-specific activity.

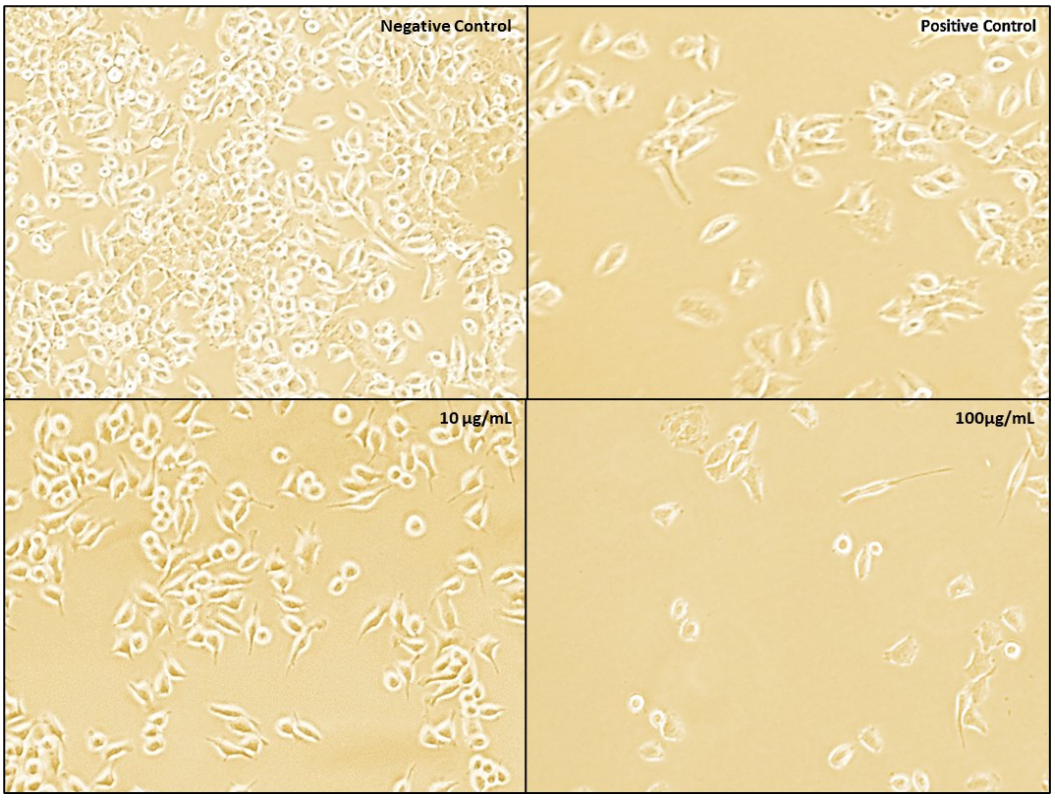
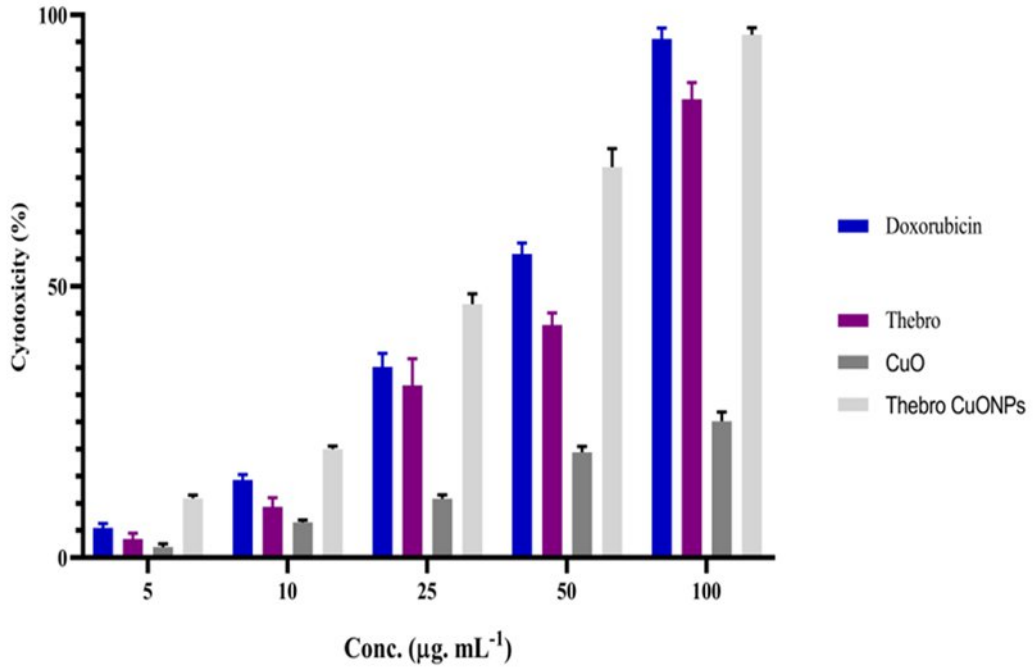


Figure :2 Microscopic visualization of KB cells treated with Theobro-CuONPs
Representative phase contrast microscopic images showing morphological changes in KB cells after 24 hour treatment with various concentrations of Theobro-CuONPs (negative control, positive control, 10µg/mL, 100µg/mL)

The MTT assay results showed an extended dose dependent cytotoxic activity of all the screened compounds against KB oral cancer cells. Doxorubicin , used as positive control , displayed higher cytotoxicity with increasing cell death from approximately 5% at the lowest level to over 96% at the highest concentration, validating the sensitivity of the assay. Theobromine alone showed poor anticancer activity with increased cytotoxicity from 2-4% at low levels to approximately 87% at the highest level, indicating that it can inhibit viability of cells by apoptotic mechanisms. In contrast, copper oxide nanoparticles alone were very cytotoxic, the maximum effect being only 27% showing negligible therapeutic use unless functionalized. Interestingly, Theobro-CuONPs were significantly more active as cytotoxic agents compared to both theobromine alone and CuO alone. Starting from 11% at lower concentrations, Theobro-CuONPs showed cytotoxicities equal to doxorubicin (97%) when used at the highest concentration. These data illustrate the synergistic action of theobromine functionalization with copper oxide nanoparticles in order to obtain improved cellular uptake, improved bioactivity, and successful killing of KB cancer cells (Figure 3).

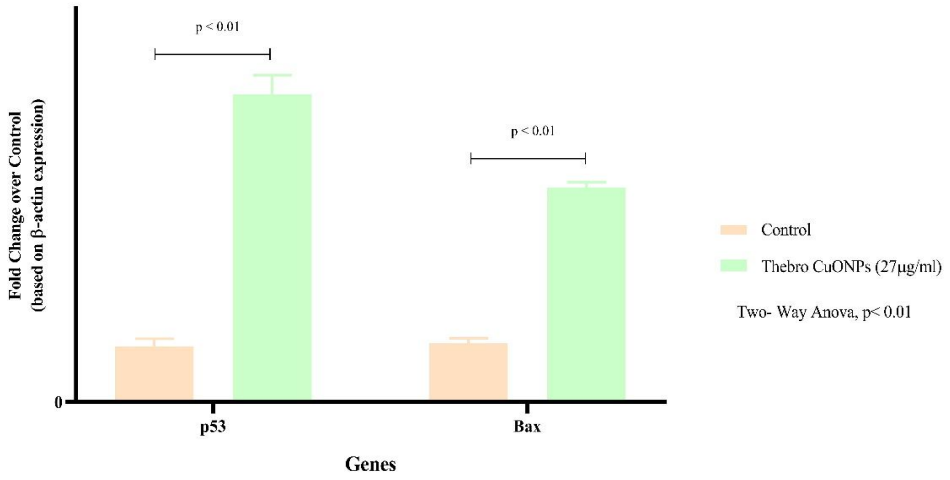


Graph 1: Represents MTT assay X axis represent Concentration in Different concentrations and Y axis represents percentage of cyotoxicity. Dose-dependent cytotoxicity of Theobro-CuONPs in KB cells determined by MTT assay

MTT assay showing KB cells viability after 24 hours of exposure with greater concentrations of Theobro-CuONPs (5, 10,25,50 and 100 µg/mL). Cell viability was evidently decreased in dose dependent manner with optimum cytotoxicity at 100 µg/mL.

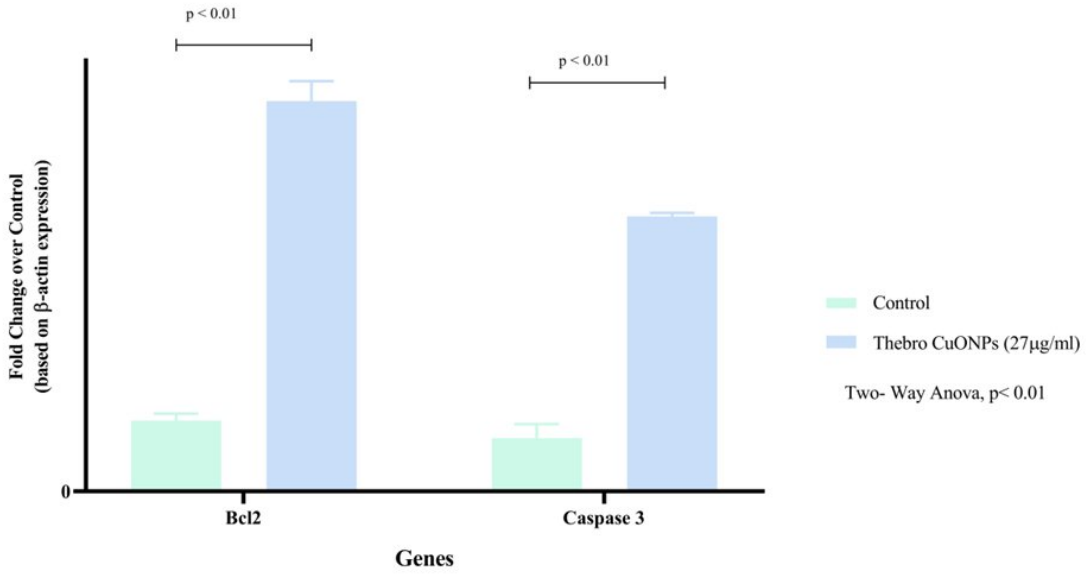
3.3 Gene Expression analysis of apoptotic and inflammatory Markers

3.3.1 Gene expression analysis of p53 and Bax



Graph :2 represents Gene expression analysis of p53 and Bax.X axis represents genes and Y axis represents expression qRT-PCR assay proved that treatment of Theobro-CuONPs resulted in elevation of p53, a tumor suppressor gene of critical significance, in KB oral cancer cells. Upregulation indicates activation of DNA damage response and pro-apoptotic pathways. At the same time, elevation of Bax, pro-apoptotic p53 and Bax downstream effector, and Bax reflects activation of intrinsic apoptotic pathways, and subsequent mitochondrial membrane permeabilization and induced programmed cell death. These were stronger than those in cells that were treated with theobromine or CuO alone, indicating the greater apoptotic potential of the nanoparticle preparation.

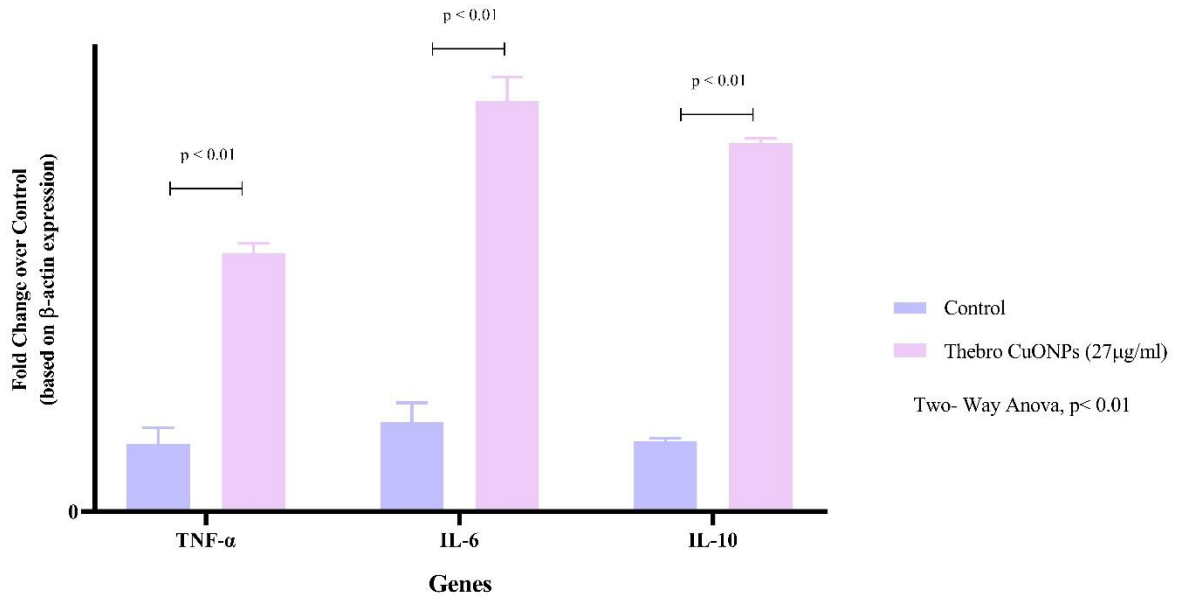
3.3.2 Gene expression analysis of Bcl-2 and caspase-3



Graph :3 represents Gene expression analysis of of Bcl-2 and caspase-3.X axis represents genes and Y axis represents expression

In contrast to pro-apoptotic upregulation observed in Bax, the prototypical anti-apoptotic gene Bcl-2 was similarly downregulated in Theobro-CuONps treated cells. Downregulation of Bcl-2 expression substantiates the shift in Bax/Bcl-2 ratio towards apoptosis. Moreover, Caspase -3, the central executioner caspase of the apoptotic pathway, was highly upregulated after nanoparticle treatment. Caspase-3 overexpression along with Bax induction and Bcl-2 suppression confirms intracellular induction of intrinsic apoptosis. All these molecular changes collectively confirm that Theobro-CuONPs induce not only cellular stress but also induce the cells to undergo apoptosis through mitochondrial as well as caspase dependent pathways.

3.3.3 TNF-α, IL-6 and IL-10 gene expression analysis



Cytokine profile of inflammation following Theobro-CuONPs treatment revealed strong modulation of the immune regulation pathways in KB cells. Tumor growth mediated by inflammation upregulated pro-inflammatory cytokines TNF-α and IL-6 , which were downregulated in treated cells. It suggests that the nanoparticles could inhibit tumour growth by suppressing it through inflammation. Conversely , downregulation of IL-10 , an anti-inflammatory cytokine but immunosuppressive cytokine , also occurred indicating the overall immunomodulatory effect. Downregulation of pro and anti-inflammatory cytokines concurrently indicates that Theobro-CuONPs would restore balance to the inflammatory tumor microenvironment rather than induce immune suppression in attributing anticancer activity.

4. Discussion

Nanotechnology-based therapeutics have gained considerable attention as potential alternatives to traditional chemotherapy owing to their improved selectivity, bioavailability, and decreased systemic toxicity.(Luddin *et al.*, 2021) Here, the gene regulation and cytotoxicity property of Theobro-CuONPs against oral cancer cells with specific reference to apoptosis and inflammation-related gene modulation was assessed by the current work. Theobromine acted as a green capping and reducing agent in the synthesis procedure that renders the resultant nanoparticle preparation biocompatible and endowed with inherent bioactivity.(Saab, 2007)

The cytotoxicity analysis by MTT assay showed considerable dose dependent inhibition of cell viability by Theobro-CuONPs , much better than theobromine or copper oxide in single use. Synergism is the most probable explanation behind reported normal physicochemical interaction at the nanolevel between phytochemical functionalized bioactive theobromine and copper ions enables enhanced cellular uptake and biological interactions. (Madkour, 2020)This observation demonstrates the therapeutic possibility of targeting cancer by utilization of phytochemical functionalized nanoparticles.

Gene expression profiling provided mechanistic data on the cytotoxicity of the nanoparticles. A sharp upregulation of p53 and Bax suggested activation of the intrinsic apoptosis pathway. p53 is a tumor suppressor gene highly central to detecting cellular stress and DNA damage , often lost or inactivated

in oral squamous cell carcinomas. (Vousden and Prives, 2009)Through restoration of p53 , Theobro-CuONPs apparently render cancer cells apoptotic-sensitive. Bax , as an immediate p53 downstream target , also supports his apoptotic signal by causing permeabilization of the mitochondrial membrane. Concurrently, downregulation of Bcl-2 was observed. Because Bcl-2 is an anti-apoptotic regulator through the fact that it preserves the integrity of the mitochondria , its suppression initiates apoptosis by tilting the balance in favor of pro-apoptotic factors.(Cory and Adams, 2002) In addition , up-regulation of caspase-3 , the terminal executor of the apoptotic cascade , directs execution of the mitochondrial apoptotic pathway. Co-regulation of Bax , Bcl-2 , and Caspase-3 supports strongly the hypothesis that Theobro-CuONPs induce p53 mediated programmed cell death.

Apart from their pro-apoptotic effect , Theobro-CuONPs were immunomodulatory. Extensive down-regulation of TNF-α and IL-6 expression suggests that the nanoparticles are anti-inflammatory. Both cytokines have been shown to promote tumor cell survival , angiogenesis , and metastasis. Inhibition of this cytokine would have the potential to inhibit weakening the pro tumorigenic microenvironment and thus sensitizing the cancer cells to apoptosis. Unexpectedly , expression of IL-10 , a predominantly anti-inflammatory cytokine , was also inhibited. While IL-10 has the ability to limit excessive inflammation , excessive overexpression within tumour microenvironments has been found to be responsible for immune suppression and poor prognosis. (Fathima *et al.*, 2020; Luddin *et al.*, 2021)Down-regulation here may reflect the balancing of immune signals rather than overall suppression. The therapeutic potential of copper based nanoparticles also stems from the ability of inducing reactive oxygen species (ROS) that are a by-product of oxidative stress and mitochondrial injury. (Deane, 2009)Theobromine , on the other hand , has antioxidative as well as anti-proliferative activity. Simultaneous administration of these two drugs can have the potential to introduce a dual mechanism system, activating ROS in cancer cells and stabilizing the supporting tissue by the effect of theobromine's bioprotection. The union of The effects possesses excellent evidence that Theobro-CuONPs possess antitumor activities against oral cancer cells with a multi-faceted process involving the activation of p53 pathway , intrinsic apoptosis, and inhibition of inflammation.(Siddiqui *et al.*, 2013) The two-prong attack on survival signaling and tumor-preferable inflammatory microenvironment imparts a never-before-seen therapeutic advantage. Interestingly, the green synthesis process also has the added value of being able to provide the guarantee that the nanoparticles are biocompactible and eco-friendly and hence ideal for upcoming translational uses.

5. Conclusion

Theobro-CuONPs synthesized via green route are highly cytotoxic against KB oral cancer cells by activating p53 mediated apoptotic pathways and inhibiting inflammatory cytokinesis. Enhanced gene regulation with significant dose-dependent cytotoxicity suggests the promising potential of Theo-CuONPs as a new biocompatible nanomedicine for treating oral cancer. Further in vivo investigations are warranted to validate these results and explore their translational applications in targeted cancer treatment.

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