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Anticancer and apoptosis-inducing effects of curcumin against gall bladder carcinoma

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Article History:	ABSTRACT
Received on: 22.01.2018 Revised on: 28.02.2018 Accepted on: 01.03.2018	<p>Curcumin, the primary bioactive component isolated from turmeric, has been shown to possess variety of biologic functions including anti-cancer activity. However, meticulous mechanism of the curcumin in gall bladder cancer has not been explored yet. Therefore, in our study, we elucidated the mechanism of the anticancer action of curcumin against human gall bladder cancer cells. It was found that the curcumin treated GBC cells decreased cell viability in a dose and time-dependent manner. Nuclear condensation, Annexin V-FITC/PI positive cells, and caspase-3 activation confirmed the apoptotic induction due to anti-proliferative action of curcumin. Furthermore, curcumin induced disruption in the mitochondrial membrane potential and increased reactive oxygen species generation which has not yet been reported in earlier studies of curcumin with gall bladder cancer. Moreover, curcumin-induced apoptosis of gall bladder cancer cells was also accompanied by significant amount of growth arrest at the G0/G1 phase of the cell cycle which has also not been documented previously. To the best part of my knowledge, this study has established curcumin as one of the promising chemotherapeutic agent against gall bladder carcinoma. Thus the present study explored a novel mechanism explaining the anti cancerous effects of curcumin, and may provide an alternative therapeutic approach which can overcome the side effects of chemotherapy.</p>
Keywords:	
Gall bladder carcinoma Curcumin Cell cycle analysis Caspase-3 Apoptosis	

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INTRODUCTION

Gallbladder cancer (GBC) is one of the most widespread malignant and lethal disease of the biliary tract (Lai *et al.*, 2008). Very few diagnostic and prognostic markers have been studied for this malignancy. Majority of patients of gall bladder cancer are diagnosed at an advanced and incurable stage due to lack of symptoms and physical signs at an

early stage (Kanthan *et al.*, 2015). Furthermore, surgical resection is the only potential option for the treatment for Gall bladder cancer. As a consequence, the overall 5-year survival rate of GBC is less than approximately 5% (Jiang *et al.*, 2014, Li *et al.*, 2014). Therefore, there is a strong need to identify natural compounds as a novel and effective agents for GBC treatment (Song *et al.*, 2017).

Through literature reviews, natural products from plants have been emerged as a potential alternate to the chemotherapeutic drugs (Ye *et al.*, 2016, Chang *et al.*, 2016). Various plant derived natural compounds such as etoposide, paclitaxel, vinblastine, and vincristine have been utilized in clinical use (Nobili *et al.*, 2009). Natural compounds exhibit their anti-cancerous potential by inducing apoptosis in cancerous cell (Fallahian *et al.*, 2017). Our study is focused on elucidating the anti-cancerous potential of curcumin in gall bladder carci-

noma. Curcumin, a natural (polyphenolic) compound is isolated from the rhizome of *Curcuma longa* (Shishodia *et al.*, 2007). Curcumin has been used as a food additive, coloring agent, and traditional medicine since ancient times. Earlier studies have reported that curcumin possesses numerous medicinal properties such as immunomodulatory, anti-tumorous, anti-microbial, anti-inflammatory and anti-diabetics (Shanmugam *et al.*, 2015). Anti-cancerous role of curcumin have been reported in various carcinomas including breast, liver, lung, oral, cervical and colon cancer (Ramamoorthi *et al.*, 2014). Curcumin demonstrated significant anti-cancerous potential either by modulating several cellular signaling pathways or by inducing cell cycle arrest and apoptosis in various cancer cell lines such as colon (Grill *et al.*, 2017), breast (Fu *et al.*, 2017), stomach (Aggarwal *et al.*, 2009), liver (Yang *et al.*, 2014, Prasad *et al.*, 2012). To the best part of my knowledge very few studies have reported the underlying mechanism of the action of curcumin in gall bladder cancer. Therefore, the aim of this study was to elucidate the anti-cancerous potential and underlying mechanism of the action of this compound against gall bladder cancer cells.

MATERIALS AND METHODS

Chemicals and reagents

Curcumin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Fetal bovine serum (FBS), RPMI-1640, and Dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). Antibiotics (penicillin and streptomycin), Hoechst 33342, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), Rhodamine123 (Rh123) and propidium iodide (PI) were procured from Sigma-Aldrich (St. Louis, USA). Annexin V-FITC Apoptosis Detection Kit was obtained from BD Bioscience, PharMingen (San Diego, CA, USA).

Primary Cell Culture

All experiments were conducted according to protocols approved by the Institutional Ethics Committee of SGPGIMS, Lucknow & IIMS&R, Lucknow. Ethical approval was obtained from the Sanjay Gandhi Postgraduate Institute of Medical Sciences (IEC Code: 2017-57-CP-96) and IIMS&R and fully informed consent from all patients before sample collection. Gall bladder cancer cells were prepared from the patient sample obtained from SGPGI, Lucknow. The primary GBC cells were then cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco, USA), 100 µg/mL streptomycin and 100U/mL penicillin (Hyclone, USA), at 37°C and 5.0% CO₂ atmosphere.

Trypan blue assay

We used trypan blue exclusion assay to assess the cellular viability (Xiong *et al.*, 2014). In brief, primary GBC cells were seeded into 96-well tissue culture plates (5x10³ cells per well) and grown in RPMI-1640 medium for 24 h. Curcumin is then added to the seeded cells at various concentrations (0-26 µM) for 24 and 48 h time periods. 40 µl of trypan blue solution was added in cell suspension to evaluate the cell death by counting on a hemacytometer. Three independent experiments were performed for the analysis.

MTT assay

MTT assay was employed to assess the Cell proliferation as described previously with some modifications (Qi *et al.*, 2010). In short, primary GBC cells were treated with different concentrations of curcumin (2-26 µM) for 24 and 48 h. After 24h of treatment, MTT solution (20 µl, 5 mg/ml in 1x PBS) was added and incubated for 4 h in an incubator. DMSO (100 µl) was then added into treated cells after discarding the media. Absorbance was recorded at 570 nm on microplate reader (Bio-Rad, Hercules, CA, USA)

Nuclear staining with Hoechst

Primary GBC cells (2x10⁵ cells per well) were seeded in 6-well plates and incubated with curcumin (2-26 µM) for 24 h. After incubation, PBS was added to GBC cells and then stained with Hoechst solution (1 µg/mL) at 37°C in the dark. Nuclear morphology was observed with a fluorescence microscope (Nikon Eclipse TE 300, Nikon, Tokyo, Japan) (Qi *et al.*, 2012).

Analysis of Intracellular ROS generation

DCFHDA fluorescent probe was used to examine the ROS (intracellular) in primary GBC cells (Mishra *et al.*, 2016, Das *et al.*, 2012). In brief, after treatment with curcumin, cells were seeded and suspended in 0.5 ml PBS containing 10 mM DCFH-DA for 15 min at 37 °C (in the dark). Cells absorb the dye and DCFH-DA gets converted into DCFH (a non-fluorescent product), which again gets converted into green fluorescent product dichlorofluorescein (DCF) by treated cells. DCF fluorescence intensity was observed by fluorimetry with excitation settings of 488 nm and emission settings of 530 nm.

Measurement of mitochondrial transmembrane potential (MMP)

MMP ($\Delta\psi$ M) was measured by utilizing Rh123 (a fluorescent cationic dye) which gets accumulated in the mitochondrial membrane in either monomeric or dimeric form depending on the mitochondrial membrane potential (Smiley *et al.*, 1991). In brief, primary GBC cells (2x10⁵ cells/well) were seeded in a 12-well plate and then incubated with

1 μM Rh123 for 30 min at room temperature in the dark. Fluorescence microscope was used to observe the changes in mitochondrial membrane potential (Enari *et al.*, 1998).

Cell cycle analysis by FACS

Primary GBC cells (1×10^6 cells per well) were seeded into 6-well tissue culture plates and then treated with selective doses of curcumin (2-26 μM) for 24. Treated cells were washed with ice cold PBS (pH 7.4) and then fixed in 70% ethanol for overnight at 4°C. Cells were then incubated with 250 μl of RNase A (100 $\mu\text{g}/\text{ml}$) for 30 min at 37°C and were finally stained with 500 μl of propidium iodide (50 mg/ml) for 1 h in the dark. FACS (BD Biosciences, San Jose, CA) was used to analyse the stained cells.

Annexin V-FITC-PI assay analysis

Curcumin induced cell death was estimated by FACS (flow cytometry) using annexin V-FITC-PI kit (Sigma). In brief, curcumin treated cells were washed with cold PBS. The cell pellet was resuspended in $1 \times$ binding buffer at a concentration of 1×10^6 cells/ml. 5 μl of Annexin V-FITC and 5 μl of PI were added to 100 μl of cell suspension and vortexed gently. Stained samples were incubated for 15 min at room temperature in dark. 400 μl of $1 \times$

binding buffer was then added to each tube. Beckman-Coulter Quanta™ SC MPL flow cytometry was used to analyse the samples.

Analysis of caspase-3 activities

Caspase-3 colorimetric kit (China) was utilized to analyse the caspase-3 enzymatic activity. Briefly, Treated Cells were washed with PBS, and then suspended in 50 μl of chilled cell lysis buffer. 50- μl final reaction buffer (1-ml reaction buffer+10 μl DTT) and 5 μl of caspase-3 colorimetric substrate (DEVDpNA) were added into the lysed cell supernatant. Plates were incubated for 2 h at 37 °C in a CO₂ incubator, and optical density was measured at 405 nm by using a microplate reader (Bio-Rad).

RESULTS

Effect of curcumin on Normal Macrophage J774A.1 Cells

MTT assay analysis was employed to find out the experimental doses and cytotoxicity of curcumin on normal mouse macrophage J774A.1 cells. After 24 h and 48h of treatment, percent cell viability of normal cells was analyzed and no significant effect was observed on cell viability of macrophages (Figure 1a). Thus, these results clearly revealed that curcumin did not show considerable cytotoxic effect in normal cells.

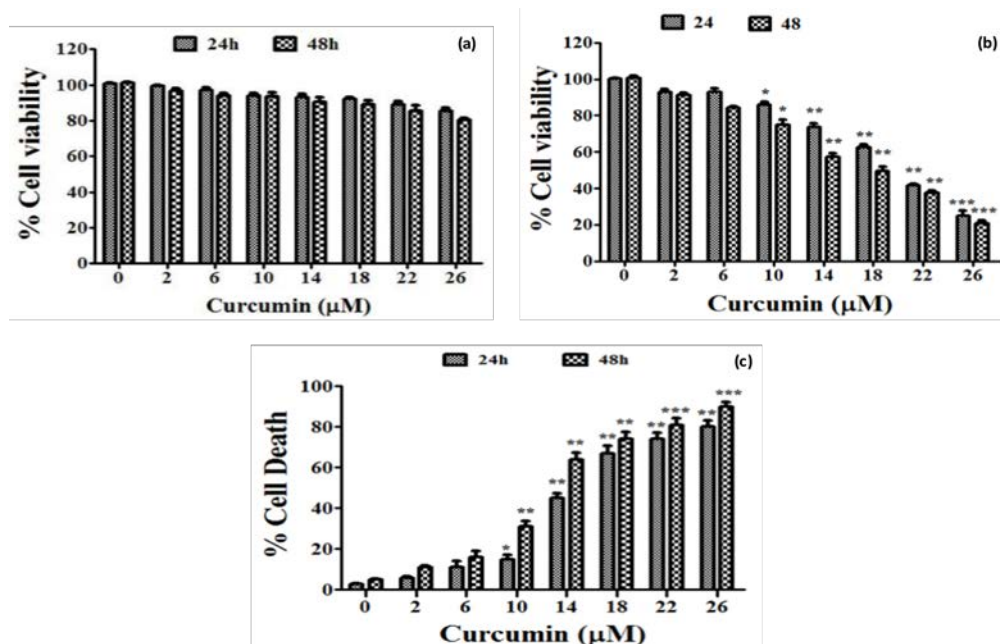


Figure 1: Time and dose dependent effect of curcumin on normal J774.1 cells and human gall bladder cancer GBC cell line. (a): Percent cell viability of J774A.1 cells treated with different doses of curcumin for 24 and 48 h assessed by MTT assay. (b): Percent cell viability of Gall bladder cancer cells (GBC) exposed with different doses of curcumin for 24 and 48 h determined by MTT assay. (c): Percent cell death of GBC cells treated with various doses of curcumin for 24 and 48 h evaluated by trypan blue dye exclusion assay. Represented results are expressed as the mean \pm SEM of three independent experiments performed in triplicate (* $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$ represent significant difference compared with control). Images shown are representative of three independent experiments.

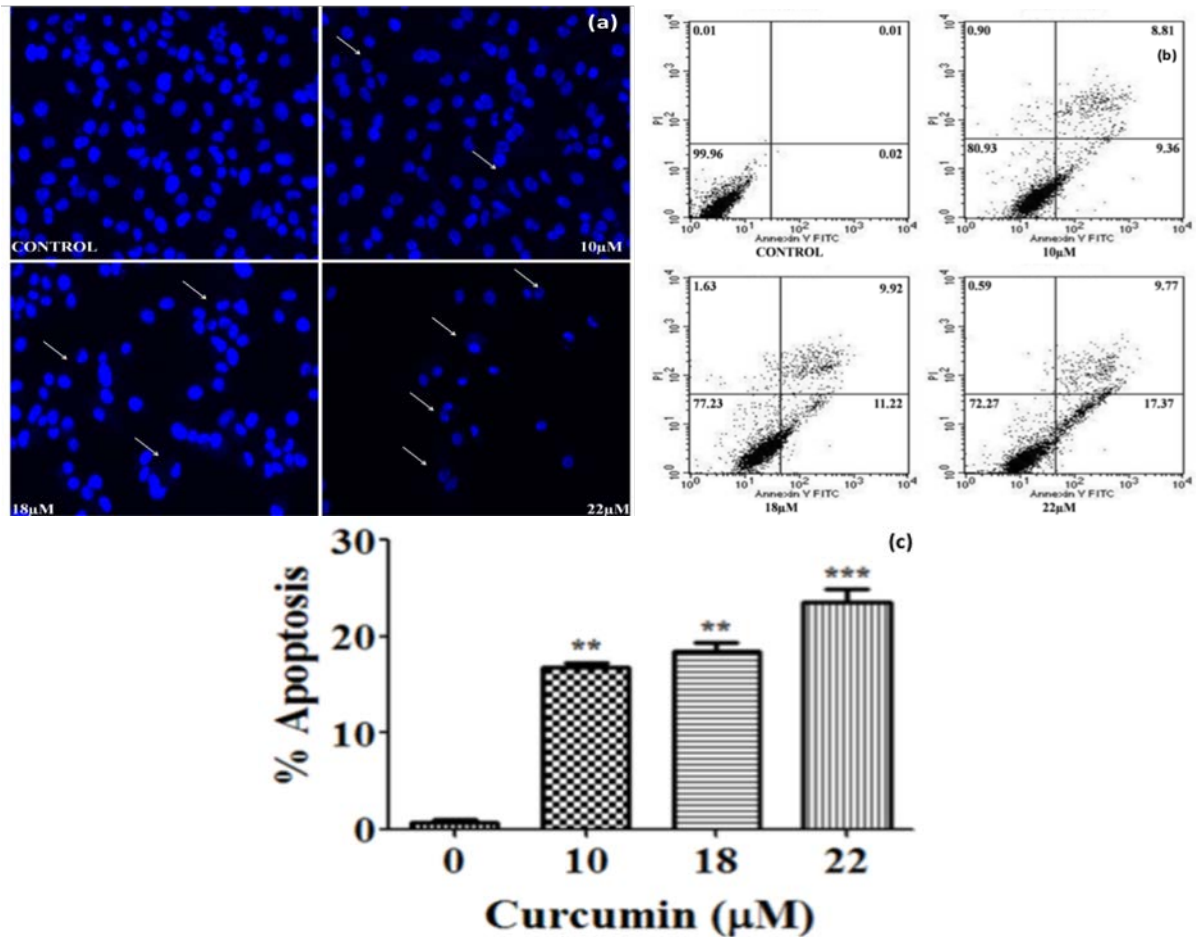


Figure 2: Curcumin-Induced Nuclear Condensation Along with Apoptosis in primary GBC Cells
 (a) Nuclear morphology of Hoechst stained nuclei of GBC cells treated with varying concentrations of curcumin for 24 h analyzed by fluorescence microscopy. Arrows indicate the condensed or fragmented nuclei. Images shown are representative of three independent experiments. (b) Percent apoptosis in Annexin V-FITC/PI-stained GBC cells treated with varying concentrations of curcumin 24 h observed by flow cytometric analysis. (c) Percent apoptosis as observed after 24h of incubation by Annexin V-FITC/PI assay. The results represented are the mean ± SEM of three independent experiments performed in triplicate (*P < 0.01, **P < 0.001, ***P < 0.0001 represent significant difference compared with control).

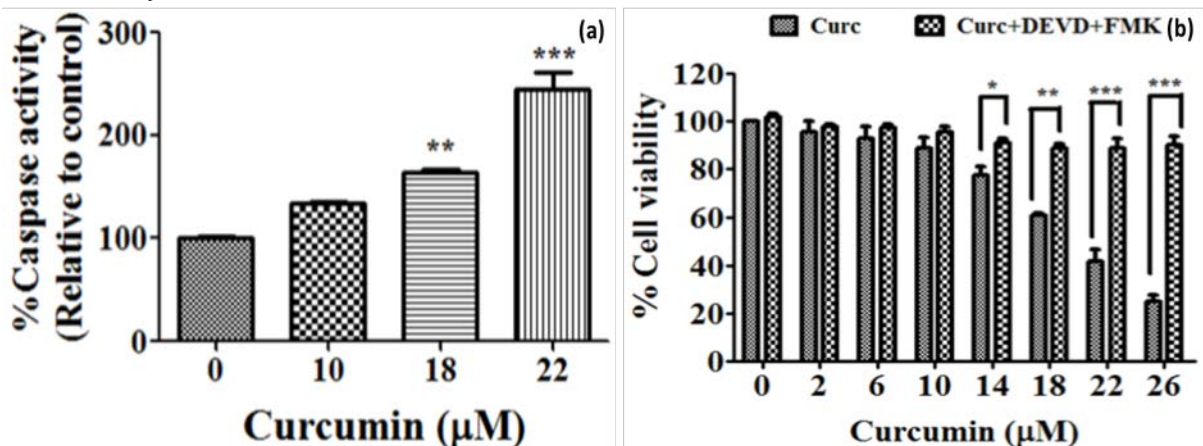


Figure 3: Curcumin-Induced Caspase Activation in gall bladder Cancer Cells.
 (a) Percent caspase-3 activity in GBC cells treated with different concentrations of curcumin for 24 h determined by caspase-3 activity assay. (b) Percent cell viability of cells pretreated with a caspase-3 inhibitor, Z-DEVD-FMK, and then treated with different doses of curcumin for 24 h assessed by MTT assay. Data represent mean ± SEM of three independent experiments performed in triplicate (*P < 0.01, **P < 0.001, ***P < 0.0001 represent significant difference compared with control).

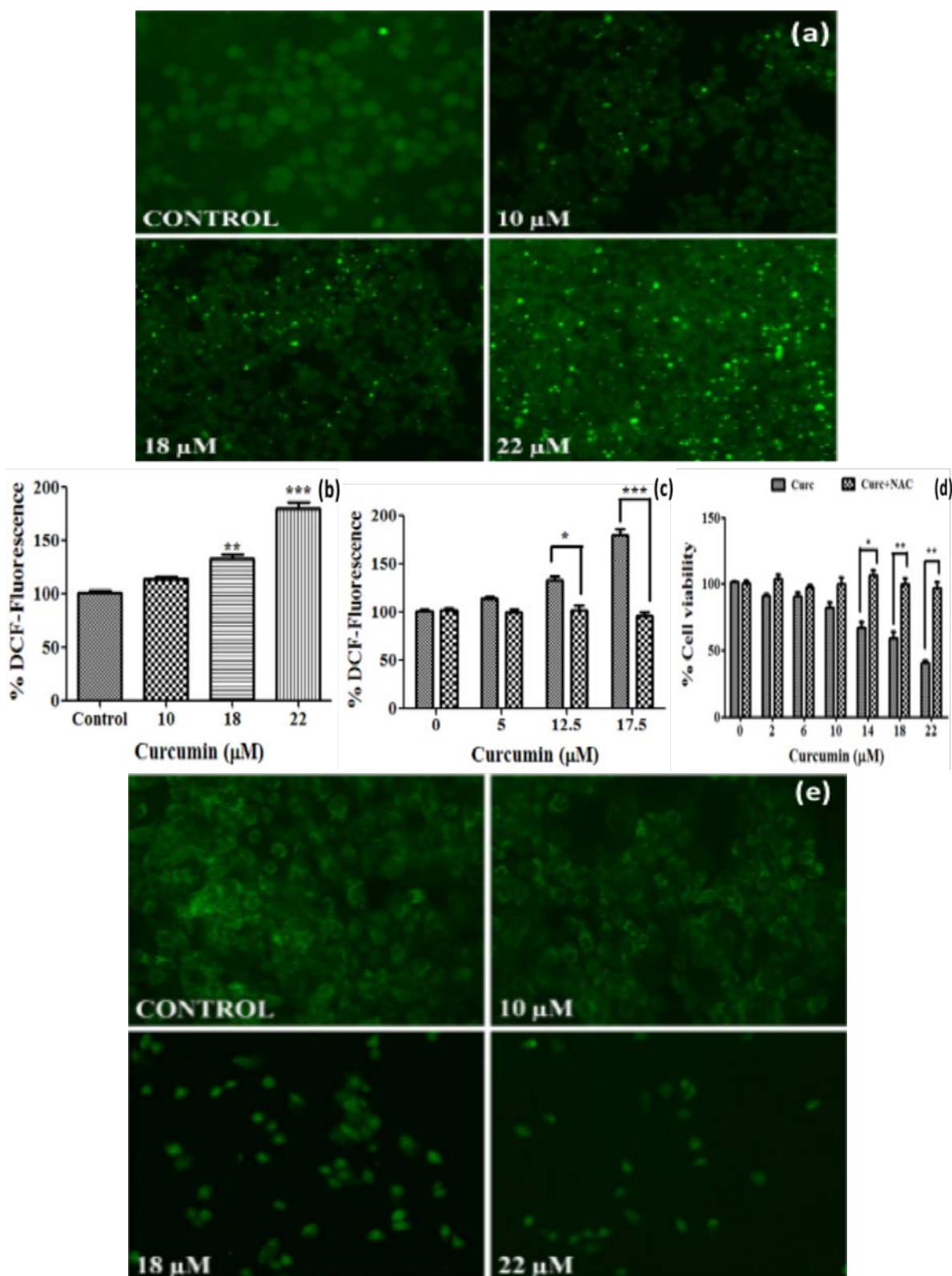


Figure 4: Curcumin Augmented Intracellular ROS Generation in gall bladder Cancer Cells

(a) Enhanced ROS generation in DCFH-DA stained GBC cells treated with different concentrations of curcumin analyzed by fluorescence microscopy. Data shown are representative of three independent experiments. (b) Quantification of ROS level in terms of percent DCFDA fluorescence in GBC cells treated with selective concentrations of curcumin. (c) Percent cell viability of GBC cells pretreated with NAC and then treated with different doses of curcumin for 24 h assessed by MTT assay. (d) ROS level in GBC cells pretreated with a ROS inhibitor, NAC and then treated with selective doses of curcumin. (e) Disruption of the mitochondrial membrane potential (DCm) of GBC cells treated with curcumin observed by staining with a fluorescent dye Rh123. Values are expressed as mean \pm SEM of three independent experiments (*P < 0.01 and **P < 0.001 as compared with control).

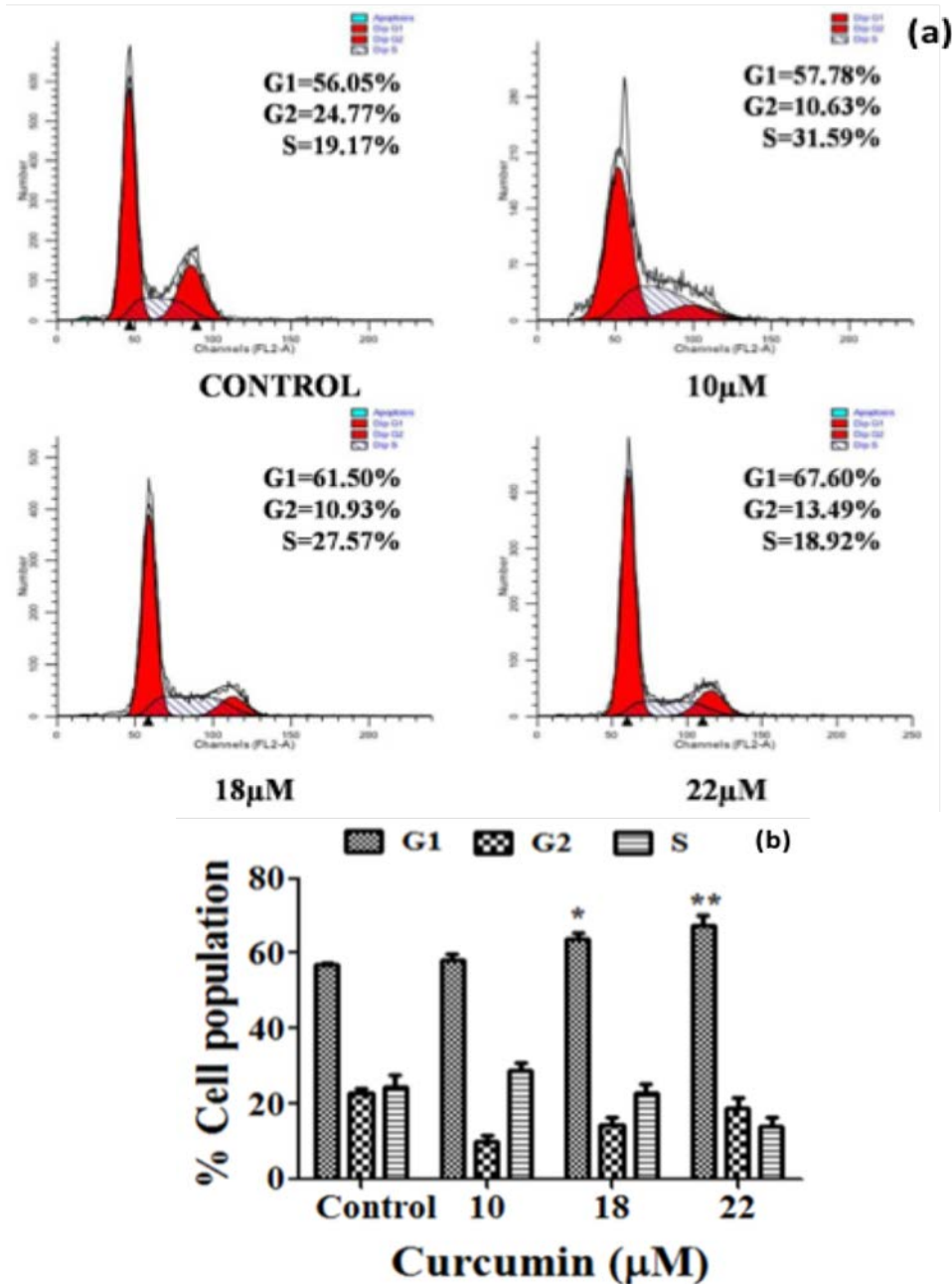


Figure 5: Curcumin induced cell cycle arrest in a dose-dependent manner

(a) Cell cycle distribution of propidium iodide-stained GBC cells treated with selective doses of curcumin for 24 h observed by flow cytometric analysis. The data shown here are from a representative experiment repeated three times. (b) Percent cell cycle distributions determined by flow cytometric analysis. Data represent mean \pm SEM of three independent experiments performed in triplicate (* $P < 0.05$ and ** $P < 0.01$ as compared to control).

Curcumin Inhibited Proliferation of primary gall bladder Cancer Cells

Cytotoxic effects of curcumin on GBC cancer cells was determined by treating cells with varying concentrations of the curcumin (2-26 μM) for 24 and 48 h. Results indicated that treatment of cells with different doses of curcumin resulted in significant inhibition of cell proliferation in comparison with control (Figure 1b). Curcumin inhibited viability of primary GBC cells in a dose and time dependent manner with IC50 of around 20.22 μM and 17.78 μM , for 24 h and 48 h respectively. Similarly,

after 48 h of treatment at (2-26 μM) curcumin, viability of GBC cells reduced significantly in a dose dependent manner in comparison with control (Fig. 1B). Hence further studies were carried out with this concentration.

In addition, we further determined the cytotoxic effect of curcumin by trypan blue exclusion assay. Curcumin treatment at different doses of 2-26 μM for 24 and 48 h resulted in significant cell death (Figure 1c). The results revealed that there was a significant cytotoxic effect in human gall bladder

cancer cells without having significant cytotoxic effect on normal cells.

Curcumin-Induced Nuclear Condensation Along with Apoptosis in primary GBC Cells

Hoechst 33342 staining was performed to identify whether curcumin-mediated inhibition of cell proliferation of gall bladder cancer cells is due to apoptosis induction. After treatment with curcumin (10, 18 and 22 μM) for 24 h, morphological changes were observed in gall bladder cancer cells. Apoptotic cells were characterized by bright-blue fluorescence condensed and fragmented nuclei when stained with Hoechst 33342 (Figure 2a). However, no significant apoptosis was observed in untreated GBC cells. Therefore these results clearly revealed that curcumin induced apoptosis in GBC cells in a dose-dependent manner.

Curcumin-Induced Apoptosis in primary GBC Cells

To determine curcumin induced apoptosis, GBC cells were treated with 10, 18 and 22 μM curcumin for 24 h and stained with Annexin-V FITC/PI. FACS (fluorescence-activated cell sorting) was used to measure the apoptotic cells. As depicted in Fig. 2C curcumin treatment leads to the reduction in the number of surviving cells thereby increasing the number of apoptotic cells in a concentration-dependent manner. The results showed that curcumin induced significant amount of apoptosis in primary GBC cells of about 17.67%, 19.92% and 23.38% after 24 h of treatment with 10, 18 and 22 μM of curcumin respectively (Figure 2b and 2c).

Curcumin-Induced Caspase Activation in gall bladder Cancer Cells

To elucidate the involvement of caspases in curcumin-induced apoptosis in GBC cells, we determined the caspase-3 activity in treated and untreated control. Results showed a significant induction of caspase-3 activity in GBC cells after treatment with different concentrations of curcumin for 24 h (Figure 3a). There was significant increase in Caspase-3 activity in comparison with untreated control in a dose dependent manner at concentrations of 10, 18 and 22 μM of curcumin, respectively.

Attenuation of Curcumin-Induced Apoptosis by Caspase-3 Inhibitor

To characterize the attenuation of curcumin-induced apoptosis in gall bladder cancer cells, GBC cells were pretreated with 50 mM Z-DEVD-FMK (a caspase-3 inhibitor) for 2 h and then treated with curcumin at various doses for 24 h. Cell viability was assessed using the MTT assay as described above. Pretreatment with caspase-3 inhibitor re-

sulted in significant decrease in the amount of cytotoxicity in primary gall bladder cancer cells in curcumin treated cells (Figure 3b). These data indicated that the induction of caspase-3 activity played a crucial role in curcumin-induced apoptosis in gall bladder cancer.

Curcumin Augmented Intracellular ROS Generation in gall bladder Cancer Cells

To elucidate the mode of action of curcumin, we further explored the curcumin effect on the intracellular redox status. Accordingly, we measured the intracellular ROS level in both curcumin-treated and untreated gall bladder cancer cells by DCFH-DA analysis. Briefly, gall bladder cancer cells were stained with DCFH-DA to identify the changes in intracellular ROS level after 12 h of curcumin treatment. The photomicrograph suggested the enhanced intracellular ROS levels in curcumin treated primary GBC cells in a dose dependent manner (Figure 4a and 4b).

In order to confirm the curcumin-mediated generation of ROS, primary gall bladder cancer cells were pretreated with a known ROS inhibitor, NAC. The quantitative analysis confirmed the attenuation of increased ROS level by pretreatment with 10 mM NAC which further confirmed that curcumin could enhance ROS generation in cancer cells (Figure 4c).

Attenuation of Curcumin-induced Cytotoxicity by N-Acetyl-L-cysteine (NAC)

To further determine the association of ROS with curcumin induced cytotoxicity in gall bladder cancer cells, we examined the effect of curcumin in GBC cells in the presence of NAC (10 mM) by MTT assay. GBC cells were treated with NAC (a ROS inhibitor) at 10 mM for 2 h and then with curcumin for an additional 24 h. Pretreatment with NAC significantly reduced the amount of cytotoxicity caused by the treatment of curcumin as seen in Figure 4d. These results indicated that the augmented intracellular ROS production plays important role in curcumin-induced apoptosis.

Curcumin Disrupted Mitochondrial Membrane Potential in primary GBC Cells

To further validate whether curcumin could disrupt the mitochondrial membrane potential, we used Rh123 dye (a mitochondria-specific and voltage-dependent dye) to identify changes in DCm in primary GBC cells. After treatment with curcumin, weakened fluorescence intensity was observed in a dose dependent manner (Figure 4e). Therefore, these results established that there is a significant reduction in the DCm of curcumin treated GBC cells in a dose dependent manner.

Curcumin-Induced Cell Cycle Arrest in gall bladder Cancer Cells

To determine the curcumin induced disruption of cell cycle progression, primary GBC cells were treated with 10, 18 and 22 μM curcumin for 24 h and subjected to flow cytometry analysis of PI-stained cells. The result showed that curcumin treatment over 24 h caused a significant cell cycle arrest in GBC cells at G0/G1 phase (Figure 5a and 5b).

DISCUSSION

Gall bladder cancer remains the leading cause of death worldwide because of its poor prognosis and diagnosis (Kanthan *et al.*, 2015). Various natural compounds have shown promising anticancer potential with due to their ability to inhibit cancer cell proliferation and metastasis with non significant side effects and thus, could be a prominent agent to reduce the cancer mortality by inhibiting the phenomenon of carcinogenesis.

Through various literatures, it has been reported that curcumin exhibited significant antiproliferative and anticancer activities in various carcinomas (Grill *et al.*, 2017) but very less studies have reported its antiproliferative role in gall bladder carcinoma. Therefore, we directed our studies towards exploring the anti-cancerous action of curcumin in gall bladder cancer cells in order to find a novel, effective anticancer agent. In our study, we elucidated the effects of the curcumin on primary gall bladder cancer (GBC) cell line. MTT and trypan blue dye exclusion assay revealed that curcumin strongly reduced the cell viability of gall bladder cancer cells. Thus it can be assumed that the treatment of curcumin significantly reduced the cell viability of gall bladder cancer cells in a dose-dependent manner. In addition we did not observe any significant cytotoxicity in normal mouse macrophages in treated cells; however, a significant reduction in cell viability was observed only at the highest dose of curcumin used in the experiment. Thus, curcumin exhibited cytotoxic effects in human gall bladder cancer cells with lesser significant cytotoxic effect on normal cells.

Earlier studies have reported that majority of the anti-tumorous agents inhibits cancer cell proliferation by apoptosis induction which can be characterized by nuclear fragmentation and chromatin condensation (Fruehauf *et al.*, 2007). Similar effects were also obtained in our experiments where curcumin induced apoptosis in gall bladder cancer cells with fragmented and condensed nuclei stained with Hoechst 33342. We further performed FITC-Annexin V assay using Annexin V (35–36 kDa Ca²⁺-dependent phospholipid-binding protein) to quantify the apoptotic induction FITCAnnexin V

assay revealed a dose-dependent increase in the Annexin V positive cells, indicating the curcumin induced induction of apoptosis in GBC gall bladder cancer cells. Apoptosis is also characterized by Caspase-3 which is responsible for the proteolytic cleavage of many cellular proteins (Trachootham *et al.*, 2009). Thus we investigated the caspase activity in curcumin treated cells. Curcumin treatment resulted in apoptosis by inducing caspase-3 activation. Further pretreatment with capase-3 inhibitor substantially reduced curcumin-induced cytotoxicity in gall bladder cancer cells, indicating activation of caspase-3 during curcumin-induced apoptosis.

ROS generation is associated with the chemotherapeutic activity of several anticancer drugs and natural anti-cancerous compounds (Scaduto *et al.*, 1999, Balaban 2006). Our results also corroborated with these reports and suggested that curcumin induced ROS-mediated apoptosis induction in GBC cells in a concentration dependent manner. However, pretreatment of cells with a ROS inhibitor, NAC attenuated the curcumin-mediated ROS generation in gall bladder cancer cells. Furthermore, pretreatment with NAC significantly reduced the amount of cytotoxicity caused by the treatment of curcumin. These data strongly confirmed the role of augmented intracellular ROS generation in curcumin-induced apoptosis which has not been reported previously in any studies. Curcumin treatment also resulted in depolarized MMP (DCm) significantly in a dose-dependent manner using Rhodamine123 (a cationic fluorescent dye) whose mitochondrial fluorescence intensity decreases with dissipation of mitochondrial transmembrane potential (Scaduto *et al.*, 1999, Balaban 2006). Curcumin-mediated disruption of mitochondrial membrane potential (DCm), which could be the probable mode of action of curcumin, has also not been reported in earlier reports.

In addition to apoptosis we further elucidated the effect of curcumin on cell cycle by using flow cytometry using PI staining. Major checkpoints of cell cycle are G1 and G2 phases and have a significant role in cell cycle progression. We observed that curcumin arrested cell cycle progression in G0/G1 by blocking the transition from G1 to S phase and subsequently lead to apoptosis. Thus, our study provides an insight to further explore the potential utility of curcumin for the treatment of gall bladder carcinoma.

CONCLUSION

In conclusion, this research provides an alternative approach for the treatment of gall bladder carcinoma. Experimental results revealed that cell death in gall bladder cancer cells was mainly due

to the induction of ROS-mediated apoptotic induction. Furthermore, curcumin was found to be responsible for inducing cell cycle arrest in gall bladder cancer cells. Therefore, it can be concluded that curcumin has a strong anti-cancerous potential which could be further utilized for drug development. Further studies are needed to elucidate the pharmacokinetic activity of curcumin.

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DECLARATION OF INTEREST

I confirm that this research paper authored by us is a genuine and original work. It has neither been published nor submitted for publication.

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