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Chemopreventive effect of corosolic acid in human hepatocellular carcinoma cells

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Anticancer effects of corosolic acid have been demonstrated earlier in human cervix adenocarcinoma and osteosarcoma cells, but the exact underlying molecular mechanisms have not been studied. Hence, an attempt was made to identify the anticancer mechanism of corosolic acid in human hepatocellular carcinoma cells [HepG2]. The anticancer activity of corosolic acid through cell growth inhibition by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, reactive oxygen species (ROS) generation, mitochondrial membrane potential and apoptotic fragmentations by fluorescence microscope was evaluated. In addition, the cleavage of PARP, NF-κB, cytochrome c (cyt. C) release, and Bax and Bcl2 expression was analyzed by Western blot. The results clearly indicate that corosolic acid was involved in the alteration of mitochondrial membrane potential resulting in the release of cyt. C from mitochondria and increased ROS generation. The corosolic acid treatment causes the induction of apoptosis in a dose dependent manner (IC$_{50}$, 40 µM). In conclusion, corosolic acid had chemopreventive effect on HepG2 cells by apoptosis.

Key words: Corosolic acid, hepatocellular carcinoma, reactive oxygen species (ROS), apoptosis, lipid peroxidation.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent life-threatening human cancers (Fanghua et al., 2010); it is an aggressive disease and the worldwide prevalence is 85% of all liver cancer (Chen et al., 2010). The most prominent etiological factors associated with HCC are chronic viral hepatitis B and C infections, exposure to environmental chemicals, alcohol and metabolic liver diseases (Zhang, 2010).

Most of the anti-neoplastic drugs induce oxidative stress which preferentially promotes death signaling pathways in tumor cells to inhibit their proliferation (Biswal et al., 2002). Oxidative modulation of transcription factors results in apoptosis of tumor cells (Biswal et al., 2003). This process may represent a mechanism by which cancer cells limit tumor progression in response to

the oxidative stress.

In most developing countries, the traditional and herbal medicines are used to enhance the primary healthcare needs. A series of compounds have been identified from plants among them are triterpenoids (Xin et al., 2008). The triterpenoids are synthesized by plants by the cyclization of squalene, and many of them have been isolated and identified (Liby et al., 2007; Harmand et al., 2005). Ursolic, pomolic and maslinic acids are well known and have been reported for their anticancer properties (Fernandes et al., 2005; Reyes-Zurita et al., 2009; Martin et al., 2007). Similarly, the present study was carried out using corosolic acid (2α-hydroxyursolic acid), a triterpenoid derivative (Figure 1), which was isolated from *Camptotheca acuminate* (Pasqua et al., 2006), *Ugni*
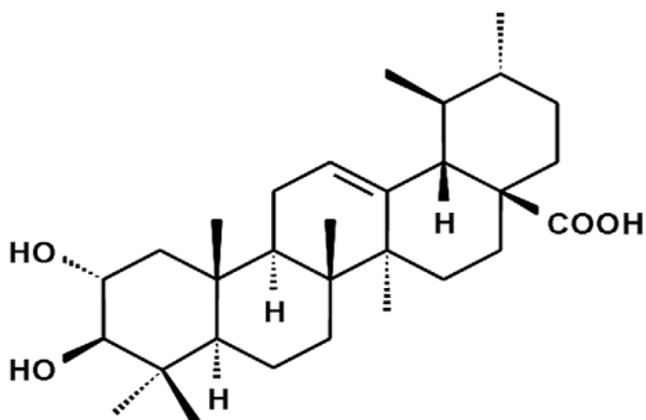
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Figure 1. Structure of corosolic acid (2a,3b-dihydroxy-urs-12-en-28-oic acid).

molinae (Aguirre et al., 2006), *Crataegus pinnatifida* (Ahn et al., 1998), *Psidium* guajava (Begum and Syed, 2002; Gutiérrez et al., 2008), *Tiarella polyphylla* (Park et al., 2002), *Eriobotrya japonica* (Li et al., 2009) and *Lagerstroemia speciosa* leaves (Hou et al., 2009). Corosolic acid have been reported to possess antidiabetic activity and improve glucose metabolism by decreasing insulin resistance in a animal model and also post-challenged plasma glucose levels in humans (Fukushima et al., 2006; Miura et al., 2006). Also, corosolic acid has been reported for its cytotoxic activities against several human cancer cell lines (Ahn et al., 1998; Yoshida et al., 2005). The aim of the present study was to evaluate the underlying molecular mechanism involved in the chemopreventive effect of corosolic acid in HepG2 cells.

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid (TBA), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), 5,5-dithiobis 2-nitrobenzoic acid (DTNB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2-7' dichlorodihydrofluorescein diacetate (H2-DCFDA), Rhodamine-123, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), ethidium bromide (EB), acridine orange (AO), fetal bovine serum (FBS), Dulbecco's modified Eagle media (DMEM), glutamine-penicillin-streptomycin solution and trypsin-EDTA were purchased from Sigma Chemicals Co., St. Louis, USA.

Cell lines and culture condition

HepG-2 (hepatocellular carcinoma cells) cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. HepG2 cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin 200 U/ml, streptomycin 100 µg/ml, kanamycin 100 µg/ml). Cells were cultured on tissues culture flask at 37°C in humidified atmosphere of 5% CO_2.

Drug treatment and dose fixation study

Corosolic acid was dissolved in sterilized dimethyl sulfoxide (DMSO). The stock solution was frozen and stored at −20°C. For treatments, the solution was diluted in cell culture medium.

Cytotoxicity of corosolic acid was determined by the MTT (3-(4,5-dimethylthiazole)-2,5-diphenyltetrazolium bromide) assay (Li et al., 2009). Cells were seeded in 96-well micro assay culture plates (8×10^3 cells per well) and grown overnight at 37°C in a 5% CO_2 incubator. Corosolic acid was added to the wells to achieve final concentrations of 5, 10, 20, 30, 40, 50, 60 and 70 µM. Control cells were prepared by addition of culture medium (100 µL) only. Wells containing culture medium without cells were used as blanks. The plates were incubated at 37°C in a 5% CO_2 incubator for 24 h. Upon completion of the incubation, stock MTT dye solution (20 µL, 5 mg mL^{-1}) was added to each well. After 4 h incubation, buffer (100 µL) containing N,N-dimethyl formamide (50%) and sodium dodecyl sulfate (20%) was added to solubilize the MTT formazan. The optical density of each well was measured on a micro plate spectrophotometer at a wavelength of 490 nm. The IC_{50} value was determined by plotting the percentage viability versus concentration of drug on a logarithmic graph and reading the concentration at which 50% of cells remain viable relative to the control. Each experiment was repeated for three times to get the mean values.

Experimental design

The HepG2 cells were divided into four experimental groups: Group 1, untreated control cells; Group 2, HepG2 cells treated with 30 µM corosolic acid; Group 3, HepG2 cells treated with 40 µM corosolic acid; Group 4, HepG2 cells treated with 50 µM corosolic acid.

Changes in mitochondrial transmembrane potential (ΔΨm) assessment

Mitochondrial membrane (ΔΨm) potential was monitored using the ΔΨm-specific fluorescent probe Rhodamine-123 (Molecular Probes Inc., Eugene, OR), a sensitive fluorescent dye (Cai et al., 2011). Briefly, the HepG2 treated with different concentrations of corosolic acid (30, 40 and 50 µM) for 24 h, were harvested with ice-cold PBS solution. Then, resuspended in DMEM medium at a density of 0.5×10^6 cells/ml, cells were permeabilized with 0.3% Triton X-100, washed with ice-cold PBS, incubated with 10 µM Rhodamine-123 for 15 min at 37°C in the dark and observed under a fluorescence microscope (Olympus BX51, Japan). Percentage fluorescence intensities was monitored using fluorescence plate reader (BD Falcon, CA) at Ex./Em. = 530/585 nm.

Determination of intracellular ROS generation

Generation of ROS was assessed by using the fluorescent probe H2DCFDA (2,7-dichlorodihydrofluorescein), a cell-permeable indicator for ROS initially shown to react with H_2O_2. As described previously (Bhosle et al., 2005), H2DCFDA was oxidized to a highly green fluorescent DCF (2,7-dichlorofluorescein) by the generation of ROS. The HepG2 cells were pretreated with corosolic acid 30, 40 and 50 µM for 24 h. Then, the cells were incubated with 100 µM H2DCFDA in PBS for 30 min. After 30 min at 37°C, DCF fluorescence (excitation, 485 nm and emission, 525 nm) was observed under a fluorescent microscope (Olympus BX51, Japan). The fluorescence intensity was measured using the fluorescence plate reader (BD Falcon, CA) at Ex./Em. = 488/525 nm.

Apoptotic morphological changes

Apoptosis was performed with a staining method utilizing acridine

orange (AO) and ethidium bromide (EB) (Spector et al., 1998). According to the difference in membrane integrity between necrosis and apoptosis, AO can pass through cell membrane, but EB cannot pass through cell membrane. Under fluorescence microscope, live cells appear green. Necrotic cells stain red but have a nuclear morphology resembling that of viable cells. Apoptosis cells appeared green and morphological changes such as cell blebbing and formation of apoptotic bodies was observed. HepG2 cells were incubated in the absence or presence of corosolic acid at concentration of 30, 40 and 50 μM at 37°C and 5% CO_2 for 24 h. After 24 h, each cell culture was stained with AO/EB solution (100 μg/ml AO and 100 μg/ml EB). Stained cells were observed under Olympus BX51 fluorescence microscope.

Propidium iodide staining

HepG-2 cells (1×10^6 cells/mL) were washed in PBS thrice and incubated for 10 min with 50 μL of propidium iodide (5 mg/mL) and mounted in slides. The slides were examined by fluorescence microscopy (Olympus BX51) (Fanghua et al., 2010).

DNA-specific fluorochrome 4',6-diamidino-2-pheny-lindole dihydrochloride (DAPI) staining

HepG2 cells treated with corosolic acid, were centrifuged at 1200 rpm for 5 min and 3.7% neutral buffered formalin was added to the cell pellet. The fixed cells were washed with phosphate buffered saline (PBS), air dried and stained with DAPI for 10 min. The slides were observed under Olympus BX51 Fluorescence microscope (Qi et al., 2012).

Measurement of lipid peroxidation

At the end of 24 h incubation, HepG2 cells were scrape harvested in ice-cold PBS and centrifuged at 2000 rpm and 4°C, the supernatant was discarded, and the cell pellets were disrupted for the measurement of TBARS, according to the procedures described by Bagchi et al. (2002).

Measurement of reduced glutathione

After 24 h incubation, corosolic acid treated HepG2 cells were used for the measurement of reduced glutathione (GSH) level. The GSH content was measured by the method of Chen et al. (2010).

Immunoblot analysis

For immunoblot analysis (Towbin et al., 1979), cells were lysed in a lysis buffer containing 20 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM b-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mg/ml leupeptin, 20 mg/ml aprotinin and phosphatase inhibitors with 100-fold dilution. After 30 min of shaking at 4°C, the mixtures were centrifuged (10,000 × g) for 10 min, and the supernatants were used as the whole-cell extracts. The protein content was determined according to the Bradford method. Proteins (50 μg) were separated on 8 to 12% sodium dodecyl sulphate (SDS)-polyacrylamide gels along with protein molecular weight standards and electrotransferred to nitrocellulose membrane. The membranes were blocked with 5% (w/v) nonfat dry milk after checking the transfer using 0.5% Ponceau S in 1% acetic acid and then probed with a relevant antibody (Bax, Bcl2, cyt.C, NF-κB and PARP at 1:1000 dilution) for 8 to 12 h at 4°C followed by detection using peroxidase-conjugated secondary antibodies and chemiluminescence. Equal protein loading was detected by probing the membrane with β-actin antibodies.

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by DMRT taking $p<0.05$ to test the significant difference between groups.

RESULTS

Corosolic acid reduced cell viability in HepG2 cells

HepG2 cells were treated with various concentrations of corosolic acid; their viability was determined by the formation of formazan dye and expressed as percent of untreated control cells (Figure 2a). The 50% growth inhibitory concentration (IC_{50}) was obtained after 24 h incubation with 40 μM corosolic acid.

In addition, cell morphology was examined using a phase-contrast microscope. Microscopic observations revealed that corosolic acid not only affect cell growth but also change cell morphology and adherent nature (Figure 2b). Exposure of HepG2 cells to 5, 10, 20, 30, 40, 50 and 60 μM corosolic acid provoked significant changes in cellular morphology. For 10 and 20 μM HepG2 cells began to shrink and retract from their adjacent cells. For 30 and 40 μM, the floating cells appeared in the culture medium and changed the cell shape. With treatment with 50 and 60 μM of corosolic acid for 24 h, most of the HepG2 cells lost their flat, polygonal shape. Meanwhile, the number of survival cells significantly decreased, as compared to the control.

Corosolic acid decreased ΔΨm in HepG2 cells

Mitochondrial transmembrane potential (ΔΨm) change in mitochondria is a crucial stage in apoptosis induced by corosolic acid. In this study, by staining with Rhd-123, the loss of ΔΨm in HepG2 cells after treatment with corosolic acid was detected. Figure 3a showed a remarkable increase in green fluorescence of Rhd-123 monomers in corosolic acid-treated cells, indicating a reduction in ΔΨm. The percentage of cells with green fluorescence increased to 42, 65 and 52% when compared with the control, respectively (Figure 3a).

Corosolic acid enhanced ROS level in HepG2 cells

Since loss of ΔΨm is associated with the generation of ROS, changes in ROS level after corosolic acid treatment are shown in Figure 3b. A gradual increase of DCF fluorescence in HepG2 cells was observed when treated with the increasing concentration of corosolic acid: 30, 40
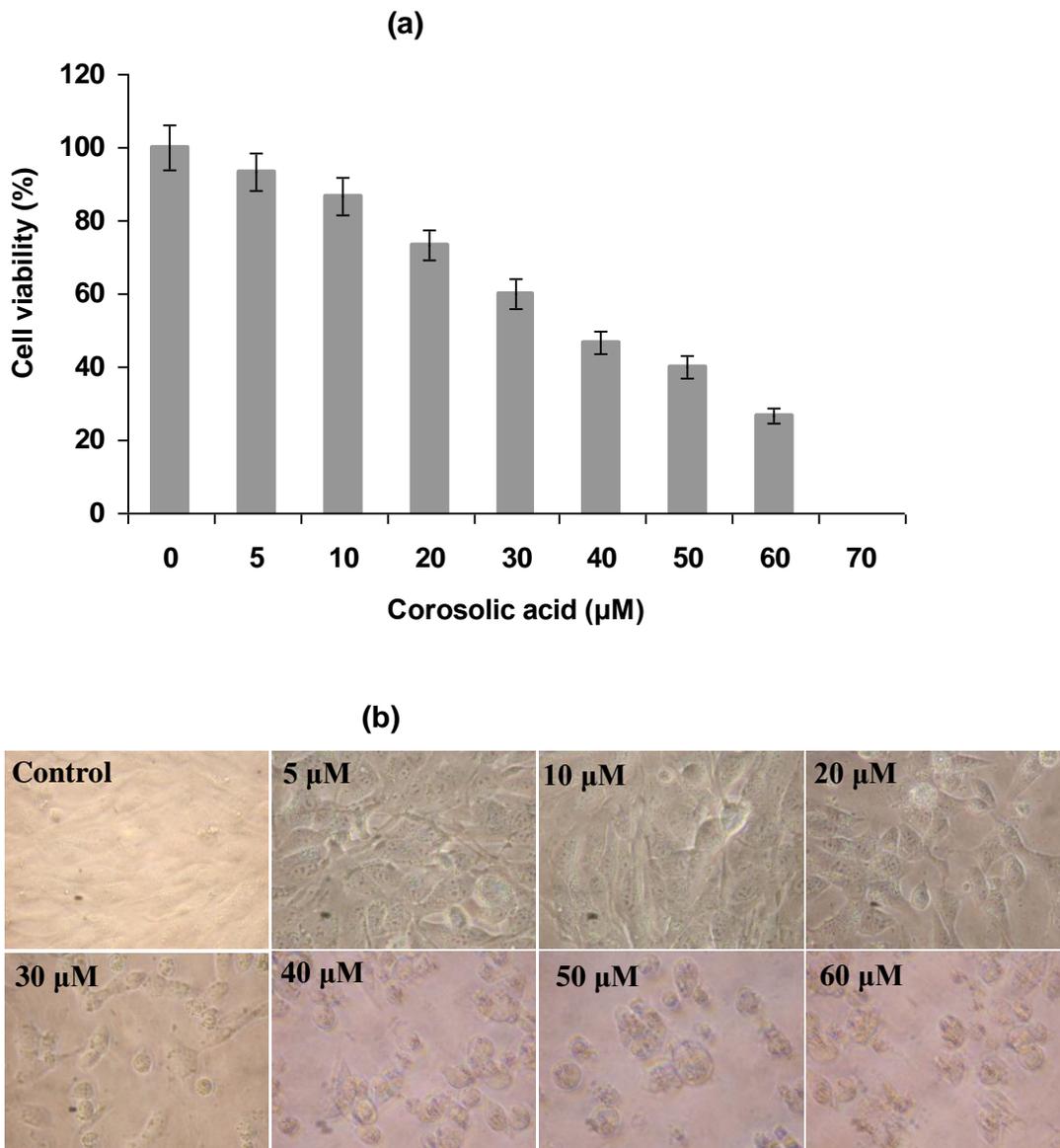
(a)

(b)

Figure 2. (a) Effect of corosolic acid on HepG2 cell proliferation. HepG2 cells were cultured in 10% FBS medium and treated with 5, 10, 20, 30, 40, 50, 60 and 70 µM corosolic acid for 24 h and cell proliferation was monitored by MTT assay. The percent cell viability was calculated in comparison with the untreated control cells taken as 100%. Values were expressed as mean ± SD of three independent experiments, each was performed in triplicate (P ≤ 0.05). (b) Microscopic photographs of corosolic acid-treated HepG2 cells. Cells were treated with or without corosolic acid (5, 10, 20, 30, 40, 50, 60 and 70 µM) for 24 h and photographs were taken using Nickon inverted microscope at 40x magnification.

and 50 µM, (Figure 3b). Further study showed that corosolic acid 30, 40 and 50 µM induced apoptosis (Figure 3c to e). This data suggested that the accumulation of ROS may play an important role in corosolic acid induced apoptosis.

Apoptosis induced by corosolic acid treated HepG2 cells

Apoptosis induced by corosolic acid is one of the

considerations in drug development. The apoptotic cells usually show apoptotic features such as nuclear shrinkage and chromatin condensation. The AO/EB staining is sensitive to DNA and was used to assess the changes in nuclear morphology. Apoptotic and necrotic cells can be distinguished from one another using fluorescence microscopy. In the presence of corosolic acid, the living cells were stained bright green in spots (Figure 3c, A), as well as red necrotic cells stained by ethidium bromide were also found (Figure 3c, B to D) in
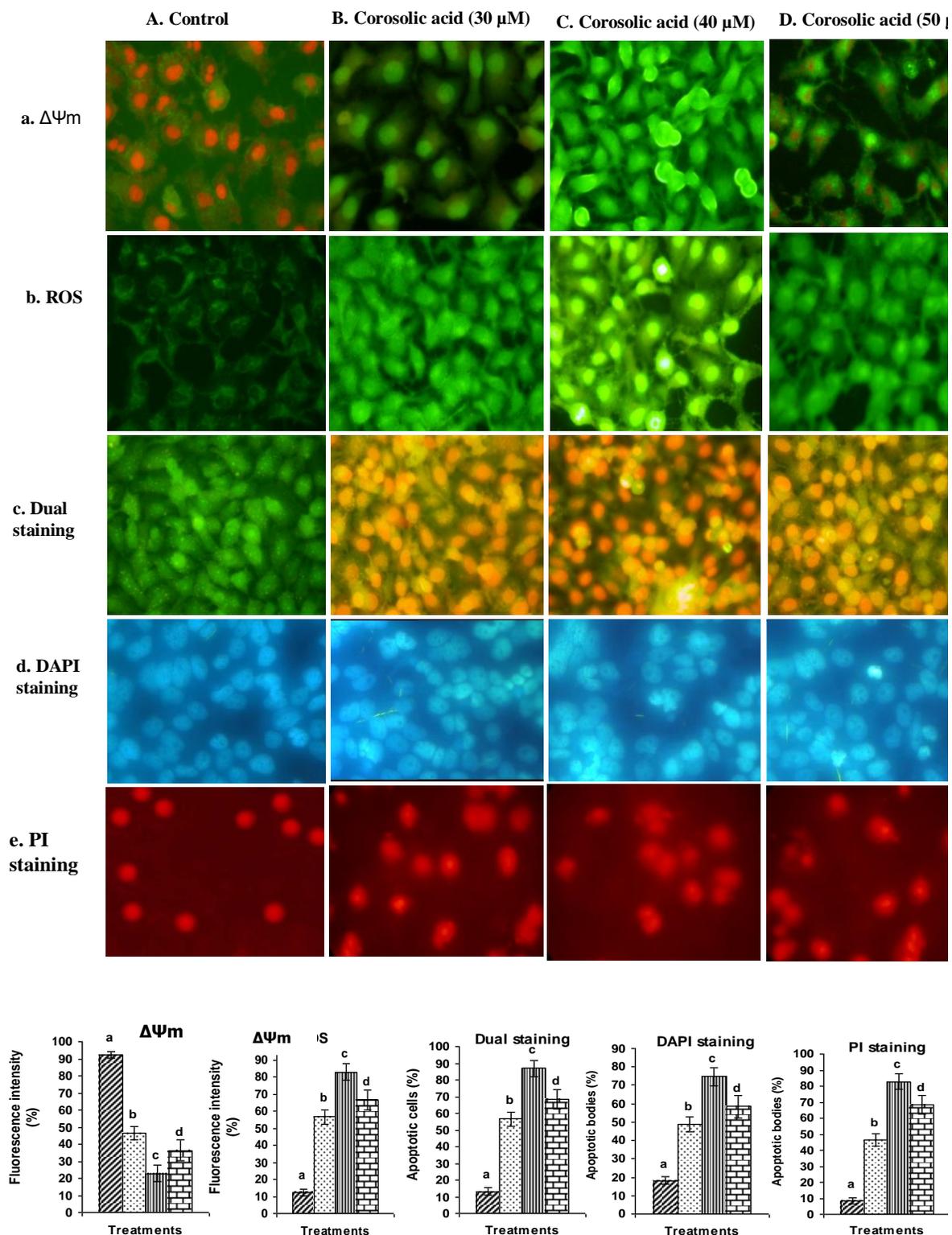
Figure 3. (a to e) Fluorescence microscopic photographs of control and corosolic acid-treated HepG2 cells. Cells were treated with or without corosolic acid (30, 40 and 50 μM) for 24 h, fixed and stained with DCFH-DA for ROS, Rhd-123 for ΔΨm, AO/EtBr for dual staining, DAPI for apoptosis and propidium iodide (PI) for DNA fragmentation and the DNA contents were photographed using Nickon inverted microscope at 40x magnification. The images were calculated as percentage fluorescence intensity in ROS and ΔΨm and percentage apoptotic bodies in dual staining, DAPI staining and PI staining of the HepG2 cells treated with or without corosolic acid. Values were expressed as mean ± SD of three independent experiments, each was performed in triplicate ($P \leq 0.05$).
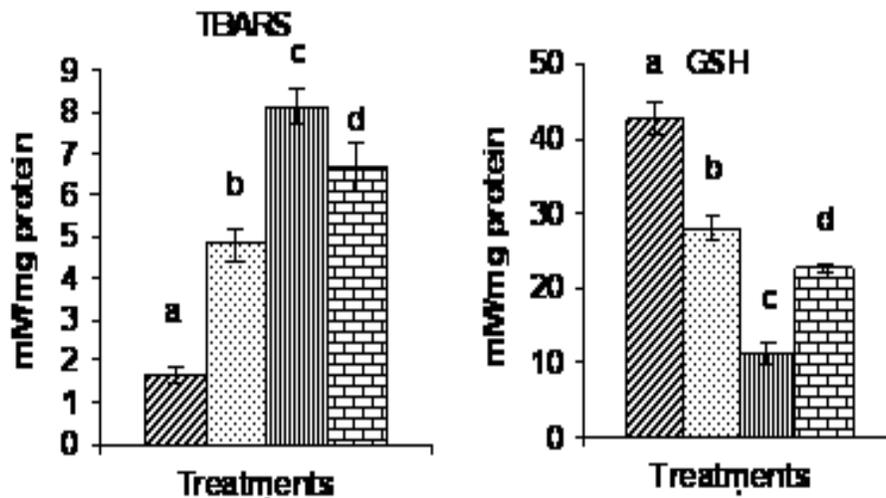
Figure 4. The TBARS and GSH levels were detected in HepG2 cells treated with corosolic acid (30, 40 and 50 µM) for 24 h. Cells were pretreated with corosolic acid for 24 h, then TBARS level was consequently increased in HepG2 cells respectively, when compared with the untreated control. Intracellular levels of GSH remarkably decreased in corosolic acid-treated cells respectively, when compared with the untreated control. Each sample was duplicated, and the figure was representative of three independent assays. Values are means ± SD for at least three independent experiments performed in triplicate (P< 0.05 when compared with vehicle control). ▨ Control HepG2 cells; ▨ HepG2 + corosolic acid (30 µM); ▥ HepG2 + corosolic acid (40 µM); ▭ HepG2 + corosolic acid (50 µM).

corosolic acid treated HepG2 cells. To further confirm the effect of corosolic acid on the morphology of apoptotic cells, DAPI and PI staining were used. After treatment with 30, 40 and 50 µM corosolic acid for 24 h, marked morphological changes in chromatin morphology and increased DNA damage such as condensation and fragmentation were observed in HepG2 cells (Figure 3d and e).

Effect of corosolic acid on TBARS and GSH in HepG2 cells

Figure 4 shows the levels of TBARS in corosolic acid treated HepG2 cells. Corosolic acid (30, 40, and 50 µM) treatment significantly increased the levels of lipid peroxidation in HepG2. Further, to evaluate the homeostasis of ROS in depletion of GSH in the corosolic acid treated cells, the effect of corosolic acid on the intracellular GSH level in HepG2 cells was tested. As shown in Figure 4, corosolic acid treatment significantly decreased the level of GSH when compared with the untreated cells, respectively.

Effect of the corosolic acid on NF-κB and PARP in HepG2 cells

Figure 5a and b represents the NF-κB and PARP protein expression levels in control and corosolic acid treated

HepG2 cells. The data shows that corosolic acid (30, 40 and 50 µM) treated HepG2 cells showed significantly down-regulation of NF-κB and up-regulation of fragmentation PARP protein expression, when compared with the control.

Effect of corosolic acid on cyt. C, Bax and Bcl-2 in HepG2 cells

As demonstrated, Bcl2, Bax and release of cyt. C in HepG2 cells treated with corosolic acid was seen. Treatment of HepG2 cells with 40 µM of corosolic acid had a concomitant increase in the cellular fraction of mitochondrial cyt. C. As seen in Figure 6a and b, corosolic acid treatment of HepG2 cells resulted in an up-regulation of Bax expression in a dose-dependent manner. The level of the anti-apoptotic protein Bcl-2 down-regulated during the corosolic acid exposure to the cells and corosolic acid alone did not affect the level of β-actin protein, used as a loading of internal control.

DISCUSSION

Much scientific effort have been made for the cure of HCC, but it is still regarded as a challenge for human health, worldwide, with cancer-related mortality estimated to double in the next 5 decades (Bakar et al., 2013). Conventional chemotherapy has been reported to cause
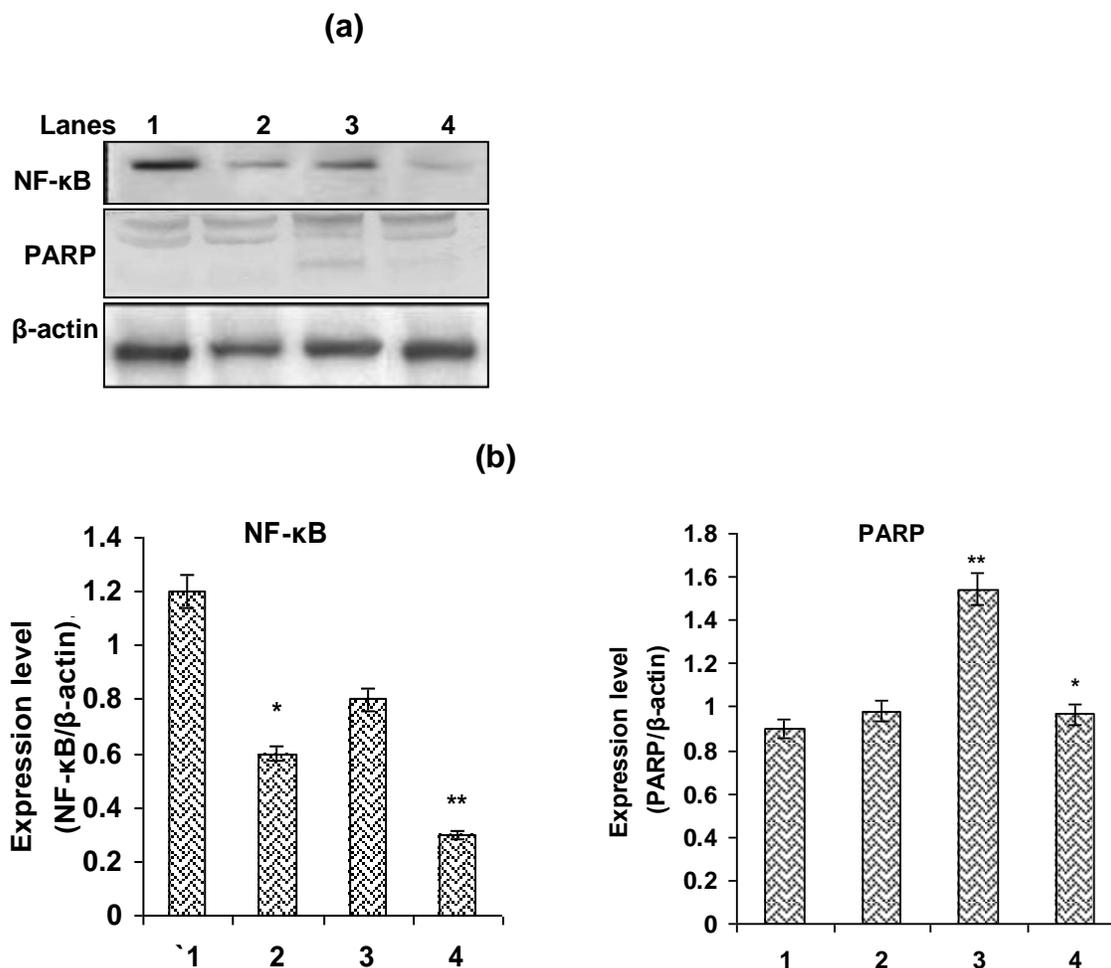
(a)

(b)

Figure 5. Effect of corosolic acid on NFκB and PARP protein expression in HepG2 cells. (a) NFκB and PARP protein expression by Western blot. (b) Band intensity scanned by densitometer. Histograms from densitometric analysis expressed as ratio of NFκB and PARP in β-actin and value are given as means ± S.D. of three experiments. *$P<0.05$ when compared with control. Lane 1, control; lane 2, corosolic acid (30 µM); lane 3, corosolic acid (40 µM); lane 4, corosolic acid (50 µM).

short- and long-term side-effects, such as nausea, stomatitis, neuropathy, fatigue and cognitive dysfunction (Partridge et al., 2001), thus compromising the quality of life of HCC cancer patients. Moreover, the increasing trend of cancer resistance against chemotherapy treatment makes it difficult to deal with these cancer patients. Thus, chemoprevention has become an important approach to mimic cancer. Therefore, exploration of new chemotherapeutic agents and more effective therapies for the treatment of HCC are still needed.

Phytochemicals are widely present in plant derived foods, beverages and in many dietary supplements or herbal remedies. A large number of epidemiological studies suggest that a daily intake of phytochemicals can reduce the incidence of several types of cancer development in humans (Russo et al., 2010). In the

present study, the anticancer property and molecular mechanism of natural triterpenoid, corosolic acid treated with HepG2 cells was analyzed. The MTT assay results show that the corosolic acid treated HepG2 cells significantly reduced the cell proliferation and enhanced apoptosis process at a low concentration (IC_{50} value is 40 µM).

In the phenomena, corosolic acid treated HepG2 cells increased ROS generation caused by apoptotic process. This shift requires considerable amounts of ROS, a drop in ATP and consequent loss of the electrochemical gradient across the inner mitochondrial membrane. The deleterious consequences of this shift reside mainly in inflammation, triggered by the rupture of necrotic cells and subsequent release of tissue-degrading enzymes (Xu et al., 2009). The present result suggests that corosolic acid treated cells gradually increased
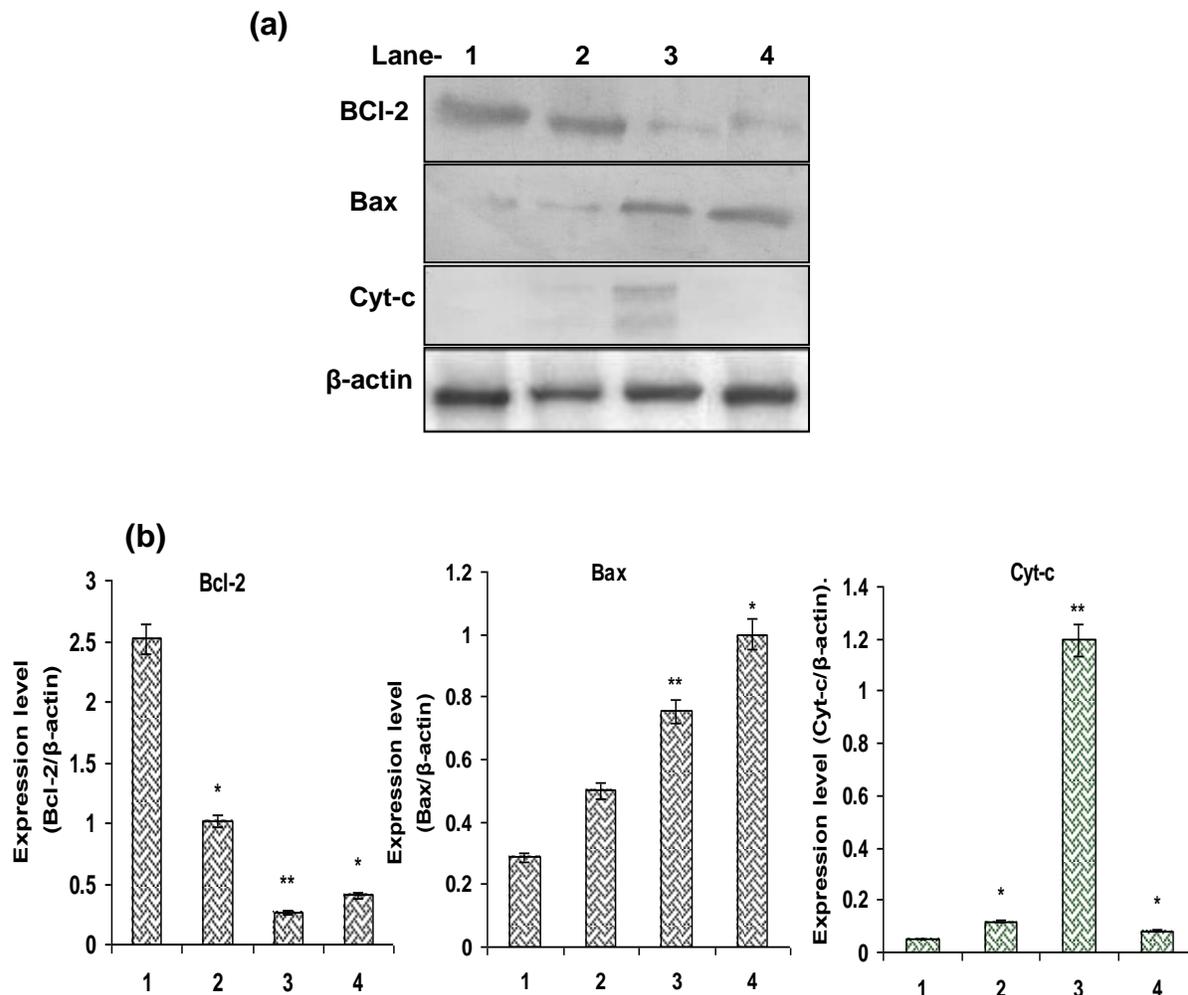
(a)

(b)

Figure 6. Effect of corosolic acid on BCl-2, Bax and cyt. C protein expression in HepG-2 cells. (a) BCl-2, Bax and cyt. C protein expression by Western blot. (b) Band intensity scanned by densitometer. Histograms from densitometric analysis expressed as ratio of BCl-2, Bax and cyt. C in β-actin and value are given as means ± S.D. of three experiments. *P<0.05 when compared with the control. Lane 1, control; lane 2, corosolic acid (30 μM); lane 3, corosolic acid (40 μM); lane 4, corosolic acid (50 μM).

generation of ROS leading to activation of inflammatory response such as activation of NF-κB and PARP. Previously, corosolic acid could suppress the M2 polarization of macrophages and tumor cell proliferation by inhibiting both signal transducer, activator of transcription-3 and nuclear factor-kappa B activation (Fujiwara et al., 2011).

Poly (ADP-ribose) polymerase catalyzes the poly ADP-ribosylation of a variety of nuclear proteins with NAD as substrate. Upon DNA damage, PARP gets activated and depletes NAD and ATP of the cell in an attempt to repair the broken DNA. The previous study also indicated that NF-κB up-regulate anti-apoptotic genes such as Bcl-2, Bcl-XL, Mcl-1 and inhibit apoptosis protein (IAP) (Bharti and Aggarwal, 2002). In this study, it was found that the level of NF-κB in the nucleus was markedly reduced in the corosolic acid-treated HepG2, when compared with

the control. This observation suggested that the corosolic acid may inhibit the translocation of NF-κB from the cytosol into the nucleus and thus contribute to apoptosis. The possible mechanisms of corosolic acid-inhibited translocation of NF-κB from the cytosol into the nucleus in HepG2 cells remain to be studied. Lee et al. (2010) reported that corosolic acid suppress human epidermal growth factor receptor expression, which in turn promote cell cycle arrest and apoptotic cell death of gastric cancer cells.

Although, with the cell morphological changes of HepG2, DNA fragmentation and involvement of apoptosis, it was related that corosolic acid caused apoptosis in HepG2 cells in a dose-dependent manner. The morphologies of corosolic acid-treated cells revealed the typical features of apoptosis. These apoptotic phenomena were further confirmed by DNA

fragmentation by PI and DAPI staining. Consequently, mitochondria-dependent apoptosis pathway may further enhance the oxidative stress that initially triggered apoptosis. Corosolic acid treated cells release cyt. C during apoptosis which disturbs the coupling efficiency of electron chain transport, results in free radical accumulation. Oxidative burst causes thiol oxidation of cellular membrane lipids and proteins, consequently mitochondrial membrane permeabilization lead to apoptosis. Additionally, an increased production of peroxides, found in cell populations containing apoptotic cells, may be the result rather than the cause of apoptosis. The observed results suggested that corosolic acid treated cells increased proapoptotic factor Bax level and decreased level of Bcl-2. Therefore, HepG2 cells proved great sensitivity toward the cytotoxic effect of corosolic acid induced apoptosis and mediate activated protein kinase activation which lead to inhibition of mammalian target of rapamycin, providing a possible mechanism to inhibit cancer cell growth and the induction of apoptosis (Lee et al., 2010).

Conclusion

The result clearly indicates that HepG2 cells are relatively sensitive to the cytotoxic effect of corosolic acid which induces apoptosis via the activation of caspase-3. Corosolic acid may be a potential chemopreventive or cancer therapeutic agent in hepatocellular carcinoma and these possibilities need further investigation.

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