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# Triterpenoids from *Cassia fistula* L. regulate p53 & ERK2 genes to induce apoptosis in HT-29 colon cancer cells

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ABSTRACT

Incidence of colon cancer is on a rise in the current decade. Though there are several modern means of medication to tackle the disease they cause several side effects. Hence, as an alternative means to reduce such risks, phytotherapeutics stands as a contemporary focus. Our study explored the anticancer efficacy of three triterpenoids, derived from ethyl acetate extract of *Cassia fistula* L., against human colon cancer cell line (HT-29). Of the three, compound 1 and 2 were found to be non-toxic on normal VERO cells but cytotoxic to cancer cells with 50 μM and 40 μM as their $IC_{50}$ concentrations respectively. Apoptotic features such as chromatin condensation, DNA fragmentation, membrane leakage and enhanced depolarization of mitochondrial membrane, with subsequent cell cycle arrest at S-phase and $G_2$/M phase were observed. RT-PCR analysis revealed that both the compounds produced upregulation of p53 expression and downregulation of ERK2 expression. The rates of invasion and metastasis were significantly decreased on exposure to the compounds. Furthermore, antioxidant activity was evident on destruction of cancer cells. Docking results showed stable interactions between the compounds and the cancer targets (p53 & ERK2). The data suggests that both the compounds exhibit high antiproliferative effect in HT-29 cells and are promising as alternative cancer therapeutics.

## 1. Introduction

Colon cancer is one of the most common cancers. It is one among the four common cancers in men, accounting for 44% of all cases, and one among the three most commonly diagnosed cancers in women (Siegel et al., 2016). Colon cancer is the third leading cause of mortality in more developed countries (Yang et al., 2008). According to Halliwell (2007) sedentary lifestyle along with changes in food habits may be the cause to bring a change in the metabolism leading to the generation of toxic free radicals. They are capable of damaging the normal homeostasis of cells and activate oncogenes that end up in cancer. Even though, various treatment modalities such as chemotherapy, immunotherapy, radiation therapy and surgery are practiced to reduce the menace of cancer, resistance to rational practices still persists and the mounting problem of side effects incurred by the use of synthetic drugs has triggered the search for alternative therapeutics.

In this context, plant-based food and medicines subscribing to pro-oxidant therapy and antioxidant supplementation have received great attention and have been a promising cure and prevention for a number of health disorders (Raskin et al., 2002). With this focus our research team has identified novel compounds from plants such as *Gymnema sylvestre* (leaves) against diabetes and prostate cancer (Daisy et al., 2009a,b); (PonNivedha et al., 2015), *Tinospora cordifolia* (stem) and *Clitoria ternatea* (leaves) against diabetes (Rajalakshmi et al., 2009); (Daisy and Rajathi, 2009), *Terminalia bellarica* (fruit) against diabetes, oxidative stress and breast cancer (Latha and Daisy, 2011); (Rajalakshmi and Sales, 2015a,b); (Rajalakshmi and Sales, 2015a,b), *Costus specioscus* (root) against breast cancer and diabetes (Eliza et al., 2009); (Roy and Manikkam, 2015), *Elephantopus scaber* (root and leaf) against diabetes and microbial infection (Daisy et al., 2009a,b); (Daisy et al., 2008) etc. *Cassia fistula* L. (*C. fistula*) (Golden shower, Indian labrum) is one such plant of interest.

*C. fistula* L., belonging to Fabaceae family, is a folklore medicinal plant in India among the South Asian countries. Cassia invites notice among researchers worldwide for its wide range of pharmacological activities. A flavanoid, catechin was isolated and identified from the

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stem bark of *C. fistula* L. and was found to possess insulin-mimetic effect and hepatoprotective effect on streptozotocin (STZ)-induced diabetic male albino Wistar rats (Daisy et al., 2010); (Pitchai and Manikkam, 2012). Furthermore, three triterpenoid compounds (**cpd-1:**17-[(E)-1′, 5′-dimethyl-2′-hexenyl]-11-hydroxy-5-(hydroxymethyl)-13, 14-dimethyl5,6,7, 11,12,13,14,15,16,17 decahydro-3H-cyclopenta[*a*] phenanthren-3-one; **cpd-2:**17-[(2E,5E)-4′,7′-diethyl-1′,8′-dimethyl-2′, 5′-nonadienyl]-11-hydroxy-5-(hydroxymethyl)-13,14-dimethyl-5,6,7, 11,12,13,14,15,16,17-decahydro-3H-cyclopenta[*a*]phenanthren-3-one; **cpd-3:**17-[(E)4-ethyl-1′,5′-dimethyl-2′-hexenyl]-11-hydroxy-5(hydroxymethyl)-13,14-dimethyl-5,6,7,11,12,13,14,15,16,17-decahydro-3Hcyclo penta[*a*]phenanthren-3-one) were isolated from *C. fistula* ethyl acetate extract and identified as novel compounds with antidiabetic activity (Indian Patent [**cpd 1**: (2880/CHE/2012), **cpd 2**: (2881/CHE/2012) and **cpd 3**: (2879/CHE/2012]).

Usually the drugs administered to treat diabetes also manage oxidative stress which in turn can control human cancer (Borek, 2004). Earlier reports have shown that triterpenoids can be used as preventive therapeutics to colon, breast, prostate and skin cancers (Patlolla and Rao, 2012); (Chaturvedi et al., 2008). Hence, the present study was aimed at the investigation of antioxidant and anticancer properties of the three triterpenoid compounds present in *C. fistula* L. stem bark via *in-vitro* analysis on human colon cancer cells (HT-29) and substantiating *in-silico* studies.

## 2. Materials and methods

### 2.1. Plant material and extraction

The bark of *C. fistula* L. was collected from Western Ghats, Idukki District, Kerala, India during the month of June and July. The species was identified and authenticated by Dr.Roseline, Plant taxonomist, Department of Botany, Holy Cross College, Tiruchirappalli, India and the voucher specimen (No. HC-15) is deposited in the herbarium of the department. The barks were chopped into small pieces, shade-dried and coarsely powdered. The powder was defatted with petroleum ether (60-80 °C) and then extracted with hexane using Soxhlet apparatus. The extract was dried under reduced pressure using a rotary vacuum evaporator (Rajalakshmi and Daisy, 2014).

### 2.2. In-vitro analysis

#### 2.2.1. Cell lines

Human colon cancer (HT-29) and normal VERO cells were procured from National Center for Cell Science (NCCS), Pune, India. Colon cancer cells were maintained in Dulbecco's Modified Eagles Media (DMEM, Himedia, Mumbai, India). VERO cells were maintained in Minimal Essential Media (MEM, Himedia). All the experiments were conducted with cells at their exponential phase of growth.

#### 2.2.2. Cell viability assay

Viability of the treated and untreated cells was measured by MTT (dimethyl triazolyltetrazolium bromide) colorimetric assay. Cells (HT-29 and VERO) were plated at a density of $5 \times 10^3$ cells/well, in a 96-well plate and incubated for 24 h under 5% $CO_2$ at 37 °C for proper attachment, after which the cells were washed with 1X PBS and then cells were incubated in serum-free medium (SFM) for 12 h. The starved cells were then added into the fresh medium containing different concentrations (10, 20, 30, 40, 50, 60 and 80 μM) of the three compounds and allowed to grow for an additional 24 h after the treatment. The medium was removed and cells were washed twice with 1X PBS after which 200 μL of 0.5 mg/mL MTT solution was added to each well and the cultures were further incubated for 24 h. Then 500 μL of DMSO was added for dissolving the formazon crystal and left undisturbed in the dark for 1 h. The intensity of coloration was measured from absorbance using an ELISA plate reader at 570 nm for each well. Cell viability was calculated as follows:

$$\% \text{ of cell viability} = \text{absorbance of treated cells/absorbance of control cells} \times 100$$

Five different observations with different concentrations of the compounds were performed and the concentration that produced a 50% reduction in the number of viable cells ($IC_{50}$) was determined (Mosmann, 1983).

#### 2.2.3. Wound healing assay

The HT-29 cells at a density of $2 \times 10^5$ cells/well were seeded into 12-well plates to grow into monolayers. The latter were wounded using a sterile 10 μL pipette. The cells were washed with 1X PBS in order to remove the debris. The cells were then incubated with the compounds at different concentrations (20, 40, 50 and 100 μM) in DMEM for 24 h. The medium was replaced with 1X PBS and photographed using phase-contrast microscope at 10× magnification (Rodriguez et al., 2005).

#### 2.2.4. Acridine Orange (AO)/Ethidium bromide (EtBr) dual staining assay

The induction of apoptosis by the triterpenoids in colon cancer cell lines was determined by AO/EtBr staining. The HT-29 cells were seeded in 6 well microplates and allowed to grow to a density of $5 \times 10^5$ cells/well by incubation at 37 °C and 5% $CO_2$ for 24 h. The triterpenoids were added to each well at their $IC_{50}$ concentrations (50 μg/mL and 40 μg/mL) and incubated for 72 h. The cells were then trypsinized, washed with ice-cold 1X PBS and cells in 20 μL of the medium were kept on a glass slide. Cells were then stained with equal volumes of AO and EtBr (100 μg/mL each). The slides were maintained at room temperature for 10min and observed under blue filter of a fluorescent microscope at 10× magnification (Olympus CK×41 with Optika Pro5 camera).

#### 2.2.5. Flow cytometric analysis of cell cycle

Two hundred microlitres of compound-treated HT-29 cells were taken in polystyrene tubes, centrifuged at 300×*g* for 5 min, washed with 1X PBS and 200 μL of ice cold 70% ethanol was added. It was kept for incubation at −20 °C for 3 h and then centrifuged. The pellet was re-suspended in 200 μL of Muse™ cell cycle reagent containing RNAse and propidium iodide and kept at room temperature for 30min. The cell suspension was then transferred to a microcentrifuge and analyzed using Muse™ Cell Analyzer (Merck, Germany) (Darzynkiewicz and Zhao, 2001).

#### 2.2.6. DNA fragmentation assay

Compounds (50 μM and 40 μM, respectively)-treated HT-29 and VERO cells were evaluated for the induction of apoptosis through formation of DNA fragments using the protocol described by Kasibhatla et al. (2006). The HT-29 cells were treated with triterpenoids at their $IC_{50}$ concentrations in DMEM for 24 h and incubated at 37 °C and 5% $CO_2$. The cells were harvested by trypsinization and lysed in a solution containing 100 mM Tris (pH 8.0), 20 mM EDTA and 0.8% N-laurylsarcosine sodium salt at 4 °C. 10 μL of RNase A (10 mg/mL) was added and incubated for 1 h at 37 °C, followed by 5 μL proteinase K and incubated at 50 °C for 30 min. Isopropanol (1 mL) was added and incubated overnight to precipitate the DNA. The pellet was collected by centrifugation, dried, dissolved in TE buffer and electrophoresed on 2% agarose gel for 3 h. Gels were visualized and photographed under UV light (Kasibhatla et al., 2006).

#### 2.2.7. Determination of mitochondrial membrane depolarization

Compounds were added to HT-29 cells seeded to 6-well plates and incubated for 24 h in a humidified incubator. Cells were harvested and stained with mitochondrial membrane potential marker JC1 (2.5 μg/well) and mixed to obtain a uniform red-violet color. The solution was kept in the dark at room temperature for 30min, washed with PBS and resuspended in 1X PBS for analysis using Muse™ Cell Analyzer (Merck,

Germany) (Rasola and Geuna, 2001).

#### 2.2.8. Determination of gene expression

HT-29 cells were cultured along with $IC_{50}$ concentrations of the respective compounds and incubated for 24 h in a humidified incubator at 37 °C. Total RNA was extracted using TRIzol® reagent (Life Technologies, USA). Cells were harvested; 1 mL of TRIzol reagent was added, mixed well and kept at room temperature for 3min. The mixture was centrifuged at 14000 rpm at 4 °C for 1min. Isopropanol (500 μL) was added to the supernatant and kept for 10 min at room temperature and the pellet was collected and dried. cDNA was synthesized from the RNA using Thermo Scientifc Verso cDNA synthesis kit and amplified using Thermo Script™ RT-PCR System (Life technologies, USA).

The primers used include ERK2 F- GAGCACCAGACCTACTGCCAG; ERK2 R- AATTTCTGGAGCCCTGTACCA; p3 F- CCAiCCATGAGCGCTGCTCA; p53 R- GCAGGGAGGGAGAGATG; GAPDH F- ACACCCACTCCTCCACCTTT; and GAPDH R- TAGCCAAATTCGTTGTCATACC. Results were normalized to experimental control (GAPDH), and the relative expression (RQ) values were calculated based on the comparative CT method (Pfaffl, 2001).

#### 2.2.9. Determination of lipid peroxidation

HT-29 cells treated with compounds overnight were harvested, lysed and 50 μL of cell lysates were taken in separate tubes. 1 mL TBA (1%) and 500 μL ethyl alcohol (70%) was added to each tube and incubated in a boiling water bath for 20min. The tubes were allowed to cool and 50 μL acetone was added to it. Polyunsaturated fatty acids broke down to produce malondialdehyde which in turn reacted with TBA (thiobarbituric acid) to form a pink-colored product whose absorbance was read at 535 nm using a UV–Vis spectrophotometer- PharmaSpec 1700 model (Okhawa et al., 1979).

#### 2.2.10. Estimation of reduced glutathione (GSH)

The supernatant of cell lysate (0.1 mL) was made up to 1.0 mL with 0.2M sodium phosphate buffer (pH 8.0). Standard GSH corresponding to concentrations ranging from 2 to 10 nM were also prepared. 2 mL of freshly prepared DTNB solution was added and the intensity of the yellow color developed was read in a spectrophotometer (Genesys 10-S, USA) at 412 nm after 10min. The values are expressed as nM GSH/g sample (Moron et al., 1979).

#### 2.2.11. Estimation of superoxide dismutase (SOD)

The assay mixture contained 1.2 mL of sodium pyrophosphate buffer, 0.1 mL of PMS, 0.3 mL of NBT, 0.2 mL of the enzyme preparation (sample) and water in a total volume of 2.8 mL. The reaction was initiated by the addition of 0.2 mL of NADH. The mixture was incubated at 30 °C for 2min and arrested by the addition of 1.0 mL of glacial acetic acid. The reaction mixture was then shaken with 4 mL of n-butanol, allowed to stand for 10min and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm in a spectrophotometer (Genesys 10-S, USA). One unit of enzyme activity is defined as the amount of enzyme that produced 50% inhibition of NBT reduction in 1min (Kakkar et al., 1995).

#### 2.2.12. Estimation of catalase (CAT)

$H_2O_2$-phosphate buffer (3 mL) was taken in an experimental cuvette to which, 40 μL of cell lysate was forcefully added and mixed thoroughly. The time required for decrease in absorbance by 0.05 units was recorded at 240 nm in the spectrophotometer. Enzyme solution containing $H_2O_2$-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units (Luck et al., 1974).

#### 2.2.13. Estimation of glutathione peroxidase (GPx)

0.2 mL each of EDTA, sodium azide, GSH, $H_2O_2$, buffer cell homogenates were mixed and incubated at 37 °C for 10min. The reaction was arrested by the addition of 0.5 mL of TCA and the tubes were centrifuged. To 0.5 mL of supernatant, 3.0 mL of phosphate solution and 1.0 mL of DTNB were added and the colour developed was read at 420 nm immediately using a spectrophotometer. Graded amount of standards were also treated similarly. GPx activity is expressed as $Unit^3$/mg protein (Rotruck et al., 1973).

### 2.3. Statistical analysis

All results were analyzed using one way analysis of variance (ANOVA) on Statistical Package for Social Sciences (SPSS) (Version 17.0) and the group means were compared by Duncan's Multiple Range Test (DMRT) (Duncan and Duncan, 1957) and $p$ value less than 0.05 was considered statistically significant.

### 2.4. In-silico analysis

The docking analysis was carried out by using the LibDock module of Accelary's Discovery studio Version 2.1. The structure of the two compounds was developed using Chemsketch software. The structure of receptors p53, ERK2 (PDB id: 1TUP_A, 5AX3_A) was retrieved from Protein Data Bank (PDB) and prepared for docking by removing the heteroatoms and water molecules in them. Crystallographic disorders and unfilled valence atoms were corrected using alternate conformations and valence monitor options and further subjected to energy minimization by applying CHARMM (Chemistry at HARvard Macromolecular Mechanics) force fields. Protein cavities were explored and active sites were identified. The prepared proteins and compounds were docked using LibDock module of Accelry's Discovery studio software 2.1. version.

## 3. Results

### 3.1. Effect of the C. fistula L. Triterpenoid compounds on cell viability

Cpd 1 and cdp 2, among the three *C. fistula* L. compounds tested in this study, produced dose-dependent inhibition of viability of HT-29 cancer cell, while cpd 3 did not affect viability of the cell to any significant level (Fig. 1). Hence, cpds 1 and 2 were subjected to further studies on HT-29 cell. The densities of attached HT-29 cells decreased with increasing concentration of the cpds 1 and 2. The cpds 1 and 2 induced cell death in HT-29 cells in a dose-dependent manner, and the $IC_{50}$ were 50 and 40 μM, respectively. There was no significant effect in the growth of normal VERO cells at these concentrations.

### 3.2. Wound healing efficacy of compounds

The anti-metastatic activity of the compounds on HT-29 cells was evaluated using wound healing assay. Both the compounds exhibited control over cellular motility. After incubation with the compounds, the distance between the wound line and the leading edge remained intact in treated cells with respect to the untreated as shown in Fig. 2. Of the various concentrations studied for finding the efficacy of the compounds (1 & 2), a dose-dependent increase in the anti-metastatic effect was noticed which was also prominent in 50 μM and 40 μM concentrations of compounds 1 and 2, respectively. Hence, further studies were carried out using the compounds at these concentrations.

### 3.3. Cell death induced by compounds

The effect of compounds to induce cell death in HT-29 cells, through changes in the nuclear morphology, was studied using AO/EtBr dual staining and the fluorescent microscopic images obtained are depicted in Fig. 3. Viable cell possessed uniform bright green nucleus whereas apoptotic cells showed early and late phase stain in bright red to orange. Condensed chromatin with nuclear fragmentation and shrunken nuclei

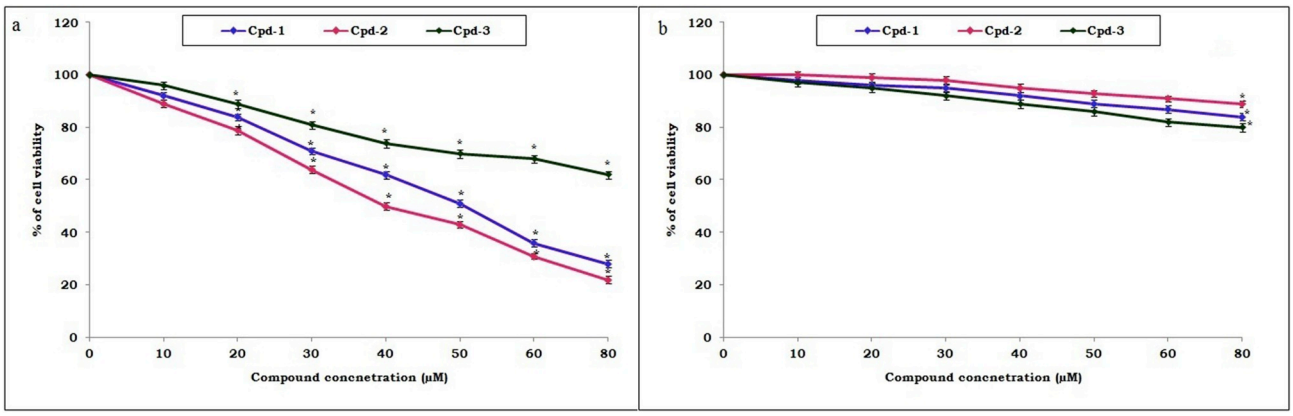


**Fig. 1.** Effect of the triterpenoids (Compound-1, 2 & 3) on a) HT-29 cell viability and b) VERO cell viability. Each value represents the mean ± SEM of five independent observations. '*' represents statistical significance between control versus compound-treated groups at $p < 0.05$.

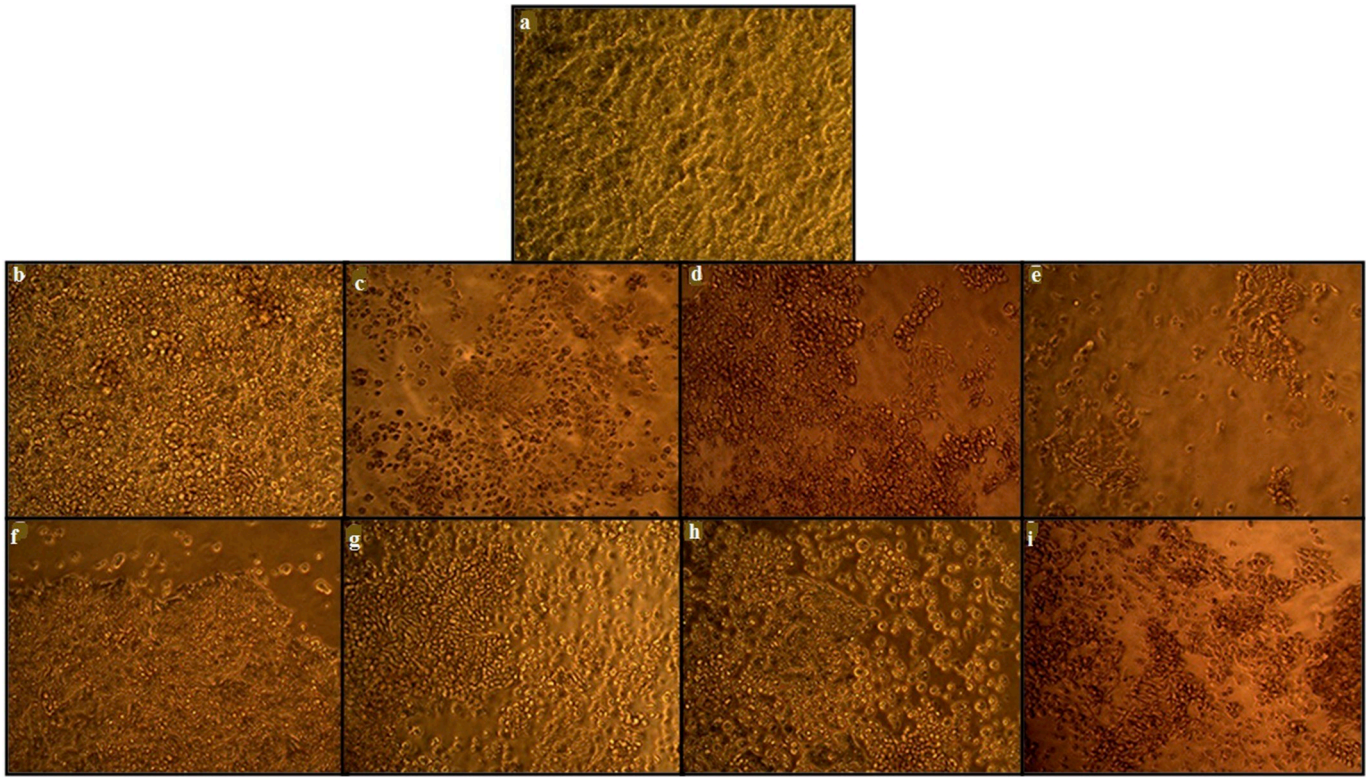


**Fig. 2.** Dose-dependent wound healing efficacy exhibited by the compounds. (a) Control; Cell survival after treatment with the compound 1 at (b) 20 µM, (c) 40 µM, (d) 50 µM, (e) 100 µM and of compound 2 at (f) 20 µM, (g) 40 µM, (h) 50 µM, (i) 100 µM on HT-29 cells overnight as seen under 10x of a phase-contrast microscope.

were identified in both the Compounds-treated colon cancer cells. These changes were also accompanied by the presence of a few apoptotic bodies; no such changes were observed in control colon cancer cells.

### 3.4. Effect of the compounds on cell cycle

Effect of the two compounds on HT-29 cell cycle progression was analyzed using flow cytometry (Fig. 4). The accumulation of cells in $G_2$ and S phases was observed in compounds-treated HT-29 cells (Table 1).

### 3.5. Effect of triterpenoids on DNA

Effect of the triterpenoids to induce DNA fragmentation in HT-29 cells was observed to be significant. Fragmented DNA was observed in both triterpenoids-treated cells which were similar to the fragmentation induced by the reference drug and no such changes were found in control DNA (Fig. 5).

### 3.6. Compounds-induced change in mitochondrial membrane potential

The compounds were found to induce significant changes in the mitochondrial membrane potential (Table 2). There was a significant decrease in colon cancer cells on treatment with the $IC_{50}$ concentrations of the compounds.

### 3.7. Compounds-mediated alteration in gene regulation

The effect of the compounds in regulating p53 and ERK2 genes to induce apoptosis was analyzed in human colon cancer HT-29 cells in comparison with their expressions in VERO cells (Fig. 6). When treated

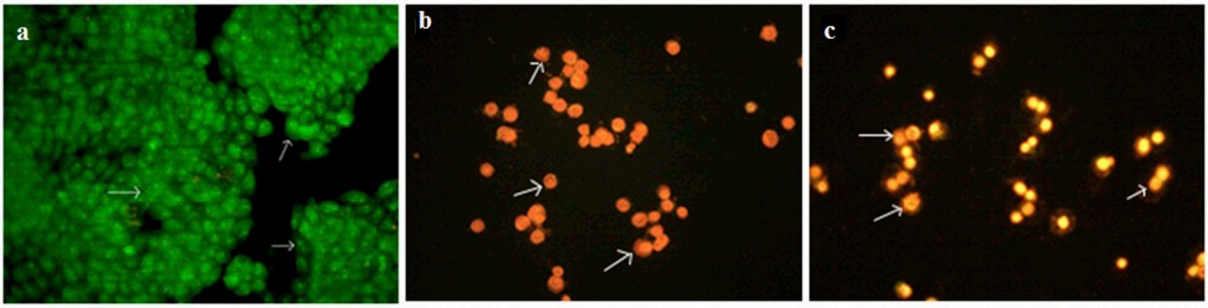


**Fig. 3.** Cell death induced by the compounds on HT-29 cells with dual staining assay observed using Olympus CKX41 with Optika Pro5 camera, 10× magnification. a. Intact nucleus with green fluorescence. b. Compound 1 induced late apoptosis with red stained nucleus. c. Early apoptopic cells obtained on treatment with compound 2. The arrows show cell membrane blebbing and DNA fragmentation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

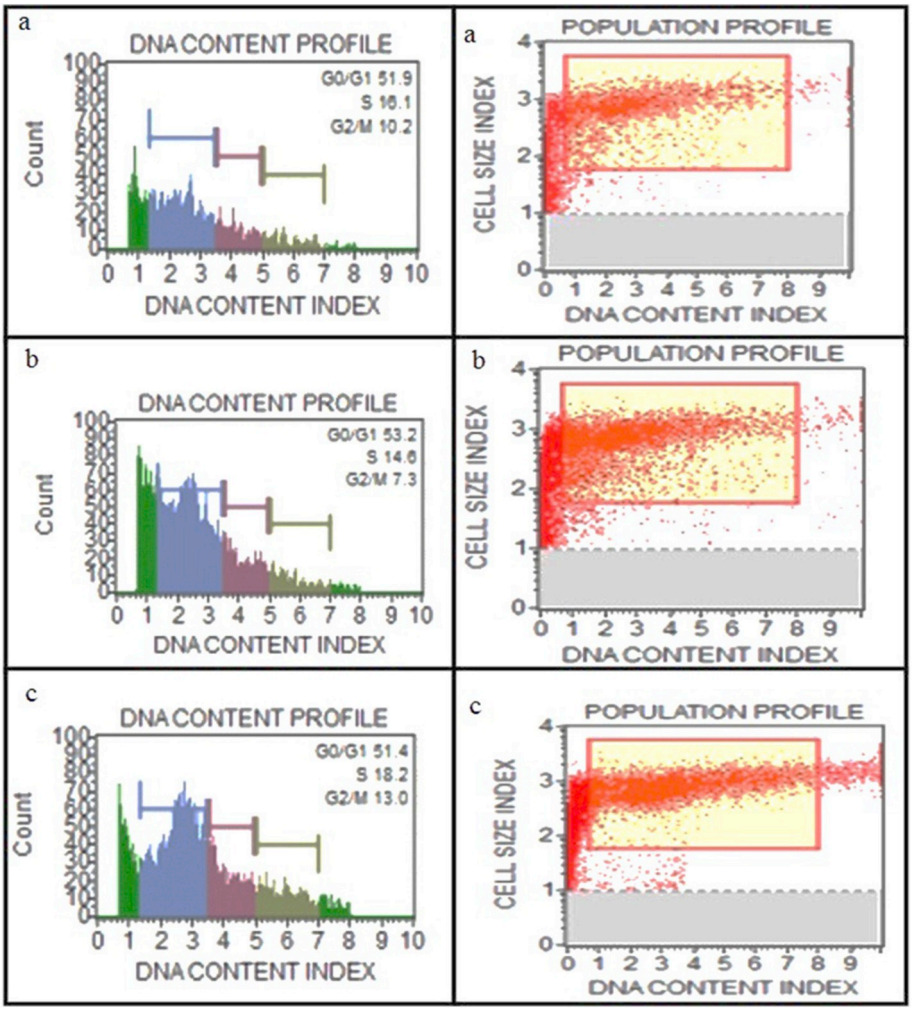


**Fig. 4.** Histogram represents the DNA distribution in each phase of cell cycle and population profile of cells. The data are obtained after 24 h post supplementation with (a) 50 μM of compound 1, (b) 40 μM of compound 2. Compound 1 caused cell cycle arrest at S phase while compound 2 blocked the progress of cell cycle from $G_2$ (c) Control untreated HT-29 cell cycle phases.

with compounds, the expression of p53 was upregulated and that of ERK2 was downregulated in HT-29 cells, when compared with the regulation of these proteins in the normal VERO cells. No such significant reduction was identified in compound treated VERO cells.

### 3.8. Antioxidant potentials of the compounds

The compounds were treated on HT-29 cells at 50 μM and 40 μM concentrations for 24 h for the determination of lipid peroxidation, reduced GSH, SOD, CAT and GPx enzyme and the results are depicted in Fig. 7. Lipid peroxidation and reduced GSH levels were doubled in HT-29 cells compared to normal cells and it was found to be normalized on

**Table 1**
Data shows cell cycle phase distribution as calculated by Muse™ Cell Analyzer (Merck, Germany).

| HT-29 cells | | $G_0/G_1$ | S | $G_2/M$ | Debris |
|---|---|---|---|---|---|
| **Compound 1-treated** | **% Gated** | 51.4 | 18.2 | 13.0 | 69.0 |
| | **Mean** | 2530.1 | 4147.9 | 5889.4 | 917.5 |
| | **%CV** | 22.5 | 10.8 | 9.4 | 288.6 |
| **Compound 2-treated** | **% Gated** | 53.2 | 14.6 | 7.3 | 69.9 |
| | **Mean** | 2330.9 | 4164.5 | 5820.2 | 213.1 |
| | **%CV** | 25.2 | 10.7 | 9.8 | 493.7 |
| **Control** | **% Gated** | 51.9 | 16.1 | 10.2 | 64.0 |
| | **Mean** | 2340.7 | 4167.4 | 5887.6 | 466.7 |
| | **%CV** | 25.3 | 10.3 | 9.5 | 383.1 |

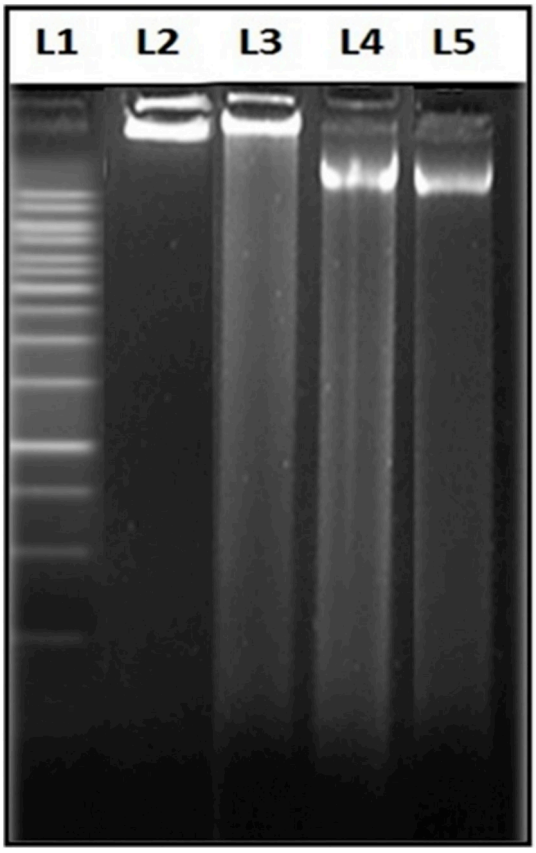


**Fig. 5.** Effect of the compounds on DNA of HT-29 cells. L1- DNA marker (10000bps), L2 - Untreated DNA control, L3 – Compound 1 treated DNA of HT-29 cells, L4- Compound 2 treated DNA of HT-29 cells, L5- Camptothecin-positive control.

treatment with both cpd 1 and cpd 2. The SOD, CAT and GPx activity of the treated cells that were significantly changed compared to VERO cells were brought to normal levels in both the compounds-treated cancer cells.

### 3.9. *In-silico interaction analysis*

The anti-cancer behavior of designed ligands on a structural basis was assessed by automated docking studies based on the binding affinities of the ligands at the active site of these receptors. The 2D structure of the cpds 1 & 2 sketched using Chemsketch is depicted in Fig. 8. Docking analysis was performed to identify the stable interaction between the compounds and cancer targets and the results of docking studies, confirmed a stronger binding affinity of cpd1 & cpd2 with p53, ERK2 shown in Fig. 9. The interactions between the compounds with the

**Table 2**
Terpenoids altered the mitochondrial membrane potential is depicted as determined using Muse™ Cell Analyzer (Merck, Germany).

| HT 29 cells | Particulars | %Gated | Cell Concentration (cells/ml) |
|---|---|---|---|
| **Compound 1-treated** | Live (LR) | 0.10% | 5.74E+02 |
| | Depolarized/Live (LL) | 95.52% | 5.49 + 05 |
| | Depolarized/Dead (UL) | 4.22% | 2.42E+04 |
| | Dead (UR) | 0.16% | 9.19E+02 |
| | Total Depolarized | 99.74% | 5.73E+05 |
| | Total Cell concentration | 5.74E+05/ml | |
| | Dilution factor | 1.00 | |
| **Compound 2-treated** | Live (LR) | 0.50% | 4.13E+02 |
| | Depolarized/Live (LL) | 85.60% | 7.07E+04 |
| | Depolarized/Dead (UL) | 11.50% | 9.50E+03 |
| | Dead (UR) | 2.40% | 1.98E+03 |
| | Total Depolarized | 97.10% | 8.02E+04 |
| | Total Cell concentration | 8.26E+04/ml | |
| | Dilution factor | 1.00 | |
| **Control** | Live (LR) | 98.56% | 2.02E+06 |
| | Depolarized/Live (LL) | 1.14% | 2.34E+04 |
| | Depolarized/Dead (UL) | nil | Nil |
| | Dead (UR) | 0.30% | 6.15E+03 |
| | Total Depolarized | 1.14% | 2.34E+04 |
| | Total Cell concentration | 2.05E+06/ml | |
| | Dilution factor | 1.00 | |

cancer targets were found to be stable. The interaction was between the active site residues of the proteins and the ligands by hydrogen bond interactions. The bond length was found to be less than 3 in all cases indicating the stability of the interaction with high Libdock score and absolute energy (Table 3).

## 4. Discussion

The ultimate goal of any anticancer drug would be to bring cell death in cancer cells without affecting the proliferation of normal cells. Biochemical indicators of cell death in almost all cells that are universally applicable are of two types. One is coagulative necrosis, characterized by the swelling proceeding to rupture of plasma and organelle membranes and dissolution of organized structure. This is normally observed after an injury by agents such as toxins and ischemia that affects cells in groups and evokes inflammation when it develops *in-vivo*. The other morphological pattern is characterized by condensation of the cell with maintenance of organelle integrity and the formation of surface protuberances that separate as membrane-bounded globules; in tissues, these are phagocytosed and digested by resident cells, there being no associated inflammation and damage to the surrounding cells (Wyllie et al., 1980).

Thus, the development of a potential cancer therapeutic drug is based on the capability of that drug to control the abnormal cell division and halt the cell cycle progression by evoking the response for apoptosis (Evan and Vousden, 2001). Most of the modern drug formulations are mainly derived from plant sources (De Smet et al., 1996). The present study investigated the effects of three triterpenoids isolated from the ethyl acetate extract of *C. fistula* L. for anticancer and antioxidant nature on human epithelial colon cancer cells (HT-29). Plants as a whole and plant-derived metabolites such as alkaloids, flavanoids, terpenoids and saponins have been reported to possess anticancer and antioxidant activities (Prakash et al., 2013). The compounds derived from *C. fistula* L. were found to effectively induce reduction in the cell viability of

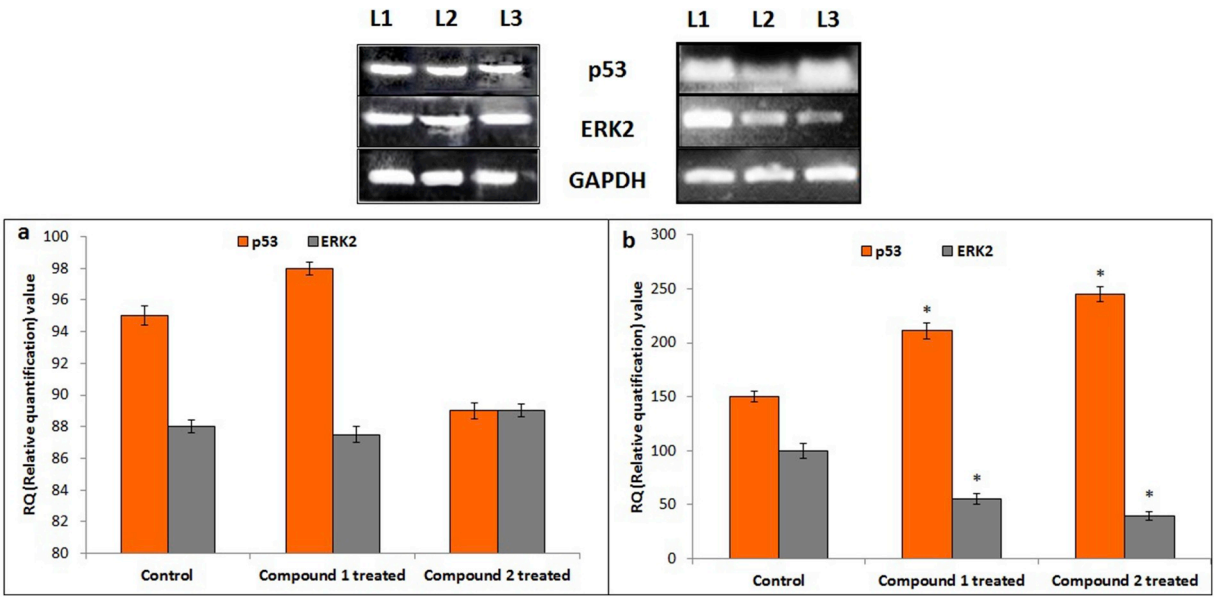


**Fig. 6.** Effect of the compounds on p53 & ERK2 mRNA expression in a)VERO and b) HT-29 cells. L1-Control untreated, L2-compound 1 treated, L3-compound 2 treated group. The ratio of p53 & ERK2/GAPDH is presented as raw data. Each bar represents the mean ± SEM of three independent observations with statistical significance between control and the treated groups at $p < 0.05$ – '*' compound treated groups compared with untreated cancer control.

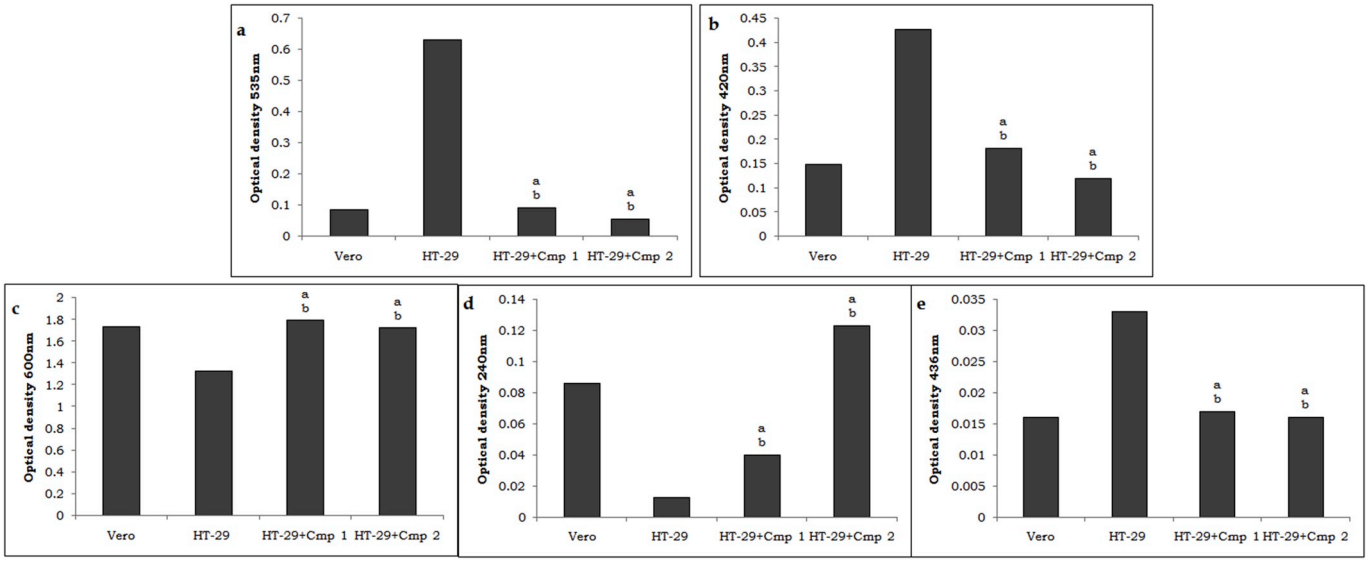


**Fig. 7.** Antioxidant potential of the compounds on HT-29 colon cancer cells. a) Lipid peroxidation b) reduced glutathione c) superoxide dismutase d) Catalase e) Peroxidase enzyme. Each value is mean ± SEM of five different observations representing cancer control and treated cells. '*' represents statistical significance between control versus compound-treated groups at $p < 0.05$.

HT-29 cells and non-toxic to normal cells.

Defective apoptosis is the hall mark of all malignant transformations (Hanahan and Weinberg, 2000). Profound changes such as membrane blebbing, cell shrinkage, chromosome condensation and DNA fragmentation on tdose-dependent response in HT-29 cells on AO/EtBr staining confirms that on exposure to the compounds 1 and 2, the cells have undergone apoptosis. Mitochondrion, the junction of all major intrinsic and extrinsic pathways, plays a pivotal role in controlling apoptosis. Increase in the outer membrane permeability favours the release of certain apoptogenic factors into the cytosol, which enhances the mitochondrial pathway of apoptosis that activates several apoptotic proteins like p53 (Kroemer and Reed, 2000); (Rottenberg and Wu, 1998); (Green and Kroemer, 2004). The promotion of apoptosis in p53 mutated cell lines using specific and multi-drugs is of present interest (Russo et al., 2016a and b). Flow cytometry data depicts that the triterpenoids decreased the mitochondrial membrane potential to a considerable extent leading to increase in the outer membrane permeability.

An increase in the level of p53 stimulates the transcription of p21 protein, which interacts with Cyclin Dependent Kinase-2 (CDK2) and inhibits cell division. Also, p53 is involved in intrinsic pathway of apoptosis in which the protein inhibits the expression of anti-apoptotic proteins (Bcl2 and Bcl-xL) but activate the expression of Bax, Bak and Fas to promote apoptosis (Zamzami et al., 1995). The expression of p53 mRNA was profoundly enhanced in presence of triterpenoids in HT-29 cells, indicating their cancer preventive efficacy.

Earlier studies of Zhu et al. (2007) have described the importance of extracellular receptor kinases (ERK-2/MAPK-1) in the progression and

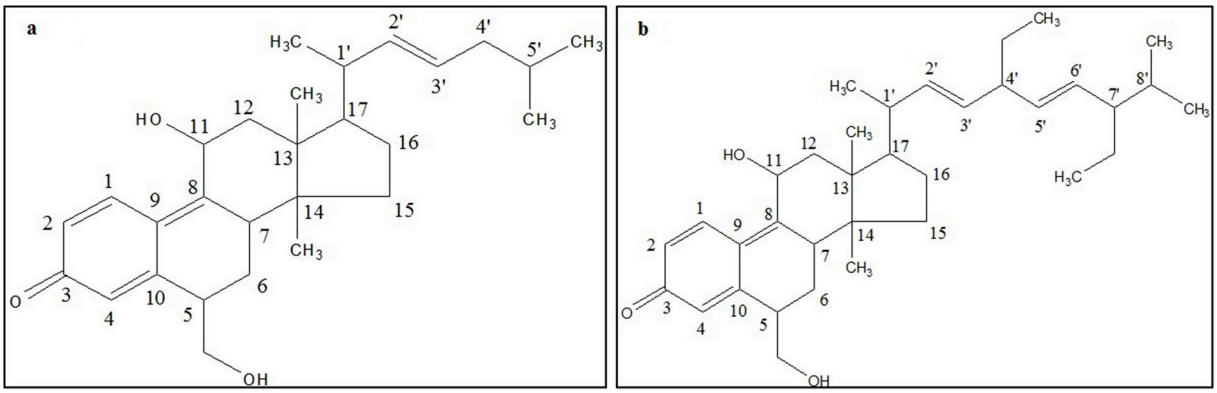


**Fig. 8.** Structure of triterpenoids isolated from the ethyl acetate extract of stem bark of *C. fistula.* a.Compound-1:17-[(E)-1′,5′-dimethyl-2′-hexenyl]-11-hydroxy-5-(hydroxymethyl)-13,14-dimethyl-5,6,7,11, 12, 13, 14, 15, 16,17-decahydro-3H-cyclo penta[*a*]phenanthren-3-one (Patent Application No.2880/CHE/2012). b. Compound-2: 17-[(2E,5E)-4′,7′-diethyl-1′,8′-dimethyl-2′,5′-nonadienyl]-11-hydroxy-5-(hydroxymethyl)-13,14-dimethyl 5,6,7,11,12,13,14,15,16,17-decahydro-3H-cyclopenta[*a*]phenanthren-3-one(Patent Application No.2881/CHE/2012).

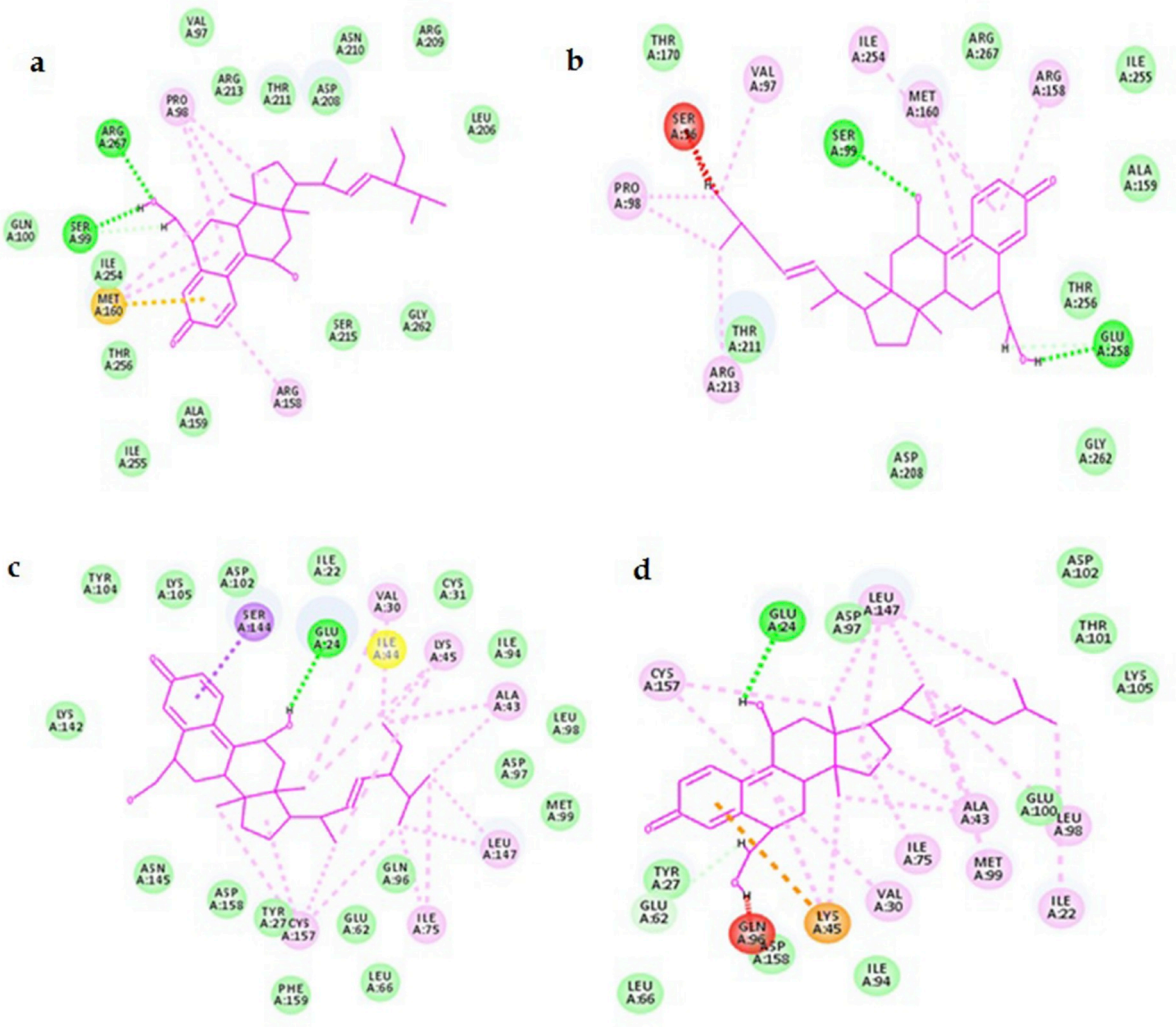


**Fig. 9.** *In-silico* interaction of cancer targets with triterpenoids a) p53-compound 1 b) p53-compound 2 c) ERK2-compound 1 d) ERK2-compound 2.

invasion of colon cancer. Activated ERKs can phosphorylate downstream kinases and induce transcription of NF-κB, which in turn activate IKK, a positive regulator of cell cycle. ERK2 also induces the expression of genes concerned with cell cycle progression such as CDKs, cyclins, growth factors, anti-apoptotic proteins and cytokines (Chang et al., 2003). Down-regulation of ERK2 genes by the triterpenoids implies that the compounds could alter the progression and invasion of cancer cells which is also substantiated through wound healing assay.

The decision to divide occurs as the cell progress through the $G_1$ phase of cell cycle check points (Pardee, 1989). Both the compounds 1

**Table 3**
*In-silico* interaction analysis report.

| Compound | Protein | No. of. Poses | Absolute Energy | Libdock Score | H-Bond | Bond Length | Residues | Interacting atom |
|---|---|---|---|---|---|---|---|---|
| Compound 1 | ERK 2 | 99 | 62.45 | 122.614 | 1 | 2.3272 | GLU24 | H52 |
| | P53 | 17 | 53.342 | 100.343 | 2 | 1.9565 | SER99 | H43 |
| | | | | | | 1.9161 | ARG267 | O13 |
| Compound 2 | ERK2 | 97 | 52.861 | 122.472 | 1 | 2.3987 | GLU24 | H45 |
| | P53 | 96 | 68.99 | 94.97 | 2 | 1.9079 | SER99 | O18 |
| | | | | | | 2.0481 | GLU258 | H41 |

and 2 effectively blocked the S phase and $G_2$/M transition in HT-29 cells respectively which may be from the inhibition exerted on CDKs (Whittaker et al., 2004). The probable reason behind the cell cycle arrest might be the downregulation of ERK2 genes on treatment with the compounds.

Also the results of AO/EtBr staining and MTT cytotoxic studies of compounds 1 and 2 showed effectiveness on cancer cells and were less toxic to normal cells, which supports the activation of mitochondrial apoptotic pathway and exhibit promising anticancer potential.

The free radicals generated on destruction of cancer cells were neutralized by the compounds as it is confirmed with the enhanced enzymatic activities of CAT and lipid peroxidation and lowering GSH levels. Damage induced by the accumulation of toxic hydrogen peroxides is nullified by CAT, the action of which was enhanced upon treatment with the compounds. Peroxidation of lipids such as polyunsaturated fatty acids (PUFA), glycolipids and cholesterol affect the integrity of the plasma membrane. p53 mutated cancer cells have also been reported to regulate oxidative stress response on treatment with drugs (Russo et al., 2017). Higher rates of lipid peroxidation were observed in cancer cells supplemented with compounds that may lead to cell damage and induce cell death.

For any drug to be efficient, targeting the root cause is essential and substantiated by *in-silico* through molecular docking (Daisy et al., 2011; Patel, 2013). A stable interaction with high libdock score observed between the triterpenoid compounds and the target proteins (ERK2, p53) substantiate the *in-vitro* results. The interaction was between the active site residues of the proteins with the ligands by hydrogen bond interactions. The bond length was found to be less than 3, indicating the stability of the interaction (Daisy et al., 2012). Thus, the results show that both the compounds (1 and 2) are efficient as anti-cancer agents against colon cancer and could be brought out as an effective cancer drug with further clinical explorations.

## 5. Conclusion

The present study demonstrates that the triterpenoid compounds 1 and 2 derived from *C. fistula* L. exhibit good anti-proliferative and cytotoxic actions in HT-29 cells *in-vitro*. Both the compounds demonstrated very high anticancer potential at low doses. Cytotoxic studies on normal VERO cells indicate that the compounds were selective to cancer cells and are less toxic to normal cells. Compounds-induced apoptosis was p53-dependent. In addition, they also induced morphological changes such as fragmentation of DNA and down-regulation of ERK2 thereby would reduce metastasis of cancer cells and also effectively inhibited cell cycle. The elimination of free radicals by activating catalase was also evident on destruction of cancer cells. Also, docking studies proved stable interaction between the compounds and p53 & ERK2. Thus drugs targeting the molecular mechanisms as of depleting over expressed cyclins and CDKs and inducing p53 expression are explored as potential anti-cancer therapeutic agents. These results suggest that both compounds 1 and 2 offer potential for treating colon cancer and hence can be used as alternative drug candidates, with further exploration on *in-vivo* aspects.

## Conflicts of interest

The authors declare that they have no conflict of interest.

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