

# ***In vitro* Investigation of Anticancer, Cell-Cycle-Inhibitory, and Apoptosis-Inducing Effects of Diversin, a Natural Prenylated Coumarin, on Bladder Carcinoma Cells**

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Chemotherapy is one of the main strategies for reducing the rate of cancer progression or, in some cases, curing the tumour. Since a great number of chemotherapeutic agents are cytotoxic compounds, *i. e.* similarly affect normal and neoplastic cells, application of antitumour drugs is preferred in cancer management and therapy. In this study, the cytotoxicity of diversin was evaluated in 5637 cells, a transitional cell carcinoma (TCC) subline (bladder carcinoma), and normal human fibroblast cells using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Chromatin condensation and DNA damage induced by diversin were also determined by means of 4',6-diamidino-2-phenylindole (DAPI) staining and the comet assay, respectively. In addition, the mechanism of action of diversin was studied in more detail by the caspase 3 colourimetric assay and flow cytometry-based cell-cycle analyses (PI staining). Our results revealed that diversin has considerable cytotoxic effects in 5637 cells, but not on HFF3 (human foreskin fibroblast) and HDF1 (human dermal fibroblast) cells. Further studies showed that diversin exerts its cytotoxicity via induction of chromatin condensation, DNA damage, and activation of caspase 3 in 5637 cells. In addition, flow cytometric analyses revealed that 5637 cells are mostly arrested at the G2 phase of the cell cycle in the presence of diversin.

**Key words:** Diversin, Anticancer, Apoptosis

## **Introduction**

Cancer, as a major cause of death worldwide, has been considered a challenging clinical issue for many years. Among several strategies, cancer chemoprevention and therapy is one of the most effective and convenient methods in cancer management. In recent years, a vast number of synthetic chemicals and biological compounds have been studied for their cytotoxic and anticancer effects *in vitro*.

Bladder cancer, which is the most prevalent cancer of the genitourinary system (Vikram *et al.*, 2009), is the 4<sup>th</sup> and 7<sup>th</sup> most common cancer in men and women, respectively. It can be grouped into different

types based on the nature of the initiating cells, the carcinogenesis process, or the extent of the tissues engaged in tumour formation, among which the transitional cell carcinoma (TCC) represents the most common form of these abnormalities.

Apoptosis and necrosis are two distinct forms of cell death in mammals. Unlike necrosis, which can invade large populations of cells, apoptosis normally triggers the death process only in a single cell. Furthermore, necrosis is an accidental cell death that occurs as a result of severe physical or chemical changes (Kerr *et al.*, 1972, 1994; Leist and Jäätelä, 2001; Saraste and Pulkki, 2000), while apoptosis not only occurs during natural fetal development (Fadeel *et al.*, 1999;

Lockshin and Zakeri, 2001; Lowe and Lin, 2000), but can be also induced by various stimulators, including drug treatment and/or other stress conditions (Ghobrial *et al.*, 2005). Since misregulation of apoptosis (Ghobrial *et al.*, 2005), including both excessive or reduced cell death, normally leads to various human diseases such as cancer (Landowski *et al.*, 1997; Reed, 2002), apoptosis-based therapies are considered new biological approaches for the treatment of such abnormalities.

Approximately three out of four anticancer drugs are obtained from natural products (Newman and Cragg, 2007). In terms of chemical properties, they fall into different chemical classes, including sesqui- and diterpenes, coumarins, and alkaloids. For instance, the genus *Ferula* is a rich source of secondary metabolites including coumarin and sesquiterpene derivatives. Various *Ferula* species are widely consumed as food in Iranian folk medicine (Barthomeuf *et al.*, 2008). We showed previously that diversin, a terpenyloxy coumarin from *Ferula diversivittata*, has promising chemopreventive effects as determined by two standard tests including the *in vitro* Epstein-Barr virus early antigen (EBV-EA) inhibition and *in vivo* two-stage mouse skin carcinogenesis assays (Iranshahi *et al.*, 2010).

The aim of the present study was to investigate the cytotoxicity and anticancer properties of diversin in cancerous 5637 and normal – HFF3 (human foreskin fibroblast) and HDF1 (human dermal fibroblast) – cell lines using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. A more detailed mechanistic description of diversin action was achieved by 4',6-diamidino-2-phenylindole (DAPI) staining, the alkaline comet method, and the assay of caspase 3 activity. Moreover, the 5637 cell-cycle distribution pattern, after diversin treatment, was also investigated through flow cytometric analyses of PI-stained cells.

## Materials and Methods

### Cell culture

5637 cells were purchased from the Pasteur Institute (Tehran, Iran), and HDF1 (human dermal fibroblast) and HFF3 (human foreskin fibroblast) cells were generously provided by the Royan Institute, (Tehran, Iran).

Low- and high-glucose Dulbecco's modified Eagle's media (DMEM; Gibco, Paisley, UK), supple-

mented with 10–15% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco), were used for cancerous and normal human cell types, respectively. Cells were grown at 37 °C in a humidified atmosphere of 5% (for 5637 cells) and 10% (for normal cell lines) CO<sub>2</sub> in air. The medium was changed every 24–48 h, and all cell lines were subcultured using 0.25% trypsin/EDTA (1 mM) (Gibco) when required.

### Extraction of diversin

Diversin [7-(3',7'-dimethyl-5'-oxoocta-3',6'-dienyl-oxy)coumarin, C<sub>19</sub>H<sub>20</sub>O<sub>4</sub>, *M<sub>r</sub>* 312; Fig. 1] was isolated from *Ferula diversivittata* as previously reported (Iranshahi *et al.*, 2010). The structure of the compound was confirmed by 1D and 2D NMR analyses (Iranshahi *et al.*, 2008), and its purity was checked by thin-layer chromatography (TLC) (Iranshahi *et al.*, 2010) and high-performance liquid chromatography (HPLC).

### MTT cell viability assay

To measure antiproliferative effects of diversin, the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma Aldrich, Munich, Germany) assay (Mosmann, 1983) was used. Briefly, after seeding the cells at a density of 8000–10,000 cells/well on a 96-well plate, various concentrations of diversin and their equivalent dimethylsulfoxide (DMSO) controls were used in triplicate for the treatment of 5637, HFF3, and HDF1 cells. After 24, 48, and 72 h of incubation, 20 μL MTT solution were added per well, and plates were kept at 37 °C for another 4 h. After replacing the contents of each well with 150 μL DMSO, the optical densities (OD) were recorded by an ELISA reader (Awareness, Palm City, FL, USA) at 495 nm. The IC<sub>50</sub> values for each time point were calculated as follows:

$$\text{half maximal inhibitory concentration (\%)} = \frac{\text{mean OD (treated cells)}}{\text{mean OD (control cells)}} \cdot 100.$$

### Morphological observations

Morphological alterations, including membrane integrity and cytoplasmic granulation, were examined under an inverted microscope (Olympus, Tokyo,

Japan). Furthermore, to determine the ability of diversin to induce apoptosis, nuclear changes were evaluated by 4',6-diamidino-2-phenylindole (DAPI) (Merck, Darmstadt, Germany) staining after 72 h in untreated 5637 cells, cells treated with 0.4% DMSO (v/v), and cells treated with 40  $\mu\text{g/mL}$  diversin. Briefly, cells were harvested and centrifuged at  $1100 \times g$  for 10 min. The cell pellets were then fixed in 4% (v/v) paraformaldehyde (Sigma Aldrich), washed with phosphate-buffered saline (PBS), and simultaneously permeabilized and stained with Triton X-100 (0.1%, v/v) (Merck) and DAPI (2  $\mu\text{g/mL}$ ). Finally, the stained cells were monitored with a fluorescent microscope (Olympus), and normal and damaged nuclei were counted.

#### Alkaline comet assay

To determine the level of DNA damage induced by diversin, single cell gel electrophoresis (comet assay) was employed. In brief, single cell suspensions were prepared by mixing the cells with PBS (1X) and low-melting point agarose (LMA) (1.5%, w/v; Fermentas, St. Leon-Rot, Germany). The suspensions were placed on microscopic slides, coated with normal-melting point agarose (0.1%, w/v) (Helicon, Moscow, Russia), and placed on ice for agarose solidification. The slides were then covered with another layer of LMA, kept in ice-cold lysis buffer (2.5 M NaCl, 100 mM  $\text{Na}_2\text{EDTA}$ , 10 mM Tris, 2% Triton X-100, pH 10) for 4 h, and incubated at 4 °C in electrophoresis buffer for 30 min. Finally, electrophoresis was carried out at 4 °C for 20 min (25 V, 300 mA). The slides were then washed with deionized water, neutralizing buffer (0.4 M Tris, pH 7.5), and ethanol, dried at room temperature, and stained with ethidium bromide (2  $\mu\text{g/mL}$ ; CinnaGen, Tehran, Iran). Using a fluorescent microscope (Olympus), photographs were taken from at least 300 cells in each group for further analyses.

#### Caspase 3 activity assay

The same treatment schedule was applied to investigate the ability of diversin to induce apoptosis via caspase 3 activation, by the use of a caspase 3 colourimetric assay kit (ab 39401; Abcam, Cambridge, UK). Cisplatin (15  $\mu\text{g/mL}$ ) was included in the experiment as a control. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. Samples containing 200  $\mu\text{g}$

protein were used for the evaluation of caspase 3 activity following the manufacturer's protocol.

#### Flow cytometric analyses of cell-cycle distribution

For cell-cycle analysis, 5637 cells treated with 35  $\mu\text{g/mL}$  diversin for 24 h were used. Adherent and detached cells (Morelli *et al.*, 2005) were washed, resuspended in cold PBS containing Triton X-100 (0.1%, v/v), sodium citrate (0.1%, v/v), RNase (100  $\mu\text{g/mL}$ ; Fermentas), and propidium iodide (100  $\mu\text{g/mL}$ ; Sigma Aldrich), and kept at 4 °C for 30 min. Subsequently, flow cytometric analyses of apoptotic cell death and cell-cycle pattern alterations were performed using a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

#### Data analyses

A statistical program (SPSS, version 17.0) was used for data analysis. The Kolmogorov-Smirnov test was used to examine the normal distribution of the data. Results are expressed as means  $\pm$  SD for each treatment. For variables with normal distribution, an independent Student *t*-test and one-way ANOVA with Tukey's test were used to compare the means between two groups or among more than two treatments for each variable, respectively. The nonparametric Mann-Whitney U test and Kruskal-Wallis H test were applied for analysis of data in variables not showing normal distribution.  $p < 0.001$  was considered a significant difference. Programs used for the comet assay and flow cytometric analyses were Tri Tek Comet Score freeware TM V 1.5 and WinMDI 2.9, respectively.

## Results

#### Cytotoxic effects of diversin on cancerous and normal human cell lines

The  $\text{IC}_{50}$  values of diversin (Fig. 1) in 5637 cells were determined at 70 and 40  $\mu\text{g/mL}$ , after 48 and 72 h of treatments, respectively, as evidenced by the

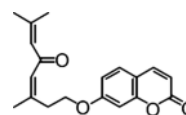


Fig. 1. The chemical structure of diversin [7-(3',7'-dimethyl-5'-oxoocta-3',6'-dienyloxy)coumarin].

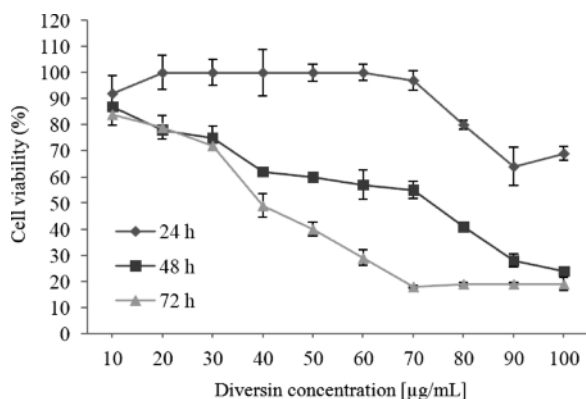


Fig. 2. Viability of 5637 cells as a function of diversin concentrations (10–100  $\mu\text{g/mL}$ ) after 24, 48, and 72 h of treatments as evidenced by the MTT assay. Data are means of three independent experiments  $\pm$  SD for each concentration.

MTT assay (Fig. 2). These results were corroborated by morphological observations showing that cells treated with 40 to 100  $\mu\text{g/mL}$  diversin for 48 to 72 h had rounded and granulated shapes, in contrast to the DMSO controls and untreated cells (Fig. 3A).

To investigate the specificity of the anticancer effects of diversin, the same concentrations were applied to two normal human cell lines, HDF1 and HFF3. The MTT assay revealed that cytotoxic effects of diversin on normal HDF1 and HFF3 cells (Figs. 4 and 5) were significantly lower than those on 5637 cells.

#### *Diversin induces chromatin condensation and DNA fragmentation in 5637 cells*

To evaluate diversin effects on the nuclei of 5637 cells, cells treated with 40  $\mu\text{g/mL}$  (128  $\mu\text{M}$ ) di-

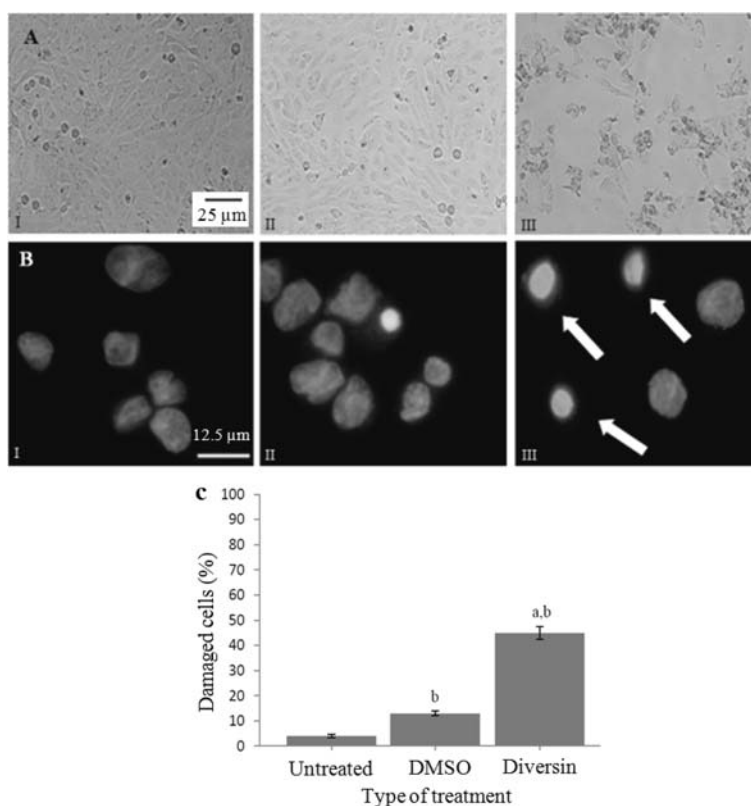


Fig. 3. (A) 5637 cell morphology of I) untreated cells, II) cells treated with 0.4% DMSO, and III) cells treated with 40  $\mu\text{g/mL}$  diversin, 72 h after treatments. (B) Fluorescent microscopic images of 5637 cells in the groups as indicated above. Damaged cells shown by arrows in B III exhibit chromatin condensation. (C) Percentages of damaged cells calculated for each treatment. (a) Significant difference ( $***p < 0.001$ ) between diversin- and DMSO-treated cells. (b) Significant difference ( $***p < 0.001$ ) in comparison with untreated cells. The results were analysed by the Kruskal-Wallis H ( $*p < 0.05$ ) and Mann-Whitney U tests.

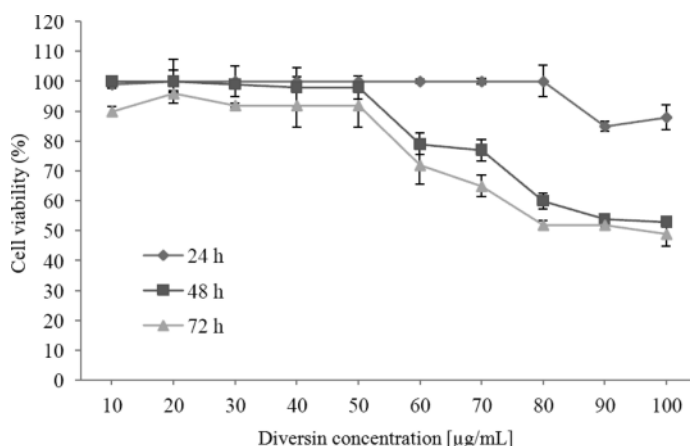


Fig. 4. Viability of HFF3 cells after 24, 48, and 72 h of treatment with diversin (10–100 µg/mL) as determined by the MTT assay. Data are means of three independent experiments  $\pm$  SD for each concentration.

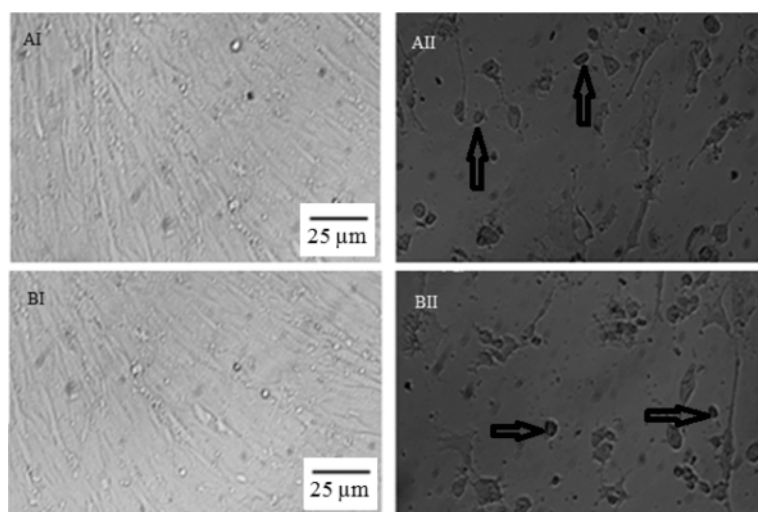


Fig. 5. Morphology of HFF3 cells after (A) 48 h and (B) 72 h of treatments with 0.9% (AI) and 0.8% (BI) of DMSO and 90 µg/mL (AII) and 80 µg/mL (BII) of diversin, respectively. Cell shrinkage and granulation of cytoplasm in diversin-treated cells are indicated by arrows. Also, as clearly observed, the cell number was decreased significantly in diversin-treated cells.

versin and an equivalent amount of DMSO were stained with DAPI. Based on the number of cells with condensed chromatin, 4, 13, and 45% of cells had apoptotic features in the untreated, DMSO-, and diversin-treated groups, respectively (Figs. 3B, C).

The comet assay revealed that the percentages of DNA migrating in a tail, as an indicator of DNA damage, were 3, 4.5, and 37 in untreated 5637 cells, in cells treated with DMSO, and in cells treated with

40 µg/mL diversin, respectively (Fig. 6G). In contrast, DNA tails were not observed in case of the normal HDF1 cells treated with the same concentration of diversin for 72 h (Fig. 6F).

#### *Diversin induces caspase 3 activity in 5637 cells*

Induction of programmed cell death by diversin was assessed by measuring the caspase 3 activ-

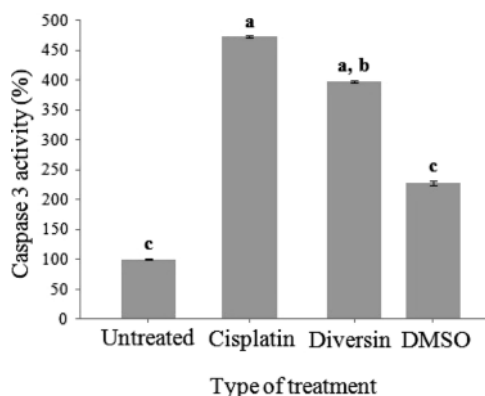
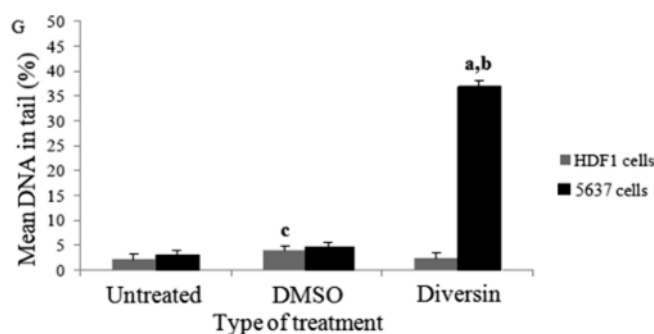
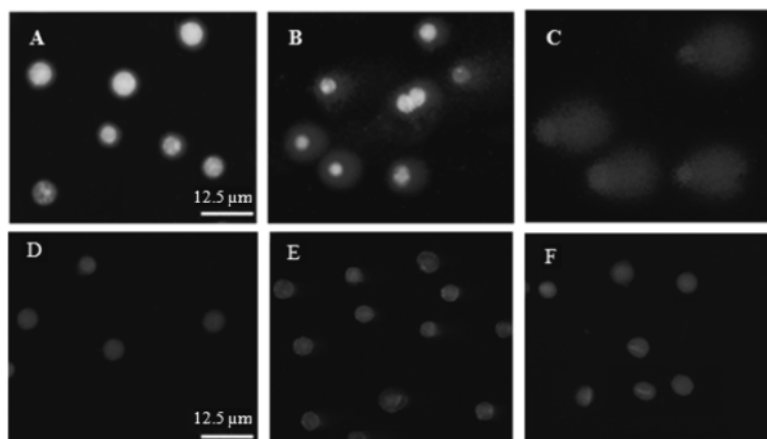


Fig. 7. Caspase 3 activity in 5637 cells. The activity of the untreated cells was considered equal to 100% and the other activities are given in relation to this value. Data are shown as means of three independent experiments  $\pm$  SD. (a) Significant ( $***p < 0.001$ ) in comparison with untreated cells. (b) Significant difference ( $***p < 0.001$ ) between diversin (40  $\mu\text{g/mL}$ )- and DMSO (0.04%) -treated cells. (c) Significant difference ( $***p < 0.001$ ) in comparison with cisplatin (15  $\mu\text{g/mL}$ )-treated cells. The results were analysed by ANOVA and Tukey's multiple comparison tests.

Fig. 6. Diversin-induced selective DNA damage visualized by the comet assay in 5637 cells. Representative cells are shown in (A, D) untreated cells, (B, E) cells treated with 0.4% DMSO, and (C, F) diversin (40  $\mu\text{g/mL}$ )-treated 5637 (upper panel) and HDF1 (lower panel) cells, respectively. (G) Percentages of DNA in the tail, reflecting DNA damage, were calculated after 72 h of treatment of 5637 and HDF1 cells. (a) Significant difference ( $***p < 0.001$ ) between diversin-treated 5637 and HDF1 cells. (b) Significant difference ( $***p < 0.001$ ) in comparison with untreated and DMSO-treated 5637 cells. (c) Significant difference ( $***p < 0.001$ ) in comparison with untreated and diversin-treated HDF1 cells. The results were analysed by the Kruskal-Wallis H ( $*p < 0.05$ ) and Mann-Whitney U tests.

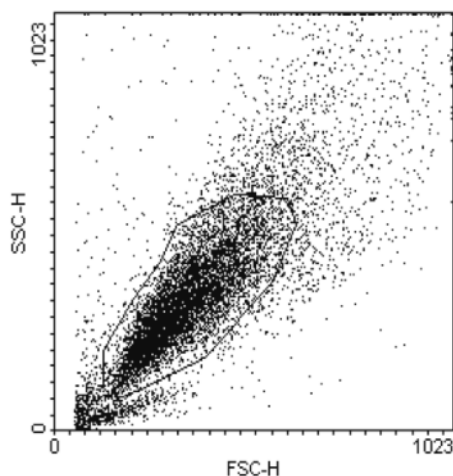
ity in untreated 5637 cells, in cells treated with DMSO (0.4%, v/v) with or without (negative control) 40  $\mu\text{g/mL}$  diversin, and cisplatin (15  $\mu\text{g/mL}$ ) (positive control), respectively. Both cisplatin and diversin increased the caspase 3 activity in 5637 cells about fourfold in comparison to the untreated cells, while DMSO alone caused a 2.5-fold increase in the caspase 3 activity (Fig. 7).

#### *Diversin treatment alters distribution of cell-cycle phases in 5637 cells*

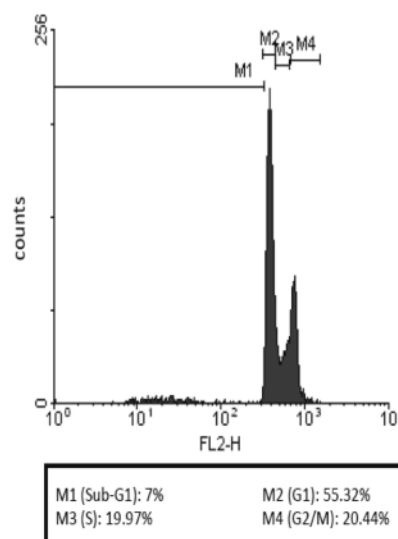
To define diversin effects on cell-cycle progression, untreated 5637 cells and cells treated with 0.35% DMSO alone or with DMSO and 35  $\mu\text{g/mL}$  diversin for 24 h were analysed by flow cytometry. The diversin-treated 5637 cells were arrested in the G2 phase of the cell cycle (Fig. 8D), while the pattern of cell-cycle phases was identical for untreated and DMSO-treated cells (Figs. 8B, C).



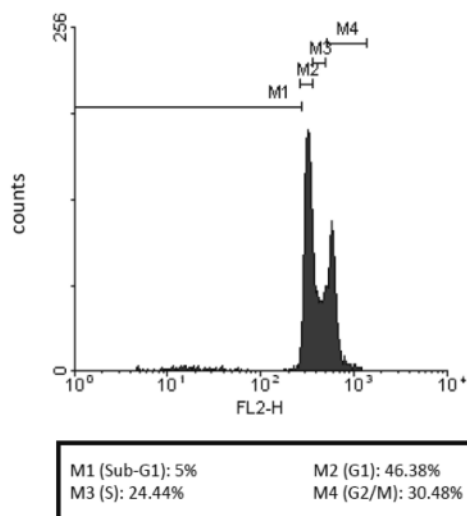
A



B



C



D

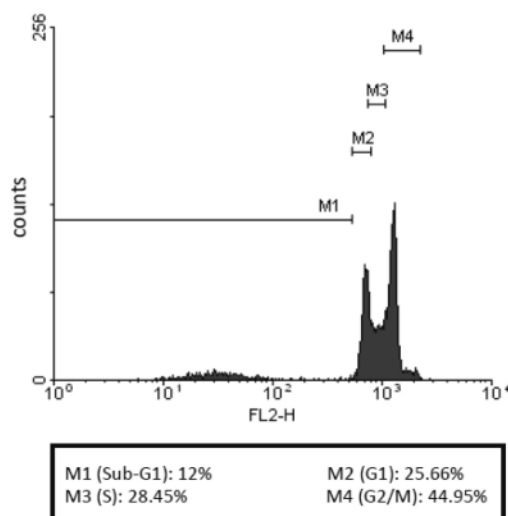


Fig. 8. Distribution of cell-cycle phases in 5637 cells, determined by propidium iodide staining and flow cytometry after 24 h of incubation. (A) DNA dot plot drawn for untreated 5637 cells. (B) Untreated cells. (C) Cells treated with 0.35% DMSO. (D) Cells treated with 35  $\mu\text{g/mL}$  diversin.

## Discussion

Coumarin derivatives are among the natural compounds that gained attention due to their numerous

and diverse biological and pharmaceutical properties (Dighe *et al.*, 2010; Fresco *et al.*, 2006; Venugopala *et al.*, 2013). Furthermore, various studies have demonstrated apoptosis-inducing effects of coumarins

in a variety of cancerous cells (Chu *et al.*, 2001; Finn *et al.*, 2003; Lopez-Gonzalez *et al.*, 2004; Madari *et al.*, 2003; Yin *et al.*, 2001).

In the present study, the cytotoxicity of diversin to cancerous and normal human cell lines was evaluated for the first time. Diversin was more toxic to 5637 cells than other simple coumarins, such as umbelliferone and herniarin (Haghighitalab *et al.*, 2014), when tested at 10 to 100  $\mu\text{g/mL}$  in the MTT assay (data not shown).

Establishing structure-activity relationships in drug screening programmes is important in the search for moieties most effective in enhancing a bioactivity. In the coumarin group, umbelliferone, a 7-OH-coumarin, is the simplest derivative of coumarin with a hydroxy group that has been shown to possess antibacterial, antifungal, cytotoxic, and antiproliferative properties, among others (Lacy and O'Kennedy, 2004). Herniarin, a methoxy derivative of coumarin, has been extracted from various plant species or was synthesized chemically (Askari *et al.*, 2009). Diversin is a 7-terpenyloxy coumarin, derived from umbelliferone, which is structurally similar to other prenyloxy coumarins, such as umbelliprenin and auraptene that have also been extracted from *Ferula* species (Iranshahi *et al.*, 2007). The positive effects of the prenyl moiety in the structure of diversin observed in our experiments, in comparison to the hydroxy and methoxy groups in umbelliferone and herniarin, respectively (Haghighitalab *et al.*, 2014), are in accordance with previous studies indicating the key role of the prenyl moiety in the enhancement of cytotoxicity and anticancer effects of various coumarins (Fraigui *et al.*, 2002; Ito *et al.*, 1999; Barthomeuf *et al.*, 2006, 2008).

Cancer chemopreventive properties of diversin were previously investigated and found to be superior to those of other terpenoid coumarins isolated from different *Ferula* species, such as auraptene, umbelliprenin, feselol, conferone, and mogoltacin (Iranshahi *et al.*, 2010). Likewise, the ability of diversin to inhibit papilloma formation was also considerably higher than that of two strong natural cancer chemopreventive agents, curcumin and quercetin, and it did not induce any local lesion or inflammation *in vivo* (Iranshahi *et al.*, 2010).

There are several reports indicating the toxicity of coumarin compounds on cancerous cells (Venugopala *et al.*, 2013). We have found here that the cytotoxic effects of diversin were more strongly pronounced in 5637 cells as compared to HDF1 and

HFF3 normal cells. These results are in agreement with previous studies indicating the selective cytotoxic effects of coumarin-based compounds on cancerous cells (Alvarez-Delgado *et al.*, 2009; Barthomeuf *et al.*, 2008; Rassouli *et al.*, 2011).

Both DAPI staining and the comet assay revealed more nuclear and DNA damage in diversin-treated 5637 cells than in the control groups and normal HDF1 cells. As seen in Fig. 6G, the mean percentage of damaged DNA is about 15-fold higher in 5637 as compared to HDF1 cells. Collectively, the results of the MTT, DAPI, and comet assays, confirmed the selective action of diversin on the cancerous 5637 cells.

The level of caspase 3 activity, one of the main effector caspases that is activated in both extrinsic (Zapata *et al.*, 2001) and intrinsic (Hockenbery *et al.*, 1990; Kroemer *et al.*, 1997) apoptotic pathways, was found to be elevated about twofold in DMSO-treated 5637 cells, which is in accordance with previous reports (Hanslick *et al.*, 2009; Liu *et al.*, 2000), while diversin provoked a fourfold increase nearly similar to that effected by cisplatin (Fig. 7).

DNA damage and induction of apoptosis are the modes of action of DNA-binding antitumour drugs including cisplatin (Eastman, 1999; Fisher, 1994; Gonzalez *et al.*, 2001). There are however, several interfering factors which may change the apoptotic pathway to necrosis (Cepeda *et al.*, 2007; Eguchi *et al.*, 1997; Leist *et al.*, 1997; Zhou *et al.*, 2002). Our observations are in agreement with those reported for caspase 3 activation by umbelliprenin and other coumarins (Barthomeuf *et al.*, 2008; Chuang *et al.*, 2007; Kim *et al.*, 2005; Willis and Adams, 2005). On the other hand, Molina-Jimenez *et al.* (2003) reported an inhibitory effect of the coumarin fraxetin on rotenone-induced apoptosis, and the investigation of the apoptotic effects of coumarin A/AA revealed that this compound did not produce caspase 3 activation after 24 h (Alvarez-Delgado *et al.*, 2009).

Arrest of the cells in the G2/M phase in response to diversin is similar to what was reported for the coumarins RKS262 and umbelliferone (Jiménez-Orozco *et al.*, 2001; Singh *et al.*, 2011), while on the other hand, umbelliprenin, a sesquiterpene coumarin, caused arrest in the G1 phase (Barthomeuf *et al.*, 2008).

In conclusion, our study revealed interesting bioactivities of the natural product diversin that may afford protection against the development and early recurrence of transitional cell carcinomas. Nevertheless, *in*



*vivo* and more *in vitro* studies are needed to better clarify the effects of diversin on biological systems.

Moreover, for enhancement of the polarity/solubility of this compound, chemically modified diversin analogues can be designed, *e. g.* by addition of hydroxy or amine groups to the coumarin nucleus or by substitution of the carbonyl by a hydroxy group. Such modifications may improve the potency of diversin as an anticancer agent for future *in vivo* or clinical studies.

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