

RESEARCH ARTICLE

Comparative Studies to Evaluate Relative *in vitro* Potency of Luteolin in Inducing Cell Cycle Arrest and Apoptosis in HaCaT and A375 Cells

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Abstract

Luteolin is a naturally occurring flavonoid present in many plants with diverse applications in pharmacology. Despite several studies elucidating its significant anti-cancer activity against various cancer cells, the mechanism of action in skin cancer is not well addressed. Hence, we investigated the effects of luteolin in HaCaT (human immortalized keratinocytes) and A375 (human melanoma) cells. The radical scavenging abilities of luteolin were determined spectrophotometrically, prior to a cytotoxic study (XTT assay). Inhibitory effects were assessed by colony formation assay. Further, the capability of luteolin to induce cell cycle arrest and apoptosis were demonstrated by flow cytometry and cellular DNA fragmentation ELISA, respectively. The results revealed that luteolin possesses considerable cytotoxicity against both HaCaT and A375 cells with IC₅₀ values of 37.1 μ M and 115.1 μ M, respectively. Luteolin also inhibited colony formation and induced apoptosis in a dose and time-dependent manner by disturbing cellular integrity as evident from morphological evaluation by Wright-Giemsa staining. Accumulation of cells in G2/M (0.83-8.14%) phase for HaCaT cells and G0/G1 (60.4-72.6%) phase for A375 cells after 24 h treatment indicated cell cycle arresting potential of this flavonoid. These data suggest that luteolin inhibits cell proliferation and promotes cell cycle arrest and apoptosis in skin cancer cells with possible involvement of programmed cell death, providing a substantial basis for it to be developed into a potent chemopreventive template for skin cancer.

Keywords: Skin cancer - luteolin - cytotoxicity - apoptosis - cell cycle

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Introduction

Skin cancer accounts for one of the major causes of deaths throughout the world (Girschik et al., 2008; Lo et al., 2011). Though Ultraviolet radiation (UVR) derived from sun exposure is a well-known important cause (Fabbrocini et al., 2010), other factors like gene polymorphisms, family history, multiple moles, sun sensitivity, immune suppression, alcohol consumption (Hsan et al., 2010), also contribute to the development of skin cancer. The three common types of skin cancer are basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma. Metastatic melanoma is the most deadly form of skin cancer, developed by the proliferation of transformed melanocytes from the basal region of epidermis. A recent report has showed that the incidence rate is increasing exponentially than other malignant diseases in United States. Approximately 123,590 new cases of melanoma is diagnosed in United States each year and 8790 people were expected to die which accounts

for 65% of all skin cancer deaths (Brady et al., 2011). However, in India, the incidence of skin malignancies is low, constituting about 1-2% of all the diagnosed cancers (Adinarayan et al., 2011). Unfortunately, despite of this higher incidence rates, efficient/safer treatment still remains critical.

The progression of cancer can be arrested by the use of chemotherapeutic agents, derived from herbal sources (Kuo et al., 2010). The cornerstone of chemoprevention has shown to prevent/halt a wide variety of cancers including skin cancers in multiple animal models by apoptotic induction (Hong and Sporn, 1997; Deorukhkar et al., 2007). At the cellular level, apoptosis is mediated by many factors of which, a dysfunction of mitochondrial membrane integrity can result in apoptotic cell death (Green and Reed, 1998). Recent literatures demonstrated that the chemotherapeutic agents are particularly be more toxic to the cancer cells than normal cells and can provoke various biological processes such as cell cycle arrest, DNA repair and apoptosis (Pelicano et al., 2004; Kang et

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al., 2010). Hence, this method of targeting cancer might provide an insight for developing much safer anticancer drugs from ethnopharmacological origin. Flavonoids are such a group of polyphenolic compounds which are present in many plants, displaying a wide range of pharmacological properties, including anti-carcinogenic and anti-inflammatory activity (Middleton et al., 2000; Chahar et al., 2011). They were known for their potentials to suppress tumors by cell cycle arrest and induction of apoptosis (Kobayashi et al., 2002).

Luteolin (3', 4', 5, 7-tetrahydroxyflavone), a common flavonoid present in many dietary sources (Ross and Kasum, 2002), and has been reported to have various pharmacological relevance including radical scavenging (Xu et al., 2009), anticancer and anti-inflammatory activity (Shi et al., 2004; Seelinger et al., 2008; Wang et al 2012). However, its activity against skin cancer cells remains inconclusive. Being less toxic in nature (Veda et al., 2002) with significant anticancer efficacies in various cancer cells, luteolin potentially has gained in importance and drawn our attention for developing safer chemotherapeutic drug from natural resources. Our focus is to evaluate its relative ability to induce apoptosis in HaCaT and A375 cells— a phenomenon universally believed to be the common downstream effector process in chemopreventive drug development irrespective of the intermediate mechanistic phenomena. Hence, the present study has investigated *in vitro* anti-proliferation, cell cycle arrest and apoptotic potentials of luteolin in HaCaT (immortalized keratinocytes) and A375 (Human melanoma) cells due to a close and important functional association between keratinocytes and melanocytes (Chung et al., 2011).

Materials and Methods

Chemicals and reagents

Ascorbic acid, phenazine methosulphate (also known as N-methylphenazonium methosulfate), Dulbecco's Modified Eagle Medium with (4.5 g/l of glucose and L-glutamine), Nutrient mixture F-12 Ham (with L-glutamine), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dulbecco's phosphate buffered saline (PBS) (Ca^{2+} / Mg^{2+} free), Giemsa stain were purchased from Himedia Laboratories Pvt. Ltd. (India). XTT {2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Apoptotic kit, Cellular DNA fragmentation ELISA (# 11 585 045 001) -Roche Diagnostics, Germany. The remaining chemicals and solvents used were of standard analytical grade unless otherwise mentioned.

Drug preparation

Stock solutions of luteolin were prepared at 6.84 mM in 100% dimethyl sulfoxide (DMSO). Final concentrations of DMSO, prepared by serial dilution, never exceeded 1% (v/v). Freshly prepared solutions were sterilized using 0.22 μm syringe filter and used for each experiment.

In vitro radical scavenging properties

DPPH• assay: The ability of luteolin to scavenge

free radicals were measured by DPPH• assay as described previously (Shimada et al., 1992) with few modifications. 900 μl of 0.1mM DPPH radical solution was prepared in ethanol, and then mixed with 100 μl of various concentrations (5-80 μM) of the luteolin dissolved in DMSO and kept in the dark for 30 min. Absorbance was recorded at 517 nm using a Cary 50 UV-Vis spectrophotometer (Varian, Inc., CA, USA). Ascorbic acid (expressed in μg) was used as a standard. The reduction in the absorbance of the DPPH• solution indicated the free radical scavenging activities of luteolin. DMSO without the sample was employed as a control. The level of percentage scavenging of DPPH• by luteolin was calculated according to the following formula: %DPPH• Scavenging=[Abs (Control)-Abs (Sample)]/Abs (Control) x100.

FRAP assay: Ferric reducing antioxidant potentials of luteolin were assessed by the protocol described previously (Benzie and Strain, 1996) with some modifications. The stock solutions prepared were 300 mM acetate buffer (3.1 g $\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$ and 16.8 ml $\text{C}_2\text{H}_4\text{O}_2$; pH 3.6), TPTZ solution (10 mM TPTZ in 40 mM HCl) and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution. Working FRAP solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml TPTZ solution and 2.5 ml of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution. The mixture was then warmed at 37°C. 150 μl of different concentrations of luteolin were allowed to mix with 2.85 ml of FRAP solution. The mixture was further incubated in the dark for 30 min. Absorbance was read at 593 nm. Percentage Fe^{3+} reduction (to Fe^{2+}) were calculated by a FeSO_4 standard calibration curve. Percentage scavenging was also evaluated with respect to ascorbic acid equivalence (AAE) in μg .

Cell lines and cell culture

HaCaT (human immortalized keratinocyte) and A375 (human melanoma) cells were obtained from the National Centre for Cell Science (NCCS, Pune, India). HaCaT cultures were initiated and propagated in DMEM with Nutrient mixture F-12 Ham in 1:1 ratio respectively. A375 cell cultures were initiated and propagated in DMEM. All the cells were supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2 at 37°C. Cells were grown in polystyrene-coated T25 (25 cm^2) cell culture flasks, and were harvested in the logarithmic phase of growth. The cells were maintained at the above-mentioned culture conditions throughout and confluent cells were used between the second and the sixth passages for all the experiments.

Determination of cell viability

XTT assay was carried out on HaCaT and A375 cell lines as described previously (Weislow et al., 1989) with minor modifications. 1×10^4 cells were seeded in each well of a 96-well plate and the cells were added with 200 μl of the respective culture medium and incubated at 37°C for a period of 24 h. The medium was then replaced with 200 μl of fresh media containing desired concentrations of luteolin. The plate was then re-incubated maintaining the same conditions for 24 h, after which, medium containing drug was substituted by 200 ml of fresh medium. 50 μl of

XTT reagent prepared in medium (0.6 mg/ml) containing 25 μ M of PMS was then added to all the wells and the plate was incubated in dark at 37°C for 4 h. After incubation, the orange coloured complex formed was read at 450 nm using a Dynex Opsys MRTM Microplate Reader (Dynex Technologies, VA, USA) with a 630 nm reference filter. Control wells with cells which are devoid of luteolin were kept and wells containing only culture medium and XTT reagent served as the blank. Percentage cytotoxicity was calculated by using the formula: %Cytotoxicity=[(OD of control cells-OD of treated cells)X100]/OD of control cells

Colony formation assay

Colony formation assay was carried out as described earlier (Dua and Gude, 2006) with minor modifications. Briefly, 6 well plates was seeded with 500 viable cells and allowed to grow for 24 h. The cells were then treated with different concentration of luteolin for 24 h. The cells were then washed with PBS and replaced by fresh medium. Plates were kept for a period of 8-10 days for incubation in CO₂ incubator and then fixed with methanol and later stained using 0.1% crystal violet solution. Colonies of 50 or more cells were counted from three independent experiments carried out. Colony forming efficiency (CFE) was calculated using the formula: %of colonies=[Number of colonies in treated/Number of colonies in control(x100)]

Cell cycle analysis

The ability of luteolin to arrest cells in different phases was assessed by flow cytometry. HaCaT and A375 cells were treated with DMSO (control cells) / luteolin treated (with IC₅₀ values obtained from XTT assay) for 24 h and cells were harvested by centrifugation at 1000 rpm for 5 min. The cells were washed with PBS and later fixed in methanol with incubation at 4°C for 2 h. Wash the cells after centrifugation with PBS and resuspend the cells in 1 ml of PBS. The cells were then exposed to 1 μ l of RNase (25 mg/ml) taken in a FACS tube with 5 min of incubation. The cells were further stained with propidium iodide (PI) (1 mg/ml) for 30 min. Immediately after that, the cells were analyzed using FACS (Becton Dickinson, San Jose, CA, USA).

Morphological studies

HaCaT and A375 cells were allowed to grow 70% confluence and treated with different concentrations of luteolin for a period of 24 h. The cells were then washed in PBS and kept in PBS/Methanol (1:1) for 2 min. Cell fixation was done after adding methanol for 10 min. After removing the methanol, the cells were stained with Wright-Giemsa for 2 min and observed under inverted phase contrast microscope at 20 X magnification after removing the excess stain.

Apoptosis detection

The ability of luteolin to induce apoptosis was analysed by cellular DNA fragmentation ELISA kit as per the supplier's instructions. Briefly, HaCaT and A375 cells were labelled with 10 μ M BrdU at 1x10⁵ cells/ml density. 100 μ l of these BrdU-labelled cells in culture medium

were treated with varying concentrations of luteolin for a period of 4 h. The cells were then lysed and the apoptotic fragments were obtained after centrifugation at 1500 rpm for 10 min and subjected for ELISA procedure. 100 μ l of this obtained sample was transferred to anti-DNA coated 96-well, flat-bottom microplates (MTPs). The plates were incubated for 90 min at 15-25°C. The DNA was then denatured by microwave irradiation (500 W for 5 min) followed by addition of 100 μ l anti-BrdU-POD conjugate solution. The plates were further incubated for 90 min and were washed 3 times with wash buffer (1X). 100 μ l substrate (TMB) solution was then added for colour development. The absorbance was read at 450 nm after addition of 25 μ l of stop solution.

Statistical data analysis

All the analytical experiments were carried out in triplicates. Data were presented as mean \pm standard deviation (SD). Statistical analyses were performed by one-way ANOVA. MATLAB ver. 7.0 (Natick, MA, USA), GraphPad Prism 5.0 (San Diego, CA, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations. Significant differences between groups were determined at p<0.05.

Results

Radical scavenging potential

DPPH• is a free radical which accepts electron or hydrogen radical to become a stable molecule (Soares et al., 1997). The radical scavenging potentials of luteolin were assessed spectrophotometrically at 517 nm. Figure 1 showed the radical scavenging response of luteolin. A concurrent increase in scavenging was observed with an increasing concentration of luteolin. The highest radical scavenging effect (43.34%) was observed at 80 μ M which is about 5.81 μ M of ascorbic acid (standard) used in this study.

Ferric reducing potential

The ability of luteolin to reduce Fe³⁺ to Fe²⁺ in the presence of TPTZ complex is performed by FRAP assay. Figure 2 illustrates the % Fe³⁺ reduction by luteolin in comparison with ascorbic acid (standard). A mild increase in Fe³⁺ reduction was observed with increasing concentration of luteolin. However, the observed result is not statistically significant in comparison with the results

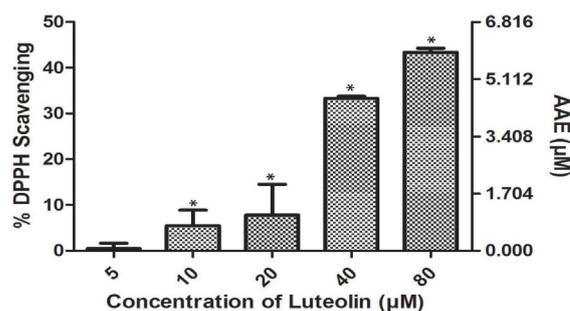


Figure 1. DPPH Radical Scavenging Activities of Luteolin Along with Ascorbic Acid Equivalence (AAE). Data expressed as mean \pm SD of n=3 samples (*P<0.05)

of the radical scavenging potentials observed in the earlier assay.

Luteolin inhibits cell proliferation

The balance between therapeutic potential and toxic effect of a compound is very important when evaluating its usefulness as a pharmacological drug (Chen and Wong, 2009). Accordingly, cells viability was assessed by XTT assay after treating the cells with various concentrations of luteolin. XTT can be metabolically reduced by mitochondrial dehydrogenase enzyme in viable cells to a water-soluble formazan product which is measured spectrophotometrically (Maioli et al., 2009). Both cells showed significant cytotoxicity with dose- dependent manner (Figure 3) among which HaCaT cells exhibited maximum toxicity with a lower IC₅₀ value of 37.17 μM in contrast to A375 cells with higher IC₅₀ value of 115.1 μM.

Luteolin inhibits colony formation efficacy

Both HaCaT and A375 cells were exposed to different concentrations of luteolin for 24 h and allowed to form colonies for 8 days after which stained with 0.1% crystal violet and counted. Luteolin significantly reduced the colony forming efficacies of both cell lines. Control cells which lack luteolin displayed maximum number of colonies. A steady decline in the number of colonies in treated wells was observed (Figure 4), attributing the efficacy of luteolin in dose-dependent manner (p<0.05). Melanoma (A375) cells exhibited higher reduction in number of colonies at similar concentration of luteolin in contrast to HaCaT cells.

Luteolin-arrested cell cycle progression

The effect of luteolin to arrest cell cycle in HaCaT

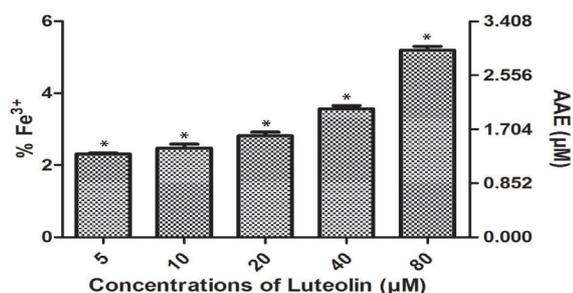


Figure 2. Percentage Fe³⁺ Reducing Potential of Luteolin Along with Ascorbic Acid. Data expressed as mean±SD of n=3 samples (*p<0.05)

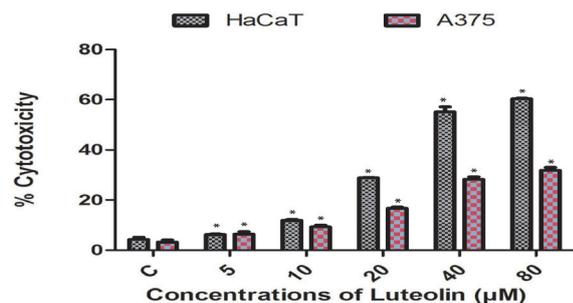


Figure 3. Dose-dependent Responses of Luteolin in HaCaT and A375 after 24 h of Treatment. Data expressed as mean±SD of n=3 samples (*p<0.05). C=DMSO control

and A375 cells was determined by FACS analysis. An accumulation of cells in G2/M (0.83-8.14%) and G0/G1 (60.40-72.56 %) phase was recorded in luteolin-treated cells after 24 h in HaCaT and A375 cells respectively, when compare to the untreated control cells (Figure 5). A significant decrease in cell density was observed in other phases in both cell lines suggested that the existence of cell cycle arrest at these phases. Among both cells, A375 cells were found to be most sensitive to luteolin when compared to HaCaT cells with identical time of drug exposure.

Morphological studies

Apoptosis or programmed cell death is a form of cell death that involves cell shrinkage, membrane blebbing, cellular disintegration, chromosome condensation, and vacuole formation (Shi and Wei, 2007). HaCaT and A375 cells were exposed to various concentrations of luteolin for a period of 24 h to study the morphological changes. Wright-Giemsa staining of both cells showed significant changes in cell morphology, loss of cell-cell contacts, membrane blebbing and vacuolization when compared to the control cells (Figure 6).

Luteolin-induced cell apoptosis

The ability of luteolin to disturb cellular integrity was further analyzed based on DNA fragments formed, which is one of the hallmarks of apoptotic cells (Kajstura et al.,

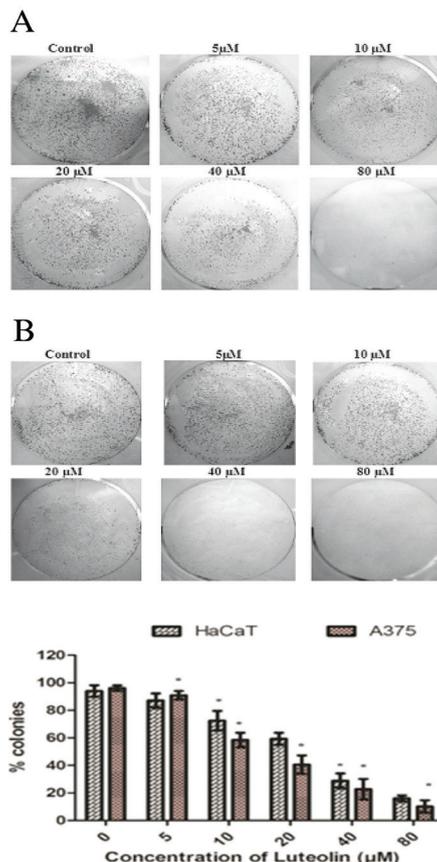


Figure 4. Luteolin Inhibits Colony Formation Efficacies of HaCaT (A) and A375 (B) Cells. Exposure of various concentrations of luteolin for 24 h showed significant reduction (*p<0.05) in percentage of colonies formed when compared to untreated control. Data expressed as mean±SD of n=3 samples

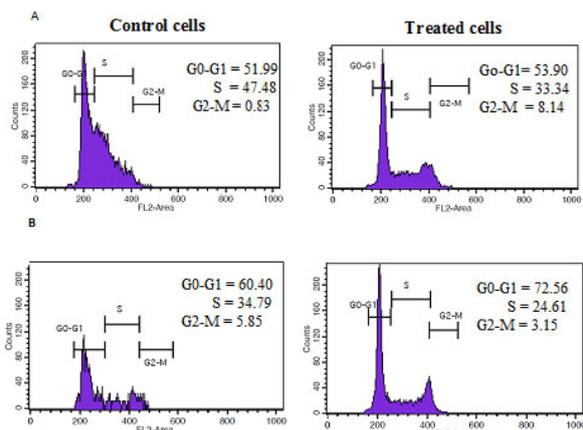


Figure 5. Luteolin Induces Cell Cycle Arrest in HaCaT (A) and A375 (B) Cells after 24 h Treatment. HaCaT and A375 cells were treated with 37.17 μM and 115.1 μM respectively to induce cell cycle arrest

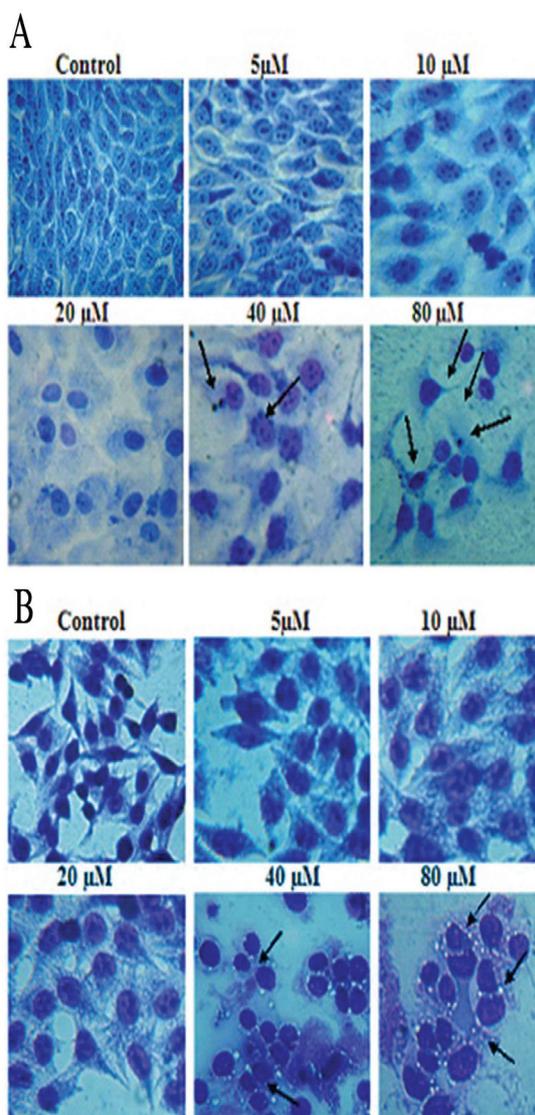


Figure 6. Morphological Changes of HaCaT Cells (A) and A375 (B) after Treatment with Luteolin, shown by Giemsa Staining. HaCaT and A375 cells showed changes in cell morphology, loss of cell-cell contacts, membrane blebbing and vacuolization (arrows) with increasing doses of luteolin when compared to control cells. Data showed are derived from three independent experiments

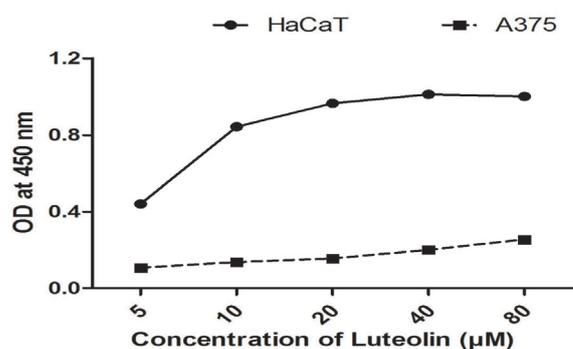


Figure 7. Apoptosis Induction in HaCaT and A375 Cells by Luteolin as Demonstrated by Cellular DNA Fragmentation ELISA with Increasing Number of DNA Fragments in Dose-dependent Manner. Data expressed as mean \pm SD of n=3

2007). Cellular DNA fragmentation ELISA kit was used as per supplier's instructions. Both HaCaT and A375 cells were subjected to different concentrations of luteolin for a period of 4 h. The result showed a dose-dependent response of luteolin with increase in BrdU labelled-DNA fragments in both cell lines which was read at 450 nm (Figure 7). However, there is a significant response to luteolin in HaCaT cells (almost double fold) with higher DNA fragmentations, when compared with A375 cells at identical concentration and time exposure.

Discussion

With the advances in the treatment and diagnostic strategies, the incidence of skin cancer still remains increasing (Garbe et al., 2011). The lack of responses to conventional chemopreventive regimens suggests a large unmet need for effective treatment technique with lesser side effects. Recently, natural metabolites have gained ample attention towards developing effective chemotherapeutic template from ethnopharmacological origin by specifically targeting programmed cell death mechanisms. Among these, plant-derived flavonoids are well known sources for their radical scavenging potentials (Shahidi, 1997). Moreover, certain types of flavonoids act as pro-oxidants and induce mitochondria-mediated apoptosis in cancer cells (Gibellini et al., 2010). Luteolin is one such common flavonoid reported to possess varied therapeutic potentials, hitherto, has not been reported against skin cancer. Hence, the present study scrutinized luteolin for its distinctive therapeutic potentials against skin cancer.

In the present study, luteolin in its antioxidant scavenging potentials, exhibited significant activity in DPPH assay with an IC_{50} value of 84.8 μM , while FRAP assay showed very moderate response ($\text{IC}_{50} > 200 \mu\text{M}$) to various concentrations of luteolin. This might be because of the differential scavenging activities of luteolin against $\text{DPPH}\cdot$ and Fe^{+3} radicals, where such differences in mechanisms of the radical-antioxidant reactions have been reported previously (George et al., 2012). The relatively lesser polarity of luteolin in comparison with its sugar derivatives, which showed more antioxidant potentials, may also be the reason for its observed non-significant

FRAP activity which was reported earlier (Ozgen et al., 2011). In addition, our antioxidant results are consistent with previous literatures (Seelinger et al., 2008).

The cytotoxic activity of chemotherapeutic compounds often coincides with the induction of apoptosis in cancer cells (Fecker et al., 2005). Hence, the cytotoxic effects of luteolin in HaCaT and A375 cells were investigated. The results indicated a strong and significant ($P < 0.05$) cytotoxicity to HaCaT cells when compared to A375 cells at identical concentrations. This might be due to the differential mechanism of luteolin towards the metastatic melanoma (Krishnamurthy and Maly, 2010), and could also be justified by the phosphorylation of signal transducer and activator of transcription (STAT) 3 and -5 in melanoma cells (Miimohammadsadesh et al., 2006). Conversely, it is also evident that luteolin has the ability to inhibit cell proliferation as reported by other investigators in many other cancer cell lines (Kang et al., 2010; Wang et al., 2010). This ability has been further confirmed by performing the colony forming assay. A dose-dependent decrease in colonies was observed as a response to luteolin treatment for 24 h, in which the proliferation efficacy of A375 cells have been greatly inhibited when compared to HaCaT cells, which justifies the ability of luteolin to halt/prevent cell proliferation.

To further clarify the mechanism of growth inhibition, its ability to arrest cell cycle has been studied. Cell cycle arrest is one of the common effects shared by many anticancer drugs. Consternation of cell cycle progression can cause severe damage to cells and may trigger apoptosis (Volland et al., 2006). The results of FACS analysis showed consistent ability of luteolin to arrest the cell cycle in the G2/M & G0/G1 phase by generating cell populations and subsequent reduction of cells in other phases. Incidentally, similar results of luteolin have been reported earlier (Chang et al., 2005), in immortalized human hepatoma cell lines which also confirmed the potentials of this flavonoid.

Induction of apoptosis in cancer cells by naturally occurring compounds has created a large impact in complementary and alternative medicine (Gu and Belury, 2005). Apoptosis is a vital phenomenon in the regulation of normal tissue turnover mechanism and it participates in the elimination of unwanted cells (Kamran and Gude, 2012). Thus, induction of apoptosis in tumor cells may be considered as a protective mechanism against development and progression of cancer (Wang et al., 2012). DNA fragmentation ELISA analysis demonstrated a dose-dependent increase in DNA fragments in cells treated with luteolin. Our results substantiate the apoptotic potentials of this flavonoid in HaCaT and A375 cells which has not been reported earlier and corroborates with the findings of others in various cell lines (Chiand et al., 2007; Kim et al., 2012). Differential level of DNA fragments observed in both cells at similar concentrations of luteolin, could possibly be due to the over expression of extracellular signal-regulated kinase (ERK)1/2 in A375 cells (miimohammadsadesh et al., 2007). Further, its higher apoptotic specificity by disturbing cellular integrity in immortalized keratinocytes was exemplified by Wright-Giemsa staining. However, the molecular events such as caspase-regulating mechanisms involved

in luteolin-mediated apoptotic cell death in HaCaT and A375 cells are yet to be analyzed.

In conclusion, our *in vitro* findings indicated that, luteolin inhibits cell proliferation and induces cell cycle arrest at G2/M and G0/G1 phases in HaCaT and A375 cells with significant apoptotic potentials. These results further added to the promising potentials of luteolin that has provided a substantial reason to develop it as an efficient and safer chemotherapeutic drug against human skin cancers than of its existing classes.

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