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Research Article

Differential Uptake of Azadirachtin by Sf9 and MCF7 Cell Lines

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ABSTRACT

Azadirachtin is the main biologically active component of Neem tree. Using MTT assay and Flow Cytometry the effect of azadirachtin on proliferation of Sf9 and MCF7 cells were evaluated. Azadirachtin inhibited Sf9 cells proliferation in concentration of 10^{-9} M, whereas affect MCF7 cells only in concentrations more than 10^{-5} M. Flow cell cytometry showed arrest of Sf9 cells but there were no changes in the cell cycle of MCF7 cell line. Using solid lipid nanoparticles to deliver azadirachtin, the combination increased the antiproliferative effect of azadirachtin on MCF7 cells by about 30%. The results of present study suggest that the differential toxicity of azadirachtin is at least partly due to difference in uptake rate, which may be improve by some kind of delivery system such as solid lipid nanoparticles. Regrading to these finding and antiproliferative effect of azadirachtin the terpenoid may also be a suitable compound in researches related to management of cancers.

Key Words: Azadirachtin, Solid Lipid Nanoparticles, MCF7 Cells, Sf9 Cells, Uptake

INTRODUCTION

Azadirachtin a tetranortriterpenoid is the main biologically active component from seed kernel of Neem tree *azadirachta indica* A.juss (Morgan *et al*, 2001).There have been many uses for different components of Neem and over 300 compounds have been isolated from this tree **(**Coventry E and Allan, E. J. 2001**)**. Neem extracts have been shown to have a range of biological activities mostly in insect pest through interaction with feeding, metamorphosis and development (Paranagama *et al*., 1993) Although most studies related to azadirachtin are in field of pest management (Linton *et al*., 1997; Raizada *et al*., 2001), different parts of this tree has long been used in both traditional and modern medicine. In India Neem, known as the ‘‘Village Pharmacy’’, (Bruneton, 1995, Norten, 2000) and in Sanskrit literature, *Neem* is known as *sarva roga nivarini*, which means the cure of all ailments (Norten, 2000).

It has been used in treatment of diabetes (Khosla *et al*, 2001), malaria and rheumatism and many other diseases (Thoh *et al*., 2011). Also there are institutions which have been involved in some investigations related to effects of neem on diseases such as AIDS and other viral infections (Norten, 2000). One of advantages of this terpenoid is that it appears to be highly specific against some cell lines while showing lower toxicity towards vertebrates including mammals. Many researchers have tried to find the mode of action of azadirachtin (Linton *et al*., 1998; Fritzsche *et al*., 1987; Salehzadeh *et al*., 2003) and some of them have found that azadirachtin has cytotoxic effect on cultured insect cell lines (Rembold and Annadurai 1993). This has been investigated on a range of cells derived from different origin and in most of these studies there have been a differential toxicity of azadirachtin on insect and mammalian cell lines. These studies have shown that azadirachtin has an antiproliferative action on insect cells which has not been seen in most mammalian derived cell lines (Salehzadeh *et al*., 2002).

Other investigations have shown that antiproliferative effect of azadirachtin on cell lines may be due to interaction of azadirachtin with some fundamental mechanisms of cell division, which is common on animal cells (Salehzadeh *et al*., 2003; Anuradha *et al*., 2007). Regarding theses findings probable difference in uptake of azadirachtin by

Insect and mammalian cells may describe this inconsistency.

A difference in the uptake of azadirachtin can be one of possibilities that causes the differences between insect and mammalian cells. So using cytotoxicity and cell cytometry assay this study was proposed to investigate the probable diferrence in uptake of azadirachtin by Sf9 and MCF7 cell lines. Also due to the sensitivity of Sf9 cell line to azadirachtin (Rembold and Annadurai 1993) the cytotoxicity of azadirachtin on MCF7 was compared with this cell line.

Also solid lipid nanoparticles was used to enhace the uptake of azadirachtin by MCF7 cells. In recent years nanoparticulate delivery systems such as solid lipid

nanoparticles have been used for drug encapsulation and drug delivery. Some advantages of this system are better bioavailability, protection of drug from degrading agent like water and also light and improved controlled drug release (Jore *et al*., 2005; Yan *et al*., 2007)

MATERIALS AND METHODS

Cell lines

Sf9 cells, derived from the ovary of *Spodoptera frugiperda* (Smith), were routinely maintained at 27 ^{o}C in 75-cm2 culture flasks in 10 ml of the insect medium TNM-FH enriched with 10 % foetal bovine serum.

MCF7 cells, derived from human breast cancer were maintained in 75-cm2 flask in 10 ml of culture medium RPMI supplemented with 10% Fetal Bovine Serum (FBS) at 37^{o}C in a humidified incubator containing 5% CO_2 / 95% air.

Chemicals

The pure azadirachtin (97%) was produced by accepted methods of extraction from the neem seeds and column chromatography (Yamasaki *et al*., 1986) in either the University of Glasgow or the University of Cambrige. Its purity was established by reverse phase HPLC and NMR spectroscopy. Propidium iodide were purchased from Sigma. Softisan® 154 (S154), or hydrogenated palm oil, was a gift from CONDEA (Witten, Germany). Lipoid S100-3 [hydrogenated soybean lecithin at 90% of phosphatidylcholine which contains the typical fatty acid composition in % to total fatty acids in Lipoid S100-3 as Palmitic acid (12-16%), Stearic acid (83-88%), Oleic acid and isomers (n.m.t. 2) and Linoleic acid (n.m.t. 1)] was a gift from Lipoid Gmbh (Ludwigshafen, Germany). Thimerosal and Sorbitol were purchased from Sigma. Oleyl alcohol, was purchased from Fluka and water was used in bidistilled quality.

Estimation of cell numbers

In the growth experiments approximately 2 $\times 10^3$ cells in 100µl of medium were placed into a 96-well plate and a solution of azadirachtin added to give the appropriate final concentrations. The cells then were allowed to grow for 3 days at the appropriate temperature and the number of cells estimated using MTT assay. After the determined period of growth, 50 µl of dye MTT (3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide) solution in phosphate-buffered saline (PH 7.2) was added to each well, and the colour allowed to develop after 45 min incubation at 37 ^{o}C due to the effect of dye. Then the dye and cells dissolved in dimethyl sulfoxide (DMSO) and the absorbance of blue formazan was estimated at 492 nm by means of an ELISA reader. The preliminary test showed a linear correlation over a range of 0.5×10^3 to 25×10^3 cells per well. All estimates of cell numbers were within these ranges.

Preparation of Azadirachtin-loaded SLN

Azadirachtin-loaded SLN was prepared using the high pressure homogenization technique (Schubert and Müller-Goymann 2005). Briefly, a mixture of S154 and Lipoid S100 at a ratio of 70:30 was grounded in a ceramic crucible. The mixture was then heated to 65 to 70°C while were stirring with a teflon coated magnet until a clear yellowish solution is obtained. This solution was the lipid matrix (LM). A solution containing 1 mL oleyl alcohol, 0.005g thimerosal, 4.75g Sorbitol and 89.25 mL bidistilled water (all w/w) with the same temperature was added to 5g lipid matrix. A preemulsion of SLN was obtained using the Ultra Turrax® (Ika, Staufen Germany) at 13000 rpm for 10 min and EmulsiFlex® -C50 (CSA10, Avestin, Ottawa, Canada) high pressure homogenizer (1000 bar and 20 cycles) at temperature of about 60°C. Azadirachtin was dissolved in DMSO and mixed with 5 mg SLN preemulsion using the Ultra Turrax® (Ika, Staufen Germany) at 13000 rpm for 10 min to obtain a mixture. This mixture was then incubated overnight at 50 to 60°C while stirring with a teflon- coated magnet at 500 rpm and then exposed to air to solidify.

Flow cytometry

Fluorescence-activated cell cytometry was performed on a Becton-Dickinson FACS cytometer. After collection, cells were washed twice in phosphate-buffered saline (PBS, pH 7.2). Cells were resuspended by trituration in 2 ml of PBS containing 10 or 20 µg propidium iodide (for staining MCF7 and Sf9 cells respectively), 0.1% saponin and 0.1U/ml RNAse and incubated for 15 min at room temperature. At least 1.5 x 10^4 cells were counted in each assay. The fraction of the total cell population presented in each cell cycle phase (G1, S, and G2/M) was obtained from DNA histograms using Cell Quest and Modfit Software. All cytometry experiments were performed on cells in log phase of growth. This was obtained by seeding cells at 0.5-1 x 10^5 cells/ml and allowing the cultures to grow for 48 h

Assessment of uptake of azadirachtin by MCF7 and Sf9 cell lines

Previous experiments had shown that azadirachtin was a potent inhibitor of insect cell replication in concentrations of as low as 10^{-9} M, whereas affected mammalian cells only at high concentrations. A difference in the uptake of azadirachtin can be one of

possibilities that causes the differences between insect and mammalian cells. Using a Becton-Dickinson FACScan cytometer the cell cytometry assay was used to investigate this difference.

Azadirachtin in concentration of 10^{-6} M was added to the flasks of two series of confluent Sf9 or MCF7 cells and incubated for 30 min (conditioned medium). Then after 30 min the conditioned medium was added to new flasks containing confluent cells. The process was repeated for 6 times. The medium from last pair of flasks changed with medium of two flasks of Sf9 cells and incubated for 20 h. To evaluate the effect of higher temperature on azadirachtin and the effect of MCF7 cells on medium also azadiarchin or medium was kept in two separate flasks for 3 h and was added to additional Sf9 flasks and incubated for 20 h.

Statistical analysis

All the data were subjected to one way analysis of variance (ANOVA) followed by Post Hoc multiple comparison and Duncan test after verification of the normal distribution of the data.

RESULTS

Figure 1 shows the effect of azadirachtin on the growth of Sf9 and MCF7 cell lines. As the figure shows despite dramatic inhibitory effect of 10^{-9} M azadirachtin on proliferation of Sf9 cells in case of MCF7 significant inhibitory effects were found only at concentrations greater than 10^{-5} M Figure 2 shows the cell cycle distribution of Sf9 cells in presence (green) and absence (blue) of azadirachtin in concentration of 10^{-5} M. As the figure shows in control (blue) most of cells (61±6%) are in G1 phase. The proportions of cells in S and G2/M, are 11 ± 6.5 and 28±3%, respectively. Adding azadirachtin has changed the cell cycle of this cells and there is an accumulation of Sf9 cells with 4NDNA and the proportion of cells in G1 is reduced to less than 10%. When the same experiment was done on MCF7 cells there was no change in cell cycle distribution.

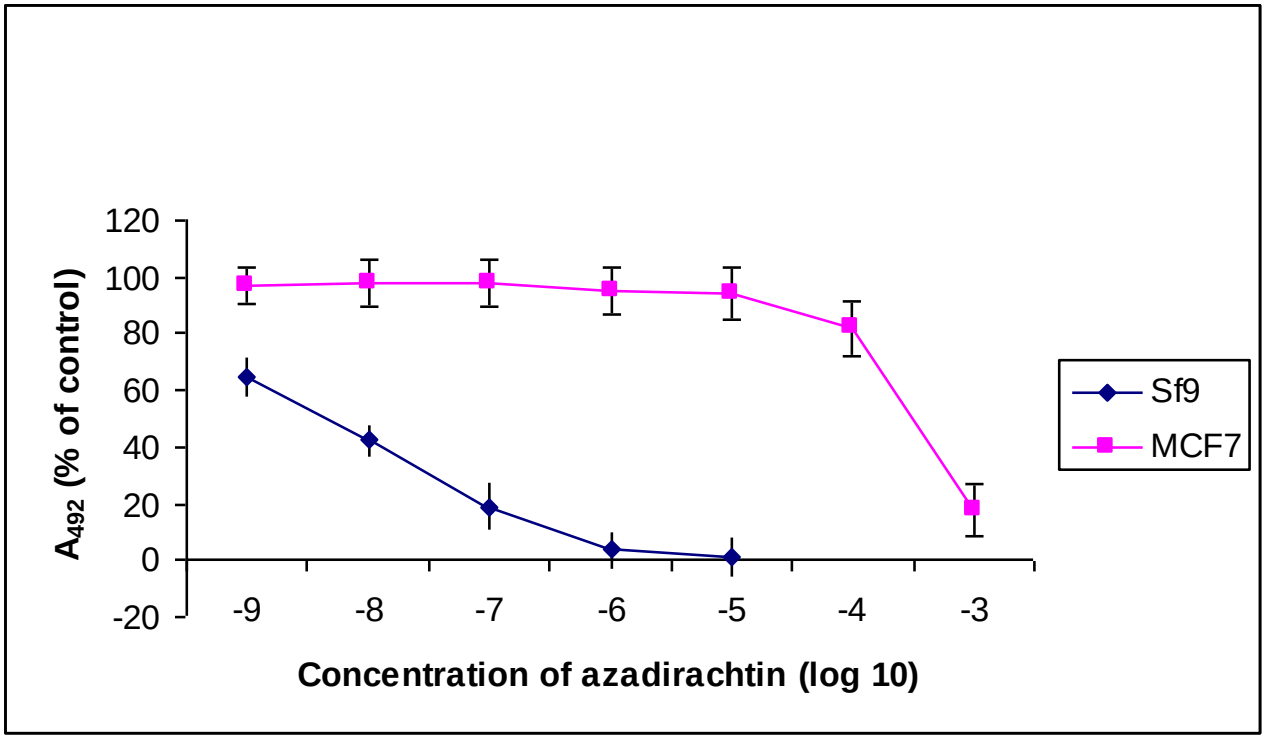

Figure.1. The effect of azadirachtin on the replication of Sf9 and MCF7 cell lines. Cells were grown under appropriate conditions in the presence of a range of concentrations of azadirachtin in 96-well plates. Control cultures contained only DMSO. Cell numbers were estimated by the reduction of MTT. Each point is mean of 5- 8 estimates. Vertical lines represent± SD. Control culture is taken as 100 % in each case.

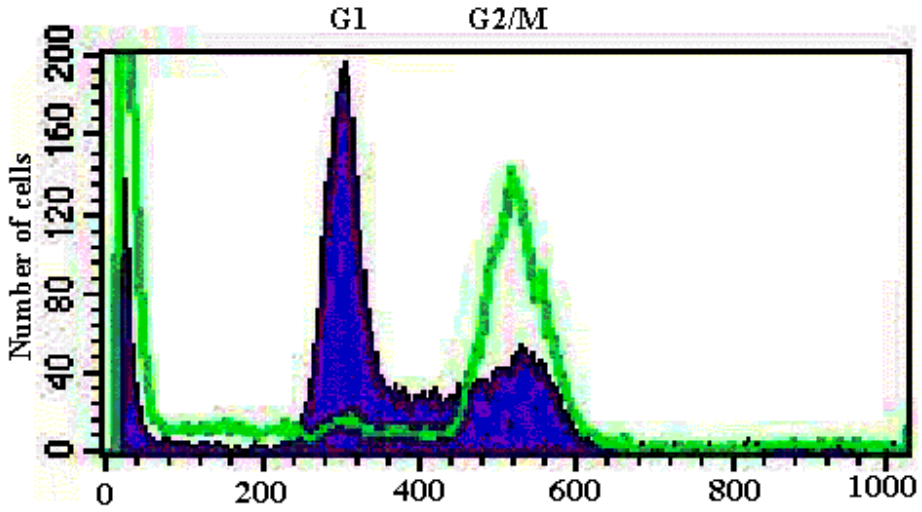

Figure 2: The effect of 10^{-5} M azadirachtin on cell cycle distribution of Sf9 cell line. The cells were incubated in presence (green) or absence (blue) of azadirachtin for 20 h.Then they were harvested and permeabilised with 0.1% saponin. The permeabilised cells were treated with 20 g/ ml propidium iodide in presence of RNAse and after 30 min incubation at room temperature, analysed by flow cytometry. Peaks corresponds to 2N (G1) and 4N (G2/M) DNA.

Table 1 summarizes the results of incubation of Sf9 cell with either conditioned or unconditioned medium containing azadirachtin. As the table shows incubation of azadirachtin with Sf9 cells has reduced its effect upon the cell cycle of Sf9 cells and the fraction of cells in G1 phase has increased from 8.9% to 19.8%, whereas incubation with MCF7 cells, or incubation in 37 °C temperature has not changed the effect of azadiarchtin significantly, so the proportion of G1 cells is almost the same as unconditioned medium containing 10^{-6} M azadirachtin. Also the effect of MCF7 cells on medium is negligible.

Sf9 cells were grown for 20 h in medium which had been subjected to a variety of conditioning procedures, and then subjected to analysis by FACS by the previously described methods. Control 1 used fresh medium containing solvent only (0.01%DMSO) and without any pre-treatment; Control 2 was to control for the effect of higher temperature at which the MCF7 cells were grown; Control 3 was eliminate any possible effect due to the MCF7 cells themselves. Unconditioned medium contained a concentration of 10^{-6} M azadirachtin without any pre-treatment. In all case the conditioning of medium was for a period of about 3 h. * Results significantly different from controls (P> 0.01).

Preliminary experiments were carried out to evaluate probable toxicity of nanoparticles on MCF7 cells. The results indicated that nanoparticles in concentration of 7.5 microgram/ml inhibit cell growth by 41 %.

. **Table 1: Relative uptake of azadirachtin by Sf9 and MCF7 cells**

Test	Azadirachtin	Conditioning of medium		Sf9 cell in G1 (%)
		Cells	Temperature (°C)	
Control **1**	-	-	-	61 ± 6
Control **2**	+	-	**37**	8.9± 2.3
Control **3**	-	MCF7	**37**	62±5
Unconditioned	+	-	-	9±2
MCF& uptake	+	MCF7	**37**	8.2±2.7

Sf9 uptake	+	Sf9	**27**	19.8±2.6 *

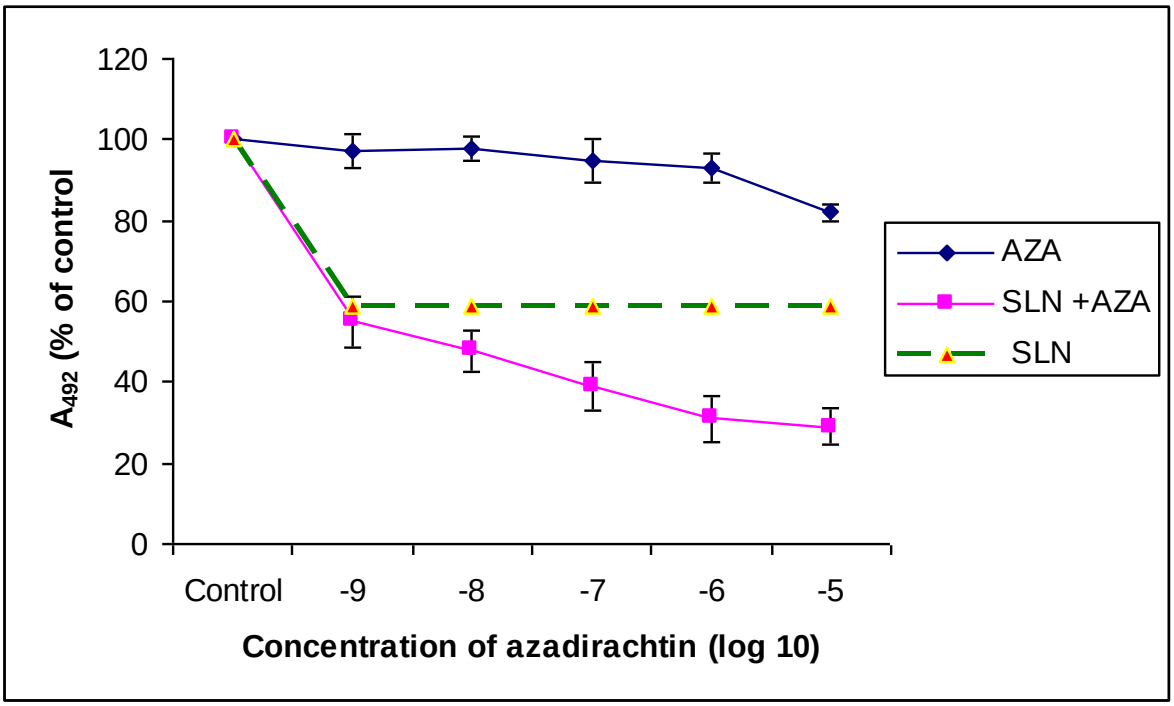

Figure 3: The effect of azadirachtin loaded nanoparticles on MCF7 cell line. Cells were grown under appropriate conditions in the presence of a range of concentrations of either azadirachtin, azadirachtin loaded nanoparticle, or Solid Lipid Nanoparticles in 96-well plates. Concentration of SLN is 7.5 microgram/ml. Control cultures contained only DMSO. Cell numbers were estimated by the reduction of MTT. Each point is mean of 5-6 estimates. Vertical lines represent± SD. Control cultures which is taken as 100% in each case.

Figure 3 compares the effect of different concentrations of azadirachtin by itself or azadirachtin loaded solid lipid nanoparticles on growth of MCF7 cells.

As figure 3 shows using solid lipid nanoparticles (SLN) has increased the effect of azadirachtin on MCF7 cells between 4 – 30%.

DISCUSSION

Since discovery of azadirachtin as a source of natural insecticide about 40 years ago many studies have been designed to evaluate its probable adverse effects on mammals (Ascher, 1993) . So far the results of most of studies have shown that the compound is relatively safe **(**Raizada *et al*., 2001**)**. Although the works on the mode of action of azadirachtin have shown that azadirachtin affects cytoskeleton and causes cell cycle arrest. In most of these experiments there has been a significant difference in effects of azadirachtin on insect and mammalian cells (Salehzaeh *et al*., 2003). The results of present study showed that azadirachtin inhibit proliferation of Sf9 cells in concentrations more than 10^{-9} M but the same concentrations of terpenoid did not affect MCF7 line derived from human breast cancer.

It is in agree with our previous works which showed differential cytotoxicity of azadirachtin on different cell lines (Salehzadeh *et al* 2002) and works of other researches such as (Reed and Majumdar., 1998)) who compared the toxicity of azadirachtin on mouse erythroleukemia and Sf9 cells.

In accordance with other studies (Salehzadeh *et al*., 2003., Thoh and Babajan 2011) using flow cell cytometry, the present study revealed that azadirachtin arrest Sf9 cells in G2M phase so in comparison to untreated cells there was a reduction in proportion of cells in G1 phase. When the same experiment was done with MCF7 cells there was no changes in cell cycle distribution (The result has been not shown). Difference in uptake may explain the differential effect of azadirachtin on different cell lines so an experiment was conducted to evaluate this hypothesis. As the table 1

shows preconditioning of azadirachtin containing medium has caused reduction in toxicity of the terpenoid on Sf9 cells, which can indicate either the relatively fast and efficient uptake of azadirachtin by insect cells or difference in binding of azadirachtin to insect and mammalian cells. Difference in uptake has been shown in others studies as well (Hasenson 2005) and may explain the resistance of some microorganisms to antimicrobial compounds (Mandel *et al*., 1984).

As the figure 3 shows using azadirachtin loaded solid lipid nanoparticles has increased the effect of terpenoid on growth of MCF7 cells by up to 30% which may indicate more or efficient uptake of azadirachtin loaded nanoparticles by these cells. Although this is the first time that nanoparticles have been used for carrying azadirachtin, the studies related to application of such system in drug delivery has been started many years ago (Fritzsche and Cleffman 1987).

Studies have shown that drug delivery system is more useful when the receptors are inside cells which is in agree with the binding of azadirachtin with actin and tubulin (Salehzadeh *et al*., 2003; Anuradha *et al*., 2007) and the result indicate in most cases such systems improve the uptake (Jong and Born., Nisha and Pramod 2008). Using such systems not only can facilitate the uptake but also it may reduce the side effects of theraupetic agents (Cao *et al*., 2009).

The Fig. 3 shows the effect of azadirachtin loaded azadirachtin on MCF7 cells. In comparison the effect of highest concentration is only about 30% more than free azadirachtin. This means that either solid lipid nanoparticle is not a suitable carrier for azadirachtin or azadirachtin affect mammalian cells with different mechanism. On the other hand the works of Akudugu have shown that in concentration about 10^{-5} M azadirachtin has inhibited the growth of glioblastoma cells effectively (Akudugu *et al*., 2001) which means in higher concentrations at least some of mammalian cells are relatively sensitive to antiproliferative effects of azadirachtin. Although this finding may slightly reduce the publicity of azadirachtin as a safe pesticides, it may also present another potential of azadirachtin as an anti-cancer agent. Because of mitotic nature of cancerous cells it is expected that azadirachtin affect this cells more than healthy cells. This is the point which has been emphasized in some new studies related to antiproliferative and apoptotic effects of Neem extracts (Kikuchi *et al*., 2011, Thoh *et al*., 2011., Priyadarsini *et al*., 2010).

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