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γ-Sitosterol from *Acacia nilotica* L. induces G2/M cell cycle arrest and apoptosis through c-Myc suppression in MCF-7 and A549 cells

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ABSTRACT

Ethnopharmacological relevance: Acacia nilotica is widely distributed in Asia. In India, it occupies an important place in the indigenous system of medicine against anti-inflammatory, antioxidant, cancers, and/or tumors.
Aim of the study: The purpose of this study is to investigate the inhibitory effect of *Acacia nilotica* leaves extract and γ-Sitosterol on cell proliferation, the apoptotic effect and cell cycle arrest in breast and lung cancer cells.
Materials and methods: GC–MS and HPLC were used to determine the chemical constituents of this extract and γ-Sitosterol respectively. Human MCF-7 and A549 cell lines were treated with *Acacia nilotica* extract and γ-Sitosterol. Cell viability was determined by MTT assay. Cell proliferation was determined by BrdU incorporation assay. Apoptosis was detected by cell morphologic observation through AO/EtBr staining, cell cycle analysis, and immunoblot analysis on the expression of protein associated with cell cycle arrest.
Results: Experimental results of bioactive compound analysis indicate that γ-Sitosterol, bioactive ingredients of *Acacia nilotica* extract. The IC_{50} value of extract on MCF-7 and A549 cancer cells was 493.3 ± 15.2 and 696.6 ± 11.5 μg/ml, respectively. *Acacia nilotica* extract and γ-Sitosterol were inhibited the cell proliferation by 54.34 ± 1.8 and 42.18 ± 3.9% for MCF-7 and 58.26 ± 1.5 and 44.36 ± 3.05% for A549 cells. The percentage of apoptotic cells observed in the MCF-7 and A549 cell lines were increased to 42.46 and 36.8% of extract; 46.68 and 43.24% for γ-Sitosterol respectively. Flow cytometric analysis results demonstrate that cells were arrested at the G2/M phase and decrease the c-Myc expression.
Conclusions: This study demonstrates *in vitro* results, which support the ethnomedical use of γ-Sitosterol against cancer. Experimental results of this study suggest that γ-Sitosterol exerts potential anticancer activity through the growth inhibition, cell cycle arrest and the apoptosis on cancer cells.

1. Introduction

The global burden of cancer continues to increase largely because of the aging and growth of the world population alongside an increasing adoption of cancer-causing behaviors, particularly smoking, in economically developing countries. Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths. Lung cancer is the leading cancer site in males, comprising 17% of the total new cancer cases and 23% of the total cancer deaths (Jemal et al., 2011).

Acacia nilotica (Linn.) belonging to the Leguminosae family and sub-family Mimosaceae has been subjected to long term clinical trials in folk medicine (El-Tahir et al., 1999), and is widely distributed in tropical and subtropical countries. Ayurvedic medicine practices use of natural medicinal plants to promote self-healing, good health and longevity, and have declared that *Acacia nilotica* can provide the nutrients and therapeutic ingredients to prevent, mitigate or treat many diseases or conditions. According to the tradition the bark, leaves, pods and flowers are used against cancer, cold, congestion, cough, diarrhea, dysentery, fever, gall bladder, hemorrhoid, ophthalmia, sclerosis, small pox, tuberculosis, leprosy, bleeding piles, leucoderma and menstrual problems. *Acacia nilotica* offers variety of bioactive components, which showed spasmogenic, vasoconstrictor, anti-hypertensive, antispasmodic, anti-inflammatory, and anti-platelet aggregatory properties (Sultana et al., 2007).

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The leaf and pod extract of *Acacia nilotica* rich in phytosterols, phenolic and flavonoid contents had potent antioxidant activity (Singh et al., 2009; Kalaivani and Mathew, 2010). It is used by traditional healers of different regions of Chhattisgarh in treatment of various cancer types of mouth, bone and skin. In West Africa, the bark and gum are used against cancers and/or tumors (of ear, eye, or testicles) and indurations of liver and spleen, the root for tuberculosis, the wood for smallpox and the leaves for ulcers. Recent scientific efforts have focused on the potential roles of extracts of traditional herbs as alternative and complementary medications for cancer treatment. In addition, *Acacia nilotica* has been reported to have anti-tumor activity with mouse breast cancer, human osteosarcoma and human prostate cancer (Kaur et al., 2005). γ-Sitosterol markedly reduced hyperglycemia in STZ-induced diabetic rats due to increased insulin secretion and inhibition of gluconeogenesis (Balamurugan et al., 2011).

In the present investigation, the leaves were chosen based on the previous reports. It was reported that the leaf extract of *Acacia nilotica* had significant chemo preventive and anti mutagenic activity than the other parts (Meena et al., 2006). To identify the several bioactive ingredients in An extract were determined. In this study we demonstrated that the, *Acacia nilotica* ethanol extract showed antitumor activity *in vitro* and could inhibit the growth of human breast and lung cancer cell line. The basic mechanism of inhibition was due to cytotoxic effects, DNA fragmentation and apoptosis. The present study examined the effects of *Acacia nilotica* and its active compound γ-Sitosterol on the cell cycle analysis and expression of cyclin E, cyclin B and c-Myc in MCF-7 and A549 cancer cells.

2. Materials and methods

2.1. Plant material

The leaves of *Acacia nilotica* (L.) *Wild. ex Delile* were collected in Western Ghats region in November 2010. Botanical identification was carried out by Prof. G.V.S. Murthy (Botanical Survey of India, Coimbatore). A voucher specimen (No. BSI/SRC/5/23/2011-12/Tech.268) has been deposited in the laboratory of Botanical Survey of India, Coimbatore.

2.2. Isolation and identification of active compounds

Air-dried leaves of *Acacia nilotica* were reduced to a fine powder (50 g), and soxhlet extracted with absolute ethanol (250 ml) for 24 h. The extract was filtered using Whatman No. 1 filter paper and the filtered ethanolic extract was concentrated to dryness under vacuum desiccator and the solvent was removed by vacuum evaporation. The solution was partitioned with diethyl ether in a 1:1 ratio using a separating funnel. The diethyl ether part was concentrated to obtain the extract. γ-sitosterol from extract of *Acacia nilotica* was isolated from fractions separated by distillation and subsequent chromatography.

2.3. High performance liquid chromatography (HPLC) analysis

All solvents used were of analytical chromatographic grade (Sigma–Aldrich). HPLC was performed with a HP Ti series 1050 liquid chromatography, equipped with a photodiode array detector (DAD, HPseries 1050). Solutions of the tested compound (5%, v/v in ethanol) was subjected to normal phase HPLC analysis carried out on a Phenomenex Hypersil 3 μm C18 BDS (100 mm × 4.6 mm) column using a mobile phase of ethanol at a flow rate of 1.0 ml/min. The injector was a Rheodyne model valve with a 20 μl loop. UV detection (DAD) at 210 nm was recorded. Elute fractions obtained from HPLC analyses were further subjected to GC–MS analysis after concentration under vacuum.

2.4. GC/MS analysis

GC/MS analysis was performed on GC–MS-QP (Shimadzu) equipped with a VF-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film coating). The injector temperature was set at 260 °C. Helium was used as carrier gas at a constant flow rate of 1.51 ml/min through the column. The column temperature was initially kept at 70 °C for 2 min, and then increased from 70 to 300 °C at 10 °C/min, where it was held for 10 min. The MS ion source temperature was set at 200 °C and the ion inlet temperature was 240 °C. Full-scan mass range of 40–1000 *m*/*z* was acquired. Sample components were identified by matching their mass spectra with those recorded in NIST08s, Wiley-8 and FAME Library.

2.5. Cell line and culture conditions

Human breast cancer MCF-7 and non-small lung cancer cell A549 cell lines were procured from National Centre for Cell Science (Pune, India). The cells were maintained in Dulbecco's Modified Eagles medium supplemented with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/l Na bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM of Na pyruate in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.6. Cell viability assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, exponentially growing cells (1×10^4 cells/well) were seeded into a 96 well plate and allowed to attach overnight. Cells were treated with the various concentration of *Acacia nilotica* extract in at least six replicate wells and left contact for 48 h. 500 μg/ml of MTT was added to each well, and the plate was further incubated at 37 °C for another 4 h. Formazan crystals formed were dissolved in DMSO. Absorbance was determined with a multiwall spectrophotometer at 550 nm. Absorbance values were expressed as percentage relative to controls.

The quantification of the γ-Sitosterol in the extract was performed using HPLC as reported previously by Gao et al. (2008).

2.7. DNA fragmentation analysis

Cells were treated with 0.5, 0.7 and 0.9 mg/ml extract concentrations based on MTT assay results. The cells were pelleted by centrifugation at 1000 × *g* for 5 min at room temperature. Aliquots from cells were resuspended in 500 μl of digestion buffer (5 mM EDTA, pH 8.0, 20 mM Tris, 0.5% SDS) containing 100 μg/ml proteinase K and RNAse followed by incubation at 37 °C for 3 h and analyzed on 1.5% agarose gel.

2.8. Cell proliferation ELISA

MCF-7 and A549 cancer cell proliferation rates in the presence or absence of varying concentrations of *Acacia nilotica* extract were compared using a 5-bromo-2′-deoxyuridine (BrdU) incorporation based ELISA. Cells were seeded at 1×10^4 cells per well (0.55×10^4 cells/cm^2) in 96-well plates and cells, grown for 48 h, and then processed according to manufacturer's instructions. The BrdU incorporation period was fixed at 16 h.

2.9. Apoptotic cell death detection

Apoptosis studies were performed with a staining method utilizing acridine orange (AO) and ethidium bromide (EB). MCF-7 and A549 cells were incubated in the absence and presence of An extract and γ-Sitosterol at concentration of 0.5 mg/ml at 37 °C and

5% CO_2 for 24 h. After 24 h, each cell culture was stained with AO/EB solution (100 μg/ml AO, 100 μg/ml EB). Samples were observed under a fluorescence microscope. Several fields, randomly chosen, were digitalized and at least 600–800 nuclei for each sample were counted and scored. Results are expressed as the relative percentages of viable and apoptotic cells to the total number of cells scored.

2.10. Cell cycle analysis

Cells (2×10^6) were treated with various concentrations of *Acacia nilotica* extract (0 and 0.5 mg/ml) and γ-Sitosterol (0.5 mg/ml) for 48 h. The cells were harvested by trypsinization. Pellet out the cells at 2500 rpm/5 min/RT. Resuspend cells in 300 μl of PBS–EDTA. Add drop wise 700 μl of chilled 70% ethanol with slow vortexing. Tap mix lightly to ensure complete mixing of ethanol, store at 0 °C over night. Add 1:100 volumes of 20 mg/ml RNase and incubate at 37 °C for 1 h. Add PI to a final concentration of 50 μg/ml incubates for 10–20 min at RT. The stained cells were analyzed for DNA histograms and cell cycle phase distribution by flow cytometry (Cell Lab Quanta™ SC MPL).

2.11. Western blotting

Western blotting was performed to detect the proteins of cyclin E, cyclin B and c-Myc. MCF-7 and A549 cells (1.5×10^6) were seeded onto 100-mm culture dishes in the presence or absence of extract and γ-Sitosterol, and were treated for 48 h. Cells were washed twice with ice-cold PBS and incubated in lysis buffer. The lysates were centrifuged at $10{,}000 \times g$ for 5 min at 4 °C, and were used as the cell protein extracts. Each extract were applied to 12% SDS polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane, and then blocked for 1 h using 10% skim milk in water. After washing in PBS containing 0.1% Tween 20 for 3 times, primary antibodies against cyclin and c-Myc or tubulin were added at a (v/v) ratio of 1:1000. After overnight incubation at 4 °C, the primary antibodies were washed away and secondary antibodies were added for 1 h incubation at room temperature. Finally, the enhanced chemiluminescence (ECL) detection reagents were used to develop the signal of the membrane.

2.12. Statistical analysis

All *in vitro* experiments were done in triplicate, and the experiments were repeated at least thrice. The statistical software SPSS version 17.0 was used for analysis. *P* values were determined using the *t* test; *P* value <0.001 was considered significant.

3. Results

3.1. Identification of active compound

The qualitative and quantitative GC/MS results are listed along with the retention indices of the twenty-six identified compounds. The HPLC analyses under normal phase conditions (CN-column and ethanol as a mobile phase) were allowed the active components to separate into the different groups of classes of increasing polarity. The qualitative analysis of γ-Sitosterol was confirmed with the assistance of HPLC (Fig. 1A). Chromatogram of high-performance liquid chromatographic analysis of extract was shown in Fig. 1B. The composition of the HPLC fractions was subsequently confirmed again by GC–MS analysis. The GC–MS spectrum revealed the presence of compound γ-Sitosterol (100%). The molecular weight of the compound was *m*/*z*: 414, 43 (100), 396, 381, 329, 303, 273 (Fig. 2). From the GC–MS analysis fraction was identified as γ-Sitosterol.

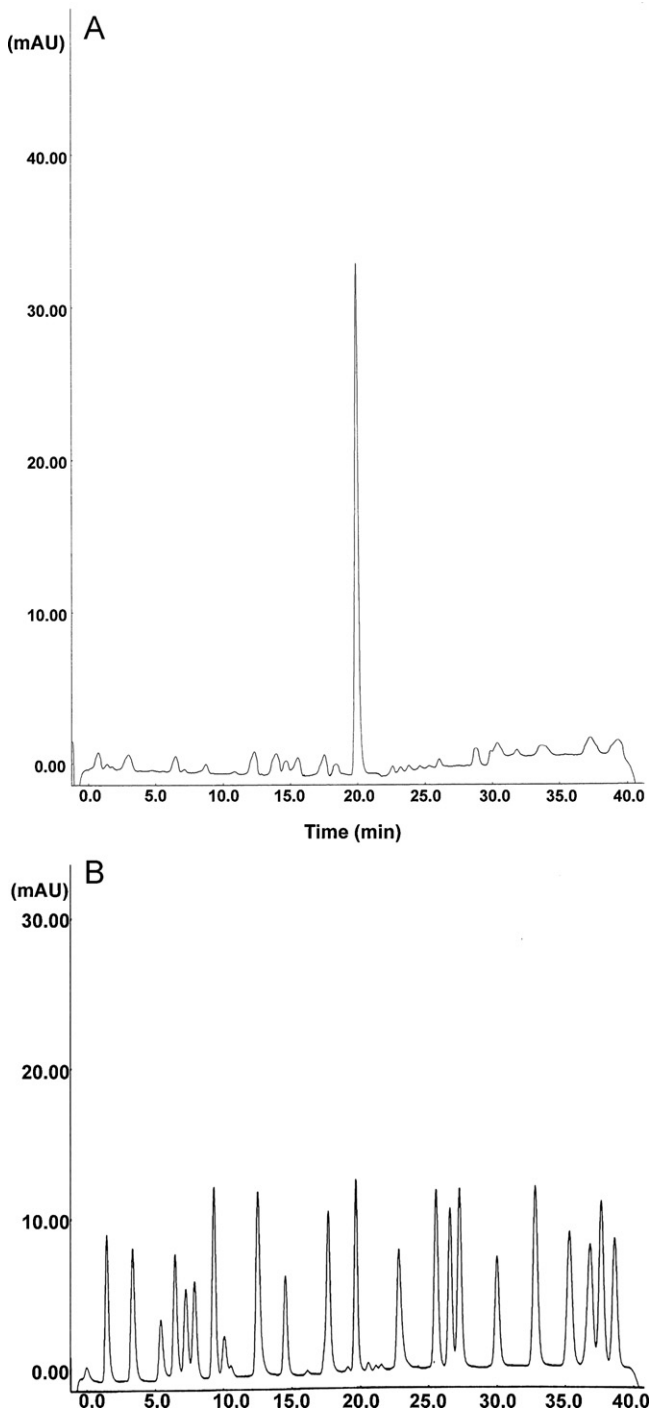

Fig. 1. Characterization of γ-Sitosterol isolated from *Acacia nilotica*. (A) Purification of γ-Sitosterol with the assistance of HPLC. (B) Chromatogram of HPLC analysis of plant extract.

3.2. Cell viability of Acacia nilotica extract on MCF-7 and A549

To investigate the potential effect of *Acacia nilotica* extract on the viability of MCF 7 and A549 cells after treatment with various concentrations (0.1–1 mg/ml) of An extract for 48 h. Doxorubicin (DXR), an effective anticancer drug, was used as a positive control. The experimental results indicate that An extract inhibited cell viability in a dose dependent manner (Fig. 3). Notably, the IC_{50} value

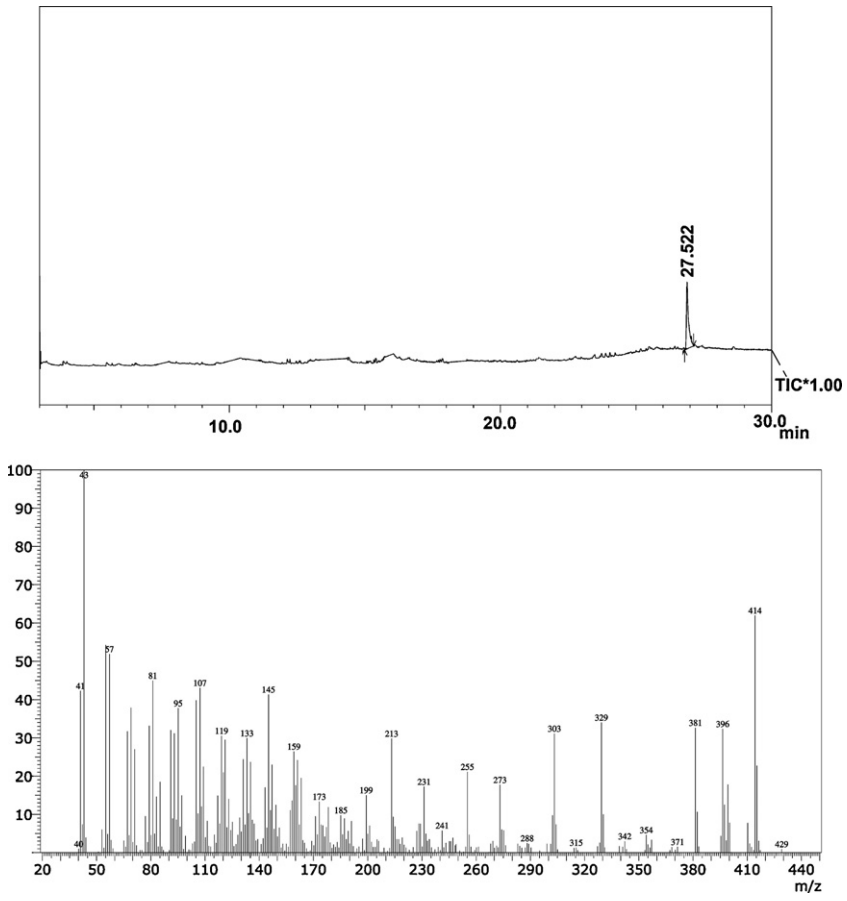

Fig. 2. GC–MS chromotograph of γ-Sitosterol and the mass spectra of γ-Sitosterol.

of An extract on MCF-7 and A549 cells determined by MTT assay, was 493.3 ± 15.2 and 696.6 ± 11.5 μg/ml, respectively.

The concentration of active compounds γ-Sitosterol was determined in either stock or working concentration of the extract as presented. 2 mg/ml stock solution contains 976 μg/ml concentration of γ-Sitosterol. 493.3 μg/ml (IC_{50} value on MCF-7) contain 240.73 μg/ml and 696.6 μg/ml (IC_{50} value on A549) contain 339.94 μg/ml content of γ-Sitosterol in the working extract.

3.3. Effect of Acacia nilotica extract on DNA and nuclear fragmentation in MCF-7 and A549 cells

One of the mechanisms by which cell growth is suppressed is apoptotic cell death. Therefore, the effect of *Acacia nilotica* extract on DNA fragmentation was examined in MCF-7 and A549 cells. The nucleosomal DNA fragmentation was observed when cells were treated with 0.5, 0.7 and 0.9 mg/ml of *Acacia nilotica* extract for 48 h. MCF-7 cells was more sensitive as even the low dose of extract induced DNA fragmentation which was further enhanced in the high dose treated group. The profile for *Acacia nilotica* extract-induced apoptosis closely correlated with its growth suppressive effects. Thus, the growth suppression induced by *Acacia nilotica* extract in MCF-7 and A549 cells may be related to the induction of apoptosis.

3.4. Inhibition of cell proliferation

The inhibitory effect of *Acacia nilotica* extract and γ-Sitosterol on MCF-7 and A549 cells was further confirmed by BrdU incorporation into the untreated and treated breast and lung cancer cells *in vitro*. *Acacia nilotica* extract and γ-Sitosterol were inhibited the cell proliferation by 54.34 ± 1.8 and 42.18 ± 3.9% for MCF-7 and 58.26 ± 1.5 and 44.36 ± 3.05% for A549 at concentrations of 0.5 mg/ml respectively (Fig. 4). The most pronounced statistically significant decreased cell proliferation in treated cell was observed as compared with control cells.

3.5. Changes in nuclei morphology after Acacia nilotica extract treatment

A double staining with a mixture of ethidium bromide and acridine orange was used to visualize and quantify the number of viable and apoptotic cells. Viable cells exhibit large green nuclei whereas apoptotic cells show signs of nuclear condensation or nuclear bead formation and are colored in orange.

3.6. Acacia nilotica extract and γ-Sitosterol induce apoptosis in cancer cells

We found that ethanolic extract of *Acacia nilotica* and γ-Sitosterol used at the concentration of 0.5 mg/ml decreased the percentage of viable cells in MCF-7 and A549 cells (Fig. 5). The percentage of apoptotic cells were increased to 42.46 and 46.68% for MCF-7 cells and 36.8 and 43.24% for A549 cells respectively, compared to untreated cells. However, treatment of *Acacia nilotica* and γ-Sitosterol significantly increased apoptosis as compared with control cells.

3.7. Cell cycle regulation

To determine whether An extract and γ-Sitosterol induced apoptosis was related to arrest cell cycle progression in MCF-7 and

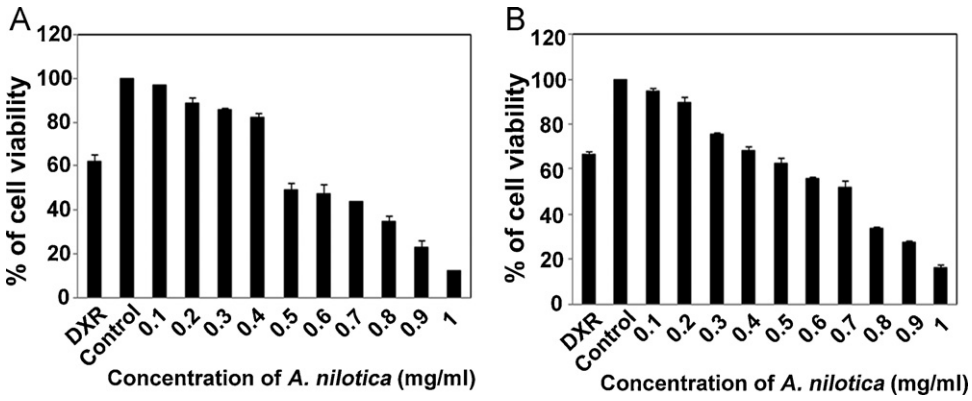

Fig. 3. Cell viability effects of different *Acacia nilotica* ethanol extract dose on (A) MCF-7 and (B) A549 cells after 48 h treatments. The doxorubicin (DXR) of 500 μg/ml was used as a positive control.

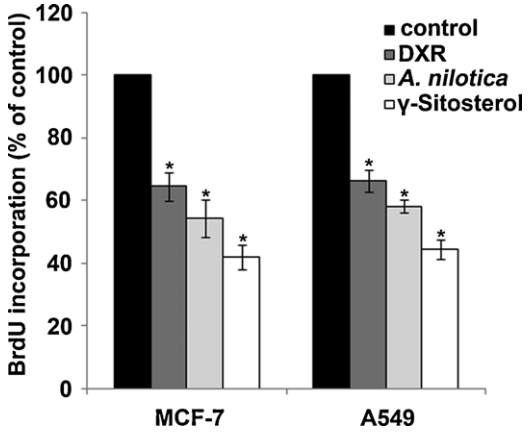

Fig. 4. Inhibition of MCF-7 and A549 proliferation by An extract and γ-Sitosterol. Quantitation of MCF-7 and A549 growth in the presence of An extract and γ-Sitosterol for 48 h ($n = 3$, ±SEM). Proliferation was determined using a colorimetric ELISA based on BrdU incorporation. *$P < 0.001$ *versus* control.

A549 cells, flow cytometry was used to analysis the cell cycle distribution under treatment with 0.5 mg/ml concentration for 48 h. The number of cells in the G2/M phase increased, and that in the G0/G1 phase decreased in An extract and γ-Sitosterol treated cells (Fig. 6). In the absence of An extract, MCF-7 and A549 showed majority of cells in G1 phase (75–78%) and low percentage of population in S phase (3–5%) and G_2/M phase (16–18%) in control. This experimental finding implies that An extract and γ-Sitosterol induced apoptosis on MCF-7 (Fig. 6A) and A549 (Fig. 6B) cells *via* G2/M cell

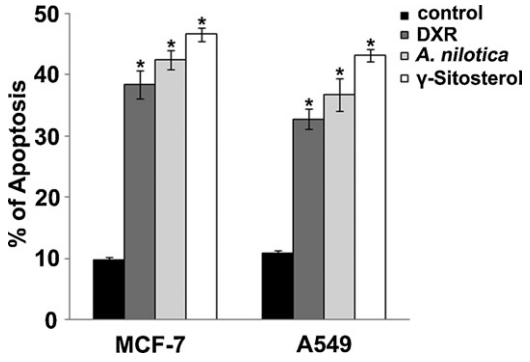

Fig. 5. An extract and γ-Sitosterol induces apoptosis of MCF-7 and A549 cells. MCF-7 and A549 cells (1×10^6 cells/ml) were treated with 0.5 mg/ml of An extract and γ-Sitosterol for 48 h staining by AO/EtBr staining. Results are mean ± SEM ($n = 3$) *$P < 0.001$ *versus* control.

cycle arrest. In addition, treatment with An extract and γ-Sitosterol resulted in a significant decrease in G0/G1 phase cell number and increase in G2/M phase cell number.

Consistent with the significant accumulation of cells in G_2/M and S phases in An extract treated cells, immunoblotting analysis showed increases in expression levels of G_2/M and S phases associated cell cycle regulators such as cyclin B and cyclin E. We also observed down regulation of c-Myc levels in An extract treated cells. Since c-Myc expression is higher at late G_1 promoting G_1 to S transition, the decreased expression of c-Myc seen in An extract treated cells may reflect the partial loss of G1 cells.

4. Discussion

In this study, isolation of γ-Sitosterol from *Acacia nilotica* extract was identified by GC–MS analysis and their anti-proliferative activities on MCF-7 and A549 cells were elucidated. An extract demonstrated significant cytotoxicity on human breast cancer cell MCF-7 and human lung cancer cell A549 with IC_{50} values of 493.3 ± 15.2 and 696.6 ± 11.5 μg/ml, respectively, at 48 h treatment (Fig. 3). γ-Sitosterol was observed to have growth inhibitory effects on both lung and breast cancer cell lines and these effects occurred over similar time and concentration ranges (0.5 μg/ml) in both cell types (Fig. 4). Awad and Fink (2000), findings that the effect of phytosterol on membrane structure function of tumor, signal transduction path way that regulate tumor growth and apoptosis. β-Sitosterol inhibits growth of HT-29 human colon cancer cells and human prostate cancer LNCaP cell line by activating the sphingomyelin cycle (von Holtz et al., 1998). Awad et al. (2003), showed that phytosterol supplementation of MDA-MB-231 human breast cancer cells increases the activities of apoptosis.

In addition, the *in vitro* data support findings that a mixture consisting of these sterols compounds exerted cytotoxic activity against human lung and breast cancer cells (Lai et al., 2010). In the present study, potential cellular mechanisms underlying phytosterol-induced apoptosis were investigated. Specific effects on cell cycle arrest and cyclin regulators were identified. Campesterol significantly inhibited the bFGF-induced proliferation and tube formation of HUVECs in a concentration-dependent manner (Choi et al., 2007). The antitumor activity of stigmasterol had mediated through the activation of protein phosphatase 2A by ceramide causing apoptosis (Ghosh et al., 2011). Additionally, β-Sitosterol exhibited good cytotoxicity against various cancer cells, including KB nasopharyngeal epidermoid carcinoma cells, MCF-7 breast cancer cells, CasKi cervical carcinoma cells, HCT 116 colon carcinoma cells and A549 cells (Malek et al., 2009). β-Sitosterol affects the amounts and activity of components of the extrinsic apoptotic pathway in human breast adenocarcinoma cells (Awada

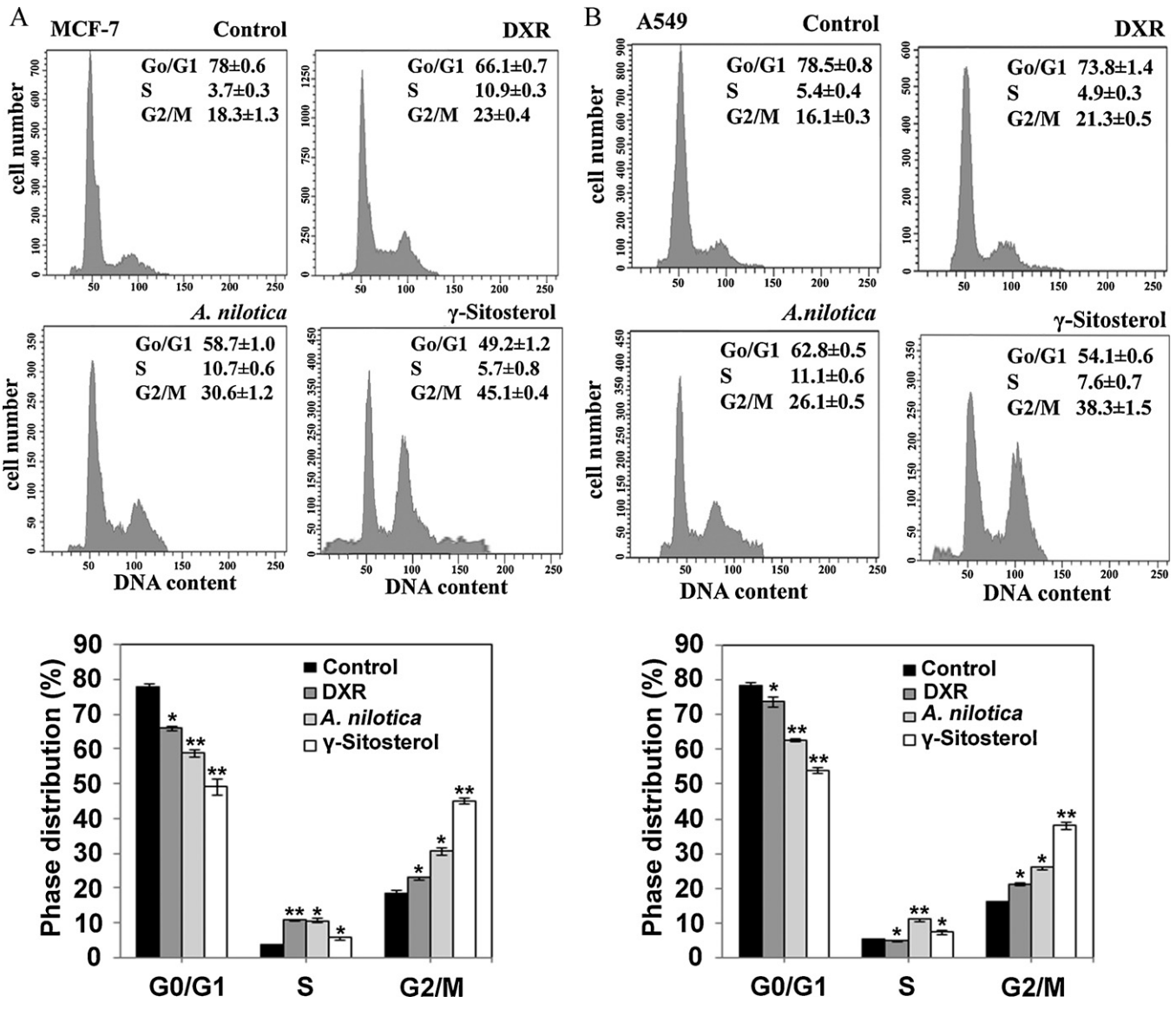

Fig. 6. Cell cycle analysis of MCF-7 and A549 cells treated with An extract and γ-Sitosterol by flow cytometry. (A) MCF-7 and (B) A549 cells (1×10^6 cells/ml) were incubated with 0–0.5 mg/ml of An extract or 0.5 mg/ml of DXR as indicated in each graph for 48 h. The percentage of each phase distribution was determined and expressed as a percentage of total cell number (*$P < 0.05$ *versus* control. **$P < 0.001$ *versus* respective control). DXR was used as a positive control.

et al., 2007). In this study, we show that *Acacia nilotica* extract and γ-Sitosterol induces several features of apoptosis, such as DNA fragmentation and apoptosis (Fig. 5).

Here the cell cycle analyses revealed that the number of cells in the G2/M phase increased, and that in the G0/G1 phase decreased in An extract and γ-Sitosterol treated cells, accompanied by a significant decrease in the G0/G1 phase (Fig. 6). Moreover, the effects on the expression of c-Myc were examined at the protein level and down regulation of c-Myc were observed in MCF-7 and A549 cells. Notably, β-Sitosterol induced G2/M arrest, endoreduplication, and apoptosis on U937 lymphoma cells and HL60 promyelocytic leukemic cells through the Bcl-2 and PI3 K/Akt signaling pathways (Moon et al., 2008).

In conclusion, study demonstrates that membrane enrichment with the γ-Sitosterol may affect the amounts and activity of components of the extrinsic apoptotic pathway in human lung and breast adenocarcinoma cells. This is a significant observation and investigation of any cause and effect relationship between these observations is warranted. The results of this study advance our understanding of the molecular mechanisms responsible for the anticancer properties of γ-Sitosterol. This study demonstrates *in vitro* results, which support the ethnomedical use of γ-Sitosterol against cancer and points to the need for further study synergistic action of the constituents of *Acacia nilotica* in combating cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2012.03.014.

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