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A novel compound β-sitosterol-3-O-β-D-glucoside isolated from *Azadirachta indica* effectively induces apoptosis in leukemic cells by targeting G0/G1 populations

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Azadirachta indica, popularly known as '*Neem*', is an important plant in the Ayurveda system of medicine. It is known to prevent about at least 40 types of diseases in home-practice. A previous report showed that the methanolic extract of this plant can effectively control proliferation of leukemia cells. Here, we explored the eEthanolic extract dried neem leaves for a new molecule with potent anti-leukemic property.Four biomolecules were isolated *viz*. rutin, isoquercetin, quercetrin and β-sitosterol-3-O-β-D-glucoside from the ethanolic extract by repeated column chromatography and HPLC.Quercetrin structures of the isolated molecules were confirmed by Mass, ^{1}HNMR, ^{13}C NMR spectra analysis. MTT assay revealed that β-sitosterol-3-O-β-D-glucosideeffectively reduced the proliferation of MOLT 4 leukemic cells in a dose and time dependent manner. DAPI staining with confocal microscopy indicated that β-sitosterol-3-O-β-D-glucoside efficiently induced nuclear DNA fragmentation in MOLT 4 cells. Finally, flow cytometry after PI staining showed that the compound has potential to check the cell cycle progression at sub G1 phase. With the available results, it can be concluded that β-sitosterol-3-O-β-D-glucoside has potential to be good therapeutic drug in leukemia treatment.

Keywords: Anti-proliferative, Cytotoxicity, Leukemia, Neem, β-sitosterol-3-O-β-D-glucoside

Leukemia continues to be an enigmatic challenge to cancer biologists and medical practitioners. Several claims have been made time to time for discovering sure molecules of leukemia, yet a dependable cure against leukemia remains a challenge. The primary reason for this is the multiple survival pathways[1,2] adopted by the leukemic cells by carrying mutations in their genome, which ensures up-regulation of anti-apoptotic proteins[3] (*e.g.* Bcl-2, Bcl-XL *etc*.) and down-regulation of pro-apoptotic proteins[4] (*e.g.* Bid, Bim *etc*.) that ultimately reduces the apoptotic events. Therefore multi-target drugs toeradicate these neoplastic blood cells with minimum side-effects are required. In recent years certain anti-leukemic herbal products, and ethanol-medicines have drawn keen attention of researchers because of their multiple targets and least side-effects.

Azadirachta indica (Neem), a member of the Meliaceae family, is commonly found in tropical countries *e.g.* India, Bangladesh, Nepal, Pakistan and African countries as well. Neem is an evergreen tree with wide spectra of biological activities. The tree is regarded as 'village dispensary' in India. Neem has been extensively used in Ayurveda, Unani and homeopathic medicine and has become a cynosure of modern medicine in different disease management[5]. In East Africa, this tree is called as Mwarobaini, which means "the tree of the 40" indicates its use for the treatment of 40 types of diseases. The entire plant is of therapeutic values as it is a complex a high number of bioactive ingredients including Nimbin, nimbidin, nimbolide, azadirachtin, different limonoids, flavonoids, *etc*. Such ingredients play vital role in disease management by modulating different genetic pathways[6-8]. Numerous biological and pharmacological activities of this plant have been reported earlier, including antifungal[9], antibacterial[10], and anti-inflammatory[6], anti-arthritic[11], anti-pyretic[12], hypoglycemic[13], anti-gastric ulcer[14] and anti-tumor[15,16] *etc.* Anti-carcinogenic properties has been demonstrated by the leaves and its components of *Azadirachta indica*[17]. The main objective of this work was to search out novel anti-leukemic molecules. In the present article, we approached to isolate biomolecules from the leaves and thoroughly screened against leukemic cell lines. One of the isolated biomolecules, showed

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anti-proliferative and nuclear deformation activity against leukemic cells.

Materials and Methods

Chemicals

RPMI 1640 and fetal bovine serum (FBS) were purchased from GIBCO/Invitrogen. Penicillin, streptomycin, DAPI, and thiazolyl blue tetrazolium bromide were purchased from SIGMA. Silica gel (100-200) and all the solvents used for column chromatography and HPLC were purchased from Merck, India.

Collection of plant materials

The leaves were collected from mature tree located in the Garmirjapur village of West Bengal during the August - October of 2014. The leaves were air dried at 35-40°C temperature for several days. Dried samples were ground into fine powder (250 g) and stored in vacuum desiccators until use.

Extraction and isolation of the compounds

Plant sample was extracted with methanol at room temperature for 3 days. This step was repeated twice. Liquid portion was filtered the extracted through Whatman filter paper and evaporated to dryness in a rotary evaporator (~50 g). Dried extract was fractioned into hexane and butanol-water (3:1). Butanol fraction was evaporated to dryness in a rotary evaporator (~10 g). Dried extract was loaded in a silica gel (100-200 mesh) column using chloroform-methanol solvent system. Fraction 3 and 4 were mixed up and further chromatographed on a silica gel column using chloroform: methanol (93-90:7-10) solvent system to yield compound β-sitosterol-3-O-β-D-glucoside. Fraction 7, 8 and 9 were mixed up and further chromatographed on silica gel column using chloroform: methanol (90-85:10-15) solvent system to yield compound isoquercetin and a mixture of isoquercetin and quercetrin. Fraction 10 was further chromatographed on silica gel column using chloroform: methanol (75-70:25-30) solvent system to yield compound rutin. Compounds isolated, were identified by analyzing their spectra (mass and NMR).

High performance liquid chromatography (HPLC)

The mixture containing compound Quercetrin was subjected to Preparative HPLC (Waters). Major compound Quercetrin present in this mixture was purified using Xtera C-18 preparative column and solvent system was methanol (40): water (59.5): acetic acid (0.5), a flow rate 20 mL/min, runtime 35 min. Compound quercetrin was amorphous, yellow powder.

Cell culture

Human leukemic cell lines MOLT 4 and U937 were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

MTT assay

Cells were seeded at a density 1×10^5 cells/well in 96-well microtitre plate and treated with each compound at different concentrations for 24 h and 48 h time points. After treatment, 20 µL of 5 mg/mL MTT solution was added to each well and incubated for 4 h at 37°C. After incubation, the medium was aspirated and for mazan crystals produced in cells were solubilized in dimethylsulfoxide. Absorbance was measured at 570 nm using a microplate reader (iMark, Bio-Rad, Japan). Results were expressed as a percentage of control.

DAPI nuclear staining

Cells were seeded at a density 1×10^5 cells/well in 96-well microtitre plate and treated with β-sitosterol-3-O-β-D-glucoside at different concentrations for 24 h. After treatment, cells were collected, washed with phosphate buffered saline (PBS) and permeabilized with methanol. DAPI (1 µg/mL in PBS) was added and incubated for 15 min at room temperature and observed under a confocal microscope (Olympus).

Cell cycle analysis

Actively growing U937 and MOLT 4 cells were treated with β-sitosterol-3-O-β-D-glucoside for 24 h time points, then washed, harvested, fixed by 70% ethanol and incubated for 30 min in PBS containing 100 µg/mL RNase and 50 µg/mL propidium iodide (PI). 1×10^4 cells were analyzed for each sample on a flow cytometer. Results were expressed as the percentage of cells in each phase of the cell cycle.

Results

Characterization of the isolated compounds

Structures of the isolated compounds were determined by analyzing the spectral data, *e.g.* mass spectrum, ^{1}H NMR and ^{13}C NMR. Spectral data of the isolated compounds were compared with the previously reported data which was almost same with the previously reported data and confirmed the isolated bio-molecules asquercetin-3-O-rutinoside (rutin), a flavone-rutinoside, molecular weight 610, amorphous, yellow powder, yielded ~ 350 mg. (0.14%); Quercetin-3-O-β-D-glucopyranoside (isoquercetin), a flavone-glycoside, molecular weight 464, amorphous, yellow

powder, yielded ~ 60 mg (0.024%); quercetin-3-O-α-L-rhamnoside (quercetrin), a flavone-rhamnoside, molecular weight of 448, amorphous, yellow powder, yielded ~ 40 mg (0.016%); β-sitosterol-3-O-β-D-glucoside, molecular weight 576, white powder, yielded ~ 20 mg (0.008%). Rutin, isoquercetin, quercetrin all have a flavonoid backbone with links sugar moiety except β-sitosterol-3-O-β-D-glucoside which is a steroidal glycoside (Fig. 1). Rutin is a ubiquitous flavonoid found in plants[18].

Differential anti-proliferative activity of β-sitosterol-3-O-β-D-glucosideagainst leukemic cells

To investigate the anti-leukemic properties of the isolated bio-molecules, we performed the MTT assay. U937 and MOLT 4 cells were treated with each compound for 24 and 48 h. The result of this assay showed that isolated bio-molecules have differential anti-proliferative actions. Compound rutin, isoquercetin, quercetrinremained inactive after treatment to the cell lines and did not show any anti-proliferative activity, rather isoquercetin accelerate the proliferation of both the cell lines tested. β-sitosterol-3-O-β-D-glucoside, a steroidal glycoside, effectively blocked the proliferation of leukemic cells MOLT 4 with IC_{50} (Fig. 2A) value

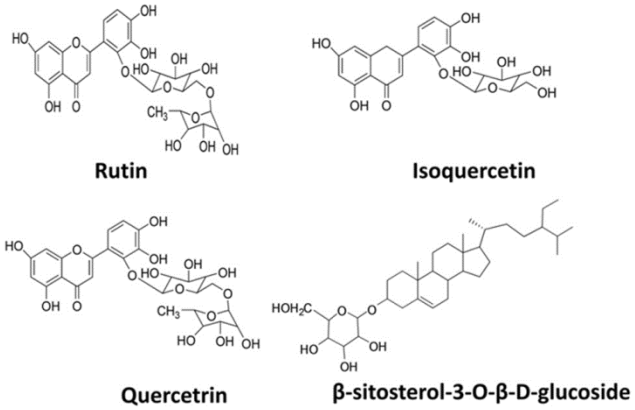

Fig. 1 — Structures of the isolated biomolecules from *Azadirachta indica*.Rutin: Quercetin-3-O-rutinoside, Isoquercetin: Quercetin-3-O-β-D-glucopyranoside**,** Quercetrin: Quercetin-3-O-α-L-rhamnoside, β-sitosterol-3-O-β-D-glucoside

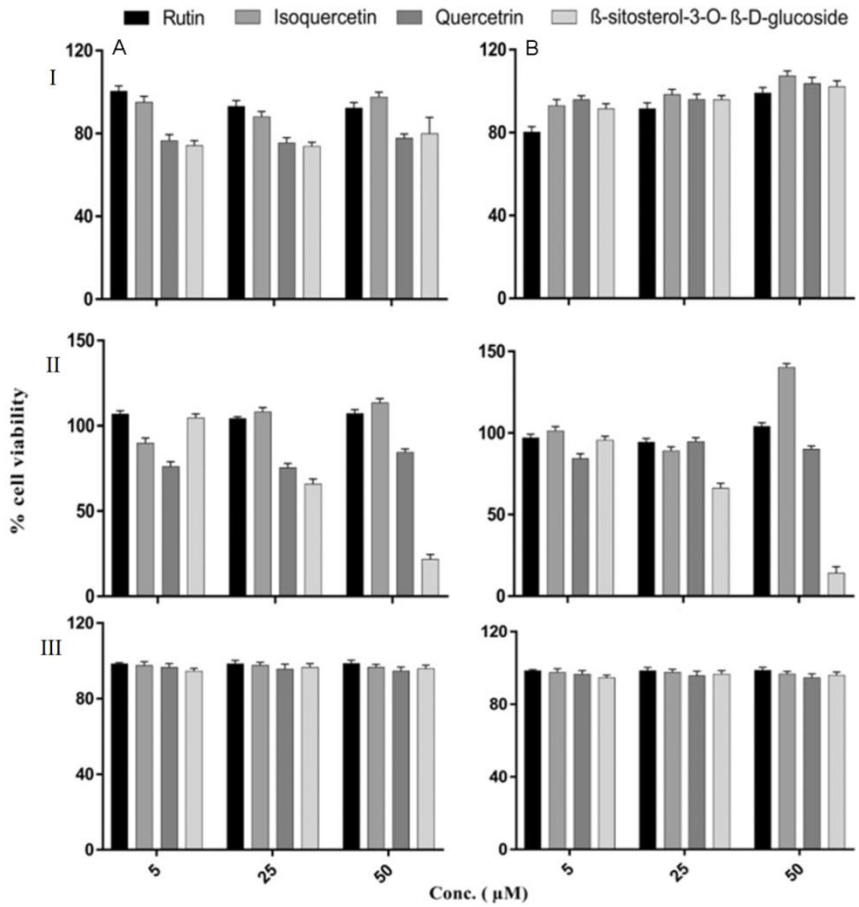

Fig. 2 — Assessment of anti-proliferative activity of the isolated compounds against (I) MOLT 4 cells; (II) U937 cells; and (III) normal PBMC. Cells were treated with each individual compound **(**Rutin, Isoquercetin, Quercetrin and β-sitosterol-3-O-β-D-glucoside**)** at different dose for 24 h (A) and 48 h (B) time points. After incubation, MTT assay was performed to evaluate the anti-proliferative capacity. Each bar graph represents Mean ± SD of three independent experiments

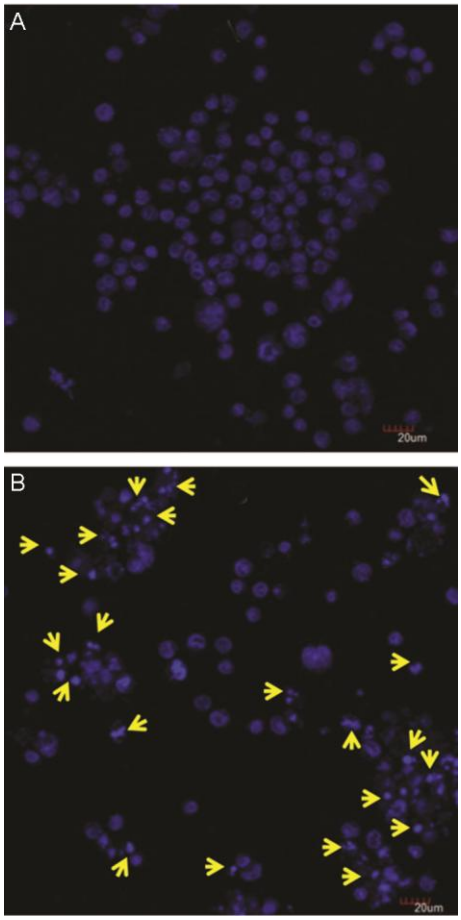

Fig. 3 — Detection of nuclearfragmentation by DAPI staining after β-sitosterol-3-O-β-D-glucoside treatment. MOLT 4 cells were treated with β-sitosterol-3-O-β-D-glucoside for 24 h. After that, nuclear DNA was stained with DAPI and cells were observed under fluorescent microscope.MOLT 4 cells showeda high degree of nuclear fragmentation

below 40 µM. This anti-proliferative activity was dose-dependent and time-dependent and cell specific, as β-sitosterol-3-O-β-D-glucoside did not show any cytotoxic activity against U937 cells (Fig. 2B). We also tested the cytotoxic activity of these bio-molecules against normal Peripheral Blood Mononuclear Cells (PBMC) and found that none of these isolated compounds showed any cytotoxicity against PBMCs (Fig. 2C).

β-sitosterol-3-O-β-D-glucosideinduced nuclear deformation and fragmentation in MOLT 4 Cells

Nuclear DNA fragmentation is one of the hallmark events of apoptosis. Therefore, to be assured about the apoptotic activity of β-sitosterol-3-O-β-D-glucoside, we treated the MOLT 4 cells with β-sitosterol-3-O-β-D-glucosideat IC_{50} values for 24 h and then stained with DAPI and observed under fluorescent microscope. We found that β-sitosterol-3-O-β-D-glucoside effectively increased the number of fragmented and deformed nuclei after treatment as compared to control set (Fig. 3).

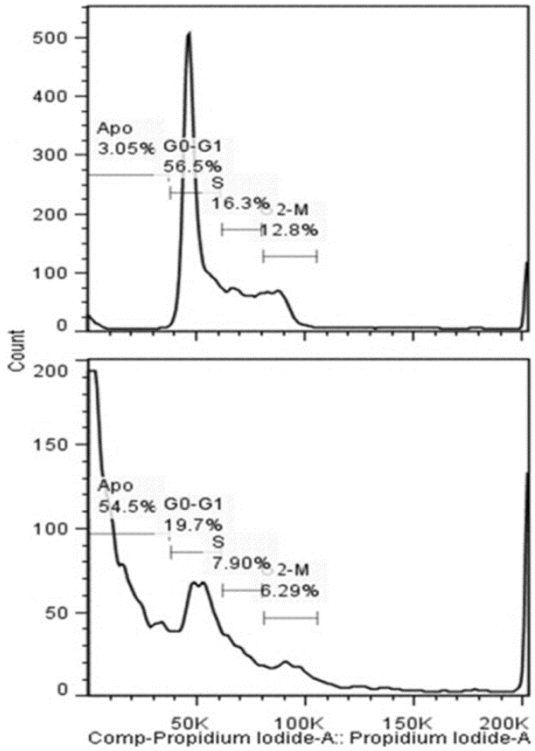

Fig. 4 — Cell cycle analysis of MOLT 4 cell line post β-sitosterol-3-O-β-D-glucoside treatment. MOLT 4 cells were treated with β-sitosterol-3-O-β-D-glucoside for 24 h and DNA was stained with propidium iodide and analyzed ona flow cytometer

β-sitosterol-3-O-β-D-glucosideincreased sub-G_1cell population of MOLT 4 cells

Cell cycle analysis of the treated MOLT 4 cells indicated that, β-sitosterol-3-O-β-D-glucoside effective increased the Sub-G_1 cell population after 24 h of treatment (Fig. 4) which further establish the apoptotic potential of the β-sitosterol-3-O-β-D-glucoside. At IC_{50} values, about 54% cells arrested at the Sub-G_1 phase of the cell cycle as compared to control set.

Discussion

Four bio-molecules has been isolated from the neem tree and their structures were determined by comparing the spectral data with previously reported data. Here we have investigated the four isolated bio molecules against two leukemic cells U937 and MOLT4 for their anti-proliferative potentiality. The result of the MTT assay indicated that out of four compounds tested, β-sitosterol-3-O-β-D-glucoside showed prominent cytotoxic activity against MOLT 4 cells but not against U937. Anti-proliferative activity in MOLT 4 cells was

dose and time dependent. All other isolated compounds did not show noticible anti-proliferaive effect. Isoquercetin showed proliferative activity in a dose dependent manner to both the cell lines tested. In this work we observed that the novel compound β-sitosterol-3-O-β-D-glucoside is much more effective against MOLT4 cell line which is a robust drug resistant cell line[19]. On the other hand β-sitosterol-3-O-β-D-glucoside showed minimal anti-proliferative effect against normal PBMC. It can be concluded that β-sitosterol-3-O-β-D-glucoside is a potent anti-leukemic agent that holds a great promise as a future drug in combating leukemia.

The nuclear DNA breakdown is an event associated with the cellular apoptosis[20]. To be assured about the apoptotic efficacy of β-sitosterol-3-O-β-D-glucoside against MOLT 4 cells, we investigated nuclear DNA breakdown event by DAPI staining. We found that at $IC_{50,}$ β-sitosterol-3-O-β-D-glucoside effectively increased the number of deformed and fragmented nuclei in the treated MOLT 4 cells. A number of cellular proteins, *e.g.* DFF, ICAD/CAD, endonuclease G, are associated with nuclear DNA breakdown[21]. Therefore, further studies will help to reveal the molecular mechanism hidden behind this observation.

Cell cycle analysis indicates that cells, those are arrested at Sub G0/G1 phase, undergo apoptosis[22]. Most of the chemotherapeutic drugs possess this potential[23]. Therefore, we studied cell cycle progression of MOLT 4 after compound treatments. We found that about 54% cells arrested at sub-G_1 phase and most of the G_0-G_1 cell population shifted to apoptotic population, which further establishes the apoptotic potential of the β-sitosterol-3-O-β-D-glucoside. However, the detail mechanism of cell cycle arrest needs to be studied in the future.

β-sitosterol-3-O-β-D-glucoside is a steroidal glycoside which has shown appreciable antiproliferative activity against MOLT 4 cells. According to previous reports, β-sitosterol-3-O-β-D-glucopyranoside which is structurally very close to β-sitosterol-3-O-β-D-glucoside selectively inhibited the activity of mammalian DNA polymerase λ[24]. Pol λ is a member of X family of DNA polymerase which performs a significant role in DNA repair mechanisms through resynthesizing missing nucleotides during non–homologous end joining during DNA double strand break. Hence, the antiproliferative activity may be achieved through several pathways including inhibiting the activity of DNA polymerase λ. Hence, blocking the DNA repair mechanism as well.

Conclusion

This study provides evidence for the therapeutic effectiveness of a novel molecule which can effectively block leukemia proliferation *in vitro.* It can efficiently increase Sub-G_1 population and fragmented nuclei in MOLT 4 cells. Further, molecular studies are required to understand the mechanism for this β-sitosterol-3-O-β-D-glucoside mediated cellular toxicity.

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Conflict of Interest

All authors declare no conflict of interest.

References

1 Hou Q, Liao F, Zhang S, Zhang D, Zhang Y, Zhou X, Xia X, Ye Y, Yang H, Li Z, Wang L, Wang X, Ma Z, Zhu Y, Ouyang L, Wang Y, Zhang H, Yang L, Xu H & Shu Y, Regulatory network of GATA3 in pediatric acute lymphoblastic leukemia. *Oncotarget*, 8 (2017) 36040.

2 Li W, Jiang Z, Li T, Wei X, Zheng Y, Wu D, Yang L, Chen S, Xu B, Zhong M, Jiang J, Hu Y, Su H, Zhang M, Huang X, Geng S, Weng J, Du X, Liu P, Li Y, Liu H, Yao Y & Li P, Genome-wide analyses identify KLF4 as an important negative regulator in T-cell acute lymphoblastic leukemia through directly inhibiting T-cell associated genes. *Mol Cancer*, 14 (2015) 26.

3 Vogler M, Walter HS & Dyer MS, Targeting anti-apoptotic BCL2 family proteins in haematological malignancies – from pathogenesis to treatment. *Br J Haematol*, 178 (2017) 364.

4 Sionov RV, Vlahopoulos SA & Granot Z, Regulation of Bim in health and disease. *Oncotarget*, 6 (2015) 23058.

5 Alzohairy MA, Therapeutics role of *Azadirachta indica* (Neem) and their active constituents in diseases prevention and treatment. *Evid Based Complement Alternat Med*, 2016 (2016) 7382506.

6 Wang L, Phan DDK, Zhang J, Ong PS, Thuya WL, Soo R, Wong ALA, Yong WP, Lee SC, Ho PCL, Sethi G & Goh BC, Anticancer properties of nimbolide and pharmacokinetic considerations to accelerate its development. *Oncotarget*, 7 (2016) 44790.

7 Rahmani AH, Almatroudi A, Alrumaihi F & Khan AA, Pharmacological and therapeutic potential of neem (*Azadirachta indica*). *Phcog Rev*, 12 (2018) 250.

8 Joy SD, Garg P, Verma A, Malik V, Maccune ER & Vasudeva A, Dentinal tubule disinfection with propolis & two extracts of *Azadirachta indica* against *Candida albicans* biofilm formed on tooth substrate. *Open Dent J*, 9 (2015) 369.

9 Akeel RA, Mateen A, Janardhan K & Gupta VC, Analysis of anti-bacterial and anti oxidative activity of *Azadirachta indica* bark using various solvents extracts. *Saudi J Biol Sci*, 24 (2017) 1.

10 Sushilkumar N & Neelavathy R, Herbs used in the treatment of arthritis-a review. *World J Pharm Med Res*, 2 (2016) 47.

11 Kumar S, Vandana UK, Agrawal D & Hansa J, Analgesic, Anti-inflammatory & anti-pyretic effects of *Azadirachta indica* (Neem) leaf extract in albino rats. *Int J Sci Res*, 4 (2015) 713.

12 Sunarwidhi AL, Sudarsono S & Nugroho AE, Hypoglycemic Effect of Combination of *Azadirachta indica* A. Juss. And *Gynura procumbens* (Lour.) Merr. Ethanolic Extracts Standardized by Rutin and Quercetin in Alloxan-induced Hyperglycemic Rats. *Adv Pharm Bull*, 4 (2014) 613.

13 Bhajoni PS, Meshram GG & Lahkar M, Evaluation of the antiulcer activity of the leaves of *Azadirachta indica*: an experimental study. *Integr Med Int*, 3 (2016) 10.

14 Moga MA, Bălan A, Anastasiu CV, Dimienescu OG, Neculoiu CD & Gavriș C, An Overview on the Anticancer Activity of *Azadirachta indica* (Neem) in Gynecological Cancers. *Int J Mol Sci*, 19 (2018) 3898.

15 Kashif M, Kim D & Kim G, *In vitro* antiproliferative and apoptosis inducing effect of a methanolic extract of *Azadirachta indica* oil on selected cancerous and noncancerous cell lines. *Asian Pac J Trop Med*, 11 (2018) 555.

16 Martelli AM, Tazzari PL, Tabellini G, Bortul R, Billi AM, Manzoli L, Ruggerei A, Conte R & Cocco L, A new selective AKT pharmacological inhibitor reduces resistance to chemotherapeutic drugs, TRAIL, all-*trans*-retinoic acid, and ionizing radiation of human leukemia cells. *Leukemia*, 17 (2003) 1794.

17 Badyal A, Sharma V, Kour N & Singh SK, Anticancer efficacy of methanolic extracts of some medicinal plants from Jammu region, Jammu & Kashmir, India. *Indian J Biochem Biophys*, 53 (2016) 51.

18 Ganeshpurkar A & Saluja A, *In silico* interaction of rutin with some immunomodulatory targets: a docking analysis. *Indian J Biochem Biophys*, 55 (2018) 88.

19 Majtnerová P & Roušar T, An overview of apoptosis assays detecting DNA fragmentation. *Mol Biol Rep*, 45 (2018) 1469.

20 Kitazumi I & Tsukahara M, Regulation of DNA fragmentation: the role of caspases and phosphorylation. *FEBS J*, 278 (2011) 427.

21 Bai J, Li Y & Zhang G, Cell cycle regulation and anticancer drug discovery. *Cancer Biol Med*, 14 (2017) 348.

22 Murad H, Hawat M, Ekhtiar A, Japawe AA, Abbas A, Darwish H, Sbenati O & Ghannam A, Induction of G_1-phase cell cycle arrest and apoptosis pathway in MDA-MB-231 human breast cancer cells by sulfated polysaccharide extracted from *Laurencia papillosa*. *Cancer cell Int*, 16 (2016) 39.

23 Mills CC, Kolb EA & Sampson VB, Development of chemotherapy with cell-cycle inhibitors for adult and pediatric cancer therapy. *Cancer Res*, 78 (2018) 1.

24 MizushinaY, Nakanishi R, Kuriyama I, Kamiya K, Satake T, Shimazaki N, Koiwai O, Uchiyama Y, Yonezawa Y, Takemura M, Sakaguchi K & Yoshida H, β-sitosterol-3-O-β-D-glucopyranoside: A eukaryotic DNA polymerase λ inhibitor. *J Steroid Biochem Mol Biol*, 99 (2006) 100.