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ORIGINAL ARTICLE

Anticancer effects of Mahanimbine alkaloid on the human bladder cancer cells are due to the induction of G0/G1 cell cycle arrest, apoptosis and autophagy

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Summary

Purpose: *The main purpose of the current research work was to investigate the anticancer effects of Mahanimbine alkaloid in human bladder cancer cells along with examining its effects on cellular apoptosis, cell cycle phase distribution, and cell autophagy.*

Methods: *Cell viability was examined by WST-1 cell viability assay. Mahanimbine-induced apoptosis was examined by fluorescent microscopy using acridine orange (AO)/ethidium bromide (EB) staining as well as using flow cytometry in combination with annexin-v/propidium iodide (PI) staining. Further, western blot assay was used to study the effects of Mahanimbine on apoptosis-related protein expressions including Bax and Bcl-2. Autophagy induction was evaluated by transmission electron microscopy (TEM) and western blot. Flow cytometry was used to study the effects on cell cycle.*

Results: *The results showed that Mahanimbine decreased the viability of the human bladder cancer cells and exhibited an IC_{50} of 32.5 μM. The test molecule also caused remarkable changes in the morphology of human bladder cancer cells and inhibited their colony forming potential. The AO/EB staining assay showed that Mahanimbine inhibits the viability of cancer cells via induction of apoptotic cell death which was associated with increase in Bax and decrease in Bcl-2 levels. The apoptotic cells increased from 5.2% in control to around 75% at 100 μM concentration. Mahanimbine also led to dose-dependent G0/G1 cell cycle arrest. Autophagic vacuoles appeared in the treated cells indicating autophagic induction by the test molecule. The Mahanimbine-triggered autophagy was also linked with increase in the expression of LC3II and decrease in p62 expression. However, no apparent effects were observed on the LC3 I expression.*

Conclusion: *Taken together, the results of this study indicate that Mahanimbine natural product has the potential to be developed as a promising anticancer agent against human bladder carcinoma but further studies are needed to this direction.*

Key words: *mahanimbine, bladder cancer, apoptosis, western blot, cell cycle*

Introduction

Bladder cancer (BC) accounts for nearly about 7% of malignancies prevailing worldwide, especially in males [1]. BC is counted among the 11 major cancers in the world and above 12 million new cases of this malignancy arise annually [2]. In past few decades, BC-associated mortality and incidence increased significantly in developing countries and Eastern European countries [3]. There is a number of reported risk factors accounting for BC incidence including genetic, high body mass index, environmental, nutritional, occupational hazards and behavioural factors [4-7]. Among the most important risk factors of BC occurrence, opium and cigarette smoking addiction are more lethal ones [8]. BC

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has two subtypes: muscle invasive bladder cancer (MIBC) and non-muscle invasive bladder cancer (NMIBC). NMIBC is associated with higher risk of mortality and poses higher number of challenges to researchers. Current effective treatments available for BC are surgical resection, chemotherapy with gemcitabine, mitomycin C and Bacillus Calmette-Guérin (BCG), immunotherapy and radiotherapy [9]. However, effective treatment regimens which would enhance patient survival in case of highly metastatic and recurrent BC are lacking [10]. Thus to curb such hurdles in the path of BC treatment, novel therapeutic approaches are the need of the hour. Natural products have been rich sources of chemotherapeutic drugs (especially plants and microbes) in the past, especially due to the huge diversity in plant species. It is supposed that they can act as a source of more potential drugs to combat many human diseases including cancer [11,12]. Alkaloids - a naturally occurring diverse class of compounds - are mostly found in higher plants belonging to Leguminosae, Ranunculaceae, Menispermaceae, Papaveraceae and Loganiaceae [13]. Alkaloids are pharmacologically active compounds with significant bio-activities including anticancer, analgesic and asthma relieving actions [13-16]. Some of these alkaloids have successfully being included as potential chemotherapeutic drugs including topoisomerase I (TopI), camptothecins (CPT) [17], vinca alcaloids and others. The current study was performed to assess the anticancer potential of Mahanimbine in human BC cells by examining induction of G0/G1 cell cycle arrest, apoptosis and autophagy.

Methods

Cell viability assay

Cell viability was performed through WST-1 cell proliferation assay using cytotoxicity assay kit (Beyotime institute of Biotechnology, China), following the manufacturer's protocol. Human BC cell line Hs172.T and normal bladder cell line RT24 were cultured in 96-well plates with 200 µl of Dulbecco's modified Eagle's medium (DMEM). Both the cell types were incubated in a 5% CO_2 incubator at 37°C for 48 h. After cells reached 70% confluence, they were subjected to Mahanimbine treatment at different doses (0, 2.5, 5, 10, 20, 40, 80 and 160 µM). Following drug treatment, 10 µl WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H tetrazolium) stock solution was added to each well of 96-well plate. Afterwards, cells were incubated at 37°C for 2 h and then the optical density (OD) was determined at 450 nm using a spectrophotometer (BioTek, USA).

Cell cycle phase distribution analysis

Flow cytometry was used for cell cycle phase distribution analysis. In brief, human Hs172.T BC cells were harvested and washed with phosphate buffered saline (PBS). Washed Hs172.T BC cells were then exposed to varying concentrations of Mahanimbine i.e. control, 5, 10 and 20 µM, followed by fixation with 70% ethanol and then again washed with PBS. Next, these treated cells were suspended in 50µl/ml of Annexin V/PI and 250 µg/ml of RNase1 solution and then subjected to incubation at room temperature for 30 min. Finally, grouping was done and 10,000 cells were placed in each group. These treated cells were then analysed through BD FACSCalibur flow cytometer.

Cell morphological changes via acridine orange/ethidium bromide (AO/EB) staining

Human Hs172.T BC cells were placed in 24-well culture plates at a density of 4×10^5 cells/well. On reaching 70-80% confluence the cells were treated with varying doses of Mahanimbine (12.5, 25 and 50 µM) followed by incubation for one day at 37°C in a 5% CO_2 incubator. Control cells were only treated with dimethyl sulfoxide (DMSO) (0.1%). Afterwards, cells were fixed with 4% formaldehyde and then stained with 10 µL of AO/EB solution for 10 min. Treated stained cells were finally observed under fluorescence microscope (Olympus Co., Tokyo, BX51TRF, Japan).

Annexin V-FITC/PI double staining for apoptosis analysis

Annexin V-FITC/PI apoptosis detection kit (Beyotime Institute of Biotechnology, China) was used as per the manufacturer's guidelines. In brief, human Hs172.T BC cells were harvested, treated with different doses of Mahanimbine (control, 12.5, 25 and 50 µM), then washed twice with PBS and binding buffer. Thereafter, treated cells were stained with annexin V-FITC/PI in the dark at room temperature for 15-20 min. Finally, flow cytometry (BD Biosciences, USA) was used to quantify fluorescence. Late and early apoptotic cells were recognised by annexin V + / PI + and annexin V + / PI– staining.

Transmission electron microscopy (TEM)

Human Hs172.T BC cells were collected and treated with different doses of Mahanimbine (control, 12.5, 25 and 50 µM). Treated cells were then fixed for 120 min with 2.5% of glutaraldehyde at 4°C. After fixation treated target cells were washed using PBS. Washing was followed by further fixation with Osmic acid (1%) for 90 min and then penetrated, embedded and dehydrated. Finally, ultra-thin slicing device was used to cut minute sections which were observed under transmission electron microscope (Olympus, Tokyo, Japan).

Western blotting analysis for determination of protein expressions

Human Hs172.T BC cells were treated with varying concentrations of Mahanimbine (control, 12.5, 25 and 50 µM), and lysed using RIPA lysing buffer. Each lysate bearing protein content was assessed through bicinchoninic acid (BCA) assay. First, protein samples were added on SDS-PAGE, followed by transference to nitrocellulose membranes. Afterwards, these mem-

branes were exposed to primary antibody treatment at 4°C for 24h, which was followed by HPR-conjugated secondary antibody treatment for one hour at 25°C. Protein bands were seen through Chemi- luminescence reagent.

Statistics

Data are shown as mean ± standard deviation and significance was analysed through one-way analysis of variance followed (ANOVA) by Student–Newman–Keuls multiple range test. P<0.05 was taken as statistically significant. All of the data was analysed through SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Cell cytotoxic effects of Mahanimbine on human Hs172.T bladder cancer cells

Cell cytotoxicity of Mahanimbine (Figure 1A) on both human BC cell line Hs172.T and normal bladder cell line RT24 were estimated via WST-1 cell proliferation assay. The results suggested that the test molecule was a powerful antiproliferative agent against BC cells. Further, on increasing drug dose concentrations (0, 2.5, 5, 10, 20, 40, 80 and 160 µM), the cytotoxic effect increased (decreasing viability from 100% to near about 5%) significantly indicating dose-dependency (Figure 1B). The cytotoxic effects of the test molecule on normal RT24 bladder cells were insignificant as compared to Hs172.T BC cells. IC_{50} value of the drug was calculated to be 32.5 µM.

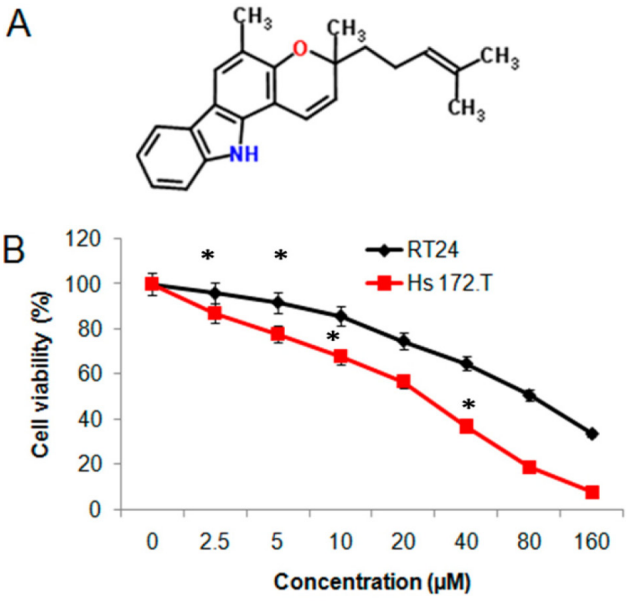

Figure 1. A: Chemical structure of Mahanimbine. **B:** Antiproliferative effect of Mahanimbine on Hs172.T BC cells as compared to normal RT24 BC cells at various dose concentrations for 24 h. All the experiments were carried in triplicate and data are expressed as mean ± SD values. *p<0.05.

Induction of cell cycle arrest in human bladder cancer cell line Hs172.T by Mahanimbine

After treatment of human BC cell line Hs172.T with varying molecule concentrations (control, 5, 10 and 20 µM), cell cycle phase analysis was performed through flow cytometry. The results showed that the number of G0/G1-phase cells increased massively with increasing drug doses as compared to G2/M-phase and S-phase cells. The percentage of G0/G1-phase cells increased from 20% to about 70%, indicating significant rise (Figure 2). To validate whether Mahanimbine indeed led to G0/G1-phase cell cycle arrest, examination of associated proteins was carried out through western blotting analysis, which clearly indicated significant alterations in the expressions of Cyclin D1, Cyclin D2, Cyclin D3 and Cyclin E. The expressions of Cyclin D1 and Cyclin D2 decreased with increasing drug concentrations and the expressions of Cyclin D3 and Cyclin E enhanced on increasing drug concen-

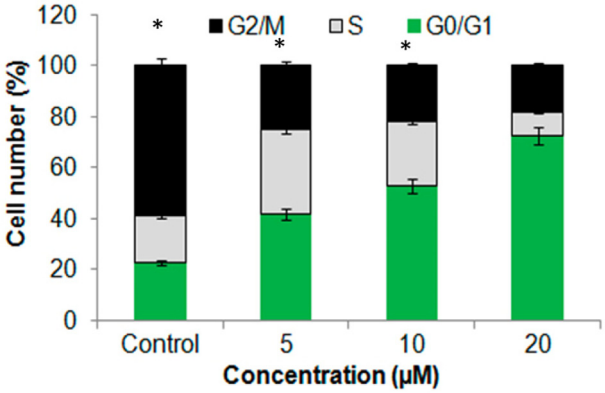

Figure 2. Cell cycle analysis of Mahanimbine-treated Hs172.T BC cells at varying dose concentrations i.e. control, 5, 10 and 20 µM for one day. All the experiments were carried in triplicate and data are expressed as mean ± SD values. *p<0.05.

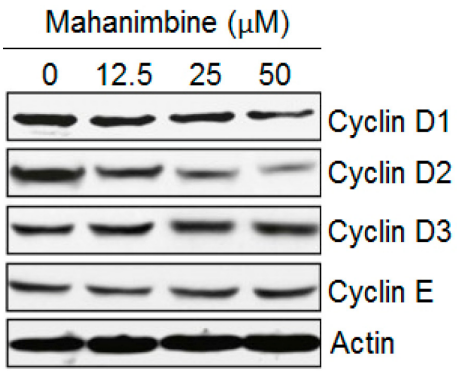

Figure 3. Effect of Mahanimbine on the expressions of Cyclin-D1, -D2, -D3 and –E. Actin was taken as loading control. The Figure shows the effects of Mahanimbine on the cell cycle related protein expression. All the experiments were carried out in triplicate.

trations (Figure 3), clearly indicating induction of G0/G1-phase cell cycle arrest by Mahanimbine.

Induction of apoptosis by Mahanimbine in human bladder cancer cell line Hs172.T

Apoptosis analysis was performed through AO/EB and annexin V-FITC/PI dual staining. AO/EB staining results after drug treatment revealed the presence of early and late stage apoptotic cells. Early stage apoptotic cells were identified through asymmetrical granular yellow-green AO or crescent-shaped nuclear staining. Late stage apoptotic cells revealed asymmetrical localized orange nuclear EB staining and further, both early and late apoptotic cell number increased on increasing drug concentrations (0, 12.5, 25 and 50 µM) (Figure 4). To quantify the effect of apoptosis induction by the test molecule annexin V/FITC/PI double staining was performed and the results revealed that the number of apoptotic cells increased tremendously on increasing drug concentrations (0, 12.5, 25 and 50 µM) (Figure 5). This was further confirmed through western blotting analysis by checking the levels of apoptosis-related proteins BAX and BCL-2. The observations revealed significant dose-dependent increase in BAX levels and decrease in BCL-2 levels (Figure 6). Thus, from the results of AO/EB staining, annexin V-FITC/PI dual staining and western blotting analysis, it is quite evident that Mahanimbine is a potential apoptosis inducer in human Hs172.T BCa cells.

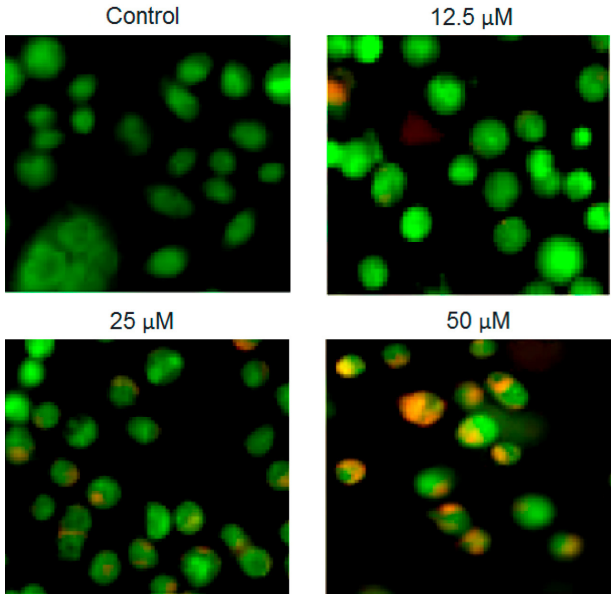

Figure 4. Morphological changes under fluorescence microscope after exposure to Mahanimbine and AO/EB staining of Hs172.T BC cells. The Figure shows induction of apoptosis in these cells by exhibiting yellow and orange fluorescence. All the experiments were carried out in triplicate.

Induction of autophagy by Mahanimbine in human bladder cancer cell line Hs172.T

Transmission electron microscopy (TEM) was used to examine autophagy induction by Mahanimbine. The results indicated that there was significant morphological changes in Hs172.T BC cells including agglutinated heterochromatin, degenerated mitochondrial vacuoles, nuclear swelling and the number of intracellular organelles declined. In addition, a major observation was the formation of autophagic vacuoles in treated BC cells. All the morphological changes were observed at control and 25 µM drug concentration (Figure 7). Furthermore, western blotting analysis was performed to check the expressions of autophagy-related proteins, and the results revealed that there was a slight enhancement in the levels of p-62 and LC3B-

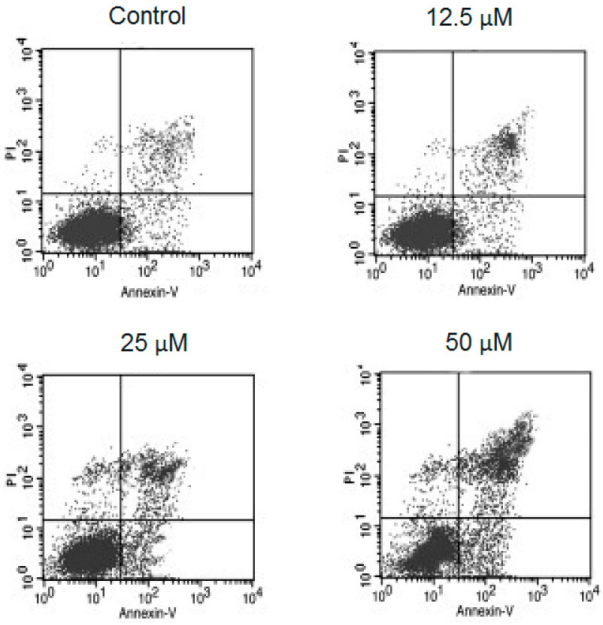

Figure 5. Apoptosis quantification after Mahanimbine treatment using annexin V-FITC/PI staining and flow cytometry. Mahanimbine induced dose-dependent induction of apoptosis. All the experiments were carried out in triplicate.

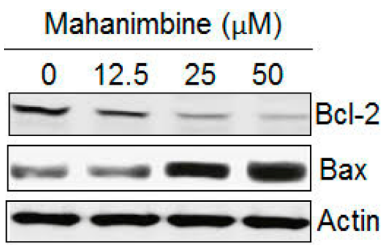

Figure 6. Effect of Mahanimbine on apoptosis-associated protein levels as indicated using Actin for internal control. The Figure shows dose-dependent decrease of Bcl-2 and increase of Bax. All the experiments were carried out in triplicate.

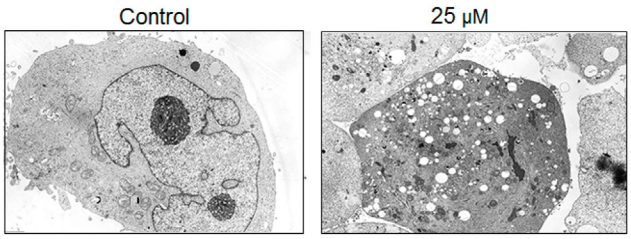

Figure 7. Effect of Mahanimbine treatment on Hs172.T BC cell morphology using transmission electron microscopy (TEM). Compared to the control group, autophagic vacuoles and degenerated mitochondria were observed in the Mahanimbine-treated group. All the experiments were carried out in triplicate.

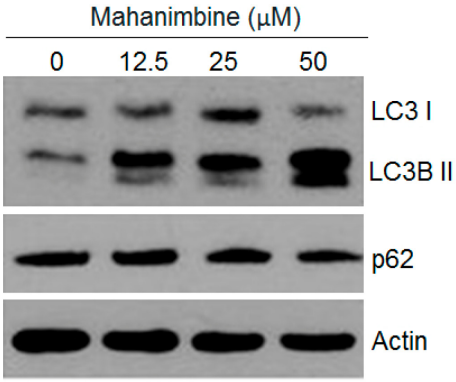

Figure 8. Mahanimbine exerted significant effects on autophagy-associated protein levels LC3-I, LC3B-II and p-62. Actin was taken as loading control. All the experiments were carried out in triplicate.

II, while no apparent effect was observed on LC3-I levels, which clearly indicates that Mahanimbine induces autophagic cell death in Hs172.T BC cells with a dose-dependent manner (Figure 8).

Discussion

Alkaloids are a major class of naturally occurring, pharmacologically active compounds with huge diversity. They have shown to play key roles in cancer cell growth suppression through topoisomerases inhibition (which play a major role in DNA replication), expression of p53 gene and apoptosis induction [18]. Some of the alkaloids bear structural resemblance with central nervous system associated neurotransmitters in humans, like acetylcholine, serotonin and dopamine. These effects of alkaloids on humans have encouraged scientists and researchers to develop potential anticancer agents and pain-killer drugs. Knowing the pharmacological significance of alkaloids and research illustrating their mechanism of action in the suppression of cancer cell growth would be helpful in framing lead molecules and drugs in the era of modern drug discovery. Mahanimbine is a naturally occurring carbazole alkaloid, found and extracted from various plant species [19]. Past studies regarding Mahanimbine have revealed its significant activity against different human cancers like leukemia and pancreatic cancer. The inhibition of pancreatic cells was mediated through apoptosis, cell cycle arrest, modulation of AKT/mammalian target of rapamycin (mTOR) activator and signal transducer of transcription 3 (STAT3) signalling pathways [20]. Herein, the anticancer activity of Mahanimbine was determined through its effect of cell cycle arrest, apoptosis induction and autophagy induction in human BC cells. WST-1 assay was performed to check the effect of the test molecule on cell viability of Hs172.T BCa cells and normal RT24 BC cells, with results indicating that the cell proliferation was suppressed significantly in case of cancer cells in a dose-dependent manner. Flow cytometry was performed to check the cell cycle phase distribution, which revealed tremendous increase in the number of G0/G1-phase cells as compared to S- and G2/M-phase cells, which clearly indicates cell cycle arrest at G0/G1-phase. The expressions of Cyclin D1 and Cyclin D2 decreased with increasing drug concentrations and the expressions of Cyclin D3 and Cyclin E increased upon increasing drug concentrations. Next, apoptosis analysis was performed through AO/EB and annexin V-FITC/PI staining, and the results showed formation of early and late stage apoptotic cells indicating apoptosis induction was dose-dependent. Apoptosis induction by this molecule was associated with increasing BAX and decreasing BCL-2 levels. Further, the autophagic effect on Hs172.T BC cells by the test molecule was evaluated by TEM, and the results showed significant morphological changes along with formation of autophagic vacuoles and it was observed that autophagy was concentration-dependent. Finally, the expression of autophagy-related proteins was determined through western blotting analysis which depicted significant alterations in p-62, LCB-II, indicating onset of autophagy.

Conclusions

In conclusion, Mahanimbine was shown to induce potent activity against Hs172.T BC cells and the results of all the above assays indicate that this molecule induces its anticancer effects via cell cycle arrest, apoptosis and autophagy. Thus it may be considered as potential therapeutic agent against human bladder cancer.

Conflict of interests

The authors declare no conflict of interests.

References

1. Afshari M, Janbabaei G, Bahrami MA, Moosazadeh M. Opium and bladder cancer: A systematic review and meta-analysis of the odds ratios for opium use and the risk of bladder cancer. PLoS One 2017;12:e0178527.
2. Ploeg M, Aben KK, Kiemeney LA. The present and future burden of urinary bladder cancer in the world. World J Urol 2009;27:289-93.
3. Chavan S, Bray F, Lortet-Tieulent J, Goodman M, Jemal A. International Variations in Bladder Cancer Incidence and Mortality. Eur Urol 2014;66:59-73.
4. Ghadimi T, Gheitasi B, Nili S, Karimi M, Ghaderi E. Occupation, smoking, opium, and bladder cancer: A case-control study. South Asian J Cancer 2015;4:111-4.
5. Ferroas J, Berbel O, Alonso-Lopez J, Garcia J, Ortega JA. Environmental non-occupational risk factors associated with bladder cancer. Actas Urol Esp 2013;37:579-86.
6. Aliramaji A, Kaseean A, Yousefnia Pasha YR et al. Age distribution types of bladder cancers and their relationship with opium consumption and smoking. Caspian J Intern Med 2015;6:82-6.
7. Lotfi MH, Farzaneh F, Mehrparvar AH, Fallahzadeh MH, Sadeghian MR. The Effect of Smoking and Opium on Bladder Cancer in Yazd Province: A Case - Control Study. JCHR 2016;5:98-109.
8. Mahmoodpoor A, Golzari SE. Epigenetics, opium, and cancer. Lancet Oncol 2014;15:e153.
9. Nargund VH, Tanabalan CK, Kabir MN. Management of non-muscle-invasive (superficial) bladder cancer. Semin Oncol 2012;39:559-72.
10. Cheng CL, Chao WT, Li YH et al. Escin induces apoptosis in human bladder cancer cells: An in vitro and in vivo study. Eur J Pharmacol 2018;840:79-88.
11. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 2016;79:629-61.
12. Harvey AL, Edrada-Ebel R, Quinn RJ. The re-emergence of natural products for drug discovery in the genomics era. Nat Rev Drug Discov 2015;14:111-29.
13. Huang L, Feng ZL, Wang YT, Lin LG. Anticancer carbazole alkaloids and coumarins from Clausena plants: A review. Clin J Nat Med 2017;15:881-8.
14. Lee MR. The history of Ephedra (ma-huang). J Royal College of Physicians of Edinburgh 2011;41:78-84.
15. Benyhe S. Morphine: new aspects in the study of an ancient compound. Life Sci 1994;55:969-79.
16. Li W, Shao Y, Hu L et al. BM6, a new semi-synthetic Vinca alkaloid, exhibits its potent in vivo anti-tumor activities via its high binding affinity for tubulin and improved pharmacokinetic profiles. Cancer Biol Ther 2007;6:787-94.
17. Huang M, Gao H, Chen Y et al. Chimmitecan, a novel 9-substituted camptothecin, with improved anticancer pharmacologic profiles in vitro and in vivo. Clin Cancer Res 2007;13:1298-1307.
18. Mohan K, Jeyachandran RD. Alkaloids as anticancer agents. Ann Phytomed 2012;1:46-53.
19. Shaikh M, Karpoormath R, Thapliyal N et al. Current perspective of natural alkaloid carbazole and its derivatives as antitumor agents. Anticancer Agents Med Chem 2015;15:1049-65.
20. Chenlin Pei, Qun He, Shuai Liang, Xuejun Gong. Mahanimbine Exerts Anticancer Effects on Human Pancreatic Cancer Cells by Triggering Cell Cycle Arrest, Apoptosis, and Modulation of AKT/Mammalian Target of Rapamycin (mTOR) and Signal Transducer and Activator of Transcription 3 (STAT3) Signalling Pathways. Med Sci Monit 2018;24:6975-83.