

Original Article

COMBINATION EFFECT OF ETHYLACETATE EXTRACTS OF *PLECTRANTHUS AMBOINICUS* (LOUR.) SPRENG. WITH DOXORUBICIN AGAINST T47D BREAST CANCER CELLS

POPPY ANJELISA ZAITUN HASIBUAN¹, JESSY CHRESTELLA², DENNY SATRIA³

¹Department of Pharmacology, ²Department of Anatomical Pathology, ³Department of Pharmaceutical Biology, University of Sumatera Utara, Medan, 20155

Email: poppyanjelisa94@gmail.com

Received: 01 Apr 2015 Revised and Accepted: 08 Aug 2015

ABSTRACT

Objective: To investigate the growth-inhibiting and apoptosis mediating effect of *Plectranthus amboinicus* (Lour.) Spreng ethyl acetate extract (PAE) in combination therapy with doxorubicin against T47D cell lines, to analyze the expression of cyclin D1 and COX-2 (cyclooxygenase-2) proteins.

Methods: The assays performed in the study were cytotoxicity assay, cell cycle assay, apoptosis induction, and immunocytochemistry of T47D cells. The cytotoxicity effects were determined by using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The effect on modulation of cell cycle and apoptosis was observed by flow cytometry assay in both single dose of PAE and its combination with doxorubicin. The expression of cyclin D1 and COX-2 proteins on T47D cell lines was identified by using immunocytochemistry.

Results: The result showed that the PAE 8 µg/ml had a synergistic effect with doxorubicin against T47D cells based on Combination Index analysis. The PAE induced apoptosis and cell accumulation at G1 phase. The combination of PAE 8 µg/ml with doxorubicin 1 µg/ml caused apoptosis induction same as its single treatment, but there was an increase in cell accumulation at G1 phase. Expression of cyclin D1 indicated that both single application and combination of PAE with doxorubicin could arrest the cell cycle of T47D cells. The expression of COX-2 showed that the combination could inhibit the metastasis of T47D breast cancer cells.

Conclusion: Based on the result that PAE could be a potential co-chemotherapeutic agent with doxorubicin on breast cancer cells.

Keywords: *Plectranthus amboinicus*, doxorubicin, T47D, cell cycle, apoptosis.

INTRODUCTION

Doxorubicin is one of chemotherapeutic agents which is widely used for breast cancers and it causes some problems such as drug resistance and toxic effects on normal tissue [1]. Cancer cells which are resistant have the ability to avoid apoptosis and may cause cell growth that is not restrained [2]. Combination of chemotherapeutics and chemopreventive agent with target cell cycle modulation and apoptosis was conducted to increase the efficacy of chemotherapeutics [3].

Plectranthus amboinicus, (Lour.) Spreng is used as a lactagogue in Batakese, North Sumatera. There are some informations about its effects such as anti-inflammatory, anti-clastogenic, nephroprotective and hepatoprotective. The previous studies had shown that the n-hexane, ethyl acetate and ethanol extracts of *Plectranthus amboinicus*, (Lour.) Spreng had antioxidant activity. The n-hexane and ethyl acetate extracts exhibited strong cytotoxic effect on T47D breast cancer cells with IC₅₀ value of 44.716 µg/ml and 37.61 µg/ml, respectively [4]. Antioxidant activity is usually correlated with cancer prevention. Thus, the extract has potential effect as a chemoprevention. A combination of chemotherapeutic agent and chemopreventive agent decrease side effect and become an effective strategy to cure cancer [2].

This research was conducted to investigate the growth-inhibiting and apoptosis mediating effect of *Plectranthus amboinicus*, (Lour.) Spreng ethyl acetate extract (PAE) in combination therapy with doxorubicin against T47D cell lines. Further, suppression of cyclin D1 and Bcl-2 on T47D breast cancer cells was analyzed.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Plectranthus amboinicus*, (Lour.) Spreng, were collected from Pematang Siantar, Simalungun regency, Sumatera Utara province, Indonesia. *Plectranthus amboinicus*, (Lour.) Spreng, was identified in Research Centre for Biology, Indonesian Institute of Science, Bogor, and the voucher specimen was deposited in

herbarium. Doxorubicin (Ebewe), DMSO (Sigma), [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma), H₂O₂ (Lab Vision Plus), chromogen 3,3'-diaminobenzidine (DAB) (Novo Castra).

Preparation of ethylacetate extract (PAE)

The air-dried and powdered leaves of *Plectranthus amboinicus*, (Lour.) Spreng (1 kg) were repeatedly extracted by cold maceration with n-hexane (3x3 d, 7.5 L). The powder was dried in the air and extracted with ethyl acetate (3x3 d, 7.5 L) at room temperature on a shaker. The filtrate was collected, and then evaporated under reduced pressure by rotary evaporator (Heidolph VV-200) to obtain a viscous extract and the concentrated extract was dried by freeze-dryer (Edwards).

Cytotoxicity assay

Cytotoxicity was determined by the MTT assay

Briefly, T47D cells were plated at 10⁴ cells/well in a 96-well plate. After incubation for 24 h at 37°C, cells were treated by *Plectranthus amboinicus* ethylacetate extract (PAE) with different concentrations and incubated for 24 h. MTT solution was added to each well and further incubated for 4 h at 37 °C, optical density was read with an ELISA reader at 595 nm [5].

Flow cytometry assay

Cell cycle inhibition assay

T47D cells (1x10⁶ cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with PAE, doxorubicin and their combination, and then incubated for 24 h. Both floating and adherent cells were collected in the conical tube using trypsin 0.025%. The cells were washed thrice with cold PBS and centrifuged 2500 rpm for 5 min. The supernatant was separated, while the sediment was collected and fixed in cold 70% ethanol in PBS at -20 °C for 2 h. The cells were washed thrice with cold PBS and re-suspended then centrifuged 3000 rpm for 3 min and

PI kit (containing PI 40 µg/ml and RNase 100 µg/ml) added to sediment and re suspended and incubated at 37°C for 30 min. The samples were analysed using FACScanflowcytometer. Based on DNA content, percentage of cells in each of stage in cell cycle (G1, S and G2/M) were calculated using ModFit Lt. 3.0. s [6, 7].

Apoptosis assay

T47D cells (5x10⁵ cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with PAE, doxorubicin and their combination, and then incubated for 24 h. Both floating and adherent cells were collected in the conical tube using trypsin 0.025%. The cells were washed thrice with cold PBS and centrifuged 2500 rpm for 5 min. The supernatant was separated, while the sediment was collected and fixed in cold 70% ethanol in PBS at -20°C for 2 h. The cells were washed thrice with cold PBS and centrifuged in 2500 rpm for 5 min. The supernatant was separated, while the sediment was collected and fixed in cold 70% ethanol in PBS at -20°C for 2 h. The cells were washed thrice with cold PBS and suspended then centrifuged 3000 rpm for 3 min and Annexin V kit added to sediment and suspended and incubated at 37°C for 30 min. The samples were analyzed using FACScan flow cytometer [8].

Immunocytochemistry

T47D cells (5x10⁴ cells/well) were seeded on cover slips in 24-well plate and incubated for 24 h. After that, the cells were treated with PAE, doxorubicin and their combination, and then incubated for 24 h. After incubation, the cells were washed with PBS and then fixed with cold methanol at 4°C for 10 min. After that, the cells were washed with PBS and blocked in hydrogen peroxide blocking solution for 10 min at room temperature, incubated using primary antibody Bcl-2 and cyclin D1 for 1 h, then washed thrice with PBS, then incubated with secondary antibody for 10 min. The cells were washed with PBS, then incubated in 3,3-diaminobenzidin (DAB) solution for 10 min, and washed with aquadest. Afterward, the cells were counter stained with Mayer-Haematoxylin for 5 min, and the cover slips were taken and washed with aquadest, and then immersed with xylol and ethanol 70%. Protein expression observed by light microscope (Nikon YS100). Cells that express a particular

protein will provide the brown colour, while the cells that does not give a specific protein will provide blue colour[2].

RESULTS

Synergistic effect of combination between PAE and doxorubicin could be occurred via cell cycle modulation (table 1 and fig. 1). Cell cycle analysis of T47D breast cancer cell lines showed that single treatment of doxorubicin induced G₀-G₁ arrest. Single treatment of PAE induced G₀-G₁ arrest, while in combination with doxorubicin induced cell accumulation in G₀-G₁ phase, as well. However, the combination doxorubicin-PAE remained lower cell accumulation on G₀-G₁ phase compared to PAE and doxorubicin single treatments. These facts indicated that the combination needs to be optimized.

Evaluation of apoptotic induction was performed by using flowcytometry assay with Annexin V. As shown in fig. 2, the cells in the upper and lower right quadrants represent late apoptotic/necrotic and early apoptotic cells, respectively. The percentage of PAE, their combination, and doxorubicin in early apoptotic was 6.56%; 15.48%; and 7.04%, respectively. Meanwhile, in late apoptotic/early necrotic the percentage was 15.82%; 1.65%; and 22.03%, and then in late necrotic were 48.20%; 3.10%; and 67.77%, respectively. The combination of PAE-doxorubicin exhibited higher in nearly apoptotic compared to PAE and doxorubicin single treatments.

The observation of expression of cell cycle regulator protein Cyclin D1 and angiogenic factor protein COX-2 was conducted in T47D cells by using PAE and doxorubicin, both a single application or in combination of them. In this study, the effects of PAE, doxorubicin and their combination on cyclin D1 and COX-2 were evaluated by using immunocytochemistry. The expression of cyclin D1and COX-2 proteins is positive characterized by brown stained nuclei in the cells (fig. 3 and fig. 4). High intensity of brown colour was shown on untreated cells (negative control) for cyclin D1 and COX-2 proteins. A single treatment of PAE decreased the cyclin D1 and COX-2 proteins expression. Single treatment of doxorubicin did not decrease cyclin D1 and COX-2 expression. However, the combination of PAE and doxorubicin was decreased these proteins expressions.

Table 1: MCF7 cells distribution after treatment of PAE, doxorubicin, and their combination for 24 h

Treatments	Concentration	Cell phase (%)		
		G ₀ -G ₁	S	G ₂ -M
control	-	33.12	26.66	15.82
PAE	8 µg/ml	36.99	25.90	10.16
Doxo-PAE	1 µg/ml-8µg/ml	34.26	27.82	12.05
Doxorubicin	1 µg/ml	58.77	19.13	3.06

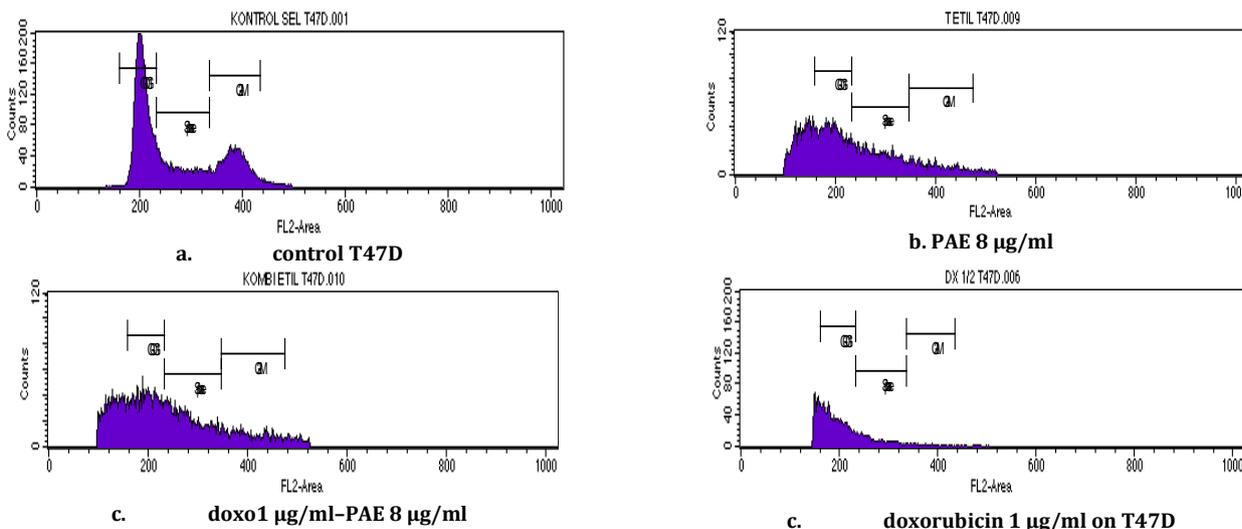


Fig. 1: Cell cycle analysis using flowcytometry. T47D cells were treated by doxorubicin, PAE, and their combination for 24 h and stained by using Propidium Iodide. (a) control cells; (b) PAE 8 µg/ml; (c) Doxo 1 µg/ml-PAE 8 µg/ml

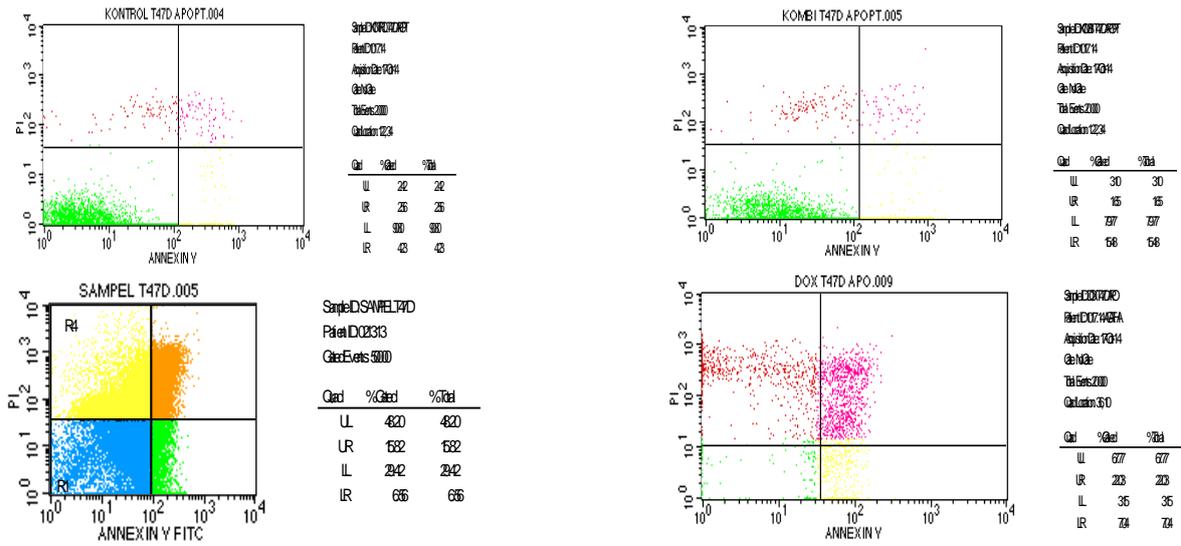


Fig. 2:Apoptotic analysis of PAE, doxorubicin and their combination on T47D cell lines. (a) cells control; (b) combination of PAE 8 µg/ml and doxorubicin 1 µg/ml; (c) PAE 8 µg/ml; (d) doxorubicin 1 µg/ml

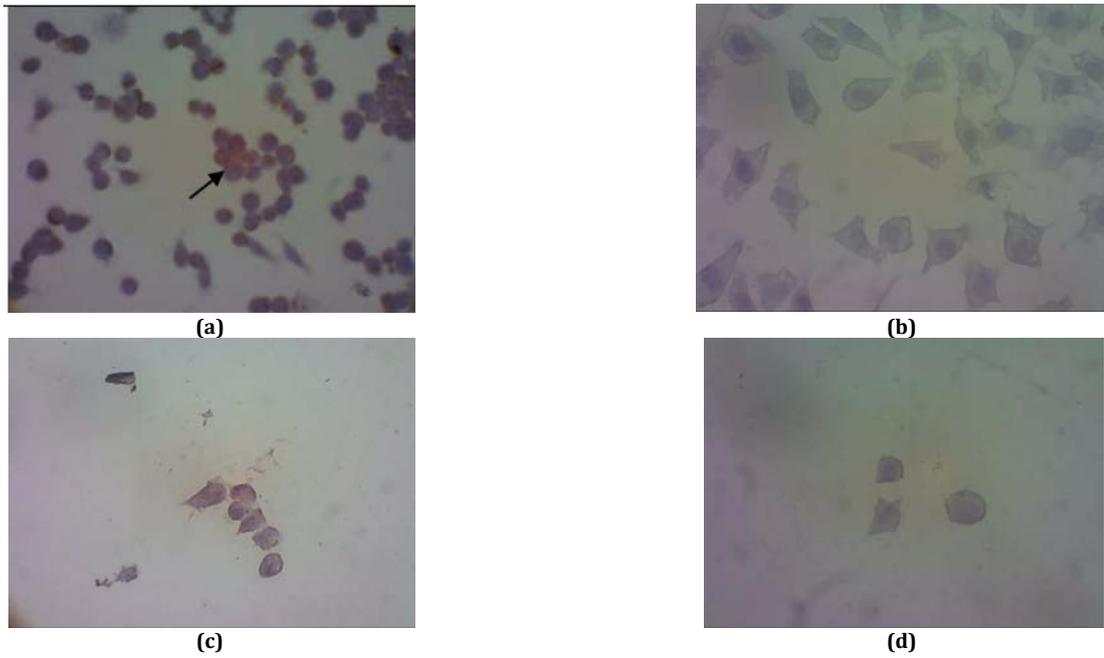


Fig. 3: Expression of cyclin D1 on T47D cells using immunocytochemistry. (a) control cells; (b) PAE 8 µg/ml; (c) doxorubicin 1 µg/ml; (d) combination of PAE 8 µg/ml and doxorubicin 1 µg/ml

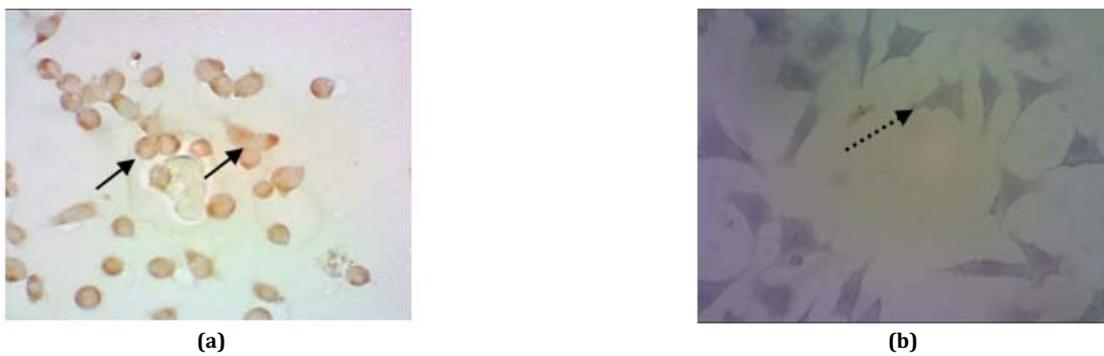




Fig. 4. Expression of COX-2 on T47D cells using immunocytochemistry. (a) control cells; (b) PAE 8 µg/ml; (c) doxorubicin 1 µg/ml; (d) combination of PAE 8 µg/ml and doxorubicin 1 µg/ml. (→ positive expression;→ negative expression)

DISCUSSION

The *Plectranthus amboinicus*, (Lour.) Spreng. leaves are used as lactagogue by native people in North Sumatera, Indonesia. One of its compounds is ursolic acid, which is known have chemotherapeutic effect [9, 10]. The result of cytotoxic assay estimated that this plant has the potential effect as anticancer[5]. Doxorubicin is one of chemotherapeutic agents which is less toxic to T47D cell lines because of the cell resistant by p53 mutation[11].

This study has shown that the PAE could induce the cell accumulation on G₀-G₁ phase but its combination with doxorubicin did not show cell accumulation. While the combination of PAE and doxorubicin showed increasing early apoptotic induction compared to late apoptotic/early necrotic and late necrotic. The cell growth retardation is possible through the cell cycle regulatory mechanisms. Both the single treatment of PAE and its combination with doxorubicin induced cell accumulation in G₁ phase, it is probably because of the ursolic acid effect. *In vitro* studies have shown that this compound in other plants reportedly inhibit the growth of numerous tumor cell lines including colon, breast, liver, prostate, and leukemia and inhibit the expression and activity of cyclo oxygenases [10, 11]. But the mechanisms need to be explored further.

The expression of cyclin D₁ of either single treatment or their combination strengthen the information about the effect of them on cell cycle accumulation especially in inhibition of cell cycle on G₀-G₁ phase. Cyclin D₁ play an important role in G₀-G₁ phase. The complex of cyclin D₁ with CDK-4 or CDK-6 controlled G₁ to S phase transition. Despite combination of PAE-doxorubicin could inhibit the cell cycle by decreasing level of cyclin D₁, it resulted inhibition of pRb phosphorylation. So, E2F cannot a part from pRb, and cells can not transcribe genes which is needed in cell cycle process or cell proliferation[7, 8].

Both single treatment and combination of PAE-doxorubicin did not express of COX-2 protein. It may be caused by ursolic acid effect which is known decrease the expression of matrix metalloproteinase-9 (MMP-9). Matrix metalloproteinase activities are involved in the remodelling of the extracellular matrix, part of the tumor micro-environment, and are thus linked to tumor invasion and increased risk of metastasis [11].

However, the molecular mechanisms of cell cycle modulation, apoptosis induction and potential anti angiogenic of this combination need to be explored more detail.

CONCLUSION

Based on the results, we concluded that the combination of ethyl acetate extracts of *Plectranthus amboinicus*, (Lour.) Spreng. with doxorubicin could increase the cell cycle arrest, inducing apoptotic, and decrease cyclin D₁ and COX-2 expressions. The extract has potential effect as co-chemotherapeutic agent for doxorubicin in breast cancer therapy.

CONFLICT OF INTERESTS

We declare that we have no conflict of interest.

ACKNOWLEDGEMENT

We gratefully thank to University of Sumatera Utara, Indonesia through "Hibah Bersaing" Research Grant 2014 for financial support in the study.

REFERENCES

- Adina AB, Goenadi FA, Handoko FF, Nawangsari DA, Hermawan A, Jenie RI, *et al.* Combination of ethanolic extract of citrus aurantifolia peels with doxorubicin modulate cell cycle and increase apoptosis induction on MCF7 cells. Iran J Pharm Res 2014;13:919-26.
- Rahmi F, Meiyanto E, Susidarti RA. Combination of doxorubicin and areca ethanolic extract induces apoptosis by increasing caspase-3 level on breast cancer (T47D) cells. Indonesian J Cancer Chemoprevention 2012;3:339-44.
- Kumala S, Meiyanto E, Ikawati M, Jenie RI. Sinergisme fraksi butanol metabolit skunder kapang endofit 1.3.11 dengan doxorubicin dalam modulasi daur sel T47D dan MCF7. J Ilmu Kefarmasian Indonesia 2010;8:1-9.
- Hasibuan PA, Rosidah Ilyas S, Nasution P. Antioxidant and cytotoxic activities of *Plectranthus amboinicus*, (Lour.) Spreng. Extracts. Int J Pharm Teach Practices 2013;4:755-8.
- Rosidah and Hasibuan PA. Cytotoxic effect of n-Hexane, Ethylacetate and ethanol extracts of *Plectranthus amboinicus*, (Lour.) Spreng. on HeLa and vero cells lines. Int J PharmTech Res 2014;6:1806-9.
- Anggraeni R, Hadisahputra S, Silalahi J, Satria D. Combinational effects of ethylacetate extract of *Zanthoxylum acanthopodium* DC. with Doxorubicin on T47D breast cancer cells. Int J PharmTech Res 2014;6:2032-5.
- Satria D, Furqan M, Hadisahputra S, Rosidah. Combinational effects of ethylacetate extract of *Picria fel-terrae* Lour. and Doxorubicin on T47D breast cancer cells. Int J Pharm Pharm Sci 2015;7:73-6.
- Hostanka K, Nisslein T, Freudenstein J, Reichling J, Saller R. Evaluation of cell death caused by triterpene glycosides and phenolic substances form cimifigua racemosa extract in human MCF-7 breast cancer cells. Biol Pharm 2004;27:1970-5.
- Kaewthawee N, Brimson S. The effect of ursolic acid on cytokine production via the MAPK pathways in leukemic T-Cells. EXCLI J 2013;12:102-14.
- Shan J, Xuan Y, Zheng S, Dong Q, dan Zhang S. Ursolic acid inhibits proliferation and induces apoptosis of HT-29 colon cancer cells by inhibiting the EGFR/MAPK Pathway. J Zhejiang Univ Sci B 2009;10:674-68.
- Neto CC. Ursolic acid and other pentacyclic triterpenoid: anticancer activities and occurrence in berries. In: Stoner, Seeram. editors. Berries and Cancer Prevention. Vol 11. Dartmouth: Springer Press; 2011. p. 41-7.