

Anticancer Effect of *Adenopus breviflorus* (Roberty) Extract Fruit on Human Breast Cancer (MCF-7) Cells

Oyedeji K.O.*¹, Danborno B.², Timbuak J.A.², Hussaini I.M.³

¹Department of Physiology, College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti, Nigeria.

²Department of Anatomy, College of Medical Sciences, Ahmadu Bello University, Zaria.

³Department of Pharmacology, Faculty of Pharmacy, University of Maiduguri, Maiduguri.

*sinaoyedeji@yahoo.com

Abstract

This study was designed to investigate the effect of ethanol extract of *Adenopus breviflorus* fruit on human breast cancer (MCF-7) cells. Cytotoxicity assay was carried out using the Trypan blue dye exclusion assay and Gentian crystal violet assay. Various concentrations of ethanol extract of *Adenopus breviflorus* (EEAB) i.e. 62.5, 125 and 250 µg/ml were prepared from stock solution. The concentration of reference standard #4 (250 µg/ml) was also prepared from stock solution. The effect of EEAB on mean cell count of MCF-7 was done using hemocytometer and the percentage cell viability was determined. The physical cytotoxic effect of EEAB on MCF-7 cells was done using Gentian crystal violet staining method. Treatment of MCF-7 cells with the reference standard #4 and extract caused significant ($p < 0.05$) reductions in the mean cell counts and percentage cell viability of viable MCF-7 cells relative to the DMSO treated cells. The untreated control MCF-7 cells and the DMSO (1.0 µg/ml) treated MCF-7 cells showed confluence, while the standard #4 (250 µg/ml) and EEAB (125 and 250 µg/ml) treated MCF-7 cells showed great reduction in cell population and cell density. It can therefore be concluded that ethanol extract of *Adenopus breviflorus* fruit probably has cytotoxic effect against human breast cancer (MCF-7) cells.

Keywords: *Adenopus breviflorus*, Human breast cancer (MCF-7), Cytotoxicity, Mean cell count.

INTRODUCTION

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body [1]. These contrast with benign tumors, which do not spread to other parts of the body. Possible signs and symptoms include a lump, abnormal bleeding, prolonged cough, unexplained weight loss and a change in bowel movements. While these symptoms may indicate cancer, they may have other causes. Over 100 types of cancers affect humans [2].

Among the medicinal plants that are in current use in Nigeria in the treatment of cancer is the fruit of the plant called *Adenopus breviflorus*.

Adenopus breviflorus belongs to the family of Cucurbitaceae. It is commonly called Wild colocynth in English language, "Ogbenwa" in Ibo language and "Tagiri" in Yoruba language [3]. It is a perennial tendril climber. It would usually lie on the ground for want of something to climb and climbs over shrubs and herbs by means of axillary tendrils. The leaves are simple, alternate and palmately veined [4].

Medicinally, the plant is used as a purgative in Tanganyika as well as a vermifuge and cathartic in Nigeria [3]. A decoction from the plant is said to be used in Nigeria for headache [3]. It is used in West Africa for a wide range of gastrointestinal disorders and measles in man. In southern Nigeria, its seed-decoction is reportedly given to pregnant women but the purpose is not stated [5]. It is used as an anticonvulsant, sedative and pain killer [6]. It is used with other medicinal plants as concoctions to aid parturition in humans [7]. Livestock farmers employ the fruit extract of the plant for the treatment of Newcastle disease and coccidiosis in animals [7]. The fruit is also used for money-making charms by Yoruba herbalists of South-Western Nigeria because of the cowrie-like inscriptions on its body [8].

Pharmacologically, it has been reported that the methanol extract of its whole fruit has anti-implantation activity [9] and abortifacient activity [10]. The ethanol extract of its whole fruit has been reported to have a broad spectrum antibacterial activity [11] as well as anti-oxidant and anti-ulcerogenic effects [12]. Its ethanol extract has been reported to have a little toxic and a lot of beneficial effects on the hematological functions and blood chemistry of male Wistar rats [13].

Since *Adenopus breviflorus* fruit has been reported by herbalists to have anticancer activity, this study therefore aims to authenticate the veracity of these claims on human breast cancer (MCF-7) cells.

MATERIALS AND METHODS

Cell culture

Human breast cancer (MCF-7) cells were supplied by the Cancer Research Laboratory, Faculty of Pharmacy, University of Maiduguri, Nigeria. They were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. The media were supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 g/ml). The cultures were maintained in a humidified 5% CO₂ incubator at 37 °C and the cells were sub-cultured every 3–4 days (with a solution of 0.05% trypsin-0.02% EDTA upon reaching 80% confluence) to maintain logarithmic growth and were allowed to grow for 24 hours before use.

Subculture of adherent cell line (MCF-7)

Cultures were viewed using an inverted microscope (Amscope, USA) to assess the degree of confluency and the absence of bacterial and fungal contaminants was confirmed. The T-25 flask was placed in laminar flow and the spent cultured medium was discarded with a sterile Pasteur pipette. The media was washed three times with

PBS to remove the serum (because without washing off the serum the trypsin will not be active) by adding 2 ml of PBS to the side of the T-25 flask opposite the cells to avoid dislodging the cells. Then, the cells were rinsed and the rinse was discarded afterwards. A total volume of 2 ml of trypsin-EDTA solution was added to the cells, ensuring the monolayer is completely covered. The T-25 flask was incubated for 2-5 minutes. The cells were examined using an inverted microscope to ensure that all the cells were detached and floated. The cells were aspirated using a micropipette into a centrifuge tube (50 ml) and fresh media was added into the centrifuge tube to inactivate the trypsin. The centrifuge tube containing the cells was centrifuged at 1000 rpm for 5 minutes. About 100-200 μ l was removed to perform a cell counting and staining. The required numbers of cells were transferred to a new labelled flask containing pre-warmed media and incubated for the cancer cell line (MCF-7).

Plant Material

Fresh samples of *Adenopus breviflorus* fruit were bought in Bodija Market, Ibadan, and were authenticated in the Taxonomy Unit of the Forestry Research Institute of Nigeria (FRIN), Jericho, Ibadan where a voucher specimen (FHI 108336) was deposited in their Herbarium.

Preparation of Crude Ethanol Extract

Large quantity (7.5 kg) of fresh specimens of the whole fruit of *Adenopus breviflorus* were washed free of debris and pulverized using mortar and pestle and air-dried for eight weeks. The resultant dried specimens (300 g) were macerated and extracted with 70 % ethanol for 72 hours at room temperature (26 - 28 °C). The resulting solution was then filtered using a wire-gauze and a sieve with tiny pores (0.25 mm). The 70 % ethanol was later evaporated using steam bath (40 - 45 °C) to give a percentage yield of 8.6 % of the starting sample. The dried sample was reconstituted in distilled water to make up test solutions of known concentration.

Experimental Design

Cytotoxicity assay

(a) Cell viability assay (Trypan blue dye exclusion assay)

It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. Trypan blue is a blue acid dye that has two azo chromophores group. The percentage of viable and non-viable cells was determined using trypan blue exclusivity stain. The cell line (MCF-7) was grown at 37 °C at humidified 5% CO₂ in RPMI 1640 medium. The RPMI 1640 medium was filtered using 0.45 μ m membrane filter. The extract (250 mg) put in 1.5 ml sterile Eppendorf tube was dissolved in 1.0 ml DMSO using a vortex (Thermolyne) and then filtered using a sterile micron filter (0.22 μ m). Various concentrations of the extract (EEAB) i.e. 62.5, 125 and 250 μ g/ml were then prepared from the stock solution. The concentration (250 μ g/ml) of the reference standard #4 was also prepared from the stock solution. Untreated control cancer cells (MCF-7) were seeded at a density of 2×10^4 cells/well into three wells (triplet) in a 24-well plate and incubated in culture

medium i.e. RPMI 1640 medium only. Treated cancer cells (MCF-7) were also seeded at a density of 2×10^4 cells/well into three wells (triplet) in a 24-well plate and they were treated with DMSO (1.0 μ l/ml), reference standard #4 (250 μ g/ml) and different concentrations of the extract (62.5, 125 and 250 μ g/ml) and incubated at 37 °C for 48 hours at 5% CO₂. After 48 hours, cells were washed three times with PBS to remove dead cells, floating cells and serum. The adherent cells were released from the plates with 0.05% w/v trypsin-EDTA (30 to 60 μ l/well) and incubated for 2 minutes and the trypsin activity was halted by the addition of equal volume (30 to 60 μ l/well) of serum-free RPMI 1640 medium. The cancer cells were then transferred into the centrifuge tubes and centrifuged at 1000 rpm for 5 minutes. After centrifuging, PBS (0.25 ml) was added and then 0.4% w/v Trypan blue (0.25 ml) was also added and mixed well using micropipette.

Fix the coverslip on the centre top of the hemocytometer, the notches should be partially exposed. Using a micropipette, 20 μ l of the suspended cell/Trypan blue solution was dispensed into the notches of the hemocytometer. The suspended cells/Trypan blue solution would pass under the coverslip by capillary action unless there is an air bubble. Make sure the notches are not overfilled and that the coverslip is not moved once it is placed on the grid and the suspended cells/Trypan blue solution is added. The hemocytometer assembly was placed on the stage of a phase contrast inverted microscope and then focus, the power of the microscope was adjusted on the hemocytometer grid until a single counting square fills the field. The observed live cells were clear in colour while the dead cells were blue in colour (Stained cells were the dead cells and unstained cells were counted as viable cells).

There are four main quadrants in haemocytometer and they are subdivided into 16 squares. For each experiment (per well), cells were counted from the four quadrants of the hemocytometer.

The percentage cell viability was determined by using the following formula:

% Cell Viability

$$= \frac{\text{Total number of extract treated viable cells} \times 100}{\text{Total number of DMSO treated viable cells}}$$

(b) Crystal violet assay

The cytotoxicity was also evaluated by the crystal violet staining method. The crystal violet assay is designed to obtain quantitative information about the relative density of adherent cells [14]. The crystal violet assay stains the nuclei of viable adherent cells.

After incubating the untreated control cancer cells, the DMSO treated cells, the reference standard #4 treated cells and the extract treated cells for 48 hours in 24-well plates as explained in (a) above, the media were aspirated with a Pasteur pipette and subsequently washed two times gently with distilled water to remove floating dead cells. Thereafter, 0.25 ml of 5% w/v Gentian crystal violet dye was added to each well for about 30 minutes. The stain was removed and the plates were rinsed using distilled water and air-dried and the cells were subsequently

photographed using a digital camera at x100 magnification.

Statistical Analysis

Data were expressed as mean ± S.E.M (Standard Error of Mean). Comparison between the control and experimental groups were done using one - way analysis of variance (ANOVA) with Duncan’s multiple range test using SPSS (IBM, Armonk, NY) software (version 23). Differences were considered statistically significant at p<0.05.

RESULTS

Effect of Treatment on Mean Cell Count and Cell Viability

Treatment of MCF-7 cells with the reference standard #4 and the extract (250 µg/ml) caused significant (p<0.05) reductions in the mean cell count and percentage cell viability of viable MCF-7 cells relative to the DMSO treated cells (Figures 1 and 2).

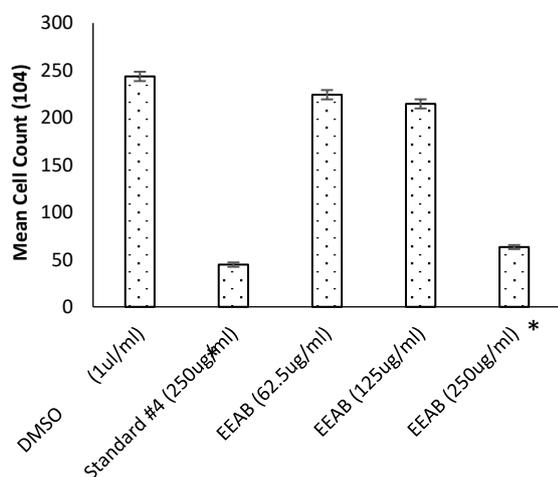


Figure 1: Effect of different concentrations of ethanol extract of *Adenopus breviflorus* (EEAB) on mean cell count of MCF-7 cells.

Data presented as mean ± S.E.M. (n=12), *p<0.05.

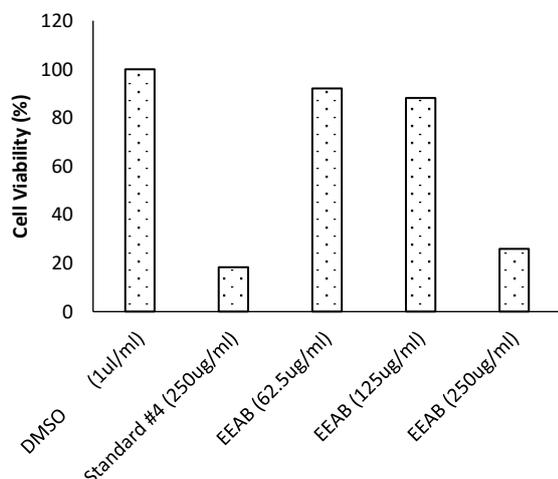


Figure 2: Effect of different concentrations of ethanol extract of *Adenopus breviflorus* (EEAB) on percentage cell viability of MCF-7 cells.

Effect of Treatment on Cell Viability

The untreated control MCF-7 cells and the DMSO treated MCF-7 cells show confluent cells (large cell number and high cell density) while the standard #4 and EEAB treated MCF-7 cells show reduction in cell number and cell density (Plates I, II, III and IV).

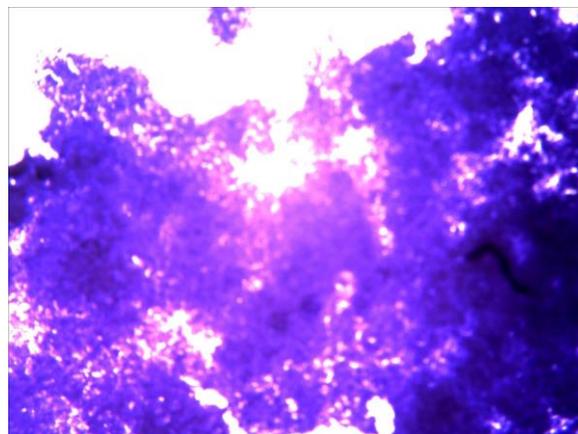


Plate I: Untreated control MCF-7 cells stained with Gentian crystal violet dye (x100).

Photomicrograph showing large cell population and high cell density.

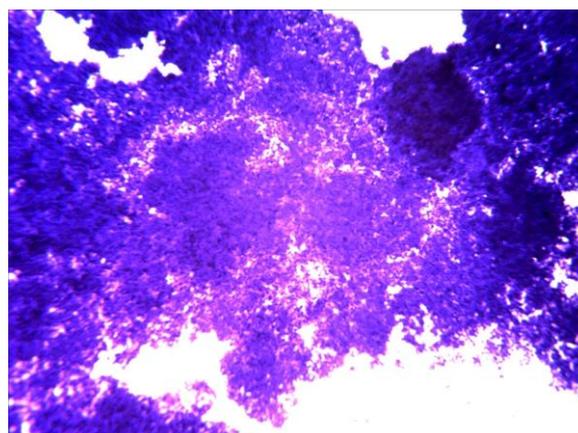


Plate II: DMSO (1.0 µl/ml) treated MCF-7 cells stained with Gentian crystal violet dye (x100).

Photomicrograph showing large cell population and high cell density.

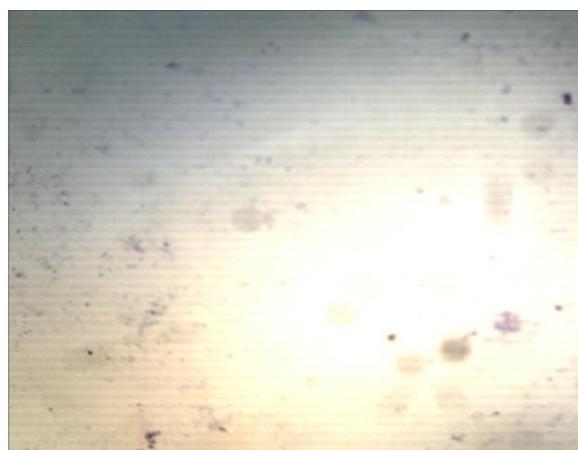


Plate III: Standard #4 (250 µg/ml) treated MCF-7 cells stained with Gentian crystal violet dye (x100).

Photomicrograph showing reduction in cell population and low cell density.

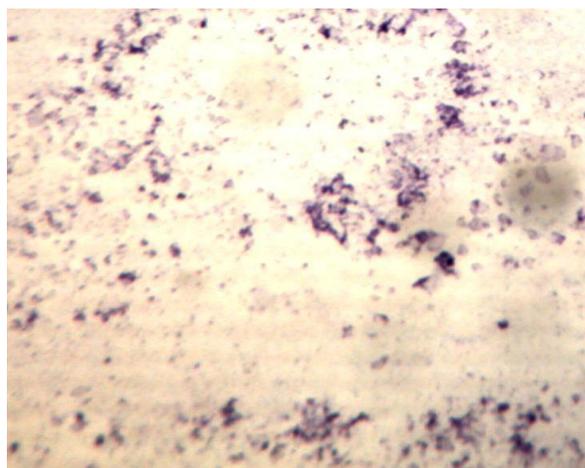


Plate IV: Ethanol extract of *Adenopus breviflorus* (250 µg/ml) treated MCF-7 cells stained with Gentian crystal violet dye (x100).

Photomicrograph showing great reduction in cell population and low cell density.

DISCUSSION

Determination of cytotoxicity, commonly used to evaluate the biological activity of natural products, is helpful to confirm whether plant extracts have potential antineoplastic properties [15]. In this study, the ethanol extract of *Adenopus breviflorus* was investigated for its cytotoxic activity against human breast cancer (MCF-7) cell lines with #4 serving as reference standard using Trypan blue exclusion and Gentian crystal violet assays.

The results have shown that the extract possess a significant cytotoxic activity against the breast cancer (MCF-7) cells by significantly reducing the mean cell count and percentage cell viability of breast cancer (MCF-7) cells. Similar result was reported by [16] in human breast cancer cells (MCF-7) treated with n-butanol extracts of *Streptomyces*. Breast cancer is the major contributor to cancer-related deaths in women and despite the advances in early detection and the understanding of the molecular basis of breast cancer biology, about 30 % of patients with early-stage breast cancer has recurrent disease [17]. To offer more effective and less toxic treatments, selecting therapies including natural agents have drawn great attention of the scientific community and the general public for their ability to suppress cancers [18].

The anticancer activity of this extract could also be due to apoptosis since it has been reported that the extracts of *Adenopus breviflorus* are capable of induction of programmed cell death (apoptosis) through the modulation of mitochondrial membrane permeability transition (MMPT) pore signalling pathway [19]. Apoptosis is a hallmark for the appraisal of potential agents for cancer prevention, and a wide variety of natural products have been known to interfere with cell proliferation or induce apoptosis [20]. The morphological characteristics of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation and apoptotic body formation [21].

The anticancer activity of this extract could be due to DNA damage. DNA fragmentation is a canonical

biochemical apoptotic feature. Many plants extracts have been shown to induce DNA damage [22]. DNA damage often involves several signaling pathways, which can also result in cell cycle arrest and eventual apoptosis [23]. DNA damage activates P53 to induce G1 and G2/M phase arrest and apoptosis [24].

The anticancer activity of this extract could be due to increasing intracellular reactive oxygen species (ROS) levels. Excessive amounts of ROS can cause DNA damage, cell cycle arrest and apoptosis [25]. Although all aerobic cells are equipped with protective enzymatic and non-enzymatic antioxidants, increased oxidative stress may overwhelm the protective mechanisms, leading to cell injury [26]. Many natural compounds, such as flavonoids and triterpenoids, have been reported to increase oxidative stress by increasing intracellular ROS levels [27]. Thus, ROS are considered an important factor of natural anticancer agents. Cells are known to thrive in low levels of ROS, but a relative increase in ROS induces cell cycle arrest and apoptosis [28]. ROS-modulating drugs are, however, being proposed as therapeutic strategies to selectively target the destruction of cancer cells [29].

In conclusion, these findings have lent some credence to the ethnomedicinal use of the *Adenopus breviflorus* in folklore medicine for the management of cancer. Its therapeutic potential is huge and can be used as alternative to or supplementation for the various therapies currently used in the treatment of breast cancer. On the basis of this study, *Adenopus breviflorus* fruit may serve as an excellent lead for the development of anticancer agent for breast cancer which can be further investigated for pharmaceutical applications and achievement of novel anticancer compounds.

Considering these findings on the effect of ethanol extract of *Adenopus breviflorus* fruit on human breast cancer (MCF-7) cells, it is recommended that people suffering from breast cancer could consume *Adenopus breviflorus* fruit as an alternative to or supplementation for the various therapies currently used in the treatments of breast cancer.

Conflict of Interest

We vehemently declare that there is no conflict of interests in this research work.

REFERENCES

- [1] World Health Organization, WHO. Cancer Fact sheet N°297. Retrieved 21 March, 2018.
- [2] National Cancer Institute. Targeted cancer therapies. www.cancer.gov. Retrieved 28 March 2018.
- [3] Ainslie JR. The list of plants used in native medicine in Nigeria, Imp. Forest. Inst. Oxford Inst., 1937; Paper 7 (mimeo).
- [4] Dutta AC. Botany for Degree Students 6th ed. Oxford University Press, Calcutta, 1995; India.
- [5] Dalziel JM. The useful plants of west tropical Africa, London: Crown agents for the colonies, 1937.
- [6] Burkill HM. The useful plants of West Tropical Africa, vol.4. The Whitefriars Press Limited, Tonbridge, Kent TN9 1QR, Great Britain, 1985.
- [7] Sonaiya EB. Family poultry and food security: Research requirements in science, technology and socioeconomics SONAIYA, 1999.
- [8] Oyedemi KO. Effect of ethanol extract of *Adenopus breviflorus* (Roberty) fruit on animal models of depression. J Pharm Sci & Res 2019; 11 (12): 3725-3731.

- [9] Elujoba AA, Olagbende SO, Adesina SK. Anti-implantation activity of the fruit of *Lagenaria breviflora* Robert. J Ethnopharmacol 1985; 13: 281-288.
- [10] Elujoba AA, Hymete A. Abortifacient activity of the fruit pulp of *Lagenaria breviflora*. Fitoter 1986; 57: 97-101.
- [11] Tomori OA, Saba AB, Dada-Adegbola HO. Antibacterial activity of ethanolic extract of whole fruit of *Lagenaria breviflora* Robert. J Anim Vet Adv 2007; 6 (Suppl 5): 752-57.
- [12] Onasanwo SA, Singh N, Saba AB, Oyagbemi AA, Oridupa OA, Palit G. Anti-ulcerogenic and *in vitro* antioxidant activities of *Lagenaria breviflora* whole fruit ethanolic extract in laboratory animals. Pharmacog Res 2011; 1 (Suppl 3): 2-8.
- [13] Oyedemi KO, Adurodija MN, Adeleye AS, Abidoye D. Effect of ethanol extract of *Adenopus breviflorus* on hematological and plasma biochemical parameters in male albino rats. Int J Pharm Sci Rev Res 2015; 35 (2): 36-40.
- [14] Kueng W, Silber E, Eppenberger U. Quantification of cells cultured on 96-well plates. Analytical Biochem 1989; 182 (1): 16-19.
- [15] Arunporn I, Peter J, Houghton E, Amoquaye E. In vitro cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. J Ethnopharmacol 2004; 90 (1): 33-38.
- [16] Maher O. Cytotoxicity of n-Butanol Extracts of *Streptomyces* against human breast cancer cells. International J Pharmacol 2017; 13 (8): 969-979.
- [17] Gonzalez-Angulo AM, Morales-Vasquez F, Hortobagyi GN. Overview of resistance to systemic therapy in patients with breastcancer. Advances in Experiment Med and Biol 2007; 608:1-22.
- [18] Aggarwal BB, Shishoda S. Molecular targets of dietary agents for prevention and therapy of cancer. Biochem Pharmacol 2006; 71:1397-1421.
- [19] Oyedemi T, Akintehinse T, Avan E, Oluwaremi S, Oluwatumininu S, Olorunsogo O. Extracts of *Adenopus breviflorus* induce opening of rat liver mitochondrial membrane permeability transition pore. Biokemistri 2017; 29: 4.
- [20] Taraphdar AK. Natural products as inducers of apoptosis: Implication for cancer therapy and prevention. Current Sci 2001; 80:1387-1396.
- [21] Rello S, Stockert JC, Moreno V, Gámez A, Pacheco M, Juarranz A, Villanueva A. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. Apoptosis 2005; 10: 201-208.
- [22] Wang Y, Cheng X, Wang P *et al*. Investigating migration inhibition and apoptotic effects of *Fomitopsis pinicola* chloroform extract on human colorectal cancer SW-480 cells. PLoS ONE 2014; 9: e101303.
- [23] Leventis PA, Grinstein S. The distribution and function of phosphatidylserine in cellular membranes. Annual Review of Biophysics 2010; 39: 407-427.
- [24] He N, Shi X, Zhao Y, Tian L, Wang D, Yang X. Inhibitory effects and molecular mechanisms of selenium-containing tea polysaccharides on human breast cancer MCF-7 cells. J Agric Food Chem 2013; 61: 579-588.
- [25] Nazirođlu M, Uđuz AC, Koçak A, Bal R. Acetaminophen at different doses protects brain microsomal Ca²⁺ ATPase and the antioxidant redox system in rats. J Memb Biol 2009; 231: 57-64.
- [26] Guney M, Ozguner F, Oral B, Karahan N, Mungan T. 900 MHz radiofrequency-induced histopathologic changes and oxidative stress in rat endometrium: protection by vitamins E and C. Toxicol Industrial Health 2007; 23: 411-420.
- [27] Huang Q, Lu G, Shen HM, Chung MC, Ong CN. Anti-cancer properties of anthraquinones from rhubarb. Medicinal Res Rev 2007; 27: 609-630.
- [28] Li H, Chen J, Xiong C, Wei H, Yin C, Ruan J. Apoptosis induction by the total flavonoids from *Arachniodes exilis* in HepG2 cells through reactive oxygen species-mediated mitochondrial dysfunction involving MAPK activation. Evidence-Based Complement Alternat Med 2014; 2014:11.
- [29] Lampiasi N, Azzolina A, D'Alessandro N, Umezawa K, McCubrey JA, Montalto G, Cervello M. Antitumor effects of dehydroxymethylepoxyquinomicin, a novel nuclear factor- κ B inhibitor, in human liver acancer cells are mediated through a reactive oxygen species-dependent mechanism. Molecular Pharmacol 2009; 76 (2): 290-300.