AENSI Journals

Advances in Environmental Biology

ISSN-1995-0756 EISSN-1998-1066

Journal home page: http://www.aensiweb.com/AEB/

Cytotoxic Effect of Red Seaweeds *Kappaphycus alvarezii* and *Kappaphycus striatum* on Hepatocarcinoma HepG2 Cell Line

[1,2]Farah Diyana Ariffin, [2]Aminah Abdullah, [1]Chan Kok Meng, [3]Shahrul Hisham Zainal Ariffin and [1]Mazrura Sahani

[1]*Environmental Health & Industrial Safety Programme, Faculty of Health Sciences, Universiti Kebangsaan Malaysia.Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*
[2]*Food Science Programme, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia*
[3]*Biochemistry Programme, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia*

ARTICLE INFO

Article history:
Received 25 July 2014
Received in revised form
8 August 2014
Accepted 15 September 2014
Available online 25 October 2014

Keywords:
Insert keywords for your paper

ABSTRACT

Background: Food antioxidants have been considered as effective agents to reduce oxidative stress which can lead to cancer. **Objective:** The aim of this study was to investigate the potential cytotoxic effect of antioxidant extracts of two commonly found seaweeds namely *Kappaphycus alvarezii* and *Kappaphycus striatum* against hepatocarcinoma HepG2 cell. **Methods:** Cell viability was evaluated by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Annexin V-FITC/PI flowcytometry assay was used to determine the cell death mode of HepG2 cells treated by *K. alvarezii* and *K. striatum* extracts. **Results:** The IC_{50} concentration of *K. alvarezii* and *K. striatum* extracts that inhibit the proliferation of hepatocarcinoma HepG2 cells was 1.8 mg/mL and 0.9 mg/mL respectively. This finding showed that the antioxidant extracts of *K. striatum* exhibited better antiproliferative effect against hepatocarcinoma HepG2 cell than the antioxidant extracts of *K. alvarezii*. However, using Annexin V-FITC/PI showed more than 80% of hepatocarcinoma HepG2 cell were viable after treatment with IC_{50} concentration of each *K. alvarezii* and *K. striatum* extract. This result suggested that cytostatic effect of *K. alvarezii* and *K. striatum* extracts to hepatocarcinoma HepG2 cells was found at high concentrations. **Conclusion:** The result of the study indicated that antioxidant extracts of *K. alvarezii* and *K. striatum* did not show cytotoxic effect to hepatocarcinoma HepG2 cells.

To Cite This Article: Farah Diyana Ariffin, Aminah Abdullah, Chan Kok Meng, Shahrul Hisham Zainal Ariffin and Sahani., Cytotoxic Effect of Red Seaweeds Kappaphycus alvarezii and Kappaphycus striatum on Hepatocarcinoma HepG2 Cell Line. ***Adv. Environ. Biol., 8(15),*** *79-84, 2014*

INTRODUCTION

Most cancer incidence attributed to DNA damage that occurs due to oxidative stress [1] arising from the continuously formation of reactive oxygen species (ROS) in large quantities through a variety of mechanisms [2]. However, there is a report stated that antioxidants may inhibit the development of cancer as an early study on legumes has inhibited the development of hepatocellular carcinoma cancer cells (HepG2 cells) [3]. Other researcher also reported that plants rich in antioxidants are capable of inhibiting the formation of ROS [4]. Phenolic antioxidants also may be able to reduce oxidative stress in vitro and in vivo, thereby preventing carcinogenesis and inhibit the proliferation of cancer cells [5].

Antioxidants have two main sources which are natural antioxidants and synthetic antioxidants [6]. However, synthetic antioxidants are associated with health effects such as the occurrence of tumours in vivo tests, asthma, eczema [7] and DNA damage [8]. Therefore, researchers are looking for antioxidants from natural sources that are cheap [9], can inhibit the oxidation process in the food system [10] and effectively inhibit free radical to replace synthetic antioxidants [11]. Nowadays, aquatic plants such as seaweeds are ranked among the most important source of natural antioxidants [10].

Seaweeds are classified based on their pigmentation to three types namely brown seaweed (Phaeophyta), red (Rhodophyta) and green (Chlorophyta) [12]. In Malaysia, seaweeds are used as food and medicine apart from its importance as a hydrocolloids source [13] because it has important components of food nutrients for

Corresponding Author: Prof. Dr. Aminah Abdullah, Food Science Programme, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
Tel: +603-89215990; E-mail: aminahsensory@gmail.com

human nutrition [14, 15], vitamins A and C [16, 17] as well as bioprotective features such as antioxidants and antimicrobials [18].

Among the compounds found in seaweed; antioxidants are substances that become a major concern for producing protection mechanisms such as antioxidant protection system that can adapt to extreme environments [19, 20]. Past research had found that seaweed extract that contains antioxidant showed antiproliferative activity against cancer cells such as cervical cancer cells (HeLa cells) [21] and breast cancer cells MDA-MB-231 [22]. Therefore, this study was conducted to assess the cytotoxic effects of the antioxidant extract of red seaweeds *Kappaphycus alvarezii* and *Kappaphycus striatum* against hepatocarcinoma HepG2 cells.

MATERIALS & METHODS

Sample preparation:

Red seaweeds *K. alvarezii* and *K. striatum* samples washed with distilled water and then, the samples were oven dried at 60^{o}C for 5 h. Dried samples were ground up into powders and were filtered using a mesh with a diameter of 250 µm to get homogenous samples.

Extraction of antioxidant:

The red seaweeds were extracted using methods reported [23, 24] with some modification. *K. alvarezii* and *K. striatum* powders were extracted by 50% acetone and 50% methanol solvent respectively. Seaweed powders were mixed with solvent in the ratio of 1g powder to 5mL solvent. The mixed samples were shaken continuously on an orbital shaker for 72 h. Then, the extracts were centrifuged for 10 minutes at 18 000 rcf (Top Refrigerated Centrifuge, Hermle Z323K, Germany). The extracts were filtered using filter paper (Sartorius Grade 292). The supernatants were oven dried for 24 h at 50^{o}C to get a brownish powder. The powders were dissolved in 21.7 mM ethanol. Then, the mixtures were kept in airtight amber bottle and stored in -20^{o}C for further analysis.

Cell Culture:

The hepatocarcinoma HepG2 cell (HepG2) were maintained in EMEM medium supplemented with 2.2 g sodium bicarbonate, 10% non essential amino acid (NEAA), 10% sodium pyruvate, 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin (100X) incubated at 37^{o}C in a constant humidified atmosphere of 5% CO_2/95% air.

MTT Assay:

Cells were seeded into 96-well plates at a density of 5 x 10^4 cell/mL per well and allowed to attach overnight in 200 µL medium incubated at 37^{o}C in 5% CO_2 in the air. The seaweed extracts in 21.7 mM ethanol were sterile-filtered prior to addition to plated cells. Seaweed extracts were added at a final concentration of 0.125, 0.25, 0.5, 1 and 2 mg/mL of medium, and the cells left to incubate in the seaweed extract containing medium for 24 h at 37^{o}C and 5% CO_2 [21]. A set of menadione (6.25, 12.5, 25, 50, 100 µM) as positive control and medium without compound as negative control were included in each microtitre plate. After incubation, traces of seaweed extract were removed by washing the cells with 200 µL PBS and applying 200 µL of fresh medium plus 20 µL of 5mg/mL MTT salt to determine the effects of the algal extracts on cell proliferation [25]. Cells were then incubated for 4 h at 37^{o}C, 5% CO_2. After 4 h, the medium was removed carefully and 200 µL of of DMSO were added to each well in order to solubilise the purple crystal formazan that were formed in the well bottom. The plate was read using an ELISA microplate reader (I Mark, Bio-Rad, USA) and the absorbance measured at 570 nm. Each concentration of the respective algal extracts was assayed in triplicate.

Flow cytometry Analysis using Annexin V-FITC/PI Staining:

Cells were plated at a density of 5 x 10^4 cell/mL in a 6-well microplate. HepG2 cells were exposed to IC_{50} of antioxidant extracts of *K. alvarezii* and *K. striatum* (obtain from MTT assay), 30 µM Goniothalamin as positive control and medium without any compound as negative control for 24 h at 37^{o}C, 5% CO_2. After 24 h, cells were collected by washed with cold PBS, trypsinisation and resuspended in PBS [26]. Then, the cells were transferred to eppendorf tube and the cells were centrifuged at 590 rcf for 5 minutes at 4^{o}C. Then, the supernatant was discarded carefully and 150 µL Annexin Binding Buffer (ABB) was added to the eppendorf tube. Then, 2.5 µL Annexin V-FITC was added to ABB for 15 minutes before 10 µL Propidium Iodide (PI) 50 µg/mL was added for 3 minutes. These procedures were performed in ice and dark. Then, 350 µL ABB was added to the mixture and the mixture was analysed using Flow Cytometre (FacsCanto II, Becton Dickinson, USA). This analysis was done in triplicate.

Statistical Analysis:

All data were expressed in mean ± SEM. Data was analyzed by Statistical Package for Social Science (SPSS, versi 15.0) software. Independent T-Test was used to determine the diffences of viabilty cell percentage between red seaweeds species for MTT assay. One way ANOVA was used to determine the differences between the mode of cell death between treated cells, positive control and negative control. The significant value for the data analyzed was set at $p \leq 0.05$.

Results:

Selection of Extraction Solvent:

The preliminary study has been done to select the best solvent for extraction of both red seaweeds species. Three different percentages of ethanol, methanol and acetone (50%, 70% and 100% v/v) were used for extraction. 50% acetone and 50% methanol was found to give the highest antioxidant value for *K. alvarezii* and *K. striatum* respectively. 50% acetone and 50% methanol was thus used for subsequent cytotoxicity assays.

MTT Assay:

The antioxidant extracts of *K. alvarezii* and *K. striatum* inhibited the viability of hepatocarcinoma HepG2 cell in a dose-dependent manner during the 24 h incubation (Fig.1). Based on Fig. 1, *K. alvarezii* antioxidant extract shows decreased of viability cell at all concentration except at 1.0 mg/mL. The IC_{50} value for *K. alvarezii* antioxidant extract is 1.8 mg/mL. As for *K. striatum* antioxidant extract, there were decreased of viability of hepatocarcinoma HepG2 cell at 0.125 mg/mL, 1.0 mg/mL and 2.0 mg/mL only. The IC_{50} value for *K. striatum* antioxidant extract is 0.9 mg/mL. Statistical test showed that there were no significant differences ($p>0.05$) in percentage of viability cell between antioxidant extracts of *K. alvarezii* and *K. striatum* at all concentrations except percentage of viability cell at the 2.0 mg/mL treatment level ($p \leq 0.05$). Based on this study, *K. striatum* could inhibit the proliferation of hepatocarcinoma HepG2 cells and/or cause a cell death of hepatocarcinoma HepG2 cells at lower IC_{50} concentration than *K. alvarezii* antioxidant extract.

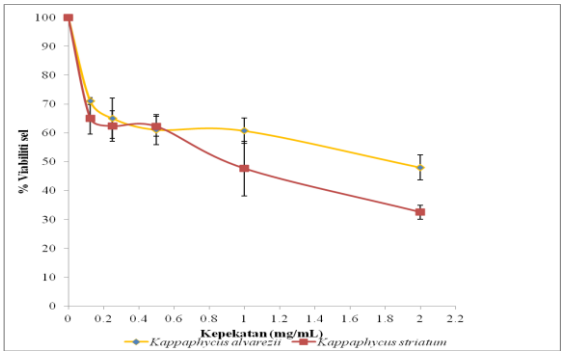

Fig. 1: Percentage of viable hepatocarcinoma HepG2 cell after treated with *Kappaphycus alvarezii* and *Kappaphycus striatum* antoxidant extracts for 24 hours using MTT assay.

Flow Cytometry Analysis using Annexin V-FITC/PI Staining:

In order to understand the mode of cell death, Annexin V-FITC/PI staining method had been employed in this study. HepG2 cells treated with IC_{50} dose of *K. alvarezii* and *K. striatum* antioxidant extracts showed higher percentage of apoptotic death compared to necrotic cells (Fig. 2). The percentage of apoptotic and necrotic cells at IC_{50} dose of *K. alvarezii* antioxidant extracts were 10.2 ± 2.4% and 2.4 ± 1.1% respectively. While the percentage of apoptotic and necrotic cells at IC_{50} dose of *K. striatum* antioxidant extracts were 11.3 ± 2.5% and 2.0 ± 1.3% respectively. However the percentage of apoptotic and necrotic cells treated with *K. alvarezii* and *K. striatum* antioxidant extracts were not significantly different ($p>0.05$) from negative control but there were significant differences ($p \leq 0.05$) with positive control. More than 80% of hepatocarcinoma HepG2 cells are viable after treated with IC_{50} dose of *K. alvarezii* and *K. striatum* antioxidant extracts. There were no significant ($p>0.05$) different between viable cells that were treated with IC_{50} dose of *K. alvarezii* and *K. striatum* antioxidant extracts with negative control.

Table 1: Percentage of hepatocarcinoma HepG2 cell death mode for treatment with IC_{50} dose of *Kappaphycus alvarezii* and *Kappaphycus striatum* antioxidant extracts, negative control and positive control.

	Apoptosis (%) ± SEM	Necrosis (%) ± SEM	Viable cells (%) ± SEM
K. alvarezii	10.2 ± 2.4	2.4 ± 1.1	87.4 ± 2.7
K. striatum	11.3 ± 2.5	2.0 ± 1.3	86.4 ± 1.9
Negative control	9.2 ± 1.7	1.1 ± 0.9	89.7 ± 2.5
Positive control	56.1 ± 1.6	1.5 ± 0.1	42.4 ± 1.7

Discussion:

Cancer ranks number four in main cause of death in Malaysia [27] and liver cancer has become the third most common cancer in the world [28]. However, various antioxidants from the diet can be considered as effective agent to reduce oxidative stress which can have an impact in cancer prevention [29]. Study on the effects of cytotoxic and antiproliferative of seaweeds extract on cancer cells has been addressed over the last few years. Previous studies found that seaweed extract is capable of giving cytotoxic effects against colon cancer cells, Caco-2 [30], human leukemia cells, U-937 [31] and breast cancer cells, MCF-7 [22] may be attributed by its antioxidant activity. This current study focused on the cytotoxic effects of antioxidant extracts of *K. alvarezii* and *K. striatum* against hepatocarcinoma HepG2 cell.

The results showed that antioxidant extracts of *K. alvarezii* and *K. striatum* are able to inhibit the proliferation and/or induce cell death of hepatocarcinoma HepG2 cells at high concentrations. However, characteristics of a good anti-cancer agent is an agent acting on cancer cells and does not affect normal cells as well as an effective agent to inhibit the proliferation and/or cause the death of cancer cells at low concentrations [32]. U.S. National Cancer Institute has also determined that crude extract cytotoxicity criteria is having IC_{50} values <30 µg/mL [33]. Previous study showed phenolic extracts *Eucheuma cottonii* (in present is known as *K. alvarezii*) can inhibit 50% of breast cancer cells (MCF-7) at low concentrations (25 µg/mL) after treatment for 24h [22].

However, the lack of MTT assay is that it cannot distinguish whether the study compounds caused inhibition of cell proliferation or increased cell death [34]. Therefore, Annexin V-FITC/PI staining assays was carried out to determine the cell death mode of hepatocarcinoma HepG2 when treated with antioxidant extracts of *K. alvarezii* and *K. striatum* at IC_{50} concentrations for 24 h. This study found hepatocarcinoma HepG2 cells treated with IC_{50} concentrations of antioxidant extracts of *K. alvarezii* and *K. striatum* did not show cell death by apoptosis or necrosis and the cell viability is above 80%. Viable cells above 80% may be due to cytostatic mechanism, rather than inducing apoptosis or necrosis. Static cells blocked in G_1/G_0 phase of the cell cycle [35].

This study findings are in contrast to the results of previous study in which the results of the study found that *E. cottonii* cause the death of breast cancer cells (MCF-7) via apoptosis [22]. They stated apoptosis is one mechanism of tumor suppression by the extracts of *E. cottonii* where it is important in maintaining the homeostasis of the cell. Extracts of *E. cottonii* cause irreversible damage cancer cells and induces apoptosis without cell cycle stationary. The difference in result between MTT assay and Annexin staining V-FITC/PI may be due to the differences in end-point by two different methods [36]. MTT assay method is based on the determination of cell viability with active mitochondria [25] while Annexin V-FITC/PI staining determine cell death via apoptosis or necrosis by morphological changes and cell membrane integrity [26]. Advantage of MTT analysis and analysis of Annexin V-FITC/PI staining is both analysis can accurately distinguish between viable cells and cell death quantitatively. However, measurements have to be done one at a time resulting in both analysis are suitable for a small number of samples only [37].

Conclusions:

This study found that antioxidant extracts of *K. alvarezii* and *K. striatum* are not cytotoxic against hepatocarcinoma HepG2 cells as IC_{50} values obtained were above 30 µg/mL. In the analysis of Annexin V-FITC/PI staining to determine the cell death mode, more than 80% of hepatocarcinoma HepG2 cells are viable after being treated with IC_{50} concentrations of antioxidant extracts of *K. alvarezii* and *K. striatum* indicate that cells become cytostatic.

ACKNOWLEDGMENTS

This research was funded by eScienceFund research grant STGL-016-2012. This study also supported by research grant STGL-007-2010 and STGL-007-2010/9. The author acknowledges the Toxicology Lab members, Faculty of Health Sciences, and Antioxidant Lab members, Faculty of Science and Technology, National University of Malaysia for the technical assistance and laboratory facilities. The authors declare that there is no conflict of interest.

REFERENCES

[1] Efferth, T. and B. Kaina, 2008. Chemical carcinogenesis: Genotoxic and nongenotoxic mechanisms. In. Toxicology and risk assessment: A comprehensive introduction. Greim, H. and Snyder, R., John Wiley & Sons Ltd, West Sussex, England, UK, 51-180.

[2] Li, Y., 2011. Antioxidants in biology and medicine: essentials, advances, and clinical applications. Nova Science Publisher. New York.

[3] Langseth, L., 1995. Oxidants, Antioxidants, and Disease Prevention. ILSI Europe. Brussels.

[4] Issa**,** A.Y., S.R. Volate and M.J. Wargovich**,** 2006. The role of phytochemicals in inhibition of cancer and inflammation: New directions and perspectives. Journal of Food Composition and Analysis, 19(5): 405-419.

[5] Xu, B. and S.K.C. Chang, 2012. Comparative study on antiproliferation properties and cellular antioxidant activities of commonly consumed food legumes against nine human cancer cell lines. Food Chemistry, 134(3): 1287-1296.
[6] Bahruddin Saad, Y.Y., Sing, Mohd Asri Nawi, Noor Hasani Hashim, Abdussalam Salhin Mohamed Ali, Muhammad Idiris Saleh, Shaida Fariza Sulaiman, Khairuddin Md Talib and Kamarudzaman Ahmad, 2007. Determination of synthetic phenolic antioxidants in food items using reversed-phase HPLC. Food Chemistry, 105: 389-394.
[7] Pussa, T., 2008. Principles of Food Toxicology. Boca Raton: CRC Press. Florida.
[8] Kashanian, S. and J.E.N. Dolatabadi, 2009. DNA binding studies of 2-tert-butylhydroquinone (TBHQ) food additive. Food Chemistry, 116: 743-747.
[9] Tachakittirungrod, S., S. Okonogi and S. Chowwanapoonpohn, 2007. Study on antioxidant activity of certain plants in Thailand: Mechanism of antioxidant action of guava leaf extract. Food Chemistry, 103: 381-388.
[10] Wang, T., R. Jónsdóttir and G. Ólafsdóttir, 2009. Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food Chemistry, 116: 240-248.
[11] Miliauskas, G., P.R. Venskutonis and T.A. van Beek, 2004. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chemistry, 85: 231-237.
[12] Chan, C.X., C.L. Ho and S.M. Phang, 2006. Trends in seaweed research. Trends in Plant Science, 11(4): 165-166.
[13] Phang, S.M., 2006. Seaweed resources in Malaysia: Current status and future prospects. Aquatic Ecosystem Health & Management, 9(2): 185-202.
[14] Ruperez, P., 2002. Mineral content of edible marine seaweeds. Food Chemistry, 79: 23-26.
[15] Gressler, V., N.S. Yokoya, M.T. Fujii, P. Colepicolo, J.M. Filho, R.P. Torres and E. Pinto, 2010. Lipid, fatty acid, protein, amino acid and ash contents in four Brazilian red algae species. Food Chemistry, 120: 585-590.
[16] Norziah, M.H. and C.Y. Ching, 2000. Nutritional composition of edible seaweed *Gracilaria changgi*. Food Chemistry, 68: 69-76.
[17] Matanjun, P., Suhaila Mohamed, Noordin Mohamed Mustapha and Kharidah Muhammad, 2009. Nutrient content of tropical edible seaweeds, *Eucheuma cottonii*, *Caulerpa lentillifera* and *Sargassum polycystum*. Journal of Applied Phycology, 21: 75-80.
[18] Devi, K.P., N. Suganthy, P. Kesika and S.K. Pandian, 2008. Bioprotective properties of seaweeds: In vitro evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. BMC Complementary and Alternative Medicine, 8: 38-48.
[19] Plaza, M., A. Cifuentes and E. Ibáñez, 2008. In the search of new functional food ingredients from algae. Trends in Food Science & Technology, 19(1): 31-39.
[20] Yangthong, M., N. Hutadilok-Towatana and W. Phromkunthong, 2009. Antioxidant Activities of Four Edible Seaweeds from the Southern Coast of Thailand. Plant Foods for Human Nutrition, 64: 218-223.
[21] Yuan, Y.V. and N.A. Walsh, 2006. Antioxidant and antiproliferative activities of extracts from a variety of edible seaweeds. Food and Chemical Toxicology, 44: 1144-1150.
[22] Farideh Namvar, Suhaila Mohamed, Samaneh Ghasemi Fard, Javad Behravan, Noordin M. Mustapha, Noorjahan Banu M. Alitheen and Fauziah Othman, 2012. Polyphenol-rich seaweed (*Eucheuma cottonii*) extract suppresses breast tumour via hormone modulation and apoptosis induction. Food Chemistry, 130: 376-382.
[23] Khalid Hamid Musa, Aminah Abdullah, Khairiah Jusoh and V. Subramaniam, 2011. Antioxidant activity of pink-flesh Guava (Psidium guajava L.): Effect of extraction techniques and solvents. Food Analytical Method, 4: 100-107.
[24] Khalid Hamid Musa, Aminah Abdullah, Bambang Kuswandi and M. Amrun Hidayat, 2013. A novel high throughput method based on the DPPH dry reagent array for determination of antioxidant activity. Food Chemistry, 141: 4102-4106.
[25] Mosmann, T., 1983. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. Journal of lmmunological Methods, 65: 55-63.
[26] Miller, E., 2004. Apoptosis Measurement by Annexin V Staining. In. Cancer Cell Culture: Methods and Protocols. SP Langdon. Humana Press, US, 183-202.
[27] Lim, G.C.C., 2002. Overview of Cancer in Malaysia. Japanese Journal of Clinical Oncology, 32(1): 37 - 42.
[28] But, D.Y.K., C.L. Lai and M.F. Yuen, 2008. Natural history of hepatitis-related hepatocellular carcinoma. World Journal of Gastroenterology, 14: 1652-1656.
[29] Khan, N., F. Afaq and H. Mukhtar, 2008. Cancer Chemoprevention Through Dietary Antioxidants Progress and Promise. Antioxidants & Redox Signaling, 10(3): 475-510.

[30] Nwosu, F., J. Morris, V.A. Lund, D. Stewart, H.A. Ross and G.J. McDougall, 2011. Anti-proliferative and potential anti-diabetic effects of phenolic - rich extracts from edible marine algae. Food Chemistry, 126(3): 1006-1012.
[31] Athukorala, Y., K.N. Kim and Y.J. Jeon, 2006. Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga, *Ecklonia cava*. Food and Chemical Toxicology, 44: 1065-1074.
[32] Drees, M., W.A. Dengler, T. Roth, H. Labonte, J. Mayo, L. Maispeis, M. Grever, E.A. Sausville and H.H. Fiebig, 1997. Flavopiridol (L86-.8275): Selective Antitumor Activity in Vitro and Activity in Vivo for Prostate Carcinoma Cells. Clinical Cancer Research, 3: 273-279.
[33] Suffness, M. and J.M. Pezzuto, 1990. Assay Related to Cancer Drug Discovery. In. Methods in Plant Biochemistry: Assays for Bioactivity. K Hostettmann. Academic Press, 6th ed, London, UK, 71-133.
[34] García-Alonso, J., G. Ros and M.J. Periago, 2006. Antiproliferative and cytoprotective activities of a phenolic-rich Juice in HepG2 cells. Food Research International, 39: 982-991.
[35] Lee S.K., B. Cui, R.R. Mehta, A.D. Kinghorn and J.M. Pezzuto, 1998. Cytostatic mechanism and antitumor potential of novel 1H-cyclopenta[b]benzofuran lignans isolated from *Aglaia elliptica*. Chemico-Biological Interactions, 115: 215-228.
[36] Chan, KM., N.F. Rajab, M.H.A. Ishak, A.M. Ali, K. Yusoff, L.B. Din and S.H. Inayat-Hussain, 2006. Goniothalamin induces apoptosis in vascular smooth muscle cells. Chemico-Biological Interactions, 159: 129-140.
[37] Wang, Y.Y. and X.X. Zheng, 2002. A flow cytometry-based assay for quantitative analysis of cellular proliferation and cytotoxicity in vitro. Journal of Immunological Methods, 268: 179-188.