

Non selective cancer cell killing by PLE extract of *Zingiber zerumbet* rhizome and its mode of cell death assesment

M.N.Norfazlina^{1,2*}, M.Y.Farida Zuraina¹, N.F.Rajab², S.Mohd Nazip¹, A.R.Rumiza¹, C.F.Suziana Zaila^{1,2}, L.Lek Mun², N.Nurshahirah^{1,2}, L.Florinsiah^{1,2}

¹Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, (MALAYSIA)

²Toxicology Laboratory, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Wilayah Persekutuan, (MALAYSIA)

E-mail : funfuzzy@gmail.com

Received: 16th November, 2013 ; Accepted: 22nd December, 2013

ABSTRACT

Zingiber zerumbet (Zz) is a medicinal herb that has been used for centuries to heal diseases. Here, the cytotoxicity of Pressurize Liquid Extraction (PLE) of Zz rhizome to human myeloid leukemia (HL60) and Chinese's hamster fibroblast (V79) cells were investigated. Treatment of HL60 cells with hexane (HEX) and ethanol (ET) extract of Zz rhizome was compared with doxorubicin (DOX). The toxicity of Zz extracts was essentially the same as that of doxorubicin to HL60 cells, but doxorubicin is much more effective to inhibit the growth of the HL60 cell line than Zz rhizome extracts. HEX extract of Zz rhizome also toxic on normal V79 cells and exhibited non selective activity. Mode of cell death regulated by HEX extract of Zz rhizome on HL60 cells were necrosis and apoptosis.

! 2014 Trade Science Inc. - INDIA

KEYWORDS

Zingiber zerumbet (Zz);
Pressurize liquid extraction
(PLE);
Human myeloid leukemia
(HL60);
Chinese hamster fibroblast
(V79);
Doxorubicin (DOX).

INTRODUCTION

Malaysia has about 1300 species from 12000 of flowering plant species that can give medicinal values^[14]. *Zingiber zerumbet* (Zz) is one of medicinal plant that very popular among Malaysian people. *Zingiber zerumbet* or locally known as 'L empyang' is a wild ginger that belongs to Zingiberaceae family^[11]. Zingiberaceae was frequently used as a raw material for formulation of traditional medicine that is commonly sold at local market^[25,30]. Even there was much research about anticancer properties of Zz rhizome extracts, but, most of them used traditional method as extraction procedure. Based on the best updated, there was no research that used Pressurized Liquid Extraction (PLE)

method for the extraction process of Zz rhizome. PLE is a recent extraction approach that was developed and meets the demand of less solvent extraction requirement and shorter extraction time^[31]. Thus, the present study used the most recent method of extraction to extract Zz rhizome components and furthermore to measure their efficiency as anti carcinogenic activity.

The feature of selective cancer cell killing by the anti carcinogenic agent is very crucial in order to reduce toxicity on normal cells^[12]. Previous research by Heny *et al.*^[15] who reported that the anticancer drug not only attack cancer cells but also causes toxicity to normal cell. It was supported by Handerson and Freil^[14], who was reported the doxorubicin is an effective anti tumor drugs with variety of activity but it also cause

Regular Paper

severe side effects on normal cell. In order to evaluate the safeties of Zz rhizome extract, the present study used untargeted normal cell which is Chinese hamster lung (V79) cells to monitor the toxicity of the treatments to the non cancerous cell.

MATERIAL AND METHODS

Collection of raw material

Fresh sample of matured Zz rhizome was purchased at Chow Kit wet market, Kuala Lumpur. The rhizomes immediately chop into a small slice and dried in the oven with temperature 45°C for 3 days. The plant sample was dried to avoid contamination by fungus and stored in dried and dark box^[24]. A human myeloid leukemia (HL60) cell line and V79 cell line were donated from the Faculty of Health Sciences, Universiti Kebangsaan Malaysia (UKM) and stored in -80 °C.

Pressurize liquid extraction (PLE) condition

In this study, Pressurize Liquid Extraction (PLE) method was applied. This method was use Acceleration Solvent Extraction (ASE) machine as equipment for extraction. This extraction method took 10 minutes of static time for one cycle with 1500psi of pressure and 80°C of temperature. The extracts produced are light, temperature and moisture sensitive; therefore the extracts were stored at -20 °C in dehydrogenaze potassium bicarbonate^[13].

Cell viability and cytotoxicity test

Suspension cell culture technique was used to maintain Human myeloid Leukemia (HL60) cell lines. HL60 cells were cultured by initially seeding 1x10⁵ cells/ml in fresh Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% Fetal Bovine Serum (FBS), 10% Penicillin-Streptomycin in a humidified atmosphere of 95% air, 5% CO₂ at 37 °C. To avoid possible effects of cell density on cell growth and survival, cells were maintained when cell density reach 8x10⁵ not exceed 1x10⁶ cells/ml. Cell were poured into centrifuge tube and centrifuged at 1500g for 5 minutes. Five ml of warm media was added in the pellet and gently re-suspended. Cells were seeded at 1x10⁵ cells/ml with daily adjustment of cell concentrations by adding fresh medium. At assay time, cells

were collect, 20ul of cell mixed with 80ul of trypan blue dye and manually counted. Actual cell numbers were calculated by multiplying diluted times compared with initial cell numbers. Each well of 96 well plates were seeded with 100ul of 1x10⁶ cells and treated with 100ul of 0-1000ug/ml of HEX and ET extract of Zz rhizome. After 24hours, 20ul of MTT solution were added into each well and put in the incubator for 4 hours. Furthermore, 200ul of DMSO were added into each well for 15 minutes. Cell viability was read using microplate reader at 570nm^[23]. Cell viability was calculated using an equation (1)^[32]. Furthermore, cytotoxicity effects were expressed by IC50 values calculated from dose responses curve. On the other hand, adherent cell culture technique was used to maintain Chinese hamster lung (V79) cells growth. The V79 cells were grown in DMEM medium supplemented with 1% penicillin-streptomycin and 10% Fetal Bovine Serum (FBS) in a humidified atmosphere in a 5% CO₂ incubator. V79 cells were exposed to HEX extract of Zz rhizome extract in order to evaluate their toxicity on normal cells.

$$\% \text{ cell survival} = (\text{At} - \text{Ab}) / (\text{Ac} - \text{Ab}) \times 100 \quad (1)$$

At = Absorbance of Test; Ab= Absorbance of Blank (Media); Ac= Absorbance of control (cells).

Apoptosis assessment

HL60 cells were treated to 60ug/ml of Zz rhizome extract in order to investigate the mode of cell death induction. Moreover, HL60 cells were treated to 5uM of Etoposide (ETO) (positive control). Apoptosis was assessed in suspension cell by annexin V-FITC^[33]. After 24 hours of treatment, the cells were harvested and centrifuged at 15000g for 5 minutes. The pellet was added with PBS and centrifuged again. The pellet was added with 150ul annexin buffer and then added with 2.5 ul annexin V-FITC and left for 15 minutes. After that, 10ul PI was added and let it for 2 minutes. After 2 minutes, 350 ul of annexin buffer was added. The percentage of annexin V positive cells was determined by flow cytometry.

Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). The Post Hoc test of Duncan was used to determine statistical significance (p≤0.05).

RESULT

Pressurize liquid extraction (PLE)

TABLE 1 showed the percentage of mass yield (mass of extract (g)/ mass o raw (g) x 100) of Zz rhizome by using different polarity of solvents. ET solvent showed the best extractor solvent because 3.86% mass of Zz extract was yielded as compared to HEX solvent produced only 7.70% of mass yield. It was seen that the extraction time using non polar HEX solvent was approximately 2 times longer than polar ET solvent. The extraction time and solvent consumption increased with the increased of weight of raw sample. On the other hand, both solvent extractors were produced aromatic dark yellow oil extract of Zz rhizome (TABLE 2). It was observed that highly non polar of HEX solvent was produced greater oily structure of extract than less polar of ET solvent.

TABLE 1 : The total volume of solvent consumption, extraction time and mass yield

Solvent	Extract Properties	Color
Hexane (HEX)	Oil	Dark yellow
Ethanol (ET)	Sticky oil	Dark yellow

TABLE 2 : Crude extract characteristic

Solvent	Volume (ml)	Time (Min)	Mass (g)		Percentage of mass yield (%)
			Raw	Extract	
Hexane (HEX)	475	122min 16s	210	0.70	0.30
Ethanol (ET)	236	61 min 8s	100	3.86	3.86

Cell viability and cytotoxicity effect

As shown in the Figure 1 indicated that HL60 viable cells more sensitive to HEX extract than ET extract of Zz rhizome. It was seen that at whole dose of HEX extract of Zz rhizome the HL60 viable cells was greater reduced as compared to ET extract of Zz rhizome ($p \leq 0.05$). The proliferation of HL60 cells was inhibited in the dose dependent manner of both solvent extracts. The cytotoxicity effects of Zz rhizome extract and DOX on HL60 and V79 cells were summarized in TABLE 3. The results showed indicated that HEX extract of Zz rhizome was greater cytotoxic on HL60 cells than ET extract of Zz rhizome with lower IC_{50} value. It was seen that both solvent extracts and DOX showed similar effect on HL60

cells, but DOX exhibited greater cytotoxic effects with lowest IC_{50} value. Moreover, HEX extract of Zz rhizome was toxic on normal V79 cells as their IC_{50} value on V79 cells lower than on HL60 cells. Both cell lines were co-tolerant with HEX extract of Zz rhizome.

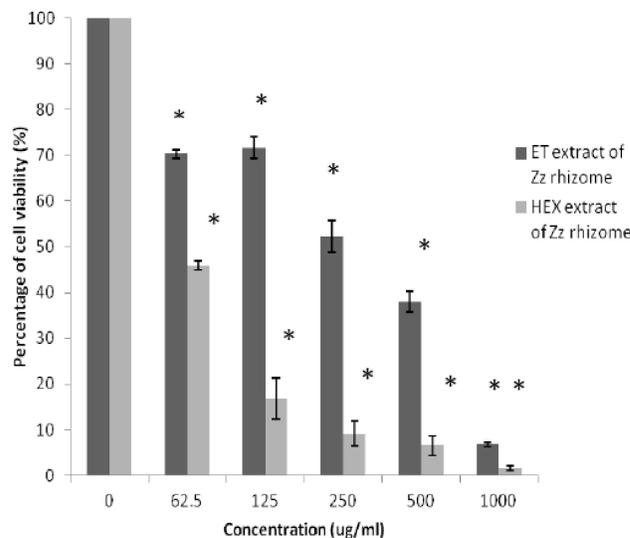


Figure 1 : Cell viability inhibitions at different concentrations of ET and HEX extracts of Zz rhizome on HL60 cell for 24 hours treated. (Note: * significant at $p < 0.05$ compared to negative control (Untreatment), n = 3 of cell viability)

Mode of cell death

The results shown in the Figure 2 indicated that HEX extract of Zz rhizome and positive control showed similar induction on the HL60 cell death mode. It was seen that both agent was induced apoptosis and necrosis mode. However, HEX extract of Zz rhizome exhibited greater apoptosis and lower necrosis induction of HL60 cells than etoposide. Untreated cells (Negative control) showed high proliferation of HL60 cells than treated cells with both HEX Extract of Zz rhizome and positive control.

Different molecular event target by MTT assay and FITC annexin-V flow cytometry

Figure 3 showed the percentage of HL60 death

TABLE 3 : The cytotoxicity effect of PLE extract of Ns seed on HL60 and V79 cell lines. DOX was used as a positive control to validate the anti cancer properties of plant extracts

Cell lines	IC_{50} value (ug/ml)		Positive control DOX
	Zingiber zerumbet (Zz)		
	HEX	ET	
HL60	63.72±5.363	345.3±34.69	0.714
V79	38	-	-

Regular Paper

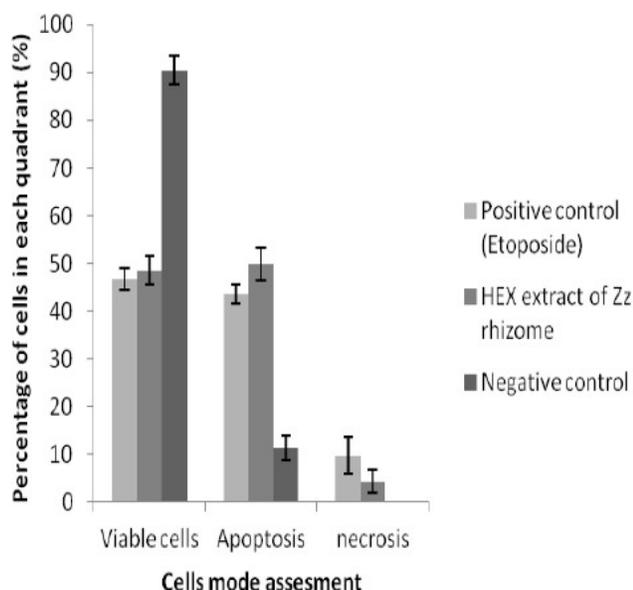


Figure 2 : The mode of cell death induction by 5uM of Etoposide and 60ug/ml of HEX extract of Zz rhizome. The HL60 cell death regulation by HEX extract of Zz rhizome was compared with positive and and negative control assessment

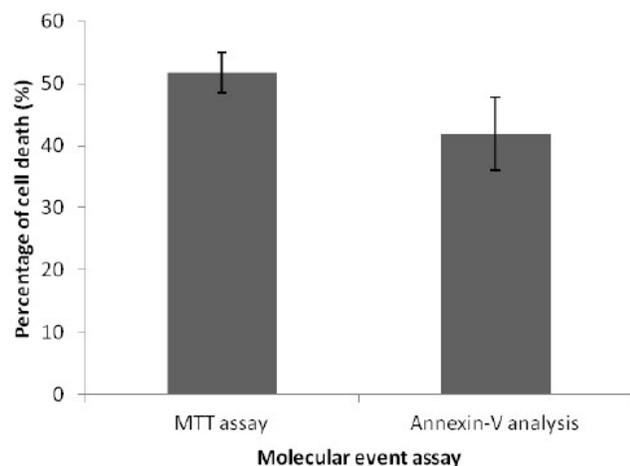


Figure 3 : Percentage of HL60 cell death after exposed to 60ug/ml of HEX extract of Zz rhizome computed using MTT assay and FIT-C annexin-V flow cytometry assay. (Note: * significant at $p < 0.05$ in comparison between both MTT and Annexin-V assay, $n = 3$ of assay)

after treated to 60ug/ml of HEX extract of Zz rhizome computed using MTT assay and VIT-C annexin-V flow cytometry analysis. Both assay showed not significant different of cell death outcome. It was seen that MTT assay resulted greater cell death consequence than annexin-V flow cytometry assay.

DISCUSSION

Plants have been exploited as medicines for thou-

sands of years. These medicines primarily used in the form of crude extracts such as powder, tea and other formulation. The utilized of medicinal plant was then developed into anticancer drugs. The crude extracts were isolated to get the compound tested on their biological activity on cancer cells. The present study was used crude extract of Zz rhizome to test on targeted HL60 and V79 cells. It was because the previous study work by Beckstrom-Sternberg and Duke^[3], was stresses the uses of whole crude extracts for pharmacological test as they was founded that the different components in crude extracts act in multiple pathway and more synergetic than a compound alone. Furthermore, Rajesh *et al.*^[26], was reported about properties of aromatic plant species is attributed in the form of oil fraction. Therefore, aromatic species of Zz rhizome was resulted yellowish oil of PLE extract. As reported by Rosnani *et al.*,^[27] the yellowish oil was due to the present of curcumin from oleoresin extracted from Zz rhizome. Curcumin is the compound that gives the yellow coloration to the crude extracts. The yellow oil produced was reported to compose the anti carcinogenic agent^[4]. On the other hand, rhizome maturation has a great influenced on the composition of natural constituent. According to Chien *et al.*,^[6], the component content of the rhizome was 3 times greater in matured rhizome as compared than younger rhizome. Therefore, the present study was used mature rhizome as a tested sample in order to grasp more natural constituent.

Pressurize Liquid Extraction (PLE) with nitrogen gas is a feasible technique for the isolation of active substances from *Zingiber zerumbet* (Zz). Previous work by Valeria *et al.*,^[35] demonstrated PLE method was utilized in elevated pressure and temperature that enable the solvents to be heated to temperature in excess of their boiling point which results in more efficient extraction process and moreover, produced high yield of crude extracts. The use of elevated temperature amplified the capability of solvent to solubilize the analyte, lessen the viscosity of liquid solvents, permitted better diffusion of the solvent into the matrix. Furthermore, the employ of elevated pressure assist the extraction of the analytes from samples by improving the solvent accessibility to the analytes that is spellbound in the matrix pores. On the other hand, high yield of crude extract mass was influenced by the solvents properties that have

different extractive capacity. As evaluated by Wahida^[36], polar solvents have unequal distribution electrical charges molecules with one of their end was positive. Therefore, polar solvent have ability to trap hydrophilic analyte which is polar component. On the other hand, non polar solvents have equally distributed electrical charges molecules and not miscible in water. For the extraction of *Zz* rhizome, polar ET solvent have better efficiency to trap their component as the yield of crude extract was greater than HEX solvent. Therefore, ET was selected as the best extractor solvent to bound more component in the matrix pore of *Zz* rhizome. The result was consistent with Rout *et al.*^[34], that showed polar solvent extractive was significant higher than non polar solvent extractive.

However, the cytotoxicity effectiveness of solvent extracts on HL60 cells was influenced by the present of anti carcinogenic agent not the amount yield of entire component. Therefore, the present study was important to measure the present of anti carcinogenic agent extracted by both solvent extractor assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test. According to Mosmann^[18], cell viability quantified based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan crystal by mitochondrial dehydrogenases. The reduction of tetrazolium salt was mediated by mitochondria uncoupling and reduce in ATP content^[28]. The results showed ET solvent was constructed high yield of crude extract than HEX solvent in PLE process, but their cytotoxicity to HL60 cells much lower than HEX extract of *Zz* rhizome. It was revealed that HEX solvent has high efficiency to trapp anti carcinogenic agent than ET solvent. It was seen that less formazon blue appeared after HL60 cells treated with HEX extract of *Zz* rhizome as compared to ET extract of *Zz* rhizome. It was indicated the increased dose of HEX extract of *Zz* rhizome strongly reduced tetrazolium salt than the increased dose of ET extract of *Zz* rhizome. This results was supported that high yield of crude extracts has no relation with the cytotoxicity activity of HL60 cells.

In term of toxicity on normal cells, *Zz* rhizome extract showed least protective effects on HL60 cells. MTT assay showed that HEX extract of *Zz* rhizome exhibit strong cytotoxicity effects on both HL60 and

V79 viable cells. The yellow color remains even after all solution was suck out. It might due, the feature of both cells allows the components of HEX extract of *Zz* rhizome to freely cross the plasma membrane, embedded inside the cell, targeted mitochondria and regulated uncoupling effects. Therefore, the results showed contradicted than previous research by Emanuela *et al.*^[18], who reported about independent effects on uncoupling miochondria in different cells type. No selective activity of *Zz* rhizome extract on normal cells resulted in this study opposite with the work by Nakamura *et al.*,^[20]. They showed that the anti proliferation effects of HEX extract of *Zz* rhizome on colonic adenocarcinoma cell lines is in a dose dependent manner and showed selective effects on normal human dormal and colon fibroblast. However, their extraction method used was different that might be extracted non toxic components. Non toxic compound was included zerumbone which exhibit pharmacology strategy with selective activity and less cytotoxic on normal cells^[19]. Therefore, the present study that used PLE extraction method might be attracted toxic component that showed no cytostatic activity, but cytotoxic activity on both HL60 and V79 cells.

DOX is an anti-cancer agent that was used clinically. As shown in the results, the anti cancer activity of DOX on HL60 cells was more proficient as compared whole extract of *Zz* rhizome. It might be their pharmacology pathway on targeted HL60 cells was more efficient as compared crude extracts of *Zz* rhizome. However, whole extracts of *Zz* rhizome showed anti-cancer activity on HL60 cells as their IC₅₀ value resulted were include in the range value of anti-cancer properties which is less than 1000ug/ml^[17].

Cell regeneration and proliferation are stabilized by cell death/apoptosis under normal health conditions. However, during tumor development this balance was swing towards proliferation. Cancer progression was due to hysterically cell proliferation and restraint of apoptosis. An effective and definite therapeutic consequence was hunted to target these crucial events^[9]. The present study was used FIT-C Annexin-V assay in order to measure the mode of cell death exhibited by HL60 cells after treated to HEX extract of *Zz* rhizome. At 60ug/ml of HEX extract of *Zz* rhizome, 50% of HL60 cells undergo apoptosis and less than 5% is undergo necrosis. It was negotiation with the work conducted

Regular Paper

by Takada *et al.*^[29], who reported the activity of the agent of *Zingiber zerumbet* rhizome to suppressed the anti apoptosis genes but upregulated the apoptosis expression. Etoposide is an established anticancer drug that was used since 1961^[16]. Etoposide was directed both apoptosis and necrosis cell death. Necrosis is the mechanism of cell death that cause inflammation and cell damage which directed by inflammation factor such as interleukin, tumor necrosis factor (TNF) and etc. Clinically, necrosis is the pathway of cell death that not necessitates^[2]. The population of necrosis cells were more responded by this drug as compared to Zz rhizome extract. Therefore, Zz rhizome extract is more outstanding because have dominant responses to apoptosis than necrosis event. The reason for the apoptosis event on HL60 cells that convey by both plant extracts is not known at present. Therefore, it was recommended to further investigation on mechanism of the apoptosis.

Cytotoxicity has been defined as the cells exterminate properties of a treatment agent independent from the mechanism of death^[10]. Therefore, the unparallel results showed by both MTT and Annexin-V assay was clarified because both assay corresponded to the different molecular target. MTT is an assay that targeted the activity of mitochondrial dehydrogenase^[5] but annexin-V flow cytometry assay is used to target the exposure of phosphotyrosine to the outer membrane^[22]. In this study, the death cell that exposed to HEX extract of Zz rhizome alone was higher when computed by MTT assay but, after computed using annexin-V flow cytometry assay HL60 death cells reduced. This might be the plant extracts was dictated the suppression activity of mitochondria but has less authority to the exposure of PS. The inclusion of this *in vitro* cytotoxicity assay provides an imperative advantage in identifying potentially cytotoxic agent^[11]. Moreover, the insertions of annexin-V flow cytometry assay provide a precise measurement of cell death^[21].

CONCLUSION

From the results, PLE method was successfully extract anti cancer agent from Zz rhizome using both ET and HEX solvent. However, HEX extract of Zz rhizome showed greater cytotoxicity effects on HL60

cells than ET extract of Zz rhizome. Moreover, HEX extract of Zz rhizome also cytotoxic on V79 cells and revealed as not selective agent. It also was declared that PLE method was successfully extract anti cancer agent from Zz rhizome. Therefore, this approach can be an alternative for researcher to extract the valuable component in the plant sample in short extraction time, less expensive and safe for environmental used. Moreover, HEX extract of Zz rhizome was targeted both mitochondrial and externalization PS to measure cytotoxic agent and mode of cell death assessment.

REFERENCES

- [1] Al-Zubairi., S.Adel, B.A.Ahmad, Y.Mohammed, I.A.Siddig, M.E.Manal, M.Syam; In vivo and In vitro Genotoxic Effects of Zerumbone. *Caryologia*, **63**, 11-17 (2010).
- [2] S.R.Balfour; Cytokines in intestinal inflammation: pathophysiological and clinical considerations. *Gastroenterology*, **106**, 533–539 (1994).
- [3] S.M.Beckstrom-Sternberg, J.A.Duke; Potential for synergistic action of phytochemicals in spices. In *Spices Herbs and Edible Fungi*. Elsevier Science: Oxford, 201–223 (1994).
- [4] M.N.I.Bhuiyan, J.U.Chowdhury, J.Begum; Chemical investigation of the leaf and rhizome essential oils of *Zingiber zerumbet* (L.) Smith from Bangladesh. *Bangladesh J.Pharmacol*, **4**(1), 9-12 (2009).
- [5] W.Bin, Z.Jin-Shui, Z. Yi, S. Wei-Ming, Z.Qiang; Predictive value of MTT assay as an in vitro chemosensitivity testing for gastric cancer: One institution's experience *World J.Gastroenterol*, **14**(19), 3064-3068 (2008).
- [6] T.Y.Chien, L.G.Chen, C.J.Lee, F.Y.Lee, C.C.Wang; Anti-inflammatory constituents of *Zingiber zerumbet*. *Food Chemistry*, **110**, 584–589 (2008).
- [7] K.Effenberger, S.Breyer, R.Schobert; Modulation of doxorubicin activity in cancer cells by conjugation with fatty acyl and terpenyl hydrazones. *Eur.J. Med.Chem.*, **45**, 1947–1954 (2010).
- [8] M.Emanuel, T.Claudia, F.Vittoria, C.Filippo, T.Valentina, P.Adriana; Critical Appraisal of the MTT Assay in the Presence of Rottlerin and Uncouplers. *Biological Procedures Online*, **11**(1), 227-240 (2009).
- [9] G.I.Evan, K.H.Vousden; Proliferation, cell cycle and apoptosis in cancer. *Nature*, **411**, 342–348 (2001).

- [10] B.Graham-Evans, P.B.Tchounwou, H.H.Cohly; Cytotoxicity and proliferation studies with arsenic in established human cell lines: keratinocytes, melanocytes, dendritic cells, dermal fibroblasts, microvascular endothelial cells, monocytes and T-cells. *Int.J.Mol.Sci.*, **4**, 13-21 (2003).
- [11] R.Hamid, Y.Rotshteyn, L.Rabadi, R.Parikh, P.Bullock; Comparison of alamar blue and MTT assays for high through-put screening. *Toxicol in vitro*, **18(5)**, 703-710 (2004).
- [12] M.N.Jha, J.S.Bedford, W.C.Cole, J.Edward-Prasad, K.N.Prasad; Vitamin E (d- α -tocopheryl succinate) decreases mitotic accumulation in gamma-irradiated human tumor, but not in normal cells. *Nutr.Cancer*, **35**, 189-194 (1999).
- [13] S.A.Hassan, W.A.Ahmed, F.M.Galeb, M.A.EL-Taweel, F.A.Abu-Bedair; In vitro challenge using thymoquinone on hepatocellular carcinoma (Hepg2) cell line. *Irianian J.Pharm.Res.*, **7(4)**, 283-290 (2008).
- [14] I.C.Henderson, E.Frei; Adriamycin cardiotoxicity. *Am.Heart.J.*, **99**, 671-674 (1980).
- [15] E.Heny, P.Eka, R.Undri; The active fraction from *Nigella sativa* and its activity against T47D cell line. *Indo.J.Chem*, **11(3)**, 217-222 (2011).
- [16] J.Mann; Natural products in cancer chemotherapy: past, present and future. *Nat.Rev.Cancer*, **2**, 143-148 (2002).
- [17] T.Meyer, I.Nindl, T.Schmook, C.Ulrich, W.Sterry, E.Stockfleth; Induction of Apoptosis by Toll-Like Receptor-7 Agonist in Tissue Cultures. *British Journal of Dermatology*, **149(Suppl. 66)**, 9-13 (2003).
- [18] T.Mosmann; Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J.Immunol.Methods*, **65(1-2)**, 55-63 (1983).
- [19] A.Murakami, D.Takahashi, T.Kinoshita; Zerumbone, a Southeast Asian ginger sesquiterpene, markedly suppresses free radical generation, proinflammatory protein production, and cancer cell proliferation accompanied by apoptosis: the a, b-unsaturated carbonyl group is a prerequisite. *Carcinogenesis*, **23**, 795-802 (2002).
- [20] Y.Nakamura, C.Yoshida, A.Murakami, H.Ohigashi, T.Osawa, K.Uchida; Zerumbone, a tropical ginger sesquiterpene, activates phase II drug metabolizing enzymes. *FEBS Lett.*, **572(1-3)**, 245-250 (2004).
- [21] J.Neuzil, I.Svensson, T.Weber, C.Weber, U.Brunck; α -Tocopheryl succinate-induced apoptosis in Jurkat T cells involves caspase-3 activation, and both lysosomal and mitochondrial destabilisation. *FEBS Lett.*, **445**, 295-300 (1999).
- [22] J.Neuzil, W.Tobias, S.Der.Andreas, L.Min, O.Georg, G.Nina, C.George, Mayne, O.Beata, N.S.Anne, S.C.Martin, J.C.Robert, W.Christian; Induction Of Cancer Cell Apoptosis By α -Tocopheryl Succinate: Molecular Pathways And Structural Requirements. *Faseb*, 403-415 (2001).
- [23] Noorashikin Md Saleh, M.Marsin; Sanagi comparison of pressurized liquid extraction with soxhlet extraction in the determination of polycyclic aromatic hydrocarbons in soil. *The Malaysian Journal of Analytical Sciences*, **13(1)**, 141-145 (2009).
- [24] I.Norsharina, I.Maznah, A.Al-Absi, G.Al-Naqeeb; Thymoquinone rich fraction from *Nigella sativa* and thymoquinone are cytotoxic towards colon and leukemic carcinoma cell lines. *Journal of Medicinal Plants Research*, **5(15)**, 3359-3366 (2011).
- [25] L.M.Perry; Medicinal plants of East and Southeast Asia. MIT Press Camb Mass USA, 436 (1980).
- [26] D.Rajesh, R.Stenzel, S.Howard; Perillyl alcohol as a radio-/chemosensitizer in malignant glioma. *J.Biol. Chem.*, **278(359)**, 68-78 (2003).
- [27] H.H.Rosnani, S.Norsaupiah, R.Mohamad, A.A.Ramlan, F.C.P.Nasrul; Extraction of Oleoresin From Zingiber Zerumbet Wiizomez Comparative Stj]Dy On Yield, Zerumbone And Curctjmin Content. Proceeding of international conference on chemical and bioprocess engineering, 1176-1179 (2003).
- [28] S.P.Soltoff; Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase Cdelta tyrosine phosphorylation. *J.Biol.Chem.*, **276(41)**, 37986-37992 (2001).
- [29] Y.Takada, A.Murakami, B.B.Aggarwal; Zerumbone abolishes NF- κ B and I κ Ba kinase activation leading to suppression of antiapoptotic and metastatic gene expression, upregulation of apoptosis, and downregulation of invasion. *Oncogene*, **24(46)**, 6957-6969 (2005).
- [30] M.Tillar; The Indonesian traditional beauty with Indonesian herbs. *FAPA Congres (Ladies Programme)*, Denpasar, Bali, Indonesia, 315-326 (1988).
- [31] Noorashikin Md Saleh., M.Marsin; Sanagi comparison of pressurized liquid extraction with soxhlet extraction in the determination of polycyclic aromatic hydrocarbons in soil. *The Malaysian Journal of Analytical Sciences*, **13(1)**, 141-145 (2009).
- [32] N.Valizadeh, H.R.Zakeri, G.Amin Asnafi, A.Shafiee,

Regular Paper

- P.Sarkhail, R.Heshmat, H.Sereshti, B.Larijani; Impact of Black seed (*Nigella sativa*) extract on bone turnover markers in postmenopausal women with osteoporosis. *DARU*, **17**, (2009).
- [33] N.H.Faujan, N.B.Alitheen, S.K.Yeap, A.M.Ali, A.H.Muhajir, F.B.H.Ahmad; Myeloid leukemia (HL-60) cell line. *African Journal of Biotechnology*, **9(38)**, 6387-6396 (2010).
- [34] O.P.Rout, K.K.Rout, R.Acharya, S.K.Mishra; Pharmacognostical and phytochemical studies on *Zingiber zerumbet* (L) SM rhizome. *IJRAP*, **2(3)**, 698-703 (2011).
- [35] F.P.Val'eria, S.Jenifer, I.S.Maria, Melecchi, C.Fernanda, Abadc, D.A.J.Ros^angela, M.Migdalia, Martinez, C.Eniz, Oliveira, B.Elina, Caram; Comparison of soxhlet, ultrasound-assisted and pressurized liquid extraction of terpenes, fatty acids and Vitamin E from *Piper gaudichaudianum* Kunth. *Journal of Chromatography A*, **1105**, 115-118 (2006).
- [36] A.F.Wahida; Extraction of *Zingiber zerumbet* (*l smith*) oil by using soxhlet extraction method. *Faculty of Chemical & Natural Resources Engineering Universiti Malaysia Pahang*, 1-32 (2010).