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In vitro cytotoxicity effects of single and combination *Nigella sativa* and *Zingiber zerumbet* extracts on human myeloid leukemia (HL60) cells and its mode of cell death

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ABSTRACT

Human myeloid leukemia (HL60) cell line is the precursor and the attractive model of human cell for studying molecular event drug evaluation and cell differentiation. This research investigate the interactive effects between *Nigella sativa* (Ns) and *Zingiber zerumbet* (Zz) treated on HL60 cell line for 24 hours at non constant ratio of combination doses. To explain cytotoxicity effect of single and combination extracts is using 3-[45-dimethylthizol-2-yl]-25-diphenyltetrazolium bromide (MTT) assay. The combination of Ns and Zz at various combination doses setting showed the antagonism interactive effects. Ns and Zz showed more potent as anti proliferative agent when treated alone as compared to co-administrations of both. As single agent the IC_{50} of Ns (Petroleum ether extract) is 654.9ug/ml which is less cytotoxic as compared to Zz (Hexane extract) that inhibit 50% of HL60 viable cells at 63.72ug/ml ($p<0.05$). In conclusion combination between Ns and Zz conveyed the antagonist interactive effects and indicated that Ns and Zz could not be combined together in order to achieve a safe drug.

INTRODUCTION

Cancer has become an important issue and occurs when a group of cells grow frenziedly abnormal and may affect almost every organ and tissue. The term cancer describes a group of more 100 different diseases and it is second leading cause of death after heart disease (WHO 2003; Huhmann and Cunningham 2005). A lot of approach was developed for cancer treatment such as modern surgery radiotheraphy and chemotherapy. However the used of radiotherapy and chemotherapy only can reduced 5 % of death (Benjamin *et al*. 1990). Plants have been a main source of greatly efficient conventional drugs for the treatment variety of cancer and was estimated that 60% of anticancer alrea dy commercialized or under development stage for clinical use (Shoeb 2006). On the other hand increasing evidence suggest that phytochemical contained within herbs are promising chemopreventive agents and may decrease the risk of malignancy. Figure 1 and figure 2 are the seed of Ns and rhizome of *Zingiber zerumbet* (Zz). Zerumbone is a food phytochemical compound that can be found abundantly in rhizomes particularly from Zz (Murakami *et al*. 2002). Zerumbone seize a great potential for use in chemopreventation and its molecular even that can inhibit the cancer progression are by suppressed the activation of NF-KappaB and NF-KappaB-regulated gene expression induced by carcinogen (Takada *et al*. 2005). Beside that zerumbone is an active component with assortment of therapeutic effects like antioxidant anti-inflammatory activity Nhareet and Nur (2003). Recently research by Rahman *et al*. (2012) reported that the antioxidant activity of Zz show the protective effect against arsenic toxicity. The other plants that has been utilized its compounds as anticancer is *Nigella sativa* (Ns).

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Fig. 1:*Nigella sativa* seed.

Fig. 2: Zingiber zerumbet rhizome.

Randhawa (2008) have reported the antioxidant activity of thymoquinone which is the main component of Ns can inhibit the development of several types of cancer. In vivo study by Rooney and Ryan (2005) revealed that the antioxidant activity of thymoquinone was able to protects rat liver against induced hepatocarcinogenesis and non toxic to the normal cells. Although the single effect of Ns and Zz extracts has promising anti cancer activities there is no study showing combinatorial effects of both plant extracts on human myeloid leukemia (HL60) cell. Combination is the promising approach used in drug discovery for increasing the effectiveness of anticancer therapy (Amy *et al*. 2009). Despite combination approach promising outcomes in pre clinically and clinical studies the therapeutic effectiveness of drug delivery systems emerges to greatly rely on the biological activity of drug payloads. Hence the concentration of the drug at the site of action and delivery schedule is the core for drug activity (Smalley *et al*. 2006). Consequently it is critical to identify the optimal combination settings for evaluation the type of interaction of both Ns and Zz extracts at the appropriate concentration.

MATERIAL AND METHODS

Sample collection

Ns seed was purchased from Klang wet market and stored in the dark at room temperature. Zz was purchased from Chow Kit wet market. HL60 cell lines were provided by UKM KL and stored in -80 ºC.

Pressurize Liquid Extraction (PLE) process

The seed of Ns was dried in oven at 40 ºC for 3 days and Ground into powder. Furthermore rhizome of Zz was chopped into small pieces and dried in the oven at temperature of 45 ºC for 3days. The dried rhizome of Zz was grounded into powder. In this study method was Acceleration Solvent Extraction (ASE). This extraction method involved 10 minutes of the static time for one cycle 1500psi of pressure and 80ºC of temperature. The extract is sensitive to light temperature and moisture therefore the extract was stored at -20 ºC in dehydrogenaze potassium bicarbonate (Valizadeh *et al*. 2009).

Stock solution preparation

The extracts of Ns and Zz produced were dissolved in the suitable solvent and made up to 200mg/ml stock solution. To make up the stock solution of PE extract of Ns 0.1g of extract was dissolved in 500ul of 100% ethanol. On the other hand 0.1 g of hexane (HEX) extract of Zz was dissolved in dimethylsulphoxide (DMSO). The stock solution was stored in several aliquots at -20 ºC to avoid repetitive freeze thaw cycles. It was then diluted further to a concentration of 1000ug/ml in IMEM by diluting 20ul of stock solution in 2000ul IMEM as working solution. It then serial diluted further to concentration 500 250 125 and 0 ug/ml in 2000 ul IMEM. Doxorubin (DOX) were used as positive control with the doses 4 2 1 0.5 0.25 and 0 uM (Halima *et al*. 2012).

Suspension cell culture

Human Leukemia cell lines HL60 cell were cultured by seeding $1x10^5$ cells/ml of fresh Dulbecco's Modified Eagle Medium (IMEM) supplemented with 20% Fetal Bovine Serum (FBS) 10% Penicillin-Streptomycin in a humidified atmosphere of 95% air 5% CO_2 at 37 ºC. To avoid possible effects of cell density on cell growth and survival cells were maintained when cell density reached $8x10^5$ 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 seeding at $1x10^5$cells/ml with daily adjusting cell concentrations by adding fresh medium (Bhuvan *et al*. 2010).

Cytotoxicity assay for sequential and simultaneous treatments

MTT assay was performed according to Stoev *et al*. (2009) in order to determine the cytotoxic potential of the single and combinations on HL60 with modification. At assay time for single effects each well of 96 well plates were seeded with 100ul of $1x10^6$cells/ml and added with 100ul of different doses of Ns and Zz extracts (0 to 1000ug/ml) and doxorubin. 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.

$$\text{Cell viability \%} = \frac{\text{average OD–blank OD}}{\text{Negative control}} \times 100$$

Subsequently the half maximal inhibitory concentration (IC_{50}) values of Ns and Zz extracts were determined. Simultaneous treatment effects were then evaluated in four combination doses

design setting (Figure 3). Combination 1 and 2 were designed to investigate the encouragement of one extract when the dose of other extract remained fixed. The range of concentration for combination 1 and 2 were lower than IC_{25}. Both Ns and Zz extracts concentration were varied in combination 3 and 4. In combination 3 the cells were exposed to several doses of less or higher than IC_{25} of both extracts. Whilst cells in combination 4 were treated at IC_{50} value of both extracts IC_{50} + IC_{25} and IC_{25} + IC_{25} of extracts (high dose range).

Ns (ug/m Ns (ug/ml) l)											
21					**1b**						
42					**2b**						
84					**3b**						
168					**4b**						
220		**1c**									
335					**5b a**		**4 a**		**3 a**	**2 a**	**1 a**
420			**2 c**								
480	**2 d**			**4 d**							
520						**3c**					
600								**4 c**			
670	**1 d**			**3 d**							
	60	45	40	30	22.5	20	11.3	10	5.25	2.81	1.41

Zz (ug/ml)

Fig. 3: Combination setting map used in this study in which HL60 cell was exposed to Ns and Zz extracts at various concentrations and non constant ratios for 24 hour. Alphabet in the box denotes as combination 1 (**a**) combination 2 (**b**) combination 3 (**c**) and combination 4 (**d**) indicate data points used to determine combination index (CI) and cytotoxic values. The numerical value 1 to 5 in box indicated as various combination doses of both plant extracts.

Data analysis

Post Hoc test of Duncan was used to analyze the data for single effect to compare the cytotoxic effects between the extracts. The interaction of the extracts whether synergy additivity or antagonism was evaluated using the theory by Chou and Talalay (1984). The Combination Index (CI) value was used to determine the synergy (CI<0.9) additivity (0.9<CI<1.1) and antagonism (CI>1.1).

RESULTS AND DISCUSSION

Plants have been exploited as medicines for thousands of years. Crude drugs like powder tea and other formulation is the early form of plant medicines before it was developed into anticancer drugs. The anticancer drug engrossed the segregation and characterization of active compound. The IC_{50} values of Ns and Zz extracts alone were determined in HL60 cell. As described in Figure 4 HEX extract of Zz were more cytotoxic with IC_{50} values at 63.72 ug/ml as compared to PE extract of Ns with IC_{50} values at 654ug/ml after 24 hour of treatment. The dose responses curve indicated that both extracts inhibit the proliferation of HL60 cell in monophasic dose responses manner. Moreover combinatorial technique became the latest discovery where they were believed to be more active than the single agent. This was supported by Raffa (2001) which noted about the increment of efficacy of the combinatorial treatments and the possibility of less toxic effect due to lower dosage used. Previous study that combined the thymoquinone (TQ) with other anticancer drugs such as doxorubin was improved the anticancer properties and reduced the incidence of multi drugs resistance and cardiotoxicity of Doxorubin (Katharina 2011). Furthermore the mixture of Ns seeds *H. indicus* roots and *S. Glabra* rhizome has powerful cytotoxic properties towards human liver cancer cells (HepG2) invitro (Thabrew *et al*. 2005).

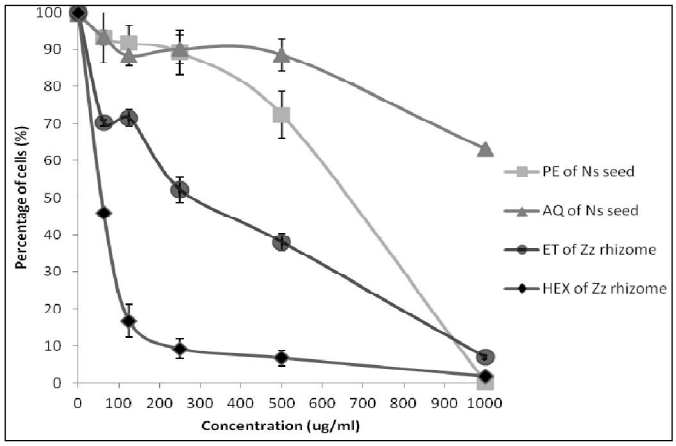

Fig. 4: Cell viability inhibitions at different concentrations of Ns seed and Zz rhizome extracts against HL60 cells for 24 hours treated. The experiment was carried out in triplicate.

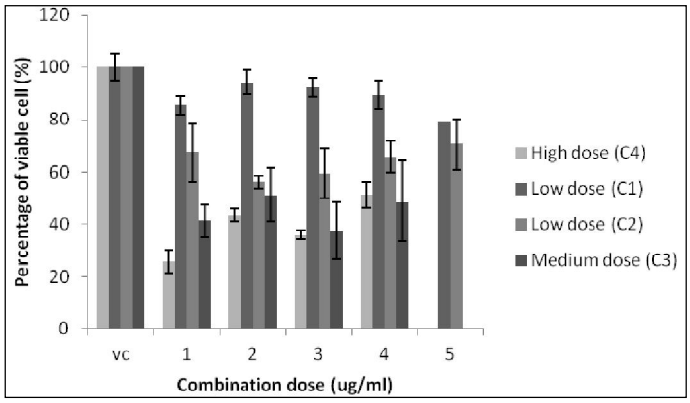

Fig. 5: MTT assay for simultaneous treatments of PE extract of Ns seed and HEX extract of Zz rhizome on human myeloid leukemia (HL60) viable cells at 24 hour. The cells was treated with various dose of both extract at combination dose design C1 (combination 1) C2 (combination 2) C3 (combination 3) and C4 (combination 4). Symbol (1-5) denoted as non constant ratio combination doses used in each combination design setting.

As reported by Eleftheria *et al.* (2012) the drug levels at the tumor site will influence the activity of the antitumor. An optimal concentration is crucial for the interaction of cytotoxic agent with its target and for inducing a pharmacodynamic effect. In addition anticancer agents have impact on pharmacokinetic profile of a cytotoxic agent throughout intracellular metabolism interaction with transporters and interaction with concomitantly. Hence we investigated the responses of HL60 cell to Ns/Zz

combination by various concentrations as shown in Figure 3. As shown in Figure 5 combination 1 and 2 has resulted insignificant different of HL60 viable cells at each combination dose as compared to negative control. Both design showed less cytotoxicity effects on HL60 cells and not selected as good delivery system. On the other hand combination 3 and 4 design treatments reduced the HL60 viable cell approximately more than 50%. However combination 3 was selected as good co-administration design even combination 4 showed more cytotoxic on HL60 cells. It was due to high dose complementary of both plant extracts in combination 4 design treatments.

In the present study an antagonistic interactive effect (CI > 1) between Ns and Zz extracts combination at various combination doses were obtained (Figure 3). Even significant and moderate cell death was obtained after treated with combination 3 and 4 design treatments all those mixing conveyed the antagonism interactive effects (Figure 6).

Ns (ug/ml)	60	45	40	30	22.5	20	11.3	10	5.25	2.81	1.41
21					•						
42					•						
84					•						
168					•						
220		•									
335					••		•		•	•	•
420			•								
480	•			•							
520						•					
600								•			
670	•			•							

Zz (ug/ml)

Fig. 6: The maps compiling combination index (CI) values. The dots indicate antagonism (CI> 1.1 black) additivity (0.9<CI<1.1 yellow) and synergism (CI<0.9 green) between Ns and Zz extracts against HL60 cell at combination setting shown in Figure 1. All combination setting showed antagonist interaction for whole level of concentrations. Experiments were performed in triplicate.

Recent drug discovery and development was focused on the therapeutic efficacy at low dose level and the used of low dose of anticancer was needed clinically to reduce the toxicity on normal cells (Farrell *et al*. 2011). However in our study the low dose level used in combination 1 and 2 showed not significant cytotoxicity effect as the percentage of cell death was less than 20% and the interaction was antagonism (Figure 6 and figure 7). The present study not supported the study by Eleftheria *et al*. (2012) that accounted the antagonism was observed at the dose levels greater than IC_{50} because in this study the antagonism interaction occurred at above and lower than IC_{50} .

Previous research was reported the inconsistent sensitivity of anticancer agent on different cell lines (Ismail *et al*. 2011). Therefore it is recommended to test Ns/Zz combination on other type of cell lines as Ns/Zz combinations are not appropriate for HL60 cells. The protective effects might be due to mode of actions of both Ns and Zz extracts. Therefore further studies are essential to elucidate the cellular mechanisms following the restricted cellular response of HL60 cell when treated with Ns/Zz combination.

Ns (ug/ml)	60	45	40	30	22.5	20	11.3	10	5.25	2.81	1.41
21					+						
42					£						
84					£						
168					£						
220		x									
335					££		£		£	£	£
420			+								
480	x			x							
520						*					
600								x			
670	*			*							

Zz (ug/ml)

Fig. 7: Drug combination cytotoxicity was differentiated using several symbols. The symbol **(*)** indicate 60-80 of cell death **(x)** indicate 40-60 of cell death **(+)** indicate 20-40 of cell death and **(£)** indicate 0-20 of cell death.

CONCLUSION

It can be conclude that the exposure of PE extract of Ns seed and HEX extract of Zz rhizome had an antagonistic effects on human myeloid leukemia (HL60) cells at lower medium and high combination dose.

REFFERENCES

Amy RN Julia D David TC Masato Y Combination of conditionally replicative adenovirus and standard chemotherapies shows synergetic antitumor effect in pancreatic cancer *Cancer Science* 2009; 100: 2181-2187

Bartkowiak D Hogner S Baust H Northdurft W Rottinger EM *Cytometr* 1999; 37: 191

Benjamin HSL Padma PT Jeffrey MT *Allium sativum* (garlic) and cancer prevention *Intl J Vitamin NutrRes* 1990;10: 937-948

Bhuvan p Raval Taxal G Shah Jignesh D Patell Bhavik A Patel Rajesh K Patel Maulik P Suthar Potent anticancer activity of *Nigella Sativa* Seeds *Archives of Applied Science Research* 2010; 2 (1): 52-56

Chou TC Talalay P Adv Enzyme Regul (1984); *27*: 105.

Eleftheria T Allison ME Younsoo B. Combination Effects of Docetaxel and Doxorubicin in Hormone-Refractory Prostate Cancer Cells *Biochemistry Research International Volume* 2012; 1-10.

Farrell D, Ptak K, Panaro NJ, Grodzinski P. Nanotechnology-based cancer therapeutics- promise and challenge-lessons learned throught the NCI alliance for nanotechnology in cancer *Pharmaceutical Research* 2011 ; 28(2): 273-278.

Halima SA, Luc V, Jeffrey FF, Johannes VS. Mutagenicity antimutagenicity and cytotoxicity evaluation of South African *Podocarpus* species *Journal of Ethnopharmacology* 2012 ; 139: 728-738.

Huhmann H, Cunningham R. Importance of nutritional screening in treatment of cancer-related weight loss The Lancet Oncology 2005 ; 6(5): 334-343.

Shoeb M. Anticancer agents from medicinal plants *Bangladesh J Pharmacol* 2006 ;1 : 35-41.

Ismail N, Ismail M, Al-Absi A, Al-Naqeeb G. Thymoquinone rich fraction from *Nigella sativa* and thymoquinone are cytotoxic toward

colon and leukemic carcinoma cell lines *Journal of Medicinal Plant Research* 2011; 5(15): 3359-3366.

Katharina EN, Rainer Schobert. Combinatorial effects of thymoquinone on the anti-cancer activity of doxorubicin Cancer Chemother Pharmacol 2011: 67:867–874.

Nhareet SM, Nur SMH. Anti-inflammatory property of ethanol and water extracts of *Zingiber zerumbet Indian journal of pharmacology* 2003; 35: 181-182.

Raffa RB. Pharmacology of oral combination analgesics: rational therapy for pain. *J Clin Pharm Ther* 2001; 26: 257-264.

Rahman M, Saud ZA, Hossain E, Islam K, Karim MR, Hoque MM, Yeasmin T, Nikkon F, Mandal A, Hossain K. The Ameliorationg Effects of *Zingiber zerumbet* Linn on Sodium Arsenite-Induced Changes of Blood Indices in Experimental Mice *Life Siences and Medicine Research* 2012; 41: 1-11.

Randhawa MA. Black seed *nigella sativa* deserves more attention *J Ayub Med Coll Abbottabad* 2008 : 20(2): 1-2.

Rooney S and Ryan MF (2005) Modes of action of alpha-hederin and thymoquinone active constituents of *Nigella sativa* Against Hep-2 cancer cells *Anticancer Res* 25 2199-2204

K S M Smalley, N K Haass, P A Brafford, M Lioni, KT Flaherty, M Herlyn. "Multiple signaling pathwaysmust be targeted to overcome drug resistance in cell lines derived from melanoma metastases" *Molecular Cancer Therapeutics* vol 2006; 5 (5): 1136–1144.

Shoieb M, Elgayyar M, Dudrick S, Bell L, Tithof K. *In vitro* inhibition of growth and induction of apoptosis in cancer cell lines by thymoquinone *Int J Onco*l 2003; 22: 107-113.

Stoev S, Denev S, Dutton M, Nkosi BJ. Cytotoxic Effect of Some Mycoto•ins and their Combinations on Human Peripheral Blood Mononuclear Cells as Measured by the MTT Assay *The Open To•inology Journal* 2009; 2: 1-8.

Takada Y, Murakami A, Aggarwal BB. Zerumbone abolishes NF-kappaB and IkappaBalpha kinase activation leading to suppression of antiapoptotic and metastatic gene expression upregulation of apoptosis and down regulation of invasion Oncogene 2005; 17: 122-130.

Thabrew MI, Ragai RM, Mohammed AM, Robin DH. Cytotoxic effects of a decoction of *Nigella sativa Hemidesmus indicus* and *Smilax glabra* on human hepatoma HepG2 cells *J Life Sci* 2005; 77: 1319-1330.

WHO. 2003 Diet nutrition and the prevention of chronic diseases WHO Technical Report Series No 916 World Health Organization Geneva

Murakami A, Takahashita D, Kinoshita T, Koshimizu K, Kim HW, Yoshihiro A, Nakamura Y, Jiwajinda S, Tereo J, Ohigashi H. Zerumbone a Southeast Asian ginger sesquiterpene markedly suppress free radical generation proinflammatory protein production and cancer cell proliferation accompanied by apoptosis Carcinogenesis 2002 ; 23: 1961-1963.

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