In vitro cytotoxicity effects of single and combination Nigella sativa and Zingiber zerumbet extracts on human myeloid leukemia (HL 60) cells and its mode of cell death

Norfazlina M.N.*,1,2, Farida Zuraina M.Y.1, Rajab N.F2, Mohd Nazip S.1, Rumiza Zaila C.F.1,2, Lek Mun L.2, Nurshahirah N.1,2, Florinsiah L.1,2

1Faculty of Applied Sciences Universiti Teknologi MARA 40450 Shah Alam Selangor Malaysia. 2Faculty of Health Sciences Universiti Kebangsaan Malaysia Jalan Raja Muda Abdul Aziz 50300 Kuala Lumpur Wilayah Persekutuan Malaysia.

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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 radiotherapy 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 already 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 chemoprevention 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).

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 IC50 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.
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 °C.

Pressurize Liquid Extraction (PLE) process
The seed of Ns was dried in oven at 40 °C 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 °C for 3 days. 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 80°C of temperature. The extract is sensitive to light temperature and moisture therefore the extract was stored at -20 °C in dehydrogenate 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 °C 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% CO2 at 37 °C. 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 1x10^5 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.

\[
\text{Cell viability} \% = \frac{\text{average OD} - \text{blank OD}}{X100}
\]

Negative control
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 \(_{25}\) value of both extracts IC \(_{50}\) + IC \(_{25}\) and IC \(_{25}\) + IC \(_{25}\) of extracts (high dose range).

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<thead>
<tr>
<th>Ns (ug/ml)</th>
<th>Zz (ug/ml)</th>
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<tr>
<td>21</td>
<td>1b</td>
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<tr>
<td>42</td>
<td>2b</td>
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<td>168</td>
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<td>220</td>
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<td>1.31</td>
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![Table 1: 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.](image)

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 doxorurbin was improved the anticancer properties and reduced the incidence of multi drugs resistance and cardiotoxicity of Doxorurbin (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) invtro (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.](image)

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).

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 IC50, because in this study the antagonism interaction occurred at above and lower than IC50.

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.

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<tr>
<td>Zz (µg/ml)</td>
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![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.](image)

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

**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.

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