Apoptotic and necrotic lymphocytes after treatment of stem bark extract of *Plumeria rubra* L invitro

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Abstract. Based on its LC50, stem bark extract of *Plumeria rubra* L was not toxic to lymphocytes. The goals of this experiment were to investigate the effect of stem bark extract of *P. rubra* L doses on apoptotic and necrotic lymphocytes. The cells were isolated from peripheral blood using density gradient configuration method to PBMCs. Then, they were cultured in well plates and treated with cisplatin and the stem bark ethanolic extract of *P. rubra* L in 0, 20, 130, 240, 350, 460, and 570 µg/mL. After 24 hours incubation, the lymphocytes in each sample were stained using annexin V and propidium iodide reagent and ran in a flow cytometer. The data gained were analysed statistically using Kolmogorov Smirnov, one way ANOVA, Lavene’s and LSD tests. The results showed that there were significant apoptotic cell percentage differences (p<0.05) among treatments (p<0.05), with the lowest of 2.22 ± 0.25% and the highest of 14.36 ± 1.06%. However, the necrosis was no different, with the lowest of 0.52 ± 0.18% and the highest of 1.85 ± 0.58%. In conclusion, the extract affect the percentage of apoptotic lymphocytes, on the contrary, did not influence the necrotic cells’.

1. Introduction

Traditional herbal drugs have been being used to prevent or treat diseases including cancer because of their safeness and cheapness. In developing herbal drugs, researches focused on their natural product, secondary metabolites (Eid et al., 2015). The metabolites were examined partially, in combination or in the form of whole plant extracts. The use of the two last forms were based on the synergic effect of metabolites (Neergheen, 2010). These lead to get many specific targets (Aung et al., 2017). In his case, synergic interactions increase the potency of getting molecular targets, especially in cancer cells. In vitro method is an initial step of finding new drugs including plant extracts (Graidist et al., 2015; Nascimento et al., 2014; Noolu et al., 2013) before preclinical and clinical tests (Lovitt et al., 2012). Examining the extract cytotoxicity on health or normal cells (Korb et al., 2010) and their apoptotic and necrotic deaths is an important procedure to identify the range of extract doses being save to the cells (Lotze and Demarco, 2004).

*Plumeria rubra* L. is a plant species that contains various secondary metabolites. The metabolites had been extracted partially. Some of them were cytotoxic on cancer cells (Kardono et al., 1990,
Hamburger et al., 1991; Rekha and Jayakar, 2011). However, its whole extract have not been examined intensively. Kuswanti et al. (2018) revealed that based on its LC50, the stem bark extract of P. rubra was toxic on cancer cells. On the other hand, it was not toxic on lymphocytes as health or normal cells. In accordance to drug management (Korb et al., 2010), to determine the range of safe doses, it is compulsory to identify the apoptotic and necrotic deaths of normal cells. Administering drug, including plant extract, had a range of optimum limit. In certain doses, the extract can be toxic on health cells (Korb et al., 2010). Apoptosis is a programmed cell death (PCD) that functions to clear the defective cells quickly, forces to remove and digests infected cells and prepare tissues renovation and repair. Necrosis is not a programmed death and generally happens when infection or injury occurs, under control of immune response and memory cells. During its process, the cells released various molecules causing inflammation, such as HMGB1, that can initiate tumour appearance (Lotze and Demarco, 2004). Following up the result of its LC50 examination on lymphocytes (Kuswanti et al., 2018), the goal of this study was to investigate the effect of the stem bark extract of P. rubra L on the apoptotic and necrotic lymphocytes.

2. Methods
2.1. Preparing secondary metabolites
The stem bark of P. rubra was got from Balongbendo District of Sidoarjo, Indonesia. The bark was sun-dried, then crushed to be powder. It was macerated in 96% ethanol during 24 hours. The sample resulted was filtered using vacuum pump, then the filtrate gained was separated in a rotary evaporator. The last three steps were conducted 3 times until a semisolid brown mass resulted. It was stored at -20°C for further use.

2.2. Preparing cell culture media
RPMI 1640 (Gibco) supplemented with 10% of Fetal Bovine Serum (FBS), and 1% of Penicillin and Streptomycin (Gemini, cat no 400-109) were prepared. They were mixed gently, then filtered using 0.2 µM millipore filter (Minisart). This procedure was conducted aseptically in a laminar air flow (LAF).

2.3. Lymphocytes isolation
Lymphocytes were isolated for PBCMs (the Peripheral Blood Mononuclear Cells). As much as 3 mL of ficoll solution (Lympho Spin Medium, cat 60-0092-10) was put into a 15 mL tube. The same volume of blood was added on the solution gently, then was centrifuged at 1,600 rpm and 25°C for 30 minutes. The supernatant was pipetted out. The lymphocyte pellet left was washed using 10 mL of PBS and centrifuged at 2,500 rpm and 10°C during 5 minutes. The pellet gained was added with 1 mL of the culture media until the scale of 1 mL. Furthermore, about 50,000 cells were plated into each well of a 96 culture well plate for further treatment.

2.4. Extract Dilution
The extract was diluted in the culture media. The extract was put into a jar with culture media, then it was stirred at 1250 rpm and at room temperature. The dilution resulted was centrifuged at 10°C and 2,500 rpm for 5 minutes. The supernatant was taken out its tube and filtered using a 0.02 µM millipore filter. The filtrate resulted was ready for treatment.

2.5. Treatments
After putting the lymphocytes in the wells, the cells were treated with cisplatin (as cancer drug) as much as 0.156/mL and the stem bark ethanolic extract in 0, 20, 130, 240, 350, 460, and 570 µg/mL. These were done in triplicate, then the cells were incubated for 24 hours.
2.6. Annexin V and PI assays
After incubation, each suspension of lymphocytes were put into a tube, then centrifuged at 2500 rpm and 10°C for 5 minutes. The pellet resulted were added with 600 µL of PBS, suspended, and centrifuged again. As much as 50 µL of annexin V and PI buffer was added to the pellet gained, then mixed and incubated. After 15 minutes, the suspensions were added with 300 µL of PBS and put into a cuvet for flow cytometry running. The cells’ survival, mortality, apoptosis and necrosis were identified using Cell Quest Pro software.

2.7. Data analysis
The data gained were analysed using Kolmogorov Smirnov for their normality, followed by Lavene’s for their homogeneity, one way ANOVA and LSD tests. The normality and homogeneity were shown by p> 0.05. The data difference significances were determined based on p<0.05.

3. Result and Discussions
Using cell quest pro software, lymphocytes’ survival, mortality, apoptosis and necrosis were shown in percentages. Their survival and mortality are shown in Figure 1.

![Figure 1. Lymphocyte survival and mortality after stem bark extract of P. rubra L. treatments. ED0= 0 µg/mL, ED1= 20 µg/mL, ED2= 130 µg/mL, ED3=240 µg/mL, ED4= 350 µg/mL, ED5= 450 µg/mL, ED6= 570 µg/mL.](image)

Figure 1 shows that there was no difference of lymphocyte survivals between cisplatin and control treatments. Compared to these first treatments, after ED1 and ED2 the cell survivals were relatively the same. However, started from ED4 they decreased. ANOVA test indicated a significant difference of cell survivals among treatments shown by p<0.05. According to LSD test, there was no differences among the survivals after cisplatin, ED0, ED1, ED2 and ED3 treatments shown by p>0.05. Cisplatin treatment and others started to differ their survivals (p<0.05) from ED4’s until ED6’s. The same trend occurred between ED0 (control sample) and the higher extract doses.

Figure 1 also exhibits the cell mortalities after cisplatin and control treatments. They were not different. Among extract treatments, compared with control treatment (ED0) the mortality of the cells after ED1 and ED2 were relatively the same. However, started from ED3 the mortality increased. ANOVA test indicated a significant difference of cell survivals among treatments shown by p<0.05. According to LSD test, there was no differences between cisplatin, ED0, ED1 and ED2 (p>0.05). The differences (p > 0.05) between cisplatin and others started from ED3, then ED4, ED5 and ED6 treatments.
The same phenomena occurred between ED0 and higher extract doses. Compared with cell survivals, lymphocyte mortalities were lower.

![Figure 2. Apoptotic and Necrotic deaths of lymphocytes after stem bark extract of *P. rubra* L. ED0= 0 µg/mL, ED1= 20 µg/mL, ED2= 120 µg/mL, ED3=240 µg/mL, ED4= 350 µg/mL, ED5= 450 µg/mL, ED6= 570 µg/mL.](image)

Treatments affected the percentage of apoptosis (Figure 2.). The differences of apoptosis was similar to survival’s, i.e. no difference between cisplatin and control treatments, between cisplatin and the deaths after ED1, ED2 and ED 3 treatments were relatively the same. In addition, started from ED4, the deaths increased. Statistical results showed a significant difference of cell apoptosis percentages shown by p<0.05 of ANOVA test. The differences between cisplatin and others were started from ED4 (p<0.05 of LSD test). The same phenomena occurred between ED0 and the higher doses. There was no differences of apoptosis (p > 0.05) between the influence of cisplatin, ED0, ED1, ED2 and ED3 treatments.

In contrast, the percentage of necrotic cells was not different shown by p (0.1) >0.05. Hence, there was no percentage differences among treatments.
Figure 3. Cell survival, mortality, apoptosis and necrosis. Cell survival (lower left), cell mortality (lower right, upper right, and upper left), apoptotic cells (lower right and upper right), and necrotic cells (upper left). ED0= 0 µg/mL, ED1= 20 µg/mL, ED2= 120 µg/mL, ED3=240 µg/mL, ED4= 350 µg/mL, ED5= 450 µg/mL, ED6= 570 µg/mL

Figure 3. displays the results of flow cytometry running that were visualised by cell quest pro software. Here, the cell survivals are displayed in lower left areas. The cell mortalities are in lower right, upper right and upper left, apoptotic cells were calculated by the cells at lower right and upper right, and necrotic cells were shown in upper left regions.

4. Discussion
Cell death may occur through two ways: necrosis or apoptosis. Apoptosis or programmed cell death (PCD) is an active and metabolic cell death in responding to physiological stimuli (Eid et al., 2015). This type of death occurs through a controlled cell elimination being important in normal tissues homeostasis (Hahm & Davidson, 1998). The final feature of the cells, known as apoptotic bodies, are engulfed by other cells including macrophages and degraded by lysozyme (Hahm & Davidson, 1998; Eid et al., 2015).
Contrary, necrosis is a passive, catabolic and pathological cell death process which generally occurs in response to external toxic factors such as inflammation, ischaemic or toxic injury (Wu et al., 2001). It generally happens when infection or injury occurs, under control of immune response and memory cells. This occurrence is needed in repairing defective tissues. During its process, the cells released various molecules causing inflammation, such as HMGB1, that can initiate tumour appearance (Lotze and Demarco, 2004).

The ethanolic extract of \textit{P. rubra} contained 42 types of secondary metabolites, including terpenoids, phenolics, and L-bornesitol, with terpenoids being the most numerous (data not shown). Referring to previous partial examinations, 29 (69%) of the compounds, i.e., 17 terpenoids, 11 phenolics, and one vitamin B complex seemed capable to treat cancer. Some of them were cytotoxic and induced apoptosis.

The 9 highest concentrating metabolites were gallic acid, kaempferol, quercitrin, protoplumericin A, caryophyllene oxide, allamcin, fulvoplumierin, quercetin and plumerubroside (data not shown). By SwissADME these compound were included as nonAMES toxic except fulvoplumierin (data not shown). It means that the most main compounds were safe for health cells.

Table 1 revealed that started from ED4 (350 µg/mL of the extract) the lymphocyte survivals decreased. Contrary, their mortality increased after ED1 (20 µg/mL). This increase was seem to be caused by the increase of apoptotic cells (Table 2). In this case, the extract’s cytotoxicity occurred via apoptosis induction. Some secondary metabolites, such as gallic acid (Jara-palacios et al., 2015; Locatelli et al., 2013), kaempferol (Sak, 2014) and quercetin (Gupta et al., 2009; Baghel et al., 2012; Jara-palacios et al., 2015) related to this phenomena.

Apoptosis seem to be induced by the appearance of some secondary metabolites contained in the extract. Rosa et al. (2016) showed that gallic acid reduced cell viability by inducing apoptotic cell death. Locatelli et al. (2015) added that the apoptosis were inversely related to glutathion (GSH) content. Gallic acid also induced caspase-3 and -8 expression, altered the Bcl-2/Bax ratio, inhibited in tyrosine phosphorylation by BCR/ALB kinase and downregulate COX-2 levels (Locatelli et al., 2015).

Kaempferol is a flavonol that could induce apoptosis (Sak, 2014) through intrinsic (Xu et al., 2008; Kang et al., 2017) and extrinsic (Kasyap et al., 2017) apoptotic signalings. Its intrinsic signaling was characterized by activating proapoptotic proteins. However, the compound inhibited antiapoptotic proteins (Bcl2) release from mitochondria (Song et al., 2015). The extrinsic signalling involved TNF cognate apoptosis-inducing ligand (TRAIL) and TNFα. Kaempferol activates the apoptosis signaling pathway by upregulating TRAIL receptors which trigger the activation of intrinsic apoptotic pathway. It involved caspase 3 (Kim and Choi, 2013).

Other compounds, caryophylene oxide increased apoptosis through ROSmediated MAPKs activation (Sun et al., 2014). Moreover, quercetin induced apoptosis and DNA damage (Baghel et al., 2012).

Referring to Figure 1, the extract influenced the mortality of lymphocytes in dose dependent manner. It started affect the mortality at ED4, that was caused by apoptosis (Table 2), as well as the metabolites.

The extract doses and cisplatin (a cancer drug) did not affect necrosis of lymphocytes (Table 2) significantly (p > 0.05), although it seem to be higher in higher doses. Necrosis can be triggered by the existence of Reactive oxygen species (ROS) (Wang and Lin 2008) and some overdose agents (Darzynkiewicz et al., 1997). The necrosis occurrence may be due to the direct effect of the extract on the ionic structure of DNA. These leads to changes in the ion distribution of the DNA group functions and disrupt DNA self-protection (Methylation) against endonuclease which may lead to DNA degradation (Qari and El-Assouli, 2016). In this case, the such process did not occurred.

One of the nine highest metabolites contained, fulvoplumierin, may be the most potent to induce necrosis, because of its AMES toxic. However, the metabolite did not seem to affect this type of death significantly until ED6 (570 µg/mL) (Figure 2). Hence, the extract was safe for the lymphocytes as healthy cells, as well as did not release substances inducing inflammation and cancer growth (Lotze and Demarco, 2004).
Recent study showed that the cell survivals were different between doses (Figure 1). This phenomena also occurred for Juglone. Although Juglone had several medicine properties, the lymphocytes percentages decreased after its treatment, followed by the increase of apoptotic and necrotic cells by 24 hrs of incubation (Barathi, 2012). Mojibi (2019) identified survival of normal and cancer cells that decreased after extract of probiotic bacteria treatment, however the levels of significancy were different. Other normal cells, vero cell line’s, decreased around 23% because of some herbal drink extract treatments in 0.31-40 mg/mL (Ruwad, 2018). Sudeep et al. (2017) examined 3 extracts of 3 traditional medicines (Annona squamosa, Datura metel, and Mentha piperita) and revealed that the mortalities of lymphocytes were higher than cell survivals in 50 μg/mL, 100 μg/mL, dan 150 μg/mL. The LC50 of the stem bark extract of P. rubra on lymphocytes was lower than on cancer cells’, Kuswanti et al. (2018), even it was in non toxic category. However, the extract influenced the cell’s apoptosis. The same tencendcy occurred during Kashif et al. (2018) work. Although LC50 of Azadirachta indica oil, known as traditional medicine to cure multiple human and animal diseases, revealed lower toxicity on normal cells than cancer ones, apoptosis might happen. The percentage of the cells started to increase in the dose of 125 μg/mL. This tendency occured in recent study. However, the extract doses causing apoptotic increase were higher in P. rubra stem bark’s, i.e. 350 μg/mL. It indicated the safer for last extract. The pattern percentage change of apoptotic lymphocytes among treatments was similar to the cell mortality’s (Figure 1 and 2). It showed that the level of cell mortality was manly determined by their apoptosis.

The level of apoptotic cells can be an aspect of consideration in the use of drugs, because apoptotic lymphocyte enhancement can lead immunodeficiency through cell loss (Rathmell and Thompson, 2002). In certain condition, anticancer drugs induced lymphocyte depletion fastly, because of direct apoptosis induction to mature T and B cells (Stahnke et al., 2001). The percentages of necrotic cells were not different among the doses (Figure 2). This means that cisplatin and the stem bark extract of P. rubra (0 until 570ug/mL) did not affect inflammation. Ngcobo et al. (2012) examined the influence of the extract of Sutherlandia frutescens, a traditional anticancer medicine, on normal T lymphocytes. They discovered that started from 500ug/mL, necrotic deaths were higher than apoptosis’s and it was getting higher for higher concentrations. In addition, Azadirachta indica oil did not cause the necrotic increase of noncancerous cells in 0, 31, 62, and 125 μg/mL (Kashif et al., 2018).

The importance of necrotic status is based on the cell potency to releases substances promoting cancer growth. One of them was HMGB1 (high mobility group protein B1). The later showed that the necrotic cells also produced other substances, such as uric acid, calreticulin, and HSP70 inducing immune response in the form of inflammation (Kazama et al., 2008). Thus, this promotes cancer growth.

The facts revealed that some cancer drugs including plant extract promoted the increase of lymphocytes’s survivals, mortality, apoptosis and necrosis in different levels. Compared with the results of previous studies, the stem bark ethanolic extract of P. rubra was safer for health cells, especially lymphocytes, in the range of 0 until 570 μg/mL, especially according necrotic level.

5. Conclusion
Apoptotic and necrotic deaths are parameters for considering the further of drug candidates examinations. Based on its safeness on health cells, the stem bark extract of P. rubra L seemed capable to be one of the candidates, referring to the level of the death types. Compared with other substances discussed, the extract seemed to be better. The safeness was supported by low apoptosis and no differences of necrotic deaths among treatments, including the control parameters. It means that the examination of the ethanolic extract of P. rubra stem bark can be continued to further suitable advance steps in relation to treat cancer.

Acknowledgement
We would like to thank the laboratory assistant of Physiology, Structure and Development of Animals Laboratory of Biology Department of Brawijaya University, Indonesia, for facilitating this research.

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