Optimal Transfection Conditions and the Safety Profiles of Dextran-Spermine/Plasmid DNA as a Potential Gene Transfer Vector to Mouse Airway

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SUMMARY

The emergence of gene therapy offers a new paradigm to the field of molecular medicine. However, current viral and non-viral gene transfer vectors are not efficient and often restricted by dose-limiting toxicity. Thus, generation of a new gene delivery vector, which is efficient and with good safety profile is highly required. In this study, optimal transfection conditions and safety profile a novel biodegradable cationic polymerdextran-spermine (D-SPM) in mouse airways were ascertained. The highest level of gene expression in the lungs of BALB/c mice was detected at D-SPM to plasmid DNA (pDNA) weight ratio (w/w) of 16, with 13.5 µg pDNA. No significant induction of pro-inflammatory cytokines in the broncho alveolar lavage fluids was observed, which implies no overt toxicity occurred in the mouse lungs. In short, these results demonstrate that D-SPM has moderate gene transfer efficiency but with acceptable safety profile in the mouse airways.

INTRODUCTION

The advancement in the gene transfer technology has made possible for the development of gene therapy. However, the main challenge for a successful gene therapy is the development of a safe and effective gene transfer vector.The viral vectors have been extensively used in gene therapy clinical trials, as they are able to deliver gene efficiently to target cells.Yet, the risk of insertional mutagenesis by the provirus poses a serious safety concern (Hacein-Bey-Albina*et al*., 2003). Due to this reason, non-viral vectors are currently emerging as a favorable option to viral vectors.Hosseinkhani*et al.,*(2004) has shown that a new biodegradable polycation,dextran-spermine (D-SPM), was efficient attransfecting cells and tissues *in vitro* and *in vivo*.However, no study has been performed to ascertain the effectiveness and the safety of this gene transfer agent exclusively in the lung of mouse via intranasal delivery. Therefore, this study aims:(1) to determine the optimum weight-mixing ratio of D-SPM to pDNA and the loading capacity ofpDNA in the complexthat can give the maximum level of gene expression, and (2)to measure the induction of pro-inflammatory cytokines in the mouse airway.

MATERIALS AND METHODS

Plasmid pCIKLux expressing luciferase reporter gene was kindly provided by the Gene Medicine Research Group, Oxford University, UK. QIAGEN EndoFree® Plasmid Mega kit (Qiagen, Germany) was used to prepare a large-scale quantity of plasmid.Complexes of D-SPM and pDNA were prepared at various D-SPM/pDNA weight-mixing ratios of 13 to 18 (µg D-SPM/ µg pDNA) (w/w). To determine the most optimal amount of pDNA, increasing amount of pDNA(9.5 to 17.5 µg) was added to D-SPM with the w/w kept at a constant ratio. Female BALB/c mice, 6 to 8 weeks of age were used in the experiment, with n=6 for each experimental group. All animal experiments were carried out in accordance to the Guidelines for Animal Experiments of Universiti Putra Malaysia.Mice were euthanized by neck-dislocation. The lung and trachea of the mice were harvested for reporter gene expression analysis. The Luciferase Assay System (Promega, USA) was used for luciferase reporter quantification on a GloMax™ 20/20 luminometer (Promega, USA).The obtained relative light units (RLUs) from luciferase assay were normalized against total protein, which was quantified using a Bio-Rad DC Protein Assay (Bio-Rad Laboratories, USA).The levels IFN-γand TNF-α in the bronchio alveolar lavage fluids (BALF) were quantified using Duoset® ELISA Development System kits (R&D Systems, USA).

RESULTS

The D-SPM/pDNA complex ranging from ratio 13 to 18 showed almost similar levels of gene expression to

the untreated group. The highest reporter gene expression was observed at weight-mixing ratio of 16, where 91.23 ± 30.18 RLU/mg protein was obtained (Fig. 1a). For the optimal amount of pDNA, the highest gene expression level was detected from the D-SPM/pDNA complex containing 13.5 µg of pDNA, accounting for 158.1 ± 79.6 RLU/mg protein. This reading was approximately 3-fold higher compared to the untreated group, although it was not statistically significant (Fig. 1b). Mice were weighed prior to the gene delivery and after they were sacrificedat day 1 post-treatment. Although the D-SPM and D-SPM/pDNA treated mice showed a massive reduction of weight (results not shown), the mice appeared healthy with no physical sign of illness. For pro-inflammatory cytokines assay, theadministration of D-SPM/pDNA resulted in no significant difference in the levels of IFN-γ and TNF-α between the D-SPM/pDNA treated group and the control untreated group (Fig. 2).

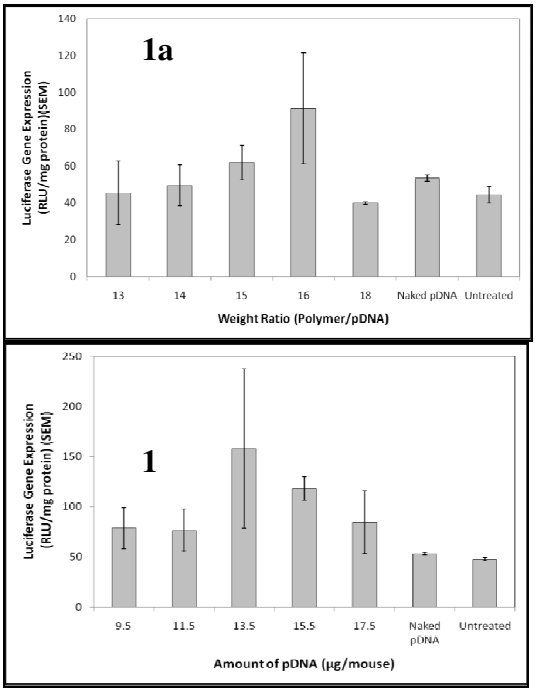

Fig. 1a. Luciferase expression in the trachea and lung of BALB/c mice following the administration of various weight-mixing ratios of D-SPM/pCIKlux complexes day 2 post-treatment.**Fig. 1b.** Luciferase expression in the trachea and lung of BALB/c mice following the administration of D-SPM with different amount of pCIKLux at day 2 post-treatment**.**

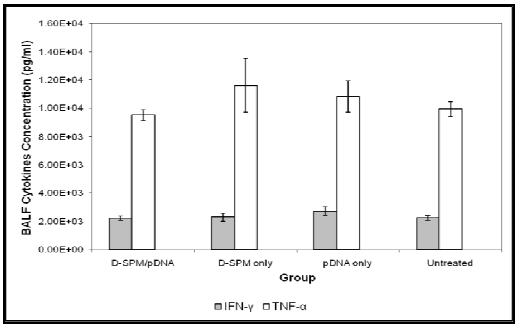

Fig. 2. Pulmonary inflammatory indicators, IFN-γ and TNF-α concentrations in the BALF of D-SPM/pDNA in comparison to naked pDNA and dextran-spermine only (D-SPM).

DISCUSSION

A modest increase of reporter gene expression by D-SPM/pDNA was observed at w/w of 16containing 13.5 µg of pDNA compared to the untreated group. No improvement of reporter gene expression was seen as the w/w or pDNA was increased. A possible reason to this is that increasing the w/w or pDNA may increase the cationic dextran moiety, resulting in the increase of the size of the complex. This eventually impedes its uptake once it reaches certain size due to the steric effect of the random charge distribution surface of the cationic carrier (Eliyahu*et al*., 2007). Although there was a massive reduction of mice weight seen in the D-SPM and D-SPM/pDNA treated groups, the doses were well tolerated and none of the mice died during the course of the experiment. No increase in the levels of TNF-αand IFN-γ was seen, implying no overt toxicity occurred in the lung of mouse following the delivery of the D-SPM/pDNA.These results suggest that although D-SPM/pDNA has moderate gene delivery ability, it was well tolerated and was relatively safe for gene transfer to the lung of mouse.

REFERENCES

1. Eliyahu, H., Joseph, A., Schillemans, J.P.,*et al*., 2007. Characterization and *in vivo* performance of dextran–sperminepolyplexes and DOTAP/cholesterol lipoplexes administeredlocally and systemically. *Biomaterials* 28(14): 2339-2349.
2. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al*. 2003. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 302:415-419.
3. Hosseinkhani, H., Azzam, T., Tabata, Y., Domb, A.J. 2004.Dextran-sperminepolycation: an efficient nonviral vector *in vitro* and *in vivo* gene transfection. *Gene Therapy*, 11: 194-203

Delivery of Plasmid Expressing Green Fluorescent Protein by PEGylated Dextran-Spermine to Acute Myeloid Leukemic Cells

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SUMMARY

Vascular endothelial growth factor (VEGF) is a potent angiogenic molecule and is overexpressed in most tumours and haematological malignancies. It is closely associated with tumor growth and metastasis. By exploiting the biological utilities of nanoparticles, we evaluated the potential of a cationic nano-sized PEGylated Dextran–Spermine (PEG-DPSM) in delivering plasmid carrying green fluorescent protein (GFP) to acute myeloid leukaemic cells. We evaluated the transfection efficiency of the PEG-DSPM/phMGFP nanoparticle in K562 and HL60 cells. The results suggest that improvements in the physiochemical property of the PEG-DSPM have to be made before it can be used to efficiently deliver therapeutic gene, such as VEGF inhibitors, to target acute myeloid leukemia.

INTRODUCTION

Angiogenesis is the formation of new blood vessels that sprout from the existing vasculature. One of most important angiogenic factors is the vascular endothelial factor (VEGF). VEGF has a central role in endothelial cell proliferation and differentiation. The VEGF-A165 isoform plays a pivotal role in tumour angiogenesis[1]. To date, 4 VEGF receptors have been identified; Flt-1 (VEGFR-1), Flk-1/KDR (VEGFR-2), Flt-4 (VEGFR-3), and neuropilin-1 (NRP- 1). The binding of VEGF-A to its receptors induces mitogenesis and chemotaxis of normal endothelial cells and increases vascular permeability, all of which contribute to new vessel formation and tumour growth[2]. The vital function of VEGF in malignant promotion suggests a potential avenue in cancer therapy by the blocking of VEGF[3]. Cationic molecular carriers are promising gene delivery systems that can be used to transport therapeutic gene which can subsequently increase the biological activity of VEGF inhibitors. They condense large genes into compact structures and mask the negative DNA charges to facilitate transfection into most cell types. A nano-sized cationic polysaccharide, Dextran-spermine (DSPM), has been shown to be highly efficient in transfecting cells *in vitro* and *in vivo*[4]. In addition, the neovasculature targeting can be improved by surface modification of the nanoparticles to incorporate a specific ligand for endothelial receptors[5]. In this study, the delivery efficacy of PEGylated dextran-spermine in acute leukemic cells was examined.

MATERIALS AND METHODS

The pcDNA3.1expressing hMGFP was generated. The hMGFP was isolated from phMGFP and the hMGFP fragment was inserted into pcDNA3.1. Sequencing was performed to confirm the sequence and orientation of the newly developed plasmid, phMGFP. GFP expression of recombinant plasmid was confirmed by transfection of K562 cells using Lipofectamine™ 2000 transfection reagent. The GFP expression was observed using fluorescence microscopy. The PEGylated DSPM/phMGFP complexes were constructed in different weight ratios (w/w) of PEG-DSPM to phMGFP. Agarose gel electrophoresis was used to determine the optimum ratio of the complex. The cytotoxic effects of these complexes were evaluated by performing the MTS assay on K562 and HL60 cells. The size and distribution of the complexes were measured by TEM and particle size analyzer. The transfection efficiency of the construct was evaluated based on GFP expression using flow cytometry.

RESULTS AND DISCUSSION

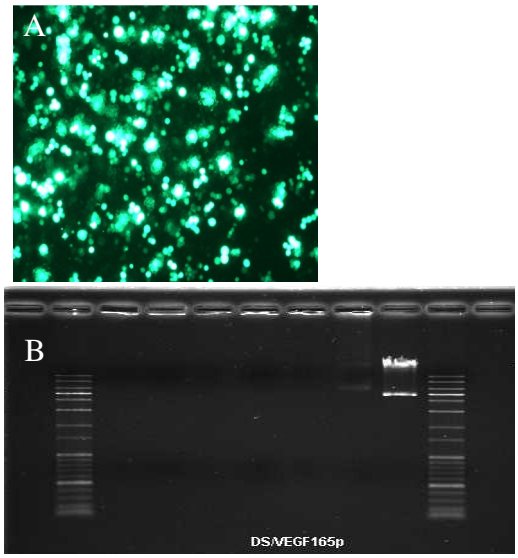
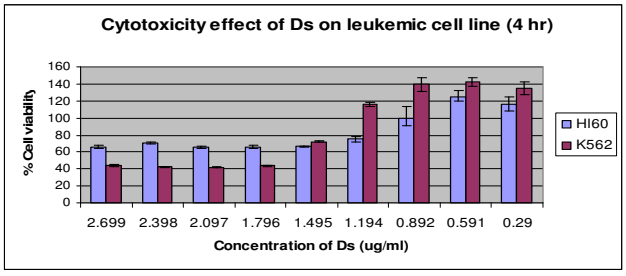


Fig 1: (A) Confirmation of GFP expression in cells transfected with phMGFP. (B) Agarose gel electrophoresis of different concentrations of PEG-DSPM/phMGFP (Lane 1-6: 500-400-200-100-50-10ug / 10ug of phMGFP, Lane 7: Uncut phMGFP). GFP expression was confirmed in transfected K562 cells at 72 hours post-transfection with lipofectamine/phMGFP complex. Agarose gel electrophoresis confirmed that the optimum ratio of the PEG-DSPM/phMGFP complex was at the ratio of 5.

A



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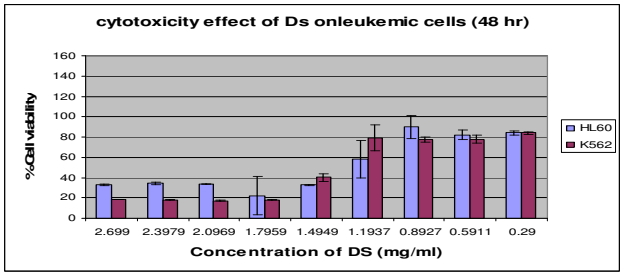

Fig 2: MTS assay analysis of cytotoxic effects of the transfection of PEG-DSPM/p***hMGFP*** complex on K562 and HL60 cells at 4 hours (A) and 48 hours (B)

The IC_{50} of the PEG-DS***PM/phMGFP*** complex was 50.1187 ug/ml at 4 hours, and 28.183 ug/ml at 48 hours.

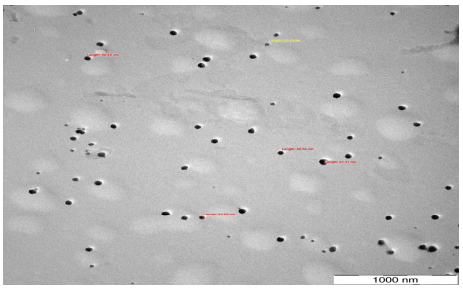

Fig3: TEM analysis of size and distribution of PEG-DSPM/phMGFP nanoparticle.

By using the TEM, we estimated the size of PEG-DSPM/phMGFP complex to be between 31.19 nm and 61.31nm.

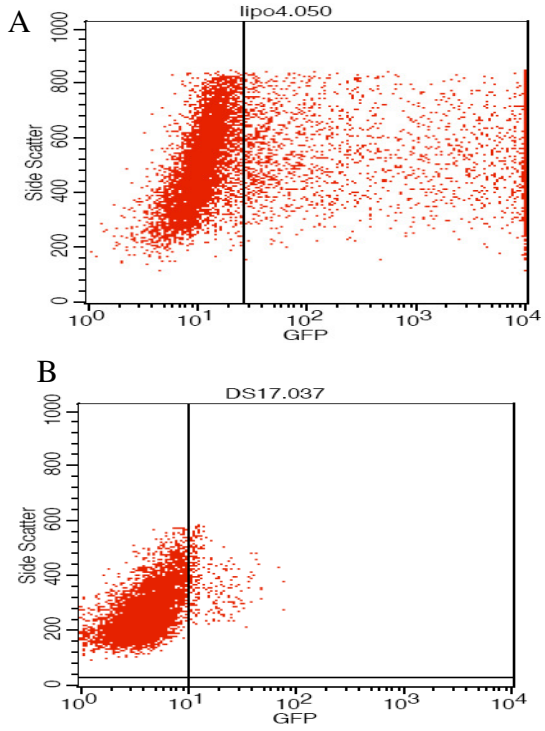

Fig 4: GFP expression analysis by flowcytometry of K562 transfected by (A) Lipofectamine/phMGFP and (B) PEG-DSPM/phMGFP

PEG-DSPM only yielded < 10% of GFP positive K562 cells as compared to >30% when Lipofectamine was used.

CONCLUSION

Our results demonstrate that although we have characterized and determined the optimum ratio of PEG-DSPM and phMGFP, the transfection efficiency of the complex is not ideal. Further modifications on the physiochemical properties of the PEG-DSPM, such as increasing the polymer length or masking the anionic charges of the complex, are required before the GFP gene can be substituted with VEGF inhibitors to target acute myeloid leukemia.

REFERENCES

1. Taniguchi, E., Sakisaka, S., Matsuo, K., et al. (2001). Expression and Role of Vascular Endothelial Growth Factor in Liver Regeneration After Partial Hepatectomy in Rats. *Histochemistry & Cytochemistry*., 49; 1: 121–129.
2. Ueda, Y., Yamagishi, T., Samata, K., et al. (2003). A novel low molecular weight antagonist of vascular endothelial growth factor receptor binding: VGA1155. *Molecular Cancer Therapeutics*., 1105-1111.
3. He, Y., Smith, S.K., Day, K.A., et al. (1999). Alternative Splicing of Vascular Endothelial Growth Factor (VEGF) - R1 (FLT-1) pre-mRNA Is Important for the Regulation of VEGF Activity. *MOL ENDO.,* 13; 4:537-544.
4. Hosseinkhani, H., Azzam, T., Tabata, Y., and Domb, A.J. (2000). Dextran-spermine polycation: An efficient none –viral vector for in vitro and in vivo gene transfection.
5. Koomagi, R., Zintl, F., Sauerbrey, A., and Volm, M. (2001). Vascular Endothelial Growth Factor in Newly Diagnosed and Recurrent Childhood Acute Lymphoblastic Leukemia as Measured by Real-Time Quantitative Polymerase Chain Reaction. *Clinical Cancer Research*. 7: 3381–3384.

Calreticulin: A Novel Biomarker of Invasive Breast Cancer

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SUMMARY

Calreticulin (CRT) is a multifunction endoplasmic reticulum protein. We investigated the expression pattern of CRT in a cohort of breast cancer tissues and through siRNA modulation of gene expression, attempted to delineate the genotype-invasive phenotype correlation. Specific overexpression of CRT in the stromal compartments of malignant tissues was confirmed by immunohistochemistry. Our CRT-knockdown cell line model implicates that breast cancer cells' migratory potential is CRT-dosage dependent.

INTRODUCTION

Breast cancer is the second leading cause of cancer deaths [1]. Invasion is an important hallmark of cancer that confers the ability of cells tometastasize [2]. Hence, finding new molecular predictors of invasion and metastasis is the key for the improvement of cancer management[3]. CRT is an endoplasmic reticulum protein. CRT is a major player in intracellular calcium storage and transfer [4]. It also plays a role in other cellular functions, namely chaperoning and adhesion[5,6]. Some studies have tried to correlate overexpression of CRT in tumourigenesis [7,8,9]. For example, CRT has been postulated as a contributing factor in thrombospondin-1's role in invasion [10]. CRT has been shown to interact with Estrogen Receptor- α and reverts hormone independent inhibition of breast cancer cell invasion [8].

MATERIALS AND METHODS

To confirm the CRT-associated breast cancer pattern of expression, immunohistochemistry analysis of paraffin-embedded breast cancer tissues of different invasive states was conducted. Using siRNA technology, we then developed a CRT-knockdown model of MCF7, breast cancer cell line and evaluated the relationship between CRT expression and the migratory potential using migration assays. Western blotting and quantitative-real time polymerase chain reaction (qRT-PCR) was used to evaluate the level of CRT expression. ß-actin was used as the house keeping gene.

RESULTS

Immunohistochemistry and meta-analysis of the histopathological results (Figure 1) confirmed that the expression of CRT was significantly higher in the stromal parts of malignant tissues when compared to those from the non-malignant samples (Table 1). Subsequently, successful siRNA-mediated CRT gene silencing in MCF7 cells was confirmed both atmRNA and protein levels by qRT-PCR and western blot, respectively. Consequently, using a migration assay, the migratory potential of CRT-deficient cells were compared with CRT-expressing cells (control) (Figure 2). The results demonstrated a significant loss in the migratory potential of CRT-deficient cells ($p<0.05$).

Fig 1: CRT expression and localization in a spectrum of breast cancer tissues. High-intensity CRT expression was found in the glandular and stromal compartments of breast cancer tissues. While expression was detectable in the nucleus, the majority of expression was cytoplasmic.

		Sum of Squares	df	Mean Square	F	Sig.
Glandular Stage	Between Groups	10.854	3	3.618	.777	.516
	Within Groups	139.617	30	4.654		
	Total	150.471	33			
Stroma Stage	Between Groups	60.128	3	20.043	4.985	.005
	Within Groups	164.850	41	4.021		
	Total	224.978	44			
Invasive Stage	Between Groups	28.301	2	14.150	3.213	.053
	Within Groups	145.338	33	4.404		
	Total	173.639	35			

Table 1: One-way ANOVA of immunohistochemistry analysis of CRT expression. The analysis showed significant higher CRT expression in stromal parts of cancer samples compared to non-malignant samples ($p<0.05$).

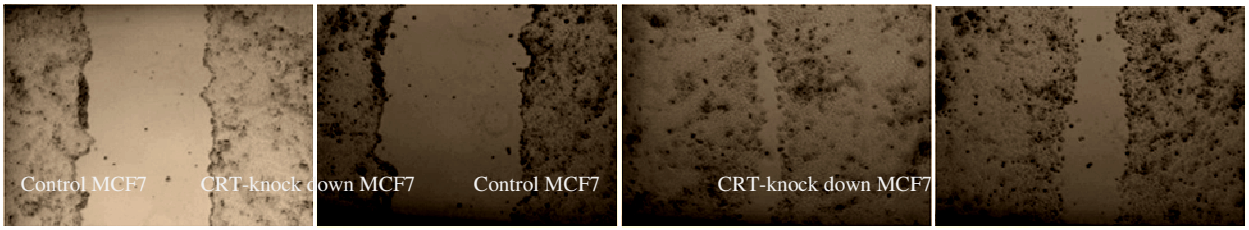

Figure 2 – **Migration assay.** The assay showed significant higher migratory potentials in control MCF7 cells as compared to CRT-knockdown cells

DISCUSSION AND CONCLUSION

Our results demonstrate that CRT, is involved (in)directly in conferring an invasive phenotype. We confirmed a higher expression of CRT in malignant tissues. By knocking down CRT *in vitro*, we were able to reduce the migratory potential of breast cancer cells. Future work will focus on defining the mechanistic role of invasion and identifying possible CRT-dependent pro-invasive targets as diagnostic, prognostic and therapeutic biomarkers of breast cancer.

REFERENCES

1. Ghafoor A, Jemal A, Ward E, Cokkinides V, Smith R, Thun M, (2004). Trends in breast cancer by race and ethnicity. CA Cancer J Clin. 2003 Nov-Dec;53(6):342-55. Erratum in: CA Cancer J Clin.54(3):181.
2. Van't Veer LJ, Paik S, Hayes DF, (2005). Gene expression profiling of breast cancer: a new tumor marker. J Clin Oncol. 10;23(8):1631-5.
3. Wittekind C, Neid M. (2005) Cancer invasion and metastasis. Oncology. 69 Suppl 1:14-6.
4. Michalak M, Corbett EF, Mesaeli N, Nakamura K, Opas M,.(1999) Calreticulin: one protein, one gene, many functions. Biochemistry Journal. 1;344 Pt 2:281-92.
5. Michalak M, Milner RE, Burns K, Opas M., (1992) Calreticulin. Biochemistry Journal. 1;285 (Pt 3):681-92.
6. Kageyama S, Isono T, Iwaki H, Wakabayashi Y, Okada Y, Kontani K, Yoshimura K, Terai A, Arai Y, Yoshiki T. (2004) Identification by proteomic analysis
7. of calreticulin as a marker for bladder cancer and evaluation of the diagnostic accuracy of its detection in urine. Clincal Chemistry. 50(5): 857-66.
8. Yoon GS, Lee H, Jung Y, Yu E, Moon HB, Song K, Lee I. (2000) Nuclear matrix of calreticulin in hepatocellular carcinoma. Cancer Research. 15;60(4):1117-20.
9. Platet N, Cunat S, Chalbos D, Rochefort H, Garcia M,. (2000) Unliganded and liganded estrogen receptors protect against cancer invasion via different mechanisms. Molecular Endocrinology.14(7):999-1009.
10. Du XL, Yang H, Liu SG, Luo ML, Hao JJ, Zhang Y, Lin DC, Xu X, Cai Y, Zhan QM, Wang MR, (2009). Calreticulin promotes cell motility and enhances resistance to anoikis through STAT3-CTTN-Akt pathway in esophageal squamous cell carcinoma. Oncogene. (42):3714-22.
11. Dai E, Stewart M, Ritchie B, Mesaeli N, Raha S, Kolodziejczyk D, Hobman ML, Liu LY, Etches W, Nation N, Michalak M, Lucas A, (1997) Calreticulin, a potential vascular regulatory protein, reduces intimal hyperplasia after arterial injury. Arteriosclerosis, Thrombo Vascular Biolology. 17(11): 2359-68.

Gender Differences in T Cell Stimulation by Mitogen Detected using Flowcytometry

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SUMMARY

Lineage identification of blood cells proliferated in culture is possible on flow cytometry unlike conventional method using labeled thymidine. We determined the percentage of proliferated T cells (CD3), monocytes (CD14) and granulocytes (CD16) after stimulation with PHA, LPS and PMA using flow cytometry. Increased proliferation of immune cells was observed after mitogen stimulation. We also observed gender differences in the percentage of proliferated cells. Flow cytometry may be useful as a tool to study functional assays of immune cells.

INTRODUCTION

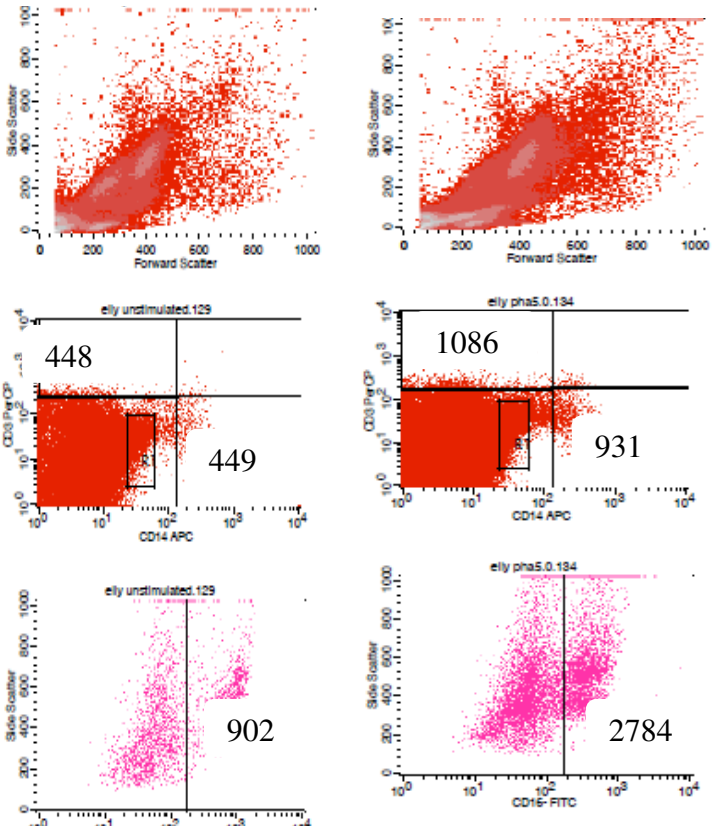
The protective function of the immune system may be compromised by various diseases and conditions. The many components of the immune system and heterogeneity in cell lineages and function make it necessary to identify the particular arm that is defective. The functional aspect of immune response may be determined by an increase in cell proliferation after stimulation with mitogens. Mitogens such as PHA (phytohemagglutinin), LPS (lipopolysaccharide) and PMA (phorbol 12-myristate 13-acetate) are used to stimulate different population of immune cells. The conventional method to measure cell proliferation involves the incorporation of isotopic thymidine into cultured PBMC isolated from blood. The drawbacks of this method include the use of radioisotopes, the need to isolate PBMC and the inability to identify the cell lineage stimulated.

MATERIALS AND METHOD

We tested an alternative method which uses a small volume of whole blood and utilizes the flow cytometry detection of surface marker CD3, CD14 and CD16 to identify T cells, monocytes and granulocytes, respectively. Cells were collected from six male and six female apparently healthy donors. Three different concentrations of PHA, LPS and PMA were incubated with cells for three days. Analysis was performed based on modified version as described by Haines and Gunner (2000). Cell proliferation was determined by the increase in the number of events of proliferated cells identified by specific CD marker after stimulation with mitogen (Figure 1).

RESULTS

We observed PHA significantly increased the percentage of T cells, monocytes and granulocytes. LPS and PMA significantly increased the percentage of T cells, suppressed monocytes but had no effect on granulocytes. Furthermore, we also found stimulation of T cells by PHA was significantly higher among male than female samples (Figure 2)



A) Before Stimulation B) After Stimulatiom

Figure 1: Flowcytometric analysis of a blood sample A) before and B) after 3-days mitogen stimulation. i) Scatter plots of whole sample. Cell events positive for ii) CD3 (y-axis) and CD14 (x-axis) and iii) CD16 were detrmined from the respective quadrants.

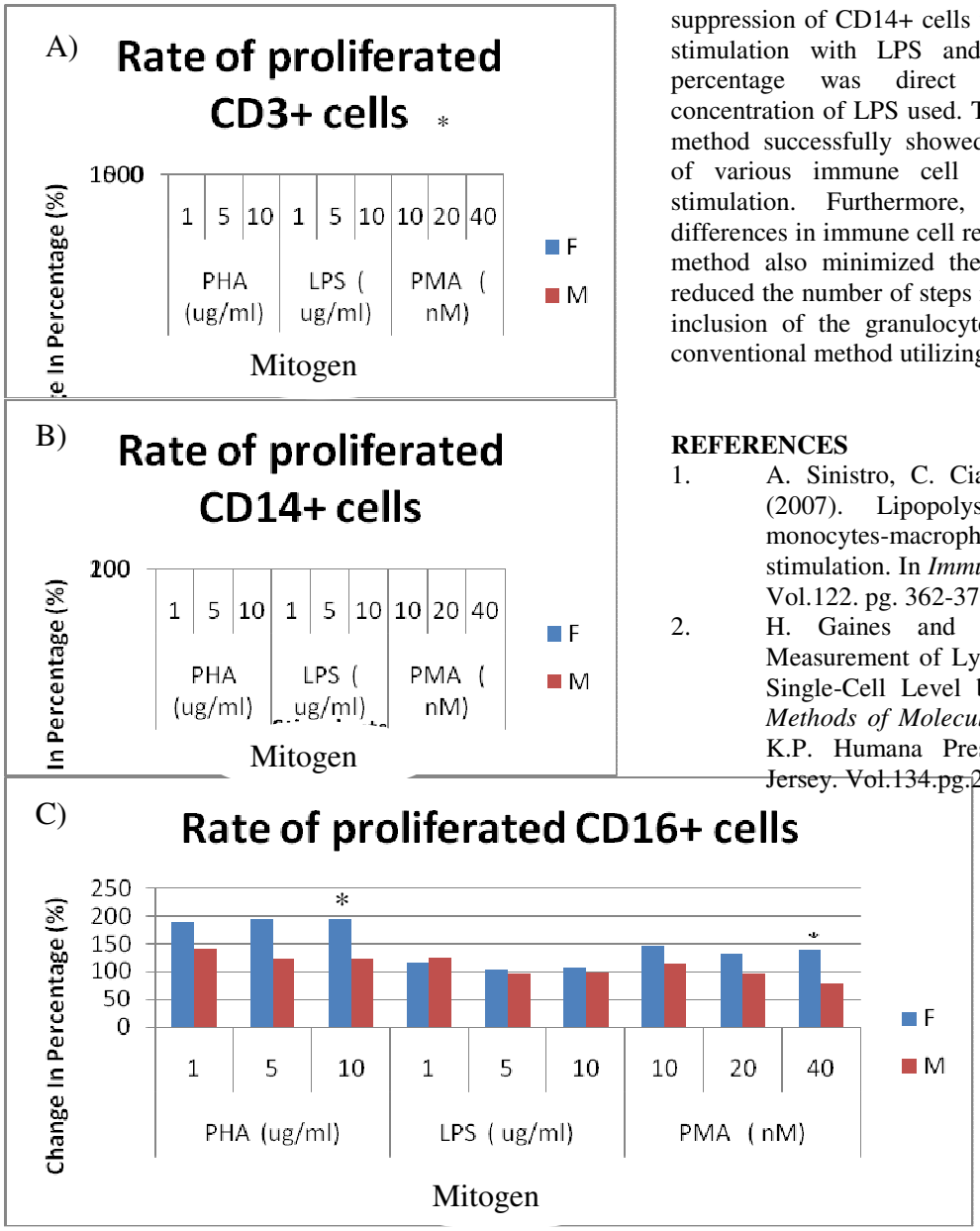


Figure 2: Percentage increase in A) CD3 B) CD14 and C) CD16 cells after stimulation with various concentration of PHA, LPS and PMA among male and female healthy donors. * Cell percentages significantly different between genders ($p<0.05$).

DISCUSSION AND CONCLUSION

The result here showed an increase in the percentage of the CD3+ after stimulation with PHA and PMA from both female and male donors. It has also been shown that monocytic cells showed a dose-dependent reduction to LPS stimulation. In the results suppression of CD14+ cells were also observed after stimulation with LPS and the decrease in cell percentage was direct proportional to the concentration of LPS used. Thus, the flow cytometry method successfully showed increased proliferation of various immune cell lineages after mitogen stimulation. Furthermore, it identified gender differences in immune cell response to mitogen. This method also minimized the risk of contamination, reduced the number of steps required and allowed the inclusion of the granulocyte population unlike the conventional method utilizing PBMC isolation.

REFERENCES

1. A. Sinistro, C. Ciaprini, S. Natoli, *et al*. (2007). Lipopolysaccharide desensitizes monocytes-macrophages to CD40 ligand stimulation. In *Immunology Original Article.* Vol.122. pg. 362-370.
2. H. Gaines and G. Biberfeld. (2000). Measurement of Lymphoproliferation at the Single-Cell Level by Flow Cytometry. In *Methods of Molecular Biology*. Ed. Kearse, K.P. Humana Press Inc., Totowa, New Jersey. Vol.134.pg.243-255.

Haematological Parameters of Leukaemic Rats Supplemented with *Morinda Citrifolia*

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SUMMARY

Morinda citrifolia (mengkudu) had been reported to have anti-tumor activity, which has been researched widely in several animal models. Sixty four eight-week-old male Sprague Dawley rats were divided into four groups of 16 rats per group namely control, MNU, *Morinda citrifolia* (MC), and MC+MNU group. The MNU and MC+MNU groups received four consecutive intraperitoneal (i.p) injections of *N*-methyl-*N*-nitrosourea (MNU) at a dose of 60 mg/kg for induction of leukaemia. Rats in the MC and MC+MNU groups were fed daily with a ration mixed with *M. citrifolia* at a dose of 5000 mg/kg body weight. The peripheral blood samples were collected at 20 weeks post MNU administration into EDTA tubes and analysed for a complete blood count. Blood smears stained with Wright's stain were prepared for a manual differential leukocyte count and examination of the leukaemic cells. The results were analysed using a one-way ANOVA. Results in this study showed that MNU group had significant lymphocytosis (66.9±98.14) compared to the other groups. The morphology of the lymphocytes in the MNU and MC+MNU groups showed a typical morphology of leukaemic cell, while the other groups had normal lymphocyte morphology. Rats in the MNU group also had anaemia with significant reduction in total erythrocyte number (6.11±2.73 x 10^{12}/L), haemoglobin concentration (131.64±21.32 g/L) and packed cell volume (37.92±8.50 L/L). The erythron parameters of MC+MNU group were comparable to the control and MC groups. In conclusion, daily supplementation of *M. citrifolia* reduced the proliferation of circulating leukaemic cells.

INTRODUCTION

Morinda citrifolia (mengkudu) had been reported to have anti-microbial and anti-tumor activities, which has been researched widely in several animal models [1,2].*N*-methyl-*N*-nitrosourea (MNU) is a model of carcinogenic chemicals based on sufficient evidence of carcinogenicity in experimental studies [3,4]. It induced both benign and malignant tumors in various organs depending on the species and the routes of administration [5]. The administration of MNU at high sub-lethal doses characteristically induced early and high incidence of leukaemia and/or malignant lymphomas in young rats [3,4]. The present study investigated the anti-tumour effects of *M. citrifolia* on experimental-induced leukaemia in male Sprague Dawley rats using the MNU.

MATERIALS AND METHODS

A total of 64 eight-week-old male Sprague Dawley rats were housed in an animal room with controlled conditions at the Animal House, Malaysian Agriculture Research and Development Institute, Serdang and the rats were provided with tap water and fed with commercial chow daily. The rats were divided into 4 groups of 16 rats per group namely control, MNU, MC and MC+MNU group. Leukaemia was induced by administrating intraperitoneally (i.p) freshly prepared MNU (Sigma-Aldrich, N4766-25G) at a dose of 60 mg/kg body weight for two consecutive weeks (total dose of 240 mg/kg body weight). Dried *M. citrifolia* fruits were ground into powder and mixed into ration. The ration was fed daily to the rats with a dose of 5000 mg/kg body weight. The rats were euthanised at 20 weeks post i.p injections of MNU by bleeding under anaesthesia. The peripheral blood samples were collected into EDTA tubes and analysed for a complete blood count using an Automated Haematology Analyser (Cell-Dyn3700®, Abott, USA). Blood smears were prepared and stained with Wright's stain for a manual differential leukocyte count and examination of leukaemic cells. The results were analysed statistically using one way-ANOVA (SPSS version 17).

RESULTS

Results in this study showed MNU group had significant lymphocytosis compared to the control, MC and MC+MNU groups (Table 1). The morphology of the lymphocytes in the MNU and MC+MNU groups showed a typical morphology of leukaemic cell, while the other groups had normal

lymphocyte morphology. Rats in the MNU group also had anaemia with significant reduction in total erythrocyte number, haemoglobin concentration and packed cell volume (PCV) (Table 2). The erythron parameters of MC+MNU group were comparable to the control and MC groups.

Table 1: Total leukocyte and differential leukocyte numbers (Mean±SD)

Group	Total leukocyte (x 10^9/L)	Lymphocyte (x 10^9/L)	Neutrophil (x 10^9/L)	Monocyte (x 10^9/L)	Eosinophil (x 10^9/L)	Basophil (x 10^9/L)
Control	11.4±2.0	8.8±1.6	2.0±0.5	0.36±0.13	0.21±0.10	0
MNU	70.3±98.9*	66.9±98.1*	3.1±1.6	0.20±0.25	0.10±0.10	0.01±0.04
MC	12.3±1.3	9.6±1.3	2.0±0.6	0.37±0.13	0.24±0.14	0
MC+MNU	16.1±10.5	13.4±10.6	2.3±1.1	0.28±0.32	0.17±0.13	0

*Significantly different at $p<0.05$

Table 2: Total erythrocyte number, haemoglobin concentration and PCV (Mean±SD)

Group	Erythrocyte (x 10^{12}/L)	Haemoglobin (g/L)	PCV (L/L)
Control	8.51±0.47	150.12±5.00	45.31±1.57
MNU	6.11±2.73*	131.64±21.32*	37.92±8.50*
MC	8.80±0.51	151.43±4.70	45.31±1.85
MC+MNU	7.62±1.77	142.47±18.34	42.18±4.24

*Significantly different at $p<0.05$

DISCUSSION

Chemical compounds extracted from *M. citrifolia* have been shown to inhibit the activity of oncogenes associated with various tumors [6]. The extract from the *M. citrifolia* fruit juice for example can prolong the life of C57 B1/6 mice implanted with lung carcinoma [7] and prevent mammary gland cancer induced by 7, 12-dimethylbenz(a)anthracene (DMBA) in female Sprague Dawley rats [8]. Similar to those findings, results in this study showed supplementation of *M. citrifolia* dried fruit reduce the proliferation of circulating leukaemic cells in rats.

REFERENCES

1. Locher CP, Burchb MT, Mowerb HF, *et al.* Anti-microbial activity and anti-complement activity of extracts obtained from selected Hawaiian medicinal plants. *Journal of Ethnopharmacology* 1995; 49: 23-32.
2. Wang MY and Su C. Cancer preventive effect of *Morinda citrifolia* (Noni). *Annals of New York Academy Science* 2001; 952: 161-169.
3. Uwagawa S, Tsuda H, Inoue T, *et al*. Enhancing potential of 6 different carcinogens on multi-organ tumorigenesis after initial treatment with *N*-methyl-*N*-nitrosourea in rats. *Japanese Journal of Cancer Research* 1991; 82: 1397-1405.
4. Locher CP, Burchb MT, Mowerb HF, *et al.* Anti-microbial activity and anti-complement activity of extracts obtained from selected Hawaiian medicinal plants. *Journal of Ethnopharmacology* 1995; 49: 23-32.
5. Wang MY and Su C. Cancer preventive effect of *Morinda citrifolia* (Noni). *Annals of New York Academy Science* 2001; 952: 161-169.
6. Uwagawa S, Tsuda H, Inoue T, *et al*. Enhancing potential of 6 different carcinogens on multi-organ tumorigenesis after initial treatment with *N*-methyl-*N*-nitrosourea in rats. *Japanese Journal of Cancer Research* 1991; 82: 1397-1405.
7. Locher CP, Burchb MT, Mowerb HF, *et al.* Anti-microbial activity and anti-complement activity of extracts obtained from selected Hawaiian medicinal plants. *Journal of Ethnopharmacology* 1995; 49: 23-32.
8. Wang MY and Su C. Cancer preventive effect of *Morinda citrifolia* (Noni). *Annals of New York Academy Science* 2001; 952: 161-169.

Toxicological Evaluation of *Phyllanthus amarus* in Rats: Haematological Profiles

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SUMMARY

A toxicity study of *Phyllanthus amarus* was conducted on female Sprague-Dawley (SD) rats in accordance to the OECD Guideline 407 for repeated dose toxicity. A total of 30 rats (10 per group) were fed crude freeze dried *Phyllanthus amarus* at a dose of 0, 2000 and 5000 mg/kg body weight/day for 28 days. Clinical observations were recorded and body weight and feed consumption measured throughout the study. At the end of the study all rats were subjected to a full necropsy and blood samples were collected for clinical pathology. Total leucocyte count (5.98 x10³/µl) and lymphocyte count (4.60 x10³/µl) were found to be significantly ($p \leq 0.05$) lower in rats of the low dose group. Nevertheless, the differences observed were within normal range of normal healthy rats and were considered to be of no toxicological significance. It was concluded that *Phyllanthus amarus* was not toxic at the highest inclusion rate of 5000 mg/kg body weight.

INTRODUCTION

Phyllanthus amarus locally known as "dukung anak" has been used in Malaysian traditional medicine (MTM) and other tropical countries for the treatment of various diseases and illnesses. Several *in vitro* and *in vivo* studies demonstrate a range of potentially beneficial effects against jaundice, constipation, diarrhoea, kidney ailments, ringworm, ulcers, malaria, genito-urinary infections, hemorrhoids and gonorrhea [1]. *Phyllanthus amarus* was also observed to demonstrate anti-inflammatory [2] and anti-viral properties [3]. The active components of *Phyllanthus amarus* such as lignans, flavonoids, alkaloids, ellagitannins, terpenes, and phenylpropanoids are believed to contribute to these medicinal effects of this plant [4]. The increased use of this plant has resulted in concern about both its efficacy and safety. Despite wide spread use of *Phyllanthus amarus* in MTM, a literature survey revealed the lack of proper toxicological evaluation of these local varieties. It is particularly important to detect toxicity occurring either after a short or prolonged exposure to *Phyllanthus amarus*. The toxicity studies also provide a preclinical safety evaluation standard expected to be performed before *Phyllanthus amarus* can be evaluated in human. This study was therefore carried out with the objective of investigating the safety of *Phyllanthus amarus* through a 28-day subacute toxicity study in Sprague Dawley (SD) rats.

MATERIALS AND METHODS

A total of 30 female Sprague-Dawley (SD) rats at 4 weeks of age, with an average body weight of 100-120 grams were used in the study. The rats were acclimatized to the housing conditions for a period of 2 weeks and the treatment commenced at the age of 6 weeks. All the rats were individually housed in polycarbonate mesh bottom cage during acclimatization period and thereafter, kept in a room maintained at a temperature of 25-27°C with a relative humidity of 40-70% in a 12-h light/dark cycle regime. The rats were weighed and randomly assigned to three groups (10 per group) namely control, low dose and high dose according to randomized complete block design (RCBD). Crude freeze dried *Phyllanthus amarus* was incorporated into rats diet at dose levels of 0 (control), 2000 (low dose) and 5000 (high dose) mg/kg body weight/ day for 28 days. Feed were given *ad libitum* and all rats had free access to water. The amounts of supplied and residual diet were weighed twice a week in order to calculate the daily food consumption. The rats were observed daily for clinical signs and mortality. At the end of the study, all the rats were subjected to a full necropsy. Blood samples were collected for clinical pathology, selected organs were weighed and tissues were preserved from all animals for histopathology. The following haematological components were analyzed: total and differential white blood cells (WBC), red blood cells (RBC), haematocrit/packed cell volume (HCT/PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets using an automated blood analyzer (Cell Dyn® 3700, Abbott Diagnostic, USA). The mean values and standard errors were calculated from the

data obtained and then statistically analyzed using SAS version 9.1. Duncan's multiple range analysis was employed to determine the differences in parameters between treatments group.

RESULTS

There was no significant difference that was attributed to the administration of the test substance between groups (Table 1) except the total leucocyte count and lymphocyte count which were significantly ($p \leq 0.05$) lower in low dose group compared to control.

DISCUSSION AND CONCLUSION

The present study was conducted to evaluate the safety of *Phyllanthus amarus* included daily in a diet of female Sprague-Dawley rats. *Phyllanthus amarus* was well tolerated and did not produce any signs of toxicity when fed at dose level 2000 and 5000 mg/kg/day. There were no adverse haematologic changes related to the treatment doses. Nevertheless, the lower leucocyte count in low dose group could not be ascertained but the value was within normal range of rats of this strain and age. In this study, significantly decreased leucocytes were contributed by the decrease in lymphocytes, nevertheless, the lymphocytes were still within the normal range of normal healthy rat. Lymphocytes are a type of white blood cell that is responsible for protecting the body against bacterial and viral infections. One of the most common causes of clinically low WBC is an underlying viral infection which can cause a temporary drop in lymphocytes as more of them are drawn away to fight infection [5]. Based on the observation made throughout the experimental period, all the rats were clinically healthy and did not show any signs of infection. Therefore, the low leucocyte and lymphocyte counts were considered to be no toxicological significance. It was concluded that *Phyllanthus amarus* was not toxic, even at the highest inclusion rate of 5000 mg/kg body weight.

Table 1: The means haematology value of treatment groups in female rats fed *Phyllanthus amarus* (mean ± S.E.M).

Parameters	Treatment Group				
	Control	Low Dose	High Dose	p-value	Normal range
WBC (x10³/µl)	8.91 ± 0.95[a]	5.97 ± 0.58[b]	7.69 ± 0.82[a,b]	0.04	(2.9-20.9)
Neu (x10³/µl)	1.41 ± 0.33	0.88 ± 0.33	0.87 ± 0.07	0.13	(0.15-7.69)
Lymph (x10³/µl)	6.73 ± 0.61[a]	4.59 ± 0.58[b]	6.19 ± 0.73[a,b]	0.05	(1.64-19.5)
Mono (x10³/µl)	0.43 ± 0.06	0.21 ± 0.05	0.39 ± 0.31	0.17	(0.00-1.61)
Eosin (x10³/µl)	0.18 ± 0.04	0.12 ± 0.03	0.21 ± 0.08	0.48	(0.00-0.71)
Baso (x10³/µl)	0.15 ± 0.06	0.17 ± 0.10	0.09 ± 0.05	0.76	(0.00-0.18)

RBC (x10^6/µl)	8.63 ± 0.17	8.75 ± 0.16	8.79 ± 0.12	0.73	(4.60-9.19)
Hb (g/dl)	16.8 ± 0.28	16.6 ± 0.36	16.8 ± 0.23	0.92	(10.0-16.9)
HCT (%)	47.2 ± 0.90	46.9 ± 1.00	47.2 ± 0.63	0.96	(34.0-53.0)

[a,b]: Means with different superscript/s within same row differ significantly ($p \leq 0.05$)

REFERENCES

1. Unander, D.W., Webster, G.L., and Blumberg, B.S. Usage and bioassays in Phyllanthus (Euphorbiaceae). IV. Clustering of antiviral uses and other effects. *Journal of Ethnopharmacology*, (1995), 45: 1–18.
2. Kiemer, A.K., Hartung, T., Huber, C., and Vollmar, A.M. Phyllanthus amarus has anti-inflammatory potential by inhibition of iNOS, COX-2, and cytokines via the NF-kappaB pathway. *Journal of Hepatology*, (2003), 38: 289–297.
3. Thyagarajan, S.P., Jayaram, S., and Valliammai, T. Phyllanthus amarus and hepatitis B. *Lancet* (1990), 336: 949–950.
4. Lesley, T. Technical Data Report for Chanca Piedra "Stone Breaker". In T. Lesley, (Ed.), *Herbal Secret of the Rainforest*, (2003), (p. 1-62) Austin: Sage Press Inc.
5. David, M.M. Hematology of the Rat (*Rattus Norvegicus*). In Feldman B.E., Zinkl J.G. and Jain N.C. (Eds.), *Schalm's Veterinary Hematology 5th ed*. (2000), (p. 1210-1218). Baltimore, Maryland: Lippincott Williams & Wilkins.

In Vitro Anti-cancer Activity of *Ficus deltoidea* Ethanolic Extract on Human Ovarian Carcinoma Cell Line

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SUMMARY

Ficus deltoidea is a Malaysian traditional herb that is known for its therapeutic value. The aim of this study is to examine the effect of ethanolic extract towards human ovarian carcinoma cell line or A2780. MTT assay was performed to see the growth pattern of this cell. The measurement of glucose uptake and LDH release has been done for further conformation. The fluorochrome staining by fluorescence microscopy using acridine orange and ethidium bromide double staining was also been performed in this study to examine the presence of apoptotic body.

INTRODUCTION

Ficus deltoidea or Mas Cotek is from Moraceae family also known as serapat, sempit-sempit, and agoluran in Sabah and tabat barito in Indonesia (Wan Hasan, 2007). The fruits are chewed to relieve headache, toothache and cold; powdered root and leaves of the plant has been applied externally to wounds and sores and around the joints to relief of rheumatism (Sulaiman et al., 2008). Some local people also believe that this herb can actually cure cancer.

MATERIALS AND METHODS

F. deltoidea was collected from Muadzam, Pahang. The ethanolic extract was prepared according to the methods by Song et al., 2007 with a slight modification. A2780 cell line was used and cultured in RPMI 1640 medium, FBS and Glutamine. Cell proliferation assay was done using slightly modified protocol by Feshney, 2005. Glucose uptake was measured by C111 Cobas according to the manufacturer's instructions. The activity of lactate dehydrogenase was measured using Biochemistry Analyzer according to manufacturer's instructions. Determination of apoptotic body was determined using fluorescence microscope and the protocols were followed as described previously by Cohen, 1993. Cisplatin was used as posititve control.

RESULTS

The IC_{50} value observed is 195.166 $\pm$ 20.21 µg/ml as shown in Figure 1. The percentage of glucose uptake shows that this extract has the lowest uptake at 1000µg/ml of concentration as shown in Figure 2. Intra-cellular and extra-cellular activity might have been blocked and the cells stop proliferating thus they stop consuming glucose. Figure 3 shows the result of LDH release after treatment with extract. The result shows that there is no significant difference as compared to negative and positive controls. Figure 4 shows the apoptotic body observation of cell line after treatment with extract. The figure shows the presence of apoptotic body. Different mechanism can be concluded, cells without any treatment release almost the same amount of LDH as cisplatin as a negative control due to 100% of glucose consumption. However, for cisplatin cells treatment, the excessive amount of LDH is caused by cell toxicity and cell lysis. The effect of 1000µg/ml on LDH release result could be signified as the cisplatin mechanism.

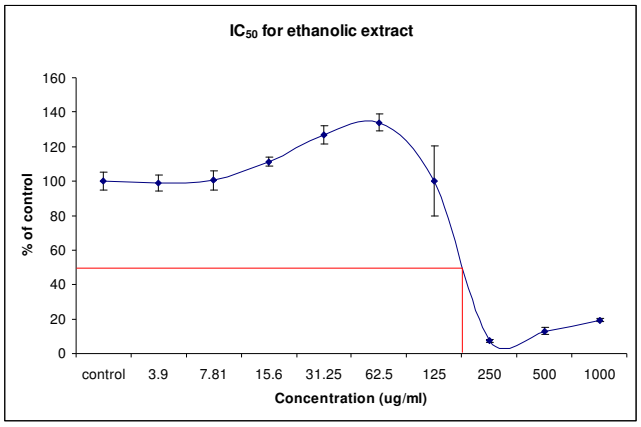


Fig 1. The percentage survival of control after treatment with extract.

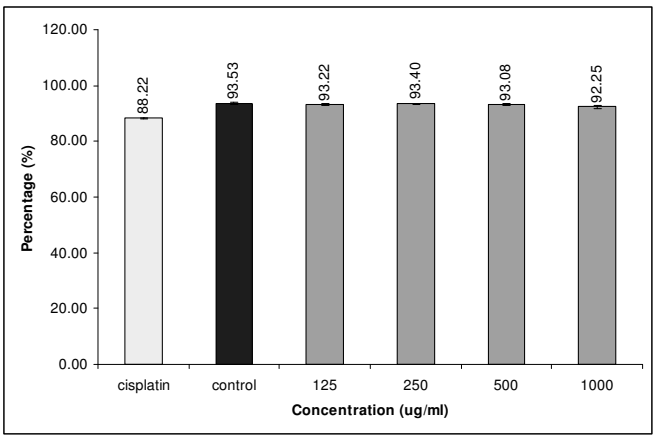


Fig 2. The percentage of glucose uptake of *F. deltoidea* extract on A2780 cell line.

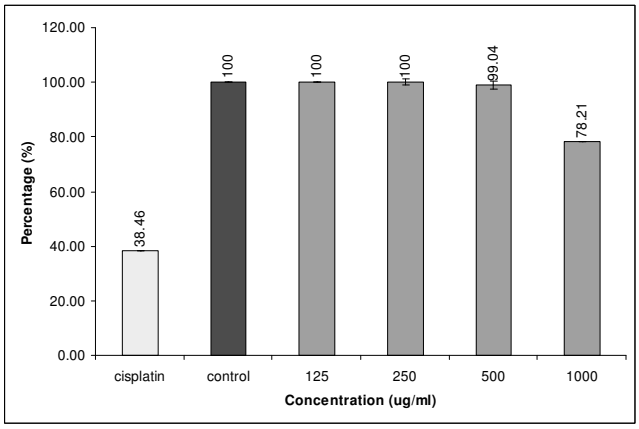
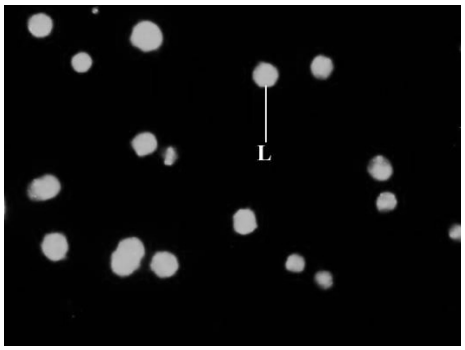


Fig 3. The percentage of LDH release of *F. deltoidea* extract on A2780 cell line

A



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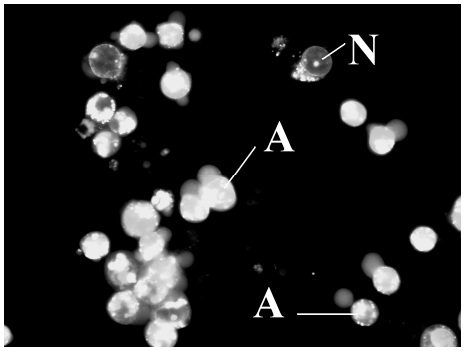

Fig 4. A comparison of apoptosis staining between negative control (A) and cells treated with 250 ug/ml ethanolic extract (B).

DISCUSSION

The result indicated that this extract may be a promising source for anticancer drug development. The least amount of glucose uptake was observed at the highest concentration of extract is supported by the fact that many types of cancer including ovarian cancer have increased glycolytic activities compared to non-malignant cells (warbug, 1956). The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid (Rae, T, 1997). The presence of apoptotic body after treatment with extract indicates that cell death was cause through apoptotic pathway.

REFERENCE

1. Rae, T. (1977). Tolerance of mouse macrophages in vitro to barium sulphate used in orthopaedic bone cement. Journal of Biomedical materials and research. Volume 11, issue 6, pages 839-846, November 1977.

Phyllanthus niruri Reduces Renal Azotaemia in Rats Induced with Chronic Renal Damage

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SUMMARY

Phyllanthus niruri is commonly used for treatment of jaundice, hepatitis, kidney stones, diuretics, influenza and antibacterial agent. This study was conducted to investigate the effects of *P. niruri* in reducing the severity of azotaemia in rats induced with chronic renal damage. A total of 32 male Sprague Dawley rats were randomly assigned into four treatment groups; Group A (control), Group B (adenine), Group C (*P. niruri*) and Group D (adenine+*P. niruri*), consisting of 8 rats each. Chronic renal damage was induced using 0.75% adenine mixed with rat diet (daily feeding for 6 weeks). *P. niruri* was supplemented daily at a dose of 3000 mg/kg body weight throughout the 10 weeks of the experimental period. Results showed that *P. niruri* reduced the severity of azotaemia in rats induced with chronic renal damage.

INTRODUCTION

Phyllanthus niruri which is locally known as Dokong anak, is a tropical herb tree that can easily be found in Malaysia. The plant is highly valued for its hepatoprotective, antidiabetic, antihypertensive, analgesic, anti-inflammatory and antimicrobial properties [1,2]. The objective of this study was to examine the effects of *P. niruri* in reducing the severity of renal azotaemia in rats induced with chronic renal damage.

MATERIALS AND METHODS

Thirty-two male Sprague Dawley rats were used in this study. The rats were randomly divided into four groups of 8 animals each; Group A (control), Group B (adenine), Group C (*P. niruri*) and Group D (adenine+*P. niruri*). Group B and D were induced with chronic renal damage by daily feeding diet containing 0.75% adenine (Sigma-Aldrich, USA) for 6 weeks. The rats were then fed with normal rat diet for another 2 weeks before they were sacrificed. Group C and D were supplemented daily with *P. niruri* (obtained from a local supplier) at a dose 3000 mg/kg body weight for 10 weeks. Blood samples were collected fortnightly (week 0, 2, 4, 6, 8 and 10) from tail vein for creatinine (Cr) and blood urea nitrogen (BUN) levels. The samples were analysed using a Dry Chemistry Analyser (scil Reflovet® Plus, Roche Diagnostics) followed as per manufacturer's instruction. At the end of the experiment (week 10), 24-hour urine samples were collected. Urine volume was recorded, urine creatinine and protein concentrations were determined using an Automated Biochemistry Analyser (Biorex, TRX7010, Manheim, Japan). Creatinine clearance (CrCl) was then calculated. Blood samples were also collected via portal vein after the rats were humanely sacrificed by bleeding under anaesthesia. Serum creatinine (Cr) concentration was determined using the Automated Biochemistry Analyser and urine protein to creatinine ratio (UPC) was calculated. Data obtained were calculated for mean values and standard deviation using one-way ANOVA (SPSS version 17.0 for windows). The differences between groups were determined by Duncan's multiple range analysis.

RESULTS

The BUN and blood Cr concentrations of all groups are shown in Figure 1 and 2, respectively. Group A and C had normal concentrations of BUN and blood Cr throughout the experimental period. Gradual increases in BUN and blood Cr concentrations (renal azotaemia) were observed in both Group B and D during the adenine treatment. The concentrations were significantly ($p<0.05$) decreased after elimination of adenine from the diet at week 8 and 10 although they were still significantly ($p<0.05$) higher than the control. Group D had insignificant ($p<0.05$) reduction in BUN concentrations at week 2, 4 and 10 compared to the Group B. It also had insignificant ($p<0.05$) reduction in blood creatinine concentrations at week 2, 6 and 8, and significant reduction ($p<0.05$) at week 10 of the experiment compared to the Group B. Consistent with azotaemia, Group B and D showed significant increase in the UPC compared to the control (Figure 3). Group B had UPC 5 times higher than the control while Group D had UPC 2 times higher than the control. Although Group D had lower UPC compared to Group B, statistically it was not significant ($p<0.05$). Similar to the UPC, the CrCl

results of Group B and D were also consistent with azotaemia. The clearance of creatinine from the body through the urine for Group B and D were significantly (p<0.05) decreased compared to the control albeit there was no significant difference between Group D and B (Figure 4).

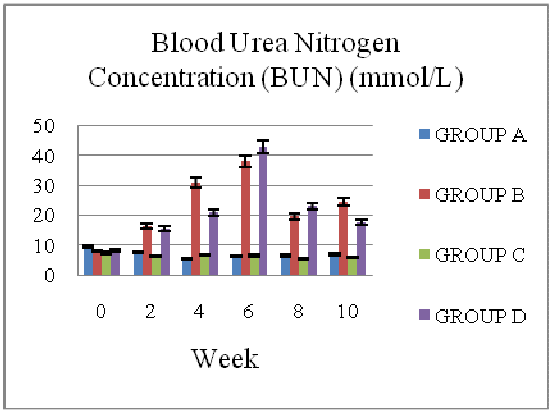

Fig 1: BUN concentrations of Group D show insignificant (p<0.05) reduction at week 2, 4 and 10 compared to Group B

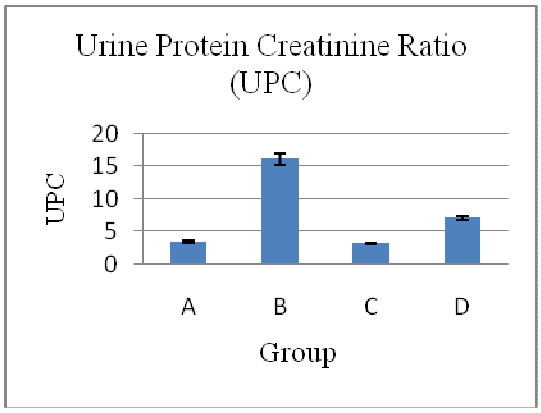

Fig 3: Group D shows insignificant (p<0.05) reduction of UPC compared to Group B

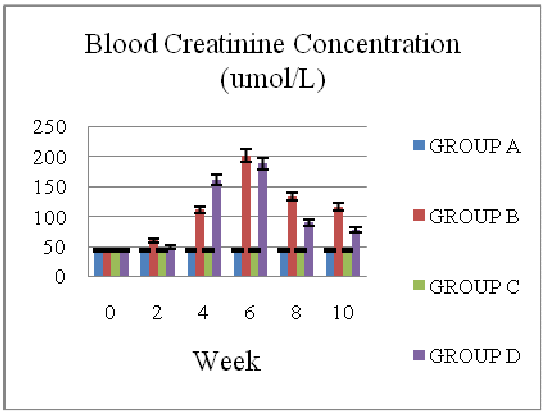

Fig 2: Blood creatinine concentrations of Group D show insignificant (p<0.05) reduction at week 2, 6 and 8, and significant (p<0.05) reduction at week 10 compared to Group B

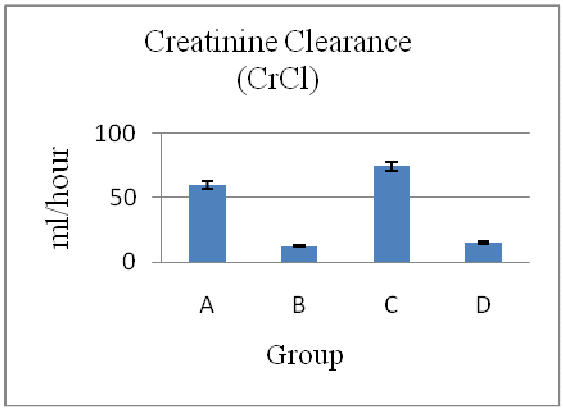

Fig 4: Group D shows insignificant (p<0.05) clearance of creatinine from the body through urine compared to Group B

DISCUSSION

P. niruri has shown protective effects on different organs in the body especially liver and kidneys [3,4]. It is used to treat a lot of conditions including kidney stones (Chopra *et al*., 1986). One of the active ingredients found in another species of this herb (*P. amarus*), quercetin (one of the bioflavonoids), has been reported can inhibit xenobiotic-induced nephrotoxicity [5,6]. Results in this study showed that daily supplementation of *P. niruri* at 3000 mg/kg body weight had shown nephroprotective activity. Generally it only mildly reduced the severity of azotaemia. Further studies should be conducted by using higher doses of dried *P. niruri* and/or using its extract.

REFERENCES

1. Ramanam MV and Sainani GS. Drugs in infective hepatitis. *Punjab Medical Journal*, 1962; 10: 607-712.
2. Adeneye AA and Arogundade MO. Immunostimulatory and haematopoietic activities of the leaf and seed aqueous extract of *Phyllanthus amarus* in normal and cyclophosphamide-treated mice. *Nigerian Journal of Health and Biomedical Sciences*, 2007; 6:53-57.
3. Tabasum N, Chatterrredi S, Grawal SS and Ahmed N. Hepatoprotective studies of *Phyllanthus niruri* on paracetamol-induced liver cell damage in albino mice. *Journal of Experimental Medicine*, 2005; 12: 211-213.
4. Nishiura J, Campos A, Boim M and Schor N. Effect of *Phyllanthus niruri* on urinary calcium levels in calcium stone forming patients. *Journal of Clinical and Laboratory Investigation of Urolithies and Related Areas*, 2005; 32, 362-366.
5. Devipriya S and Shyamaladevim CS. Protective effect of quercetin in cisplatin induced cell injury in the rat kidney. *Indian Journal of Pharmacology*, 1999; 31: 422.
6. Annie S, Rajagopal PL and Malini S. Effect of *Cassia auriculata* Linn. root extract on cisplatin and gentamicin-induced renal injury. *Phytomedicine*, 2005; 12: 555-560.

Effects of *Morinda citrifolia* on Early Stage of Leukaemia in Rats

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SUMMARY

We have studied the effects of *Morinda citrifolia* on haemogram parameters in rats with early stage of leukaemia. A total of 32 male Sprague Dawley rats were divided into four groups (*n*=8) namely control, *Morinda citrifolia* (MC), *N*-methyl-*N*-nitrosourea (MNU) and MC+MNU group. Leukaemia induced rats were provided with died *M. citrifolia* fruits incorporated into rat diet at a dose of 3000 mg/kg body weight were given daily to the MC and MC+MNU groups. Results showed there were no significant differences in the leukon and erythron parameters in all groups. Although lymphocytosis was not observed in the MNU and MC+MNU groups, examination of the blood morphology showed a typical morphology of leukaemic cell. Seventy-five and sixty percent of rats in the MNU and MC+MNU groups, respectively, had leukaemia. Our results indicate that examination of blood smear provides an important tool for diagnosis of early stage of leukaemia and daily supplementation of *M. citrifolia* at the dose of 3000 mg/kg reduced the incidence of early stage of leukaemia in rats.

INTRODUCTION

Morinda citrifolia offers a potential pharmaceutical values. The entire plant was used by the Polynesians as folk medicine to treat common illnesses. It was reported that the fruit juice is able to stimulate significant body's immune responses [1] and therefore was mainly consumed for health maintenance [2]. One of the carcinogenic chemical that able to induce lymphoma or leukaemia in rats is *N*-methyl-*N*-nitrosourea (MNU) [3, 4]. This study was conducted to examine the effects of *M. citrifolia* on early stage of leukaemia in rats induced using MNU.

MATERIALS AND METHODS

Thirty-two, 8-week old male Sprague Dawley rats were placed under controlled conditions and randomly divided into four groups (*n*=8) namely control, *N*-methyl-*N*-nitrosourea (MNU), *M. citrifolia* (MC) and MC+MNU group. The control and MC groups were injected with normal saline. The MNU and MC+MNU groups were injected intraperitoneally (i.p) with freshly prepared MNU (Sigma-Aldrich, USA), bi-weekly for 2 consecutive weeks at a dose of 60 mg/kg body weight per administration. The MC and MC+MNU groups were fed daily with ground dried *M. citrifolia* fruit at a dose of 3000mg/kg body weight mixed with commercial ground rat diet. At 12 week of post MNU administration, bloods were collected via cardiac puncture and analysed for a complete blood count using an Automated Haematology Analyser (Cell-Dyn® 3700, Abbott Diagnostics, USA). Blood smear was prepared and stained with Wright's stain for a manual differential leukocyte count and examination for the presence of leukaemic cells. Packed cell volume (PCV) was determined manually using a standard method. Results were analysed using SPSS version 17.0 for windows and differences were considered significant at $p<0.05$.

RESULTS

Results for the haematology are shown in Table 1 and Table 2. The leukon and erythron parameters showed no significant differences in all the groups. Examination of the leukocyte morphology revealed 75% and 60% of rats in the MNU and MC+MNU groups, respectively, had leukaemia (Table 3).

Group	Leukaemia (%)
Control	NA
MC	NA
MNU	75
MC+MNU	60

Table 3: The incidence of leukaemia in all groups

Table 1: The leukon parameters in all groups (Mean±SD)

Group	Leukocytes ($x10^9$/L)	Lymphocyte ($x10^9$/L)	Neutrophil ($x10^9$/L)	Monocyte ($x10^9$/L)	Eosinophil ($x10^9$/L)	Basophil ($x10^9$/L)
Control	10.1±2.7	7.73±2.20	0.60±0.81	0.25±0.14	0.05±0.03	0.31±0.06
MC	10.0±4.0	7.30±4.37	0.21±0.03	0.34±0.32	0.04±0.01	0.36±0.09
MNU	8.4±1.1	6.53±0.82	0.20±0.02	0.27±0.25	0.05±0.05	0.20±0.16
MC+ MNU	9.7±1.2	7.41±1.49	0.18±0.04	0.28±0.30	0.07±0.06	0.24±0.18

Table 2: The erythron parameters in all groups (Mean±SD)

Group	PCV (L/L)	RBC ($x\ 10^{12}$/L)	Haemoglobin (g/L)
Control	52.5±4.1	9.90±1.10	184.8±20.55
MC	49.3±0.6	8.72±0.27	163.0±10.66
MNU	51.3±2.5	8.73±0.50	167.3±4.65
MC+MNU	51.8±2.1	8.83±0.65	167.5±10.66

DISCUSSION

According to Hutheyfa *et al*. [3,4], administration of MNU at the dose of 60 mg/kg body weight per injection twice a week for two consecutive weeks (with a total dose of 240 mg/kg body weight) successfully induced 30% leukaemia with marked lymphocytosis and 100% lymphoma in young male Sprague Dawley rats after 20 weeks of injections. Our results showed 75% of male Sprague Dawley rats administered with MNU using similar method described by Hutheyfa *et al*. had leukaemia without lymphocytosis after 12 weeks of injections. It is suggested that examination of blood smear is important for diagnosis of early stage of leukaemia. Daily supplementation of *M. citrifolia* to the rats at the dose of 3000 mg/kg body weight reduced the incidence of early stage of leukaemia to 60%. Administration of *M. citrifolia* at a higher dose (5000 mg/kg body weight) has significantly reduced the incidence of leukaemia at 20 week post MNU administration [5].

REFERENCES

1. Hirazumi AY. *Antitumor Studies of a Traditional Hawaiian Medicinal Plant, Morinda citrifolia (noni), in vitro and in vivo* [doctoral dissertation]. University of Hawai'I, Honolulu, Hawaii. 1990.
2. Wang MY, Brett JW, Jarakae J, Diane N and Gary A. *Morinda citrifolia* (Noni): A Literature Review and Recent Advances in Noni Research. *Acta Pharmacology Sinaca* 2002, 12: 1127-1141.
3. Hutheyfa AH, Hazilawati H, Rosly SM, Jasni S, Noordin, MM and Shanmugavelu S. *N*-methyl-*N*-nitrosourea induced leukaemia in male Sprague-Dawley rats. *Proceedings of the International Conference on Animal Health and Human Safety, 2009 December 06-08; Putrajaya, Malaysia.* Faculty of Veterinary Medicine, Universiti Putra Malaysia-Airlangga University, Indonesia-Department of Veterinary Services, Malaysia 2009a, 210-213.
4. Hutheyfa AH, Hazilawati H, Rosly SM, Jasni S, Noordin M M and Shanmugavelu S. Development of lymphomas in male Sprague-Dawley rats exposed to *N*-methyl-*N*-nitrosourea. *Proceedings of the International Conference on Animal Health and Human Safety, 2009 December 06-08; Putrajaya, Malaysia.* Faculty of Veterinary Medicine, Universiti Putra Malaysia-Airlangga University, Indonesia-Department of Veterinary Services, Malaysia 2009b, 214-218.