CYTOTOXICITY OF LEUKEMIC CELLS BY NYPA FRUTICANS THROUGH REGULATION OF ADIPONECTIN EXPRESSION

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ABSTRACT

Introduction: Acute lymphoblastic leukemia (ALL) is the most common leukemia subtypes among paediatrics in Malaysia. Although treatment options are available but some patients remain incurable, some undergo relapse and many experiences adverse effects by the conventional therapies. Thus, we aim to investigate possible treatment alternative by studying the antileukemogenesis properties of concentrated *Nypa fruticans* sap called *nisaan* by focusing on adiponectin expression.

Method: Our study model was CCRF-CEM, an acute lymphoblastic leukemia cell lines. The cells were treated with *nisaan* at a range of concentration and treated for 24, 48 and 72 hours followed by determination of the leukemic cells viability using tryphan blue method. Effective *nisaan* concentrations that significantly reduced the cells viability were again treated to the cells followed by determination of the cell proliferation using BrdU colorimetric kit and adiponectin level using adiponectin ELISA kit.

Results: The results showed that, increase concentration of *nisaan* treatment reduced the cells viability and cells proliferation and enhance the adiponectin level in the leukemic cells.
Conclusion: This preliminary data suggest that *Nypa fruticans* might has the anti-leukemogenesis effect on acute lymphoblastic cells by regulating the adiponectin expression.

KEY WORDS: *Nypa fruticans, adiponectin, acute lymphoblastic leukemia, cells cytotoxicity*

INTRODUCTION

Adiponectin is a hormone secreted by the adipocytes. It involved in metabolic pathways regulation by down-regulates genes that involved in gluconeogenesis pathway (Berg, et al., 2001), the uptake of glucose, the fatty acid metabolism and also lactate synthesis in

myocytes (Yamauchi, et al., 2002). Decreased plasma adiponectin level was shown to lead to insulin resistance in type 2 diabetes (Kubota, et al., 2002) and atherosclerosis (Okamoto, et al., 2002). Apart from metabolic diseases, adiponectin may also involve in the pathogenesis pathway of several type of cancers. Previous studies had shown adiponectin had antiproliferative effect on breast cancer cells in vitro by activating the adiponectin receptors (Körner, et al., 2007). Apart from the breast cancer cell lines (Takahata et al. 2006), the receptors were shown to be expressed in prostate cell lines (Mistry, et al., 2006) and hepatocellular (Miyazaki, et al., 2005) carcinoma cell lines. In addition, the expression of adiponectin receptors, AdipoR1/R2 was shown to be inversely correlated with plasma insulin concentrations and decrease in pathologic conditions (Tsuchida, et al., 2004).

Leukemia is a group of heterogenous disease of the bone marrow due that lead to formation of unusually high numbers of immature blood cells called blasts. The blast cells are able to proliferate uncontrollably but lack the functions of normal blood cells. Overcrowding of the bone marrow with the leukemic cells lead to less production of normal blood cells including platelet to carry out normal physiological functions. Further, the leukemic cells also able to metastase to other part of the body such as lymph nodes, kidneys, adrenals and the heart (Trendowski, 2014).

This group of disease is a health concern globally. In 2010, approximately 281,500 people died due to leukemia (Lozano, et al., 2012). Leukemia is also a major concern in Malaysia. ALL is the major subtypes among Malaysian pediatrics (MASPHO, 2009) and many patients experience remission after undergoing the conventional therapy (Conter, et al., 2010). The exact cause of leukemia is unknown. Factors involved could be inherited or could be environmental (non-inherited).

Most forms of leukemia are treated with a multi-drug chemotherapy regimen, others with radiation therapy or BM transplant (NCI). Prognosis and response towards therapy varies in patients. Some patients are cured, others might not and some undergo remission. Moreover, many patients experience side effects such as loss of hair, loss of appetite, fatigue and also damage the blood-producing cells in bone marrow due to the conventional therapy. The success of treatment depends on the type of leukemia and other factors. Thus, lots of studies are undergoing to better understand and eventually find effective alternative in treating the disease.

One possible alternative with fewer side effects could lies in the herbal medicine or possibly components from natural resources that might possess the medicinal properties. The usage of herbal medicine to treat diseases could be traced back to 5000 years ago by the Sumerian and in many culture, herbal remedies were used to treat many diseases. Nowadays, advancement in herbal medicine researches had led to the development of pharmaceutical agents derived from natural resources such as plants, animals, marine organisms and also microorganisms. Vincristine for example, is a plant-derived compound and is used to treat cancer (Guérritte, et al., 2005).

Malaysia is rich with natural resources that promote the interests and advances in medicinal plant research. Many had shown promising effect towards certain type of diseases and had led to discovery of biologically active compounds to be used as chemical templates to produce new drug candidates (Jantan, 2004). *Nypa fruticans* or locally known as *nipah* palm is one of the species of palm native to the coastlines and estuarine habitats that were claimed by the natives to have several medicinal properties but lacking in scientific data to prove the claim. The palm produces fruit with edible endosperm (Teo, et al., 2010). The flower cluster or inflorescence can be tapped before it blooms to yield edible sap and collected as local alcoholic beverage called *tuak* or *nira*. *Nira* could be stored to produce a type of vinegar called *nipah* vinegar. The *nira* is boiled to make it into concentrate to yield *nisaan* which is used in many traditional dishes.

The local claimed that *nira* and vinegar able to treat many conditions such as diabetes, high blood pressure, gout and kidney stone (Mat Satar, et al., 2011). Preliminary scientific studies had shown that methanolic extract of leaf and stem of nipah had antiglycemic properties based on a study conducted on glucose loaded hyperglycemic mice (Reza, et al., 2011). A preliminary study had indicated that the methanol extract of nipah fruit to be cytotoxic against MCF-7, the breast cancer cells (Nurul Huda, et al., 2011).

To date, there are limited scientific studies to further support the local claims on this important underutilized fruit notably in cancer. Therefore, we aim to screen for possible antileukemogenesis properties of nipah palm produce which is *nisaan* and its possible involvement in adiponectin expression.

METHOD

Maintaining the culture

The acute lymphoblastic cell line, CCRF-CEM cells (purchased from ATCC) were cultured in RPMI supplemented with 10% FBS and 0.1mg/ml penicillin and streptomycin. The cultures were maintained at 37^{o}C in a humidified incubator with 5% CO_2.

Sample

The *nisaan* was concentrated product from nipah sap. It was provided by a local from Pulau Gajah, Kelantan who collected the nipah sap from around the area. The sap was then heated to make it in a concentrated form and this step produced the syrup. The syrup was dried in an oven for 2 weeks and weighed several times during the drying process to achieve the constant weight.

Sample Preparation

The *nisaan* was weighted and directly diluted in complete culture medium to prepare the working concentrations.

Cell Viability Assay

Screening for effective *nisaan* concentrations towards the CCRF-CEM cells was done by tryphan blue exclusion assay according to protocols described previously (Strober , 2001). Briefly, the cells were treated with five *nisaan* concentration i.e 0, 3.125,6.25,12.5,25 & 50 mg/mL for 24, 48 and 72 hours. Then, the cells were mixed with the dye and visually examined. Viable cells were able to exclude the dye and appear bright under the inverted microscope while non-viable cells took up the dye and stained blue. Then, the percentage of viable cells was calculated.

B**rdU Cell Proliferation ELISA Kit (Abcam)**

The method was employed to confirm the cells viability and proliferation. Two *nisaan* concentrations that significantly affected the cells viability were used to treat the CCRF-CEM cells and cells without treatment were used as control. After 24 hours, the cells proliferation rate was determined using BrDu. Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) was a synthetic analog of the nucleoside thymidine. The compound was incorporated into replicating DNA in dividing cells, thus labelled the i*n vitro* dividing cells. Anti-BrdU antibodies were added and visualized with HRP conjugated secondary antibodies and DAB.

Determination of Adiponectin Level

Adiponectin expression after treatment with *nisaan* was determined by ELISA method using the adiponectin Assay kit (Adipogen). The cells were treated with effective *nisaan* concentration (12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml) with non-treated cells as control for 24 hours. Then, the cells were collected and lysed using a 1% Triton in PBS in a 21-mm gauge needle. The ELISA assays were carried out according to the manufacturer protocols.

Statistical Analysis

The data were analyzed by non-parametric test Kruskal-Wallis using SPSS software and *p* value less than 0.05 was taken as statistically significant.

RESULTS

Nypa fruticans syrup extract was screened for its effect on viability of the CCRF-CEM acute lymphoblastic leukemic cell line at concentrations of 25 mg/ml, 50 mg/ml, 100 mg/ml, 200 mg/ml and 400 mg/ml for three different incubation times of 24 hours, 48 hours and 72 hours (figure 1). The lowest *nisaan* dosage, 25 mg/ml reduced 10 % of the cell viability and it was significantly lower compared to control group. The cell viability was found as 84.8%, 68.4% and 66.8%, respectively. Similar trend was shown for 48 and 72 hours of treatment. To confirm the cells toxicity, cell proliferation assays using BrDU was conducted. Treatment with 3.13 mg/ml and 12.5 mg/ml cells showed significant reduction in cells proliferation compared to control (figure 2). As for adiponectin assays, the results showed that adiponectin expression in both treatment groups were significantly higher than the control group (figure 3). This result was inversely correlated to the cells toxicity study.

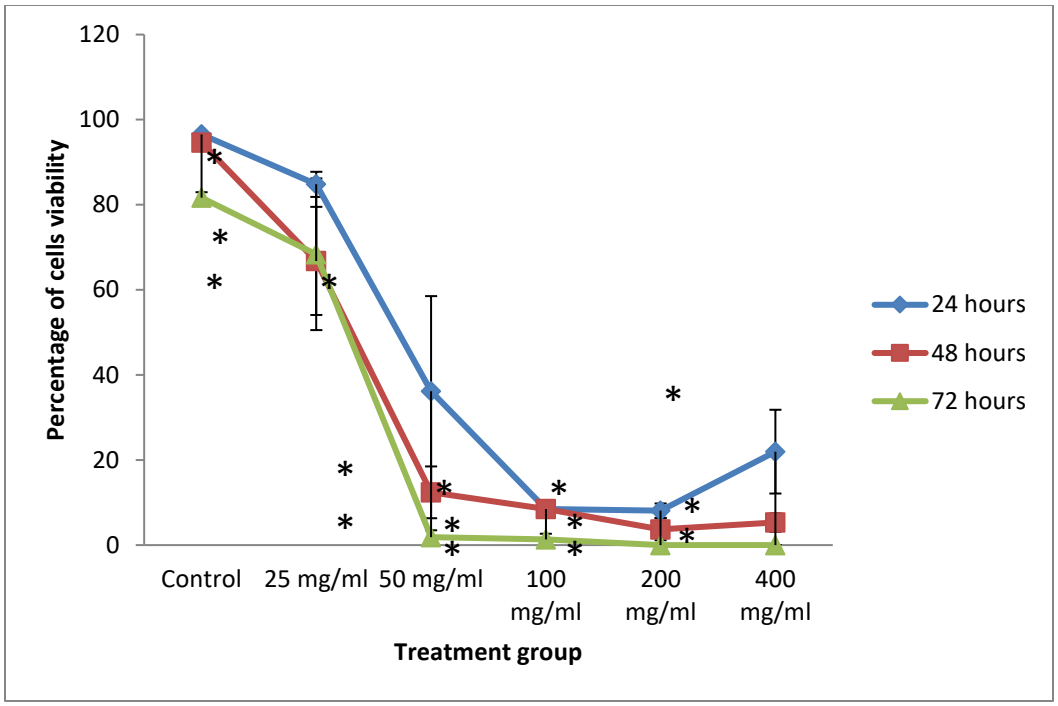

Figure 1 *The figure shows the percentage of CCRF-CEM cells viability after being treated with 5 different nisaan concentrations at 24, 48 and 72 hours (n =3). All treated groups showed significant viability reduction compared to control (p<0.05). The treatment also showed significantly reduced cells viability in a dose-dependent manner. Similar pattern was shown in the three different incubation times.*

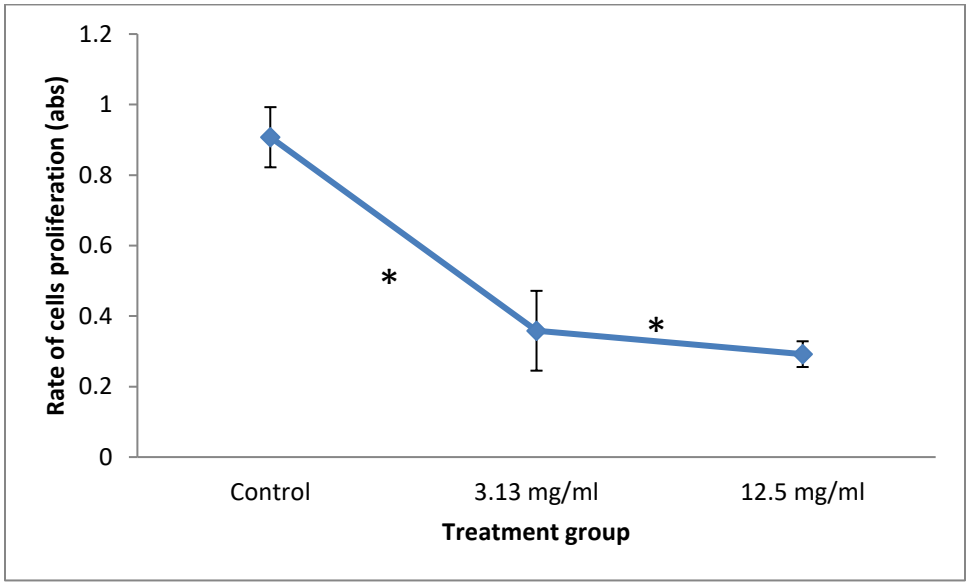

Figure 2 *The figure showes the rate of CCRF-CEM cells proliferation after treatment with selected nisaan concentrations at 24 hours (n=3). * refer to p < 0.05 compared to control group. Two lowest concentrations of nisaan that significantly reduced the leukemic cells viability were treated at 24 hours and these concentration were also shown to significantly reduce the cells proliferation using BrDU assay.*

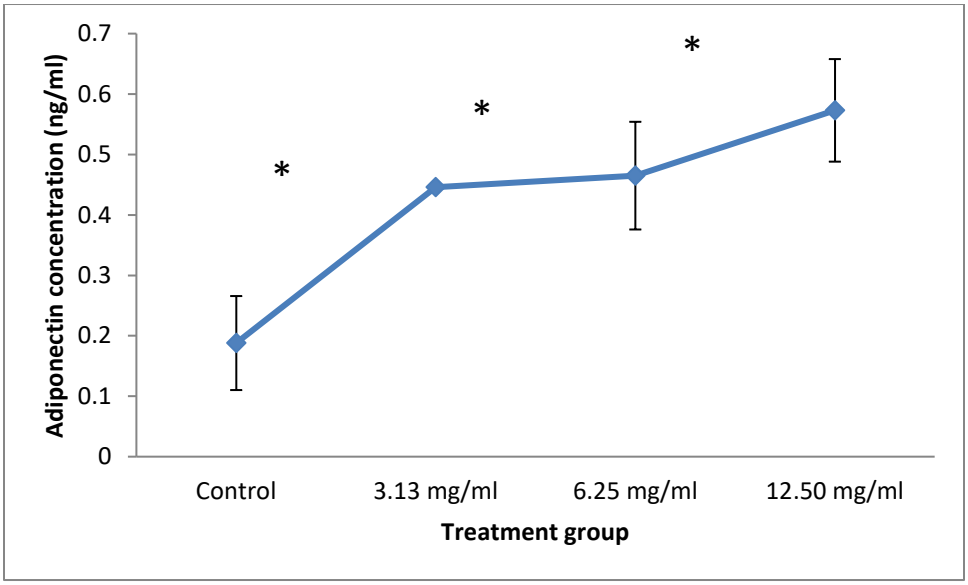

Figure 3 *CCRF-CEM cells adiponectin expression after treated with nisaan at selected concentrations. * refer to p < 0.05 compared to control group. Treatment with nissaan significantly increased adiponectin level in the CCRF-CEM cells in a dose-dependent manner.*

DISCUSSION

Previous studies on *nipah* showed that it had antimicrobial activities against *Vibrio alginolyticus* (Shamsuddin, et al., 2013). The midveins, husks and leaves extracts of this plant were shown for to have antimicrobial property against *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus epidermidis* and *Staphylococcus aureus* (Ebana, et al., 2015). More studies had shown that *nipah* extract had antihyperglycemic activities. Methanolic extract from the leaf and stem of *nipah* were also shown to possess anti-hyperglycemic activity on glucose loaded hyperglycemic and antinociceptive activity on mice model (Reza, et al., 2011). Furthermore, food product from *nipah* called *nipah* vinegar possesses antihyperglycaemic activities comparable to the metformin in rat model. The findings also suggest that antioxidant compounds of the vinegar do not contribute much towards the overall observed antidiabetic effect (Yusoff,

et al., 2015). In addition, *nipah* vinegar was shown to be able to suppress postprandial hyperglycemia following glucose, sucrose and starch load (Yusoff, et al., 2015).

Very limited studies were conducted to investigate the possible anticancer properties of *nipah*. Recent study showed that the methanol extract of *nipah* showed cytotoxicity activity against breast cancer cell lines, MCF-7 (Nurul Huda, et al. 2011). In this study, we had showed that *nipah* also showed cytotoxicity activity against the acute lymphoblastic leukemia cell lines, CCRF-CEM. To further understand the possible mechanism involved, we had also investigate the level of adiponectin expressed in the cells upon treatment with *nipah* and found that the leukemic cells viability decrease upon treatment but on the other hand, showed increment of adiponectin expression.

Previous study had characterized the phytochemical content of *nipah* fruits extract. Chlorogenic acid was identified as major compound in the extract. Unripe endosperm extract of *nipah* fruits showed high antioxidant capacities, total flavonoid and total phenolics, content as compared to ripe endosperm extract (Prasad, et al., 2013) and might play a major role in pathological conditions related with free-radical generated disease.

Chlorogenic acid, the major compound in *nipah* extract might also be the phytochemical responsible in contributing to its medicinal properties. Chloregenic acid is a phenolic acid present in many types of fruits, vegetable and seeds. Previous study had shown that chlorogenic acid decreased fasting plasma glucose and HbA1c level in diabetes and also improve kidney fibrosis possibly by modulating the adiponectin receptor signaling pathways in diabetic mice model (Jin, et al., 2015). Furthermore, studies had also shown that consumption of coffee rich in chlorogenic acid decrease plasma glucose (van Dijk, et al., 2009, Johnston, et al., 2003, Olthof, et al., 2011), reduce weight loss in obese people (Thom, 2007), lower plasma glucose and C-reactive protein levels and also improve liver function in diabetic patients (Herrera-Arellano, et al., 2004, Abidov, et al., 2006).

Furthermore, this compound was shown to have antidiabetic properties in animal models (Karthikesan, et al., 2010, Rodriguez & Hadley, 2002, Zhang, et al., 2011, Li, et al., 2009). Another study showed that chlorogenic acid significantly reduced FPG and HbA1c, accompanied by increased adiponectin level in adipose tissues of type 2 diabetic mouse model. Their data also indicated that the increased of adiponectin receptors, ADPNR-2 in the liver and muscle of the animal models (Jin, et al., 2015). ADPNR-2 is involved in the activation of adiponectin signaling pathway and reduction of the receptor

expression lead to reduce adiponectin sensitivity and this function relies on adiponectin (Yamauchi & Kadowaki, 2008 , Peters, et al., 2013, Yamauchi, et al., 2007).

Adiponectin was also showed to be the inhibitor that capable to prevent cancer progression and invasion via their receptors. Evidences from previous studies showed that the level of plasma adiponectin was inversely associated with the risk of cancer development correlated with the obesity body weight index (BMI) status and insulin resistance including endometrial cancer, breast cancer, prostate, colon and gastric cancer and also leukemia (Shehzad, et al., 2012).

In determining the association of adiponectin in progression of cancer specifically for leukemia or blood diseases, the level of adiponectin and presence of adiponectin's receptors could be a significant indicator of the state of diseases. In leukemia, lymphomas or myeloproliferative diseases, studies have observed the disturbances of the level of circulating adiponectin due to these diseases (Bastard & Fève, 2012). Furthermore, in study reported by Ozturk *et al.* (2012), the levels of serum adiponectin were inversely associated with the types of childhood leukemia including childhood acute myeloblastic leukemia (AML). Moreover, it has been found that, the circulating levels of adiponectin were lower for both chronic lymphoproliferative and myeloproliferative diseases. The study also suggested that adiponectin could promote the migratory characteristic in cancer cells by which contributing to their ability to spread.

Based on study by Lang & Ratke (2009), despite the fact that adiponectin exclusively secreted by adipocytes, this protein able to work on tumor cells directly for various cancer cells which express AdipoR1 and adipoR2 receptors. This finding is supported with studies of Dalamaga & Koumaki (2014) which stated that, expression of adiponectin receptors have been found on various cancer cell lines by which suggested that the adiponectin able to exert direct actions on these cells. Besides, based from Grossman & Clearly (2012), also stated that in chronic stages of disease where the cancers invade aggressively, the lack of adiponectin able to build a compensatory mechanism and a complicated counter play among adiponectin and cellular environment that might influence the metastasize of tumor.

CONCLUSION

Based on our results, treatment of the ALL cells with *nipah* lead to increase cells cytotoxicity followed by increment of adiponectin level. We postulate that chlorogenic acid, which is the major compound found in nipah might increase the cells cytotoxicity through adiponectin signaling pathway by up-regulating the adiponectin receptor. Further study needed to be done to validate the hypotheses.

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