Preliminary Study on Anti-proliferative Activity of Methanolic Extract of *Nephelium lappaceum* Peels towards Breast (MDA-MB-231), Cervical (HeLa) and Osteosarcoma (MG-63) Cancer Cell Lines

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ABSTRACT: Cancer is a very serious public health in Malaysia with increasing incidence and mortality rates every year. This study was conducted to determine the extraction yield and potential of *Nephelium lappaceum* peel extract (NLPE) as anti – proliferative agent towards breast cancer cells (MDA-MB-231), cervical cancer cells (HeLa) and osteosarcoma cancer cells (MG-63). *N.lappaceum* (NL) belongs to family Sapindaceae and tropical native fruit of the Southeast Asia. Previous studies have shown that NL to pose therapeutic activities such as antioxidant, antibacterial, antivirus and anti-hyperglycemic. Two varieties of NL peels, yellow and red have been utilized in this study. Cytotoxicity in vitro of NLPE against various cancerous cell lines compared with normal cell line, MDCK were assayed by using Methylene Blue Assay. Cisplatin was used as positive control. The IC_{50} were calculated and the optical density values were read at 655nm. The methanolic yellow NLPE exhibited promising activity against MDA–MB–231 and MG-63 with IC_{50} value 5.42±1.67 µg/ml and 6.97±1.02 µg/ml, respectively. Both varieties NLPE did not showed any anti-proliferative activity towards HeLa. From this, methanolic yellow NLPE showed most potent cytotoxic activity against several tested cancerous cell line and further morphological changes should be performed in order to identify apoptosis in such cancer cell lines.

Keywords: anti-proliferative, *Nephelium lappaceum*, cancer cells

Introduction

Cancer is a major health problem and cause of mortality that increase annually. In the year 2000, there were 10.4 million new cancer cases and it is expected that this number will be doubled in 2030 (Khaorrek *et al*., 2012). The most common cancers occur among women are breast cancer and cervical cancer whereas osteosarcoma cancer occurs mostly on children and young adult. Breast cancer is a notable cause of morbidity and mortality among women in the worldwide (Lu and Sererro, 2001). The osteosarcoma is a type of cancer that starts in the bones and not a common cancer. Osteosarcoma is a primary malignant tumor of bone that is characterized by the production of osteoid or immature bone by the malignant cells.

Hidayati *et al*., (2011) reported that cancer therapies that utilizing the natural products such as plants is a relatively new to prevent cancer but it is a promising strategy where it suppress, delay, reverse, or retard the process of carcinogenesis. This kind of therapies also known as cancer chemoprevention which applies specific natural compound to inhibit or reverse carcinogenesis and help to suppress the development of cancer from premalignant lesions (Sankar and Li, 2007). Malaysia is rich in plant biodiversity and therefore it is wide opportunity to find new leads for drug discovery. There is a need for this kind of plant to be explored by investigating the bioactivities of plants that already used for traditional or even alternative medicine. Plants can be said as a producer that gives out a diverse range of bioactive molecules and this making them as an utmost source of different types of medicine. Thus, discovery on efficient anti – cancer drugs based on natural products that tending to inhibit growth of the cancer cells is urgent.

The *Nephelium lappaceum* L. (NL) is one of the common plants that have been used for its medicinal properties. This plant belongs to family of Sapindaceae as other sub-tropical fruit and it is native to Southeast Asia (Marisa, 2005). This fruit is an important commercial crop in Asia, where it is taken freshly or processed. In Southeast Asia, the dried fruit peel has been used in traditional medicine for centuries. Additionally, the peel is used in cooking and the production of soap (Palanisamy *et al*., 2011). Previous studies have shown that NLPE exhibit high anti-oxidant activity (Palanisamy *et al*., 2008), antibacterial activity (Thitilertdecha *et al*., 2008), anti-proliferative activity against human cell lines (Okonogi *et al*., 2010) and anti-Herpes Simplex

virus type 1 (Nawawi *et al*., 1999). NLPE also has an anti-hyperglycemic potential (Palanisamy *et al*., 2008)**.** As far as our literature survey could ascertain, no studies on anti-proliferative activity and anticancer activity has been previously published specifically using both varieties of NL peels. Thus, the objective of this study is to determine the anti-proliferative activity of yellow and red NL peels towards breast cancer cells (MDA-MB-231), cervical cancer cells (HeLa) and osteosarcoma cancer cells (MG-63).

Materials and Methods

Plants collection and preparation

The two varieties of NL peels (Yellow and Red) were collected from Pasir Tumboh, Kelantan, Malaysia. The samples were authenticated by botanist of Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia. The peels were first washed using tap water and were dried in oven at 50°C. The dried peels were then blended into powder form.

Successive extraction of plants

25 gram of each NL powder was subjected to successive extraction using Soxhlet instrument with few modifications (Hasmah *et al*., 2006). Both peels of NL were extracted using methanol. Extraction is completed when the solvent became clear. All the extraction products were concentrated through vacuum using rotary evaporator and were left dried in fume hood to eliminate solvent residual. Then, the weights of the extracts were measured. A concentration series of plant extracts and Cisplatin (positive control) at concentration range of 0.39 – 99 µg/ml were prepared by dilution with DMSO and stored at 4°C for further uses.

Cell culturing

Three types of cancer cell lines were used which are cervical cancer cell line (HeLa), breast cancer cell line (MDA-MB-231) and osteosarcoma cell line (MG-63). Non-malignant cell line, Madin-Darby canine kidney cell line (MDCK) was used as a control. Cells from stocks were

cultured separately on cell culture flask in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin). Cells were incubated at 37°C humidified incubator supplemented with 5% (v/v) CO_2.

Sub-culturing cell lines

When cells were 80-90% confluence, old medium was discarded and cells were washed with PBS to remove dead cells. 500 µl of 0.25% (v/v) trypsin was added into flask and incubated for 5 minutes in 5% (v/v) CO_2 incubator at 37°C. New medium were added into flask and divided into new culture flasks. Cells were then incubated in a humidified atmosphere of 5% (v/v) CO_2 incubator at 37°C. Cell culture stocks also established from the healthy cells and stored at -80^{o}C.

Cell treatment

Cultured cancer cells with 80-90% confluence were used for plating. The adherent cells were trypsinized to detach cells. 100 µl of cells were seeded into each well of the 96-wells microtiter plates (1-5 × 10^4 cells per well). The plate was maintained at 37^{o}C in a humid incubator with an air mixture containing 5% (v/v) CO_2 for 24-48 hours until 80-90% confluence. Then, old medium were discarded and 200 µl of new medium were added. Next, the cells were treated with 2 µl of series dilution of plant extracts (Table 1). The cells were also treated with 2 µl of series dilution of cisplatin as positive control and DMSO as negative control. The plate was returned to incubator for 72 hours. All treatments were done in triplicate. The same procedure was also done on non-malignant cell lines.

Table 1: Final concentration in wells of plant extract and cisplatin used for treatment of cells.

Extract concentration (mg/ml)	Final concentration in wells (mg/ml)	Final concentration in wells (µg/ml)
10.000	0.0990	99.00
5.000	0.0495	49.50
2.500	0.0248	24.80
1.250	0.0124	12.40
0.625	0.0062	6.19
0.313	0.0031	3.09
0.156	0.0016	1.55
0.078	0.0077	0.77
0.039	0.0039	0.39

Methylene blue assay

After 72 hours treatment, anti-proliferative activity of plant extracts were studied using methylene blue assay (Lin & Hwang, 1991). 22.5 µl of 25% glutaraldehyde was added into each well to fix the viable cells to bottom of the well and run off on shaker for 15 minutes. Then, glutaraldehyde and old medium were removed and dead cells were washed away with 100 µl 0.15 M sodium chloride (NaCl) for three times. Subsequently, the viable cells were stained with 100 µl of 0.05% methylene blue dye and run off on shaker for 15 minutes. The balance of methylene blue was then removed and rinse with 0.15 M NaCl for three times. 200 µl of 0.33 M HCl was added and placed on shaker for 15 minutes to get good colour elution. OD was read using ELISA reader at 660 nm.

IC_{50} determination

Inhibitory Concentration (IC) was defined by IUPAC Compendium of Chemical Terminology as a concentration of a substance that causes a defined inhibition of a given system. IC_{50} is the median concentration that causes 50% inhibition. IC_{50} value of the extract was determined from the plot of viable cells percentage and final concentration of the extract. Viable cells percentage was counted as:

$$IC_{50}\text{ determination} = \left[\frac{\text{Mean of optical density of treated cells}}{\text{Mean of optical density of control cells}} \times 100\%\right]$$

Statistical Analysis

The absorbance means have been calculated using the Excel® software (Microsoft, US) and the standard error of the mean (SEM) are calculated for 3 wells in triplicate sampling. After all the data have been collected, statistical analysis with ANOVA by using SPSS software version 18 was carried out. Probability values $P<0.05$ were considered statistically significant.

Results and Discussion

Extraction yields

This current preliminary research study describes the extraction yield and the anti-proliferative properties of NLPE from yellow NL peels and red NL peels. From 25 gram of each varieties of NL dried powder, each extraction produced a different extraction yields (Table 2). Types of solvent used in the extract preparation greatly influenced the bioactive compound extraction (Pinelo *et al*., 2005). In this study, the extraction of NLPE was depends solely on methanol extraction since it was reported that methanol can efficiently extract compound that might contain important secondary metabolites such as tannins and ellagitannins (Mans *et al*., 2000). The isolated ellagitannins as the principal components of NLPE could be further utilized as a medicine since it have been reported to exhibit antioxidant, apoptotic and cytoprotective

properties (Thitilertdecha *et al*., 2010). It was found out that the extraction peels of the plants with methanol gave the highest yield between 16% to 26% is in line with the findings that extraction of pomegranates peel with methanol gave the highest yield (Singh *et al*., 2002). In this study, the methanolic red NLPE and yellow NLPE achieved percentage of final extraction of end product higher than the other peels extraction study which is 29.2% and 27.2%, respectively. Okonogi *et al*., (2010) reported that the extraction yield of NLPE with methanol gives out 25.5% which is highest compared to other solvent and the extracts obtained with relatively polar solvent (methanol or aqueous methanol mixture) had the highest content of polyphenolic compound (Dufour *et al*., 2007).

Table 2: Yield percentage of final NLPE (Yellow and Red)

Extracts	Weight before extraction (g)	Weight after extraction (g)	Percentage of final extraction of end product (%)
Yellow NLPE	25.0	6.8	27.2
Red NLPE	25.0	7.3	29.2

From the result, after 72 hours incubation of cell lines with methanolic yellow and red NLPE, these extracts showed efficient anti-proliferative effects towards MDA-MB-231 cell line and MG-63 cell line in concentration dependent manner (Figure 1 and Figure 2). The IC_{50} values for MDA-MB-231 and MG-63 cancer cell lines that been treated with methanolic yellow were 5.42±1.67 µg/ml and 6.97±1.02 µg/ml, respectively and the IC_{50} values for MDA-MB-231 and MG-63 cancer cell lines that been treated with red NLPE were 12.4±1.99 µg/ml and 13.95±1.38 µg/ml, respectively (Table 3) which shown acceptable anti-proliferative properties towards tested cancer cell lines as the extract that has IC_{50} value ≤ 20 µg/ml has a significant anti-proliferative value (World Health Organization, 1972). Both yellow NLPE and red NLPE treatment did not showed any anti-proliferative effect on HeLa cancer cell lines.

Table 3: IC_{50} value of NLPE and Cisplatin on all cell lines tested for 72 hours.

Extract	IC_{50} value for cell lines (µg/ml))±SEM			
	HeLa	MDA-MB-231	MG-63	MDCK
Yellow NLPE	-	5.42±1.67	6.97±1.02	37.15±2.51
Red NLPE	-	12.4±1.99	13.95±1.38	37.15±7.99
Cisplatin	-	18.60±2.22	3.87±1.16	15.50±2.62

Anti-proliferative activity of NLPE on tested cancer cells

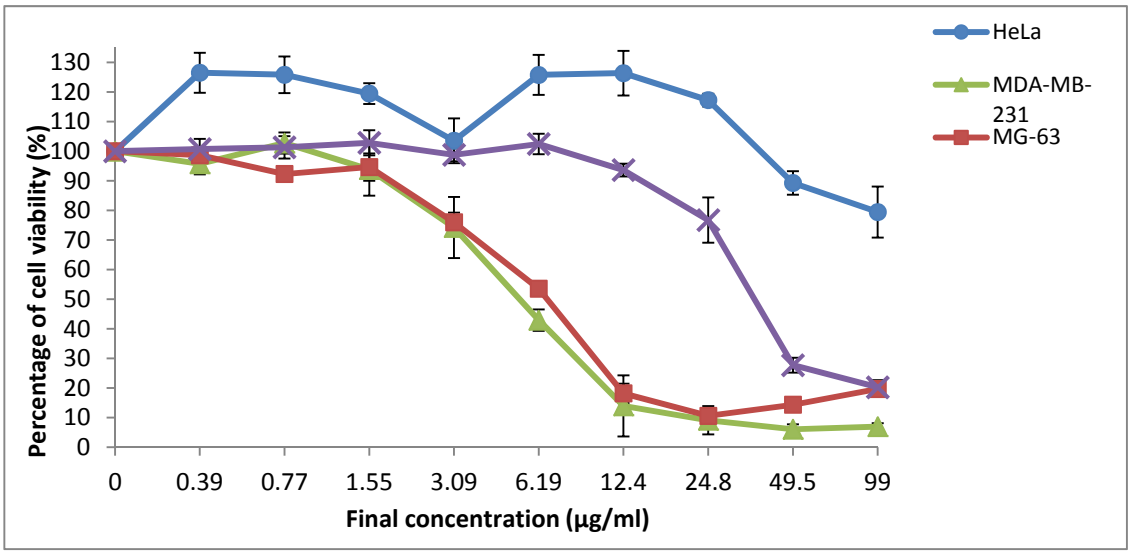

Figure 1: Anti-proliferative activity of methanolic yellow NLPE on HeLa, MDA-MB-231, MG-63 and MDCK cell lines at different final concentrations of 0.39-99 µg/ml for 72 hours. Each point showed the percentage of viable cells. n=3, $P<0.05$.

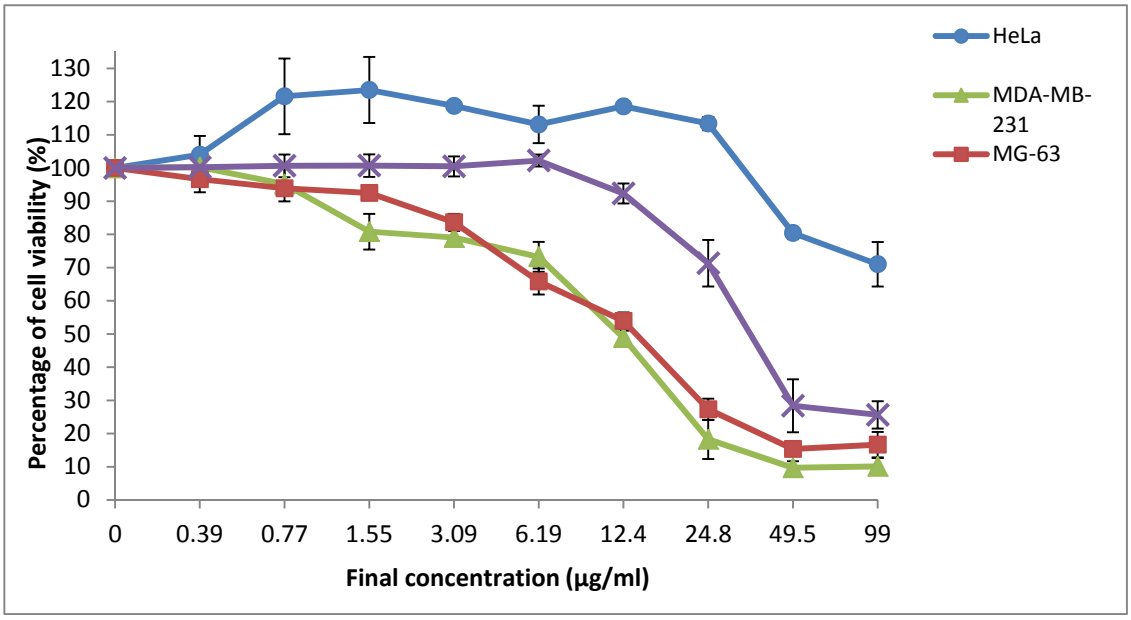

Figure 2: Anti-proliferative activity of methanolic red NLPE on HeLa, MDA-MB-231, MG-63 and MDCK cell lines at different final concentrations of 0.39-99 µg/ml for 72 hours. Each point showed the percentage of viable cells. n=3, $P<0.05$.

Both samples also were tested against non – malignant cell line, Madin-Darby canine kidney, MDCK. Based on the result, it showed that both samples having low anti-proliferative properties and cytotoxic effect on MDCK. This can be suggested that the test on the other non – malignant cells must be carried out to screen NLPE side effect on normal cells. There was a finding reported that methanolic NLPE and buthanolic NLPE showed a dose that produces the desired effect in half of a population (ED_{50}) value of 9.8±3.4 µg/ml and 15.7±6.9 µg/ml, respectively on KB cell, human oral squamos carcinoma cell which indicate that methanolic NLPE have more potential as anticancer agent compared to buthanolic NLPE due to effective dose needed only 9.8 µg/ml (Okonogi *et al*., 2010). Those researchers also found out that methanolic NLPE having IC_{50} values more 100µg/ml and no ED_{50} value detection towards Caco-2 cell, human colon adenocarcinoma cell.

The drug control, cisplatin in our study showed excellent indication of cancer cell inhibition on MG-63, MDA-MB-231 and MDCK cancer cells lines with IC_{50} values of 3.87±1.16 µg/ml, 18.6±2.22 µg/ml and 15.5±2.62 µg/ml (Table 2 and Figure 3) but not towards HeLa. Majumdar *et al*. (2001) reported that treatment of established cancer drugs such as cisplatin, demonstrated

inhibitory effect on HeLa cells multiplication at lower concentration but somehow show toxicity at higher concentration and longer treatment duration.

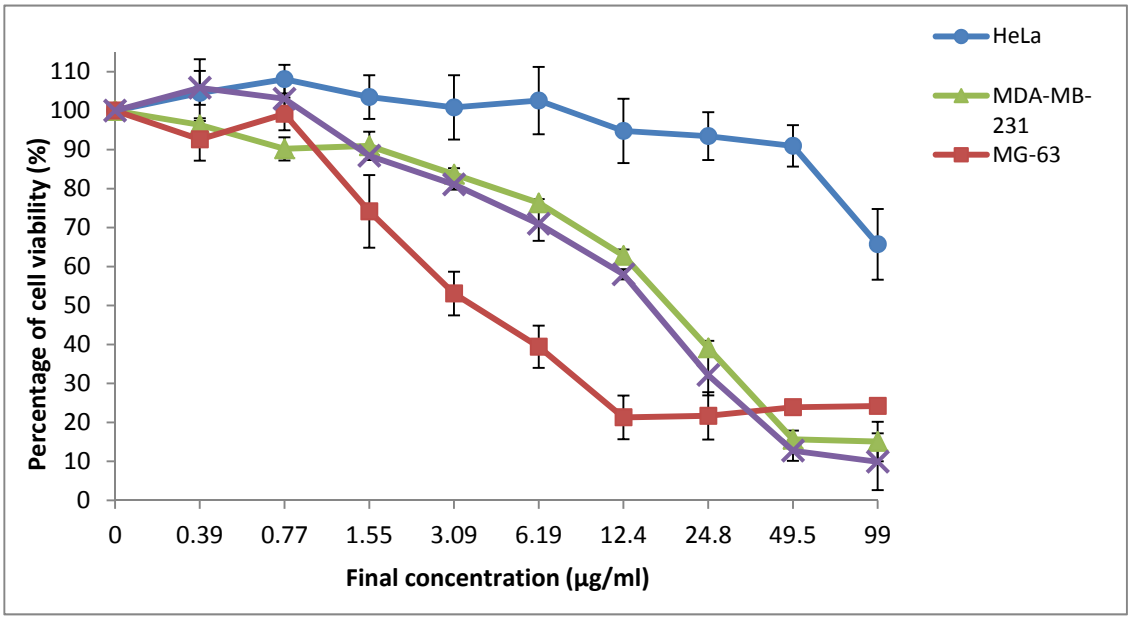

Figure 3: Anti-proliferative activity of cisplatin on HeLa, MDA-MB-231, MG-63 and MDCK cell lines at different final concentrations of 0.39-99 µg/ml for 72 hours. Each point showed the percentage of viable cells. n=3, $P<0.05$.

A major problem with present cancer chemotherapy is the deficiency of active drugs for the curative therapy of tumors (Kinghorn *et al*., 2003). Since conventional treatments available for cancer disease such as chemotherapy or radical surgery eventually fails to exert control on the disease, cancer chemoprevention brings the alternative way for cancer treatment and thus, by utilizing the waste product from plants and fruits in Malaysia might have beneficial effect towards cancer chemopreventive study (Hidayati *et al*., 2011). Plants play an important role as a source of effective anti-cancer agents where currently over 60% of used anti-cancer agents are derived from natural sources including plants and marine organisms (Newman *et al*., 2003). Vast quantities of agricultural-food waste are produced annually worldwide and the disposal of this waste can have a serious environmental effect (Thitilertdecha *et al*., 2010). NL has historically and continually been investigated for promising new leads in pharmaceutical development (McChesney *et al*., 2009). However, no previous study was done on the anti-proliferative activity specifically utilizing both varieties of NL peels.

The highest total phenolic content of methanolic NLPE that have been reported is 2.05±0.11 GAE (mg/ml) which compared with usage of other solvent (Okonogi *et al*., 2010). The rind and leaf of NL peels have high phenolic content and strong antioxidant activity that are seen to have correspondingly better radical – scavenging activity than vitamin C and grape seed (Palanisamy *et al*., 2008). Recently, many studies reported that plant extract with high level of polyphenolic compound have anticancer activity due to neutralization of biological reactive oxygen species (ROS). As biological reactive oxygen species are involved in cancer development, compounds with high ROS reduction activity are likely able to prevent cancer incidence which have been shown that both synthetic and natural antioxidant play an important role in ROS reduction, resulting in prevention and treatment of ROS-induced tissue degenerations (Moongkarndi *et al*., 2004; Russo *et al*., 2005).

Conclusion

Based on our findings, yellow NLPE is the best candidate for further research as anticancer agent especially in breast cancer diseases. Thus, our results recommend that yellow NLPE may represent an experimental therapeutic approach for the cancer treatment mainly for breast cancer. A further investigation on their molecular mechanism is worth in understanding the anti-cancer drug development.

Acknowledgement

This study was financially aided by Universiti Sains Malaysia Short Term Grant Scheme, 304/PPSK/61310030.

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