

## Proximate Analysis and Anti-Proliferative Properties of *Vitex negundo* L. (Analisis Proksimat dan Sifat Perencatan Proliferatif *Vitex negundo* L.)

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### ABSTRACT

The study of the proximate analysis on the leaves of *Vitex negundo* L. (VN) was done to gain the preliminary data of the anti-proliferative properties on cancer cell lines. Aqueous and organic extracts of the leaves of VN were used to identify its cytotoxic effect on six types of cancer-origin and normal cells, namely hormone-dependent breast cancer cell line (MCF-7), non-hormone-dependent breast cancer cell line (MDA-MB-231), ovarian cancer cell line (Caov-3), cervical cancer cell line (HeLa), liver cancer cell line (HepG2) and human foreskin fibroblast cell line (Hs27). The anti-proliferation activities of these extracts were investigated by employing colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)2,5 diphenyltetrazolium bromide) assay through time periods of 24, 48 and 72 h. Preliminary results showed that the methanol extracts had significant effects ( $p < 0.05$ ) on MDA-MB-231 with  $IC_{50}$  values 65.38  $\mu\text{g/mL}$ . The nutritional composition of the leaves provides a strong basis for emphasizing the nutritional value of *Vitex negundo* L.

**Keywords:** Cancer cell line; MTT assay; proximate analysis; *Vitex negundo* L.

### ABSTRAK

Kajian mengenai analisis proksimat ke atas daun *Vitex negundo* L. (VN) dilakukan bagi mendapatkan data permulaan mengenai sifat perencatan proliferatif terhadap sel kanser selanjara. Ekstrak akues dan organik daun VN digunakan untuk mengenal pasti kesan sitotoksik terhadap enam jenis sel kanser dan normal, iaitu sel selanjara kanser payudara yang bergantung pada hormon (MCF-7), sel selanjara kanser payudara yang tak bergantung pada hormon (MDA-MB-231), sel selanjara kanser ovari (Caov-3), sel selanjara kanser serviks (HeLa), sel selanjara kanser hepar (HepG2) dan sel selanjara fibroblast kulit manusia (Hs27). Aktiviti anti-proliferatif kedua-dua ekstrak dikaji dengan menggunakan asai MTT (3-(4,5-dimethylthiazol-2-yl)2,5 difeniltetrazolium bromida) melalui tempoh masa 24, 48 dan 72 jam. Data awal menunjukkan ekstrak metanol memberi kesan signifikan ( $p < 0.05$ ) terhadap sel MDA-MB-231 dengan nilai  $IC_{50}$  65.38  $\mu\text{g/mL}$ . Komposisi pemakanan daun ini memberi asas yang kuat bagi menekankan nilai pemakanan yang ada pada *Vitex negundo* L.

**Kata kunci:** Analisis proksimat; asai MTT; sel kanser selanjara; *Vitex negundo* L.

### INTRODUCTION

Cancer is a leading cause of death worldwide (World Health Organization 2013). In Malaysia, in the year 2007, 18217 cancer incidences were reported for both male and female patients (Zainal & Nor Saleha 2011).

The health benefits associated with the use of traditional plants are attributed to the presence of phytochemicals with their bioactivities. Hence the potential use of natural products as anticancer treatment has been explored intensively by the scientists (Wan-Nor Izzah et al. 2009).

There are 270 known species of *Vitex*, few species may be found in temperate zones. Phytochemical screening of the leaves of *Vitex trifolia* showed the presence of saponins, tannin, flavonoids and glycosides in different types of solvents extract (Geetha et al. 2004). Phytochemical constituents of *Vitex negundo* (VN) leaves extract such as alkaloids, flavonoids and phenolic acids had been reported to be important compounds in many other medicinal plants (Panda et al. 2009). The plant was shown to possess potent

preferential cytotoxic activity against human pancreatic cancer cells (PANC-1) (Awale et al. 2011). The leaves of this plant commonly consumed in the preparation of a popular food (nasi lemak) in Malaysia, are believed to have medicinal benefits, in promoting proliferation. The present study seeks to assess the cytotoxic properties of aqueous and methanolic extracts of the leaves of VN on cancer-origin cell lines.

### MATERIALS AND METHODS

#### PLANT AND MATERIALS

Leaves of VN were purchased from Kampung Seronok, Bayan Lepas, Pulau Pinang, Malaysia in October 2010. The leaves were identified by Mr. Adnan Jaafar. Voucher specimen (USM Herbarium 11461) was deposited in the herbarium of School of Biological Sciences, Universiti Sains Malaysia. The plant is easily recognized by the purplish bottom part of the leaf (Chopra et al. 1956).

The Hs27 (ATCC® CRL-1634™, human foreskin fibroblast cell line), MCF-7 (ATCC® HTB-22™, hormone-dependent breast cancer cell line), MDA-MB-231 (ATCC® HTB-26™, non-hormone-dependent breast cancer cell line) Caov-3 (ATCC® HTB-75™, human ovary cancer cell line), HeLa (ATCC® CCL-2™, human cervical cancer cell line) and HepG2 (ATCC® HB-8065™, human liver cancer cell line) were purchased from the American Type Culture Collection (ATCC), USA. Phosphate Buffer Solution (PBS) tablets were obtained from AMRESCO INC, Cleveland, Ohio, USA. Dulbecco's modified eagle medium (DMEM with low and high glucose) and Foetal Bovine Serum (FBS), penicillin –streptomycin and trypsin were from Gibco®, Invitrogen™, USA. MTT (3-(4,5-dimethylthiazol-2-yl)2,5 diphenyltetrazolium bromide) labelling reagent was obtained from Molecular Probes®, Invitrogen™, Oregon, USA.

#### SAMPLE PREPARATION

Leaves of VN were separated from its stem and dried at room temperature (24°C±5°C) for 4 days prior blending them for 5 min. Subsequently, plant powders were kept in an air tight polyester container at -20°C before use to avoid changing in substance and also to prevent microorganism growth (Saleem et al. 2002).

#### PROXIMATE ANALYSIS

Proximate analysis was carried out to determine moisture, ash, fat, protein and fibre contents as well by using the method stipulated by Association of Analytical Chemists (AOAC), 1990. The percentage of carbohydrate content was determined by difference, which is 100 - (% ash+ % fat+ % protein + % fibre content).

#### SAMPLE EXTRACTIONS

The polarity and viscosity of the solvents used plays a major role in the extractability of plant bioactive compounds (Wijekoon et al. 2011). Hayouni et al. (2007) concluded that using higher polarity solvents resulted in extracted compounds with higher antioxidant capacity relative to using lower polarity solvents.

The water extract was prepared as described by Huang et al. (2003), achieved by soaking the leaves powder of VN extract in boiling water (100°C) in the proportion of 1:20 (w/v) for 4 h. According to Li et al. (2006), increasing the extraction temperature resulted in an increase in total phenol recovery. This is possible as higher extraction temperature may improve the recovery because extraction in hot water can extract some pectic polysaccharides from the cell wall and thus weaken cell wall integrity (Hayouni et al. 2007). The resulting crude extracts were filtered and lyophilized by freeze drier. The methanol extract was obtained by maceration of powdered VN leaves in 95% methanol for 24 h. Chlorophyll of the leaves weren't removed. The methanol fraction was collected and the residual solvent eliminated by reduced pressure at 40°C

using a rotary evaporator. The residue obtained was dried in a desiccator until it reached a constant weight (Wicaksono et al. 2009). The extract was diluted in PBS and sterilized before assays. Final dilution was made in low glucose DMEM containing 20% FBS but Hs27 was grown in DMEM supplemented with additional amount of glucose means 4.5 g/L (high glucose).

#### GROWTH OF CELL LINES

The cells were cultured in the appropriate medium, supplemented with 10% FBS and 1% penicillin-streptomycin, CO<sub>2</sub> using 25 cm<sup>2</sup> tissue culture flasks in 37°C incubator (NUAIRE Incubator Model NU-4750E, NuAire Laboratory Equipment Supply, USA) with 5% CO<sub>2</sub> (complete media = basal medium + 10% FBS + 1% penicillin-streptomycin). All cell culture work was carried out in a biological safety cabinet (NUAIRE biological safety cabinet model NU-425-400E, NuAire laboratory equipment supply, USA). Reagents were pre-warmed by using a water bath (Grant Water Bath Model GR150, Fisher Scientific, Malaysia) prior to use (Freshney 1994).

#### MEASUREMENT OF THE GROWTH INHIBITORY EFFECT

Each of the cancer cell lines was grown in a 96-well Microtiter plate (Nunc, Denmark) in a volume of 60 µL culture medium per well. The stock solution was serially diluted (with DMEM containing 20% FBS) to a concentration of 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 µg/mL. The crude extract demonstrated active when the IC<sub>50</sub> was below 100 µg/mL and the crude extract also has potential to purification of active compound and have strong antiproliferative activity.

Each well contained 3×10<sup>3</sup> cells/mL and was incubated for 24 h. The cancer cells (MCF-7, MDA-MB-231, Caov3, HeLa and HepG2) and normal cell Hs27 (control) were then treated with 60 µL extracts of VN leaves which contained a serial dilution at doses of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL and the temperature was maintained at 37°C with CO<sub>2</sub> for 24-72 h. The cells in the first row of the 96-well microtiter plate were fed with fresh growth medium for control. After 24-72 h of incubation, about 24 µL of MTT was added to each well and incubated for another 4 h. Excess MTT was removed after incubation and the remaining was solubilised with 100 µL of acidified-isopropanol. One hundred microlitre distilled water was added into each well for further colour development. The absorbance of viable cells was measured immediately using a spectrophotometric plate reader (Multiskan spectrum, Thermo Electron Co., Waltham, Massachusetts, USA) at 570 nm because the product is unstable. Absorbance for cell proliferation is usually between 500 and 600 nm (Wu 2010; Zachary 2003). To calculate the IC<sub>50</sub> the processes were as follows:

$$\text{Cell viability (\%)} = \frac{\text{OD of drug} - \text{test sample} - \text{OD of Blank}}{\text{OD of Control} - \text{OD of Blank}} \times 100.$$

## STATISTICAL ANALYSIS

The results for percentage cell viability were reported as means  $\pm$  standard error of triplicate measurements. Significant differences for multiple comparisons were determined by one-way analysis of variance (ANOVA) followed by Duncan test with  $\alpha=0.05$  by SPSS statistical package (ver. 19.0).

## RESULTS AND DISCUSSION

The results of the proximate analysis of VN are presented in Table 1. The parameters determined were moisture content, ash, crude fat, crude protein and crude fibre. The leaves contained appreciable amounts of crude fibre (11.61 $\pm$ 0.14) and fat (7.05 $\pm$ 0.03). Low-fat foods are known to reduce the cholesterol level (Gordon & Kessel 2002). A dietary pattern containing low-fat and high-fibre products has been associated with reduced risks of breast cancer (Kushi et al. 2012). The presence of these essential nutrients implies that VN leaves can be consumed as a nutritional and health ingredient for the human body.

Figure 1 shows the percentage growth inhibition exerted by the methanolic extract on various human cancer-origin cell lines such as MCF-7, MDA-MB-231, Caov-3, HeLa and HepG2 and normal cell line (Hs27). As shown in the figure, growth inhibition of cancer-origin cell lines increased steadily with increasing concentrations of the methanolic extract. Growth inhibition of the cancer origin cell line was most significant at 72 h. The methanolic extract had no effect on normal cell growth. The  $IC_{50}$  values obtained from the methanolic extract are presented in Table 2. The most significant growth inhibition by the methanolic extract was on MDA-MB231 cells with an  $IC_{50}$  of 65.38  $\mu$ g/mL at 72 h ( $p<0.05$ ). The  $IC_{50}$  values at 72 h for MCF-7, HeLa, HepG2 and Caov3 cells were 165.14  $\mu$ g/mL, 167.23  $\mu$ g/mL, 226.52  $\mu$ g/mL and 298.05  $\mu$ g/mL, respectively.

Figure 2 emphasizes the growth inhibitory effect of the water extract on MDA-MB-231 cell lines ( $IC_{50}$  = 301.63  $\mu$ g/mL) at 48 h and MCF-7 cell lines ( $IC_{50}$  = 422.89  $\mu$ g/mL) at 72 h ( $p<0.05$ ). In contrast, the water extract had no effect on the growth of Caov-3, HeLa and HepG2 (Table

TABLE 1. Proximate analysis of VN leaves

Moisture	Ash	Fat	Protein	Crude Fibre	Carbohydrate
53.45 $\pm$ 0.15	8.07 $\pm$ 0.17	7.05 $\pm$ 0.03	0.87 $\pm$ 0.01	11.61 $\pm$ 0.14	18.95 $\pm$ 0.05

( $n=3 \pm$  S.D.)

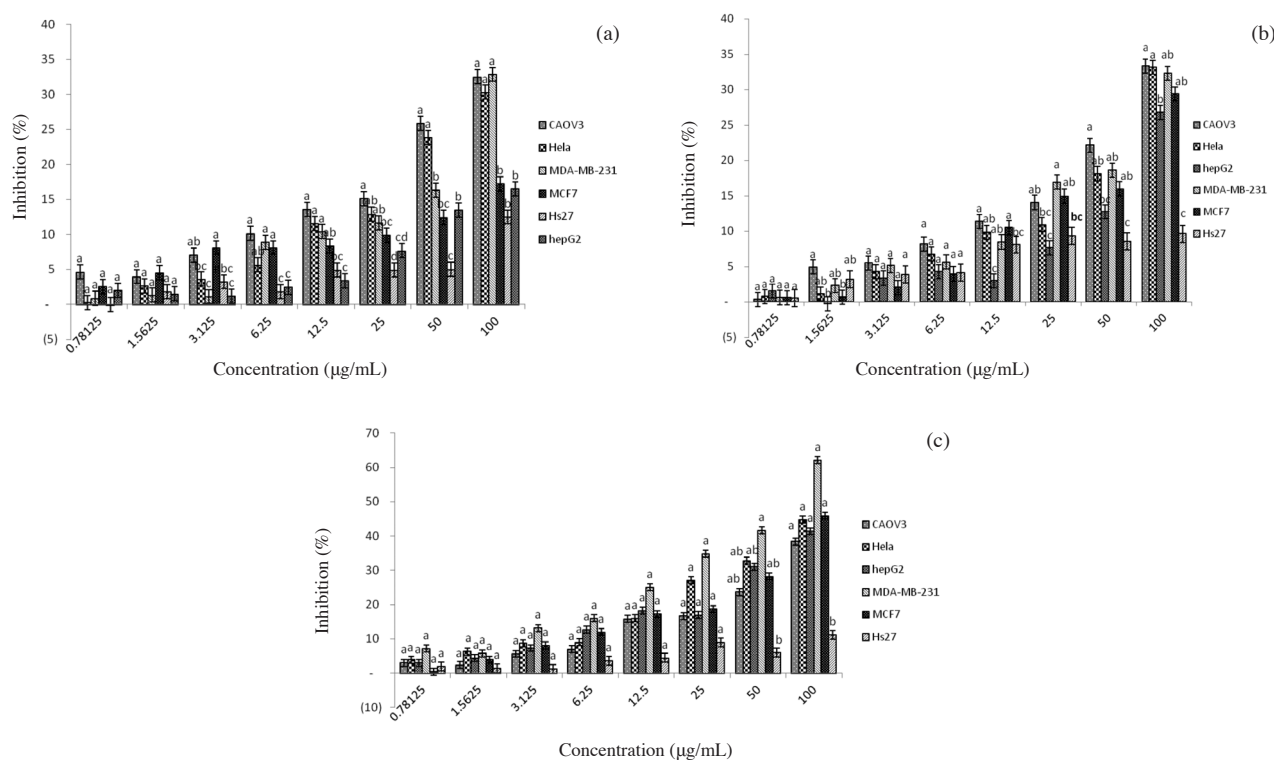


FIGURE 1. Inhibition of methanol extract of VN on origin-cancer cell lines. (a) Treated MCF-7, MDA-MB-231, Caov3, HeLa, HepG2 and Hs27 in 24 h, (b) treated in 48 h and (c) treated in 72 h. Values are expressed as mean  $\pm$  standard error (SE) of triplicate measurements; dry basis and are expressed in percentage (%)

TABLE 2. The IC<sub>50</sub> of methanol extract from leaf of VN on cancer cell lines

Cell lines	24 h	48 h	72 h
Caov-3	602.9	436.03	298.05
HeLa	383.6	427.1	167.23
HepG2	2447.1	398.4	226.52
MDA-MB-231	403.38	424.3	65.38
MCF7	70930.1	446.9	165.14

Using Probit analysis on a Finney computer program Bio Stat™ 2009.

Probit analysis is a type of regression used to analyze binomial response variables

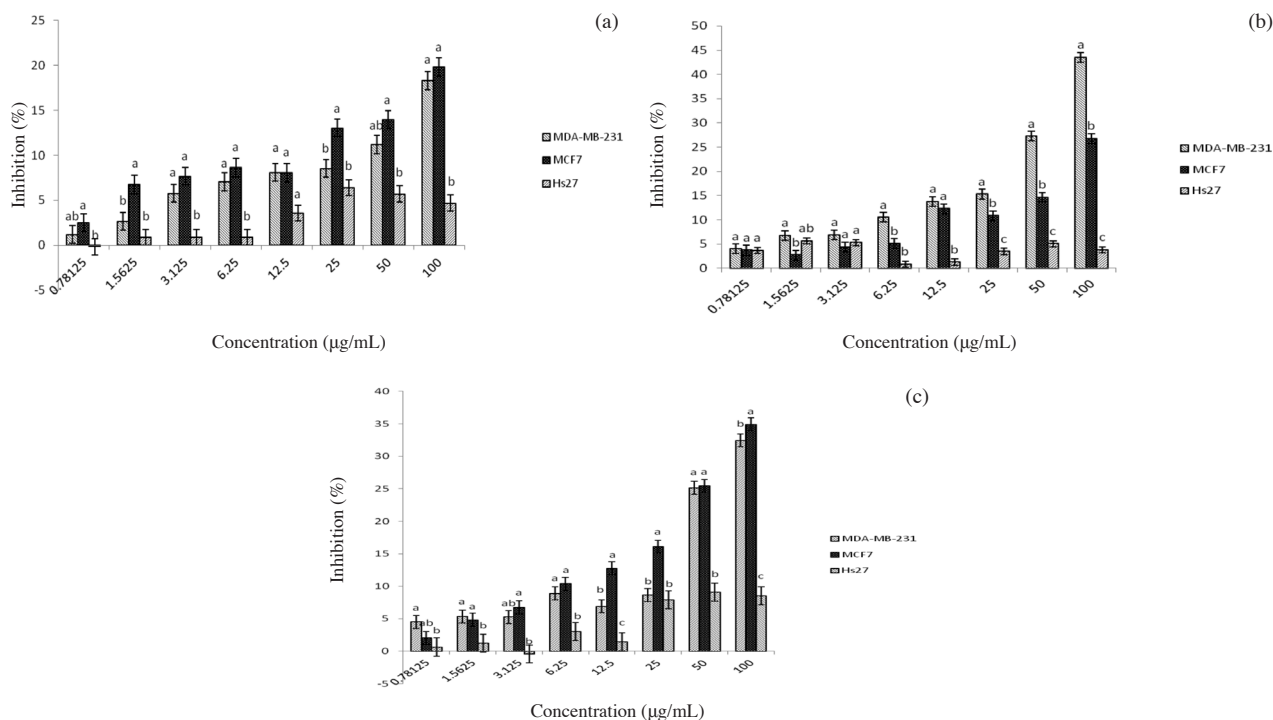


FIGURE 2. Inhibition of water extract of VN on MCF-7, MDA-MB-231 and Hs27, respectively (a) treated MCF-7, MDA-MB-231 and Hs27 in 24 h, (b) treated in 48 h and (c) treated in 72 h. Values are expressed as mean  $\pm$  standard error (SE) of triplicate measurements; dry basis and are expressed in percentage (%)

TABLE 3. The IC<sub>50</sub> of water extract from leaves of VN on cancer cell lines

Cell lines	24 h	48 h	72 h
Caov-33	11587.4	5677.7	1815.1
HeLa	82765.7	4366.2	2546.6
HepG2	5109027	16583.1	4218.1
MDA-MB-231	9226.9	301.6	948.4
MCF7	14778.8	1711.1	422.8

Using Probit analysis on a Finney computer program Bio Stat™ 2009.

Probit analysis is a type of regression used to analyses binomial response variables

3). The methanolic extract of VN was found to have a stronger antiproliferative effect in comparison with the water extract ( $p < 0.05$ ). This may be due to the presence of more active compounds in the methanolic extract.

Several studies have shown that the cytotoxicity and anticancer properties of natural plants are mainly due to the presence of flavonoids. Phenolic compounds, including flavonoids are especially promising candidates

for cancer prevention (Bravo 1998). Guha et al. (2010) reported that methanolic extracts from VN had high concentrations of polyphenolic compounds. This could have contributed to the susceptibility of the cells to methanolic VN extract. However, further screening test and *in vivo* study is needed to confirm our findings and evaluating an actual anti-proliferative property in the leaves of VN.



## CONCLUSION

Conclusively, better inhibitions of cancer cell lines were observed in the methanol extract as exemplified by an IC<sub>50</sub> of 65.38 µg/mL in the case of the MDA-MB-231 cell line. Knowing the exact compounds responsible for the plant's anticancer properties will help in formulating anticancer agents. In addition, the results from the proximate analysis in the plant have provided the pertinent information for food formulations. Lastly, *in vivo* and clinical evaluations need to be performed for the successful commercialization of this plant to benefit both the food and pharmaceutical industries.

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