

Investigation of Anticancer Activity of *Lindernia crustacea* (L.) F. Muell. var. *Crustacean*[†]

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Abstract

Lindernia crustacea (L.) F. Muell. var. *crustacean* or “Ya Kap Hoi: YKH” was misunderstood as “Ya Yad Nam Kang” and cancer curative. This study aimed to investigate anticancer activity by the analysis of chemical constituent in extracts and *in vitro* screening of biological activities of the extract in several aspects such as cancer cell lines cytotoxic activity, immune cell proliferating activity, reducing power activity, alkylation activity. The HPLC analysis showed the absence of plumbagin peak observed in the HPLC chromatograms of both YKH aqueous and YKH ethanolic extracts. The YKH ethanolic extract yielded more chemical constituents than that of the YKH aqueous extract. The YKH aqueous extract was inactive against all cancer cells tested. Interestingly, YKH ethanolic extract caused cancer cell death in HCT116 colon cancer, HepG2 liver cancer, and Jurkat leukemic cancer cell lines in the concentration dependent manner. The following IC₅₀ concentrations of the YKH ethanolic extract that possessed 50 % cell death after 24 h exposure in HCT116, HepG2, and Jurkat cell line were 195.4 ± 12, 171.7 ± 8.7, and 48.8 ± 5.7 µg/mL, respectively. Both aqueous and ethanolic extracts of YKH showed high antioxidant activity based on reducing power activity but did not have alkylation activity. At high concentration (250 µg/mL), YKH ethanolic extract can inhibit immune cells proliferation activity more than the YKH aqueous extract. An unexpected but critical outcome of our studies was the finding that anticancer activity is promised by selecting the plant extraction solvent with less polarity. Potential anticancer constituents were extracted from YKH using ethanol and these constituents cannot be found in aqueous solution of YKH.

Keywords: Alkylation, cell proliferating, cytotoxic, *Lindernia crustacean*, reducing power, Ya Kap Hoi

Introduction

Ya Kap Hoi (YKH) found as grass in Thailand and have been used as edible vegetable in Northeastern part of Thailand. In 2012, YKH was misunderstood as “Ya Yad Nam Kang” and cancer curative and published in Thai newspapers [1]. The similar Thai local name between “Ya Yad Nam

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Kang” and “Ya Nam Kang” caused public controversy, although these 2 plants were not identical. Then YKH was collected by the Department of Development of Thai Traditional and Alternative Medicine, Ministry of Public Health and was investigated by the Department of Medical Sciences at that time. The scientific name of the identified plant was *Lindernia crustacea* (L.) F. Muell. var. *crustacea* [2,3] and *Drosera indica* L. for “Ya Nam Kang” [3,4]. Based on literature search, no study associated with cancer therapy was found for *L. crustacea* (L.) F. Muell. var. *crustacea*. Whereas, the ethanolic and aqueous extract of *D. indica* L. was reported to be effective on metabolic syndrome in Dalton lymphoma ascites (DLA) bearing mice [5], and synergistic use with anticancer chemotherapy [5-7]. Despite no confirmation of YKH in clinical use, the claimed uses of it for anticancer, antibacterial activity and treatment of joint pain was reported in the newspaper. For example, Thai traditional uses stem for treatment of venereal disease and leaves decoction for postpartum conditions, in India, the dry powder was infused in aqueous for drinking in morning for detoxification purpose, chewing leaves with lemon is used to increasing the biliary flow. The skin application was also reported to be for the treatment of ringworm, scalding, burning, antifungal activity and antibacterial activity. In Malaysia it is used for the treatment of scalds and burn, anti-inflammatory specifically from tick and also for postpartum conditions [1].

There has been no information reported on anticancer activity of YKH. This study aimed to investigate anticancer activity by the analysis of chemical constituent in extracts and *in vitro* screening of biological activities of the extract in several aspects such as cancer cell lines cytotoxic activity in various cell lines (4 types of cell lines), immune cell proliferation activity, reducing power activity, alkylation activity.

Materials and methods

Plant preparation and extraction

Lindernia crustacea (L.) F. Muell. var. *crustacea* or female “Ya Kap Hoi : YKH” was provided by Kamphaeng Phet Provincial Public Health Office. The YKH were extracted and prepared using the following method. The crude 50 % ethanol-aqueous extract was obtained as previously described [8,9]. The fresh weight was 200.40 g and dried for 1 - 2 h. The dried YKH was ground and homogenized with 500 mL water, and filtered through gauze cloth. The filtrate was centrifuged at 13,000 rpm for 10 min and freeze-dried and then frozen at -20 °C. The obtained dried residue after freeze-drying was the YKH aqueous extract (9 g). The remaining residue was subjected to oven dry at 50 °C for 24 h. The obtained dried residue was 29.78 g which was macerated in 100 % ethanol for 7 days with concessionary shaking. The filtration was done using gauze cloth and filter paper, respectively. The filtrate was evaporated under vacuum in 40 °C until the dried YKH ethanolic extract (0.13 g) was obtained. Two parts of YKH crude extracts were dissolved in dimethyl sulfoxide (DMSO) at 20 mg/mL as stock solutions. The stock YKH extracts were freshly diluted in fresh medium at various concentrations before use.

Plumbagin standardization by High performance liquid chromatography (HPLC) technique

Plumbagin standardization by HPLC was used to identify the YKH in this study since plumbagin was reported to be composed in Ya Nam Kang [4]. The HPLC system was comprised of 1100 Agilent series (Minnesota, USA) with a pumping system model G1310A, a manual injection model G1328B and detector model G1314A. The chromatographic determination was performed as previously described [8]. The stationary phase was a C18 (25 cm × 4.6 mm, 5 µm) reverse phase column (HiQ sil, Japan), with a 5 µm particle size (KYA TECH Corporation, Japan). The reverse phase HPLC was conducted using an isocratic mobile phase of 90 % methanol in Milli-Q water and 5 mM NaH₂PO₄ with a flow rate of 0.8 mL/min. The detector was set at 265 nm wavelength. The ethanolic extract stock solution was prepared in ethanol and aqueous extract stock solution was prepared in water. The plumbagin standard which was provided by Professor Putalun W, Faculty of Pharmaceutical Sciences, Khon Kaen University, was dissolved in ethanol as a 10 µg/mL and was used as the reference standard to confirm its presence in the extract fraction.

Human cancer cell cytotoxic activity by NR assay

Human cancer cell cytotoxic test was performed for detection of anticancer activity. Four types of human cancer cell lines; leukemic cells (Jurkat at 5×10^5 cells/mL), liver cancer cells (HepG2 at 4×10^5 cells/mL), lung cancer cells (SK-LU1 at 4×10^5 cells/mL) and colon cancer cells (HCT116 at 3×10^5 cells/mL) were used as cell models and normal African green monkey kidney cell line (Vero 6×10^5 cells/mL) was used as normal cell control. The stock at 20 mg/mL YKH in DMSO which were freshly diluted before use with fresh culture medium to desired concentrations ranging from 50 to 500 μ g/mL. The final concentration of DMSO in each concentration did not exceed 1 % v/v to ensure less than 10 % toxicity. The determination of cancer cell cytotoxic activity was performed *in vitro* by using NR assay as previously described [9,10]. Briefly, 100 μ L/well of each cells were seeded into 96-wells plate and incubated for 24 h. Cancer cell lines were treated with the YKH extracts at various desired concentrations (50 - 500 μ g/mL) for 24 h and untreated cell was a control cell group. The 96-wells plate was centrifuged at 492 g for 10 min. Supernatant (180 μ L for floating cells and 200 μ L for adherent cells) was removed. And 100 μ L of 50 μ g/mL of NR solution in media was added into each well. The cells were incubated for 2 h at 37 °C in 5 % CO₂ incubator. The 96-wells plate was centrifuged at 492 g for 10 min and supernatant was removed and cells were washed twice with 1x PBS (150 μ L). Cells were solubilized with 100 μ L of 0.33 % HCl in isopropanol. The viable cells with NR were measured for absorbance with dual wavelengths at 520 nm and 650 nm (reference wavelength). The % cytotoxicity was calculated and the concentration causing 50 % cell death (IC₅₀) was determined from a linear equation obtained from the plot between the %cytotoxicity and the concentration of YKH.

Immune cell proliferating activity assay by CFSE staining

The cell proliferating test was performed for detection of toxic activity of the YKH extracts on immune cell. The detection was *in vitro* performed on peripheral blood mononucleated cell (PBMC) represented as immune cells. The PBMC was isolated as previously described [11]. Cell proliferating test was performed by succinimidyl ester of carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described [12]. Briefly, fresh venous blood was diluted with PBS and centrifuged at 400g for 35 min on Ficoll-Paque Plus. Then lymphocyte interface was collected and washed twice in RPMI-1640 and resuspended with 10 % foetal calf serum in RPMI-1640, 20 mM HEPES, 100 U/mL penicillin, 100 μ g/mL streptomycin. Cell number and viability were counted and checked by trypan blue exclusion assay. Fresh isolated PBMC was exposed to YKH extracts for 24 h. Then PBMC was stained with CFSE at 37 °C for 30 min in 24 well plate and the stained cells were measured for fluorescence intensity by flow cytometer represented as the proliferating activity.

Reducing power activity using ferric reducing antioxidant power (FRAP) assay

The *in vitro* determination of reducing power activity by Ferric Reducing Antioxidant Power (FRAP) assay as previously described [13]. Briefly, FRAP reagent was freshly prepared before use by mixing 100 mL of 300 mM acetate buffer pH 3.0, 10 mL of 10 mM TPTZ in 40 mM HCl, 10 mL of 20 mM FeCl₃ · 6H₂O and 12 mL water and incubated at 37 °C for 4 min. The ferric-tripyridyltriazine (Fe³⁺-TPTZ) complex was reduced by the reducing agent (antioxidant agent) at low pH resulting in a colored ferrous-tripyridyltriazine (Fe²⁺-TPTZ) complex which could be detected at 593 nm. The standard curve was set up from the absorbance of various concentration of FeSO₄ · 7H₂O. The standard curve was linear between 25 and 40 in μ M Trolox (μ MTE/mg).

Alkylation activity assay

The determination of alkylation activity was measured *in vitro* as DNA model generating nucleophilicity by using 4-(4'-nitrobenzyl) pyridine (NBP) reagent. Thus the NBP indicates the mechanism of anticancer activity as an *in vitro* alkylation. Alkylating activity of the test sample could be determined based on the colored compound produced from the chemical interaction between the test sample and the NBP at a specific condition. NBP produces a violet color after undergoing alkylation. The change of absorbance in the presence and the absence of test compound were measured by using UV-VIS

spectrophotometer at 600 nm at various time points. The NBP assay was performed as previously described with some minor modification [14]. Briefly, different concentrations of the mixture solutions of the test sample and buffer pH 4.00 solutions were prepared. This solution mixture was then incubated in a dry oven pre-heated at 70 °C with a solution of 4-(4-nitrobenzyl) pyridine in centrifuge tubes and thoroughly mixed. At each time point, 90 µL of the mixture solutions were pipetted and added into the 96-well plate which already contained a mixture of an absolute ethanol and 0.1 N NaOH and placed in ice bath. The blue colored of alkylation interaction was measured as the absorbance at each time point by using UV-VIS spectrophotometer at 600 nm.

Data analysis

SPSS was performed to analyze statistical differences between cells treated with jujube extracts and untreated group (one-way ANOVA). Statistical significance was considered within the 95 % confidence interval when $p < 0.05$.

Results and discussion

Plant extract

The physical form and the extraction yield of YKH extracts were shown in dark brownish-black powder with 4.5 % (w/w) fresh weight from aqueous extraction. This is compared to dark green viscous residue with 0.44 % (w/w) dry weight from ethanolic extract in **Figure 1**. The extract characteristics seem to reveal that no distinct yellow color was visually observed in both of aqueous and ethanolic YKH extracts compared to standard plumbagin compound which composed in *Drosera indica* L. or “Ya Nam Kang” [3,4]. However, the standardization of both aqueous extraction and ethanolic extract with HPLC analysis was further conducted to ensure the absence of plumbagin.

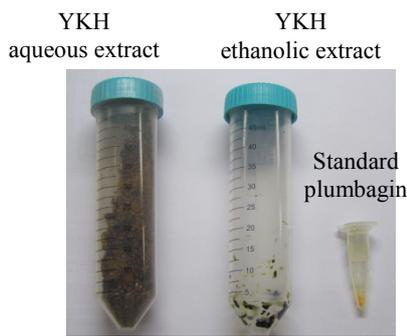


Figure 1 The photos of extract characteristic of YKH aqueous and ethanolic extracts as compare to standard plumbagin compound.

Majority of chemical constituents are non-polar and absence of plumbagin compound in extracts

Based on the HPLC chromatograms in **Figure 2**, the peak of standard plumbagin which HPLC retention time was eluted at 5.468 ± 0.04 min (**Figure 2c**). Neither is plumbagin peak observed in aqueous extract HPLC chromatograms (**Figure 2a**) or ethanolic extract HPLC chromatograms (**Figure 2b**). The YKH aqueous extract possessed less peaks than the YKH ethanolic extract, indicated that the YKH ethanolic extraction technique yielded more chemical constituents than the YKH aqueous extraction technique. The evidence reveals that majority of YKH chemical constituents are non-polar. Additional testing of the cytotoxic activity of the plant extracts was further conducted.

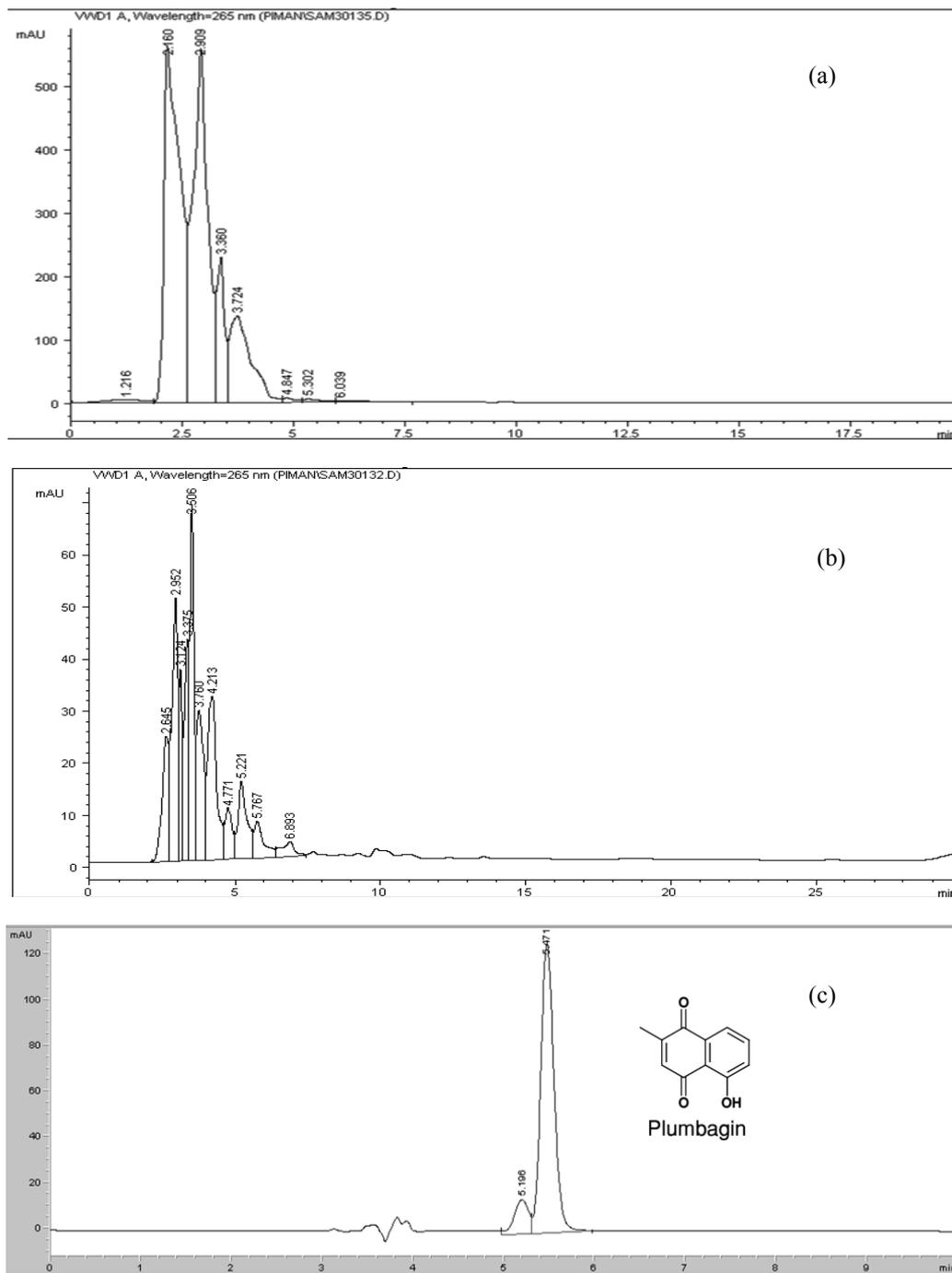


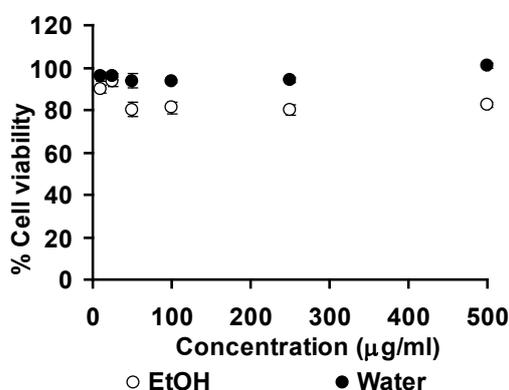
Figure 2 HPLC chromatograms of *Lindernia crustacea* (L.) F. Muell. var. *crustacean* (YKH); a) 10 mg/mL aqueous extract dissolved in aqueous, b) 1 mg/mL ethanolic extract dissolved in ethanol compared to c) 10 µg/mL standard plumbagin dissolved in ethanol.

Anticancer activity *in vitro*

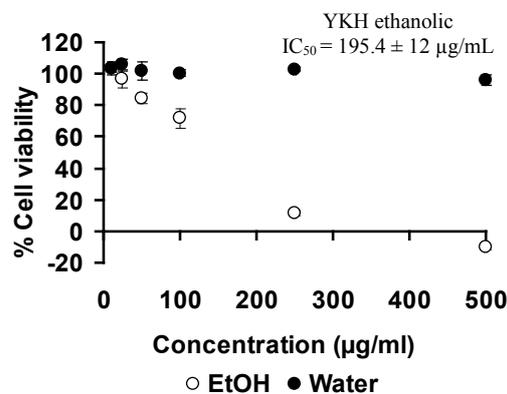
The YKH extracts were tested for the cytotoxicity effect on various human cancer cell lines. The IC_{50} represented the concentration of the plant ethanolic extract that possessed 50 % cell death after 24 h exposure in Jurkat, HepG2, HCT116, and SK-LU1 were $48.8 \pm 5.7 \mu\text{g/mL}$, $171.7 \pm 8.7 \mu\text{g/mL}$, $195.4 \pm 12 \mu\text{g/mL}$ and $> 500 \mu\text{g/mL}$, respectively. Whereas the IC_{50} value cannot be calculated even used the maximum concentration up to $500 \mu\text{g/mL}$ of YKH aqueous extract in all tested cancer cell lines as was demonstrated in **Figure 3**. The IC_{50} value cannot be calculated even used maximum concentration ($500 \mu\text{g/mL}$) is considered to be inactive. The results showed that only the YKH ethanolic extract exerted cytotoxicity against cancer cell in Jurkat leukemic cancer cell, HepG2 liver cancer and HCT116 colon cancer but not toxic to SK-LU1 lung cancer cell line and Vero normal cell line.

This study is the first formal investigation of the chemical constituents in extracts and *in vitro* screening of biological activities which leads to further understanding of the plants' potential relative to *in vitro* anticancer activity. Based on the compound/s that possessed cytotoxicity IC_{50} value $< 100 \mu\text{g/mL}$ is classified as a potentially cytotoxic compound [15]. Hence, only the plant ethanolic extract showed potential cytotoxicity against Jurkat leukemic cancer cell line and moderate cytotoxicity in HepG2 liver cancer and HCT116 colon cancer cell lines with high selectivity. Based on the HPLC chromatograms, the plant extraction method in this study either by using aqueous or ethanolic extraction did not yield the cytotoxic plumbagin compound. Hence, the following cytotoxic effects observed from the plant ethanolic extract may be attributed to the other chemical constituents. Moreover, that observed cytotoxic constituent/s were not present in the aqueous extract as the HPLC chromatograms of both extracts were different. This finding seems to show that the presence of chemical constituents which have less polar or non-polar properties in extracts convinced us of the compounds' cytotoxic activity [16]. However, the analysis to identify the exact chemical constituent/s in both extracts is recommended need to be elucidated further.

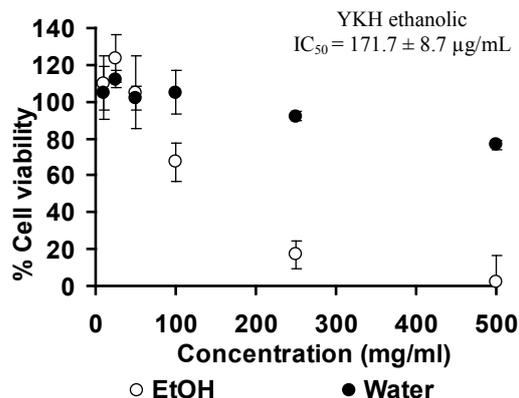
(a) Vero normal cell line



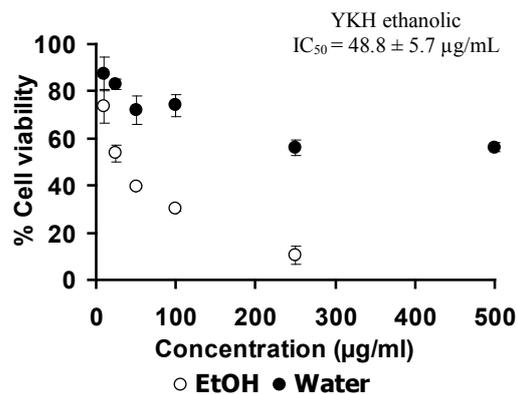
(b) HCT116 colon cancer cell line



(c) HepG2 liver cancer cell



(d) Jurkat leukemic T cell



(e) SK-LU1 lung cancer cell line

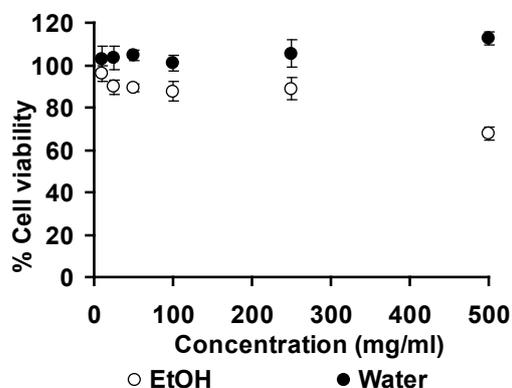


Figure 3 Cytotoxicity activity study of *Lindernia crustacea* (L.) F. Muell. var. crustacean (YKH); ethanolic extract (○) and aqueous extract (●) after 24 h exposure on various human cancer cells; a) Vero normal cell line, b) HCT116 colon cancer cell line, c) HepG2 liver cancer cell line, d) Jurkat leukemic T cell line and e) SK-LU1 lung cancer cell line

Effect of the extract on immune cell proliferation

The plant extracts were determined for the immune cell proliferation effect. Both ethanolic and aqueous extracts did not have any immune cells proliferation activity at less than 50 µg/mL concentration when compared to control. However, when the extract concentration was increased at 250 µg/mL, both ethanolic and aqueous extracts significantly inhibited immune cells proliferation activity when compared to the untreated cells (Table 1). Additionally, the inhibitory activity of the ethanolic extract was superior as compared to the aqueous extract. Although at the present the exact potential chemical constituents in the extracts. This finding strongly supports the assumption that the less polar or non-polar chemical constituents regulate immune cell proliferation activity in same manner that effect on cancer cells [16].

Table 1 Immune cell proliferation activity of YKH extracts.

Final Conc. ($\mu\text{g/mL}$)	Immune cell proliferation (%)			
	YKH ethanolic extract		YKH aqueous extract	
	mean	SD	mean	SD
0	100	0.96	100	0.96
10	107.5	1.69	108.5	0.96
50	105.7	0.72	102.6	1.21
250	42.6*	2.78	70.2*	4.31

*p < 0.05 vs. untreated cells

Antioxidant activity of the extract

The antioxidant activity of the plant extracts was determined based on the reducing power as measured by Ferric Reducing Antioxidant Power (FRAP) assay. FRAP values were obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration and represented as Trolox equivalent. Both ethanolic and aqueous extracts showed strong antioxidant activity in dose dependent manner as shown in **Figure 4**. However, the reducing power of the plant ethanolic extract was not significantly higher from the aqueous extract. The result contrast with cancer toxicity. This finding strongly supports that antioxidant activity is not solely act for cancer cytotoxic and this evidence also implies that the extracts may not play the major role as the potential compound for *in vitro* activity such as immune cell proliferation activity and cytotoxic activity [11], and anticancer activity as well [17]. The chemical constituents possessing less polarity or no polarity do not always counterpart with strong antioxidant property. This phenomenon supports previous report which did not show any relation or association between antioxidant level and cytotoxicity of various cancer cell lines [18].

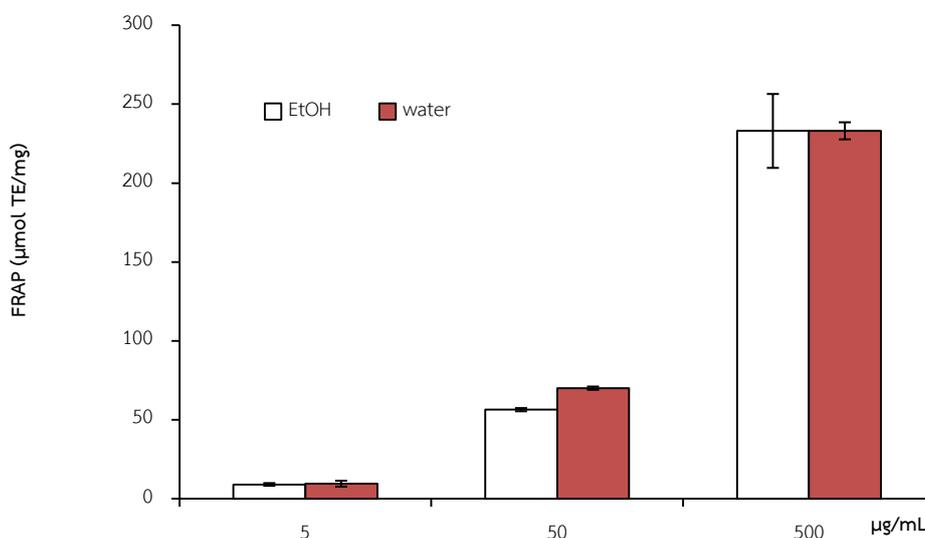


Figure 4 Reducing power activity study of *Lindernia crustacea* (L.) F. Muell. var. crustacean (YKH); ethanolic (□) and aqueous (■) extracts by ferric reducing antioxidant power (FRAP) assay determination. The experiments were done in triplicates.

Alkylation activity of the extract

Alkylation is a mechanism of anticancer activity indicating the reaction of the test compound with the nucleophilic site of DNA base. As a consequence, the DNA strand will be fragmented. In this study, the reagent 4-(4'-nitrobenzyl) pyridine (NBP) was used as DNA model generating nucleophilicity. NBP produces a violet color after undergoing alkylation. The change of absorbance in the presence and the absence of the test compound were measured by using UV-VIS spectrophotometer at 600 nm at various time points. Results showed that both ethanolic and aqueous plant extracts did not have any alkylation activity when compared to melphalan (positive control) as shown in **Figure 5**. This evidence suggests that the potential chemical constituents which exert *in vitro* activity such as immune cell proliferation, cytotoxic activity, and also anticancer activity with non-alkylation mechanism is distinct from the alkylating drug melphalan [19,20]. Melphalan inhibits DNA and RNA synthesis *via* formation of interstrand cross-links with DNA, likely binding at the N position of guanine [21], cell cycle phase-nonspecific, and also has immunosuppressive properties [22].

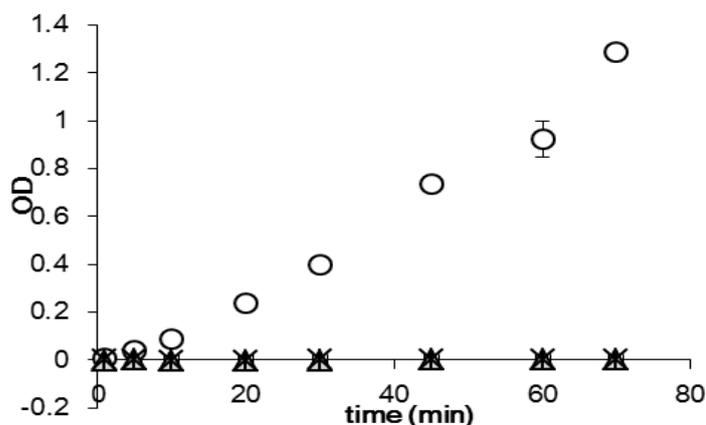


Figure 5 Alkylation activity study of *Lindernia crustacea* (L.) F. Muell. var. crustacean (YKH); ethanolic extract (Δ) and aqueous extract (*) as compared to melphalan positive control (\circ). The experiments were done in triplicates.

Important of Non-polar Chemical constituents in Anticancer activity

Although, both YKH ethanolic extract and aqueous extract showed strong antioxidant activity in dose dependent manner but the antioxidant activity did not show any correlation with HPLC chromatogram as shown in **Figure 2**. But this study found very impressive result that YKH ethanolic extract showed potential cytotoxicity against Jurkat leukemic cancer cell, and moderate cytotoxicity in HepG2 liver cancer and HCT116 colon cancer cell lines with high selectivity. These evidences indicated the following conclusion: 1) The non-polar property of YKH are highly associated to cytotoxicity than the antioxidant property, 2) Jurkat leukemic cancer cell, HepG2 liver cancer and HCT116 colon cancer cell lines possibly have similar survival pathways but different of YKH response level, 3) SK-LU1 lung cancer cell line survival pathway is different from other 3 cancer cell lines or low of YKH response level. 4) Non-polar property of YKH are also linked to normal white blood cell, the evidence support for Jurkat leukemic cancer cell is the most sensitive to YKH cytotoxicity. Siriamornpun *et al.* [18] studied bioactive compounds in various jujube fruits extract. The data showed antioxidant activity was not correlated with most of cancer cell line cytotoxicity. However, only Jurkat leukemic cancer cell responded with some jujube extracts. The evidence supported with the Taechakulwanijya *et al.* [23] found jujube seed ethanolic extract active cytotoxic on Jurkat leukemic cancer cell line, but inactive for water extract. The cytotoxic activity was specified apoptosis induction [23]. The polarity property of plants resulted to affect cancer

cell cytotoxicity in specified manner via apoptosis induction in many cell types were reported such as HepG2 liver cancer cell lines [8,9,16,17,24,25] and HCT116 colon cancer cell lines [17]

In summary, the public has been misinformed about the *Lindernia crustacea* (L.) F. Muell. var. crustacea (YKH) as “Ya Yad Nam Kang” by local Thai people in recent times. We found this neglected plant offers very interesting potentials with several *in vitro* activities demonstrated such as strong antioxidant property, immune cell proliferation inhibition and potent cytotoxic activity which has been reported for first time in this study. The ethanolic extract demonstrated potential as a source of anticancer agent with potentially cytotoxicity and high selectivity against Jurkat leukemic cancer cell line and moderate cytotoxic in HepG2 liver cancer and HCT116 colon cancer while the aqueous extract did not. The extracts having less polar or non-polar chemical constituent which play a major role for anticancer activity still need more specific elucidation. The potential chemical constituent(s) that play a major role for *in vitro* anticancer activity seem to demonstrate less polar properties *via* non-alkylating mechanism.

Conclusions

There has been no information available about anticancer activity of YKH. Our study able to provide the first time informations on biological activities such as immune cell proliferating activity, reducing power activity, alkylation activity, *in vitro* anticancer activity in various cell lines (4 types of cell lines). An unexpected but critical outcome of our studies was the finding that anticancer activity is promised by selecting less polar solvents for plant extraction. The candidate chemical constituent(s) with less polarity or non-polarity, which play a major role for anticancer activity, *in vitro* screening of biological activities of the extract in several aspects such as cancer cell lines cytotoxic activity, immune cell proliferation activity, reducing power activity, non-active alkylation activity was purposed. Therefore, the traditional way of decoction of the plant might not yield sufficient bioactive compounds. Since this study can only provide some basic information, detailed experiments should be conducted in future in order to confirm the anticancer potential of this plant.

Acknowledgements

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