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ANTI-PROLIFERATIVE AND ANTIOXIDATIVE ACTIVITIES OF THAI NONI/ YOR (*MORINDA CITRIFOLIA* LINN.) LEAF EXTRACT

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Abstract. In this study the leaves of the Thai noni/Yor, (*Morinda citrifolia* Linn.) were extracted by several methods and evaluated against human cancer cell lines: KB (human epidermoid carcinoma), HeLa (human cervical carcinoma), MCF-7 (human breast carcinoma) and $HepG_2$ (human hepatocellular carcinoma) cell lines as well as a Vero (African green monkey kidney) cell line, employing the MTT colorimetric method, comparing it to damnacanthal, rutin, and scopoletin. The dichloromethane extract of the fresh leaf showed a better inhibitory effect against KB and HeLa cells with IC_{50} values of 21.67 and 68.50 µg/ml, respectively. The dichloromethane extract of dried leaves revealed cytotoxicity against the KB cell line with an IC_{50} value of 39.00 µg/ml. Other extracts, as well as rutin and scopoletin, showed reduced anti-proliferative effects on all cancer cell lines (IC_{50} 103 to over 600 µg/ml). Interestingly, the damnacanthal had potent cytotoxicity against all cancer cell lines and Vero cell lines. These results suggest Thai noni extracts may be safer than the pure compounds, due to their higher safety ratios, which is a good indicator for possible cancer treatment. Several non-aqueous extracts from the leaves showed antioxidant properties, giving IC_{50} values of 0.20-0.35 mg/ml. It can be concluded the leaves of *M. citrifolia* may have benefit as a food supplement for chemoprevention against epidermoid and cervical cancers.

Key words: *Morinda citrifolia* Linn., Thai noni, leaf extract, anti-proliferative effects, anti-oxidative activity, cancer cell lines

INTRODUCTION

Thai people have been using Thai noni or *Yor Ban* (*M. citrifolia* Linn.) for up to 10,000 years or more (Spriggs and Anderson, 1993; Anderson *et al*, 2006). Thai noni/ *Yor* grows naturally and can be found everywhere in Thailand. Thai people use the leaves for daily cooking in a recipe called "*Hor Mhok Bai Yor*" and "*Bai Yor Rice*". The fruits are used to prepare "*Tum Yor*", which is similar to "*Som Tum*" or papaya salad. It has been used in Thai traditional medicines for over thousand years, either alone or in combination with other products (Phadungcharoen, 1981; Bunyapraphatsara and Chokechaicharoenporn, 2000).

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Noni (*M. citrifolia* L.) is a tree in the family Rubiaceae. Parts of the plant have been traditionally used for treatment of various complaints, including use for analgesia (Younos *et al*, 1990), antibacterial effects (Jayaraman *et al*, 2008), anti-inflamatory effects (Jensen *et al*, 2001; McClatchey, 2002; Wang *et al*, 2002), anticancer effects (Hirazumi *et al*, 1996), antifungal effects (Wang *et al*, 2002), as an antidiabetic drug (Jensen *et al*, 2005), for cancer chemoprevention (Tepsuwan and Kusamran, 1977; Wang and Su, 2001) and for immune stimulation (Hirasumi and Furusawa, 1999; Pansuebchue *et al*, 2002; Pattanapanyasat *et al*, 2003). Noni fruit juice has emerged on the USA market as a safe and popular food supplement during the past decade, having been supported by research in the USA (McClatchey, 2002; Wang *et al*, 2002; Walker, 2003). The leaves were investigated and reported to have several polyphenolic compounds, including ursolic acid, quercetin, kaempferol and rutin (Sang *et al*, 2001, 2003; Takashima *et al*, 2007). An ethanolic extract of noni leaves posses wound healing activity (Shivananda *et al*, 2007) and has been shown to be safe in acute, sub-acute and sub-chronic oral toxicity tests on mice (West *et al*, 2007). Rutin has been reported to have antioxidant, anti-diabetic, anti-inflammatory and anticancer activity (Sang *et al*, 2003; Marzouk *et al*, 2007). Rutin appears to be a marker of the antiproliferative and antioxidant properties of noni. If these activities of Thai noni/Yor leaf extract were established then the leaf extract would have potential as a food supplement or as chemoprevention.

MATERIALS AND METHODS

Plant materials

The leaves of Thai noni were collected from Amphoe Ban Rai, Uthai Thani Province, Thailand in 2007. The plant was authenticated by Assoc Prof Omboon Vallisuta, Head of Pharmacognosy Department, Faculty of Pharmacy, Mahidol University, where the herbarium specimen No. WT01.2007 was deposited.

Chemicals

Authentic Damnacanthal, a reference compound isolated from the root of *M. citrifolia*, was provided by Assoc Prof Dr Pluemchitt Rojanaphan, Pharmacy Department, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. Rutin hydrate, scopoletin, gallic acid, 3, [4, 5-dimethylthiazol-2yl]-2,5-diphenyl) tetrazolium bromide (MTT) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich Chemicals (St Louis, MO). All solvents were analytical grade, obtained from Lab Scan Asia (Bangkok, Thailand). De-ionized water was used in all preparations.

Preparation of crude extracts from fresh leaves

Freeze-dried juice and ethanolic extracts of fresh leaves. Fresh Thai noni leaves (1 kg) were crushed in a juice extractor filtered and lyophilized, to give a freeze-dried juice extract of fresh leaves (3.72%, F1). The crushed leaves were then macerated with ethanol at room temperature for 72 hours. The filtrate was evaporated to dryness by a rotary evaporator to give an ethanolic extract of fresh leaves (2.6%, F2).

Aqueous extract of fresh leaves. Fresh Thai noni leaves (500 g) were chopped into small pieces, boiled in distilled water (1:3, w/v) at 100ºC for 2 hours. The aqueous extract was filtered, concentrated by a rotary evaporator and then lyophilized until dryness, to give an aqueous extract of fresh leaves (7.19%, F3).

Ethanolic extract of fresh leaves. Fresh Thai noni leaves (500 g) were chopped into small pieces and blended with 95% ethanol (500 ml) in a blender for 5 minutes and macerated for 72 hours. The filtrate was evaporated to dryness in a rotary evaporator, to give an ethanolic extract of fresh leaves (8.37%, F4).

Dichloromethane and methanolic extracts of fresh leaves. Fresh Thai noni leaves (500 g) were chopped and minced in a blender and then macerated with dichloromethane (CH_2Cl_2) at room temperature for 72 hours. The extract was filtered and evaporated to dryness in a rotary evaporator, yielded a dichloromethane extract of fresh leaf (1.57%, F5). The marc was then macerated with methanol (MeOH) in the same condition. The filtrate was then evaporated to dryness, yielded a methanolic extract of fresh leaf (4.22%, F6).

Preparation of crude extract from dried leaves

Fresh Thai noni leaves (1 kg) were chopped into pieces and dried at room temperature for 24 hours. The air dried leaves were kept at 50-55ºC in a hot air oven for 24 hours and then ground into powder. The dried, powdered leaves (150 g) were extensively extracted with a Soxhlet apparatus using dichloromethane (1:50, w/v) for 48 hours. The filtrate was concentrated to dryness under reduced pressure. This yielded a dichloromethane extract of dried leaves (0.79%, D1). This was then extracted with methanol, concentrated to dryness, which yielded a methanolic extract of dried leaves (1.46%, D2).

Cell culture

Four human cancer cells, KB (human epidermoid carcinoma); HeLa (human cervical carcinoma); MCF-7 (human breast adenocarcinoma), HepG2 (human hepatocellular carcinoma) and non-tumorigenic Vero cells (African green monkey kidney), were cultured on Modified Eagle Medium (MEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin and 100 µg/ml of streptomycin sulfate. The cells were maintained at sub-confluence in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC.

Cell proliferation assay

Inhibition of cell proliferation by Thai noni leaf extracts was determined by a MTT (3, [4, 5-dimethylthiazol-2yl]-2, 5-diphenyl) tetrazolium bromide colorimetric assay. Briefly, four human cancers (KB, HeLa, MCF-7 and HepG2) and non-tumorigenic Vero cells, were placed in a 96-well culture plate (Costar, Cambridge, MA) at a density of $3x10^3$ cells/well in 200 µl of serum containing media. After 24 hours pre-incubation, the cells were treated with various concentrations of Thai noni leaf extracts (0-1,000 µg/ml) for 48 hours. The medium and 0.1% DMSO were used as negative controls. At the end of incubation, 20 µl MTT solution [(3, [4, 5-dimethylthiazol-2yl]-2, 5-diphenyl) tetrazolium bromide, 5 mg/ml in PBS] was added to each well and further incubated at 37ºC for 3 hours. After centrifugation at 1,400 rpm for 5 minutes at 4ºC, the medium was aspirated and the formozan product was dissolved in 100 µl DMSO in each well. The absorbance was measured using a Microplate reader (Benchmark 550, Bio-Rad, USA) at 550 nm wavelength. Each concentration of drugs was repeated in six wells for three independent experiments. The 50% inhibition concentration (IC_{50} value) was determined by plotting the percentage of cell viability versus the drug concentration.

Antioxidant activity measured by a DPPH free radical scavenging assay

A DPPH (1, 1-diphenyl-2-picryl-hydrazyl) radical scavenging assay was

Table 1
Antiproliferative activity of Thai noni leaf extracts against human cancer cells and non-tumorigenic Vero cells.

Thai noni/*Yor* leaf extracts	Antiproliferative activity (IC_{50} values, μg/ml)				
	KB	HeLa	MCF-7	HepG2	Vero
Fresh leaf extracts					
F1 Freeze-dried juice extract	169.17±13.20	310.00±7.07	>600	496.67±64.70	318.00±8.37
F2 Ethanolic extract	313.75±7.50	390.00±9.35	>600	>600	467.50±23.40
F3 Aqueous extract	>300	>600	>600	>600	>600
F4 Ethanolic macerated extract	270.00±6.12	230.83±15.94	>600	>600	248.00±2.74
F5 Dichlorometane macerated extract	21.67±1.54	68.50±2.43	266.67±7.53	190.00±13.78	187.50±4.18
F6 Methanolic macerated extract	103.83±16.61	188.00±2.47	296.00±21.33	240.00±10.49	174.17±5.85
Dried leaf extracts					
D1 Dichloromethane extract	39.00±6.67	207.50±10.84	455.00±11.18	205.00±23.87	324.00±23.82
D2 Methanolic extract	186.25±4.79	270.83±10.68	245.00±8.37	256.00±23.82	202.50±2.74
Reference compounds					
Rutin	167.00±6.71	265.00±13.69	>600	395.00±59.69	345.83±20.60
Scopoletin	120.00±6.12	179.00±2.24	416.00±13.42	362.50±15.08	202.50±5.24
Damnacanthal	6.50±0.5	22.00±3.52	17.17±0.26	23.63±1.60	17.20±2.39

KB, human epidermoid carcinoma; HeLa, human cervical carcinoma; MCF-7, human breast carcinoma; HepG2, human hepatocellular carcinoma; Vero, African green monkey of kidney.

performed for the antioxidant activity. Briefly, various concentrations of the Thai noni leaf extract were dissolved in DMSO, and added to 0.15 mM DPPH in ethanol. The reaction mixture was shaken vigorously and left at room temperature for 30 minutes in the dark. DMSO 0.1 % was used as a negative control and gallic acid as a positive control. Absorbance was measured at 515 nm using an ultraviolet spectroscope (Bio-Rad, USA). The percent scavenging activity was determined by comparison with the DMSO negative control. Concentrations that produced 50% radical scavenging (IC_{50}) were extrapolated from plots of % residual radicals and concentrations.

Statistical analysis

Data were expressed as means ± SD Significant differences ($p<0.05$) between means of controls and treated cells were analyzed by ANOVA analysis.

RESULTS

Thai noni leaf extracts

The maceration of fresh Thai noni leaves with ethanol (F4) gave the highest yield (8.37%w/w), whereas extraction of the dried leaf with dichloromethane (D1) gave the lowest yield (0.79%w/w). Rutin was present in fresh and dried extracts, but not the dichloromethane extracts.

Antiproliferative activity of leaf extracts against four human cancer cells and non-tumorigenic Vero cells

The inhibitory effects of *M. citrifolia* leaf extracts and the three reference compounds on four human cancer cells KB

Table 2
Antioxidative activity of Thai noni/*Yor* (*M. citrifolia* Linn.) leaf extracts.

Thai noni/*Yor* leaf extracts	DPPH radical scavenging activity(IC_{50}, mg/ml)
Fresh leaf extracts	
F1 Freeze-dried juice extract	0.35
F2 Ethanolic extract	0.35
F3 Aqueous extract	>0.60
F4 Ethanolic macerated extract	0.31
F5 Dichlorometane macerated extract	0.30
F6 Methanolic macerated extract	0.28
Dried leaf extracts	
D1 Dichloromethane extract	0.20
D2 Methanolic extract	0.26
Standard compounds	
Rutin	0.01
Scopoletin	0.25
Gallic acid	0.002

Table 3
Safety ratios calculated from the ratio of IC_{50} values between Vero cells and other cancer cell lines for each extract and standards.

Test sample	Ratio of IC_{50} values			
	V/KB	V/HeLa	V/MCF-7	V/HepG2
F1	1.88	1.00	0.53	0.64
F2	1.49	1.20	0.78	0.78
F3	2.00	1.00	1.00	1.00
F4	0.92	1.07	0.41	0.41
F5	8.65	2.73	0.70	0.98
F6	1.68	0.93	0.59	0.72
D1	8.31	1.56	0.71	1.58
D2	1.09	0.75	0.83	0.79
Damnacanthal	2.65	0.78	1.00	0.73
Rutin	2.07	1.30	0.58	0.89
Scopoletin	1.69	1.13	0.49	0.56

(human epidermoid carcinoma), HeLa (human cervical carcinoma), MCF-7 (human breast carcinoma) and HepG2 (human hepatocellular carcinoma)) and the non-tumorigenic Vero (African green monkey of kidney) cells were measured using a MTT colorimetric assay. As shown in Table 1, the dichloromethane extracts from fresh leaves (F5) and methanolic extract from dried leaves (D2) had the greatest antiproliferative effect on KB cells with IC_{50} values of 21.67±1.54 and 39.00±6.67 µg/ml, respectively. Moreover, the dichloromethane extract from fresh leaves

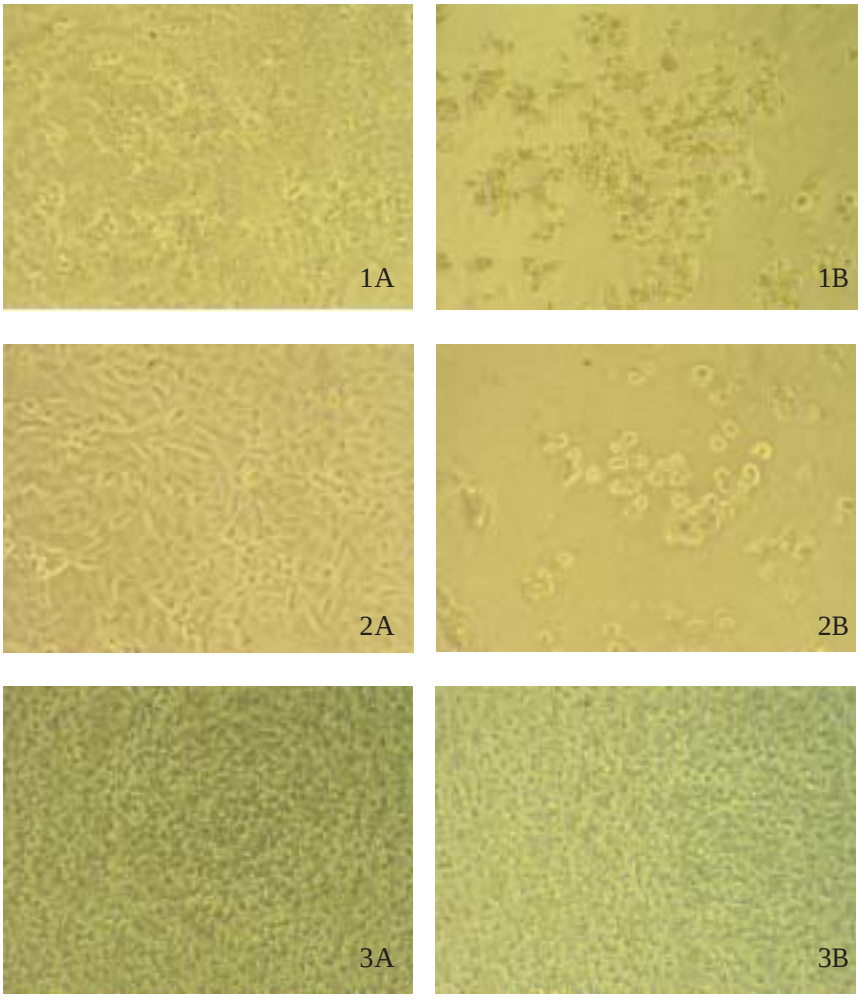

Fig 1–Antiproliferative effects of dichloromethane extract from Thai noni/*Yor* fresh leaves (F5).
(1) Against KB (human nasopharyngeal carcinoma)
(2) Against HeLa (human cervical carcinoma)
(3) Against Vero (African green monkey kidney) cell lines
A: Treated with DMSO as a control
B: Treated with 10 mg/ml of Thai noni/*Yor* fresh leaf dichloromethane macerated extract (F5)

(F5) had moderate activity against HeLa cells with an IC_{50} value of 68.50±2.43 µg/ml, and the methanolic extract (F6) had weak activity against KB cells with an IC_{50} value of 103.83±16.61 µg/ml. None of the extracts had inhibitory effects on MCF-7, HepG2 or non-tumorigenic Vero cells. Among the reference compounds, damnacanthal had the most potent antiproliferative effect on KB cells with an IC_{50} value of 6.50±0.5 µg/ml, with slightly activity against HeLa, MCF-7, HepG2 and Vero cells with IC_{50} values of 22.00±3.52, 17.17±0.26, 23.63±1.60 and 17.20±2.39 µg/ml, respectively. Rutin hydrate and scopoletin had a slight inhibitory effect on all tested cells

(IC_{50} values >100 µg/ml).

Antioxidant activity of leaf extracts

The antioxidant activity of all Thai noni leaf extracts are summarized in Table 2. Dichloromethane extract from dried leaves (D1) was found to be the most potent antioxidant (IC_{50} value of 0.20 mg/ml), followed by the methanolic extracts of dried (D2) and fresh (F6) leaves, with IC_{50} values of 0.26 and 0.28 mg/ml, respectively. The aqueous extract from fresh leaves (F3) was less active (IC_{50} value > 0.60 mg/ml). Rutin had potent antioxidative activity at an IC_{50} value of 0.01 mg/ml, but was less active than gallic acid (IC_{50} value of 0.002 mg/ml) and more active than scopoletin (IC_{50} value of 0.25 mg/ml).

DISCUSSION

There was not much difference in the antioxidant activities among the various non-aqueous extracts of Thai noni leaves (IC_{50} value 0.20-0.35 mg/ml), however, the aqueous extract was very much less active (IC_{50} value > 0.6 mg/ml). This finding supports the retention of antioxidant properties in Thai noni leaf-containing food with coconut milk.

The extracts showed different antiproliferative properties against the 4 types of cancer cell lines. Only the dichloromethane extract of the fresh leaves (F5) and the methanolic extract of the dried leaves (D2) showed selective antiproliferative activity against the epidermoid cancer cell line. These two extracts had a higher safety ratio than damnacanthal (Fig 1, Table 3) which is a good indication for use as a cancer treatment *ie*, the extracts inhibit the growth of cancer cells but not normal cells.

This is the first time such antiproliferative activities of Thai noni leaf extracts have been reported. It also suggests the benefit of these extracts against human nasopharyngeal carcinoma and human cervical carcinoma. The leaf extract may have potential as a functional food for chemoprevention against epidermoid and cervical cancers. The results of this study support further development of Thai noni leaves as a nutraceutical or drug.

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