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Der Pharmacia Lettre, 2016, 8 (6):238-244
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Phytochemical study of *Ruellia tuberosa* chloroform extract: antioxidant and anticholinesterase activities

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ABSTRACT

Ruellia tuberosa is a tropical plant in Acanthaceae family that has been used as folk medicine and widely distributed in South East Asia. This study was conducted to determine phytochemical content, antioxidant and anticholinesterase activity of chloroform extracts including leaves, stem and root of R. tuberosa, collected from Chiang Mai, Thailand. Total phenolic, flavonoid, tannin and alkaloid contents were determined for all of the extracts.1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, nitric oxide scavenging assay, reducing power and ferric reducing antioxidant power (FRAP) were used as an antioxidant models. Anticholinesterase activity was determined using Ellman's assay. The results showed that R. tuberosa leaves extract displayed the highest phenolic, flavonoid, tannin and alkaloid contents with 0.16±0.01 µg gallic acid equivalent/g extract, 1.55±0.13 µg quercetin equivalent/g extract, 0.20±0.04 µg tannic acid equivalent/g extract and 1.62±0.01 µg strychnine equivalent/g extract, respectively. Leave extract also had the highest potential to scavenge DPPH and nitric oxide radical while stem and root extract could inhibit both acetylcholinesterase and butyrylcholinesterase at concentration 200 µg/ml with low free radicals scavenging.

Keywords: *Ruellia tuberosa*, phytochemistry, antioxidants, anticholinesterase, free radical scavenging

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INTRODUCTION

Imbalance between free radicals and antioxidants leading to oxidative stress which are caused of non-communicate diseases including cancer, cardiovascular diseases, diabetes and Alzheimer's disease[1,2]. Alzheimer's disease (AD) is a chronic disease and neurodegradative disorder of the brain leading to change in cognition and behavior in elder population. Higher levels of protein oxidation and lipid peroxidation could found in AD patients because the brain contains high unsaturated fatty acid and ferrous ion levels which important keys catalyst of brain oxidative stress[3,4]. Many reports indicated that phytochemicals containing in dietary and herbal medicine can scavenge excess free radicals in cells. Synthetic substances especially, butylated hydroxytoluene and butylated hydroxyanisole, have been used as an antioxidant substances in food industry but they can induce toxic response to the liver and carcinogenesis. Natural antioxidants has been interested due to their safety and cheaper cost than synthetic antioxidants[5]. 40-80% of population in each countries have been used traditional medicine especially, herbal medicine and their active compounds for their primary health care[6].

Ruellia tuberosa, a tropical plantin Acanthaceae family, is widely distributed in South East Asia and has been used as folk medicine. In traditional medicine, it has been used as anti-diabetic, antipyretic, analgesic, antihypertensive, anticancer, and antidotal toxic agents[7-9]. Previous studies showed that this plant contained steroids, terpenoids,

long-chain aliphatic compounds and flavonoids [10-12]. Moreover, the aerial part extracts showed antioxidant and anti-inflammatory activities. In Northern Thailand, this plant has been used for anti-inflammation, detoxification of poisons and prevent diseases, but no evidences to support their properties. The objective of this study was to determine phytochemical contents, antioxidant and anticholinesterase activities of *R. tuberosa*.

MATERIALS AND METHODS

Chemicals

DPPH (2, 2- diphenyl-1-picrylhydrazyl), quercetin, Folin-ciocealtue reagent 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 2, 4, 6-tris (1-pyridyl)-5-triazine (TPTZ), acetylcholine iodide were purchased from Sigma- Aldrich (St. Louis, MO,USA).

Plant material

R. tuberosa was collected from Hang Dong district, Chiang Mai province, Thailand. The plant was authenticated by Mr. J. F Maxwell, Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University, Thailand. A voucher number is 023195.

Preparation of plant extract

Dried materials including leaves, stem and root of the plant materials were cut into small pieces and ground to powder. The powder was extracted 3 times with chloroform, then filtrated with What man No.1 and the chloroform extract was evaporated under vacuum to obtain crude extract.

Determination of phytochemical contents

Total phenolic content

Total phenolic content was determined using Folin-Ciocalteu reagent according to Kusirisin method[13]. Briefly, 0.5 ml of plant extract and 4 ml of 1 M sodium carbonate was added in 5 ml 10%Folin-Ciocalteu reagent. The mixture was allowed to stand for 15 min in the dark and the absorbance was measured at 765 nm. The total phenolic content was calculated and expressed as µg of gallic acid equivalent per g extract.

Total flavonoid content

Total flavonoid content was measured following the method of Kusirisin et al[13]. Briefly, the mixture contained with 1.5 ml of plant extract and 2.8 ml of distilled water in tubes. Then, 0.1 ml of 10% aluminium chloride and 1 M potassium acetate were added. The mixture was stood at room temperature in the dark for 30 min. The absorbance was measured at 415 nm and compared with a standard curve of quercetin solution. The result was expressed as µg of quercetin equivalent per g extract.

Total tannin contents

Total tannin content was estimated using spectrophotometer with modified the method of Kusirisin *et al* [13]. Briefly, 0.5 ml of each the extracts were mixed with 5 ml 10% Folin-Ciocalteu reagent and 4 ml of 0.075% sodium carbonate. The reaction mixture was allowed to stand for 30 min at 40°C. Subsequently, absorbance was measured at 765 nm and total tannin was expressed as tannic acid equivalent.

Total alkaloid content

Total alkaloid content was determined by modified the method described by Kasempitakpong, *et al*[4]. Briefly, 1 ml of the plant extract solution was transferred to separatory funnel. Then, 5 ml of phosphate buffer pH 4.7 and 5 ml of bromocresol green solution were added. The absorbance of the complex in chloroform layer was measured at 470 nm. The result was calculated and expressed as µg of strychnine equivalent per gram extract.

DPPH radical scavenging assay

DPPH radical scavenging assay was determined according to the method of Jaikang*et al*[14]. Briefly, various concentrations of the extracts were added into 2.7 ml of 0.004% DPPH solution in methanol. The reaction mixture was incubated in the dark for 30 min. The absorbance was measured at 515 nm and used quercetin as a positive control. The results were calculated and expressed in percentage of inhibition.

Nitric oxide scavenging assay

Nitric oxide scavenging assay was measured by spectrophotometer which described by Kumaran *et al* [15]. Briefly, various concentrations of extracts were mixed with 10 mM sodium nitroprusside and phosphate buffer saline pH 7.4 to make volume up to 3 ml. The solutions were incubated at 25ºC for 150 min. 2 ml of Griess reagent (1% sulfanilic acid and 0.1% naphthylethylenediamine dichloride in 2.5% phosphoric acid) was added into 0.5 ml of the solutions

and stood at 25ºC for 30 min. The absorbance was measured at 540 nm and used quercetin as a positive control. The results were calculated and expressed in percentage of inhibition.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was measured according to the method of Benzie and Strain [16]. Briefly, FRAP reagent was contained with 300 mM acetate buffer pH 3.6, 10 mM 2, 4,6-tripyridyl-S-triazine (TPTZ) solution in 40 mMHCl and 20 mMferric chloride. The plant extracts were mixed with 3 ml of the FRAP reagent and stood at 37°C for 10 min. The absorbance of reaction mixture was measured at 593 nm and quercetin was used as positive control.

Reducing power assay

The reducing power was determined by the method of Oyaizu [17]. Different concentrations of the extracts were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 1 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Then, 1.5 ml of 10% trichloroacetic acid was added and followed by centrifugation at 3000 rpm for 10 min. 1 ml of the upper solution was mixed into 1 ml of distilled water and 0.5 ml of 0.1% ferric chloride. The absorbance of the mixture was measured at 700 nm and quercetin was used as positive control.

Acetylcholinesterasse Inhibition

The acetylcholinesterase activity was measured using human serum and human red blood cell membrane as a source of acetylcholinesterase. The protocol was approved according to the Ethical Committee for Human Research, Faculty of Medicine, Chiang Mai University (Research ID: 1032/Study code No. FAM-12-1032-EX). Protocol was modified Ellman's reaction [18]. In this study, chloroform extracts of different parts of *R. tuberosa* were examined for their effects on the AChE activity. The assay mixture was contained 0.2 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in 50 mM phosphate buffer pH 7.4 and 20 µl of human serum or RBCs's membrane which prepared as previous method of Srivastava N*et al*[19]. 100 µl of acetylthiocholine iodide (ATI) was added into the mixture and mixed rapidly. The changing of absorbance at 405 nm was recorded 30 sec interval for 2 min. The activity was calculated follow as equation: AChE (U/L) = $\Delta A \times 23{,}400$

The reaction mixture containing all the components except the plantextracts were used as a control.

Statistical Analysis

All experiments were studied in triplicates and data were expressed as mean ± standard deviation (SD). The half maximal inhibitory concentration (IC_{50}) value was calculated using GrapPad Prism 5program.Analysis of Variance (ANOVA) following with multiple comparison by Tukey's test were performed to analyze the different between groups. A *p*-value less than 0.05 ($p<0.05$) was regarded as significance.

RESULTS AND DISCUSSION

Extraction and phytochemical contents

Air dried and ground plant material of different part was extracted at room temperature with chloroform. After removing of the solvent, leaves of *R. tuberosa* gave the highest yield extract with 0.52% mg/g dried weight and part of stem and root were found to be 0.24 and 0.22% given, respectively. Chen and his colleagues extracted the stem of *R. tuberosa* from Taiwan with chloroform giving 0.3% of yield extract[20] and Rajan and group workers extracted leaves of *R. tuberosa* collected from Tamil Nadu, India gave1.11% yield extract[21].

The chloroform extracts were determined phytochemical contents including total phenolic, total flavonoid, total tannin and total alkaloids. The results of phytochemical contents are shown in Table 1.The leaves fraction was displayed the highest total phenolic, total flavonoid, total tannin and total alkaloid contents. There are several studied on dietary polyphenol, especially phenolic, flavonoid, tannin and alkaloids which have been used to decrease the diseases caused from free radicals. *R. tuberosa* was composed of flavonoid, steroid, triterpenoid and alkaloid. The distribution of phytochemicals in solvents show that terpenoid and flavonoids are mainly found in chloroform extracts[22]. Cirsimaritin, cirsimarin, cirsiliol 4'-glycoside, sorbifolin and pedalitin are flavonoids that found in *R. tuberosa* extract[23].

Phytochemical substances in chloroform extract contained carbohydrate, glycoside, flavonoid, steroids, tannin and phenolics ,but alkaloid, amino acid but not saponin[21]. Difference of phytochemical contents may depended on weather, season [24]or location[25].

Table 1: Bioactive compounds of chloroform extract of *R. tuberosa*

Parameters	Part		
	Leaves	Stem	Root
Yield extraction (%mg/g DW)	0.52	0.24	0.22
Total phenolic (µg GAE/g)	0.16±0.01	0.03±0.03	0.09±0.01
Total flavonoid (µg QE/g)	1.55±0.13	0.29±0.04	0.21±0.03
Total tannin (µg TE/g)	0.20±0.04	0.08±0.01	0.10±0.01
Total alkaloid (µg SE/g)	1.62±0.01	0.18±0.01	0.04±0.01

Data are presented as the mean ± standard deviation, (n = 3)

DW = dry weight, GAE = Gallic acid equivalent, QE = Quercetin equivalent, TE = Tannic acid equivalent, SE = Strychnine equivalent

Free radical scavenging activity

The antioxidant activity of various part of *R. tuberosa* extracts were evaluated in vitro model. Each models based on one feature of antioxidant activity. The scavenging activity of *R. tuberosa* extracts were dose-response manner and the results are showed in Fig.1. The IC_{50} values were calculated using linear equation. Quercetin was used as a positive control for free radical scavenging due to quercetin can be found in several plants and had IC_{50} value 14.10 µg/ ml. The leaves extract was the highest DPPH radical scavenging activity with IC_{50} value was 4.71 mg/ml and root and stem extract were 10.06 and 21.24 mg/ml, respectively.

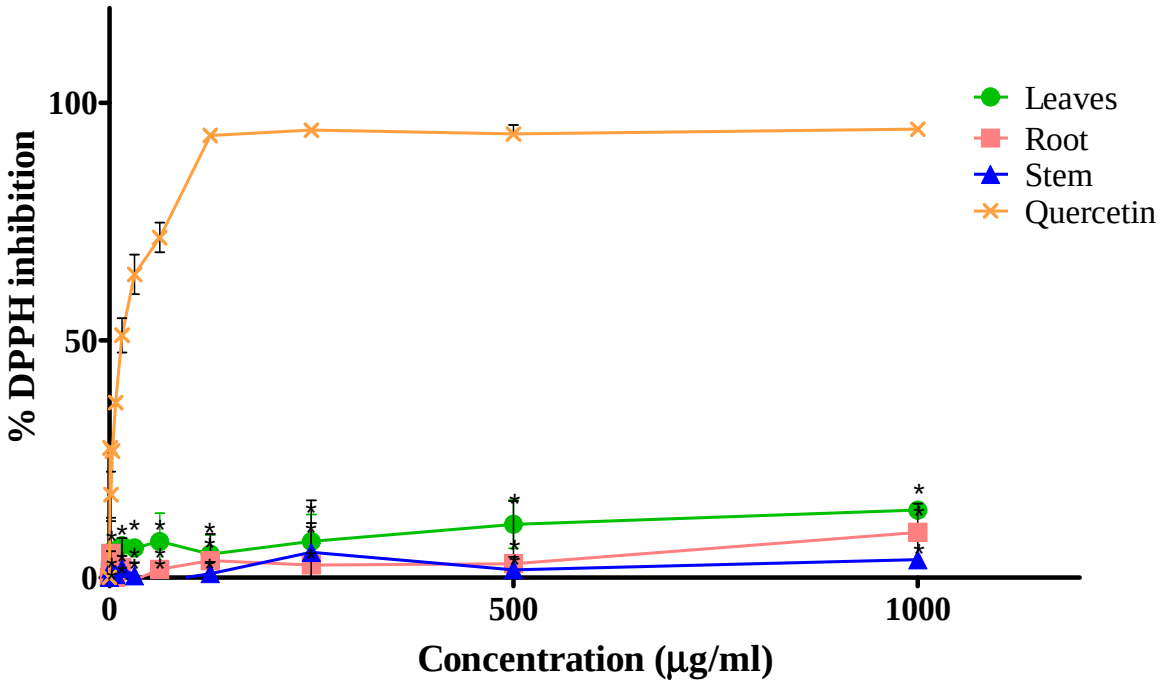

Figure 1: DPPH radical-scavenging activity of chloroform extracts of *R. tuberosa*

Nitric oxide is an important chemical involved in the regulation of various physiological processes and biological systems including immunological, neuronal and cardiovascular systems. Increasing of NO level is related to several diseases. All the extracts of *R. tuberosa* were low potency to scavenge nitric oxide (data not shown).The leaves extract at concentration 1 mg/ ml was presented percent NO scavenging with value 36.08±0.33%, the stem extract was scavenge 12.77±2.37% while, the root extract was no potency to scavenge NO at the same concentration. Quercetin was high potency to scavenge NO radical with value 0.94±0.02 mg/ ml.

FRAP assay is used for determination of antioxidant power to reduce ferric ion to ferrous ion. A single electron from plant extract was transferred to ferric ion. The results showed that the leaves, stem and root fractions were potential to transfer electron to ferric ion with value 11.93± 0.48, 13.60±0.66 and 12.46±0.81 µg quercetin equivalent, respectively. The results are shown in Table 2.

Table 2: FRAP (Fe^{2+} equivalent mM)of the chloroform extract of *R. tuberosa*.

Concentration (µg/mL)	Absorbance value at 593 nm			
	Leaves	Root	Stem	Quercetin
15.625	0.064±0.006[a]	0.075±0.005[a]	0.066±0.003[a]	0.432±0.081[b]
31.25	0.063±0.001[a]	0.080±0.005[a]	0.065±0.001[a]	0.690±0.142[c]
62.50	0.068±0.001[a]	0.085±0.003[a]	0.071±0.003[a]	0.858±0.062[c]
125	0.070±0.004[a]	0.089±0.016[a]	0.076±0.003[a]	1.522±0.126[d]
250	0.084±0.018[a]	0.090±0.006[a]	0.090±0.003[a]	2.689±0.279[e]

Samples represented with different small alphabets are significantly different from other samples ($p<0.05$)

Reducing power method is used for measuring the electron-donating capacity of antioxidant or plant extracts. An antioxidant reaction was conversed Fe(III)/ ferricyanide complex to Fe(II) form. The results are shown in Table 3. The chloroform extract of *R. tuberosa*had low potency to scavenge free radical or donating electron because of low flavonoid and phenolic contents[26-28].

Table 3:Reducing power of chloroform extract of *R. tuberosa*

Concentration (µg/ml)	Absorbance at 700 nm			
	Leaves	Root	Stem	Quercetin
15.625	0	0	0	0.26±0.16[c]
62.50	0	0	0	0.78±0.01[d]
250.00	0.02±0.01[a]	0.01±0.01[a]	0.02±0.01[a]	1.02±0.03[e]
1000.00	0.06±0.03[b]	0.06±0.03[b]	0.09±0.03[b]	1.15±0.04[f]

Samples represented with different small alphabets are significantly different from other samples ($p<0.05$).

Anticholinesterase activity

Acetylcholinesterase is an enzyme that hydrolyses acetylcholine in cholinergic synapses system. Inhibition both acetylcholinesterase and butyrylcholinesterase activity have been accepted as a model for treatment or management AD, senile dementia, ataxia and myasthenia gravis. Acetylcholinesterase is presented in erythrocyte membrane and cholinergic system and its function is damage neurotransmitter, acetylcholine, to choline and acetate. Butyrylcholinesterase or pseudocholinesterase is expressed in plasma and can hydrolyze toxic substances especially, organophaosphate and carbamate[29]. Decreasing of butyrylcholinesterase activity may increase adverse effects of the toxins.

The chloroform extract of the stem and root of *R. tuberosa*at concentration 200 µg/ml had ability to inhibit both AChE and BChE activities significantly when compared with control group ($p<0.05$). For, the leaves extract inhibited BChE activity but did not inhibit AChE activity and the results are shown in Fig. 2. Low polyphenol contents might cause of less potency to inhibit the enzymes. However, the low ability of butyrylcholinesterase inhibition might meant low side effects to this enzymes after consumption.

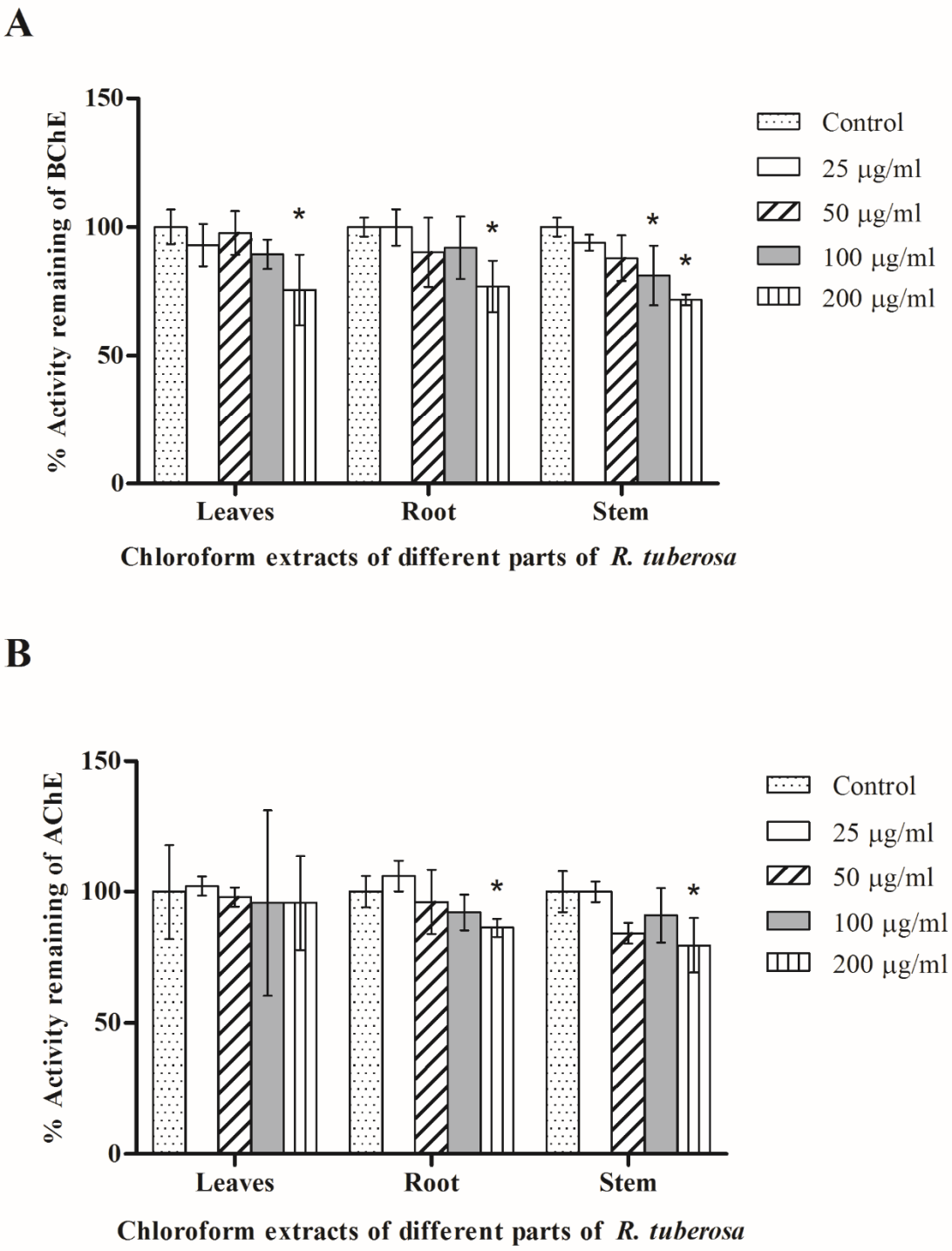

Figure 2: Acetylcholinesterase and butyrylcholinesterase activity remaining after treatment with chloroform extract of *R. tuberosa*.

CONCLUSION

Our results showed that the chloroform extract of *R. tuberosa*grown in Northern Thailand had phytochemicals contents including, phenolic, flavonoid, tannin and alkaloid contents. The chloroform extract of the stem and root could inhibit both acetylcholinesterase and butyrylcholinesterase at concentration 200 µg/ml but low activity in free radicals scavenging. Therefore, the chloroform extract may not suitable for AD drug development. Another solvent extracts of *R. tuberosa* needs to be investigated and identified phytochemical compounds.

Acknowledgements
This study was greatly acknowledge grants from Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

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