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In vitro antioxidants and anticancer activity of crude extract isolates from Euphorbiaceae in Northern Thailand

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ABSTRACT

The properties of *Croton persimilis* Müll.Arg. and *Antidesma puncticulatum* Miq.— interesting plant in the family Euphorbiaceae were investigated. The ethanol crude plant extracts were established for phytochemicals screening, antioxidants activities, anticancer in five important human cancer cell lines (MCF-7, MDA-MB-231, HeLa, HepG2, and NCI-H187), and cytotoxic activity in normal cell line (hTERT-HME1). The leaves extracts of *C. persimilis* Müll. Arg. contained flavonoids, saponins, terpenoids, and tannins. The total phenolic content properties were found in 43.40 ± 1.36 mg Gallic Acid Equivalents (GAE)/g crude extract. They also showed lowest antioxidant activity through 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay (IC_{50}>1,000 µg/ml) and 2.4.2 ferric reducing antioxidant power (FRAP) assay (369.84 ± 2.39 µM/g of crude extract). Whereas, the raw fruits of *A. puncticulatum* Miq. contained saponins and tannins. The total phenolic content properties were found in 193.47 ± 4.63 mg GAE/g crude extract. The antioxidant activity showed better IC_{50} via DPPH assay (IC_{50} = 51.50 ± 3.30 µg/ml) and FRAP assay (964.67 ± 5.88 µM/g of crude extract). The leaves extracts of *C. persimilis* Müll Arg had the greatest inhibition activity against MCF-7 and $CaCO_2$, while the raw fruits extract of *A. puncticulatum* Miq. had low growth inhibition against in all cancerous cells. Thus, both extracts are potentially sources of natural antioxidants. *C. persimilis* Müll Arg may be a potential compound in anticancer.

Keywords: Euphorbiaceae, *Croton persimilis* Müll.Arg., *Antidesma puncticulatum* Miq., Antioxidant activity, Anticancer activity

INTRODUCTION

Cancer is the world's most important cause of globally death, accounting for estimated the annual global burden is 9.6 million deaths. Lung, prostate, colorectal, stomach, and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervical, and thyroid cancer are the most common among women.[1] At present, chemotherapy, surgery, and radiotherapy are the gold standard treatment of cancer. Although these therapies have more effective to cure and control cancers, the severe side effects seriously affect the patient's tolerance to treatment. Therefore, it is a challenge to find more diverse and effective plant-derived natural are relatively low-toxic.

Medicinal plants still play an important role in the ecosystem service all over the world directly implicated in human well-being and alleviate ailments and diseases. The World Health Organization estimated that 91% of populations from South East Asia use traditional medicine as the primary prevention and alternative treatment.[2] In Thailand, medicinal plants are mainly used as a form of self-medication or prescribed by folk medicine. In addition to being used for health care, it is also used as an alternative to treating serious diseases such as cancer. A previous study was reported; various traditional medicines are taken by cancer patients that indicated an improvement in quality of their life.[3]

Euphorbiaceae family commonly known as a wide variety of chemical compounds such as terpenoids,

alkaloids and flavonoid, which are the empirical inhibiting cell proliferation,[4-7] and also inducing apoptosis and cell cycle arrest.[8,9] Previous preliminary survey in the Li River Basin, Lamphun province found the interesting plant in the family Euphorbiaceae which are *C. persimilis* Müll.Arg. and *A. puncticulatum* Miq. It is possible to have the pharmacological activity to inhibiting the growth of cancer cells according to folk medicine report[10] and several publications. The leaves extracts of *Croton macrobothrys* Baill., *Croton zambesicus* Müll. Arg., *Croton lechleri* Müll. Arg., and *Croton* matourensis Aubl. showing *in vitro* cytotoxicity against human cervix carcinoma cells and non-small cell lung cancer cells[5,11-13] and *in vivo* indicated tumor mass inhibition rates of the essential oil from the leaves of C. matourensis Aubl. (40 and 80 mg/kg/day) were 34.6–55.9%.[14] *Antidesma thwaitesianum* Müll. Arg. and *Antidesma bunius* L. extract have high levels of antioxidant activity.[15,16] and has the effect of inhibiting the growth of lung cancer cells.[16] Inhibiting the growth of MCF7 breast cancer cells at medium level.

In the present study carried out, *in vitro* screening of anticancer and cytotoxic activity of the Thai plants in Euphorbiaceae family from Northern Thailand, most importantly *C. persimilis* Müll. Arg. and *A. puncticulatum* Miq. collected from Northern Thailand. These plants are candidates for further phytochemical profiling, drug research, and development.

MATERIALS AND METHODS

Plant Material

Leaves of *C. persimilis* Müll. Arg. and *A. puncticulatum* Miq. located in Li watershed, Northern Thailand, were collected during July–August 2018 and August–September 2018, respectively. The plant material was botanically identified by Dr.Akharasit Bunsongthae, Department of Biology, Chiang Mai Rajabhat University.

Extraction Procedure

Croton persimilis Müll. Arg. extraction

Dried and powdered leaf material (120 g) was extracted by stirring with 95% ethanol (1 g: 20 ml) for 6 h, maceration at room temperature for 24 h then filtered through a paper membrane (Whatman No.1 Filter paper). Crude extract was concentrated using rotary evaporator under reduce pressure at 46°C. The resulting were weighed in vials and stored at −20°C before phytochemical analysis. The yield of fresh crude extract was 10.02% w/w (5.01 g).

A. puncticulatum Miq. extraction

Dried of raw fruit material (50 g) was grinded and extracted by stirring with 95% ethanol (1 g: 20 ml) for 6 h, maceration at room temperature for 24 h then filtered through a paper membrane (Whatman No.1 Filter paper). Crude extract was concentrated using rotary evaporator under reduce pressure at 46°C. The resulting were weighed in vials and stored at −20°C before phytochemical analysis. The yield of fresh crude extract was 8.43% w/w (10.12 g).

Phytochemical Screening

Phytochemical constituents of the crude extract was performed according to the methods of previous studies with slightly modification Ayoola *et al.* (2008).[17] General reactions used to identify the natural potential groups such as terpenoids, flavonoids, saponins, alkaloids, and tannins using different solvents. Appearance and disappearance of coloration revealed the presence or absence of such potential groups. Total phenolic content was determined by Folin-Ciocalteu method as described by Thomas *et al.* (2012).[18] Total phenolics were determined as Gallic Acid Equivalents (GAE) in mg/g of crude extract. The data were presented on average ± stander deviation (SD) for the triplicates.

Determination of Antioxidant Activity

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability assay

Free radical scavenging effect was determined using the stable scavenger DPPH with modifications of the method described by Sharma, *et al.* (2014).[19] 50 µL of crude extracts with 50–1000 µg/ml concentrations were mixed with 100 µL Working DPPH and standard ascorbic acid solution (1–100 µg/ml). The mixture was incubated for 30 min in the dark at room. Finally, the free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution at 517 nm. The ability to scavenge the DPPH radical was expressed as percentage inhibition and calculated. The data were presented on average ± SD for the triplicates.

Ferric reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions of sample was described by Sharma, *et al.* (2014)[19] with some modification. 30 µL of crude extracts with concentration 1000 µg were mixed with 270 µL of FRAP reagent. The mixture was incubated for 30 min in the dark at room. Finally, the absorbance was measured at 593 nm. Ascorbic acid was used as positive reference standard. The ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of sample. The data were presented on average ± SD for the triplicates.

Cell Culture

The cell lines breast cancer cells (MCF-7), colon cancer cells (CaCO2), liver cancer cells (HepG2), lung cancer cells (NCI-H187), and normal cell (hTERT-HME1) were purchased from ATCC®. The cells were subcultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/ml of penicillin, 100 µg/ml of streptomycin and incubated in a 5% CO_2 incubator at 37°C. When a cell line reaches about 80% confluence, trypsinization was done, the cell count was done and the cell viability was tested by trypan blue using a hemocytometer. A known number of cells (1.5×10^4 cells/well in 100 µl of medium) were seeded into 96-well plates respectively for carrying out a MTT assay.

MTT Cell Viability Assays

The cell viability test of breast cancer cells (MCF-7), colon cancer cells (CaCO2), liver cancer cells (HepG2), lung

Table 1: Phytochemical screening of methanolic extracts of *Croton persimilis* Müll. Arg. and *Antidesma puncticulatum* Miq.

	Alkaloids	Flavonoids	Saponins	Tannins	Terpenoids	Total phenolic content Mean±SD (mg GAE/g crude extract)
C. persimilis Müll Arg	+	++	++	++	++	43.40±1.36
A. puncticulatum Miq.	+	+	+++	+	++	193.47±4.63

+: present in mild, ++: present in moderate, +++: present in massive, and -: present in absent. *C. persimilis*: *Croton persimilis*, *A. puncticulatum*: *Antidesma puncticulatum*

cancer cells (NCI-H187), and normal cell (hTERT-HME1) were performed using the MTT method according to Mossmann's method with modification.[20] The cells were treated with various concentrations (0.1, 1, 2.5, 5, 7.5, 10, 25 and 50 µg/mL) and incubated in a 5% CO_2 incubator at 37°C for 24 h and 48 h. Then, MTT at a concentration of 5 mg/mL in 0.1 mM Phosphate-buffered saline pH 7.4 was added and incubated at 37°C for 4 h. DMEM supplemented with 10% FBS was used as the negative control and 2% Triton-X100 as the positive control. Viable active cells reduced yellow MTT salt to insoluble purple formazan, which was dissolved using Dimethyl sulfoxide. The absorbance of the colored solution was measured at a wavelength of 570 nm using micro-plate multi-detection instrument (Thermo Electron Corporation, Vantaa, Finland). The obtained absorbance at 570 nm of all treated cells, controls, and normal. All samples were assayed in triplicate, and the mean for each experiment was calculated. The mean of triplicate experiments for each dose was used to calculate the concentration of drug required for 50% inhibition of cell viability (IC_{50}) as determined using the Calcusyn™ version 1.1 program (BioSoft, Cambridge, UK.).

RESULTS AND DISCUSSIONS

Phytochemical Profiling

Table 1 showed the preliminary phytochemical screening of the ethanolic extracts of *C. persimilis* Müll. Arg. and *A. puncticulatum* Miq. showed that they are presented of various phytochemicals such as alkaloids, flavonoids, saponins, tannins, and terpenoids. The results concluded that the leaves extracts of *C. persimilis* Müll. Arg. contained appreciable middle amount of flavonoids, saponins, terpenoids, and tannins while alkaloids content was almost negligible. In the croton species, these basic findings are consistent with Antonio *et al.* (2007)[21] and André *et al.* (2020)[22] showing that terpenoids was the predominant secondary metabolite constituents while flavonoids, saponins, and tannins was virtually ubiquitous and alkaloids are notable. There are however some differences in phytochemical screening of *Croton oblongifolius* from northeastern Thailand that revealed presence of alkaloids, flavonoids, phytosterols, triterpenoids, diterpenoids, and tannins with an absence of saponins and phenols.[23] A concise appraisal of total phenolic content properties were found in promising amount in 43.40 ± 1.36 mg GAE/g crude extract that similar to Chatatikun and Chiabchalard (2017)[24] were found total phenolic content 19.41 ± 0.81 mg GAE/g dry material in *Chrysophyllum roxburghii* N.P.Balakr and 16.28 ± 0.29 mg GAE/g dry material in *Croton sublyratus* Kurz. However, these findings showed less nearly half of total phenolic content (79.97–91.41 mg GAE/g crude extract) than the previous studies.[25,26]

Table 2: Antioxidant activity as IC_{50} (µg/ml) for DPPH assay and FRAP (µM/g of crude extract) of methanolic extracts of *C. persimilis* Müll Arg and *A. puncticulatum* Miq**.**

	DPPH IC_{50} (µg/ml)	Mean±SD FRAP value µM/g crude extract
C. persimilis Müll. Arg.	>1000	369.84±2.39
A. puncticulatum Miq.	51.50±3.30	1905.88±13.04
L-ascorbic acid	14.5±0.78	590.24±2.67
Gallic acid	6.46±0.48	964.67±5.88
BHT	400.98±3.39	559.61±3.13

C. persimilis: *Croton persimilis*, *A. puncticulatum*: *Antidesma puncticulatum*

The raw fruits of *A. puncticulatum* Miq. contained high consequential amount of saponins, and middle amount of tannins while flavonoids, terpenoids, and alkaloids content was almost negligible. However, the results of the previous studies were consistent that showed tannins was found to be common in the *A. bunius* L. fruit crude extract and fractions in while flavonoid and alkaloid were absence.[27] Nevertheless, another study showed steroids, flavonoids, and tannins were widely distributed in *A. bunius* L. fruit while saponins were absence.[27] A concise appraisal of total phenolic content properties was found in promising amount in 193.47 ± 4.63 mg GAE/g crude extract that showed greater total phenolic content (85.77–120.818 mg GAE/g crude extract) than the previous studies.[28,29]

Antioxidant Activity

Table 2 displays the percent antioxidant activity of ethanolic extracts of *C. persimilis* Müll. Arg. and *A. puncticulatum* Miq. using DPPH assay and FRAP assay. It displayed the raw fruits extract of *A. puncticulatum* Miq. had exceptional antioxidant activity (IC_{50} = 51.50 ± 3.30 µg/ml) which was lower than BHT (IC_{50} = 400.98 ± 3.39 µg/ml) via DPPH assay. It also showed highest Fe3+-TPTZ reducing power (1905.88 ± 13.04 µM/g of crude extract) than ascorbic acid (964.67 ± 5.88 µM/g of crude extract), Trolox (559.61 ± 3.13 µM/g of crude extract), and BHT (590.24 ± 2.67 µM/g of crude extract) via FRAP assay. The results showed stronger antioxidative power that obtained similar to Tuyoien and Arunporn (2010), Dechayont *et al.* (2012), Bhanuz *et al.* (2012), and Basak *et al.* (2013)[30-33] exhibited highest DPPH value (EC_{50} = 2.42–11.73 µg/ml) than BHT (EC_{50} = 12.12–13.36 µg/ml) and highest FRAP value was recorded in *A. ghaesembilla* (2114 µM AEAC/g dw). While the lowest antioxidant activity is reported for leaves extract of *C. persimilis* Müll. Arg. through DPPH assay (IC_{50}>1000 µg/ml) and FRAP assay (369.84 ± 2.39 µM/g of crude extract). The results of previous studies were consistent that showed lowest scavenging ability was detected. Alves *et al.* (2013)[34] exhibited the free radical scavenging activity by DPPH assays with EC_{50} values >1000 µg/mL.

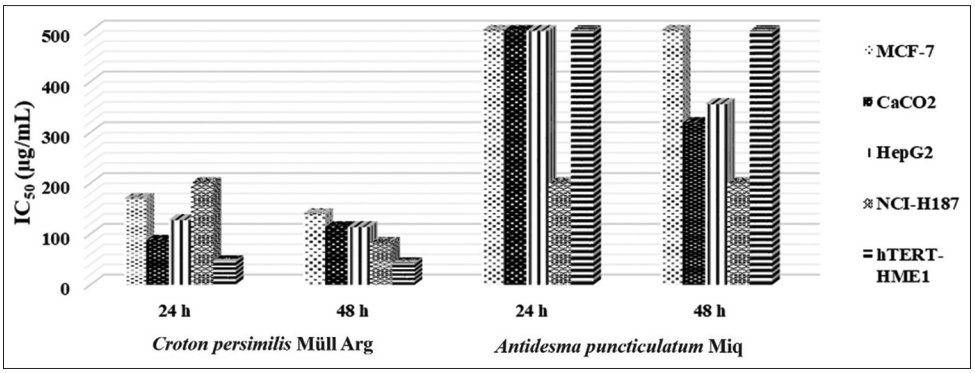

Figure 1: The comparison graph of IC_{50} values of leaves extract of *Croton persimilis* Müll. Arg. and the raw fruits extract of *Antidesma puncticulatum* Miq. in non-cancerous and cancerous cells

Table 3: Half maximal inhibitory concentration (IC_{50}) values of cytotoxic activity of leaves extract of *C. persimilis* Müll. Arg. and the raw fruits extract of *A. puncticulatum* Miq**.**

	IC_{50} (µg/mL)									
	***Croton persimilis* Müll. Arg.**		***Antidesma puncticulatum* Miq.**		**5 - FU**		**Doxorubicin**		**Cisplatin**	
	24 h	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
MCF-7	169.28±1.95	138.73±10.55	>500	>500	>100	91.74	16.21	2.24	-	-
$CaCO_2$	86.86±2.31	113.32±1.24	>500	318.58±12.01	>100	>100	>100	7.91	-	-
$HepG_2$	127.34±8.61	113.63±12.20	>500	355.76±7.86	-	-	-	-	2.71	1.19
NCI-H187	>200	82.59±1.81	>200	>200	-	-	-	-	3.63	1.31
hTERT-HME1	47.19±1.84	42.64±0.91	>500	>500	-	-	-	-	-	-

In vitro Anticancer and Cytotoxic Activity

Table 3 presented half maximal inhibitory concentration (IC_{50}) values of cytotoxic activity of leaves extract of *C. persimilis* Müll. Arg., and the raw fruits extract of *A. puncticulatum* Miq. for 24 h and 48 h. 5-Fluorouracil, Doxorubicin and Cisplatin were used as the positive control. The leaves extract of *C. persimilis* Müll. Arg. displayed an IC_{50} value of 169.28 ± 1.95 µg/mL and 138.7 ± 10.55 µg/mL for MCF-7, 86.86 ±2.31 µg/mL and 113.32 ± 1.24 µg/mL for $CaCO_2$,127.34 ± 8.61 µg/mL and 113.63 ±12.20 µg/mL for $HepG_2$, >200 µg/mL and 82.59 ± 1.81 µg/mL for NCI-H187, and 47.19 ± 1.84 µg/mL and 42.64 ± 0.91 µg/mL for hTERT-HME1, respectively [Figure 1]. The SI [Table 4] of leaves extract of *C. persimilis* Müll. Arg. shows SI value <2 indicates non selective and general toxicity in any cell line. The same observation was made with the ethanol leaf extract of fresh leaves of the Croton species from South Africa and Southern Brazil. The results revealed *C. pseudopulchellus* Pax and *C. sylvaticus* Mull. Arg. were more cytotoxic on both non-cancerous and cancerous cells with lowest IC_{50} values (1.75–106.52 µg/mL) obtained against MCF-7, HeLa, Caco-2 and A549 cell lines.[35] C. macrobothrys extract exhibited activity against cancerous cells with lowest GI_{50} values in NCI-H460 (GI_{50} 6.08 µg/mL) and K5662 (GI_{50} 7.45 µg/mL).[36] Nevertheless, there is an enormous difference in anticancer activity in breast cancer cell line that showed *C. oblongifolius* exerts greater toxic against breast cancer cell lines (MDA-MB-213) and acts more selective (SI = 2.8).[23] While the raw fruits extract of *A. puncticulatum* Miq. revealed an IC_{50} value of >500 µg/mL both 24 h and 48 h for MCF-7, >500 µg/mL and 318.58 ± 12.01 µg/mL for $CaCO_2$, >500 µg/mL and 355.76 ±7.86 µg/mL for $HepG_2$, >200 µg/mL and >200 µg/mL for NCI-H187, and >500 µg/mL and >500 µg/mL for hTERT-HME1, respectively [Table 3]. Consistent with previous results that showed the ethanol extracts *A. thwaitesianum* Mull. Arg. dried fruits had low growth inhibition against A549, COR-L23, PC-3, HeLa, MCF7, LS174T cell lines with a similar range of IC_{50} values (>100 µg/mL).[37] The highest SI values of raw fruits extract of *A. puncticulatum* Miq. were observed that mean there is more than twice and half more cytotoxic to NCI-H187 as compare with the normal cell line. The findings are in line with previous studies that have shown that leaves extract of *A. bunius* L. revealed cytotoxic activity against A549 human lung adenocarcinoma[38] and *A. thwaitesianum* wood extract also

Table 4: Selectivity index (SI) of leaves extract of *C. persimilis* Müll. Arg. and the raw fruits extract of *A. puncticulatum* Miq**.**

	Selectivity index (SI)			
	***Croton persimilis* Müll. Arg.**		***Antidesma puncticulatum* Miq.**	
	24 h	**48 h**	**24 h**	**48 h**
MCF-7	0.278768904	0.307359619	1	1
$CaCO_2$	0.54328805	0.376279562	1	1.569464499
$HepG_2$	0.370582692	0.375253014	1	1.405441871
NCI-H187	0.23595	0.516285265	2.5	2.5

C. persimilis: *Croton persimilis*, *A. puncticulatum*: *Antidesma puncticulatum*

exhibited the highest cytotoxic against COR-L23 lung cancer cells.[30]

CONCLUSION

Our results indicate that leaf extracts of *C. persimilis* Müll. Arg., and raw fruits extract of *A. puncticulatum* Miq. shows promise as potential ingredients for antioxidant-rich plants possess belonging to the groups of saponins, terpenoids, tannins, and flavonoids. *A. puncticulatum* Miq. had exceptional antioxidant activity through DPPH and FRAP and possible potential of against human lung adenocarcinoma. Although the leaves extract of *C. persimilis* Müll. Arg. exhibit both cellular antioxidant activities through DPPH and FRAP, it is toxic to both normal cells and cancer cells. Thus, both extracts are recommended as a good source of natural antioxidants. C. persimilis Müll. Arg. may serve as a potential source of compounds for anticancer.

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