Pharmacogn J. 2019; 11(3): 531-535
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Effect of Agricultural Model of using Fertilizer, Harvesting Time and Extraction Method on Phytochemical Contents and Antioxidant Activities from Mulberry Leaves Grown in Maha Sarakham Province, Thailand

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ABSTRACT

Aims: Phytochemical contents and free radical scavenging of Mulberry leaf extracts by using different fertilizer, time of harvesting and solvent extraction were evaluated. **Materials and Methods**: Dried Mulberry leaves were extracted by using different solvent including aqueous, 50% ethanol and 95% ethanol. The phytochemical screening were determined by Total Phenolic Compounds (TPC) and Total Flavonoid Contents (TFC). The anti-oxidation were tested by using 2,2-diphenyl-1-picrylhydrazy (DPPH) radical scavenging and 2,2 -azinobis-(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^+$) assay. **Results**: This experimental study found that the mulberry leaf extract were given extraction with 95% ethanol, all of fertilizer and at Week 4 showed highest total phenolic contents espectcially BET2 was high amount of TPC (124.444±0.609 mgGE/gExt). The harvest time, all of groups at the Week 4 were significantly higher than all of groups at the Week 2. On the other hand, total flavonoid contents, the DET1 (Fertilizer formula 15-15-15, extraction with 95% ethanol at the Week 2; 110.913±3.208 mgQE/gExt) showed highest amount. The Antioxidant activities, DPPH free radical scavenging activity, The groups were given extraction with 95% ethanol, all of fertilizer at the Week 4 including CHT2 (IC_{50} =0.00459±0.00001 mg/mL), BHT2 (IC_{50} =0.00487 ±0.00005 mg/mL), AHT2 (IC_{50} =0.00499±0.00007 mg/mL), DHT2 (IC_{50} =0.00499±0.00005 mg/mL) and EHT2 (IC_{50} =0.00667 ±0.00039 mg/mL) were more potent on free radical scavenging higher than all of groups. The $ABTS^+$ assay, at the Week 2 of all fertilizer groups were given with all solvent extraction including BHT1 (IC_{50} =0.03191±0.00257 mg/mL), CHT1 (IC_{50} =0.03247±0.00044 mg/mL), AHT1 (IC_{50} =0.03320±0.00120 mg/mL), EHT1 (IC_{50} =0.03342±0.00116 mg/mL) and AAT1 (IC_{50} =0.03792±0.00076 mg/mL) showed free radical scavenging activity not different from standard substances, ascorbic acid (IC_{50} =0.00699 ±0.00004 mg/mL) and Trolox (IC_{50} =0.01594±0.00116 mg/mL). **Conclusion**: The study was undertaken to investigate it's fertilizer use, harvest time and extraction method for biologically activities also chemical composition contents and their antioxidant potentials. Therefore, our data might be help to good cultivation and harvesting practice selection in order to produce better of mulberry leaf production.
Key words: Fertilizer, Harvesting, Phytochemistry, Antioxidation, Mulberry leaves.

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History
- Submission Date: 17-12-2018;
- Review completed: 09-01-2019;
- Accepted Date: 12-02-2019

DOI : 10.5530/pj.2019.11.85

Article Available online
http://www.phcogj.com/v11/i3

INTRODUCTION

In Thailand, the mulberry is widly cultivated within promotion and support by government agencies especially Sakolnakorn variety. Thai farmer used their leaves for feed silkworms and the fruits are eaten raw or processed as juice, mulberry fruit wine and supplementary food. Norteastern of Thailand, Thai rarul has been favorite bring them for a local cuisine called "Boiled chicken" indicated to folklore cooking wisdom which this will make the food useful and tasteful.[1]

Mulberry (*Morus alba* L.) is belonging to the family Moraceae, is a short-lived, fast-growing, small to medium sized mulberry tree, which grows to 10–20 m tall. The plant can grow in a wide range of climatic, topographical and soil conditions which can affect the chemical composition and nutritional status.[2-4] The mulberry leaves have been widely used to produce various functional foods such as mulberry leaf carbonated beverages, health beverages and mulberry leaf tea. Phytochemical investigation has indicated that there are many active constituents such as flavonoids, alkaloids, phenolics, steroids,[5] amino acids, simple phenylpropanoids[6] and polysaccharides.[3] The plant has been used to treat various illnesses since ancient times. The herb was described as being able to eliminate cold and heat in the body, promote perspiration and have detoxifying properties.[6] It has been exhibit multiple therapeutic effects

Cite this article: Chanhan P, Konsue A, Nammatra R. Effect of Agricultural Model of using Fertilizer, Harvesting Time and Extraction Method on Phytochemical Contents and Antioxidant Activities from Mulberry Leaves Grown in Maha Sarakham Province, Thailand. Pharmacog J. 2019;11(3):531-5.

including anti-diabetic, anti-inflammation, anti-cancer effects,[5] antihyperglycemic, anti-hyperlipidemia activities,[3] hypoglycemic, antidepressant, antioxidant and hepatoprotective effects.[4]

Herbal preparation have gained good attention toward the treatment of various diseases on the globe. Phytopharmaceuticals have revealed the efficacy and toxicity associated with herbal agents and have become more popular among public.[7] Traditional extraction methods including soxhlet appalatus extraction, heating reflux extraction and organic solvent maceration have some disadvantages. The effects of main operating extraction yields were investigated. The extraction method is an important factor for the use of plant active components.[5] The free radical scavenging properties postulated to be as a result of the synergistic action of antioxidant compounds including carotenoids, flavonoids, moracins and others present in the mulberry leaves. Oxidative stress,which results from an improper balance between Reactive Oxygen Species (ROS) and their metabolites and antioxidant defence, is a factor in the pathogenesis of various diseases such as cardiovascular disorders, neurological conditions, Parkinson's disease, rheumatoid arthritis and ageing.[8]

However, though many literatures were reveal some biological activities, chemical compositions, cultivation and havesting, but there is no yet any report to the appropriately agricultural model in Thailand Farming. Therefore, the propose in this study were detemined to fertilizer use, time of harvesting and solvent extraction on phytochemical compositon and effect of antioxidant activities to appropriately agricultural selection which will be help to increase productivity.

MATERIALS AND METHODS

Sample Collection

The white mulberry (Variety: Sakolnakorn) were cultivated at the Experimental Farm of the New Innovation Excellence Center, Mahasarakham University, Maha Sarakham, Thailand. The plants were given to different fertilizer including chicken dung fertilizer, Urea fertilizer formula 46-0-0, chemical fertilizer formula 15-15-15 and mix of all (w:w:w;1:1:1) after trim of branch. The fresh leaves were harvested at Week 2 and Week 4 growing period. They were cleaned and dried at 60°C for 48 hr in a hot air oven then powdered.

Preparation of Extracts

The aquoes extract was prepared by distilled water for 30 min at 60°C in sonication bath (1:10 w/v). The ethanolic extracts were macerated with 50% ethanol and 95% ethanol for 7 days (1:5 w/v). The residue powder was excluded by using filter papers. The filtrate was evaporated using by a rotary evaporator (Heidolph Laborota 4000, Germany) and freeze-dried to obtain dark brown extract. The extracts were kept in the fridge at -4°C until be used.

Total phenolic content assay

Total phenolic content was determined according to a modified procedure.[9] Sample (100 µL) will be oxidized with 500 µL of 0.2 N Folin-Ciocalteu's reagent and neutralized by adding 400 µL of 7.5% Na_2CO_3. The absorbance measured at 765 nm after mixed and incubated in room temperature for 30 min. The results were expressed as gallic acid equivalents (mgGE/gExt).

Total Flavonoid Content Assay

Flavonoid content was estimated using the aluminum chloride colorimetric method.[10] The extracts from recipe will be mixed with 100 µL of 5% aluminum chloride (w/v), 400 µL of 2.5% $NaNO_2$ After 5 min, 500 µL of 5% $AlCl_3$. The mixture will be allowed to stand at room temperature for 10 min. The solution was mixed 2,000 µL distilled water. The results was measured at 415 nm. The TFC was calculated from a standard quercetin equivalent (mgQE/gExt).

DPPH free radical scavenging activity

2,2-diphenyl-1-picrylhydrazy (DPPH) radical scavenging capacities of wheat extracts were estimated by the reduction of the reaction color between DPPH solution and sample extracts as previously described by prior method.[11] DPPH was dissolved in ethanol to a 0.039 mg/mL. The plant extract at various concentrations was diluted with distilled water to get sample solution.100 µL of the sample solution following which 900 µL DPPH (0.1 mM) working solution. After a 30 min reaction kept in the dark at ambient temperature then absorbance of the solution was measured at 515 nm. In this study, will be used Trolox® and ascorbic acid as standard substances. Blanks were run in each assay. DPPH radical ability was expressed as IC_{50} (mg/mL) and the inhibition percentage calculated using the following formula: DPPH scavenging activity (%) = $(A_0-A_1)/ A_0$ x 100 where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

$ABTS^+$ Radical Scavenging Activity

In ABTS assay, the plants extract will be allowed to react with $ABTS^+$, a model stable free radical derived from 2,2-azinobis (3-ethylvenzothiazolin-6-sulphonic acid) ($ABTS^+$) assay was performed.[12] The $ABTS^+$ (900 µL) was added to the extracts (100 µL) and thoroughly mixed. The mixture was held at room temperature for 6 min and absorbance was immediately measured at 734 nm. Trolox® and ascorbic acid solution in 80% ethanol was prepared and assayed under the same conditions. ABTS scavenging ability was expressed as IC_{50} (mg/mL) and the inhibition percentage calculated using the following formula: ABTS scavenging activity (%) = $(A_0-A_1)/ A_0$ x 100 where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Statistical Analysis

All assays were expressed as mean± Standard Error of Mean (SEM) from three separate experiments (n = 3). Statistical analysis was carried out using One-Way Analysis of Variance (ANOVA) followed by Duncan's multiple range tests. Differences at $p<0.05$ were considered to be significant.

RESULTS

Phytochemical Compositions

Total Phenolic Contents

As shown in Table 1, the plant were given extraction with 95% ethanol, all of fertilizer and at Week 4 shown highest total phenolic contents, espectcially BET2 was high amount of TPC (124.444±0.609 mgGE/gExt) than DET2 (116.074±0.588 mgGE/gExt), AET2 (115.118±0.567 mgGE/gExt), CET (85.741±0.937 mgGE/gExt) and EET2 (75.639±1.445 mgGE/gExt) respectively. The harvest time, all of groups at the Week 4 were significantly higher than all of groups at the Week 2.

Total Flavonoid Contents

As shown in Table 1, the DET1 (fertilizer formula 15-15-15, extraction with 95% ethanol at the Week 2; 110.913±3.208 mgQE/gExt) showed highest total flavonoid contents highest amount than AET1 (88.398±2.905 mgQE/gExt), EHT (88.280±1.171 mgQE/gExt), BET (80.860±2.009 mgQE/gExt) and CET1 (72.663±2.156 mgQE/gExt) respectively. The harvest time, all of groups at the Week 2 were not different than all of groups at the Week 4.

Table 1: Phytochemical Composition Showed Total Phenolic Contents (TPC) and Total Flavonoid Contents (TFC) from Mulbery Leaf Extract.

	Groups	TPC (mgGE/gExt)		TFC (mgQE/gExt)	
		Mean	Std. Error	Mean	Std. Error
1	AAT1	1.951^{ab}	±0.118	36.378^{j}	±0.464
2	BAT1	1.582^{ab}	±0.163	13.740^{cd}	±0.419
3	CAT1	2.582^{abc}	±0.024	38.683^{l}	±1.029
4	DAT1	1.747^{ab}	±0.051	31.783^{hi}	±1.553
5	EAT1	1.758^{ab}	±0.020	15.888^{d}	±0.068
6	AHT1	2.751^{bc}	±0.162	49.123^{m}	±0.847
7	BHT1	1.2441^{ab}	±0.013	60.533^{n}	±1.401
8	CHT1	2.3741^{bc}	±0.061	60.550^{n}	±0.397
9	DHT1	1.8841^{ab}	±0.020	41.515^{l}	±0.997
10	EHT1	3.7141^{c}	±0.143	88.280$^{q\#}$	±1.171
11	AET1	0.401^{a}	±0.019	88.398$^{q\#}$	±2.905
12	BET1	0.302^{a}	±0.029	80.860$^{p\#}$	±2.009
13	CET1	0.352^{a}	±0.004	72.663$^{o\#}$	±2.156
14	DET1	0.311^{a}	±0.015	110.913$^{r\#}$	±3.208
15	EET1	0.446^{a}	±0.010	75.270^{o}	±1.657
16	AAT2	14.114^{d}	±0.594	7.935^{a}	±0.116
17	BAT2	15.059^{d}	±0.387	9.730^{ab}	±0.126
18	CAT2	23.925^{g}	±0.104	16.203^{de}	±0.221
19	DAT2	22.488^{g}	±0.490	11.895^{bc}	±0.147
20	EAT2	17.073^{e}	±0.746	11.288^{abc}	±0.719
21	AHT2	29.434^{i}	±1.035	25.025^{f}	±0.237
22	BHT2	26.769^{h}	±0.464	29.540^{gh}	±0.316
23	CHT2	28.157^{hi}	±1.553	32.320^{hi}	±0.372
24	DHT2	26.873^{h}	0.397	28.910^{gh}	±0.590
25	EHT2	19.375^{f}	±0.168	19.625^{e}	±0.173
26	AET2	115.118$^{l\#}$	±0.567	32.483^{hi}	±0.896
27	BET2	124.444$^{m\#}$	±0.610	42.170^{l}	±0.621
28	CET2	85.741$^{k\#}$	±0.937	19.588^{e}	±0.577
29	DET2	116.074$^{l\#}$	±0.588	34.688^{ij}	±0.197
30	EET2	75.639$^{j\#}$	±1.445	26.673^{fg}	±0.435

Groups: The first capital were diffirent fertilizers; A was a control group, B was given with chicken dung fertilizer, C was given with Urea fertilizer formula 46-0-0, D was given with chemical fertilizer formula 15-15-15 and E was given with mix of all fertilizer (w:w:w;1:1:1). The second capital were different extraction methods; A was extracted with aquoes, B was extracted with 50% ethanol and C was extracted with 95% ethanol. The third capital were time of harvesting; T1 was harvested at Week 2 and T2 was harvested at Week 4. TPC was measured with gallic acid equivalents (mgGE/gExt). TFC was measured with quercetin equivalent (mgQE/gExt). Different letters indicated significantly difference at *p*-values less than 0.05. # ingradient contents as top of five from different groups.

Table 2: Antioxidant Activities Showed Inhibitory Concentration (IC_{50}) of different Extracts from Mulbery Leaves.

	Groups	DPPH (mg/mL)		$ABTS^{+}$ (mg/mL)	
		Mean	Std. Error	Mean	Std. Error
1	AAT1	0.13681^{d}	±0.00091	0.03792$^{a\#}$	±0.00076
2	BAT1	0.16073^{f}	±0.00301	0.06137^{a}	±0.00187
3	CAT1	0.13874^{d}	±0.00171	0.03879^{a}	±0.00120
4	DAT1	0.11951^{d}	±0.00159	0.04175^{a}	±0.00022
5	EAT1	0.14014^{d}	±0.00144	0.05201^{a}	±0.00311
6	AHT1	0.07715^{b}	±0.00086	0.03320$^{a\#}$	±0.00120
7	BHT1	0.15454^{e}	±0.00209	0.03191$^{a\#}$	±0.00257
8	CHT1	0.13915^{d}	±0.00118	0.03247$^{a\#}$	±0.00044
9	DHT1	0.14263^{d}	±0.00285	0.06647^{a}	±0.00040
10	EHT1	0.10621^{c}	±0.00125	0.03342$^{a\#}$	±0.00116
11	AET1	0.23183^{i}	±0.02663	0.04570^{a}	±0.00096
12	BET1	0.23409^{i}	±0.01890	0.05372^{a}	±0.00211
13	CET1	0.27159^{k}	±0.02507	0.06922^{a}	±0.00060
14	DET1	0.47942^{l}	±0.01444	0.07247^{b}	±0.00108
15	EET1	0.50773^{l}	±0.04121	0.08160^{c}	±0.00102
16	AAT2	0.28296^{k}	±0.00118	0.79605^{j}	±0.01583
17	BAT2	0.26324^{j}	±0.00067	0.67562^{i}	±0.00693
18	CAT2	0.25276^{j}	±0.01145	0.41878^{e}	±0.01293
19	DAT2	0.14529^{d}	±0.00123	0.36976^{d}	±0.02510
20	EAT2	0.26149^{j}	±0.00335	0.48170^{g}	±0.01750
21	AHT2	0.00499$^{a\#}$	±0.00007	0.35238^{d}	±0.02437
22	BHT2	0.00487$^{a\#}$	±0.00005	0.44635^{f}	±0.01734
23	CHT2	0.00459$^{a\#}$	±0.00001	0.37508^{d}	±0.00592
24	DHT2	0.00499$^{a\#}$	±0.00005	0.45111^{f}	±0.01218
25	EHT2	0.00667$^{a\#}$	±0.00039	0.61084^{h}	±0.02278
26	AET2	0.21447^{h}	±0.01491	0.65082^{h}	±0.02265
27	BET2	0.14216^{d}	±0.00497	0.67521^{i}	±0.02154
28	CET2	0.18630^{g}	±0.02010	0.83239^{j}	±0.07338
29	DET2	0.12485^{d}	±0.00218	0.90744^{k}	±0.03162
30	EET2	0.18622^{g}	±0.00314	1.30769^{l}	±0.03071
31	ascorbic acid	0.00018^{a}	±0.00001	0.00699^{a}	±0.00004
32	trolox®	0.05156^{b}	±0.00016	0.01594^{a}	±0.00017

Groups: The first capital were diffirent fertilizers; A was a control group, B was given with chicken dung fertilizer, C was given with Urea fertilizer formula 46-0-0, D was given with chemical fertilizer formula 15-15-15 and E was given with mix of all fertilizer (w:w:w;1:1:1). The second capital were different extraction methods; A was extracted with aquoes, B was extracted with 50% ethanol and C was extracted with 95% ethanol. The third capital were time of harvesting; T1 was harvested at Week 2 and T2 was harvested at Week 4. DPPH radical scavenging and $ABTS^{+}$ assay were used ascorbic acid and trolox® as standard substances. Different letters indicated significantly difference at *p*-values less than 0.05. # influence of free radical scavenging as top of five from different groups.

Antioxidant Activities

DPPH Free Radical Scavenging Activity

As Table 2 shown in this study, DPPH free radical scavenging activity, standard substances, ascorbic acid and Trolox® were showed more potent than all of groups. The groups were given extraction with 95% ethanol, all of fertilizer at the Week 4 including CHT2 (IC_{50} =0.00459±0.00001 mg/mL), BHT2 (IC_{50} =0.00487 ±0.00005 mg/mL), AHT2 (IC_{50} =0.00499±0.00007 mg/mL), DHT2 (IC_{50} =0.00499±0.00005 mg/mL) and EHT2 (IC_{50} =0.00667 ±0.00039 mg/mL) were more potent on free radical scavenging higher than all of groups.

ABTS$^+$ Radical Scavenging Activity

As Table 2 shown by using ABTS$^+$ assay, at the Week 2 of all fertilizer groups were given with all solvent extraction including BHT1 (IC_{50} =0.03191±0.00257 mg/mL), CHT1 (IC_{50} =0.03247±0.00044 mg/mL), AHT1 (IC_{50} =0.03320±0.00120 mg/mL), EHT1 (IC_{50} =0.03342±0.00116 mg/mL) and AAT1 (IC_{50} =0.03792±0.00076 mg/mL) showed free radical scavenging activity not different from standard substances, ascorbic acid (IC_{50} =0.00699 ±0.00004 mg/mL) and Trolox® (IC_{50} =0.01594±0.00116 mg/mL). All groups of the Week 2 were significantly free radical scavenging acitivity on IC_{50} less than all the groups of Week 4.

DISCUSSION

Mulberry leaves contain a wide range of bioactive compounds, such as flavonoid and phenolic compound contents of white mulberry leaves depended on the harvest time which are responsible for beneficial effects on human health.[13] The mulberry leaves have been extensively investigated for their various health benefits, including antioxidative, hypolipidemic, antihyperglycemic and antiatherogenic effects.

This studies have indicated that mulberry leaves exhibited significant scavenging effects on free radicals both DPPH and ABTS$^+$ which protected low-density lipoprotein against oxidative damage. The reports suggested that polyphenols and flavonoids present in mulberry contribute to these health benefits.[14] In the present study, the free radical scavenging activities, phenolic compound and flavonoid contents of the extract were significantly different according to the fertilizers, harvest times and extraction methods. The phenolic compound contents by 95% ethanolic extraction related with harvesting time. The study showed that Week4 were highest phenolic compound contents exert than other method, particular environment and has a direct bearing on the composition of the plants found there.[15] The methods applied in this study considered the antioxidant properties of the mulberry extract as determined by different group testing methods.[16] It has been reported that mulberry contains a lot of bioactive compound, polysaccharides, but in this study did not extracted on a Box–Behnken design, polysacharide function isolation. It is well known that the biological functions including antioxidant activity of polysaccharides are intimately related to their structure features such as chemical components, molecular weight, monosaccharide composition and glycosidic linkage. A comprehensive study of purification, characterization and antioxidant activities of mulberry leaves will provide useful information on the relationship between antioxidant activity and structure features.[17-18] Some report was reveal that this in animal model can be used in functional food studies, where information on the absorption and bioavailability of dietary antioxidants is still limited. The reported mechanisms for the absorption of flavonoids probably due to the presence of antioxidants with higher glucose moieties in the extract.[19]

Therefore, we report here the fertilizer use, harvesting time and extraction were applied to optimize conditions on phytochemical content and antioxidation activity and the resulting extraction conditions were used to prepare cultivation through harvest and extraction method precipitation. Mulberry leaves harvested on Week 4, ethanolic maceration had the highest total phenolic contents. On the other hand, total flavonoid contents had highest on Week 2 by using hydroethanolic extraction. It considered to be a better model concerning phenolic and flavonoid contents on antioxidant activity, respectively.[20] These methods may not produce plants with the highest levels of the targeted functional components and so some modifications of the agricultural model may be necessary. Based on the results of the current study, formulation of fertilizer, time of harvesting and method of extraction appear to be valid indicators for determining the best practice model to obtain the highest amounts of each targeted functional component.[21]

However, the current study was undertaken to investigate its fertilizer use, harvest time and extraction method for biologically activities also chemical constituent contents and antioxidant potentials. In addition, isolation, purification and identification of active compounds obtained from mulberry leaves were also researched.

CONCLUSION

This study showed that the mulberry leaves form Sakolnakorn cultivar were nutritionally rich, highest total flavonoid and phenolic compound contents were found in the leaves. Our results might be help to select the good cultivation and harvesting practice in order to produce better also quantitative or/and qualitative mulberry leaf production.

ACKNOWLEDGEMENT

The study was partially financial supported by the Development Research Division, Mahasarakham University, Maha Sarakham, Thailand and National Research Council of Thailand (NRCT) Bangkok, Thailand.

CONFLICT OF INTEREST

There are no conflicts of interest.

ABBREVIATIONS

TPC: Total phenolic compounds; **TFC:** Total flavonoid contents; **DPPH:** 2,2-diphenyl-1-picrylhydrazy assay; **ABTS+:** 2,2 -azinobis-(3-ethylbenzothiazoline-6-sulphonate) assay.

Groups: The first capital were diffirent fertilizers; A was a control group, B was given with chicken dung fertilizer, C was given with Urea fertilizer formula 46-0-0, D was given with chemical fertilizer formula 15-15-15 and E was given with mix of all fertilizer (w:w:w;1:1:1). The second capital were different extraction methods; A was extracted with aquoes, B was extracted with 50% ethanol and C was extracted with 95% ethanol. The third capital were time of harvesting; T1 was harvested at Week 2 and T2 was harvested at Week 4.

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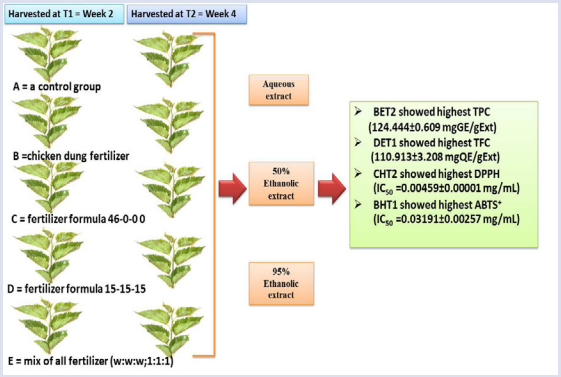
GRAPHICAL ABSTRACT



SUMMARY

- This study showed that the mulberry leaves form Sakolnakorn cultivar were nutritionally rich, highest total flavonoid and phenolic compound contents were found in the leaves.

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Cite this article: Chanhan P, Konsue A, Nammatra R. Effect of Agricultural Model of using Fertilizer, Harvesting Time and Extraction Method on Phytochemical Contents and Antioxidant Activities from Mulberry Leaves Grown in Maha Sarakham Province, Thailand. Pharmacog J. 2019;11(3):531-5.