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Assessment of total phenolic and flavonoid contents, antioxidant activity, and anti-acetylcholinesterase activity from *Codiaeum variegatum* (L.) Blume leaves found in Thailand

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ABSTRACT: *Codiaeum variegatum* (L.) Blume contained many cultivars with different leaf shapes and coloration. Most cultivars of *C. variegatum* were widely used as vegetables in northern Thailand, in which two cultivars (spirale and broad leaf) were selected for the investigation of the phytochemical profiling and the biological activity related to the prevention of Alzheimer's disease (AD). The ethanolic extracts of spirale and broad cultivars of *C. variegatum* (ESC and EBC) contained total phenolic content about 17.99±0.02 and 30.35±0.10 mg GAE/g extract, respectively. Total flavonoid content of the ESC and EBC were 39.30±0.20 and 34.72±0.67 mg QE/g extract, respectively. Quantitative estimation revealed that flavonoids, alkaloids, steroids, terpenoids, and cardiac glycosides are presented in both ESC and EBC. Both ESC and EBC showed weak scavenging activity against 2,2-diphenyl-l-picrylhydrazyl (DPPH) with IC_{50} values of 8.00±0.60 and 6.88±0.40 mg/mL, respectively. This study was considered the first report on the anti-acetylcholinesterase activity of *C. variegatum* leaves extracts. The microplate assay revealed that the spirale cultivar exhibited potent AChE inhibition with an IC_{50} value of 551.36 µg/mL while broad cultivar was inactive. Active compounds of spirale cultivar were supposed to be further investigated.
Keywords: alzheimer's disease; croton; phenolic compounds; phytochemicals screening

INTRODUCTION

Codiaeum variegatum (L.) Blume (croton) is the second-largest genera (c. 700 species) of the family Euphorbiaceae. *Codiaeum variegatum* (L.) Blume is a popular ornamental foliage plant that displays an anomalous range of variations in its leaf size, shape, and color pattern (Mollick et al., 2011). The *C. variegatum*, one of the oldest known edible plant, which is cultivated through the tropical forest and also known for its medicinal properties, such as antioxidant, antifungal, antiviral, and cytotoxic activities (Saffoon et al., 2010; Awoyinka et al., 2012; Saffoon et al., 2014; Larson et al., 2014; Njoya et al., 2014; Anim et al., 2016). Most cultivars of *C. variegatum* are widely used as vegetables in northern Thailand, such as ingredient of curry (namely keang-kea-kai) (Pharmacy, CMU).

It is widely accepted that diets rich in fruits and vegetables which contain polyphenolic compounds can lower the risk of age-related chronic diseases, including cardiovascular disease, neurodegeneration, and cancer (Parke, 1999; Rio et al., 2013). Blueberry, strawberry, pomegranate, papaya, apple, green tea, walnut, saffron, cinnamon, garlic, and ginger are among edible plants exhibiting neuroprotective effects against Alzheimer's disease (AD) (Lomarat et al., 2015). Alzheimer's disease is the leading neurodegenerative disease characterized by an age-dependent loss of memory and an impairment of multiple cognitive functions. The exact cause of AD is still

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Received: date; May 19, 2020 Accepted: date; October 12, 2020 Published: date April 5, 2021

uncertain. (Anekonda and Reddy 2005; Lomarat et al., 2015; Rahman 2017). The previous studies presented many qualitative data about secondary metabolites of *C. variegatum* leaves, such as alkaloids, anthraquinones, cardiac glycosides, saponins, phlobatannins, tannin, cardenolides, steroids, flavonoids, phenols, phyllates, and phytic acid (Ogunwenmo et al., 2007). However, an extensive study to investigate the total polyphenolic and biological activities related to AD has not been carried out on *C. variegatum* leaves in Thailand.

This study aimed to perform the qualitative and quantitative phytochemical analyses and determine antioxidant and anti-acetylcholinesterase activities of *C. variegatum* leaves extracts both spirale and broad cultivar from Thailand. This results could promote *C. variegatum* to functional foods for decreasing the risk of degenerative diseases related to the prevention of AD.

MATERIALS AND METHODS

Plant material: The broad and spirale cultivars of *Codiaeum variegatum* (L.) Blume leaves was collected from Lamphun province, northern of Thailand. The collected plants lies at the latitude of 18.515884, the longtitude of 98.956655, and the elevation of 298 m.

Enzymes and chemicals: 6-Hydroxy-2,5,7,8-tetra methylchroman-2-carboxylic acid **(Trolox)**, 2,2-diphenyl-1-picryl hydrazyl **(DPPH)** were purchased from Sigma-Aldrich. Analytical grade methanol was purchased from Merck. Acetylthiocholine iodide **(ATCI)**, lyophilized powder of AChE (a purified enzyme from eel [Electrophorus electricus] type VI-s, 425.94 units/mg, 687 U/mg protein), 5,5′-dithiobis[2-nitrobenzoic acid] **(DTNB)**, galantamine and bovine serum albumin **(BSA)**, were obtained from Sigma-Aldrich. Fifty mM Tris-HCl (pH 8.0) was used as a buffer throughout the experiment. The lyophilized enzyme was prepared in the buffer to obtain 100 U/mL. It was then further diluted with the buffer containing 0.1% BSA to yield 0.22 U/mL for the microplate assay. β-Amyloid 1-42 and thioflavin T **(ThT)** was purchased from American Peptide Company (Sunnyvale, CA, USA) and Sigma-Aldrich, respectively. A stock ThT solution (1 mg/mL; 3.14 mM) was prepared in deionized, distilled water. Forty microliters of the ThT solution were added to 50 mM Tris buffer, pH 7.4, to yield a final 25 mL of 5.0 μM ThT solution. The β-Amyloid stock solution was prepared by dissolving β-amyloid in 10 mM NaOH (0.5 mg/mL) and stored at -70°C until used. β-Amyloid stock solution (216.4 μL) was added to the Tris-HCl buffer (738.6 μL) to yield a final concentration of 25 μM β-amyloid solution.

Preparation of plant extract

C. variegatum (spirale and broad) leaves were dried in an oven at 45°C and then ground with a Moulinex® grinder. The powder (1.5 kg) was successively extracted with 95% ethanol with a maceration method. Each extract was filtered and dried by evaporating under vacuum and stored at 4°C for further research. The extraction was carried out in triplicate.

A B

Figure 1 Leaves of broad (A) and spirale (B) cultivars of *Codiaeum variegatum* (L.) Blume

Qualitative phytochemical tests

The phytochemical qualitative tests were slightly modified the standard methods of Bijekar, and Gayatri (2015).

Test for alkaloids

The test solution of the extracts was dissolved in 95% ethanol, and the solution was extracted with diluted HCl or H_2SO_4 and the acid layer was taken and tested for the presence of alkaloids. To 2 mL of the acid layer of the test solution, add 2 mL of Dragendroff's, Marme's, Mayer's, Tannic Acid, Valser's, and Wagner's Reagent.

Test for anthraquinone (Borntrager's test)

The reaction mixture contained 6 N freshly prepared HCl (3 mL) and the extract then heat for 10 min, 3 mL dichloromethane was added and shaken for 5 min. The extract was filtered, and the filtrate was shaken with an equal volume of 10% ammonia solution. A pink violet or red color in the ammoniacal layer (lower layer) indicates the presence of anthraquinone.

Test for carbohydrates (Molisch's test)

The extract mixed with 1mL of the **α**-napthol solution, and added concentrated H_2SO_4 through the sides of the test tube. Purple or reddish-violet color at the junction of the two liquids revealed the presence of carbohydrates.

Test for cardiac glycosides

The cardiac glycosides consisted of three structures, basically steroids, unsaturated lactone ring, and deoxy-sugar. The alcoholic extract and 0.5 mL of strong lead acetate solution were added, shaken, and filtered. The filtrate was extracted with an equal volume of dichloromethane. **Steroid Backbone (Liebermann-Burchard's Test):** The dichloromethane extract was evaporated to dryness, and the residue was dissolved with acetic anhydride and added concentrated H_2SO_4 through the sides of the evaporating disc. **Unsaturated Lactone Ring (Kedde's Reagent):** The test solution of the extracts was dissolved in 95% ethanol. The extract was evaporated to dryness, and Kedde's Reagent A (2% 3,5-dinitrobenzoic in ethanol) and B (5% KOH in ethanol) were added to the residue. Purple or reddish-violet color reveals the presence of an unsaturated lactone ring. **Deoxy-Sugar (Keller-Kiliani Test):** The dichloromethane extract was evaporated to dryness, and residue was dissolved in 3 mL of glacial acetic acid, followed by an addition of a few drops of $FeCl_3$ solution. The resultant solution was transferred to a test tube containing 2 mL of the concentrated H_2SO_4. The reddish-brown layer was formed, which turns bluish-green after standing due to the presence of deoxy sugar.

Test for coumarins

In a test tube, 1 g of each of the extracts was placed and covered with filter paper moistened with 20% NaOH, then heated on a water bath for 10 minutes. The filter paper was examined under UV light; yellow fluorescence indicated the presence of coumarins.

Test for flavonoids (Shinoda's test)

The test solution of the extracts was dissolved in 95% ethanol. To this, a small piece of magnesium foil metal was added; this was followed by 3-5 drops of the concentrated HCl. The intense cherry red color indicated the presence of flavonoids.

Test for tannins (Ferric chloride Test)

The 95% ethanolic extract was treated with a 1% ferric chloride test solution. The resultant color was investigated. A blue color indicated the presence of hydrolyzable tannin. Into 10 mL of freshly prepared KOH in a beaker, 0.5 g of the extract was added and shaken to dissolve. A dirty precipitate observed indicates the presence of tannin.

Test for terpenoids and steroids (Liebermann-Burchard's Test)

The dichloromethane extract was evaporated to dryness and the residue was dissolved with acetic anhydride. The concentrated H_2SO_4 was then added through the sides of the evaporating disc. A reddish-brown coloration of the interface indicated the presence of terpenoids. A blue coloration of the interface indicated the presence of steroids.

Quantitative phytochemical test

Determination of total phenolic content

The total phenolic content of the 95% extracts was determined using the Folin and Ciocalteu reagent, followed the method as described by Sankhalkar and Vernekar (2016) with slight modifications. Sample and standard readings were made using a microplate reader (M200, Tecan, Switzerland) at 765 nm against the reagent blank.

The test sample (20 µL) was mixed with 100 µL of Folin-Ciocalteu's phenol reagent. After 5 min, 80 µL of saturated sodium carbonate solution (7.5% w/v in water) was added to the mixture, and the volume was made up to 280 µL with distilled water. The reaction was kept in the dark for 30 min, and after centrifuging, the absorbance of blue color from different samples was measured at 765 nm. The phenolic content was calculated as gallic acid equivalents (mg GAE/g extract) base on a standard curve of gallic acid. All determinations were carried out in triplicate.

Determination of total flavonoid content (Aluminium chloride colorimetric method)

The 95% extracts (100 µL) were separately mixed with 100 µL of 10% aluminum chloride. After mixing, the solution was incubated for 10 min at room temperature. The absorbance of the reaction mixtures was measured against blank at 415 nm wavelength with a microplate reader (M200, Tecan, Switzerland). The concentration of total flavonoid content in the test samples was calculated from the calibration plot, quercetin was used to make the standard calibration curve and expressed as mg quercetin equivalent (mg QE)/g of extract. All the determinations were carried out in triplicate (Sankhalkar and Vernekar, 2016).

DPPH radical scavenging assay

The antioxidant potency of the tested extracts and compounds was assessed by using DPPH in a microplate, which was modified based on a method of Phanthong et al. (2015). DPPH in MeOH (1 mM) was added to the reaction mixture in triplicate and incubated in the dark at room temperature for 10 min. The absorbance was measured at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation.

$$\%\text{inhibition} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

Where Abs is the absorbance

Determination of anti-acetylcholinesterase activity

The microplate assay was performed by the modified Ellman's colorimetric method using 96-well plates. Briefly, 125 µL of 3 mM DTNB in buffer containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$, 25 µl of 15 mM ATCI in deionized water, 50 µL of buffer containing 0.1 % BSA and 25 µL of the sample (in buffer containing 10 % methanol) were added to the wells followed by 25 µL of 0.22 U/mL AChE. Methanol, which has no effect on the assay, was used as the solvent. The absorbance was measured at 405 nm before adding the enzyme and was measured every 45 s for 5 cycles again, after adding the enzyme, by a microplate reader (M200, Tecan, Switzerland). The assay was done in triplicate. Galantamine was used as a positive control (Ellman, 1961). The AChE inhibitory activity was calculated from the differences between the absorbance values of the control and the sample and expressed as percentage inhibition. The percentage of enzyme inhibition was calculated as

$$\%\ \text{AChE inhibition} = \{[(A-B) - (C-D)]/(A-B)\} \times 100$$

Where A is the absorbance of the control (reagent + methanol + enzyme)

B is the absorbance of blank of control (reagent + methanol)

C is the absorbance of the sample (reagent + sample + enzyme)

D is the absorbance of blank of the sample (reagent + sample + methanol)

The concentration that inhibited 50 % of AChE activity (IC_{50}) was obtained from a graph that was plotted between the percentage of AChE inhibition and the concentration of the sample.

Statistical analysis

The results of the total phenolic content, total flavonoid content, the DPPH free radical scavenging activity, and anti-AChE activity are presented as the average and standard deviation (mean ± SD).

RESULTS AND DISCUSSION

The ethanolic extracts of spirale and broad cultivar of *C. variegatum* (ESC and EBC) were filtered and dried by evaporating under vacuum to yield 13.31±0.54 and 13.68±0.50 % of dry weight, respectively. The results of the qualitative phytochemical analysis were presented in **Table 1**. The results of the qualitative phytochemical test of ESC and EBC supported the results from previous report which indicated that *C. variegatum* contained many phytochemicals, such as flavonoids, alkaloids, steroids, terpenoids, and cardiac glycosides (Bijekar and Gayatri, 2015).

Table 1 Qualitative phytochemical analysis of the ethanolic extract of spirale and broad cultivar of *C. variegatum* (ESC and EBC)

Phytochemical	Test	EBC	ESC
Flavonoids	Shinoda's Test	+	+
Tannins	Ferric Chloride Test	ND	ND
Alkaloids	Dragendorff's	+	+
	Marme's	+	+
	Mayer's	+	+
	Valser's	+	+
	Wagner's	+	+
	Tannic acid	+	+
	Vapor NaOH Reaction	+	+
Coumarins	Modified Borntrager's Test	ND	ND
Anthraquinones	Liebermann-Burchard Test	ND	ND
Terpenoids	Liebermann-Burchard Test	+	+
Steroids		+	+
Unsaturated Lactone Rings	Kedde's Reagent	ND	+
Deoxy-Sugar	Keller-Kiliani Test	+	+
Carbohydrates	Molisch's Test	ND	ND
Saponins	Froth Test	ND	ND

ND = not detected

Due to the complex nature of phytochemicals, this phytochemical screening analysis was considered a primary screening. Polyphenols, including flavonoids, need to be determined. Both ESC and EBC contained amount of total phenolic at 17.99±0.02 and 30.35±0.10 mg GAE/g extract, respectively (**Table 2**). Total flavonoid content of ESC and EBC were 39.30±0.20 and 34.72±0.67 mg QE/g extract, respectively. The phytochemicals such as flavonoids and phenols, which were rich in both ESC and EBC may contribute to providing the medicinal properties to the plant. The ESC and EBC were subjected to antioxidant activity assays. In the DPPH radical scavenging assay,

the ESC and EBC possessed moderate antioxidant activity with IC_{50} = 8.00±0.60 and 6.88±0.40 mg/mL, respectively, which was comparable with that of Trolox. The results, including total phenolic and flavonoids content and antioxidant activity, were in accordance with those from the study of Saffoon et al. (2014). The mechanism of action of phenolics and flavonoids is through scavenging or chelation and its effects on membrane permeability. The also acted on membrane-bound enzymes, like ATPase and phospholipase (Tsao, 2010).

The ESC exhibited potent anti-AChE activity with IC_{50} = 551.36 µg/mL, while EBC was inactive (**Table 2**). This was considered the first report on anti-AChE activity in *C. variegatum* leaves. The previous studies revealed that the potential of polyphenols, including flavonoids to improve neurological health, were releted to several mechanisms. These involved with their abilities to interact with intracellular neuronal and glial signaling, to influence the peripheral and cerebrovascular blood flow, and to reduce neuronal damage and losses induced by neurotoxins and neuroinflammation (Rio, 2013). It was interesting to perform a further study on the spirale cultivar to determine its activity related to the prevention of chronic diseases, including cardiovascular disease, neurodegeneration, and cancer as well as its toxicity.

Table 2 Evaluation of total phenolic content, total flavonoid content, DPPH radical scavenging activity, and anti-AChE activity in *C. variegatum* (L.) extracts

***C. variegatum* (L.) cultivars**	**ESC**	**EBC**	**Positive control**
Total phenolic content (mg GE/g extract)	17.99±0.02 (26.51 mg/100g fresh weight)	30.35±0.10 (43.71 mg/100g fresh weight)	-
Total flavonoid content (mg QE/g extract)	39.30±0.20 (58.56 mg/100g fresh weight)	34.72±0.67 (50.00 mg/100g fresh weight)	-
DPPH radical scavenging activity IC_{50} (mg/mL)	8.00±0.60	6.88±0.40	0.0041 (Trolox)
anti-AChE activity IC_{50} (µg/mL)	551.36	ND	1.71 (Galantamine)

Values are presented as the mean ± SD (n = 3), IC_{50} = the concentration of test sample that produces 50% inhibition, NA = not assessed, ND = not determined, due to the inhibition is below 50% at the concentration tested.

CONCLUSION

It can be concluded from this study that the *C. variegatum* leaves were rich in flavonoids and phenolics, and they expressed antioxidant and anti-AChE activities. It was interesting to perform further studies on its biological studies, active compounds, and toxicity to support the use of the spirale leave cultivar of *C. variegatum* as a useful agent for the prevention of AD.

ACKNOWLEDGMENTS

The grants funded by University of Phayao supported this research. The authors would like to thank the Faculty of Pharmacy, University of Phayao, Chiang Mai University, and Mahidol University for providing instruments.

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