Songklanakarin J. Sci. Technol.
42 (5), 1084-1090, Sep. - Oct. 2020

http://www.sjst.psu.ac.th

Original Article

A green and effective method using oils to remove chlorophyll from *Chromolaena odorata* (L.) R.M. King & H. Rob

Suppalak Phaisan[1], Gorawit Yusakul[1, 2*], Attapon Sakdamas[1], Nattapon Taluengjit[1], Seiichi Sakamoto[3], and Waraporn Putalun[4, 5]

[1] *School of Pharmacy, Walailak University, Tha Sala, Nakhon Si Thammarat, 80160 Thailand*

[2] *Drug and Cosmetics Excellence Center, Walailak University, Tha Sala, Nakhon Si Thammarat, 80160 Thailand*

[3] *Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, 8128582 Japan*

[4] *Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand*

[2] *Research Group for Pharmaceutical Activities of Natural Products Using Pharmaceutical Biotechnology, Khon Kaen University, Mueang, Khon Kaen, 40002 Thailand*

Received: 8 February 2019; Revised: 7 July 2019; Accepted: 17 July 2019

Abstract

Chromolaena odorata (L.) R.M. King & H. Rob has excellent wound healing effects. Therefore, it is useful for cosmeceutical purposes; however, the dark-greenish appearance of its extract limits its attractiveness for product development. This study aimed to develop an oil-based system for chlorophyll removal. Dried ethanolic extracts were dissolved in different aqueous EtOH solvents, and then the chlorophyll was partitioned to the oil phase. The efficiency of chlorophyll removal was monitored using a spectrophotometer. The recoveries of flavonoid and phenolic contents and the antioxidant activity were determined after the treatment. When the solutions of extract were prepared in 25% (v/v) - 75% (v/v) EtOH in water, the efficacy of chlorophyll removal by oil was higher than 85%. A higher concentration of EtOH resulted in a lower chlorophyll removal efficiency; however, the recoveries of phenolic and flavonoid contents and antioxidant activity improved. Palm oil showed efficiency higher than hexane for chlorophyll removal and high recoveries of the beneficial phytochemicals. This system can be applied in the fields of natural products-based health product development and in phytochemical studies.

Keywords: *Chromolaena odorata*, chlorophyll removal, oil, antioxidant, flavonoids

1. Introduction

Chromolaena odorata (L.) R.M. King & H. Rob, called Siam weed (“Sap suea” in Thai), has received much attention due to its various pharmaceutical activities. In Thailand and other tropical countries, fresh leaves of *C. odorata* have traditionally been used to stop bleeding and enhance wound healing. Scientific investigations revealed that the extract of *C. odorata* exhibited various activities involved in wound healing. For the reasons mentioned above, the extract of *C. odorata* may be useful as an active ingredient of wound healing and cosmeceutical products.

The leaves of *C. odorata* contain various classes of compounds such as flavonoids (Phan *et al.*, 2001), phenolics (Phan *et al.*, 2001), alkaloids (Biller, Boppré, Witte, & Hart mann, 1994), and essential oils (Pisutthanan *et al.*, 2006). An

*Corresponding author
Email address: gorawit.yu@wu.ac.th

ethanol extract of *C. odorata* was fractionated via column chromatography, and the flavonoids' and phenolic fractions showed strong antioxidant activity in protecting cultured skin cells against oxidative damage. The phenolic fraction was composed of *p*-coumaric acid, *p*-hydroxybenzoic acid, ferulic acid, and vanillic acid (Phan *et al.*, 2001), whereas tamarixetin, scutellarein tetramethyl ether, quercetin, aromadendrin 4'-methyl ether, eriodictyol 7,4'-dimethyl ether, naringenin 4'-methyl ether, taxifolin 4'-methyl ether, sinensetin, and kaempferol were identified in the flavonoid fraction (Vijaya raghavan, Rajkumar, Bukhari, Al-Sayed, & Seyed, 2017). Similar to the traditional use of *C. odorata*, the extract of *C. odorata* leaves exhibited various mechanisms involved in the wound healing enhancing effect. When taken orally, a water extract of *C. odorata* showed beneficial effects against inflammation (Owoyele, Adediji, & Soladoye, 2005). Further investigation indicated that the flavonoid fraction of *C. odorata* ethanol extract exhibited analgesic, anti-inflammatory, and antipyretic activities (Owoyele *et al.*, 2008). Additionally, pharmacological activities directly related to wound healing, such as antioxidant activities, were also reported to protect fibroblasts and keratinocytes and to enhance cutaneous wound healing (Phan *et al.*, 2001; Thang, Patrick, Teik, & Yung, 2001). This effect was confirmed in an *in vivo* investigation using topical application of a *C. odorata* leaf aqueous extract. *C. odorata*-treated groups showed a faster reduction in wound area than the control and Betadine-treated groups, and it was found that the use of *C. odorata* extract increased collagen synthesis and its stabilization at the wound site (Vijayaraghavan, Rajkumar, & Seyed, 2017). Overall, *C. odorata* extracts have shown high potential for topical use to promote healing of excisions, lesions, and burns.

For cosmetic or cosmeceutical product development of topical formulations, the formulation appearance is important to consumers, particularly in cosmetics for external use. Therefore, formulation development for an attractive appearance of the product is considered as much as its therapeutic efficiency. Some pharmacologically active natural products are contained in the plant leaves; however, chlorophyll content in the extract may diminish the appeal of these natural products.

For phytochemical analysis, chlorophyll also interferes in analytical procedures. Therefore, the sample preparation must remove chlorophyll or discoloration of chlorophyll before analysis, for improved accuracy of the results. In a recent study (Scheepers, Malan, Du Preez, & Dyk, 2018), UV radiation and activated charcoal were used for sample pre-treatment, and the results showed that UV irradiation was more effective for sample bleaching before HPLC analysis. In another study (Rosaria, Bresciani, Lami, & Morabito, 2017) chlorophyll a was extracted using 90% acetone and phosphate buffer for more accurate analysis of phycocyanin and allophycocyanin by spectrophotometer. Although many methods have been developed for chlorophyll removal from extract to reduce interference in analyses, these methods are not completely effective, and the procedures are cumbersome.

A previous study (Bahmaei, Sadat Sabbaghian, & Farzadkish, 2005) reported a method for removing chlorophyll from canola oil using sulfuric and phosphoric acids that can reduce the chlorophyll content from up to 30 ppm to less than 0.01 ppm. Another study (Li *et al.*, 2016) used a saponification method for chlorophyll removal. Chlorophyll can be saponified in the presence of sodium hydroxide resulting in higher hydrophilicity of chlorophyll and then can be easily separated from the lipid, enabling the removal of chlorophyll from the system. Sand and a drying agent were used in the process of grinding the leaves, and then they were directly extracted with acetone. The chlorophyll was extracted from spinach leaves extract. After that, the chlorophyll was separated from the extract by strong ion-exchange resin, which interacts with magnesium ions of the chlorophyll molecules. This method was easy to perform and decreased the interference by chlorophyll in thin layer chromatography (Quach, Steeper, & Griffin, 2004).

These abovementioned methods are difficult to apply for preparing natural products because these extreme treatments affect the stability of bioactive compounds. Activated charcoal was also used to adsorb chlorophyll and other pigments from the extract of *Centella asiatica* (Puttarak & Panichayupakaranant, 2013). Activated bentonites are another type of adsorbents for removing chlorophylls from oils (Makhoukhi, Didi, Villemin, & Azzouz, 2009). In natural product research and development, the use of activated bentonites and charcoal agents has the drawback of non-specific adsorption that results in losses of desired bioactive compounds. Additionally, organic solvents such as hexane and carbon tetrachloride are typically used for separating chlorophylls from the compounds of interest. Although hexane and carbon tetrachloride are utilized for removing chlorophyll from plant extract on the scale of experimental work or in the laboratory, they are toxic to humans and to the environment and have low efficacy in chlorophyll removal. Effective and safe methods are needed for industrial production of cosmeceutical ingredients from plant leaves. Because the dark-greenish color of *C. odorata* extract is caused by chlorophyll a less polar, lipophilic vegetable oil can separate chlorophyll from the extract via a "like dissolves like" concept. This approach does not require organic solvents as the previous methods, so it can be safe and free from solvent residues in the extract. Moreover, vegetable oils are friendly to humans and the environment.

Therefore, it is crucial to develop a green system for the effective removal of chlorophyll in the preparation of natural products. This system supports the development of bioactive compounds originating from plant leaves for external cosmetic products and reduce interference in phytochemical analyses. In this study, we use an oil-based system for removing chlorophyll from the extract of *C. odorata*. This requires simple processing based on the partitioning technique; the procedure is easy to perform, does not affect the content of bioactive flavonoids, and is more effective than the conventional partitioning using hexane. Besides, the method is cheap and friendly to humans and the environment.

2. Materials and Methods

2.1 Chemical reagents

C. odorata leaves were identified and collected at Walailak University, Nakhon Si Thammarat, Thailand by Dr. Gorawit Yusakul. The Folin-Ciocalteu reagent was obtained from Merck (Darmstadt, Germany). Rutin (RU, ≥97%) was

purchased from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). Gallic acid (GA, 97.5-102.5%), quercetin (QUE, ≥95%), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). EtOH and hexane of analytical reagent grade were supplied by RCI Labscan Limited (Bangkok, Thailand). All of the other chemicals used in this study were analytical grade. Vegetable oils, including coconut, palm, soybean, corn, sunflower, rice bran, and canola oils were commercial products used for food.

2.2 Extraction of *C. odorata*

The dried and powdered *C. odorata* leaves (100 g) were extracted with EtOH (2 L, 95%) by maceration for 24 hours at room temperature (30 °C approximately). Then, the extract was collected via filtration. The remaining residue was extracted again using the same procedures. Then, all of the extract solutions were pooled and concentrated by a vacuum rotary evaporator, and then the mixture was lyophilized. The crude extract was obtained.

2.3 Chlorophyll removal by liquid-liquid extraction with oils

The EtOH extract (0.5 g) of *C. odorata* was dissolved in various solvents including water, and 25% (v/v), 50% (v/v), and 75% (v/v) EtOH in water. Then, these solutions were mixed using vortex with palm oil at the volume ratio of 1:1. The mixture was separated into two phases by centrifugation at 4500 rpm for 10 min at room temperature. The aqueous phase was collected, and the oil phase was discarded. For the only extract dissolved in 75% (v/v) EtOH in water, the aqueous phase was partitioned for further two times with fresh oil. The efficiency of chlorophyll removal from the extract was analyzed using a spectrophotometer. Then, the treated extract was dried, and the yield of the extract after treatment was recorded. The contents of flavonoids and phenolic compounds in the obtained extracts were determined. Also, the antioxidant activity of the treated extract was investigated using the DPPH scavenging method.

2.4 Determination of chlorophyll removal efficiency

The chlorophyll removal efficiency was determined following the method of (Lichtenthaler & Buschmann, 2001) with some modifications. The absorbance (at 660 nm) of extracts was recorded using a microplate spectrophotometer (Eon™, BioTek Instruments, Inc., VT, USA), and the absorbances of the extract recorded before and after the treatment with oil were compared. Chlorophyll removal efficiencies were calculated using the following equation:

$$\text{Chlorophyll removal efficiency (\%)} = \frac{Abs_B - Abs_A}{Abs_B} \times 100 \quad (1)$$

where Abs_B is the absorbance of the extract prior to the chlorophyll removal and Abs_A is the absorbance of the extract after chlorophyll removal.

In addition, the efficiencies for chlorophyll removal using hexane and palm oil were compared. The *C. odorata* extract was dissolved in 75% (v/v) EtOH in water and then partitioned with 4 changes of fresh palm oil or hexane. For each partitioning step, the amount of the chlorophyll remaining in the extract was monitored.

2.5 Determination of total phenolic content

The total phenolic content (TPC) was measured using the Folin-Ciocalteu colorimetric method (Rabie *et al.*, 2016) with some modifications. Gallic acid (15.6 – 500 µg/ml) was used as a reference standard. The *C. odorata* extract was prepared in a series of concentrations (0.78 – 6.25 mg/ml in 50% (v/v) EtOH in water) as the working solution for TPC determination. Twenty microliters of the sample or gallic acid solutions were added in each well of a 96-well plate (n=3) and then mixed with the Folin-Ciocalteu reagent (100 µl) and 7% (w/v) Na_2CO_3 (80 µl). The mixtures were then incubated at room temperature for 30 min, and the absorbance was measured at 760 nm. The TPC values were calculated from the calibration curve of GA and expressed as mg GA equivalence (eq.)/mg of extract. Using the below equation, the TPC was calculated according to the yields (mg unit) of the extract obtained after partition, and TPC after treatment was referred to crude extract amount (0.5 g) used for the partition. Therefore, the TPC is the recovery yield of phenolic compounds after the partition that can be compared to the TPC of the nonpartitioned extract.

$$\text{TPC} = \frac{\text{Content (mg GA eq./ mg of extract)} \times \text{yield of partitioned extract (mg)}}{\text{Amount of extract used for partition (g)}} \quad (2)$$

2.6 Determination of total flavonoid content

The total flavonoid content (TFC) was determined by the aluminum chloride colorimetric method (Rabie *et al.*, 2016) with some modifications. Briefly, sample solutions were prepared at the concentrations of 0.31-25 mg/ml using 50%(v/v) EtOH in water as the solvent. Standard RU (3.90 – 500 µg/ml) and QUE (0.781 – 100 µg/ml) solutions were prepared using the same solvent. Each sample or the standard concentration (100 µl) was added to each well of a 96-well plate (n=3). Next, $NaNO_2$ (20 µl, 5% (w/v) in water) was added to each well and mixed. After incubation for 6 min, $AlCl_3 \cdot 6H_2O$ (35 µl, 10% (w/v) in water) was added. The mixture was mixed again and then was allowed to stand for 6 min. The absorbance was measured at 430 nm using a spectrophotometer. TFCs were calculated according to the calibration curves of both RU and QUE and were expressed as mg RU eq./mg of extract and mg QUE eq./mg of extract, respectively. Using the following equation, the TFC was calculated according to the yields (mg unit) of extract after partitioning treatment and initial crude extract (0.5 g) used for the partition. Finally, the recovery yield of the flavonoid compound could be compared to the TFC of the nonpartitioned extract.

$$\text{TPC} = \frac{\text{Content (mg RU eq./ mg of extract)} \times \text{yield of partitioned extract (mg)}}{\text{Amount of extract used for partition (g)}} \quad (3)$$

$$TPC = \frac{\text{Content (mg QUE eq./ mg of extract) x yield of partitioned extract (mg)}}{\text{Amount of extract used for partition (g)}} \quad (4)$$

2.7 Determination of antioxidant activity

The antioxidant activity of the extract was determined by the DPPH assay. The extracts were prepared with the concentrations of 0.198 – 1.50 mg/ml in 50% (v/v) EtOH in water. The solutions of QUE and RU (0.39 – 25 µg/ml) were used as the reference antioxidant compounds. Briefly, each solution of extract or reference compounds (100 µl, 0.198 – 1.50 mg/ml) were mixed with a DPPH solution (100 µl, 0.2 mM), and then incubated in the dark at room temperature for 30 min. Then, the absorbance of the mixture was measured at 517 nm. The ability of the sample to scavenge the DPPH radical was calculated according to

$$\text{DPPH Scavenging effect (\%)} = \frac{Abs_{DPPH} - (Abs_s - Abs_{sb})}{Abs_{DPPH}} \times 100 \quad (5)$$

where Abs_{DPPH} is the absorbance of the DPPH solution without the extract. Abs_s and Abs_{sb} are the absorbances of the samples in the presence and absence of DPPH solution, respectively. The relationship between the sample concentrations and the percentage of scavenging was plotted and analyzed by linear regression. Then, the extract concentration providing a 50% DPPH scavenging effect was calculated and is reported as the IC_{50}.

2.8 Statistical analysis

The values are expressed as means from triplicate experiments ± SD. The significance threshold was taken as $p < 0.05$ in one-way analysis of variance (ANOVA), followed by Tukey's honest significant difference test to post hoc assess the differences between groups.

3. Results and Discussion

3.1 Effect of solvents on chlorophyll removal using oils

The EtOH extraction via maceration of *C. odorata* yielded 7.45% (w/w) by dry weight. One-half gram of the extract was dissolved in 20 ml of various solvents, and then the extracts were partitioned with palm oil by equal volume. For the extracts dissolved in water, 25% (v/v) EtOH, and 50 (v/v) EtOH in water, a single partition is sufficient to remove the chlorophyll (>80%), while the extract prepared in 75% (v/v) EtOH in water requires the use of two further partitions to increase the chlorophyll removal efficiency. The results indicate that the compounds of *C. odorata* extract precipitated when the extract was dissolved in water. By contrast, the compounds are dissolved when the solvent contains EtOH. This may be due to the low water solubility of flavonoid aglycones in *C. odorata* (Phan *et al.*, 2001). The phytochemicals of *C. odorata* are soluble in EtOH, but the higher EtOH portion in the solvent resulted in poorer chlorophyll removal efficacy. Therefore, repeated partitioning was required for the extract prepared in the 75% (v/v) EtOH in water. The oil-treated extracts were collected and dried using a vacuum rotary evaporator and a lyophilizer. The yields of *C. odorata* extracts obtained after partition and drying were calculated by comparison with the initial content (0.5 g) of the extract (Table 1). The extraction yield ranged from 24.08% to 58.23%. The solvent system with 50% EtOH showed the highest extraction yield over those with water, 25% (v/v) EtOH and 75% (v/v) EtOH in water. Therefore, this solvent mixture of EtOH and water may be optimal between hydrophilicity and lipophilicity, giving rise to high extraction yield. Although the yield after oil partition using 75% (v/v) EtOH in water as the solvent is quite low due to three partition steps, the highest total flavonoid content and the highest antioxidant activity were obtained using this system.

The TPC of *C. odorata* extract after chlorophyll removal by palm oil in various solvents was determined using the Folin-Ciocalteu reagent, and the results are presented in Table 1. The calibration curve was obtained using a series of

Table 1. Effect of the solvents on the partition yield, chlorophyll removal efficiency, total flavonoid content, and total phenolic content of *C. odorata* extract after chlorophyll removal using palm oil.

Solvent for partition[a]	Partition yield (%)	Chlorophyll removal efficacy (%)	TPC	TFC	
			[mg GA eq./ 0.5 g extract]	[mg RU eq./ 0.5 g extract]	[mg QUE eq./ 0.5 g extract]
Water	45.20	81.40 ± 0.73	21.70 ± 1.80*	1.76 ± 0.08*	0.35 ± 0.01*
25% EtOH	24.08	97.30 ± 0.03	6.91 ± 0.61*	2.74 ± 0.02*	0.54 ± 0.01*
50% EtOH	58.23	92.54 ± 0.03	11.21 ± 0.28*	12.31 ± 0.17*	2.47 ± 0.07*
75% EtOH	35.50	85.35 ± 0.44	14.89 ± 0.41*	22.08 ± 0.11**	4.40 ± 0.02**
Control[b]	-	-	33.44 ± 1.37	20.86 ± 0.30	3.88 ± 0.09

The values are presented as the mean of three replicates ± standard deviation (Mean ± SD).
[a]All samples were dissolved in the specified solvent, and then a partition step was performed with palm oil. The extract dissolved in 75% EtOH was partitioned an additional 3 times with fresh oil.
[b]The nonpartitioned ethanol extract of *C. odorata* was used as the control.
*Significant decrease ($p < 0.01$ level) compared with the control.
**Significant increase ($p < 0.01$ level) compared with the control.

GA concentrations. Linear regression with the GA concentrations in the range of 15.6 µg/ml – 0.5 mg/ml gave y= 4.272x+0.0375, R^2=0.9997, where x is the concentration of GA solution, and y is the absorbance at 760 nm. The TPC of the nontreated EtOH extract was used as a control to evaluate the recovery of TPC after the partition processes. In this study, only the aqueous ethanol phase was of interest and was determined for TPC and TFC, because phenolic and flavonoid compounds of *C. odorata* extract were mostly soluble in water and ethanol. Many previous studies have reported that both phenolics and flavonoids were more soluble in polar solvents than in non-polar solvents (Do *et al.*, 2014; Widyawati, Budianta, Kusuma, & Wijaya, 2014).

When the extract was dissolved in various solvents and partitioned with palm oils, the TPC in the obtained extract was lower than that for the control. This indicated that a portion of TPC was lost after the partition. The preparation of extract in water gave the highest phenolic content retention. When 25% (v/v) EtOH, 50% (v/v) EtOH and 75% EtOH (v/v) in water were used as the solvents, lower recovery of TPCs was obtained (Table 1).

The TFCs of *C. odorata* extracts processed for chlorophyll removal by palm oil are presented in Table 1. The calibration curves were obtained using RU and QUE as the reference standards, and the results were calculated and expressed as mg RU eq. per 0.5 gram of extract (mg RU eq./0.5 g extract) and mg QUE eq. per gram of extract (mg QUE/0.5 g extract). Linear regression for the concentration range 7.81–250 µg/ml RU gave y=0.0035x+0.0121, R^2 = 0.9992; and for 0.781–50.0 µg/ml QUE it gave y = 0.0182x + 0.0012, R^2 = 0.9998. TFCs in the extracts partitioned with oil were determined. The results showed that TFC recovery increased with EtOH proportion in the extraction solvent. This result is consistent with the results for TPC when 25–75% (v/v) EtOH mixtures were used as the solvents. Since phenolic compounds include components that are soluble and insoluble in water, the phenolic compounds of *C. odorata* may be mainly soluble in water. Based on the TFC results obtained in this study, the best solvent for the partition system was 75% (v/v) EtOH in water. Using this system, the TFC in the extract after chlorophyll removal was similar to the TFC in the extract prior to chlorophyll removal. Therefore, it was proved that the partition processing did not decrease the flavonoid content of the *C. odorata* extract. Therefore, the *C. odorata* extract dissolved in 75% (v/v) EtOH in water was partitioned with other oils to compare the chlorophyll removal efficiencies and retention of phytochemicals.

3.2 Effect of oils on chlorophyll removal

As indicated in the above section, 75% (v/v) EtOH in water was found to be the best for chlorophyll removal and recovery of bioactive flavonoids of *C. odorata*. Then, this solvent system (75% (v/v) EtOH in water) was chosen to study the effects of various oils on the extraction yields, chlorophyll removal efficiency, and recovery of *C. odorata* phytochemicals.

The chlorophyll removal efficiency is shown in Table 2. The extraction yields had the following order: soybean oil> palm oil > coconut oil > corn oil > canola oil > rice bran oil > sunflower oil (Table 2). These results indicate that choice of oil influences the recovery yield of the extract after the partition. TPCs were analyzed in the extracts that were dissolved in 75% (v/v) EtOH in water and partitioned with various oils, with results summarized in Table 2. The results indicate that the TPC recovery was highest with palm oil, soybean oil, and sunflower oil. The chlorophyll removal efficiency of sunflower oil was quite low. For all oils, chlorophyll removal results and the loss of TPC were compared to control. This study demonstrated that palm oil and soybean oil showed over 80% chlorophyll removal and high TPC recovery, and were therefore useful for chlorophyll removal. Table 2 also shows the TFC of *C. odorata* extracts after the chlorophyll removal by each oil. The TFC retention values for the extracts treated with various oils were in the rank order palm oil > canola oil > soybean oil > rice bran oil > sunflower oil > coconut > corn oil. The partitions with palm oil, canola oil, and soybean oil did not decrease the flavonoid content. Overall, based on the obtained results for the chlorophyll removal efficiency and TPC and TFC recoveries, palm and soybean oils were considered the best for chlorophyll removal from *C. odorata* extract.

Table 2. Effect of oil on the partition yield, total flavonoid content and total phenolic content of *C. odorata* extract after chlorophyll removal.

Oil for partition	Partition yield (%)	Chlorophyll removal efficacy (%)	TPC	TFC	
			[mg GA eq./0.5 g extract]	[mg RU eq. /0.5 g extract]	[mg QUE eq./0.5 g extract]
Coconut	32.35	85.59 ± 0.42	9.03 ± 0.11*	11.49 ± 0.09*	2.36 ± 0.06*
Palm	35.50	85.35 ± 0.44	14.89 ± 0.41*	22.08 ± 0.11**	4.40 ± 0.02**
Rice Bran	19.94	67.48 ± 0.63	8.63 ± 0.17*	19.13 ± 0.07*	3.77 ± 0.01
Soybean	50.81	80.52 ± 0.17	12.32 ± 0.15*	20.87 ± 0.15	3.87 ± 0.03
Corn	29.47	86.46 ± 0.89	4.65 ± 0.19*	7.13 ± 0.05*	1.44 ± 0.02*
Sunflower	19.39	61.87 ± 1.74	9.77 ± 0.48*	15.72 ± 0.08*	3.30 ± 0.18*
Canola	26.64	62.18 ± 0.45	4.43 ± 0.19*	21.47± 0.08**	4.29 ± 0.05**
Control[a]	-	-	33.44 ± 1.37	20.86 ± 0.30	3.88 ± 0.09

The values are presented as the mean of three replicates ± standard deviation (Mean ± SD).
*Significant decrease ($p < 0.01$ level) compared with the control.
**Significant increase ($p < 0.01$ level) compared with the control.
[a]The nonpartitioned ethanol extract of *C. odorata* was used as the control.

3.3 Determination of antioxidant activity

Antioxidant activities of *C. odorata* extracts play a major role in their pharmacological activity and wound healing effects. Therefore, the DPPH assay was applied to ensure that the chlorophyll removal by partitioning with oils does not degrade the pharmacological activity of *C. odorata*. In this study, the antioxidant activities are reported as IC_{50} in Table 3. The antioxidant activities were in the following rank order: 75% (v/v) EtOH in water > 50% (v/v) EtOH in water > 25% (v/v) EtOH in water > water. Based on these results, antioxidant activity increased with EtOH content. The activity was consistent with the results for TFC and lipophilic TPC. Therefore, it may be inferred that the antioxidant activity of *C. odorata* extract was due to both TFC and TPC. Overall, chlorophyll removal by oil did not result in the loss of antioxidant activity of *C. odorata* extract.

Table 3. Antioxidant activity of *C. odorata* extract after chlorophyll removal with palm oil.

Solvent for the partition[a]	Antioxidant activity (IC_{50}) (µg/ml)
Water	818.06
25% EtOH	445.71
50% EtOH	443.92
75% EtOH	409.20
Control[b]	528.64
QUE	14.34
RU	34.61

[a]Solvent utilized for the partition
[b]The nonpartitioned ethanol extract of *C. odorata* was used as the control

3.4 Comparison of chlorophyll removal efficiency with a conventional method

The efficiencies of chlorophyll removal using palm oil and hexane were compared and are presented in Figure 1 The extracts were partitioned with palm oil in parallel with hexane with four changes of fresh solvents. For every round of partition, chlorophyll retention in the extract was monitored using a spectrophotometer. The results showe that chlorophyll removal using palm oil was better than that with hexane for every partition round. The partition by palm oil shows increasing removal efficiency with a greater number of partition cycles. On the other hand, the removal efficiency of chlorophyll was stable after the second partition with hexane. The results show that chlorophyll removal with palm oil is more efficient than that with hexane. Hexane is an organic solvent used in the conventional method for chlorophyll removal from plant extracts (Ramluckan, Moodley, & Bux, 2014; Widodo, Khoiruddin, Ariono, Subagjo, & Wenten, 2018); however, it is toxic for humans and the environment. Any residual hexane remaining in the extract may give rise to toxicity. In this study, hexane was replaced by palm oil for chlorophyll removal. Palm oil is sufficiently inexpensive for development as an alternative solvent for chlorophyll removal from plant extracts. Therefore, this method will not only reduce the human efforts and environmental impacts of chlorophyll removal but also will increase the utility of palm oil, which is currently produced in excess of demand.

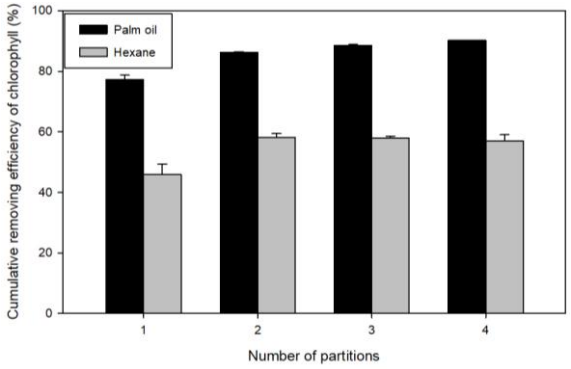

Figure 1. Comparison between palm oil and hexane of chlorophyll removal efficiency.

4. Conclusions

The recovery yield of *C. odorata* extracts obtained after partition and drying ranged from 24.08% to 58.23%. The lowest extraction yield was produced by 75% (v/v) EtOH in water; however, this system had high chlorophyll removal efficiency, high recovery of TPC and TFC and the highest antioxidant activity of the *C. odorata* extracts. Palm oil and soybean oil used as the solvent gave high TPC and TFC and high chlorophyll removal efficiencies (> 80%). Palm oil had higher efficiency than hexane, which is used as the solvent in the conventional chlorophyll removal method. Therefore, palm oil is of interest for the development of chlorophyll removal from plant extracts, due to its low cost, high efficiency, and safety.

Acknowledgements

We would like to acknowledge the support from The Thailand Research Fund [grant number IRN 61W0005] and School of Pharmacy, Walailak University, Thailand**.** This research was also partially supported by the Research Fund [grant number WU61313] from the Institute of Research and Innovation, Walailak University**.** The authors also would like to thank the facilities and support of The Center for Scientific and Technological Equipment, Walailak University, Thailand**.**

References

Bahmaei, M., Sabbaghian, E. A., & Farzadkish, E. (2005). Development of a method for chlorophyll removal from canola oil using mineral acids. *Journal of the American Oil Chemists' Society, 82*(9), 679-684. doi:10.1007/s11746-005-1128-8

Biller, A., Boppré, M., Witte, L., & Hartmann, T. (1994). Pyrrolizidine alkaloids in *Chromolaena odorata*. Chemical and chemoecological aspects. *Phytochemistry, 35*(3), 615-619. doi:10.1016/S0031-9422(00)90573-9

Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., Huynh, L. H., Soetaredjo, F. E., Ismadji, S., & Ju, Y. H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *Journal of Food and Drug Analysis, 22*(3), 296-302. doi:10.1016/j.jfda.2013.11.001

Li, T., Xu, J., Wu, H., Wang, G., Dai, S., Fan, J., *et al.* (2016). A saponification method for chlorophyll removal from microalgae biomass as oil feedstock. *Marine Drugs, 14*(9), 162. doi:10.3390/md14090162

Lichtenthaler, H. K., & Buschmann, C. (2001). Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. *Current Protocols in Food Analytical Chemistry, 1*(1), F4.3.1-F4.3.8. doi: 10.1002/0471142913.faf0403s01

Makhoukhi, B., Didi, M. A., Villemin, D., & Azzouz, A. (2009). Acid activation of bentonite for use as a vegetable oil bleaching agent. *Grasas y Aceites, 60*(4), 7. doi:10.3989/gya.108408

Owoyele, B. V., Oguntoye, S. O., Dare, K., Ogunbiyi, B. A., Aruboula, E. A., & Soladoye, A. O. (2008). Analgesic, anti-inflammatory and antipyretic activities from flavonoid fractions of *Chromolaena odorata*. *Journal of Medicinal Plant Research, 2*(9), 219-225.

Owoyele, V. B., Adediji, J. O., & Soladoye, A. O. (2005). Anti-inflammatory activity of aqueous leaf extract of *Chromolaena odorata*. *Inflammopharmacology, 13*(5-6), 479-484. doi:10.1163/156856005774649386

Phan, T.-T., Wang, L., See, P., Grayer, R. J., Chan, S.-Y., & Lee, S. T. (2001). Phenolic compounds of *Chromolaena odorata* protect cultured skin cells from oxidative damage: implication for cutaneous wound healing. *Biological and Pharmaceutical Bulletin, 24*(12), 1373-1379. doi:10.1248/bpb.24.1373

Pisutthanan, N., Liawruangrath, B., Liawruangrath, S., Bara mee, A., Apisariyakul, A., Korth, J., *et al.* (2006). Constituents of the essential oil from aerial parts of *Chromolaena odorata* from Thailand. *Natural Product Research, 20*(6), 636-640. doi:10.1080/14786410500462678

Puttarak, P., & Panichayupakaranant, P. (2013). A new method for preparing pentacyclic triterpene rich *Centella asiatica* extracts. *Natural Product Research, 27*(7), 684-686. doi:10.1080/14786419.2012.686912

Quach, H. T., Steeper, R. L., & Griffin, G. W. (2004). An improved method for the extraction and thin-layer chromatography of chlorophyll a and b from spinach. *Journal of Chemical Education, 81*(3), 385. doi:10.1021/ed081p385

Rabie, K., Mourad, K., Meryem, E. J., Zouhra, D., Abdelaziz, B., Yahia, C., *et al.* (2016). Antioxidant activities, total polyphenolic compounds and HPLC/DAD/MS phenolic profile of argan oil derived from two differents methods of extractions. *Journal of Chemical and Pharmaceutical Research, 8*(11), 173-179. doi:10.15761/iptg.1000 115

Ramluckan, K., Moodley, K. G., & Bux, F. (2014). An evaluation of the efficacy of using selected solvents for the extraction of lipids from algal biomass by the soxhlet extraction method. *Fuel, 116*, 103-108. doi: 10.1016/j.fuel.2013.07.118

Rosaria, L., Bresciani, M., Lami, A., & Morabito, G. (2017). Chlorophyll a interference in phycocyanin and allophycocyanin spectrophotometric quantification. *Journal of limnology, 77*(1), 169-177. doi:10.4081/jlimnol.2017.1691

Scheepers, J. C., Malan, S. F., Du Preez, J. L., & Dyk, S. V. (2018). The high performance liquid chromatography (HPLC) analysis of ultraviolet (UV) irradiated chlorophyll a and secondary plant compounds. *African Journal of Biotechnology, 10*(74), 16976-16985. doi:10.5897/AJB11.1999

Thang, P. T., Patrick, S., Teik, L. S., & Yung, C. S. (2001). Anti-oxidant effects of the extracts from the leaves of *Chromolaena odorata* on human dermal fibroblasts and epidermal keratinocytes against hydrogen peroxide and hypoxanthine-xanthine oxidase induced damage. *Burns, 27*(4), 319-327. doi:10.1016/S0305-4179(00)00137-6

Vijayaraghavan, K., Rajkumar, J., Bukhari, S. N., Al-Sayed, B., & Seyed, M. A. (2017). *Chromolaena odorata*: a neglected weed with a wide spectrum of pharmacological activities (Review). *Molecular Medicine Reports, 15*(3), 1007-1016. doi:10.3892/mmr.2017.6133

Vijayaraghavan, K., Rajkumar, J., & Seyed, M. A. (2017). Efficacy of *Chromolaena odorata* leaf extracts for the healing of rat excision wounds. *Journal of Veterinary Medicine, 62*(10), 565-578. doi:10.17221/161/2016-VETMED

Widodo, S., Khoiruddin, Ariono, D., Subagjo, & Wenten, I. G. (2018). Membrane separation for non-aqueous solution. *Materials Science and Engineering C-Materials for Biological Applications, 285*(1), 012008.

Widyawati, P. S., Budianta, T. D. W., Kusuma, F. A., & Wijaya, E. L. (2014). Difference of solvent polarity to phytochemical content and antioxidant activity of Pluchea indicia less leaves extracts. *International Journal of Pharmacognosy and Phytochemical Research, 6*(4), 850-855.