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Comparison Study on Antioxidant Capacity of *Chromolaena Odorata* Leaves Extract Obtained from Maceration and Ultrasound-assisted Maceration

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Abstract: *Chromolaena odorata L.* can been considered as one of the medicinal plants that are highly valuable because of its significant antioxidant properties that can greatly enhance the wound healing process. The excellent healing capacity of *C. odorata* is mainly contributed by its high content of bioactive compound called antioxidants. Antioxidants can be obtained from plant through various extraction process, however the study to determine the optimum method for highest antioxidant activity per extract yield have not much been pursued in the research scene, especially extraction method that make use of two different extraction techniques. Therefore, this study aims to explore the effectiveness of applying two different extraction techniques (ultrasound-assisted extraction and maceration extraction) on the extract yield and antioxidant capacity. In this study, the bioactive compound of *C. odorata* was extracted by maceration and ultrasound-assisted maceration at two different extraction times, 48 hours and 120 hours. The extraction process was conducted using distilled water as solvent and was maintained at 34 °C. Yield assessment were then conducted through the dry weigh of the extracts. For this study, the extracts were qualitatively assessed for their antioxidant capacity by DPPH and FRAP assay. Based on gallic acid and ascorbic acid standard curve, the antioxidant capacity of every extract was visually graphed for comparison. In this study, ultrasonic-assisted maceration has been shown to be more efficient in extracting process compared to maceration alone as the yields are higher for both extraction time. When comparison between the extraction time applied, the 120 hours extraction time successfully extracted more bioactive compounds from the *C. odorata* at 87.36 % and 27.70 % for the ultrasound-assisted maceration and maceration respectively, when compared to the 48 hours extraction time with 65.94 % for ultrasound-assisted maceration and 13.26 % for maceration. The results for antioxidant assay was observed and all four

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extracts exhibited antioxidant capacity with increasing order from maceration (48 hours), followed by maceration (120 hours), ultrasound assisted maceration (48 hours) and lastly is ultrasound assisted maceration (120 hours). Quantification of gallic acid and ascorbic acid were done using spectrometry to determine the antioxidant content in the extracts. It is established that gallic acid and ascorbic acid made up of about approximately one-fifth of *C. odorata*. This study shows that *C. odorata* has the potential as antioxidant source and the findings may be used for the development of medicinal products from this useful weed.

Keywords: *C. odorata*, Maceration Method, Ultrasound-Assisted Maceration, Extraction Time, Gallic Acid, Ascorbic Acid

1. Introduction

The problem of wound not able to heal properly is becoming major trouble that is currently affecting the health care systems around the world. Wounds that do not show any sign of healing after three months are called non-healing wounds [1]. Most of non-healing wounds are the resulted from prolong aggressive pro-inflammatory response by the body to combat pathogens which can be cause by high level of reactive oxygen species (ROS). In a severe condition, high level of ROS often lead to alarming diseases such as atherosclerosis, arthritis and cancer. As such, a new highly effective strategy at treating wound has to be researched in order to tackle the problematic nonhealing and chronic wounds that is currently plaguing the world.

Antioxidants generally are known as materials that are capable to inhibit or significantly slows down the oxidation process. Antioxidants acts as a reducing agent that donates in own electrons to eliminate the free radical electrons vacancy, reducing the oxidative stress [2]. This helps in the wound healing process since oxidative stress cause by high level of reactive oxygen species (ROS) can lead to a pro-inflammatory effect that will damage healing tissues [3]. Due to this, antioxidant can be used as a strategy for tackling wound healing problem, making antioxidant a precious component for producing medicine. Antioxidant can be found in abundance in plants since plants contain phenolic compound such as flavonoids and carotenoids which exhibits antioxidant capacity [4].

C. odorata is a is a flowering plant species of perennial shrub that belongs to the Asteraceae family, that is more commonly known as Siam Weed. The *C. odorata* have been categorised as one of the highly serviceable plants in the medicinal field because of its exceptional flexible healing usage for treating various ailments ranging from cancer therapy to hepatoxicity care [5]. The many utilities of the Siam weed in the medicinal field are contributed by its numerous healing properties especially the antimicrobial, anti-inflammatory and antioxidant [5]. The extract of *C. odorata* has been determined in many researches to contained high amount of antioxidant agents that can help in bolstering wound-healing process [6].

In order to utilise the bioactive constituents that is contained within medicinal plants for usage of either research or medicine production, a suitable sample must first be procured by preparing the medicinal plant through extraction process [7]. As such, proper extraction has to be conducted in order to produce high quality extract that is rich in bioactive compound. Two of the most prevalent methods for extraction are ultrasonic-assisted extraction (UAE) and maceration because of their cost effective and simple procedure. Despite that, the study to determine the optimum method for highest antioxidant activity per extract yield have not much been pursued in the research scene [8][9]. Moreover, studies that make use of two different extraction techniques in *C. odorata* extract are limited. Hence, the aim of this study is to evaluate the effect of ultrasonic-assisted maceration when compared to only using maceration especially for antioxidant capacity.

2. Materials and Methods

In this project, different extraction techniques were used maceration extract left for 48 hours and 120 hours as well as maceration extract that was assisted by ultrasonication then left for 48 hours and 120 hours. The obtained extracts were dry weighted to evaluate the yield comparison. Then, the four extracts were used for the study of antioxidant capacity with DPPH assay and FRAP assay in order to evaluate the oxidative stress that can be alleviate by the antioxidants in the extracts. Finally, the quantification of antioxidant is conducted after the antioxidant assay to determine the concentration of gallic acid and ascorbic acid in the extracts.

2.1 Materials

Chromolaena odorata L. leaves, DPPH (Sigma-Aldrich, USA), Ethanol (Merck Millipore, USA), Ferric Chloride (Sigma-Aldrich, USA), TPTZ (Sigma-Aldrich, USA), Ascorbic acid (Sigma-Aldrich, USA), Gallic acid (Sigma-Aldrich, USA).

2.2 Preparation of *C. odorata* sample

Collected *C. odorata* leaves were first cleaned by flowing tap water in order to eliminate extraneous substances such as dirt and dust. Then, the leaves were put inside a drying oven at temperature 30°C for drying purposes until the weight is constant, to make sure that water has been completely removed from the leaves sample. Then, the dried sample were grinded into fine powder using ball mill grinder with the parameters set at 200 rpm for 1 hour and 30 minutes. The powders were then later kept in an air-tight container for storage until used.

2.3 Preparation of *C. odorata* extract

In this method, 10 g of powdered *C. odorata* sample were added into a 250 mL glass conical flask which were then be immersed with 150 mL of distilled water. Parafilm were used to seal the conical flask to make it air-tight and aluminium foil will be wrapped around the conical flask. The conical flask was afterward put in a mechanical shaker at room temperature for 48 hours. Later, a vacuum pump was used to filter the extract. A rotary evaporator is then use with temperature set to 34 °C to concentrate the extract. The extract gathered was then weighted and stored at a temperature of 4 °C in a refrigerator. Additionally, for the ultrasound-assisted extraction, the conical flasks are immersed in an ultrasonic bath at 40 °C for 15 minutes with intensity of the ultrasound set at about 1 W/cm^3 and the frequency fixed at 25 kHz before being put into the mechanical shaker for extraction. The steps were then repeated for 120 hours extraction time.

2.4 Evaluation of antioxidant capacity of fractionate *C. odorata* extract

2.4.1 DPPH assay

The first step in DPPH assay was to obtain the DPPH working solution (6×10^{-5} M) by immersing 2.37 mg of DPPH into 100 mL of methanol. Then, 1 ml from the DPPH working solution was taken for mixing with 33 µg fractionated extract using the highest dissolved methanol concentration. The sample solution was incubated for 20 minutes at room temperature afterwards. The absorption level (As) of the reaction mixture was measured at wavelength 517 nm using a UV-Vis spectrophotometer. A blank sample consisting of methanol and DPPH working solution were used as a control sample for blank absorbance in this study. Gallic acid was used as a standard. As level comparison between the extracts and gallic acid were conducted to determine the antioxidant capacity of the extracts.

2.4.2 FRAP Assay

FRAP assay started with the synthesis of FRAP working solution three reagents [10]. The first reagent was acetate buffer (300 nM, pH 3.6) which consist of 3.1g sodium acetate.$3H_2O$ and 16 mL glacial acetic acid in 1000mL buffer solution. The second reagent was 2, 4, 6-triphridyl-s-triazine (TPTZ) (10 nM) in 40 mM HCl and the last reagent was $FeCL_3$. $6H_2O$ (20 mM) in distilled water. The three reagents were mixed in ratio 10:1:1 respectively to produce FRAP working solution. Ascorbic acid was used as a standard for calibration. Then 3 mL from the FRAP working solution was added to 100 µL of the fractionated extract at different concentration of 20, 40, 60, 80 and 100 µg/mL and was mixed and allowed to stand for 4 minutes. The measurements were made using the calibration curve of the tested ascorbic acid as standard in parallel process at 593 nm in 37 °C [10].

2.5 Quantification of Gallic Acid and Ascorbic Acid

Firstly, an accurately weighed 100 mg of pure gallic acid and ascorbic acid was transferred to 100 mL volumetric flask. Then, 30 mL distilled water was added to it. The solution was shaken for 5 minutes to solubilize compound and final volume was made up to mark with distilled water [11]. For this procedure, gallic acid and ascorbic acid standard curve was constructed representing the antioxidants at five different concentration; 5.0 µg/ml, 7.5 µg/ml, 10.0 µg/ml, 15.0 µg/ml and 20.0 µg/ml. The wavelength on the spectrometer was selected to be the maximum absorbance for each standard, which for gallic acid is at 256 nm while for ascorbic acid is at 258 nm [11]. The standard curves were used to identify the concentration of gallic acid and ascorbic acid contained the extract by substituting the absorbance of diluted sample into the standard curve regressing line (trendline) equation. The percentage of gallic acid and ascorbic acid is calculated afterwards.

3. Results and Discussion

3.1 Extract Yield from *C. odorata*

The yield of C. *odorata* was determined for the two methods of extraction: maceration and ultrasonic assisted maceration (15 minutes ultrasonication) that were proposed to compare which method will yield the best extract when left at the same temperature of 34°C. This study also employed two different extraction time: 48 hours and 120 hours to see which solvent will yield more extract as well as the antioxidant capacity. The extracts were filtered using a vacuum pump and then dried using a rotary evaporator in order to remove the solvent and obtain the dry extracts. Then, the final weight readings were taken and used for the calculation of percentage yield.

Table 1: Dry weight of *C. odorata* final extracts

	Maceration alone	**Ultrasonic assisted maceration**
48 hours extraction time	0.663 g	3.297 g
120 hours extraction time	1.385 g	4.368 g

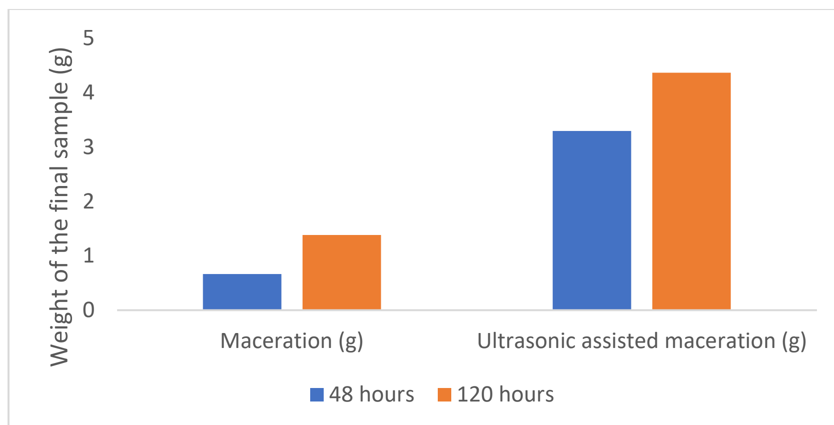

Figure 1: Dry weight of *C. odorata* extracts

Figure 1 shows the weight of *C. odorata* extracts after being removed from excess solvent at the same time the samples were concentrated. The highest yield of sample extraction is by ultrasonic assisted maceration method when left for 120 hours of extraction time with 4.368 g while the lowest yield of sample extraction is by maceration method when left for 48 hours with 0.663 g. The ultrasonic assisted maceration method gains more yield from extraction of *C. odorata* than maceration method and the difference is very significant, especially for the 120 hours extraction time procedure with the difference in sample dry weight being 2.983 g. This shows that the ultrasonic assisted maceration extraction has better yield of extract than the maceration extraction. Additionally, the extraction will yield more extract if the extraction time is increase. Table 2 shows the percentage of yield of extraction.

Table 2: Percentage of yield of extraction

	Maceration alone	**Ultrasonic assisted maceration**
48 hours extraction time	13.26 %	65.94 %
120 hours extraction time	27.70 %	87.36 %

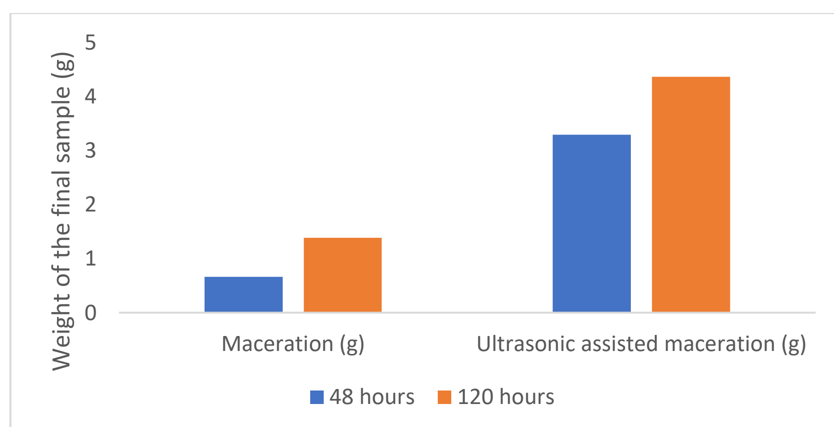

Figure 2: Yield percentage from extraction of *C. odorata*

Figure 2 shows the graph of yield percentage from extraction of *C. odorata*. The ultrasonic assisted maceration technique yields 52.68 % more extract than maceration technique in the 48 hours extraction run while for the 120 hours extraction, the difference becomes marginally bigger at 59.66 %. Additionally, when the extraction time is increased by 72 hours, the yield percentage increase by 14.44 % for the maceration method while for the ultrasonic assisted maceration the increase is by 21.42 %.

From this study alone, it can be observed that the optimum extraction technique is ultrasound assisted maceration with 120 hours of extraction time.

Ultrasound extraction offers a significantly higher amount yield when compared to using conventional method such as maceration extraction. This is because through ultrasonication thick and rigid structure of sample, such as cell wall in plant, are able to be breakdown to allow for easier access for penetration of solvent through the sample. This makes the extraction process become much more efficient and faster as the less penetration is required making it easier to extract bioactive components from samples [12]. Whereas, maceration extraction takes more time to penetrate the surface of sample resulting in a longer time to extract bioactive components from sample. When left with the same extraction time, the ultrasonic assisted maceration extraction will be able to gain a significantly more extract yield because of its faster bioactive components extraction rate when compare to maceration extraction [13].

Other than that, extraction time plays an important role in gaining the extract yield from an extraction process. The longer the sample is exposed to the solvent, more extract yield will be produced if no limiting factor is in play [14]. This is because the solvent will be continuously absorbing the bioactive components in the sample until the factor is reached such as if the sample is exhaust of any bioactive compound (sample factor) and if the solvent is completely used up for extraction (solvent factor). Since the sample or the solvent is not fully exhausted within in the first 48 hours of extraction, the extraction process still continues until either is exhausted or until the 120 hours is complete. For a more thorough study, extraction with a much varying extraction time such as applying for 48 hours, 72 hours, 96 hours, 120 hours and 144 hours of extraction time can be conducted to observe the limiting factor of a *C. odorata* extraction with 10 g of sample in a 100 mL distilled water solvent.

3.2 Assessment on Antioxidant Activities

3.2.1 DPPH assay

From the assay, every extract is observed to exhibit an effective free radical scavenging activity according to their absorbance values. Lower value of absorbance signifies that higher antioxidant capacity since there are more free radical scavenging by the antioxidants [15]. The absorbance value for gallic acid at 1.0 µg/ml concentration is much lower than every of the extract. This expresses that 1.0 µg/ml of gallic acid exhibits a much greater antioxidant capacity when compared to the *C. odorata* extracts. Additionally, the result shows that extracts from ultrasound assisted maceration techniques have a lower absorbance value than that of maceration technique extract. Another factor that was studied is extraction time, which shows that the absorbance value is significantly less in 120 hours extract. The order of antioxidant capacity was found as maceration (48 hours) > maceration (120 hours) > ultrasound assisted maceration (48 hours) > ultrasound assisted maceration (120 hours), which was expected.

Table 3: Absorbance of standard gallic acid and *C. odorata* extract for DPPH assay

		Absorbance reading at 517nm		
		1	**2**	**Mean**
Standard:	**Gallic acid (1.0 µg/ml)**	0.411	0.419	0.415
***C. odorata* extracts**	**Maceration (48 hours)**	0.658	0.630	0.644
	Maceration (120 hours)	0.578	0.598	0.588
	Ultrasound-assisted maceration (48 hours)	0.547	0.547	0.547

	Ultrasound-assisted maceration (120 hours)	0.511	0.515	0.513

For FRAP assay, the lower absorbance value signifies greater antioxidant capacity [13]. From Table 4, every extract is observed to exhibit an effective reduction of Fe (TPTZ)2 (III) into Fe (TPTZ)2 (II) according to their absorbance values. The absorbance value for ascorbic acid at 1.0 µg/ml concentration is lower than every of the *C. odorata* extract. This expresses that 1.0 µg/ml of ascorbic acid exhibits a much greater antioxidant capacity when compared to the *C. odorata* extracts. Additionally, the result shows that extracts from ultrasound assisted maceration techniques have a lower absorbance value than that of maceration technique extract. Another factor that was studied is extraction time, which shows that the absorbance value is significantly less in 120 hours extract. The order of antioxidant capacity was found as maceration (48 hours) > maceration (120 hours) > ultrasound assisted maceration (48 hours) > ultrasound assisted maceration (120 hours), which was expected.

Table 4: Absorbance of standard ascorbic acid and *C. odorata* extract for FRAP assay

		Absorbance reading at 593nm		
		1	**2**	**Mean**
Standard:	**Ascorbic acid (1.0 µg/ml)**	0.852	0.848	0.850
***C. odorata* extracts**	**Maceration (48 hours)**	1.280	1.280	1.280
	Maceration (120 hours)	0.937	0.845	0.941
	Ultrasound-assisted maceration (48 hours)	0.931	0.933	0.932
	Ultrasound-assisted maceration (120 hours)	0.867	0.867	0.867

3.3 Quantification of Antioxidant Capacities Based on Gallic Acid and Ascorbic Acid Standard Curve

Estimation of gallic acid and ascorbic acid can be through various method such as chromatography and capillary electrophoresis. However, the simplest yet precise method is by spectrophotometric method for estimating the gallic acid and ascorbic acid content in herbal formulations [11]. The wavelength on the spectrometer was selected to be the maximum absorbance for each standard, which for gallic acid is at 256 nm while for ascorbic acid is at 258 nm [16][17]. For the standard curve, five different concentration of were used; 5,7.5,10,15 and 20 µg/mL as shown in Figure 3.3 and Figure 3.4.

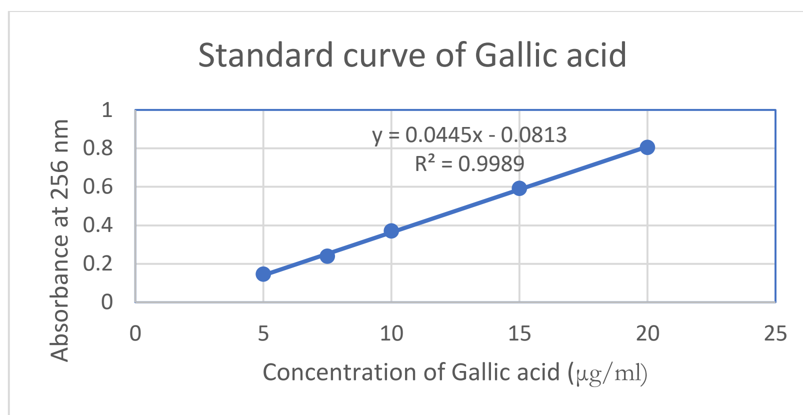

Figure 3: Standard curve of gallic acid at 256 nm

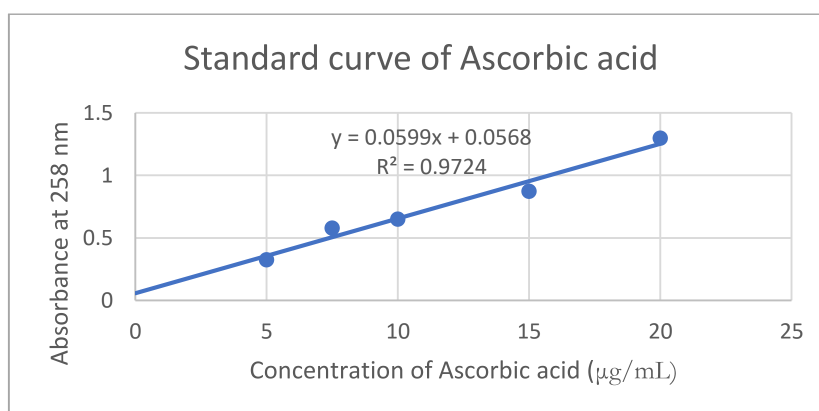

Figure 4: Standard curve of ascorbic acid at 258 nm

Gallic acid has shown linear absorbance over the concentration range of 5 – 20 µg/mL with 0.9989 as the R^2 value. This y = 0.0445x + 0.08133 was used for the determination of concentrations of gallic acid in the extracts. As for ascorbic acid, a linear absorbance can also be observed over the concentration range of 5 – 20 µg/mL with the R^2 value 0.9724. The equation y = 0.0599x + 0.0568 obtained from the standard was used for determining the concentration of ascorbic acid in the extracts. The data for the concentration of gallic acid and ascorbic acid were shown in Table 5 and Table 6.

Table 5: Concentration of gallic acid in each extract

Sample	Concentration of extract (µg/ml)	Absorbance	Concentration of Gallic acid in extract (µg/ml)	Percentage of Gallic acid content in extract (%)
Maceration (48 hours)	10	0.002	1.87	18.7
Maceration (120 hours)	10	0.006	1.96	19.6

Ultrasound assisted maceration (48 hours)	10	0.008	2.00	20.0
Ultrasound assisted maceration (120 hours)	10	0.015	2.16	21.6

Table 6: Concentration of ascorbic acid in each extract

Sample	Concentration of extract (μg/ml)	Absorbance	Concentration of Ascorbic acid in extract (μg/ml)	Percentage of Ascorbic acid content in extract (%)
Maceration (48 hours)	10	0.002	1.87	18.7
Maceration (120 hours)	10	0.005	1.93	19.3
Ultrasound assisted maceration (48 hours)	10	0.006	1.96	19.6
Ultrasound assisted maceration (120 hours)	10	0.009	2.02	20.2

From Table 5 and Table 6, it can be observed that each extract contained a noteworthy amount of ascorbic acid which makes up of approximately one-fifth of the extracts' concentration for both gallic acid and ascorbic acid content. Ultrasound assisted maceration (120 hours) contain the highest amount of both gallic acid and ascorbic acid content which is at 21.60 % concentration for gallic acid and 20.20 % for ascorbic acid. Meanwhile, the extraction with the lowest gallic acid and ascorbic acid count is maceration (48 hours) that contain 18.70 % of both gallic acid and ascorbic acid concentration. Additionally, extracts produced with 120 hours of extraction time can also be seen containing a higher amount of gallic acid and ascorbic acid. The order of content for gallic acid and ascorbic acid was

determined to be the same which is maceration (48 hours) > maceration (120 hours) > ultrasound assisted maceration (48 hours) > ultrasound assisted maceration (120 hours).

The concentration of gallic acid and ascorbic acid in the extract can be tied back to the antioxidant capacity results. Gallic acid and ascorbic acid are strong antioxidant and free radical scavenger that protects tissues, cell membranes, and DNA from oxidative damage. Hence, the concentration of the antioxidant gallic acid and ascorbic acid directly correlates to the antioxidant capacity of an extract. This is because the if the level of antioxidants in an extract is high, more electrons from antioxidant will be released to neutralized free radicals inhibiting oxidation process [18][19]. It is shown in this study that observed extract with high contain of gallic acid and ascorbic acid such as the ultrasound assisted maceration extraction (120 hours), exhibits a much higher antioxidant capacity in the DPPH and FRAP assay. The presence of gallic acid and ascorbic acid have contributed to the antioxidant capacity in *C. odorata*. However, since both only make up of only approximately 20.00 % of bioactive compounds contain in C. *odorata*, it cannot be said for certain that either bioactive compounds are the major antioxidant component in *C. odorata*. Moreover, the amount of gallic acid and ascorbic acid contained in *C. odorata* is about the same with gallic acid have marginally more concentration. Thus, a more in-depth study has to be conducted in order to determine the major antioxidant compound in *C. odorata.*

4. Conclusion

Overall, this study found that ultrasonic assisted maceration and longer extraction time has a significant advantage in maximizing the rate of *C. odorata* extraction. The largest extract yield was obtained from the ultrasonic-assisted maceration at 120 hours of extraction time. Additionally, extracts from ultrasound assisted maceration also exhibit a significantly higher level of antioxidant especially at 120 hours extraction time. Ascorbic acid and gallic acid were discovered in all *C. odorata* extracts with the highest concentration is in ultrasonic-assisted maceration at 120 hours of extraction time.

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