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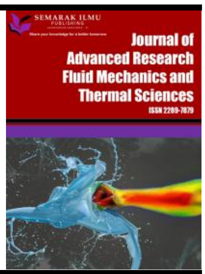

Quantification of Polyphenols Content and Antioxidant Activity of *Euphorbia tirucalli* L. Extracted using Maceration and Soxhlet Method

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ABSTRACT

Euphorbia tirucalli L. *(E. tirucalli*) has gained attention for these past few years in terms of its phytochemical studies due to the antioxidant attribution of polyphenols source as its bioactive compounds in extracts. In order to extract important plant compounds, conventional extraction methods such as maceration and Soxhlet are still widely incorporated for plant extraction techniques due to their convenient application in laboratory settings. This study focused on the two conventional techniques; maceration and Soxhlet on polyphenols extraction of *E. tirucalli* using various extraction solvents. In order to provide insight into its potential for polyphenols extraction, yield percentage (%) and phytochemical tests such as Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant activity (%) were assessed. In terms of yield percentage, Soxhlet methanolic extract was found to be the best. The findings also suggest that both maceration and Soxhlet techniques are effective in extracting a significant amount of phenolics and flavonoid content in which highest value recorded were 17.26 ± 0.23 mgGAE/100g and 50.08 ± 1.13 mgQE/100g for maceration while 16.17 ± 0.21 mgGAE/100g and 43.02 ± 0.01 mgQE/100g for Soxhlet. Maceration with methanol solvent appears to be more effective for TPC and TFC while Soxhlet acetonic extract is chosen as the best for antioxidant activity with 75 .79 ± 0.04 % of radical scavenging activity in *E. tirucalli* extract. Thus, this study indicates the noteworthy potential of both maceration and Soxhlet techniques for polyphenols extraction.

Keywords:
Euphorbia tirucalli L.; maceration; Soxhlet; polyphenols; total phenolic content; total flavonoid content; antioxidant activity

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1. Introduction

Phytochemistry comprises a study of phytochemicals which are known as plant chemicals; its secondary metabolites play a crucial role in self-defense against insects, pests, pathogens, herbivores, ultraviolet exposure, and environmental hazards [1]. A comprehensive understanding of this subject is significant for drug discovery and the innovation of novel therapeutic agents against major diseases. Phytochemicals were classified into phenolics, terpenes, N (organonitrides), and S (organosulfides) containing compounds [1]. They are accumulated in fruits, vegetables, whole grains, spices, legumes, herbs, shrubs, and trees. One of the traditional plants which is been studied for its various remarkable bioactive properties in these recent years is *E. tirucalli* as shown in Figure 1 [2-5]. It belongs to the *Euphorbiaceae* family and is very widely used for popular medicinal uses originating from Africa and America [6].

Fig. 1. *E. tirucalli* plant (Right – close up image)

Antioxidants are molecules that account to neutralize the free radicals in biological cells as these imbalanced radicals could be a major threat to living organisms due to oxidative stress [7]. It is worth mentioning that *E. tirucalli* extracts are considered a source of natural antioxidants due to the identification of polyphenolic compounds in the plant extract [8]. The incorporation of various extraction approaches is important to extract the target compounds from plants as they produce a wide range of bioactive constituents [9]. How efficient the method is whether it is conventional or non-conventional methods will govern by its input parameters, plant matrix nature, the properties of the bioactive compounds, and the operator expertise [10]. Most importantly, the quantity and quality of the polyphenols in a particular plant are immensely affected by the extraction method [11].

Maceration, sonication, and Soxhlet are several methods of extraction chosen by the researchers, especially for conventional techniques such as maceration and Soxhlet, both are generally practical at small-scale research [12-14].

As any extraction method will also rely on many crucial parameters for extracting high-quality of bioactive compounds, one of the parameters is the solvent as it may decide the condition of the extract and what compounds are found in that extract [15]. The solubility of phenolic compounds is proven to be influenced by the nature of the solvent and its polarity [16]. The roles of extracting solvents on polyphenols content and antioxidant activities have been studied extensively by different authors such as hexane, ethyl acetate, chloroform, and methanol, particularly for *Euphorbia hirta* (dudhi) L. and other high polar solvents including water, methanol, and ethanol for different type of vegetables [17]. A very recent study focused on phytochemical screening and evaluation of the antioxidant properties and antimicrobial activity of *E. tirucalli* extracts [7]. They reported that a good amount of polyphenols were found in the extracts using the maceration technique. However, the extraction time was longer, the volume of solvent used was huge, and there was no specific comparison between which conventional method was better equipped in polyphenols extraction for a small-scale study.

Similarly, Heya *et al.,* [3,18] proposed two different studies of *E. tirucalli* extracted using Soxhlet for *in vitro* antifungal and antibacterial activity from the extracts. The findings suggest the presence of polyphenols such as flavonoids in the extracts without quantifying the amount of polyphenols accordingly. In addition, the use of pure solvent in large amounts might be detrimental due to its toxic properties as well as the fact that pure solvent is not effective for polyphenol extraction due to its polarity difference [19]. These conditions will limit the polyphenolic extraction and need to be addressed. The focus of *E. tirucalli* study on polyphenolic content and its antioxidant potential with the incorporation of conventional extraction methods is among the gaps that this study should address. Hence, we focused on the extraction of different conventional methods; maceration and Soxhlet using different impure solvents for the quantification of polyphenols and their antioxidant activity in *E. tirucalli*.

2. Methodology
2.1 Materials and Sample Preparation

E. tirucalli fresh stems were harvested and donated by a local farm in Kangar, Perlis with a weight was approximately 1.5 kg. The plants were scissored into smaller parts and cleaned with water to remove the excess soil and other impurities. The cutting stems were rinsed multiple times with distilled water and kept dry using paper tissues. Finally, the stems were further dried in the oven at 65°C for 3 days to completely remove the water composition. To obtain the powder of plants, the dried stems were ground or pulverized into smaller particles using an electrical mill. The powder was weighed and kept inside an air-tight container in the refrigerator under 4°C until use.

The extraction solvents included methanol, acetone, and petroleum ether. Reagents used for Total Phenolic Content (TPC) analysis included Folin-Ciocalteu reagent, gallic acid, and sodium carbonate (Na_2CO_3), while Total Flavonoid Content (TFC) analysis utilized quercetin, aluminum chloride ($AlCl_3$), and sodium nitrate ($NaNO_3$); all were purchased from Chemiz , Malaysia. Antioxidant activity was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma Aldrich, USA) reagent.

2.2 Extraction Preparation
2.2.1 Maceration

The powder samples of *E. tirucalli* (10 g) were extracted for 60 minutes using 80% methanol, 80% acetone, and petroleum ether as the solvents with occasional stirring at room temperature [10]. The solvent-to-sample ratio used was 10:1 with 100 mL of the solvent volume. The extract solution was filtered using filter papers (Smith Filter Papers 102 Qualitative, 150mm) to separate the solid matrix from the extract solution. Next, the extract was evaporated using a rotary evaporator (Eyela N-1200A) at 60°C under reduced pressure (58 mbar). To further dry the solvent, the extract was placed in a vial and kept in the oven under 55°C for 24 hours. After the solvent was dried, the crude supernatant was kept at 4°C for further phytochemical testing.

2.2.2 Soxhlet extraction

A total of 10 grams of the powdered sample was extracted in an assembled Soxhlet apparatus with 300 mL of 80% methanol [10]. The temperature level of the heating mantle was adjusted to the heating of 60°C-80°C for 4-5 hours till the extraction solution became colorless. The resulting dark-green extract was then concentrated using a rotary vacuum evaporator (Eyela N-1200A) under reduced pressure (58 mbar) at 60°C. Crude extracts were collected when a thick and viscous paste of extract was visible. The oven-drying process was done for 24 hours under 55°C. Subsequently, the gel-like crude was then kept in an air-tight container to maintain its phytochemical elements at 4°C for further analysis. The process was then repeated separately using 300 mL of 80% acetone and 300 mL of petroleum ether. The calculation for extract yield percentage from both methods is calculated using Eq. (1) as follows:

$$Yield\ percentage\ (\%) = \frac{Weight\ of\ extract\ (g)}{Weight\ of\ dried\ powder\ (g)} \times\ 100 \tag{1}$$

2.3 Determination of Total Phenolic Content (TPC)

TPC was determined using a variation of the Folin-Ciocalteu assay [10]. The Folin reagent is a pre-prepared solution. Approximately 0.1 mL of the sample extract was mixed with 0.2 mL of Folin-Ciocalteu reagent. The solution added was mixed thoroughly and incubated for 3 minutes. 1 mL of 20% sodium carbonate was added to maintain the pH and the mixture was kept in a dark place for 30 minutes. The absorbance was measured at 765 nm using UV-Vis spectrophotometer (Shimadzu UV-1280, Kyoto, Japan) for each triplicate sample. The standard curve of gallic acid was plotted by preparing the absorbance versus the different concentrations of the gallic acid graph in the range of 0.1- 0.4 mg/mL using stock gallic acid. A blank sample without gallic acid was prepared as a control variable. The obtained absorbance value for the test sample is referred to as the standard gallic acid calibration curve (y = mx + c).

2.4 Determination of Total Flavonoid Content (TFC)

The aluminum chloride colorimetric method was used to determine the TFC in *E. tirucalli* extract [10]. Approximately, 0.1 mL of the sample extracts was added to 2 mL of distilled water. Then, the mixture was incubated for 5 minutes after 0.15 mL of 50% sodium nitrate ($NaNO_3$) was added to the mixture. Next, 0.15 mL of aluminum chloride ($AlCl_3$) was added and the absorbance was recorded at 415 nm after 15 minutes of incubation. The TFC was expressed as mg quercetin equivalent (QE)/100g of the sample. Quercetin standard solutions in the range of 0.1-0.4 mg/mL were prepared for the standard curve equation. The absorbance was measured at a wavelength of 415 nm against the blank solution using a UV-Vis spectrophotometer (Shimadzu UV-1280, Kyoto, Japan).

2.5 Evaluation of Antioxidant Activity: DPPH Method

The colorimetric 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was carried out according to the protocol modified from the previous study [20]. A volume of 2.5 mL of DPPH methanol solution (6×10^{-5} M) was combined with 0.1 mL of plant extract. A blank solution (pure methanol) was prepared as a control solution for calculating the inhibition percentage. The mixtures were thoroughly mixed and incubated for 30 minutes. Absorbance was measured at 517 nm. Antioxidant activity (%) is calculated using Eq. (2).

$$Antioxidant\ activity\ (\%) = \left(1 - \frac{Absorbance\ of\ sample\ at\ 517\ nm}{Absorbance\ of\ control\ at\ 517\ nm}\right) \times\ 100 \quad (2)$$

2.6 Statistical Analysis

Exploratory data analysis is mentioned as means ± SD of three parallel measurements. The statistical analysis was done using Minitab and Microsoft Excel (Version 16.75).

3. Results

3.1 Yield Percentage

The extraction method can result in an abundant yield of the extracts as well as minimal differences in the biological needs the extracts would provide [16]. This would require a great deal of effort to analyse which technique is convenient to be applied for a particular extract. Hence, a similar amount of *E. tirucalli* dried powders was extracted using two different conventional methods, namely maceration and Soxhlet extraction with methanol, acetone, and petroleum ether (pet-ether) based on different polarities as the solvent.

Table 1 shows the findings for the yield percentage of *E. tirucalli* extracts. The extracts produced by maceration technique recorded 10.24%, 3.39%, and 0.42% of yield for methanol, acetone, and pet-ether solvent respectively. On the other hand, Soxhlet extraction resulted in a higher amount of yield percentage; 43.30%, 14.04%, and 12.74% for similar solvents. The highest yield was obtained using Soxhlet methanolic extract. In general, the higher yield percentage shown by Soxhlet was in agreement with the previous study as they compared the most suitable method for plant aerial extract [10]. Soxhlet extraction nature is the repetition of contact between the sample and the extractant, resulting in the facilitation of transfer equilibrium displacement as well as the effective solubility due to the high temperature during extraction [20]. Nevertheless, a study led by Heya *et al.,* [18] proposing a similar technique yields a lower amount of extract using pure methanol. As for the solvent used, this condition could be attributed to the polarity variation between the solvent and the plant extract [21].

Table 1
Yield percentage of *E. tirucalli* extracts

Extracts		Yield (%)
Maceration	Methanol	10.24
	Acetone	3.39
	Pet-ether	0.42
Soxhlet	Methanol	43.30
	Acetone	14.04
	Pet-ether	12.74

3.2 Total Phenolic Content (TPC)

Total phenolic content (TPC) was determined using Folin-Ciocalteu assay with absorbance measured at 765 nm. The standard calibration curve (y=0.1663x - 0.0873; R^2 = 0.9901) was plotted using the concentration of standard gallic acid (100-500 ug/mL). Figure 2 shows that total phenolic content for both maceration and Soxhlet was comparable with a slight difference using methanol which is 17.26 $\pm$ 0.23 mgGAE/100g and 16.17 $\pm$ 0.21 mgGAE/100g respectively. Meanwhile, Soxhlet method was more efficient for phenols extraction using acetone with 16.10 $\pm$ 0.10 mgGAE/100g compared to maceration which is around 11.90 $\pm$ 0.34 mgGAE/100g. Both maceration and Soxhlet recorded low TPC values when using pet-ether; 0.20 ± 0.03 mgGAE/100g and 1.15 ± 0.13 mgGAE/100g respectively.

A recent study reported by Das *et al.,* [12] showed a consistent outcome for phenolic content in maceration that can be higher than Soxhlet. Despite producing a higher extract yield, the main drawback for Soxhlet is a longer extraction time [22]. As a high temperature is necessary during the extraction process, this can lead to thermal degradation which explains the lower yield of phenols [23]. It is very crucial to note that temperature alone failed to explain this condition as it may also be due to other important polyphenols extraction conditions [23]. A very insignificant phenolic amount extracted from both methods using pet-ether is highly expected as less polar solvents are more prominent for non-polar compound extraction such as waxes and oils [24,25].

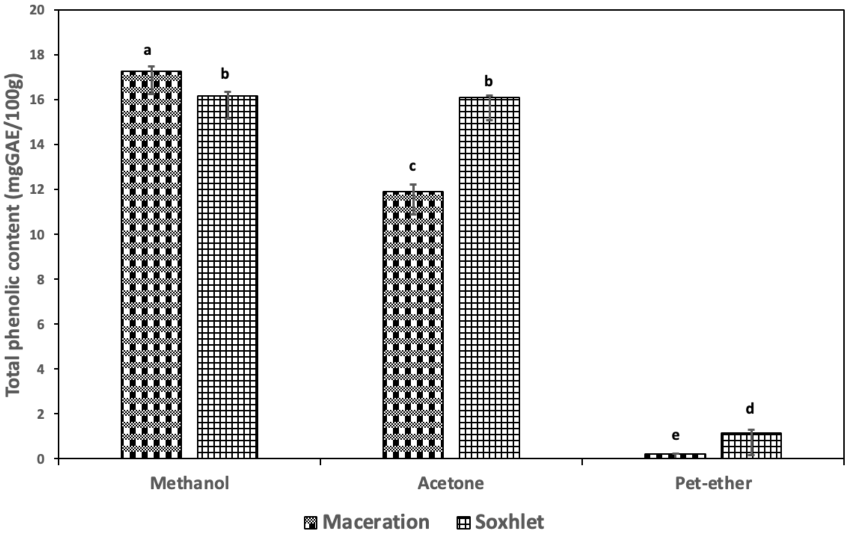

Fig. 2. Total phenolic content of *E. tirucalli* extracts using two conventional methods; maceration and Soxhlet with three different solvents. Results were expressed as mean ± standard deviation of three means (n = 3). Means that do not share a letter are significantly different ($p < 0.05$) using Tukey's HSD test

3.3 Total Flavonoid Content (TFC)

Total flavonoid content (TFC) recorded for both extraction methods is shown in Figure 3; using quercetin standard calibration curve (y=0.051x +0.0075; R^2 = 0.995). All extracts measured different scales of absorbance for TFC. The highest flavonoid content was observed at 50.08 ± 1.13 mgQE/100g extracted by maceration using methanol. Both extracts using acetone and pet-ether with similar method recorded 11.89 ± 1.02 mgQE/100g and 0.45 ± 0.02 mgQE/100g respectively. Meanwhile, a similar variation is shown by using Soxhlet which is 43.02 ± 0.01 mgQE/100g for methanol, 37.21 ± 0.01 mgQE/100g for acetone, and 1.14 ± 0.003 mgQE/100g for pet-ether.

This variability may be due to the sensitivity of flavonoids to the heat treatment as occurred with Soxhlet technique which is high temperature resulted in the degradation of total flavonoids [26]. As a matter of fact, the rotary evaporation step which required the extracts to be heated up to their boiling point of solvent explained that temperature may be the parameter that affects TFC as methanol exhibits a higher boiling point than acetone [27]. Comparing our study with the highest TFC shown using the maceration method, Le *et al.,* [7] reported a lower amount of TFC from *E. tirucalli* extracted using a similar method with ethyl acetate, but with a longer extraction time. These findings suggest that extended extraction time, especially under alkaline conditions, can trigger the loss of polyphenols [22]. Meanwhile, the incorporation of different extraction solvents for each method shows that polarities difference plays a crucial role in extracting high amounts of polyphenols [28].

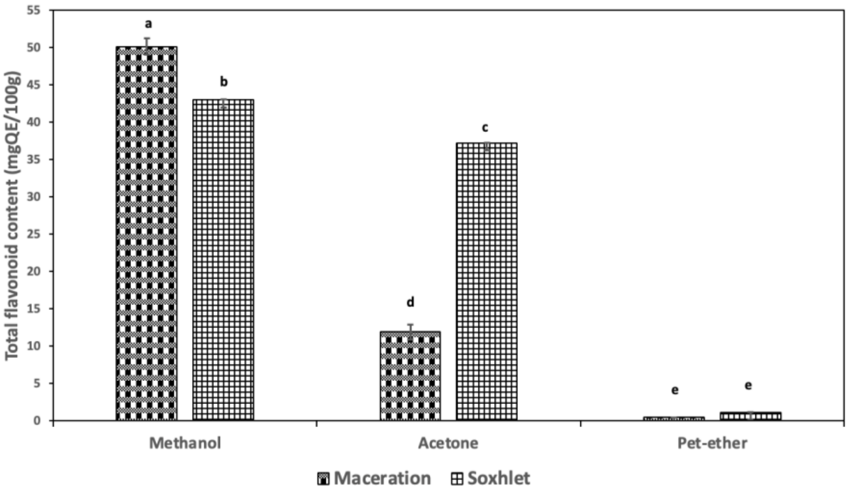

Fig. 3. Total flavonoid content of *E. tirucalli* extracts using two conventional methods; maceration and Soxhlet with three different solvents. Results were expressed as mean ± standard deviation of three means (n = 3). Means that do not share a letter are significantly different ($p < 0.05$) using Tukey's HSD test

3.4 Antioxidant Activity

The antioxidant activity of *E. tirucalli* was determined using DPPH radical scavenging assay which requires only a particularly short time. The mechanism of this assay is indicated by a colour change from purple to yellow, resulting from the reduction of the stable free DPPH radical to 1,1-diphenyl-2-picrylhydrazine. Hence, a decrease of absorbance will be detected which is explained by the ability of the extract to donate hydrogen/electrons [28].

The radical scavenging activity is shown for both extraction methods in Figure 4. The results explained that Soxhlet method was more effective in extracting the crude for *E. tirucalli*; this technique was able to record higher radical scavenging activity compared to the maceration technique using all different solvents with different polarity regardless of the variation in TPF and TFC. Soxhlet method recorded antioxidant activity (%) in the range of 63.06 $\pm$ 0.04 %, 75.79 $\pm$ 0.04 %, and 54.16 $\pm$ 0.11 % for methanol, acetone, and pet-ether respectively. Meanwhile, maceration technique shows a similar trend in the percentage of antioxidant activity; 56.11 $\pm$ 2.37 %, 65.09 $\pm$ 4.38 %, and 19.71 $\pm$ 2.39 % for methanol, acetone, and pet-ether accordingly. Quercetin was observed as the standard, which shows 76.52 $\pm$ 0.08 % of antioxidant activity. As a previous study suggested, this attribution was related to the reflux extraction condition of Soxhlet, which happened to release some bound phenolics that can increase the antioxidant activity [10,29]. However, a study conducted by Le and his fellows shows otherwise with higher radical inhibition activity (%) using maceration technique [7]. It is very crucial to consider that a reflux technique such as Soxhlet can contribute to thermal decomposition as well [30]. Thus, despite producing a good amount of antioxidant activity, Soxhlet technique may affect the polyphenolic content in the extract as a result of high temperature and longer extraction time [22,23]. Further improvements should be considered for this method such as the extraction parameter optimization.

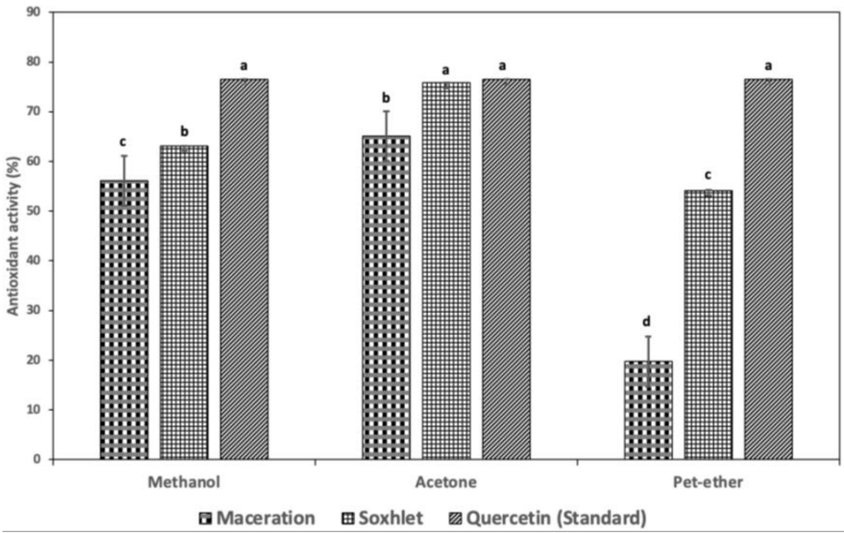

Fig. 4. Antioxidant activity (%) of *E. tirucalli* extracts using two conventional methods; maceration and Soxhlet with 3 different solvents. Results were expressed as mean ± standard deviation of three means (n = 3). Means that do not share a letter are significantly different ($p < 0.05$) using Tukey's HSD test

4. Conclusions

This study was conducted to compare the impact of different extraction methods used to extract polyphenols from *E. tirucalli* using conventional techniques, namely maceration and Soxhlet using various solvents. Polyphenols content was determined by phytochemical tests such as TPC, TFC, and antioxidant activity. Results revealed that all extracts with different extraction methods obtained a significant amount of phenolic and flavonoid content; varying due to different solvent polarities except petroleum ether extracts. The highest extraction yield was shown using Soxhlet employing methanol solvent. Maceration method employing methanol is the most effective for TPC and TFC. Meanwhile, Soxhlet extraction technique employing acetone as the solvent is considered the best option for the antioxidant activity from *E. tirucalli* plant. In the future, this knowledge can be further investigated regarding the role of other extraction parameters on the polyphenols extraction and how to optimize the extraction conditions through the optimization study.

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