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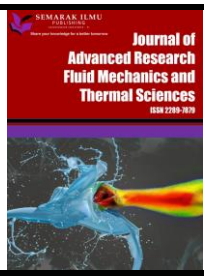

The Total Phenolic Content, Total Flavonoid Content and Antioxidant Properties of *E.tirucalli* L. Extract Partitioned using Different Solvents

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ABSTRACT

E.tirucalli L. is a succulent plant native to Africa renowned for its medicinal properties and antioxidant activity. This study aimed to evaluate the total phenolic and flavonoid content as well as the antioxidant activity of *E.tirucalli* L. extracts obtained using different solvents. Aqueous ethanol, hexane, and dichloromethane were used for extraction through Soxhlet extraction and partitioning. The total phenolic content was highest in the aqueous ethanol extract (2.501 mg GAE/g), followed by hexane (0.110 mg GAE/g) and dichloromethane (0.050 mg GAE/g) extracts. Similarly, the total flavonoid content was highest in the aqueous ethanol extract (1.307 mg QE/g), followed by hexane (0.164 mg QE/g) and dichloromethane (0.061 mg QE/g) extracts. The DPPH assay demonstrated that the aqueous ethanol extract exhibited the highest radical scavenging activity (RSA) with an IC_{50} value of 36.89 ± 0.05 µg/mL, followed by dichloromethane (50.94 ± 0.39 µg/mL) and hexane (62.42 ± 1.34 µg/mL) extracts. These findings indicate that aqueous ethanol is an effective solvent for extracting phenolic and flavonoid compounds with potent antioxidant activity from *E.tirucalli* L. extracts.

1. Introduction

E.tirucalli L. is a species of succulent plant that is native to Africa and is also commonly known as "Pencil Cactus" [1]. It is a unique plant that is able to withstand harsh conditions and is often grown for ornamental purposes due to its pencil-like stems. *E.tirucalli* L. is a plant that has been traditionally utilized in African and Indian traditional medicine for various therapeutic purposes. This plant has been historically employed to address a wide array of health issues, including wound healing,

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digestive ailments, respiratory disorders, inflammation, and certain types of cancer [2]. Recently, scientific investigations have focused on exploring the potential health benefits of *E.tirucalli* L., primarily due to its recognized antioxidant properties [3].

Hydroxyapatite (HA), a naturally occurring mineral form of calcium apatite, is widely used in biomedical applications due to its bioactivity and biocompatibility, particularly in bone grafting and dental implants [4]. The functionalization of HA with bioactive agents, such as polyphenols from plants, has garnered significant interest due to the synergistic effects that may enhance its antimicrobial and antioxidant properties. Since hydroxyapatite lacks antimicrobial properties, plant extract from *E.tirucalli* L. is used in this study to determine the antioxidant properties that will be able to combat bacterial growth in implants that is made up of hydroxyapatite alone.

The emerging interest in the potential health advantages associated with *E.tirucalli* L. is largely linked to its antioxidative attributes. Free radicals develop various diseases, such as cancer, cardiovascular ailments, and neurological disorders. Antioxidants are known for their ability to counteract these harmful radicals, safeguarding cells and tissues from oxidative harm. Within *E.tirucalli* L., phenolic compounds such as triterpenoids, phenylpropanoids, diterpenoids, and flavonoids, have been identified, displaying antioxidant effects by counteracting free radicals and averting cellular and tissue oxidative stress. Among the plant kingdom, phenolic compounds are an extensive class of organic substances.

Phenolic compounds cover various of organic molecules that can be categorized into distinct subgroups such as phenolic acids, lignans, flavonoids, stilbenes, and tannins. Each subgroup exhibits unique attributes and biological functions. Nevertheless, a comprehensive spectrum of biological activities, encompassing anti-inflammatory, antibacterial, antiviral, and antifungal properties, is attributed to phenolic compounds as a whole [5]. In the context of plants, phenolic compounds play pivotal roles in defence mechanisms against pathogens and environmental stresses, often contributing to the synthesis of pigments, fragrances, and secondary metabolites [6]. In human health, phenolic compounds are believed to confer protection against oxidative stress, inflammation, and also contribute to their antimicrobial capacities. Fruits, vegetables, coffee, tea, and wine are just a few examples of the foods and beverages that contain phenolic compounds. Catechins in green tea, resveratrol in red wine, and ferulic acid in whole grains are a few typical examples of phenolic compounds. Pathogens, stress, and sunshine are a few environmental elements that can affect a plant's ability to produce phenolic compounds. For instance, exposure to environmental stress, such as drought or high temperatures, can enhance the production of phenolic compounds in some plants because they aid in plant defence against stressors. It is also worth noting that some plants can produce phenolic compounds in response to pathogen attack, as these compounds can help defend the plant against diseases caused by bacteria, viruses, or fungi. In summary, the production of phenolic compounds in plants is a complex process that involves the interaction of several metabolic pathways and can be influenced by environmental factors [7].

On the other hand, flavonoids are crucial components of a healthy diet since it provides many fruits, vegetables, and flowers their vibrant colours. The existence of a distinctive flavonoid backbone, which consists of two aromatic rings joined by a three-carbon bridge, distinguishes flavonoids from other organic compounds. The subclasses of flavonoids include isoflavones, anthocyanins, flavones, flavanols, flavanones, and flavanols [8]. Each subclass has a distinct structure and has various biological behaviours. Furthermore, certain flavonoids have been demonstrated to have estrogenic activity and may be advantageous for the health of women. For instance, a subclass of flavonoids called isoflavones has been demonstrated to have estrogenic actions and may help lower the incidence of breast cancer and osteoporosis [9].

The DPPH test is a widely recognized method for evaluating the antioxidant capabilities of a substance. Antioxidants play a crucial role in counteracting the damaging effects of free radicals by providing an electron or hydrogen atom, thereby preventing oxidative stress-induced harm to cells and tissues [9]. The DPPH assay operates on the principle of diminishing the stable free radical DPPH through the intervention of an antioxidant agent. This interaction leads to the formation of a colourless solution and a non-radical DPPH molecule. The degree of inhibition of the DPPH radical directly reflects the antioxidant potential of the tested substance. The DPPH assay is favoured over alternative methods due to its simplicity, speed, and versatility [10].

Consequently, the primary objective of this research is to evaluate the comprehensive content of phenolic and flavonoid compounds, along with conducting antioxidant assessments on the aerial segments of the *E.tirucalli* L. plant. This will be accomplished by utilizing the Folin-Ciocalteu, aluminium chloride colorimetric, and DPPH assays to quantify the levels of these compounds. The extraction of antioxidant constituents will be conducted through the Soxhlet extraction technique, followed by the partitioning of the extracted components employing a diverse range of polar and nonpolar solvents.

2. Methodology

2.1 Sample Collection

E.tirucalli L. aerial parts were obtained from Kangar, Perlis, Malaysia. The aerial parts were cleaned thoroughly to remove dirt or brown spots and washed three times using distilled water. The next step was to dry them for 2 to 3 days in an oven set to 50^{o}C. The parts were dried, then blended, and a fine powder was obtained by sieving them under a 250 nm sieve. In order to preserve the powder's phytochemicals for later investigation, the powder was then put into an airtight container and placed in a table freezer set at -21^{o}C, according to the findings of [6,11].

2.2 Soxhlet Extraction

To fit within the Soxhlet extractor, the *E.tirucalli* L. powder was weighed at about 10 g on cellulose paper and secured with a string. Next, 200 mL of ethanol was measured and added to the Soxhlet apparatus's flask with a flat bottom. To condense the ethanol that had evaporated, the condenser was attached, and water was routed through the pipes. The extraction process was aided by heating the ethanol to a temperature just a few degrees over its boiling point, which allowed the evaporated solvent gas to condense, and the cycle was repeated 20 times. In this step the excess solvent was evaporated in a rotary evaporator operating at 45^{o}C to create a concentrated crude extract. The volume of the final crude extract was calculated by weighing the round-bottom flask used to hold the crude extract prior to rotational evaporation. To prevent the crude extract from sticking to the flask's round bottom, the crude extract was not overly evaporated in the rotary flask. The final volume of the crude extract was calculated which has some solvent contained in it, then it was oven dried to eliminate any leftover ethanol [12,13].

2.3 Partition

Partition was done by taking 20 mL of 25% aqueous ethanol to dissolve 1 g of the ethanolic crude extract. After adding 20ml of n-hexane to the 25% aqueous ethanol in a separatory funnel, the mixture was agitated vigorously for two minutes and the various separated layers were collected. The partitioned n-hexane layer and the layer of partitioned aqueous ethanol were collected, and the

process was repeated using di-chloromethane instead of n-hexane. Following rotational evaporation, all three solvent extracts were kept at -21^{o}C for additional analysis. During the solvent partitioning process, the mixture was dissolved in one of the solvents and then mixed with the other solvent. The mixture was then vigorously shaken or stirred to ensure thorough mixing and partitioning of the different components in the mixture between the two solvents. All the three solvents were dried in an oven at 50^{o}C to eliminate any leftover solvent [13].

2.4 Determination of Total Phenolics

The research approach employed in this study was adopted from De Araújo *et al.,* [6] and Abu *et al.,* [13]. Using a modified Folin-Denis technique, the total phenolic content of the extracts was determined. To do this, 0.5 mL of the extract or a gallic acid standard was combined for 3 minutes with 8.0 mL of deionized water and 0.5 mL of Folin-Denis reagent. Then, 1 mL of saturated Na_2CO_3 solution was added, and the mixture was incubated for 60 minutes at room temperature in the dark. Using a blank of 0.5 mL of the blank, the absorbance was measured at 720 nm. Gallic acid standards were used at different concentration to create a calibration curve. Each sample was examined three times, and the results were given as milligrams of gallic acid equivalent (GAE) per gram of dry material.

2.5 Determination of Total Flavonoid

The aluminium chloride colorimetric method described by De Kumar *et al.,* [14] and Safdar *et al.,* [15] was used to determine the total flavonoid content of the extracts. A mixture of 0.5 mL of the extract or a quercetin standard, 2.0 mL of deionized water, and 0.15 mL of Sodium Nitrate solution was incubated for 5 minutes. Then, 0.15 mL of aluminium chloride solution was added, and the mixture was incubated for 15 minutes in the dark at room temperature. Using a blank of 0.5 mL of the solvent, the absorbance was measured at 720 nm. Gallic acid standards were used to create the calibration curve at different concentrations. Each sample was examined three times, and the results were given as milligrams of quercetin equivalent (QE) per gram of dry matter.

2.6 Determination of 1,1, dipheny-2-picrylhydrazyl (DPPH) Radical Scavenging Activities

1 mL of each extract was added to a test tube for the DPPH radical scavenging activity test. Then, 1.5 mL of a DPPH• ethanol solution (6×10^{-5} M) was combined with 0.5 mL of ethanol with different concentrations of each extract. After 30 minutes of room temperature dark storage, the combination was tested for absorbance at 517 nm. Blank solutions, consisting of 2.5 mL of ethanol, were used as a baseline. The absorbance of the control solution was represented by Abs blank, and the absorbance of the extract-DPPH solution was represented by Abs sample. By extrapolating from the plot of the inhibition percentage, the IC_{50} values which represented the extract concentration that gave 50% inhibition were calculated. A total of three runs of each experiment were run, and the average result was calculated. Using the provided equation, the DPPH radical scavenging activity was calculated.

$$\%\text{Radical scavenging activity} = [(\text{Abs blank} - \text{Abs sample})/\text{Abs blank}] \times 100\% \quad (1)$$

2.7 Statistical Analysis

Experimental data will be analysed using the Statistical Package for the Social Sciences version 28.0 software (IBM SPSS, Version 28, Chicago, IL, USA). The results from this study will be represented as the mean values of three individual replicates ± the standard deviation. The One-Way Anova will be used to determine the significant differences between the mean values at a significance level of $p < 0.05$.

3. Results and Discussion

The identification of new sources of bioactive compounds is great importance to the pharmaceutical and food sectors, especially in replacing synthetic compounds or overcoming challenges such as antibiotic resistance in microorganisms [12,16]. As such, this experiment aims to determine the total phenolic content, total flavonoid content, and antioxidant properties of various extracts of *E.tirucalli* L. using dried aerial parts of the plant and solvents of different polarities, including aqueous ethanol, hexane, and dichloromethane, through the DPPH assay.

3.1 Determination Total Phenolic compound and Total Flavonoid Content

According to Table 1, the total phenolic content in the extracts of *E.tirucalli* L. aqueous ethanol extract had highest phenolic concentrations which is 2.501 mg GAE/g compared to the hexane and dichloromethane extract at level ($p < 0.05$).

Table 1
Total phenolic content present in different extracts of aerial part of *E.tirucalli* L., reported in gallic acid equivalents (GAE)

Samples	Total Phenolic Content (mg GAE/g sample)
Aqueous Ethanol (25%)	2.501mg/g ± 0.50
Hexane Extract	0.110mg/g ± 0.12
Dichloromethane Extract	0.050mg/g ± 0.01

* Values are shown as mean and standard deviation

Table 2 shows that aqueous ethanolic extracts exhibited significantly higher flavonoid content than the control which is 1.307 mg QE/g at level $p < 0.05$.

Table 2
Total flavonoid content present in different extracts of aerial part of *E.tirucalli* L., reported in quercetin equivalents (QUERCETIN)

Samples	Total Flavonoid Content (mg QE/g sample)
Aqueous Ethanol (25%)	1.307mg/g ± 0.11
Hexane Extract	0.164mg/g ± 0.03
Dichloromethane Extract	0.061mg/g ± 0.02

* Values are shown as mean and standard deviation

In this study, evaluated that the total phenolic content using three different solvents: aqueous ethanol (25%), hexane, and dichloromethane. The results revealed significant variations in the total phenolic content depending on the solvent used for extraction.

Among the solvents tested, aqueous ethanol (25%) exhibited the highest total phenolic content of 2.501 mg GAE/g ± 0.50. This finding is consistent with previous studies that have reported higher

phenolic content using polar solvents. For instance, De Kumar *et al.,* [14] and Le *et al.,* [17] found a total phenolic content of 19.74 mg GAE/g in *E.tirucalli* L. latex when extracted with methanol. The higher phenolic content observed in polar solvent extracts can be attributed to the ability of these solvents to dissolve a wider range of polar compounds, including phenolic acids and flavonoids.

In contrast, the hexane and dichloromethane extract of current study yielded significantly lower total phenolic content values of 0.110 mg GAE/g ± 0.12 and 0.050 mg GAE/g ± 0.01, respectively. These findings are consistent with the expected outcome of non-polar solvents, which have limited solubility for polar compounds like phenolics. The lower phenolic content obtained from these extracts aligns with the results of other studies. For example, De Kumar *et al.,* [14] reported total phenolic content values of 15.25 mg GAE/g and 20.52 mg GAE/g in water and methanol (80%) extracts of *Citrus reticulate* L. peel. These observations collectively highlight the influence of solvent polarity on the extraction efficiency of phenolic compounds.

The choice of solvent for extracting bioactive compounds is critical in obtaining accurate assessments of their potential health benefits. The variation in total phenolic content observed among different solvents underscores the importance of employing appropriate extraction methods that align with the specific class of compounds being targeted. Polar solvents are generally more effective in extracting polar compounds such as phenolics, non-polar solvents are preferred for lipophilic compounds.

In a previous study the ethyl acetate extract of *E.tirucalli* L. was used to measure the phenolic and flavonoid content, resulting in a range of 16.65-106.3 mg GAE/g and 0.97-0.45 mg QE/g, respectively [17]. However, in the current study, the total flavonoid content using aqueous ethanol was slightly higher which is 1.307 mg QE/g. The previous study on citrus peels highlights key findings relevant to the study of *E. tirucalli L.*, particularly the influence of solvent type on the extraction of bioactive compounds [18]. It emphasizes that polar solvents like aqueous ethanol are more effective in extracting phenolic and flavonoid compounds, aligning with the results of this study where aqueous ethanol extract exhibited the highest total phenolic and flavonoid content, as well as the strongest antioxidant activity. The solvents used for plant extraction in this study were hexane, dichloromethane, and aqueous ethanol using partitioning extraction. The choice of extraction method can significantly impact the yield and composition of the extracted compounds. Solvents with different properties, such as polarity, boiling point, and solubility, can affect the extraction efficiency of phenolic compounds. Aqueous ethanol, a polar protic solvent, effectively extracts phenolic and flavonoid compounds due to its high polarity, similar to methanol and acetone, which are commonly used in antioxidant studies. Methanol, slightly more polar than ethanol, is widely used for phenolic extractions, while acetone, though slightly less polar, has demonstrated efficacy for flavonoid isolation [19]. Hexane, a non-polar solvent effective for lipid extraction, shares characteristics with petroleum ether and cyclohexane, which are also suited for non-polar compound isolation. Dichloromethane, a moderately polar solvent, has comparable properties to chloroform and ethyl acetate, both of which are effective for extracting semi-polar compounds such as flavonoids [20]. These solvents provide a broad polarity spectrum, enhancing the efficiency of compound extraction depending on their chemical nature. In this study, the aqueous ethanol extract had the highest total phenolic and flavonoid content. Ethanol, which is a polar solvent, is commonly used for the extraction of phenolic compounds from plant materials due to its high boiling point and its ability to dissolve a wide range of polar and non-polar compounds, including phenolic compounds. Ethanol has high polarity making it a suitable solvent for extracting polar phenolic compounds, such as flavonoids, which are known for their high antioxidant activity [1].

Hexane and dichloromethane, on the other hand, are non-polar solvents that are commonly used in the extraction of non-polar compounds, such as lipids and terpenoids. These solvents have a lower

boiling point and are less effective at dissolving polar compounds, such as phenolic compounds [20]. As a result, the yield of phenolic compounds extracted using hexane and dichloromethane is lower in this study compared to ethanol. Hexane and dichloromethane are able to extract a wide range of non-polar compounds, such as latex, lipids and terpenoids, from plant materials [1]. In the case of *E.tirucalli* L., which is high in latex, hexane and dichloromethane can be used to extract the lipophilic antioxidant compounds present in the latex, such as terpenoids and fatty acids however the hexane and dichloromethane extract shows significantly lower phenolic and flavonoid content at level $p < 0.05$ than aqueous ethanol extract.

3.2 Determination of 1,1, dipheny-2-picrylhydrazyl (DPPH) Radical Scavenging Activities

Different solvents - hexane, aqueous ethanol, and dichloromethane and then tested for their antioxidant activity using the DPPH assay, which is a common method for measuring the potential of compounds to act as antioxidants. The results of the DPPH assay using different solvent are shown in Table 3.

Table 3
Percentage of Radical Scavenging Activity (% RSA), and IC_{50} of different extracts of aerial part of *E.tirucalli* L., determined by DPPH method

Concentration (ug/ml)	50	100	150	200	250	300	350	IC50
Samples								
HEXANE	57.96 ± 0.12	65.59 ± 1.90	67.29 ± 0.12	70.85 ±0.38	73.59 ±1.31	74.71 ± 0.26	77.49 ± 0.13	62.42 ± 1.34[c]
DICHLOROMETHANE	67.58 ± 1.21	75.50 ± 0.25	79.31 ± 0.34	83.29 ± 0.19	85.32 ± 0.13	84.66 ± 0.72	84.74 ± 0.31	50.94 ± 0.39[b]
AQUEOUS ETHANOL	89.18 ± 0.13	89.53 ± 0.12	90.55 ± 0.25	90.63 ± 0.07	91.04 ± 0.13	90.69 ± 0.50	90.88 ± 0.26	36.89 ± 0.05[a]
QUERCETIN (STANDARD)	88.39 ± 0.19	88.64 ± 0.19	89.22 ± 0.50	89.72 ± 0.07	89.84 ± 0.07	90.05 ± 0.12	90.96 ± 0.26	37.40 ± 0.13[a]

* Values are shown as mean and standard deviation

The IC_{50} value and radical scavenging activity of DPPH are two commonly used methods to evaluate the antioxidant activity of natural products. In this study, the IC_{50} value and DPPH radical scavenging activity were determined for three different samples and a standard: hexane, dichloromethane, aqueous ethanol, and quercetin. The results of the IC_{50} value from Figure 1 shows that the concentration required to inhibit 50% of the DPPH radical activity for aqueous ethanol was significantly lower than that of hexane and dichloromethane. Figure 2 shows the IC_{50} values for aqueous ethanol was 36.89 ± 0.05 µg/ml, respectively, while those for hexane and dichloromethane were 62.42 ± 1.34 and 50.94 ± 0.39 µg/ml, respectively.

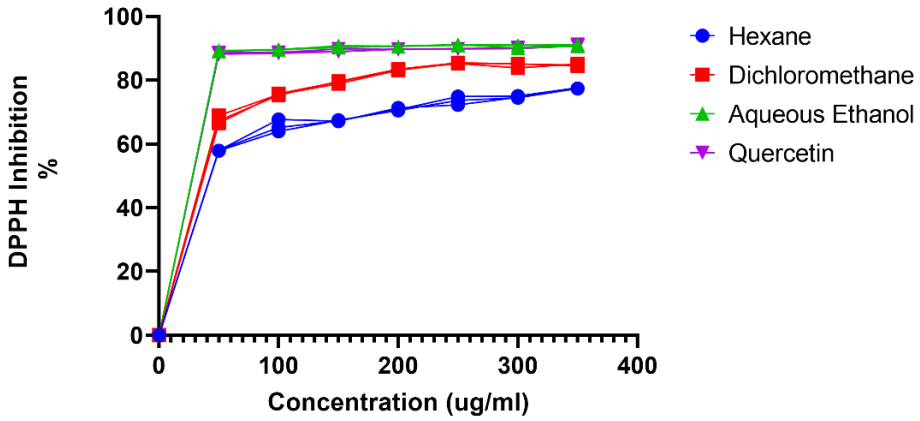

Fig. 1. The IC_{50} graph of DPPH assay using different solvents on *E.tirucalli* L.

HX = 62.42 ± 1.34
DCM = 50.94 ± 0.40
AQ.ETOH = 36.89 ± 0.05
QE = 37.40 ± 0.13

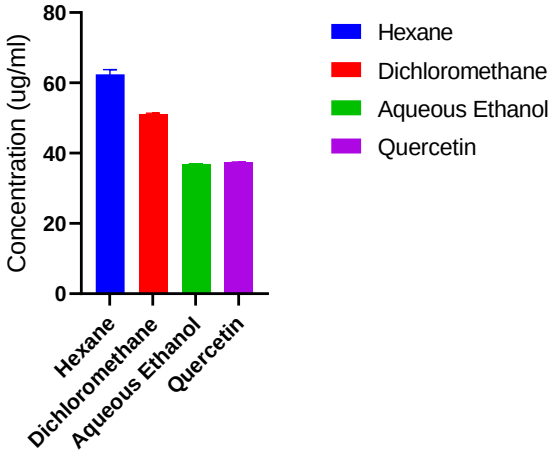

Fig. 2. The One-way Anova bar data of DPPH assay using different solvents on *E.tirucalli* L. (Hx-Hexane, DCM- Dichloromethane, AQ. EtOH- Aqueous ethanol, QE- Quercetin)

The results of the DPPH radical scavenging activity also showed that aqueous ethanol had significantly higher antioxidant activity against DPPH than hexane and dichloromethane. The percentage of DPPH radical scavenging activity for aqueous ethanol was consistently higher than that of hexane and dichloromethane at all concentrations tested. For instance, at the concentration of 350 µg/ml, the DPPH radical scavenging activity for aqueous ethanol and quercetin was 90.88 ± 0.26% and 90.96 ± 0.26%, respectively, while that for hexane and dichloromethane was 77.49 ± 0.13% and 84.74 ± 0.31%, respectively.

The results of this study suggest that aqueous ethanol could be potential sources of best solvent for extraction of natural antioxidants with strong antioxidant activity against DPPH for *E.tirucalli* L. extract. The high Radical Scavenging Activity (RSA) activity and low IC_{50} value of the aqueous ethanol extract suggest that it contains a high concentration of antioxidant compounds. This is likely due to the polar nature of ethanol, which can extract a wider range of polar compounds from the plant. On the other hand, the lower RSA activity and higher IC_{50} value of the hexane extract may be attributed to its non-polar nature, which may limit its ability to extract polar compounds with antioxidant

properties in the latex of the plant. For instance, the IC_{50} values of *Euphorbia royleana* extract in a study by Nowalid *et al.,* [18] were 29.1 µg/ml for the ethanolic extract, which is lower than current aqueous ethanol used, but higher than dichloromethane extract.

In terms of RSA activity, previous studies also found that Euphorbia plants possess potent antioxidant properties, which is consistent with current results. For example, the RSA values for Euphorbia tirucalli extract in the study by Mali *et al.,* [16] were 93.2% for the ethanolic extract, which is higher than current aqueous ethanol extracts, but lower than hexane extract. Additionally, the RSA values for Euphorbia extract in the study by Le *et al.,* [17] were 84.4% for the methanolic extract, which is comparable to current aqueous ethanol and quercetin extracts. Overall, the results from current study and previous studies indicate that Euphorbia plants contain compounds with potent antioxidant properties that can be extracted using different solvents [1]. The variations in the IC_{50} and RSA values obtained from different studies may be attributed to various factors such as differences in plant species, parts used, extraction methods, and geographical locations [1,20]. Nonetheless, the potential use of Euphorbia plants as a natural source of antioxidants should be further explored given the increasing demand for natural antioxidants as alternatives to synthetic ones.

In conclusion, the results of this study indicate that aqueous ethanol have strong antioxidant activity against DPPH, as demonstrated by the low IC_{50} values and high DPPH radical scavenging activity. These findings suggest that aqueous ethanol could be promising sources solvent to extract natural antioxidants for potential use in the food and pharmaceutical industries.

4. Conclusion

In conclusion, the results of the study on the total phenolic, total flavonoid content and DPPH assay of *E.tirucalli* L. extracts suggest that the aqueous ethanol extract contains a higher amount of these compounds and radical scavenging activity compared to hexane and dichloromethane extracts. This indicates that ethanol is an effective solvent for the extraction of phenolic and flavonoid compounds from *E.tirucalli* L. The high content of these compounds in the aqueous ethanol extract is significant because phenolic and flavonoid compounds have been shown to have numerous health benefits, including antioxidant and antimicrobial activities. These findings suggest that *E.tirucalli* L. may be a potential source of biologically active compounds with medicinal properties. Further studies are needed to fully understand the properties and potential applications of these compounds in the treatment of various diseases and health conditions.

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