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The Total Phenolic Content, Total Flavonoid Content and antioxidant properties of Euphorbia tirucalli L. extract partitioned with different solvents.

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ABSTRACT

The objective of current study is to measure the amounts of total phenolics, total flavonoids compound and DPPH assay from partitioned Euphorbia tirucalli dried aerial parts using different solvents. A total of three solvents were used in the extraction process: aqueous ethanol, hexane and dichloromethane. The results of the study showed that the aqueous ethanol extract had the highest phenolic content, at 2.501 mg GAE/g as well as the highest flavonoid content, at 1.307 mg QE /g. The IC50 values for hexane, dichloromethane, aqueous ethanol, and quercetin were 62.42 ± 1.34, 50.94 ± 0.39, 36.89 ± 0.05, and 37.40 ± 0.13, respectively. These results suggest that aqueous ethanol have the highest radical scavenging activity among the tested samples. This proves that aqueous ethanol is the most competent solvent for the extraction of phenolic and flavonoid compounds from Euphorbia tirucalli in this study. These findings have important implications for the potential use of Euphorbia tirucalli as a source of biologically active compounds with medicinal properties. Further studies are necessary to fully understand the properties and potential applications of these compounds in the treatment of various diseases and health conditions.

1. Introduction

Euphorbia tirucalli is a species of succulent plant that is native to Africa and is also commonly known as "Pencil Cactus" 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 (Kuster et al., 2015). Euphorbia tirucalli is a plant that has been traditionally used in African and Indian medicine for various purposes. Euphorbia tirucalli has been used to treat a variety of conditions,

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including wound healing, digestive disorders, respiratory disorders, inflammation, and even some types of cancer (Appedino et al., 2016). In recent years, it has been the subject of scientific research due to its potential health benefits due to its antioxidant activity.

The potential health benefits of Euphorbia tirucalli, which are attributed to its antioxidant activity, have drawn attention recently. Free radicals have been connected to a number of diseases, including cancer, cardiovascular disease, and neurological disorders. Antioxidants are well known for their capacity to neutralise these radicals and shield cells and tissues from oxidative damage. Numerous phenolic substances, including triterpenoids, phenylpropanoids, diterpenoids, and flavonoids, are found in Euphorbia tirucalli and display antioxidant effects by scavenging free radicals and preventing oxidative damage to cells and tissues. A vast variety of organic compounds known as phenolic compounds are found throughout the plant kingdom. They are distinguished by the presence of one or more phenol functional groups, which are made up of a hydroxyl (-OH) group attached to an aromatic group.

Phenolic compounds are a diverse group of organic compounds that can be classified into various subgroups, such as phenolic acids, lignans, flavonoids, stilbenes, and tannins. Each subgroup possesses distinct characteristics and biological functions. However, a wide spectrum of biological activities, including anti-inflammatory, antibacterial, antiviral, and antifungal capabilities, are recognised for all phenolic compounds (de Arajo et al., 2014). In plants, phenolic compounds play important roles in defense against pathogens and environmental stress, and are often involved in the production of pigments, fragrances, and secondary metabolites (Safdar et al., 2017). In humans, phenolic compounds are believed to provide protection against oxidative stress, inflammation, and also contributes antimicrobial properties.

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 (Dobias et al., 2010). 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 (Maroyi, 2018)

On the other hand, flavonoids are crucial components of a healthy diet since it provides many fruits, vegetables, and flowers their vibrant colours (Moroyi, 2018). 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, flavonols, flavanones, and flavanols (Khan et al., 2021). 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 (Le et al., 2021).

The DPPH test is a popular technique for assessing a compound's antioxidant properties. By contributing an electron or a hydrogen atom to a free radical, antioxidants can neutralise free radicals and stop them from harming cells and tissues through oxidative stress. The DPPH assay is based on the reduction of the stable free radical DPPH by an antioxidant agent, which creates a colourless solution and a non-radical DPPH molecule. The amount of the DPPH radical that is inhibited directly relates to the substance's antioxidant activity. The simplicity, rapidity, and adaptability of the DPPH assay make it superior to other antioxidant assays (Safdar et al., 2017).

Plants are rich in phytochemicals, such as phenolic compounds and flavonoids, which have a variety of biological functions including antioxidant, anti-inflammatory, and antibacterial characteristics (Thi et al., 2018). Phenolic and flavonoid compounds can hinder bacterial growth through various mechanisms, such as interfering with metabolic pathways, cell walls, and membranes. They can also act as antioxidants to neutralize reactive oxygen species produced by bacteria, reducing oxidative damage to host cells (Le et al., 2021). Moreover, some phenolic compounds can interfere with bacterial signaling and induce oxidative stress, reducing bacterial virulence and preventing the formation of biofilms. The effectiveness of phenolic and flavanoid compounds as antimicrobial agents can depend on several factors, including the specific compound, the concentration, and the type of microorganism being targeted. Therefore, the purpose of this study was to assess the total phenolic, flavonoid content, and antioxidant assay in the aerial part of the Euphorbia tirucalli plant using the Folin-Ciocalteu, aluminium chloride colorimetric, and DPPH assay methods, respectively. Soxhlet extraction will be used to extract antioxidant compounds, and this will be followed by partitioning the extracted compounds using various polar and nonpolar solvents.

2. Methodology

2.1 Sample collection

A total of 1090g of Euphorbia Tirucalli aerial parts were obtained from Kangar. 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 72 hours in an oven set to 50C. The parts were dried, then blended, and a fine powder was obtained by sieving them under a 250 nm siever. The final powder, which had been dried and sieved, weighed 121.65g. 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 -21C, according to the findings of de Arajo et al. (2014).

2.2 Soxhlet extraction

To fit within the Soxhlet extractor, the Euphorbia Tirucalli powder was precisely weighed at 9g on cellulose paper and secured with a string. Next, 200mL 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 (90C), which allowed the evaporated solvent gas to condense. Until the solvent was no longer coloured, the cycle was repeated 20 timesThe ethanol sample was extracted, and some of the solvent was removed in a rotary evaporator operating at 45ºC to create a 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 dried in an oven at room temperature to eliminate any leftover ethanol.For the Soxhlet extraction, a thimble containing the solid material is placed inside the Soxhlet apparatus, and the apparatus is filled with the solvent of choice, which is heated to boiling. The solvent vaporizes and condenses in a condenser, dripping back into the flask containing the solid material. This process is repeated continuously, with the solvent dissolving and extracting the desired compounds from the solid material. The Soxhlet apparatus is designed to prevent the solvent from coming into contact with the heating source, reducing the risk of solvent degradation or contamination (de Arajo et al., 2014).

2.3 Partition

Abu et al. (2017) provided the methodology that was employed in this investigation, with a few minor changes. A 20ml of 25% aqueous ethanol were used to dissolve 1g of the ethanolic crude extract. After adding 20ml of n-hexane to the 25% ethanolic extract in a separatory funnel, the mixture was agitated vigorously for two minutes. After 2 hours of settling the shaken mixture, the various separated layers were collected. The n-hexane layer and the layer of 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 -21C 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 (Abu et al., 2017)..

2.4 Determination of Total Phenolics

The research approach employed in this study was adopted from de Arajo et al. (2014). 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 Na2CO3 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 ranging from 0.01 to 0.1 mg/mL in 25% ethanol, hexane, and dichloromethane, respectively, were used to create a calibration curve. Each sample was examined three times, and the results were given as milligrammes of gallic acid equivalent (GAE) per gram of dry material.

2.5 Determination of Total Flavonoid

The aluminum chloride colorimetric method described by Cho et al (2020) 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 aluminum 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 concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1 mg/mL in 25% ethanol, hexane, and dichloromethane, respectively. Each sample was examined three times, and the results were given as milligrammes 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 (2.5, 5, 10, 20, and 40 µg/mL) 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 IC50 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 analyzed 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 use of herbal products to treat diseases has become increasingly popular due to their numerous phytometabolic contents with diverse chemical structures and biological activities. Phenolic compounds are molecules that are widely present in plants and are produced during the secondary metabolism of fruits, herbs, and vegetables. Research has demonstrated that an increase in the consumption of these foods can have positive health impacts. These compounds contribute to plant growth, reproduction, and pigmentation, while in foods, they help maintain oxidative stability and influence flavour and astringency.

The identification of new sources of bioactive compounds is highly valuable in the pharmaceutical and food industries, especially in replacing synthetic compounds or overcoming challenges such as antibiotic resistance in microorganisms. As such, this experiment aims to determine the total phenolic content, total flavonoid content, and antioxidant properties of various extracts of Euphorbia 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

According to Table 1, the total phenolic content in the extracts of Euphorbia tirucalli L. aqueous ethanol extract had highest phenolic concentrations which is 2.501 mg/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 Euphorbia tirucalli L., reported in gallic acid equivalents (GAE).

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

* Values are shown as mean and standard deviation, Different letters (a, b,c) mean significant differences ($p < 0.05$).

3.2 Determination of Total Flavonoid Content

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

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

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

* Values are shown as mean and standard deviation, Different letters (a, b,c) mean significant differences ($p < 0.05$).

In general, the best results in terms of either the phenolic content and flavonoid content were obtained from aqueous ethanolic extracts. According to de Araujo et al. 2014, the phenolic content of dried acetonic extract of Euphorbia tirucalli was higher than fresh acetonic extract of this plant which was 0.30 mg GAE/ g and 0.07 mg GAE/g respectively. The literature has extensively acknowledged and reported the impacts of drying fresh herbs, particularly rosemary, oregano, sage, marjoram, basil, and thyme, prior to extracting their constituents. Drying plant material before extraction is a common practice as it serves several purposes. Firstly, drying helps to preserve the plant material as fresh plant material has a high moisture content that promotes the growth of microorganisms and causes it to spoil (de Souza et al., 2019). Secondly, drying helps to concentrate the bioactive compounds present in the plant material, making them more potent and easier to extract (Khan et al., 2021). Thirdly, some plant compounds are not very soluble in water, but are more soluble in organic solvents. Drying the plant material can make these compounds more accessible and easier to extract with organic solvents (Mali and Panchal, 2017). Lastly, drying enhances the stability of the bioactive compounds, making

them less susceptible to degradation during storage and handling (Safdar et al, 2017). Overall, drying the plant material prior to extraction improves the quality and quantity of the bioactive compounds extracted from the plant material, which is crucial in various industries including food, cosmetics, and pharmaceuticals.

In a previous study by Le et al. (2021), the ethyl acetate extract of Euphorbia tirucalli 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. However, in the current study, the total flavonoid content using aqueous ethanol was slightly higher at 1.307 mg QE/g compared to the range reported by Le et al. (2021) of 0.97-0.45 mg QE/g. 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. 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.

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 (Dobias et al., 2010). These solvents have a lower boiling point and are less effective at dissolving polar compounds, such as phenolic compounds. As a result, the yield of phenolic compounds extracted using hexane and dichloromethane is lower in this study compared to ethanol. Hexane and dichloromethane is able to extract a wide range of non-polar compounds, such as latex, lipids and terpenoids, from plant materials.In the case of Euphorbia tirucalli, 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.

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

This discussion examines how effective extracts from Euphorbia tirucalli are as antioxidants. The extracts were made using 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 assay are discussed in relation to the potential use of Euphorbia tirucalli as a source of antioxidants.

Table 4. Percentage of Radical Scavenging Activity (% RSA), and iC50 of different extracts of aerial part of Euphorbia 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	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, Different letters (a, b,c) mean significant differences ($p < 0.05$)

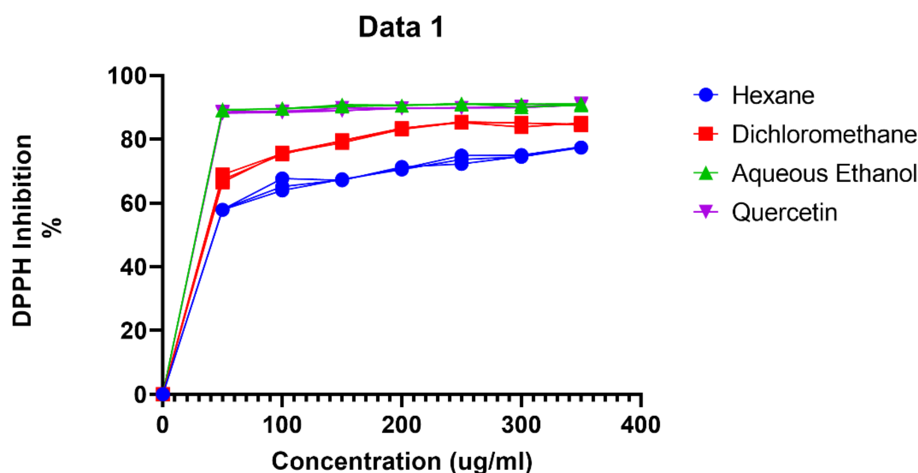

Figure 1.The IC50 graph of DPPH assay using different solvents on *Euphorbia Tirucalli* L.

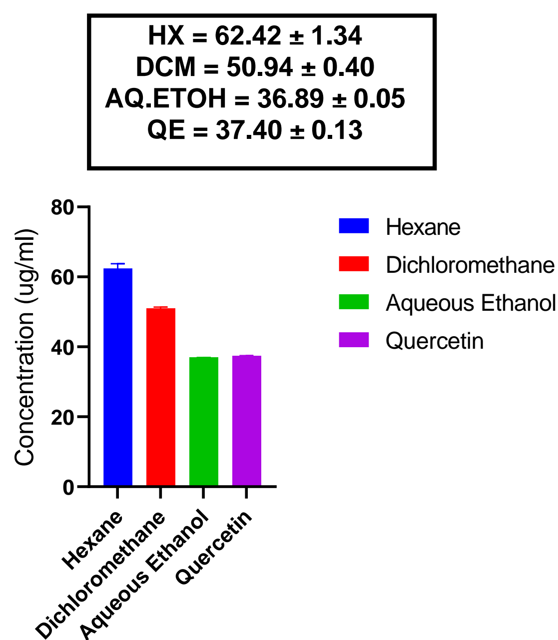

Figure 2. The One-way Anova bar data of DPPH assay using different solvents on *Euphorbia Tirucalli* L.

The IC50 value and radical scavenging activity of DPPH are two commonly used methods to evaluate the antioxidant activity of natural products. In this study, the IC50 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 IC50 value from Figure 1 showed that the concentration required to inhibit 50% of the DPPH radical activity for aqueous ethanol was significantly lower than that of hexane and dichloromethane. The IC50 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.

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 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 Euphorbia Tirucalli extarct. The high RSA activity and low IC50 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 IC50 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 IC50 values of Euphorbia royleana extract in Li et al. (2018) study were 29.1 µg/ml for the ethanolic extract, which is lower than current aqueous ethanol used, but higher than dichloromethane extract. Similarly, the IC50 values of Euphorbia helioscopia extract in Khan et al. (2013) study were 39.3 µg/ml for the methanolic extract, which is comparable to current aqueous ethanol 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 royleana extract in Li et al. (2018) study 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 helioscopia extract in Khan et al. (2013) study 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. The variations in the IC50 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. 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 IC50 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 Euphorbia tirucalli 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 Euphorbia tirucalli. 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 Euphorbia tirucalli 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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