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ORIGNAL ARTICLE

Phytochemical Characterization and Evaluation of Antioxidant and Antidiabetic Activities of *Euphorbia hirta*

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Conflict of Interest
All the authors have no conflict of interest

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Abstract
Background: For centuries, different countries have employed the genus "*Euphorbia*" to cure a variety of diseases. *Euphorbia hirta (EH)* has been traditionally employed in South Africa and tropical America for the management of inflammatory disorders and has been marketed as a crude medicinal preparation for conditions such as diabetes, bronchitis, asthma, wound healing, and pregnancy-related complications. In view of these ethno medicinal claims, the current study intended to determine the phytochemical composition, total phenolic content, potential of it antioxidant capacity, and to appraise the anti-diabetic potential of the methanolic extract and its different solvent fractions obtained from *Euphorbia hirta (EH)* leaves.
Methodology and results: Phytochemical screening was carried out using standard qualitative chemical assays. The presence of phenols, saponins, steroids, and flavonoids was confirmed in the *n*-hexane, methanol, aqueous, and ethyl acetate fractions of *EH* leaves. Among all fractions, the methanolic extract unveiled the maximum total phenolic content (89.2 ± 2.5 mg GAE/g extract). At a concentration of 100µg/ml, the plant's methanolic fraction demonstrated the greatest antioxidant activity, inhibiting DPPH radicals by 84.7%. The D glucose-6-phosphatase inhibition assay was used to measure in vitro antidiabetic activity. The methanolic fraction demonstrated notable inhibitory effect, attaining 59.1% glucose-6-phosphatase inhibition at 100µg/ml.
Conclusion: *Euphorbia hirta (EH)* leaves exhibit significant antioxidant and antidiabetic activities, particularly in the methanol extract due to its high phenolic contents. This is the first study to evaluate these bioactivities in this plant. The results demonstrated its potential as a source of natural therapeutic agent.
Keywords: *Euphorbia hirta (EH)*; phytochemical analysis; Antidiabetic activity; Antioxidant activity.

1. Introduction

From prehistoric times, plants both cultivated and wild type have been subjugated as source of medicine all across the world (Endesew & Amsalu, 2024). Different part of these plants e.g. berries, leaves, rhizome, seeds, roots, stem, bark, or flowers of plants one has to utilize to prepare such remedies. There are several benefits of employing plants as medication for a variety of medical ailments. Plants have pharmacological activities stem from phytochemical, contained in various parts of plants that have specific therapeutic effect on interacting with body (Nwozo, Effiong, Aja, & Awuchi, 2023). According to the reports of WHO, (World Health Organization), around 75%-80% of people globally trust on these herbal remedies. The growing popularity is largely attributed to several factors e.g., cost effectiveness of herbal medicines as compared to prescribed medicines, synthetic medications side effects, non-toxic nature and lower cost of herbal remedies, and the increased accessibility of health information (Organization, 2019). Plants are provider of many distinct kinds of secondary metabolites. These substances are employed not only as direct medicinal agents but also as models for pharmacologically active molecules or as building blocks for drug production (Abegaz & Kinfe, 2019). Many of these chemicals have pharmacological actions and are used to treat a variety of diseases, including depression, inflammation, cardiovascular disease, prostate issues, immune system enhancement, and antioxidant

qualities. Flavonoids, phenolic acid, tannins, and other chemicals with hydroxyl functionality, as well as phenols and their oxygen-substituted derivatives, have antioxidant action (Papuc, Goran, Predescu, Nicorescu, & Stefan, 2017).

EH leaf extracts were used in this study to assess their antioxidant and antidiabetic properties, based on the conventional aspects of herbal therapy. *EH* also known as Tawa-Tawa, is a member of the Euphorbiaceae family of plants. It's annual hairy erect herb that grows in arable lands, waste areas, roadsides and pathways. Among the many chemical components found in *EH* and other species are phenols, dicarboxylic acids, saponins, flavonoids, fatty acid esters, steroids and coumarins [7]. Numerous pharmacological activities, such as capillary permeability inhibition, antioxidant, antibacterial, antidiabetic effects and anticancer were investigated for Euphorbiaceae plants [8]. In our investigation into the phytochemical analysis of therapeutic plants, we discovered that leaf extracts and fractions *EH* have antidiabetic and antioxidant qualities (Ghosh et al., 2019).

2. Material and methods

2.1 Materials

The solvents like methanol, ethyl acetate, sodium hydroxide (NaOH), ammonia (NH_3), sulfuric acid (H_2SO_4), hydrochloric acid (HCl), dichloromethane, and glacial acetic acid were procured from Sigma-Aldrich. All solvent and chemical agents used were of analytical grade. The Folin-Ciocalteu reagent, HgCl, DPPH (1, 1-diphenyl-2-picrylhydrazyl), ascorbic acid, Dragendorff's reagent, anhydrous sodium carbonate (Na_2CO_3), gallic acid, potassium iodide (KI), and iodine all were procured from Sigma-Aldrich. Using a Rotatory evaporator, the solvents were recovered. To measure absorbance, a UV-Visible spectrophotometer (Shimadzu UV-1800; Japan) in the wavelength range of 200-800 nm was used.

2.2 Collection of Plant material

EH leaves were gathered in the month of April, 2024 from the Bahauddin Zakariya University's (BZU) Botanical Garden, Multan. The plant was identified by a botanist from the Department of Botany, BZU, Multan. A sample specimen was deposited in the herbarium with voucher number 3842F for future reference. The collected leaves were dried in the air, coarsely powdered, and stored in a sealed container for experimental examination.

2.3 Extraction and fractionation

Approximately 1 kg of *EH* leaves were taken and cut into small pieces and allowed to dry in an open air at room temperature for a few days. After drying, the pieces were crushed, weighed, and soaked in 95% methanol. Five extractions were done, with three days of rest between each one. After percolation, Whatman filter paper was used to filter. The whole alcohol-based extract was concentrated using a Rotatory-evaporator set to 150 rpm at 45°C. Using methanol, the extract (concentrate) was taken out from round-bottom flask and subsequently put into weighed beakers. The alcohol-based solvent was then permitted to evaporate until it was dried. After that, the solid dried extract was gathered, weighed, and separated. After treating 50 g of methanolic extract with *n*-hexane by trituration, the *n*-hexane insoluble portion was dispersed in water and then extracted using 3×500 ml of methanol and 3×500 ml of ethyl acetate. As a result, fractions of methanol (20.5 g), water (8.2 g), and ethyl acetate (12.7 g) were produced.

2.4 Phytochemical screening

All extracts and fractions underwent phytochemicals screening using with some minor adjustments, the method outlined by (Momoh, Olaleye, Sadiq, & Ahmed, 2022), Phenolic acids, flavonoids, steroids, and saponins were all searched for during the screening. Analytical testing included precipitate formation and color intensity. The qualitative findings are denoted by (+) for phytochemical presence and (-) for phytochemical absence.

2.5 Estimation of the total phenolic content

Using a Folin-Ciocalteau reagent the total soluble phenolic contents in the extract were determined through UV-Vis spectrophotometer, by following the protocols described (Neo et al. 2008). Gallic acid was employed as the standard. To prepare the stock solution, 10 ml of methanol were used to dissolve one mg of gallic acid (100µg/ml). This was then further diluted to 8µg/ml, 4µg/ml, 2µg/ml, and 1µg/ml. Distilled water (10 ml) was used to dilute a 1 ml sample of each dilution. The Folin-Ciocalteu reagent (3ml) was later added, and the whole mixture was incubated for about 5 min at room temperature. Every model of the sample received ~2ml of 20% (w/w) sodium carbonate (Na_2CO_3) and was allowed to remain at room temperature for 30 min. the absorbance was measured using a UV-Vis spectrophotometer at 765 nm, in comparison to distilled water as a blank (Zhou et al., 2019).

2.6 DPPH assay

The results were described in mg of gallic acid equivalent (GAE) per 100 grams of the dry material. The value of the coefficient of determination was found to be r = 0.997. Using the previously established methodology, the DPPH antioxidant capacity was measured. The sample was found to be the cause of a drop in the absorbance of a colored DPPH solution in methanol at 517nm wavelength. The reference standard used was ascorbic acid. In short, 1 ml of the sample solution was mixed with 5ml of methanol and DPPH (0.1 mM) at concentrations of 5, 10, 25, 50 and 100µg/ml as concentrates. These mixtures were allowed to incubate at room temperature for about 30 min. The absorbance was then calculated at 517 nm wavelength using UV-Vis spectrophotometer against methanol used as a blank. All fractions were analyzed for the DPPH assay (Okawa, Kinjo, Nohara, & Ono, 2001). The given formula was employed to determine the DPPH radical's inhibition:

$$\% \text{ Inhibition of (DPPH)} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

Where;

Absorbance $_{(control)}$ = Absorbance of DPPH radical and methanol.

Absorbance $_{(sample)}$ = Absorbance of DPPH radical extract/standard.

2.7 D-glucose-6-phosphatase assay

To assess how the plant extracts affected the metabolism of liver glucose, we performed a glucose-6-phosphatase assay. This enzyme is crucial for gluconeogenesis and glycogenolysis. Male Wistar strain rats' livers were taken to perform the experiment. The animals had unobstructed access to food and water ad libatum, while being kept at a constant temperature of (24±1) °C, humidity of (55-65%), and a 12-hour light/dark cycle. Before being used in experiments, the animals were acclimatized to the standard laboratory conditions for at least one week. All protocols used in the study were approved by the Animal Ethical Committee of the University via a letter 24-PEC/2024. Using a Potter Elvejhem glass homogenizer with a Teflon pestle, a 10% homogenate was made in 150 mM KCl. After centrifuging the homogenate for 10 minutes at 1500 rpm, the supernatant was decanted and employed as a source of enzymes. By pre-incubating the chemical for 15 minutes in a 1ml reaction system and subsequently calculating the remaining glucose-6-phosphatase activity using Hubscher and West's technique, the effects of extract and fractions were investigated (Aboy-Pardal et al., 2024). A 200mM glucose-6-phosphate, around 0.3M citrate buffer having (pH = 6.0), 28mM EDTA, 14mM NaF, and enzyme protein were all included in 1ml of assay apparatus. After 30 minutes of incubation at 37°C, the mixture was supplemented with 1 ml of TCA (10%). The inorganic phosphates (P_i) quantity in the supernatant that is protein-free was estimated using the following approach (Tanveer, Singh, & Khan, 2017). The rate of inorganic phosphate release, measured in µmol P_i min^{-1} mg^{-1} protein, was used to evaluate the activity of glucose-6-phosphatase.

2.8 Statistical analysis

All the data were presented as means± SD and are used to express the values. The experiments were conducted in triplicate. The prism 8.0.2 (GraphPad Prism software, inc., USA) was utilized to examine the data via one-way ANOVA and subsequent Dunnett's test for multiple comparisons. In the experiments, *P* values below 0.05 i.e. $P<0.05$ were considered significant.

3. Results and Discussion

3.1 Phytochemical screening

Phenols, saponins, steroids and flavonoids were among the secondary metabolites found in *EH* leaf extracts (Table 1) after a preliminary phytochemical screening. The methanol and aqueous fractions of plant extract were found to be rich in most of the secondary metabolites like flavonoids, saponins, phenols with fewer steroids while ethyl acetate fraction moderately contained the phenols, steroids with a smaller number of flavonoids. *n*-hexane fraction was found to be active only for a smaller number of steroids as mentioned in table-1.

Table 1: Results of Phytochemical screening of *Euphorbia hirta* leaf extract.
Reaction intensity is shown as +++ (strong), ++ (medium), + (weak), and − (not observed).

Sr.	Phytochemicals	Ethyl acetate Fraction	*n*-Hexane Fraction	Aqueous Fraction	Methanol Fraction
1.	Saponins	-	-	+++	+++
2.	Phenol	++	-	++	+++
3.	Steroids	++	+	-	+
4.	Flavonoids	+	-	+++	++++

3.2 Extraction yield

According to the findings of the extraction yield, *EH* various extracts amount vary depending on the type of solvent and ranges from 8.2 to 44.0% in decreasing order: methanol > ethyl-acetate > water > *n*-hexane (Table 2). While the extraction yield with solvent like *n*-hexane was little when equated to other solvents. The extraction of methanol produced the greatest number of total extractable chemicals. Methanol may have a higher extraction yield because it readily passes through cell membranes to remove intracellular components from plant (Saini, Prasad, Shang, & Keum, 2021). According to these findings, *EH* methanolic extract contains a higher concentration of polar secondary metabolites compared to ethyl acetate, aqueous, and *n*-hexane extracts.

3.3 Total phenolic content

The Folin-Ciocalteu chemical was utilized to quantify the amount of total phenol. The gallic acid standard curve was used to represent the total phenols as mg/g GAE (Gao, Xu, He, Sun, & Zeng, 2019). Methanol fractions had the highest phenolic concentration (89.2 ± 2.5mg GAE/g extract) while the ethyl acetate fraction moderately contained the phenolic contents; 22.7 ± 1.6mg GAE/g. There were no phenolic contents found in the *n*-hexane and aqueous fractions. These findings unequivocally show that the polarity of the solvent determines the amount of phenolic compounds; the more polar the solvent, the higher the phenolic compounds present (Zengin et al., 2022). Furthermore, these results indicate that *EH* leaves are a significant source of phenolic compounds as mentioned in table-2.

Table 2: Results of extraction yield and total phenolic content of the *Euphorbia hirta* extracts.

Sr. No	Fraction	Extraction yield (%w/w)	Total phenolic content (mg GAE/g extract)
1	n- Hexane	8.2 ± 0.5	ND
2	Ethyl acetate	24.3 ± 2.3	22.7 ± 1.6
3	Methanol	44.0 ± 1.8	89.2 ± 2.5
4	Aqueous	17.3 ± 1.9	ND

Tests were conducted in triplicate and result is expressed as mean ± standard deviation (SD).
ND= Not detected

3.4 Antioxidant activity

The free radical scavenging activity can be best described by estimation of DPPH activity. Many antioxidant assays describe the capacity of the tested chemical or other products to hunt free radicals. It is used for the study of compounds that capable of donating electrons and hydrogen, such as phenols and flavonoids (Munteanu & Apetrei, 2021). The antioxidant potential of other fractions was evaluated via using DPPH % free radical scavenging test at varied doses ranging from 5-100µg/ml for each fraction. At all doses, the methanol fraction exhibited the highest percentage of free radical scavenging, with 84.7% inhibition at 100µg/ml. The ethyl acetate has a considerable scavenging activity ranging from 24.9% at 5µg/ml to 36.7% at 100µg/ml. The *n*-hexane fraction displayed less antioxidant activity with maximum 27.4% inhibition at 100µg/ml concentration. In this test, the aqueous fraction did not exhibit any detectable inhibition. For the current study, ascorbic acid was utilized as the standard. These results suggest that the methanol fraction has the highest capacity to scavenge free radicals, most likely because of its higher phenolic concentration as presented in table-3.

Table 3: Results of DPPH assay of various fractions of *Euphorbia hirta.*

Sr No.	Concentration (µg/ml)	DPPH% inhibition of free radicals			
		***n*-Hexane Fraction**	**Ethyl acetate Fraction**	**Methanol Fraction**	**Aqueous Fraction**
1	5	19.9	24.9	69.8	ND
2	10	22.8	28.3	78.3	ND
3	25	23.7	31.7	79.9	ND
4	50	26.8	33.3	80.1	ND
5	100	27.4	36.7	84.7	ND

ND= Not detectable.

3.5 Antidiabetic activity

Diabetes is associated with increased oxidative stress due to the production of free radicals produced from oxygen. Free radical generation in diabetes mellitus can lead to lipid peroxidation by oxidative damage, non-enzymatic glycation of proteins, and the breakdown of glucose (Black, 2024). Through the production of phosphate groups and free glucose upon hydrolysis, glucose-6-phosphatase functions as a hydrolyzing enzyme and is essential for the homeostatic control of blood glucose levels (Tan et al., 2024). The inhibitory activity of glucose-6-phosphatase was assessed in each fraction using sodium orthovanadate, a common drug. Two fractions like *n*-hexane and ethyl acetate showed inhibition of the glucose-6-phosphatase enzyme with 21.3% and 34.7%, respectively. The methanol fraction showed the most active inhibition of the enzyme at 100µg/ml mentioned in the figure-1 below. In this experiment, the aqueous fraction showed no detectable inhibition. These results of aqueous fractions suggest that bioactive components responsible for observed antidiabetic activity are extracted more effectively using organic solvents rather than in aqueous medium.

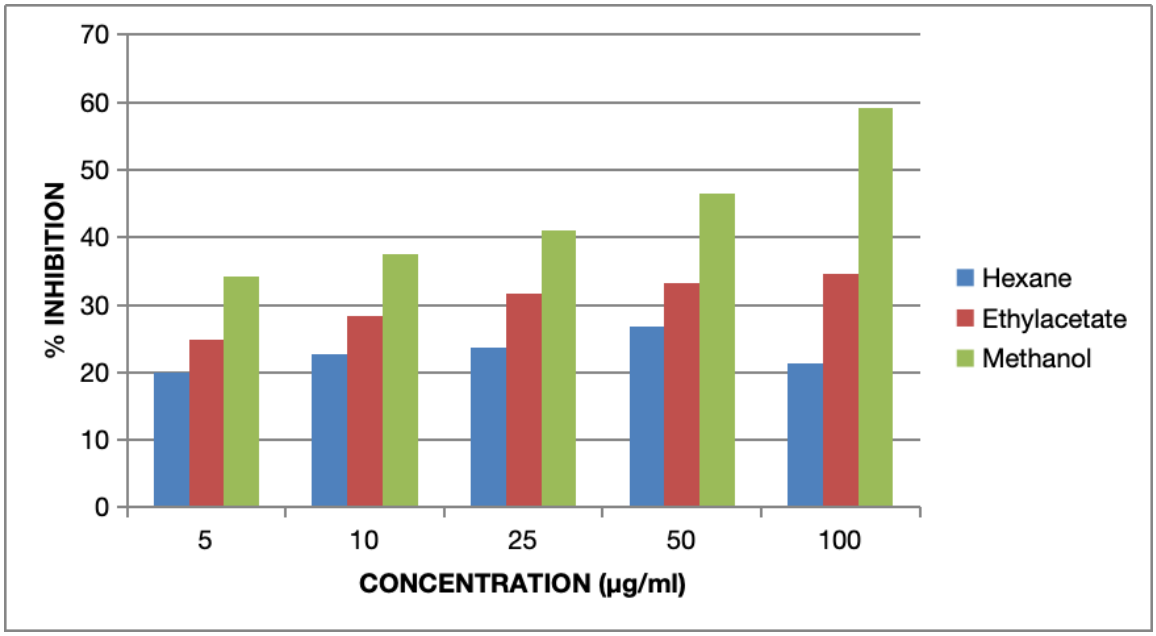

Figure 1: Results of antidiabetic activity of various extracts of *Euphorbia hirta*. Here the X-axis indicates the concentration of plant extract and Y-axis indicates percentage inhibition of glucose-6-phosphatase.

4. Conclusion

This study provides scientific validation for the traditional use of *Euphorbia hirta.* Phytochemical screening confirmed the presence of phenols, flavonoids, saponins, and steroids in the leaf extracts. The methanol fraction demonstrated the highest phenolic content (89.2 ± 2.5mg GAE/g extract) and exhibited the most potent bioactivities. At 100µg/ml, it scavenged 84.7% of DPPH free radicals, demonstrating a strong antioxidant ability, and notable antidiabetic potential by inhibiting 59.1% of glucose-6-phosphatase enzyme activity at 100µg/ml. The strong correlation between the high phenolic contents and bioactivities suggests that these compounds are likely responsible for the observed effects. This plant is evaluated for the first time for the antioxidant and antidiabetic potential and these findings confirm that *Euphorbia hirta* leaves are a promising source of natural antioxidants and antidiabetic agents, warranting further investigation to isolate and characterize the specific bioactive compounds for potential therapeutic development.

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