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Assessment of Phenolic Contents, Cytotoxic, and Anti-Oxidant Potential of *Euphorbia royleana*

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ABSTRACT

Aim and Objective: The study was designed to assess the cytotoxic, phenolic contents and anti-oxidant potential of *Euphorbia royleana*.

Methods: The organic extracts were obtained by extraction of whole dried plant with n-hexane, dichloromethane and methanol. Pytochemical screening was carried out by using standard methods. Total phenolics contents were measured by using the Folin–CioEcalteu reagent method. Cytotoxic action was evaluated by brine shrimp lethality bioassay. DPPH radical assay was employed for anti-oxidant activity.

Results: Phytochemical investigations confirmed the presence of anthraquinones, saponins, cardiac glycosides and flavonoids. Highest phenolic content (276.41 ± 64 mg GAE/g dry extract wt) was observed in methanol extract. Significant antioxidant potential was exhibited by methanol extract by means of 78.8% inhibition with IC_{50} value of 35.71µg/ml. In brine shrimp lethality bioassay, methanol extract demonstrated prominent cytotoxicity at highest level of dose with LD_{50} 471.05.

Conclusion: The study concluded that *Euphorbia royleana* contains significant antioxidant and cytotoxic potential. Further investigation is suggested for isolation of secondary metabolites responsible for reported biological activities.

Keywords: *Euphorbia royleana, Total phenolics, Anti-oxidant, Cytotoxic.*

INTRODUCTION

A large number of higher plants hoard extractable secondary metabolites in adequate quantity to be utilized to cure a variety of disorders. Plants have been employed for pharmacological purposes since the start of human civilization. An extensive collection of bioactive molecules had been produced by medicinal plants. Scientific studies of these molecules have resulted in production of medicinal substances with biological actions in opposition to infection and ailments [1]. Herbal medicines are used by more than seventy five percent of word population as, described by data of W.H.O. [2].

Over time there is considerable enhancement in the tactic and instrumentation employed for depiction, synthesis and isolation of natural products. Recent developments in this regard may be beneficial, resulting in advancement in exploration of secondary metabolites. Literature survey reports that plant derived natural products have vital role in production of reliable and affluent supply of drugs. Structural diversity of natural products is one of the prominent features, which provides the researchers many

opportunities, leading to discover novel lead structures with improved pharmacological actions. Plants and microorganism are major source of natural products. According to literature survey up till now, only ten percent of plants have been investigated in this regard. That's why there is immense potential for researchers to discover new secondary metabolites with significant biological potential [3].

The plant family "Euphorbiaceae" belongs to *Phylum "Anthophyta".* "Euphorbiaceae" is a very large family which comprises almost 326 genera and almost 9100 species. Plants from this family are distributed all over the world. The plants of this family are found in tropical regions, with the majority of the species in the Indo-Malayan region. Almost 25 genera of Euphorbiaceae exist in Pakistan the genus "*Euphorbia"* hold almost two thousand species. The discrepancy within this genus is surprising, as it contains both low-growing garden weeds called "spurges" and gigantic, cactus-like succulents. Majority of Euphorbia succulent is present in temperate climates.

Euphorbia royleana, also known as Dandathor in Pakistan, is an important medicinal plant. It's a spiky shrub that grows up to 1.8–2.4 metres tall. It has long been used to cure a variety of diseases, including paralysis, ear ache, and sluggish motions [4]. Its latex has been shown to have anti-inflammatory and anti-arthritic properties. *Euphorbia royleana* stem bark extract possesses antiacetylcholinesterase action [5]. According to a literature review; plants in the Euphorbiaceae family have a variety of biological activities. Bhuvaneshwar *et al.* in a study described that *E. tirucalli* is used as traditional medicine to combat various ailments including rheumatism, asthma, arthritis, neuralgia, warts, cough, cancer and gonorrhea. Latex of the plant *Euphorbia nerrifollia* has been found effective for the treatment of tumors and abdominal disturbance. Study also reveals that the latex of *Euphorbia hirta* is employed as purgative, carminative, whooping cough, asthma and management of jaundice and kidney stones. Leaves of the plant have been used for handling abdominal pain, bronchitis, tumors and various inflammatory disorders [6].

Free radicals have been proved to affect human health, by contributing to progression of different chronic ailments including diabetes, hypertension, cancer heart and other degenerative disorders. Free radicals are produced via metabolic process in body. Anti-oxidants intake can help our body to cut down the bad effects of free radicals. Remarkable interest in use of antioxidants has been observed in recent years, to prevent the unsafe effects of free radicals to human. Preference is given to use of anti-oxidants obtained from natural source rather than form semi-synthetic source [7].

Recent study was conducted to explore phyto-chemical screening, phenolic contents, and anti-oxidant action of various extract from *Euphorbia royleana.* Outcome of this research work may add on the whole to therapeutic potential of the selected plant.

MATERIALS AND METHODS

The research was carried out in the pharmaceutical chemistry laboratory of B.Z.U. Multan's Faculty of Pharmacy. The materials and processes are depicted in great detail below.

Collection of *Euphorbia royleana*.:

Euphorbia royleana was collected from WAPDA grid station in District Sahiwal. Prof. Dr. Sameena recognized the plant as *Euphorbia royleana*. The specimen voucher # 452CV2 was placed in the herbarium of B.Z. University Multan's department of applied biology.

Extraction of *Euphorbia royleana*:

Whole plant material was dried for 15 days in the shade for adequate extraction. It was dried, then ground and weighed in a grinding mill. The extraction of Euphorbia royleana was done by maceration in succession. A determined volume of n-hexane was introduced to 500 gm of powdered material in an extraction bottle. This mixture was shaken for a while before being homogenised in an ultrasonic bath to determine the effectiveness of the extraction. After a 24-hour period, the mixture was filtered. Then, following the same technique, marc was macerated again with n-hexane. The marc was extracted with dichloromethane and methanol in the same conduct after the third filtration of the extract. The rotary evaporator was used to concentrate the solvent extracts. The extracts were quantified and allocated ERH (Euphorbia royleana n-hexane), ERD (Euphorbia royleana dichloromethane) and ERM (Euphorbia royleana methanol) codes, accordingly.

Preliminary Phytochemical Analysis:

Chemical tests are performed to determine the elements of a drug sample and to identify them.

These tests might be very specific for a single component or very comprehensive for a collection of compounds, such as alkaloids. Majority of tests result in the development of color or turbidity. Color should be matched to an actual specimen, and turbidity in the sample tube should be compared to the reagent-containing test tube alone in the event of precipitation reactions. Generally, these assays can be used on both extracts and isolated components [8].

Tests for Cardiac Glycosides:

Detection of cardiac glycosides was carried out by using Keller Kilini test. In a test tube, 1g of the ground drug under investigation was placed. Alcohol was added to it. Then mixture was heated for 2 minutes on water-bath and then strained. Filtrate was diluted with twice volume of distilled water. Lead acetate was added, followed by filtration of solution that removed chlorophyll and other pigments. The filtrate was subjected to extraction by using 10ml of chloroform or carbon tetrachloride. Chloroform layer was separated. It was evaporated by using china dish over water bath. Few ml of 3.5% ferric chloride in glacial acetic was added to residue in order to dissolve it. Later on transferred to test tube after few minutes. Then sulfuric acid was poured down, causing the inferior layer to separate. On standing, the presence of cardiac glycosides was confirmed by the appearance of pale green hue in the top layer and brown color at the junction [9].

Test for Anthraquinones Glycosides (Borntrager's Test):

Borntrager's test was employed to identify the above mentioned secondary metabolite**.** On the study, 0.5g of pulverized drug was taken and extracted for 10 minutes using ten ml of hot water. Later on subjected to filtration. It was cooled, later on extracted by using ten ml of CCl_4. CCl_4 layer was taken off; 5 ml of water was added to test tube. Afterwards, a5ml diluted ammonia solution was added and thoroughly mixed. As a result of the lack of colour, no free anthraquinones were discovered (pink to cherry-red).

For testing of bound anthraquinones separate test was conducted. 0.4g of powdered drug was extracted using ten ml $FeCl_3$ solution and 7ml HCl. It was heated for 15 mints in a water bath. The filtrate was chilled after the solution was filtered. After that, 10ml of CCL4 was used to extract it. After separating the carbon tetrachloride, 5ml of distilled water was used to wash it away. It was mixed with 5ml of dil. NH4 solution. The absence of colour (deep pink to cherry-red) in the medication under investigation revealed no anthraquinone glycosides [10].

Tests for Saponin Glycosides:

0.5g of powdered drug was added to water. Persistent froth were formed which indicated presence of saponins [11].

Tests for Alkaloids:

3g of the powdered material under research was heated for one minute in 10ml of dil. HCl. It was cooled, and the debris was given time to settle. The liquid from the supernatant was transferred to another test tube. In 1ml of filtrate, 4 drops of Dragendorff's reagent were added. Turbidity was noticed, which confirmed the presence of alkaloids.

By adding a weak ammonia solution to the filter, it was rendered alkaline. It was then transferred to a separating funnel and extracted with 10 mL chloroform and gentle shaking. There were two levels identified. With 10ml of dil. CH_3COOH, the chloroform layer (bottom) was extracted.. The extract was equally separated into four test tubes. 2-5 drops of reagents were added to 3 test tubes, one by one. Each tube was compared to untreated test tube, for examination of turbidity or precipitate, which ultimately reconfirmed the presence of alkaloids in test sample [12].

Determination of Total Phenolics:

Polyphenolic compound are among significant plant constituents having redox properties responsible for antioxidant action. Presence of ployhydroxyl groups in secondary metabolites of plant extracts participate vital task in facilitating free radical scavenging action. Plant extracts were taken in different test tubes. 5 ml Folin-Ciocalteu reagent was added to each tube. 4 ml of 7.5% sodium carbonate (Na_2CO_3) was added to each tube after few minutes. After a few minutes, 4 ml of 7.5 percent sodium carbonate (Na_2CO_3) was added to each tube. For 3 hours, the sample was maintained at room temperature. Spectrophotometers were used to calculate absorbance at 766 nm. Absorbance of each sample was taken thrice. Standard curve of gallic acid solution was prepared using the parallel course of action. Total phenolic content of plant extracts was stated as mg GAE/100 g extract sample.

Anti-oxidant Activity:

Because of its ease of use and low cost, DPPH is the predominant approach for determining anti-oxidant

potential. Antioxidant prospective of plant extracts was evaluated by free radical scavenging using DPPH method. Free radical scavenging action of plant extracts on DPPH radical was studied to evaluate the anti-oxidant potential. Anti-oxidant evaluation was carried out by scheme recommend via Akowiuah *et al.* (2005) [13].

A test tube was filled with 200 µl of sample extracts and 0.8 ml of methanol. After that, each test tube was filled with a 0.1mM DPPH methanolic solution. Blend was shaked well; test tubes were kept for one hour in dark. The control was prepared by mixing 1 ml methanol and 2 ml of DPPH. The absorbance of each sample was measure at 517 nm with the help of micro plate reader spectrophotometers. Each reading was taken thrice. One hundredth of DPPH actions was pre-meditated by formula given below.

% inhibition of DPPH = [Abs control –Abs sample / Abs control] x 100.

Brine-Shrimp Lethality Assay:

Many secondary metabolites are found to be toxic for shrimp larvae. The Brine-Shrimp lethality assay is one of the rapid, cost-effective techniques for evaluation and scrutinizing of bioactive natural products [14].

Procedure: In a liter of distilled water, 3.8 g of sea salt was mixed and then filtered in order to make artificial sea water. Shrimp eggs covered with aluminum foil in larger slot of tank. These tanks were filled with artificial water prepared as mentioned above. Shrimp eggs were hatched and mature in 48 hours at controlled temperature of 22-29 ºC. 3 replicate of every extract were prepared. For this purpose 20mg of each sample was dissolved in 2ml of suitable organic solvent. Afterwards these were shifted to 500µl, 50µl or 5µl vials correspondingly. Organic solvent was allowed to evaporate at room temperature. Insoluble content was added to DMSO 5ml artificial sea water and ten shrimps/ vial were added after 48 hours of maturation of larvae, with the assistance of a Pasteur pipette the vials were kept in a lit environment. With the use of a 3x magnifying glass, after 24 hours, number of extant shrimps was then counted and recorded.

RESULTS

Phytochemical Screening:

Secondary metabolites profile of the plant was studied by using standard phytochemical screening methods. Result of detection of secondary metabolites is sum up in Table **1**.

Total phenolic contents:

The result is represented as, gallic acid equivalent. Result of total phenolic contents in plant extracts is given below in Table **2**.

Antioxidant activity:

The DPPH free radical scavenging assay was used to determine the anti-oxidant capacity of the plant extracts. Both extracts demonstrated concentration-dependent increase in radical scavenging capacity. ERM showed 78.8% inhibition with IC_{50} value of 35.71µg/ml, at highest concentration (100 µg/ml). Whereas the dichloromethane extract exhibited 71.82% inhibition at dose of 100 µg/ml with IC_{50} of 41.88 µg/ml. n-hexane extract exhibited non-significant anti-oxidant potential. Result of antioxidant activity of plant extracts is shown in Figure **1**. Ascorbic acid was used as standard in DPPH assay.

Table 1. Result of Detection of Secondary Metabolites *in Euphorbia royleana:*

Plant Extract	Alkaloids	Anthraquinones	Saponins	Cardiac glycosides	Flavonoids
ERH	−	−	+	−	+
ERD	−	+	+	+	+
ERM	−	+	+	+	+

(+) = Present (−) =Absent

Table 2. Total Phenolic Contents in The Plant Extracts (Expressed in Terms of Gallic Acid Equivalent (mg of GA/g of Extract)

Plant Extract	TPC (mg GAE/g dry extract wt)
ERH	04.67± 0.71
ERD	106.23 ± 0.41
ERM	276.41 ± 64

TPC: total phenol content GAE: Gallic acid equivalents

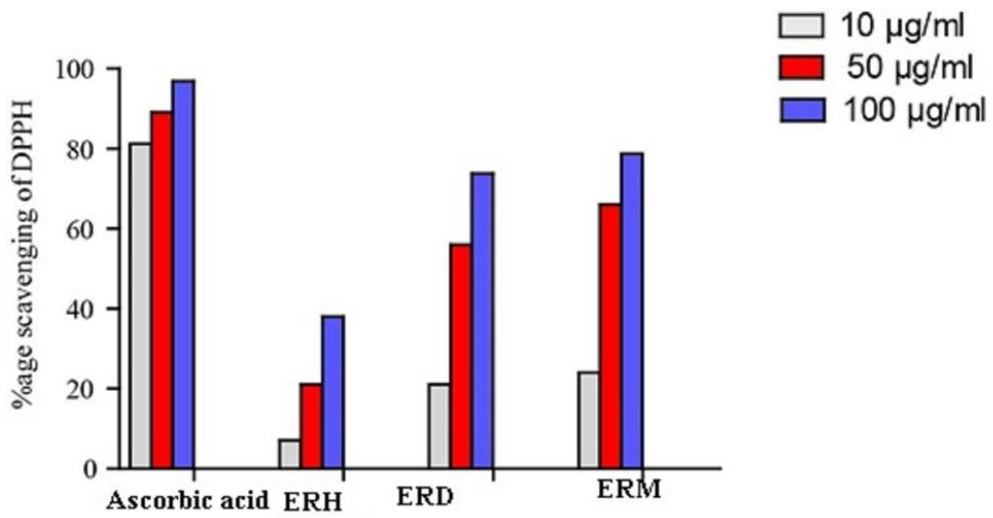

Figure 1. DPPH free radical scavenging activity of different extracts of *Euphorbia royleana*.

Table 3. The Result of N-hexane, Dichloromethane, and Methanol Extracts of *Euphorbia royleana* on the Lethality of Brine Shrimp.

Extracts	Dose (µg/ml)	No. of shrimps	No. of survivors	LD50 (µg/ml)	Standard Drug	LD50 (µg/ml)
ERH	1000	30	24	42564.1	Etoposide	7.4625
	100	30	28			
	10	30	29			
ERD	1000	30	23	22563.3	Etoposide	7.4625
	100	30	26			
	10	30	29			
ERM	1000	30	02	471.5	Etoposide	7.4625
	100	30	25			
	10	30	28			

Brine shrimp (*Artemia salina*) lethality bioassay:

Plant extracts of *Euphorbia royleana* were investigated for cytotoxic prospective by employing Brine shrimp lethality test. Methanol extract was found to be cytotoxic at the highest dose level, having an LD_{50} of 427.18. Non significant activity was shown by n-hexane and dichloromethane extract. Result is described below in table 3 mentioned below.

DISCUSSION

Due to their redox properties, phenolic compounds are important plant components with anti-oxidant action. The hydroxyl groups in plant extract aid in free radical scavenging. Flavonoids are one of the important secondary metabolites which hold considerable anti-oxidant and chelating actions. Substitution pattern of hydroxyl groups in structure of any flavonoid contribute to its anti-oxidant action [15].

Polyphenolic compounds from plant source have been reported as potent anti-oxidants, attractants for insects, UV screens (flavonoids), and in production of defense response chemicals. Various flavonoids play an important role in signal pathway. Phenolic compounds have a key role in human defense responses, such as anti-oxidants, anti-aging, anti-proliferative and anti-inflammatory actions [16]. Study conducted by Zunli *et al* has validated the antioxidant, anti-inflammation, anti-aging potential of various flavonids derived from plant source [17].

Human body produces certain unstable molecules as a reaction to various environmental and stress factors. Anti-oxidants which are also called as “free

radical scavengers" are those substance which can avert or slow down harm to human body cells by free radicals.

Free radicals smash up the cell of any organism by damaging the DNA. Oxidative stress by a series of reactions is also produced by these free radicals. Therefore, biological evaluation of crude extracts regarding anti-oxidant potential from plant origin is also increasing rapidly [18, 19]. The brine shrimp lethality technique is a swift, low-cost, and easy bioassay for assessing plant extract bioactivity which is usually associated with cytotoxic and anti-tumor activities. It's a preliminary toxicity test in preparation for more research on mammalian animal models. Several studies have demonstrated that the brine shrimp assay is a useful tool for preliminary toxicity assessments, screening medicinal plants commonly used for a variety of reasons, and monitoring the isolation of a wide range of biologically active chemicals [20].

CONCLUSIONS

Euphorbia royleana contains high phenolic content, which most likely is contributing factor for its anti-oxidant potential. Plant extract also exhibited significant cytotoxic potential at highest tested dose. Further investigation is suggested for isolation of secondary metabolites responsible for reported biological activities.

CONFLICT OF INTEREST

There are no conflicts of interest declared by any of the author.

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