CODEN [USA]: IAJPBB ISSN : 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES
SJIF Impact Factor: 7.187
http://doi.org/10.5281/zenodo.5140796

Online at: http://www.iajps.com *Research Article*

EVALUATION OF PHENOLIC CONTENTS, CYTOTOXIC, AND ANTIOXIDANT POTENTIAL OF EUPHORBIA NESEMANII

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Article Received: June2021 **Accepted:** June2021 **Published:** July2021

Abstract:

Aim and Objective:

The aim of study was to evaluate the cytotoxic, phenolic contents and antioxidant potential of Euphorbia nesemanii.

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 antioxidant 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 nesmanii contains significant antioxidant and cytotoxic potential. Further investigation is suggested for isolation of secondary metabolites responsible for reported biological activities

Key words: Euporbia nesemanii, Total phenolics, Antioxidant, Cytotoxic.

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Please cite this article in press Khuram Ashfaq et al., ***Evaluation Of Phenolic Contents, Cytotoxic, And Antioxidant Potential Of Euphorbia Nesemanii****.., Indo Am. J. P. Sci, 2021; 08(07).*

1. INTRODUCTION:

A large number of higher plants hoard extractable secondary metabolites in adequate quantity to be used for treatment of various ailments. For very beginning of human civilization, plants had been effectively used for pharmacological actions. 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 about 9000 species. Plants from this family are distributed all over the world. This family occurs mainly in the tropics, 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. Majorty of Euphorbia succulent are present in temperate climates.

"Euphorbia nesemanii" is an upright succulent. It is a cactus like fleshy shrub with least number of leaves. New leaves come out at some point in the showery season. Its flowers are yellow and come into view at some stage in the summer. [4]. Literature survey reports several Euphorbiaceae plants, teas and fresh latex are used these days in different alternative folk medicines. Bhuvaneshwar et al. in a sudy described that *E. tirucalli* is used as traditional medicine to combat various ailments including rheumatism, asthma, arthritis, neuralgia, warts, cough, cancer and gonorrhea [5].

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 [6]. Free radicals are produced via metabolic process in body. Antioxidant's 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 harmful effects of free radicals in human body. Preference is given to use of antioxidants obtained from natural source rather than form semi-synthetic source [7].

Recent study was conducted to investigate phytochemical screening, phenolic contents, and antioxidant action of various extract from *E. nesemanii.* Outcome of this research work may add on the whole to therapeutic potential of the selected plant.

2 MATERIALS AND METHODS:

The research study was performed in pharmaceutical chemistry laboratory, Faculty of Pharmacy, B.Z.U. Multan. Detailed depiction of materials and methods is illustrated below.

2.1. Collection of Euphorbia nesemanii:

Euphorbia nesemanii, was collected from forest park of Perrowal distric Khanewal. Prof. Dr. Zaffrullah identified the plant as Euphorbia nesemanii. The specimen voucher # 38FCV1 was deposited in the herbarium of department of pure and applied biology, B.Z. U. Multan**.**

2.2. Extraction of Euphorbia nesemanii:

For effective extraction, whole plant material was kept under shade for drying for 15 days. When dried, it was grinded by grinding mill and weighed. The extraction of Euphorbia nesemanii was carried out by successive maceration. 500 gm of powdered material was taken in extraction bottle and measured volume of, n-hexane was added to it. In order to ensure the maximum possible extraction, this mixture was shaken after some time then homogenized in ultrasonic bath. Filtration of this mixture was carried out after 24 hrs. Then marc was macerated again by n-hexane using same procedure. After third filtration of extract, the marc was extracted by, dichloromethane and methanol in the same manner. Rotary evaporator was used to concentrate the solvent extracts. Extracts were weighed and assigned with codes as ENH, END and ENM respectively.

2.3. Preliminary Phytochemical Analysis:
Chemical tests are used for evaluation and identification of constituents in drug sample. These tests are very particular for any single compound or general for a definite group of constituents i.e. alkaloids. Color or turbidity is developed in many tests. Color should be matched with an authentic specimen while turbidity in sample tube, in case of precipitation reactions, is compared with reagent containing test tube alone. Mostly, these tests can equally be applied for extracts and isolated components [8].

2.3.1. Tests for cardiac glycosides:
Detection of cardiac glycosides was carried out by using Keller Kilini test. 1g of ground drug under study was put in test tube. Ten ml of seventy percent alcohol was added to it. Then mixture was boiled for 2 minutes on water-bath and then filtered. Filtrate was diluted with double volume of distilled water. Lead sub acetate solution was added. Solution was filtered again that remove 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. 3ml 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 added (along the wall of test tube) which separated as the lower layer. Cardiac glycosides were confirmed by appearing of pale green colour at upper layer and Brown color at interface on standing [9].

2.3.2. Test for anthraquinones glycosides (Borntrager's test):
0.5g of ground drug on study was taken and extracted with 10ml of hot water for 5 minutes. Later on filtered while hot. It was cooled and extracted by using 10ml of CCl_4. CCl_4 layer was taken off; 5 ml of water was added to tube. Afterwards 5ml of dilute ammonia solution was added and shaked well. No free anthraquinones were revealed as absence of color (pink to cherry-red).

For testing of bound anthraquinones separate test was conducted. 0.4g of powdered drug was extracted with 10ml of $FeCl_3$ solution and 5ml hydrochloric acid. It was heated on water bath for 15 minutes. The solution was filtered, and the filtrate was allowed to cool. It was then extracted with 10ml of CCL_4. The carbon tetrachloride was separated and washed with 5ml of water. and 5ml of dilute ammonia solution was added to it. No anthraquinone glycosides were revealed as absence of color (intense pink to cherry-red) in drug under study [10].

2.3.3. Tests for saponin glycosides:
0.5g of powdered drug was added to water. Persistent froth was formed which indicated presence of saponins [11].

2.3.4. Tests for alkaloids:
3g of powdered drug under study was taken and boiled with 10ml of dilute HCl for one minute. It was cooled: the debris was allowed to settle down. Supernatant liquid was transferred to other test tube. 4 drops of Dragendorff's reagent were added to 1ml of filtrate. Appearance of turbidity was observed which indicates presence of alkaloids.

Leftovers of the filtrate were made alkaline by adding dilute ammonia solution. It was transferred to separating funnel, extracted with 10 ml of chloroform with mild shaking. Two layers were observed. Chloroform layer (lower) was extracted with 10ml of dilute acetic acid. The extract was divided into four portions. Few drops of Wagner's reagent, Mayer's reagent and Dragendorff's reagent were added to three portions 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].

2.4. Determination of total phenolics:
Polyhenolic compound are among significant plant constituents having redox properties responsible for antioxidant action. Presence of ployhydroxyl groups in secondary metabolites of plant extracts play important role in facilitating free radical scavenging action [13]. Total phenolic contents of plant extracts were determined using Folin-Ciocalteu reagent as described by Singlaton and Rossi (1965). 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. Sample was kept at room temperature for 3 hours. The absorbance was measure at 765 nm using microplate reader spectrophotometers (Molecular devices, VERSAmax tunable, California, USA). Abosrbance of each sample was taken thrice. Standard curve of gallic acid solution (10, 20, 40, 60, 80 and 100 ppm) was prepared using the similar procedure. Total phenolic content of plant extracts was stated as mg GAE/100 g extract sample.

2.5. Antioxidant Activity:
Antioxidant potential of plant extracts was evaluated by free radical scavenging using DPPH method. Free radical scavenging action of plant extracts on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was studied to evaluate the antioxidant potential.

Antioxidant evaluation was carried out by method proposed by Akowuah *et al*. (2005).

200 µl of sample extracts was taken in test tube and 0.8 ml of methanol was added to it. Then 0.1mM DPPH methanolic solution was poured to each test tube. Mixture 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 ofeach sample was measure at 517 nm with the help of microplate reader spectrophotometers. Each reading was taken thrice. Percentage of DPPH scavenging activity was calculated by formula given below.
% Inhibition of DPPH = [Abs control –Abs sample / Abs control] x 100.

2.6. 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: 3.8 g of sea salt was dissolved per liter of distilled water and then filtered in order to make artificial sea water. Shrimp eggs covered with aluminum foil e 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. Three replicates of each 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 dissolved in dimethyl sulfoxide (DMSO). 50µl/5ml of artificial sea water was also added to vials. 5ml artificial sea water and ten shrimps/ vial were added after 48 hours of maturation of larvae, with the help of Pasteur pipette. Vials were kept under illumination. After 24 hours, with the help of a 3x magnifying glass; the number of surviving shrimps were counted and recorded. Recorded data was investigated by using software (Probit analysis) in order to find out LC50 and 95% confidence intervals values.

3 RESULTS AND DISCUSSION:

3.1. Phytochemical screening:
The phytochemical profile of the plant was studied by using standard phytochemical screening methods. Result of detection of secondary metabolites is summarized in Table 1.

Table 1: Result of detection of secondary metabolites *in Euphorbia nesemanii:*

Plant Extract	Alkaloids	Anthraquinones	Saponins	Cardiac glycosides	Flavonoids
ENH	–	–	+	–	+
END	–	+	+	+	+
ENM	–	+	+	+	+

(+) = Present **(–) =Absent**

3.2. Total phenolic contents:
The total phenolic contents in the examined plant extracts using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent. The values obtained for the concentration of total phenols are expressed as mg of GA/g of extract. Result of total phenolic contents in plant extracts is given below in table 2.

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)
ENH	04.67± 0.71
END	106.23 ± 0.41
ENM	276.41 ± 64

TPC: total phenol content GAE: Gallic acid equivalents

3.3. Antioxidant activity:

Antioxidant potential of the plant extracts was evaluated by using DPPH free radical scavenging assay. Plant extracts showed concentration-dependent increase in radical scavenging capacity. ENM 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 antioxidant potential. Result of antioxidant activity of plant extracts is shown in figure 1. Ascorbic acid was used as standard in DPPH assay.

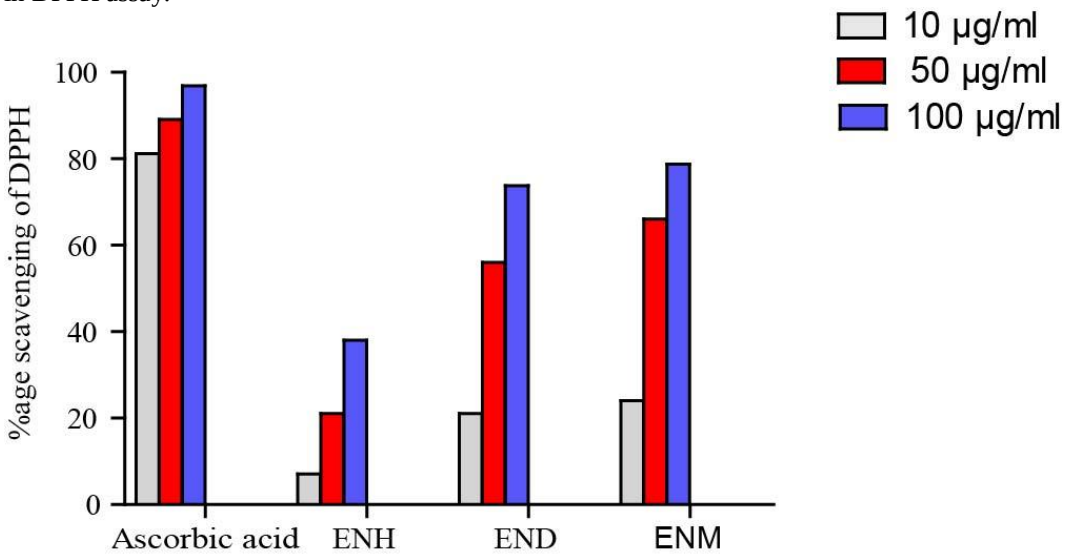

Figure 1: DPPH free radical scavenging activity of different extracts of Euphorbia nesemanii.

3.4. Brine shrimp (*Artemia salina*) lethality bioassay:

Plant extracts of *Euphorbia nesemanii* were investigated for cytotoxic prospective by employing Brine shrimp lethality test. Methanols extract demonstrated cytotoxicity at highest level of dose with LD_{50} 427.18. Non significant activity was shown by n-hexane and dichloromethane extract. Results are shown below in table below 3.

Table 3: Results of Brine shrimp (*Artemia salina*) lethality bioassay of n-hexane, dichloromethane and methanol extract of *Euphorbia nesemanii*.

Extract	Dose (µg/ml)	No. of shrimps	No. of survivors	LD50 (µg/ml)	Standard Drug	LD50 (µg/ml)
ENH	1000	30	24	42564.1	Etoposide	7.4625
	100	30	28			
	10	30	29			
END	1000	30	23	22563.3	Etoposide	7.4625
	100	30	26			
	10	30	29			
ENM	1000	30	02	471.5	Etoposide	7.4625
	100	30	25			
	10	30	28			

DISCUSSION:

Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity. The hydroxyl groups in plant extracts are responsible for facilitating free radical scavenging. Flavonoids are one of the important secondary metabolites which hold considerable antioxidant and chelating actions. Substitution pattern of hydroxyl groups in structure of any falvonoid contribute to its antioxidant action. [15].

Polyphenolic compounds from plant source have been reported as potent antioxidants, 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 antioxidants, anti-aging, anti-proliferative and anti-inflammatory actions. [16].

Human body produces certain unstable molecules as a reaction to various environmental and stress factors. Antioxidants 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 antioxidant potential from plant origin is also increasing rapidly [17, 18]. The brine shrimp lethality assay symbolizes a fast, low-cost and straightforward bioassay for testing plant extracts bioactivity which in most cases associates convincingly with cytotoxic and anti-tumor properties. It's a preliminary toxicity screen for further experiments on mammalian animal models. Several studies have shown that brine shrimp assay has been an excellent method for preliminary investigations of toxicity, to screen medicinal plants popularly used for several purposes and for monitoring the isolation a great variety of biologically active compounds [19].

4. CONCLUSIONS:

The study revealed that Euphorbia nesemanii contains high phenolic content, which most likely is contributing factor for its antioxidant potential. Plant extract also exhibited significant cytotoxic potential at highest tested dose. To the best of our knowledge this is the first ever report of cytotoxic and antioxidant study of Euphorbia nesemanii. Further investigation is suggested for isolation of secondary metabolites responsible for reported biological activities.

5. Conflict of interest:

All authors declare that there is no conflict of interest.

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