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Original Research Article

Botanical Features and Anti-cancerous Potential of *Euphorbia nivulia* Buch.-Ham

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ABSTRACT

Euphorbia nivulia – Buch.-Ham. *(En)*, one of the members of Euphorbiaceae family, is rich in various phytoconstituents including flavonoids and polyphenolics. Many species of the genus *Euphorbia* have been reported to possess varying biological activities, including anti-proliferatory, cytotoxic, anti-inflammatory and antimicrobial activities. This medicinal plant species indigenous to the Cholistan desert of Bahawalpur, Pakistan, was evaluated for its botanical features and anti-cancerous potential. The species was evaluated for botanical features through anatomical evaluation and powder microscopy. Standard conventional procedures were employed for preliminary qualitative phytochemical screening of *En* (crude extract) and various fractions, named as *En* hexane (*En* hex), *En* chloroform (*En* ch), *En* butanol (*En* bt) and *En* aqueous (*En* aq) to identify various phytoconstituents. Toxicity to *Artemia salina* (Leach shrimp) larvae was assayed through Brine Shrimp Lethality Assay (BSLA) using Etoposide as standard. Anticancer activity of the plant against 3T3 and HeLa cell lines was assayed using the tetrazolium salt reduction assay. Doxorubicin and Cyclohexamide were used as standard. *En* showed optimum activity, 73.33% mortality against *Artemia salina*. Maximum cytotoxic activity against 3T3 and HeLa cells was shown by *En* and *En* hex. The current study may be helpful in proper identification of the plant species and suggests that *En* may be a suitable anticancerous candidate.

Keywords: *Euphorbia nivulia*, Botanical features, Anti-cancerous potentials, 3T3, HeLa cells.

Introduction

Traditional medicines have been used for thousands of years in countries such as Egypt, India, China, and many other countries. Herbal products are still used as primary health care products in most third world countries.[1] Plants are an enormous reservoir of biologically active phytochemicals/compounds with diverse chemical structures and possessing protective/preventive properties against various diseases. Therefore, recent researches are devoted to the phytochemical investigation of higher plants with ethno-pharmacological relevance.[2] Today, the use of novel bioactive plant extracts in drug production for cancer prevention is dominating over synthetic chemicals.[3,4]

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Plant-derived biologically active compounds could act as protective agents against human carcinogenesis. Indo-Pakistan medicinal plants have a great history for their utility as a remedy for the treatment of a variety of ailments. The impact of medicinal plants and their isolated pure compounds is in a variety of therapeutic areas, but the major contribution is in the field of anti-infective and anticancer drug discovery.[5] In the past, plants have provided leads for the development of potential novel anti-cancerous agents like Taxol, paclitaxel, vinblastine and vincristine, etc., for a wide variety of leukemia, ovarian, breast and non-small-cell lung carcinomas. Screening of plant extracts has been of great interest to scientists to discover new anti-cancerous drugs.[6] Cancer is one of the most dangerous diseases in humans and presently demanding new anticancer agents from natural products. Over the last half-century most of the plant secondary metabolites and their derivatives have been used towards combating cancer.[7,8] Smoke, smoking, dietary imbalances, hormones, chronic infections/inflammations, plastic, artificial colors, etc., are the major causes of cancer. One of the major reasons of using herbs/plants as a source of anti-cancerous drug is that they are relatively simple, readily available, effective, economical and least toxic. To properly study the natural bioactive compounds of plant origin, development of certain bioassay techniques are necessary. In vitro methods allow the screening of large number of compounds for cytotoxicity against

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many types of cancer cell lines. 9 It is not always possible to test against cancer in animal models due to the system complexity. Therefore, in vitro assays are more sensitive to most anti-tumor agents than in vivo assays and usually requires less test material, time and money. [10] Crude plant extracts can be first assayed for particular activities and the active fractions then analyzed phytochemically. [2] A variety of cytotoxicity bioassays are now available. [11] Brine shrimp lethality assay (BSLA) is a general bioassay that appears capable of detecting a broad spectrum of bioactivity. This method provides a front-line screen that can be backed up by more specific and expensive bioassays, once the active compounds have been isolated. BSLA is predictive of cytotoxicity and pesticidal activity.[12] BSLA study cytotoxicity to reveal new anticancer compounds. Taxol [TM], an antitumor drug approved by FDA for treatment of ovarian, breast and non-small-cell lung carcinomas was discovered using the BSLA assay.[13] Ethno-pharmacological and scientific investigation has revealed anticancerous activity displayed by various members of *Euphorbia*, to justify the use of plants in ethno-medicine for the treatment of cancer and other various diseases. The Euphorbiaceae family, also known as the spurge family (the 4th largest family of the angiosperms), is composed of about 340 genera and over 8000 species, [14] which are distributed in several types of vegetation and habitats, mainly in the tropics. Approximately 195 species of one of its genus *Euphorbia* have been recorded from India. [15] *Euphorbia nivulia* – Buch.-Ham. is one of the members of the genus *Euphorbia* that belongs to the Euphorbiaceae family. It is rich in various phytoconstituents including flavonoids and polyphenolics. Preliminary phytochemical investigation of the plant showed the presence of phenolics and flavonoids as well as other several phytoconstituents.[16] Many species of the genus *Euphorbia* have been reported to possess varying biological activities, including anti-proliferation, cytotoxic, anti-inflammatory and antimicrobial activity, etc. These biological activities are attributed to the presence of specific classes of macro- and polycyclic diterpenes.[17, 18] Ethnopharmacologically, a few species of the genus *Euphorbia* have been used for the treatment of skin diseases, gonorrhea, migraine, intestinal parasites and as wart cures. [19] Generally, Euphorbia spp. has been used to treat asthma, leukemia, warts, as a laxative and diuretic, and have antiviral, antibacterial and antifungal activities.[20-22] As anticancer and antitumor, some Euphorbia species have shown activity against some cancers and tumors, like malignant melanoma, squamous cell carcinoma and lung cancer.[23] It has been reported that some species of Euphorbia have potential cytotoxic effect against different cell lines.[24] Presence of carcinogenic compounds, skin irritants, tumor promoters, anti-cancer agents and agents for overcoming multidrug resistance[25] is an incentive for detailed studies of other *Euphorbia* spp. Interest in natural products from *Euphorbia* species exhibiting biological activities was initiated with the isolation and structure elucidation of the tumour-promoting phorbol-12,13-diester of *Croton tiglium* L. (Euphorbiaceae) in 1968[26] and the description of the anti-tumour activity of a diterpene isolated from *Euphorbia esula* L. in 1976.[27] Release of ingenol mebutate (Picatos), a diterpene isolated from *Euphorbia peplus* L. used for the topical treatment of actinic keratosis[28] stimulated interest in natural products from Euphorbia species. Lathyrane diterpenoids isolated from *E. nivulia*, showed significant cytotoxic activity against Colo 205, MT2, and CEM cell lines.[29] The present work is an attempt to study the botanical features and anti-cancer activity of *Euphorbia nivulia.*

Materials and Methods

Plant material

Aerial parts (stem, branches, leaves and flowers) of fresh, well grown *Euphorbia nivulia* plant were collected in the months of March and April, 2015 from the premises of Hasilpur Road and adjoining areas of Bahawalpur region, Pakistan. The plant was authenticated by Ghulam Sarwar, a taxonomist in the Department of Botany, The Islamia University of Bahawalpur. A voucher specimen (EN-AP-05-12-041) was deposited in the herbarium of Pharmacology Research Lab, Department of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur, Pakistan. The collected plant parts were chopped into pieces and spread on filter paper under shade for drying at room temperature for forty days. Then, these were powdered by an electric grinder, and sieved through mesh No. 60. The powder was kept in closed amber coloured glass bottles. The fresh material was used for organoleptic evaluation, while the dried form was used for the physicochemical analysis and other parameters.

Chemicals

All the chemicals and solvents used were of analytical grade and purchased from Sigma Aldrich, B. D. H. and Merck. Organic solvents (methanol/ethanol/acetone/water, DMSO), distilled water, test samples, *Artemia salina* (brine shrimps eggs), sea salt (38 g/L of D/W, pH 7.0, 3T3 (mouse fibroblast cells), HeLa cells, tetrazolium salt (obtained from Dr. Panjwani Center for Molecular Medicine and Drug Research and H.E.J Research Institute of Chemistry, University of Karachi, Karachi), hatching tray with perforated partition, lamp to attract brine-shrimp larvae, micro pipette (5, 50, 500 µL), vials tray, 9 vials samples for storage of *Artemia salina* eggs, flask, measuring cylinder, weighing balance, petri dish rotary evaporator, refrigerator, filter paper what-man # 1, binocular stereomicroscope (Labomad, USA).

Botanical Evaluation

Botanical evaluation of the fresh aerial parts was performed as per WHO guidelines.[30,31]

Microscopic evaluation

Powder m*icroscopy*

Small quantity of fine powder of the sample was placed on glass slide and 1 to 2 drops of mounting media were added. Digital binocular stereomicroscope **(**Labomed, USA) was used to observe the tissues and their organisations initially at 10x and 40x magnifications corresponding to low and high-power lens. The observed microscopic structures were identified by comparing with the previously reported standard.[32, 33]

Anatomical evaluation

Free hand transverse sections were prepared from leaves, stems and flowers of *Euphorbia nivulia*. Each specimen was placed on the glass slide and covered with cover slip, while binocular research microscope was used to observe the slide.[34] Safranin solution was used for the staining of thick-walled tissues like xylem, collenchyma, sclerenchyma cells, etc., while malachite green was used for the staining of thin-walled tissues like phloem, parenchyma cells, etc.

Extraction and fractionation

Dried powdered plant material (10 kg) was macerated in 30 L of 70% aqueous ethanol at room temperature for 15 days with occasional stirring. The macerated mixture was filtered three times with muslin cloth separately and then further filtration was done by Whatman filter paper number 01. The filtrate was then evaporated under reduced pressure (-760 mmHg) and controlled temperature on the rotary evaporator (Heidholph Laborota 4000-efficient Germany and Buchi Rotavapor R-20). A thick, semisolid, and dark brown gummy mass was obtained which was then placed in the oven (Memmert Beschichung Loading Model 100-800). The dried material was weighed, labeled and then stored at 4^{o}C in an airtight container. The percentage yield was calculated. The condensed extract was used for further experiments. Moreover, n-hexane, chloroform and n-butanol was used in increasing order of polarity for partitioning of the extract into various fractions as previously described.[31]

Preliminary phytochemical screening

Preliminary qualitative phytochemical screening of the *En* crude extract as well as various fractions, named as, *En* Hexane (*En* hex), *En* Chloroform (*En* ch), *En* Butanol (*En* bt) and *En* aqueous (En aq) fraction to identify the phytoconstituents like alkaloids, glycosides, flavonoids, tannins, saponins and phenols, etc., was carried out by using standard conventional procedures.[16,30,31] Details of various tests performed is summarised in Table 3.

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Brine shrimp lethality assay
The procedure for BSLA as described by *Solis et al.*[35] with little modification was adopted. Each sample (20 mg) was dissolved in 2.0 mL of ethanol and used to prepare the concentrations of 10, 100, 1000 µg/mL by transferring 5, 50 and 500 µL respectively to each vial. Three vials were made for each concentration, then the solvent was allowed to evaporate overnight.[35] Data was analyzed with Finney computer program to determine LD_{50} values with 95% confidence intervals.[36]

Cytotoxicity assay (3T3)
Cytotoxic activity of phytoconstituents was evaluated in 96-well flat-bottomed micro plates by using the standard MTT (3-[4, 5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay. MTT assay is potentially useful for assaying antiproliferative activities of materials, because of its specificity for living cells.[37] For this purpose, 3T3 (mouse fibroblast) cells were cultured in Dulbecco's Modified Eagle's Medium. The extent of MTT reduction to formazan within cells was calculated by measuring the absorbance at 540 nm, using a micro plate reader (Spectra Max plus, Molecular Devices, CA, USA). The cytotoxicity was recorded as concentration causing 50% growth inhibition (IC_{50}) for 3T3 cells. The percent inhibition was calculated using following formula:

% inhibition = 100-((mean of O.D of test compound – mean of O.D of negative control) / (Mean of O.D of positive control – mean of O.D of negative control)*100).

Results (% inhibition) were processed by using Soft- Max Pro software (Molecular Device, USA). The following concentrations were used for the calculations of IC50): 30 µM, 15 µM, 7.5 µM, 3.75 µM, 1.875 µM, 0.937µM, 0.468 µM and 0.234 µM

Anticancer activity (HeLa cells)
Cytotoxic activity of phytoconstituents was evaluated in 96-well flat-bottomed micro plates.[37] For this purpose, HeLa cells were cultured in Minimum Essential Eagle's Medium. The extent of MTT reduction to formazan within cells was calculated by measuring the optical density (O.D) absorbance at 570 nm, using a micro plate reader (Spectra Max plus, Molecular Devices, CA, USA). The cytotoxicity was recorded as concentration causing 50% growth inhibition (IC_{50}) for HeLa. The percent inhibition was calculated by using following formula:

% inhibition = 100-(mean of O.D of test compound – mean of O.D of negative control) / (Mean of O.D of positive control – mean of O.D of negative control)*100).

The results (% inhibition) were processed by using Soft- Max Pro software (Molecular Device, USA).

Results and Discussion

Currently, different sophisticated modern research techniques and tools are available for the identification of drugs of plant origin, but macroscopic and microscopic methods are still one of the simplest, reliable, precise and economical methods to confirm the correct identity of the plant source. According to the World Health Organization,[30] the macroscopic and microscopic description of a plant is the first criteria to establish the identity and the degree of purity of material and should be done before any tests are conducted. Botanical /macroscopic evaluation is a qualitative test based on the study of morphological characters of crude drugs and serves as diagnostic tool. The current research was conducted on aerial parts of an indigenous medicinal plant of Cholistan Desert of Bahawalpur, Pakistan. Botanical features of *Euphorbia nivulia* reveal that the plant is a herb, 3-9 m in height. Stem is hard with straight, often whorled branches along with straight paired, stipulary spines often blackish. Leaves are 8.5-21.5 cm long and 4-5 cm wide. The fresh leaves are simple, fleshy, succulent, dark green with an herbaceous odour and characteristic taste; crowded at the end of branches, obviate oblong or spathulate and glabrous. Both dried and fresh leaves are cuneate shapes, the base is acute, and apex is sub-acute. Venation is pinnate. Lamina is entire and flat. The only midrib is visible in normal conditions. Secondary and tertiary veins are not visible due to their fleshy nature [Figure. 1(A,B,C,D,E)].
For microscopic study, fine powder mounted in chloral hydrate, glycerin and iodine revealed that the aerial parts contain fragments of epidermal tissues composed of thick-walled polygonal cells. Stomata are anomocytic type but infrequent. There are numerous rosette and needle-shaped calcium oxalate crystals and starch granules. Annular vessels showing pits on their secondary walls, and spiral vessels were also present. The cell walls of the fairly abundant (xylem) fibers are thickened or lignified. Multicellular, uniseriate, glandular trichomes are found scattered and attached to fragments of the epidermis with blunt ends [Figure. 2 (A, B, C, D, E, F)].
TS of the leaf showed the single-layered thick rectangular epidermal cells; epidermis is covered with cuticle. Cells of ground tissues are thin and spherical to irregular in shape. Laticifers are distributed in mesophyll region around the vascular bundles. Lamina is differentiated into mesophyll, upper and lower epidermis. On surface view both the epidermal cells appear polygonal in shape. Mesophyll tissue consists of thin-walled parenchyma cells differentiated into elongated palisade and spongy cells. Midrib is composed of bundle sheath, collenchyma and vascular bundles. Vascular bundles (VB) consist of lignified xylem (pink colour) whereas none lignified phloem (green colour) along with parenchyma. Calcium oxalate crystals were seen frequently among loosely arranged spongy mesophyll.
TS of young stem revealed normal dicot structures. Epidermis is thick, and below epidermis, there is a layer of collenchyma (Hypodermis), which is thick walled but still living. Secondary growth is extensive, and secondary phloem is distributed in distinct patches; the pith is large. Vascular bundles are composed of (pink) xylem and (green) phloem. The cortical region is rich in laticifers which secrete white thick milky latex.
TS of the flower showed that the outermost layer is epidermis. Below the epidermis ground tissues are composed of parenchyma cells. Inside ground tissues laticifers are present. Around central pith, vascular bundles are present [Figure. 3 (A, B, C, D)].
The microscopic studies of the powder and transverse sections showed the presence of various histological structures like epidermal cells, stomata, starch granules, vessels of different types, fibers, vascular bundles, laticifers and unicellular blunted and glandular trichomes.[16]

Table 1: Phytochemical evaluation (*En* cr and fractions)

Class	ENH	ENC	ENB	ENA
Alkaloids	-	-	+	-
Glycosides	-	-	-	-
Tannins	-	-	+	+
Flavonoids	-	+	+	+
Saponins	-	-	+	+
Phenolic contents	-	+	+	+

+ = Present; - = Absent

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The use of different stains or reagents helps to differentiate between various cellular structures based on their chemical composition.[38]
The current study aimed to evaluate the *in vitro* cytotoxic activity of hydro-alcoholic (70%) *En* and various fractions, named as *En* hexane, *En* chloroform, *En* butanol and *En* aqueous. The evaluation was performed using Brine Shrimp Lethality Assay (BSLA), 3T3 and HeLa cell lines in comparison to anticancer drugs. Results of BSLA are shown in Figure 4. The extract (*En*) showed the optimum activity against Brine Shrimp while its two fractions, i.e., *En* hexane and *En* butanol also showed activity. Etoposide was used as standard. Results of cytotoxicity assay (3T3) are shown in Figure 6. *En* showed good activity against 3T3 cells, while its fraction, *En* hexane also showed good activity. Doxorubicin was used as standard. Results of anticancer activity (on HeLa cells) are shown in Figure 5. *En* showed good activity against HeLa cells while its fractions, *En* hexane also showed activity. Cyclohexamide was used as standard.

Figure 1: *Euphorbia nivulia* A: Plant habit, B: Phyllotaxy**,** C: Fresh leaves**,** D: Stem with black spines**,** E: Bark & Wood (TS Stem)

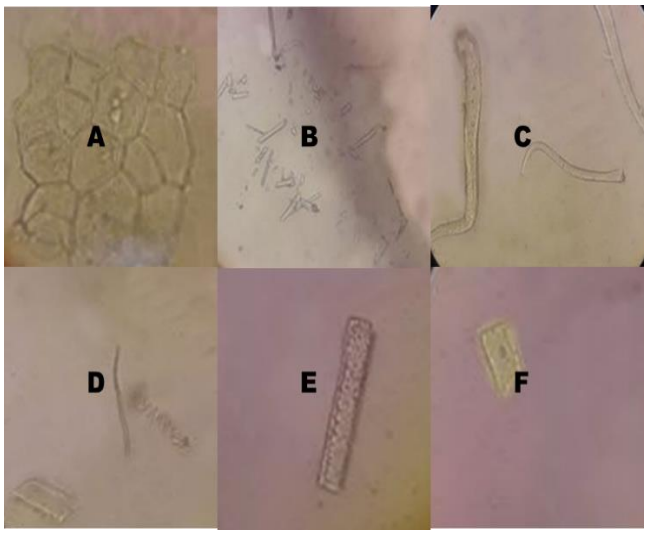

Figure 2: Powder Study A: Parenchyma cells, B: Calcium oxalate crystals,C: Trichomes, D: Spiral vessel & fiber, E: Pitted vessel, F:Mesophyll

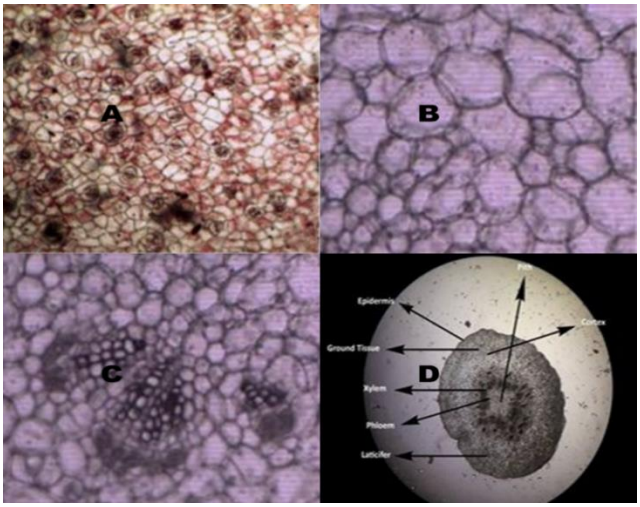

Figure 3: Anatomy A: TS Leaf (Upper Epidermis), B: TS Stem (Pith), C: TS Stem (Vascular Bundle), D: TS Flower (Pedicel)

Qualitative phytochemical screening tests were performed to detect phytochemicals in the crude extract and its fractions. Phytochemical analysis showed the presence of various phytochemicals like phenols, flavonoids, terpenoids, glycosides, alkaloids, saponin, steroids and tannins, etc. Brine shrimp lethality assay is commonly used in the primary screening of the crude extracts as well as fractions/isolated compounds to assess the toxicity and cytotoxic properties of the test samples. BSLA is used as a marker for general toxicity screening as it is a simple, efficient and inexpensive method for detection and isolation of insecticidal, pesticidal, cytotoxic, anti-neoplastic, antimalarial, and antifeedant compounds from plant extract, and is a suitable method to correlate the cytotoxic and other biological properties.[39-41] Many antitumor and pesticide natural products have been isolated using this bioassay.[42, 43] The variation in BSLA results may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins, flavonoids, triterpenoids, or coumarins) present in the crude extracts and fractions. Results showed decreased cell viability and cell growth inhibition in a dose-dependent manner. Furthermore, significant lethality of the crude extract (73.33% mortality) and *En* hexane and *En* butanol to brine shrimp indicates potent cytotoxic constituents. Cytotoxicity potentials of *En* against (3T3 and HeLa) cell line are due to various phytochemicals. Among these bioactive constituents, phenols, flavonoids, saponins, phenolic compounds and tannins, etc. possess strong antioxidant potential.
These antioxidants may prevent and cure cancer and other diseases by protecting the cells from damage caused by ROS/free radical**s**, the highly reactive oxygen compounds. Furthermore, phytochemicals including vitamins (A, C, E and K), carotenoids, terpenoids, flavonoids, polyphenol, tannins, saponins, pigments, enzymes and minerals display anticancer activities. These chemicals block various hormones and metabolic pathways associated with the development of cancer.[44] Several studies evaluated the relationships between the anti-proliferative activity of plant products and their phenolic content. A correlation exists between the structural oxidation state, position, number and nature of the substituents of the polyphenolic compounds and their anti-proliferative effects.[45] Phenolics may act as anti-proliferative agents to liquidate the tumor cells by disrupting the cell cycle.[46] Moreover, triterpenes and saponins may contribute to strong anticancer activities.[47] Similarly, some of steroidal saponins had antimicrobial and cytotoxic activity.[48,49] Hydroxycoumarins are typical phenolic compounds that are made up of fused benzene and a-pyrone rings, and possess antioxidant, hepatoprotective, antiviral, and anticarcinogenic activities, therefore act as potent metal chelators and free radical scavengers.[50] Recent reports show that tannins may have potential value as cytotoxic and/or antineoplastic agents.[51]

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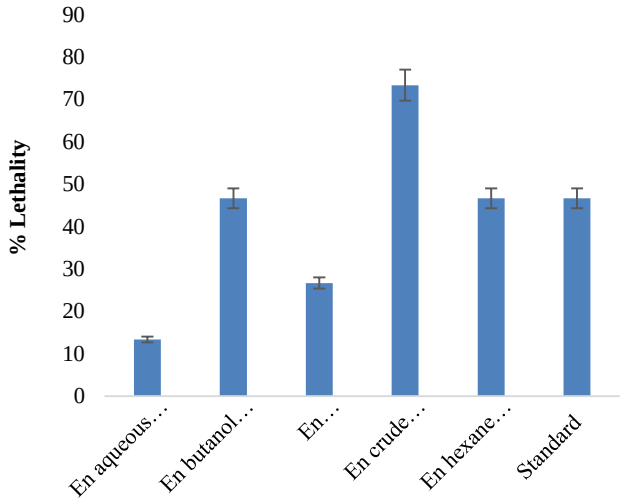

Figure 4: Lethality of various fractions (1000 µg/mL) of *E.nivulia* on Brine Shrimp eggs against Etoposide

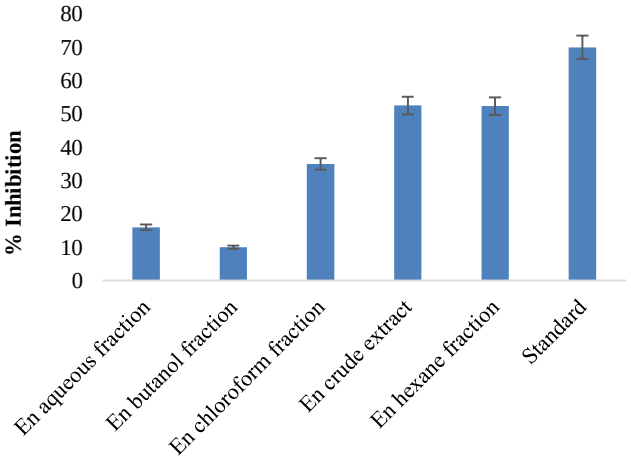

Figure 5: Cytotoxicity studies of various fractions (30 µg/mL) of *E.nivulia* on Hela cells against Doxorubicin

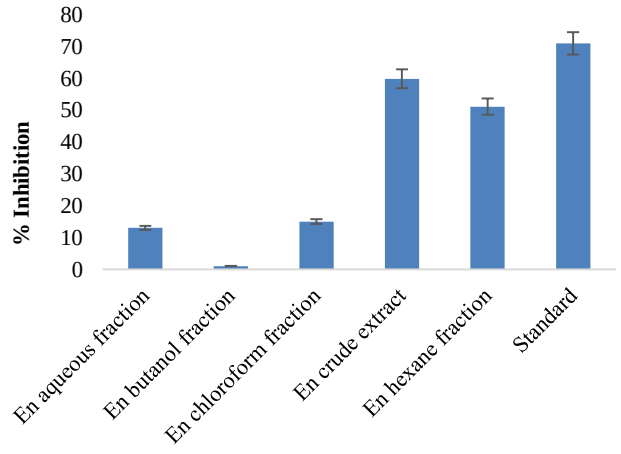

Figure 6: Cytotoxicity studies of various fractions (30 µg/mL) of *E.nivulia* on 3T3 cells against Cyclohexamide

Conclusion

The current study is a step towards the identification of the plant species, i.e., *Euphorbia nivulia* (En) belonging to Cholistan Desert of Pakistan. The present study also revealed that *En* is very rich in various phytochemicals including polyphenolic compounds like phenolic acids and flavonoids with excellent antioxidant properties. Moreover, the study recommends that *En* may be a good candidate as anticancerous agent for developing new formulation(s).

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and any liability for claims relating to the content of this article will be borne by them.

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