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Antioxidant, antimicrobial, antitumor, and cytotoxic activities of an important medicinal plant (*Euphorbia royleana*) from PakistanAisha Ashraf ^{a,*}, Raja Adil Sarfraz ^{a,b}, Muhammad Abid Rashid ^a,
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ABSTRACT

The aim of present study was to evaluate antioxidant, antimicrobial, and antitumor activities of methanol, hexane, and aqueous extracts of fresh *Euphorbia royleana*. Total phenolic and flavonoid contents were estimated as gallic acid and quercetin equivalents, respectively. Antioxidant activity was assessed by scavenging of free 2,2'-diphenyl-1-picrylhydrazyl radicals and reduction of ferric ions, and it was observed that inhibition values increase linearly with increase in concentration of extract. The results of ferric reducing antioxidant power assay showed that hexane extract has maximum ferric reducing power (12.70 ± 0.49 mg gallic acid equivalents/g of plant extract). Maximum phenolic (47.47 ± 0.71 μ g gallic acid equivalents/mg of plant extract) and flavonoid (63.68 ± 0.43 μ g quercetin equivalents/mg of plant extract) contents were also found in the hexane extract. Furthermore, we examined antimicrobial activity of the three extracts (methanol, hexane, aqueous) against a panel of microorganisms (*Escherichia coli*, *Bacillus subtilis*, *Pasteurella multocida*, *Aspergillus niger*, and *Fusarium solani*) by disc-diffusion assay, and found the hexane extract to be the best antimicrobial agent. Hexane extract was also observed as to be most effective in a potato disc assay. As hexane extract showed potent activity in all the investigated assays, it was targeted for cytotoxic assessment. Maximum cytotoxicity (61.66%) by hexane extract was found at 800 μ g/mL. It is concluded that investigated extracts have potential for isolation of antioxidant and antimicrobial compounds for the pharmaceutical industry.

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1. Introduction

Plants are friends of mankind. They contribute strongly to fulfill necessities of life such as food, medicine, clothing, and

construction. The World Health Organization has catalogued 20,000 plant species studied for medicinal purposes [1]. Bioactive components of plants include array of compounds (e.g., tannins, lignans, coumarins, quinones, stilbenes, xanthenes, phenolic acids, flavones, flavonols, catechins, anthocyanins,

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and proanthocyanins) that could delay or inhibit the inception of degenerative diseases [2] and increase life expectancy. Infectious diseases are also the major cause of mortality worldwide. About 50,000 people die worldwide every day because of infectious disease. Literature surveys show that plant-based drugs play a promising role in the treatment of infectious diseases [3]. Efficacy of plant extracts against plant pathogens such as *Agrobacterium tumefaciens* is also well documented [4]. The bioactive compounds in plants lead to their fundamental role in modern drug development.

Euphorbia royleana is an important medicinal plant, known as *Dandathor* or *Dozakhimeva* in Pakistan. It is a spiny shrub usually up to 1.8–2.4 m tall. It is traditionally used for treatment of many ailments including paralysis, ear pain, and loose motions [5]. Its latex is documented for anti-inflammatory and anti-arthritis attributes [6]. Stem bark extract of *E. royleana* has piscicidal and antiacetylcholinesterase activity [7]. The ethyl acetate fraction of *E. royleana* has immunosuppressive characteristics [8]. *E. royleana* belongs to the family *Euphorbeaceae*, different plants of which are traditionally used for the treatment of skin problems, asthma, jaundice, anemia, cough, and constipation [9]. *Euphorbeaceae* is scientifically reported for its antiviral [10], antimicrobial [11], anticancer [12], cytotoxic, and antitumor activities [13].

On the basis of the strong medicinal background of *Euphorbiaceae*, *E. royleana* was targeted for investigation. Although work has been done to explore some pharmacological properties of *E. royleana*, there is a complete gap of knowledge about the medicinal attributes of fresh *E. royleana*. As fresh *E. royleana* is used in most of traditional remedies [5], the ultimate goal of the present study was to explore antioxidant, antimicrobial, antitumor, and cytotoxic activities of fresh *E. royleana*.

2. Materials and methods

2.1. Plant material

Plant material was collected from the Botanical Garden, University of Agriculture, Faisalabad, Pakistan. Fresh plant material was washed with distilled water and cut into cubes of 2 cm × 2 cm × 2 cm with a stainless steel knife (Global, Chiba, Japan). Cubes of fresh plant material were immediately used for extraction. Cubes of fresh plant material (20 g) were placed in a 500 mL flask, mixed with 200 mL of methanol, plugged with cotton swab, and tightly wrapped with aluminum foil. Extraction was carried out using an orbital shaker at 350 rpm for 72 hours [14], after which the suspensions was filtered using Whatman No.1 filter paper. The filtrate was evaporated at 65°C using a vacuum drying oven (Memmert GmbH, Dusseldorf, Germany), maintained at 69°C for hexane and 100°C for water, to get dry extract. Solvent free extract was transferred to extract vials and stored at 4°C for further use. Similar practice for extraction was done with hexane and water solvents individually.

2.2. Chemicals

Potassium ferricyanide and agar powder were from Daejung Chemicals and Metals (Shiheung, Korea), nutrient broth was

from Lab M (Bury, Lancashire, UK), and ferric chloride from BDH laboratory supplies (Lutterworth, Leicestershire, UK). Aluminum chloride hexahydrate, trichloroacetic acid, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), gallic acid (GA), quercetin, and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Methanol, hexane, and chloroform used for extraction purpose were purchased from Merck KGaA (Darmstadt, Germany). Folin–Ciocalteu's phenol reagent, sodium carbonate and nutrient agar were acquired from Applichem, GmbH (Darmstadt, Germany).

2.3. Total phenolic content

Total phenolics were determined by the Folin–Ciocalteu process [15,16]. Briefly, 1 mL of each extract (methanol, hexane, water) solution at concentration of 800 µg/mL was mixed with 7.5 mL of double deionized water, 500 µL of Folin–Ciocalteu reagent and 1 mL of 5% Na₂CO₃. The mixture was incubated for 90 minutes at room temperature. After the incubation absorbance was measured at 760 nm using a spectrophotometer (Lambda EZ 201, Perkin Elmer, Waltham, MA, USA). The content of phenolics expressed as µg GA equivalents (GAEs) per mg of extract.

$$\text{Absorbance} = 0.005 \mu\text{g GA} + 0.074 \quad (R^2 = 0.99) \quad (1)$$

2.4. Total flavonoid content

Total flavonoids were determined by the AlCl₃ method [17,18]. A 2 mL sample of each extract (methanol, hexane, water) solution at concentration of 800 µg/mL was mixed with 2 mL of aqueous AlCl₃·6H₂O (0.1M). The mixture was incubated at room temperature for 10 minutes and absorbance was measured with the spectrophotometer at 417 nm. The content of flavonoids expressed as µg quercetin equivalents (QEs):

$$\text{Absorbance} = 0.040 \mu\text{g quercetin} + 0.051 \quad (R^2 = 0.99) \quad (2)$$

2.5. DPPH assay

The free radical scavenging capability of each extract solution on DPPH radicals was determined as described previously [19]. Briefly, 4 mL of methanol solution of DPPH (0.1mM) was mixed with 1 mL of each of extract (methanol, hexane, water) solution at different concentrations (25–800 µg/mL). The reaction mixture was incubated in a dark room for 30 minutes and the free radical scavenging ability was estimated by measuring the absorbance at 515 nm with the spectrophotometer. The reaction was carried out in capped glass test tubes that were tightly wrapped with aluminum foil. The DPPH radical stock solution was freshly prepared every day for the reaction, and precautionary measures were taken to reduce the loss of free radical activity during the experiment. The inhibition percentage of DPPH radicals was calculated as:

$$\text{Inhibition (\%)} \text{ of DPPH radicals} = \frac{AC - AS}{AC} \times 100 \quad (3)$$

where Ac is absorbance of the control reaction (all reagents except plant extract) and As is absorbance of the sample (plant extract).

2.6. Ferric reducing antioxidant power assay

The ferric reducing antioxidant power assay [20] was used to assess the reducing capacities of extracts of fresh *E. royleana*. Different dilutions (25–800 µg/mL) of each plant extract (methanol, hexane, water) were added to 2.5 mL of phosphate buffer and 2.5 mL potassium ferricyanide (1% W/V). The mixture was incubated at 50°C for 25 minutes, after which 2.5 mL trichloroacetic acid solution was added to stop the reaction. Then 2.5 mL of this mixture was mixed with 2.5 mL of water and 500 µL of ferric chloride solution. Absorbance was measured at 700 nm after 30 minutes.

$$\text{Absorbance} = 0.033 \mu\text{g gallic acid} + 0.201 (R^2 = 0.99) \quad (4)$$

2.7. Antimicrobial activity

The agar disc diffusion method [21] was used for determination of diameters of inhibition zones made by methanol, hexane, and aqueous extracts of fresh *E. royleana* against various bacterial and fungal strains (*Escherichia coli*, *Bacillus subtilis*, *Pasteurella multocida*, *Aspergillus niger*, and *Fusarium solani*). Sterile nutrient agar was inoculated with 100 µL suspension of tested bacteria and sterile potato dextrose agar was inoculated with 100 µL of tested fungi. The inoculated nutrient agar and potato dextrose agar were then poured into sterilized petri plates individually. Sterile filter discs impregnated with 50 µL of sample solution were placed in inoculated petri plates using sterile forceps. Rifampicin and terbinafine were used as positive control in bacterial and fungal inoculated plates, respectively. The plates were incubated at 37°C for 24 hours and at 27°C for 48 hours for maximum bacterial and fungal growth, respectively. Antibacterial and antifungal activities were evaluated by measuring diameter (mm) of inhibition zones using a zone reader.

2.8. Antitumor assay

Antitumor activities of methanol, hexane, and aqueous extracts of fresh *E. royleana* were evaluated by the potato disc method using Vincristine sulfate as positive and DMSO as negative control [22]. *A. tumefaciens* from storage culture was inoculated into sterilized growth medium using aseptic techniques. The culture was vigorously shaken and then placed in a shaker for 48 hours at 28°C. Red skinned potatoes were surface sterilized in 10% sodium hypochlorite for 20 minutes and extensively washed with autoclaved distilled water and cut into 4 mm thick discs of 8 mm diameter using cork and surgical blades sterilized by γ -irradiation (2.5 MRads). Agar was prepared by dissolving agar powder in autoclaved distilled water. Agar was poured into sterilized petri plates and allowed to solidify in laminar air flow. Eight potato discs were placed on each agar plate using sterilized forceps (Rolzem International, Sialkot, Pakistan). A 50 µL sample of inoculum was placed on the surface of each disc. Plates were wrapped with parafilm and incubated at 27°C for 21 days. After 21 days, discs were stained with Lugol solution (10% KI+5% I₂) for 20 minutes and tumors were counted on each disc using a dissecting microscope. Twenty-eight tumors were counted in negative control (DMSO). Percent

inhibition was calculated using the formula described by Kanwal et al [4].

2.9. Brine shrimp lethality test

The brine shrimp cytotoxicity assay was performed according to a previously described method [23]. Brine shrimp (*Artemia-nauplii*) eggs were hatched in a rectangular tank (19.5 cm length × 19.5 cm width × 20.3 cm height) containing 1 L of artificial sea water [mixture of commercial salt (Harvest Co. Hong Kong) and distilled water] and subjected to continuous gentle aeration. Brine shrimp eggs were spread in the dark part of container. Mature nauplii (age 48 hours) were collected from the other half of the container that, which was exposed to continuous illumination (60 W). Methanol extract of fresh *E. royleana* was dissolved in water to make final concentrations of 25 ppm, 50 ppm, 100 ppm, 200 ppm, 400 ppm, and 800 ppm. A 0.5 mL aliquot of each concentration was taken in graduated vial and subjected to evaporation overnight. Twenty shrimps were added into each vial and the volume was made up to 5 mL with seawater. Vials were kept at 25°C under illumination for 24 hours. After 24 hours the number of live nauplii was counted using a 5 × magnifying glass. Median lethal dose was calculated by linear regression analysis [13].

2.10. Statistical analysis

Minitab software version 16 (Statistical software, Minitab Inc., State College, PA, USA) was used to perform analysis of variance (ANOVA) and to determine significant differences ($p < 0.05$).

3. Results and discussion

3.1. Total phenolic contents

Phenolic compounds are health benefactors because they act as antioxidative agents [19]. In the present study, the total phenolic contents of methanol, hexane, and aqueous extracts of fresh *E. royleana* were determined by the Folin–Ciocalteu method and results are shown in Table 1. This method involves the reaction of phosphomolybdate and phosphotungstate with all phenolic compounds in the sample [24]. This reaction generates a blue pigment that has extensive light

Table 1 – Total phenolic and flavonoid contents of methanol, hexane and water extracts of fresh *Euphorbia royleana*.

E. royleana extracts	Total phenolic content	Total flavonoid contents
	(µg GAE/mg of plant extract)	(µg QE/mg of plant extract)
Methanol	56.97 ± 0.27 ^a	43.98 ± 0.79 ^e
Hexane	63.68 ± 0.43 ^b	47.47 ± 0.71 ^d
Water	30.65 ± 0.46 ^c	18.89 ± 0.41 ^f

Data are presented as mean ± SD of three individual determinations. Means with different letters in the same column indicate significant ($p < 0.05$) differences among the solvents used. GAE = gallic acid equivalents; QE = quercetin equivalents.

absorption at 760 nm [25]. Total phenolic contents of the three extracts ranged from 30.65 ± 0.46 μg GAEs/mg of plant extract to 63.68 ± 0.43 μg GAEs/mg of plant extract, with hexane extract exhibiting the highest value. A similar result for hexane extract was previously reported [26]. Phenolic contents of fresh *E. royleana* are not previously reported. Amount of phenolic contents in methanol extract of fresh *E. royleana* is higher than previously reported values of *Euphorbiaceae* [27].

3.2. Total flavonoid contents

Flavonoids (polyphenolic plant compounds) possess a wide range of pharmacological properties. They do a lot for human health by scavenging free radicals [2]. In the present investigation, the aluminum chloride colorimetric method was used for detection of flavonoid contents. In this method, reaction of aluminum chloride with adjacent keto or hydroxyl groups of flavones or flavonols generates acid-stable complexes [28], which show maximum absorption at 415 nm. Methanol, hexane, and aqueous extracts of fresh *E. royleana* showed significant ($p < 0.05$) amount of flavonoid contents (Table 1). The highest amount of flavonoids was observed in hexane extract (47.47 ± 0.71 μg QE/mg of plant extract) followed by methanol (43.98 ± 0.79 μg QE/mg of plant extract) and aqueous (18.89 ± 0.41 μg QE/mg of plant extract) extracts. The flavonoid contents of fresh *E. royleana* extracts are not previously published, but are comparable with other medicinal flora of Pakistan [29].

3.3. DPPH assay

The antioxidant potential of methanol, hexane, and aqueous extracts of fresh *E. royleana* was evaluated on the basis of their ability to scavenge stable free DPPH radicals. This test is based on change in color of DPPH solution from purple to yellow, due to scavenging of stable free DPPH radicals [30]. A stronger yellow color indicates a greater ability of the extract to scavenge free DPPH radicals and stronger antioxidant potential. In the present study, the antioxidant potential of the three extracts was explored in a dose-dependent (25–800 $\mu\text{g}/\text{mL}$) manner as shown in Table 2. An increase in DPPH scavenging ability was observed with increase in concentration of extracts. This trend is in agreement with a previous study [19]. The DPPH scavenging ability of hexane extract ($60.52 \pm 0.39\%$) was higher than methanol extract ($56.78 \pm 1.37\%$), and least ($51.64 \pm 0.46\%$) in aqueous extract. The DPPH scavenging ability of methanol extract is in agreement with literature [31].

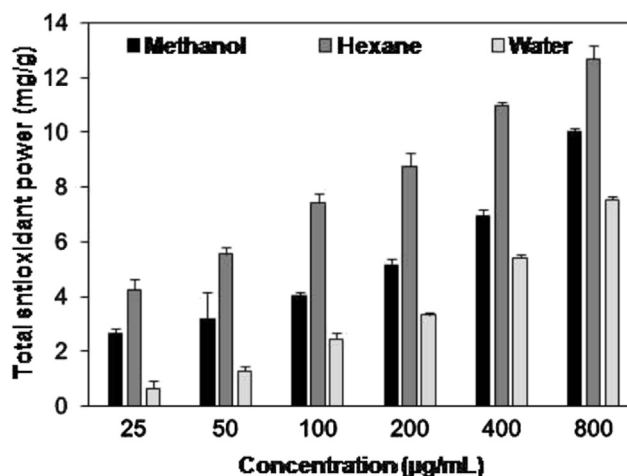


Fig. 1 – Total antioxidant power of methanol, hexane, and water extracts of fresh *Euphorbia royleana*. Values are presented as mean \pm standard deviation of three individual determinations.

3.4. Total antioxidant activity assay

Reducing power is an important parameter for assessment of antioxidant activity. Antioxidant compounds reduce reactive radicals to stable species by donating electrons [32]. Reducing capacities of methanol, hexane and aqueous extracts of fresh *E. royleana* ranged from 0.651 ± 0.25 mg GAEs/g of plant extract to 12.70 ± 0.49 mg GAEs/g of plant extract (Fig. 1). Reducing capacity of the three investigated extracts increases with increase in concentration (25–800 $\mu\text{g}/\text{mL}$). This relation is also supported by previous studies [33]. A significant difference ($p < 0.05$) among reducing activities of each extract was observed at different concentrations (25–800 $\mu\text{g}/\text{mL}$). Reducing power of the hexane extract was significantly ($p < 0.05$) higher than of methanol and aqueous extracts. Significantly ($p < 0.05$) lower reducing ability was observed in aqueous extract. The reducing power of fresh *E. royleana* is not reported previously in the literature.

3.5. Antimicrobial activity

The results of antimicrobial potential of methanol, hexane, and aqueous extracts of fresh *E. royleana* against five pathogenic microorganisms are shown in Table 3. The three

Table 2 – Free radical (DPPH) scavenging (%) activities of methanol, hexane and water extracts fresh *Euphorbia royleana*.

Extracts	Concentration ($\mu\text{g}/\text{mL}$)						IC ₅₀ (mg/mL)
	25	50	100	200	400	800	
Methanol	18.22 ± 1.11^a	23.46 ± 0.64^b	28.22 ± 1.46^c	33.51 ± 1.30^d	46.71 ± 1.25^e	56.78 ± 1.37^f	0.58
Hexane	16.09 ± 0.51^c	24.89 ± 0.68^d	31.19 ± 0.38^e	39.38 ± 1.19^f	50.96 ± 0.44^g	60.52 ± 0.39^h	0.38
Water	11.18 ± 0.77^a	17.47 ± 1.49^b	20.18 ± 0.96^c	25.95 ± 1.51^d	36.68 ± 0.29^e	51.64 ± 0.46^f	1.13

Data are mean \pm SD of three individual determinations. Means with different superscript letters in the same row indicate significant ($p < 0.05$) difference among concentrations tested. Means with different subscripts in the same column indicate significant ($p < 0.05$) difference among solvents used.

IC₅₀ = half maximal inhibitory concentration.

Table 3 – Antimicrobial activity of methanol, hexane and water extracts of fresh *Euphorbia royleana*.

Tested microorganisms	Methanol	Hexane	Water	Diameter of inhibition zone (mm)	
				Rifampicin	Terbinafine
<i>Escherichia coli</i>	5.67 ± 0.57 ^b	7.00 ± 1.00 ^b	6.00 ± 1.00 ^b	21.66 ± 1.41 ^a	—
<i>Bacillus subtilis</i>	10.67 ± 0.57 ^b	12.00 ± 0.57 ^b	8.33 ± 0.00 ^c	24.66 ± 0.47 ^a	—
<i>Pasteurella multocida</i>	8.33 ± 0.57 ^c	10.33 ± 0.57 ^c	8.00 ± 1.00 ^c	23.33 ± 1.69 ^b	—
<i>Aspergillus niger</i>	11.66 ± 0.57 ^c	14.00 ± 1.00 ^b	9.33 ± 0.57 ^a	—	25.66 ± 1.69 ^d
<i>Fusarium solnai</i>	10.00 ± 1.00 ^a	10.33 ± 0.57 ^a	8.66 ± 0.57 ^a	—	24.00 ± 0.82 ^b

Data are mean ± SD of three individual determinations. Means with different superscript letters in the same row indicate significant ($p < 0.05$) differences among the solvents used.

investigated extracts exhibited considerable antimicrobial effects against all tested microorganisms. Methanol and hexane extracts showed the excellent activities against *A. niger* and with inhibition zones of 11.66 ± 0.57 mm and 14.00 ± 1.00 mm, respectively. A smaller zone of inhibition (5.67 ± 0.57 mm) was observed by methanol extract against *E. coli* than previously reported values of inhibition zones by methanol extracts of *Euphorbia hirta*, *Euphorbia tricholi*, and *Euphorbia nurofila* [11]. The highest activity of aqueous extract was observed against *A. niger* with inhibition zone of 9.33 ± 0.57 mm. A previous study [11] showed that methanol extract of *E. hirta* has inhibition a zone of 9.00 ± 0.00 mm against *B. subtilis*, which is smaller than our value of 10.67 ± 0.57 mm by methanol extract of fresh *E. royleana*. In the present study, antibacterial and antifungal potential may be attributed to bioactive components present in the plant [34]. Overall, the hexane extract showed better antibacterial activities than the other two extracts.

3.6. Potato disc assay

E. royleana is used for the treatment of paralysis, bladder stone, earache, inflammation, and other diseases [5], but the antitumor potential of plant has not been investigated previously. Therefore, different concentrations (25–800 µg/mL) of

methanol, hexane, and aqueous extracts of fresh *E. royleana* were investigated by bench top potato disc assay. The results (Fig. 2) indicate that percent tumor inhibition increase with increases in concentration of plant extracts. A similar relation between percent tumor inhibition and concentration of plant extract was previously reported [4]. Maximum tumor inhibition was investigated in hexane extract ($63.49 \pm 1.81\%$) followed by methanol ($56.01 \pm 1.34\%$) and aqueous ($45.87 \pm 2.71\%$) extracts. The effect of concentration on tumor inhibition was statistically significant ($p < 0.01$), and the results reveal that the three investigated extracts have considerable antitumor activity.

3.7. Brine shrimp lethality test

The brine shrimp lethality test is a potent, precise, and economical method for assessment of cytotoxic, phototoxic, pesticidal, and many other activities [35]. In the present study, the brine shrimp lethality test was used to assess cytotoxic potential of hexane extract of fresh *E. royleana*. The results obtained are shown in Fig. 3. Minimum and maximum mortality (6.66% and 61.66%) was found at 25 ppm and 800 ppm, respectively. These values are lower than previously reported values of percent mortality for *E. hirta*, *E. heterophylla*, and *E. nerifolia* [12]. Median lethal dose (598.8 µg/mL) of hexane extract of fresh *E. royleana* is in comparison with other medicinal flora of Pakistan [36].

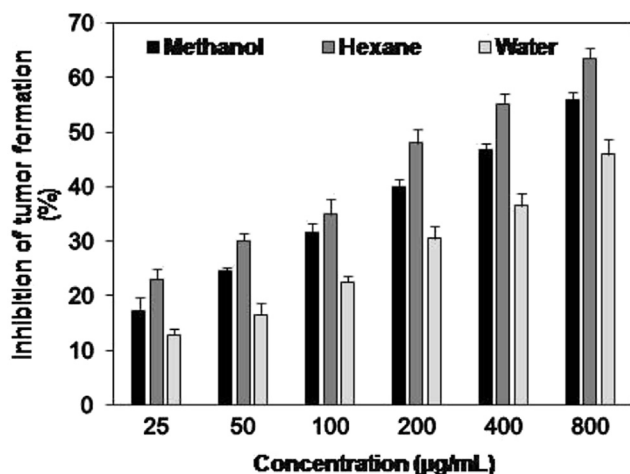


Fig. 2 – Inhibition of tumor formation by methanol, hexane and water extracts of fresh *Euphorbia royleana*. Values are presented as mean ± standard deviation of three individual determinations.

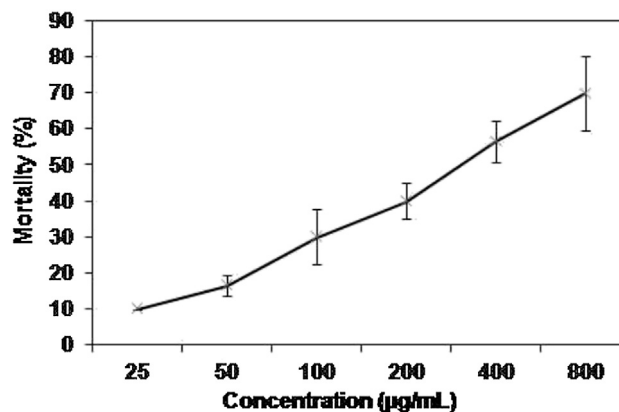


Fig. 3 – Cytotoxic activity of hexane extract of fresh *Euphorbia royleana*. Values are presented as mean ± standard deviation of three individual determinations.

4. Conclusion

The present study was carried out to explore antioxidant, antimicrobial, antitumor, and cytotoxic potential of methanol, hexane, and water extracts of *E. royleana*. Maximum antioxidant and antitumor activities were observed for hexane extract, which might be correlated to its leading phenolic and flavonoid contents as compared to its methanolic and aqueous counterparts. Significant difference ($p < 0.05$) in antimicrobial activity was noted in all of the investigated extracts. On the basis of our pharmacological findings (antioxidant, antimicrobial, antitumor) of hexane extract, it was selected to assess cytotoxic activity. In future, work will be done to isolate bioactive constituents of fresh *E. royleana* extract to locate potential pharmacological agents.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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