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Research Article

Pharmacological profiling and phytochemical analysis of fractionated extracts of *Euphorbia royleana*

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Abstract

Euphorbia royleana belongs to family Euphorbiaceae, with great therapeutic potential. The present study is aimed to validate its traditional uses. GC-MS analysis of *Euphorbia royleana* crude and fractionated extracts were performed. Cytotoxicity was evaluated by Brine shrimp lethality (BSL) assay. Plant extract antioxidant activity was performed through *in vitro* multidimensional assays. Plant elemental analysis was performed through atomic absorption spectroscopy. In addition, the extract antibacterial activity against two gram negative i.e. *Staphylococcus aureus*, *Xanthomonas campestris,* and two gram-positive bacterial strains i.e. *Escherichia. coli*, *Klebsiella pneumoniae* with agar well diffusion assay was performed. GC-MS analysis of n-hexane fraction revealed the presence of 16 phytocomponents. Phytochemical investigation led to identification and quantification of phenols, glycol cyanide, tannins, saponins, alkaloids and flavonoids. Highest phenol and flavonoid content (1.886±0.02 µg/mg, 0.855±0.01 µg/mg, 0.551±0.01 µg/mg and 0.090±0.01 µg/mg respectively) was quantified in plant extract and in n-hexane fraction. Crude and fractionated extracts (n-hexane, chloroform, ethylacetate and methanol) exhibited moderate cytotoxicity 81%, 71%, 52.5%, 57.5% and 51.5% respectively against brine shrimp nauplii with LD_{50} values of 168.46 and 220.30%. Plant extracts also showed scavenging activity ranging from 23-61% at 25-400 µg/mL. *E. royleana* consist of various compounds and minerals, namely K^{+}, Na^{+}, Fe^{+2}, Co^{+3}, Mn^{+2}, Cu^{+3}, Cr^{+3}, and Cd^{+2}. The antimicrobial activity revealed that the plant crude extract and n-hexane fraction comparatively exhibited the highest antibacterial activity against *Staphylococcus aureus* and *Xanthomonas campestris*. This study evaluated the plant's potential as a source of antimicrobials and antioxidants for future application in treating infectious disorders.

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Keywords: antioxidant potential; antibacterial activity; bioactive compounds; cytotoxicity

Introduction

Humans have had an intimate relationship with plants since the beginning of time, as plants and their products are fully integrated into human lives and run the need wheel smoothly. More than 35,000 plant species have been utilized as natural repositories of bioactive substances, producing a wide spectrum of phytochemicals with potential medicinal qualities (Benjamaa *et al*., 2024). Many Euphorbia species including *E. roylena, E. fischeriana* and *E. marginata*, *E. peplus* yielded secondary metabolites in significant quantities, (e.g., tannins, lignin, coumarins, quinones, stilbenes, xanthones, phenolic acids, flavones, flavonols, catechins, diterpenoids, triterpenoids, anthocyanins, and proanthocyanins) in their aerial parts which can be utilized for treating various dermatological, digestive, respiratory, migraine, and inflammatory diseases (Zaghlol *et al*., 2024; Ji et al. 2023; Tripathy 2015).

Infectious diseases are also a leading cause of death worldwide. Every day, over 50,000 people worldwide die as a result of various infectious diseases. According to the literature, plant-based medications have a promising role in the treatment of infectious disorders (Agarwal *et al*., 2021). Over 1,340 plants have been identified with defined antimicrobial activity, and researchers have isolated over 30,000 antimicrobial compounds from plants. Additionally, it's estimated that 14-28% of higher plant species possess medicinal properties, and a significant 74% of bioactive plant-derived compounds have been discovered based on ethnomedicinal use (Vaou *et al*., 2021; Xiang *et al*., 2023).

Euphorbia royleana Boiss. belongs to family Euphorbiaceae, with great medicinal importance. Euphorbiaceae, found in Pakistan, hosts the genus *Euphorbia* with close to two thousand species. This genus surprises with its diversity, ranging from small garden weeds known as "spurges" to towering succulents resembling cacti (Ullah *et al*., 2023). *E. royleana Boiss*. is originated from China, India and Pakistan; presently distributed in southwest part of the China and Himalayan region of Pakistan and widely grow in arid and subarid regions of the world (Şafak Odabaşı, 2024). *E. royleana* is present in Subtropical rain shadow valleys; on rocky slope, its own communities have been identified (Alyas *et al*., 2020). It has various vernacular names in different regions of the world (Table 1). *E. royleana* typically grows to heights of 5-7 meters, displaying green coloration, with stems featuring 5-7 angles, and numerous branches originating from the upper sections. It is a shrub that grow on the slopes of the western Himalayas at an altitude of 900-1500 m above sea level (Biswas *et al*., 2013; Radi *et al.*, 2024). Plant is a thorny succulent shrub, leaves are sessile, oblanceolate, alternate, apically crowded, prickle-like stipules, paired spines, somewhat juicy, attenuate shaped base, smooth margin, apex is obtuse or sub-truncate; inconspicuous vascular bundle (Poudeyal *et al*., 2020). In *E. royleana* the Inflorescence is Cyathia, that is terminal and axillary, yellow peduncle; cyathophylls as extended as involucre, membranous; diagonally elliptic, dark yellow. Flowering and fruiting in May–July, fruit is Capsule, smooth and glabrous. Seeds brown, adaxially striate; caruncle absent (Zahra *et al*., 2014; Paudel *et al*., 2021). For instance, *E. royleana* is used to cure ear pain, loose motions, paralysis, skin problems and body pain skin problems, constipation and asthma, jaundice, anemia, cough (Al-Ansi *et al*., 2024; Paudel *et al*., 2021). The specialized inflorescence and milky latex are prominent features of the *Euphorbia* genus, latex released upon injury and traditionally used, to cure various ailments including skin issues, asthma, jaundice, anemia, cough, and constipation (Akram *et al*., 2020; Adil *et al*., 2024).

Current study was designed to investigate *E. royleana* stem for preliminary qualitative and quantitative phytochemical analysis. As a result, antioxidant, antibacterial, and cytotoxic properties of crude methanol extracts of stem and polarity-based fractions were investigated. Furthermore, active fraction elemental and GC-MS analysis was also performed.

Table 1. *E. royleana* common names around world

Sr. No.	Country	Common name	References
1	Pakistan	'Thuhar', 'Dandathor' or 'Dozakhimeva'	Sabeen and Ahmad (2009)
2	China	Takterak, 'Sulla', 'Chhuien', 'Ba Wang Bian'	Kichu *et al*. (2015); Bijalwan and Madan (2013); Zhang and Wei (2023)
3	India	Siudhi'	(Tiwari and Singh, 2004)
4	Nepal	Naga,	(Kichu *et al*., 2015)

Materials and Methods

Plant collection and identification, drying and extraction

E. royleana stem material was collected in July from PMAS Arid Agriculture University Rawalpindi and identified by Prof. Rehmatullah Qureshi, an expert Taxonomist in the Department of Botany PMAS Arid Agriculture University Rawalpindi. Fresh *E. royleana* stem was chopped and shade dried, ground to fine powder. Plant samples were stored in sealed bags until they were needed. Cold maceration methodology described by (Bibi *et al*., 2012) was used for extraction.

Fractionation

The method of (Ali *et al*., 2013) was followed for Crude extract fractionation. Scheme for fractionation of crude extract of *E. royleana* is shown in Figure 1.

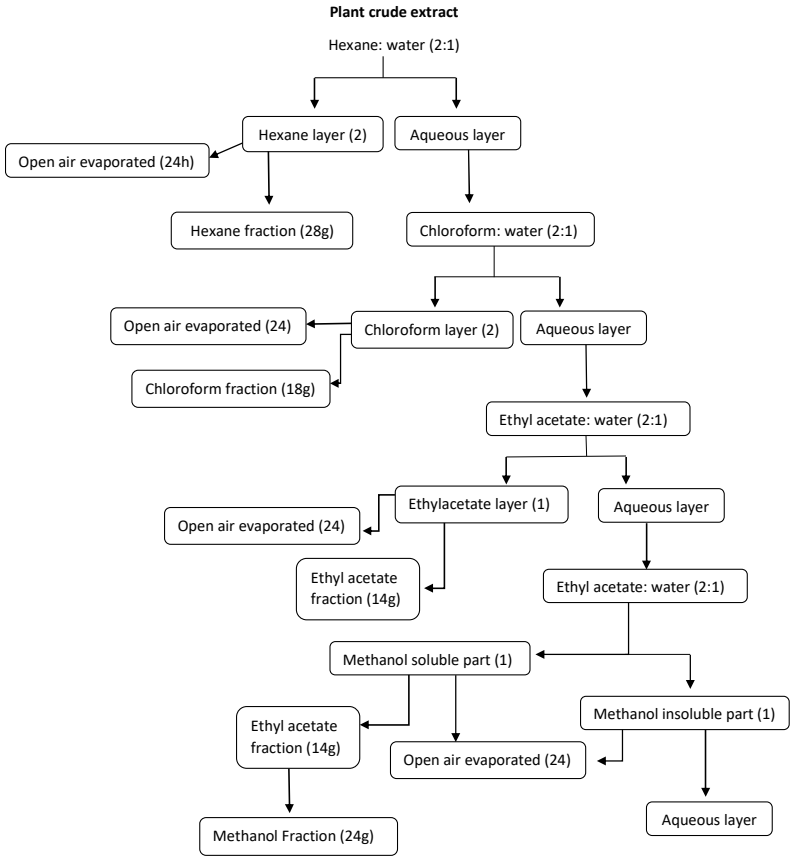

Figure 1. Scheme used for fractionation of crude stem extract of *Euphorbia royleana*

Preparation of n-hexane extract

2 µL of the n-hexane fraction of *E. royleana* stem was employed for GC-MS analysis.

GC-MS analysis

The isolated phytochemicals from *E. royleana* stem were analyzed using a GC-MS Agilent Technologies-7820A GC system. Agilent Technologies-5977MSD Gas Chromatogram linked with Mass Spectrometer fitted with an Agilent Technologies GC-MS capillary column HP-5MS (30 m × 0.25 mm ID × 0.25 m) comprised of 5% diphenyl 95% dimethyl polysiloxane. A 70 eV ionizing energy electron ionization device was employed. Helium gas (99.99%) was utilized as the carrier gas at a continuous flow rate of 1 mL/min with an injection volume of 1 mL at a split ratio of 50:1, injector temperature of 60 °C, and ion source temperature of 250 °C. A voltage of 70 eV was used to record mass spectra. The relative percentage quantity of each component was estimated by comparing its average peak area to the total areas, using GC-MS Mass Hunter software to analyze spectra and chromatograms.

Phytochemical analysis

Qualitative analysis

Numerous standards protocol were followed to evaluate the presence or absence of various phytochemicals in crude extract and fractions (Doss, 2009).

Quantitative analysis

The total phenolics content of *E. royleana* stem was calculated by utilizing the Folin ciocalteau procedure of (Tongco *et al*., 2015). Gallic acid were used as standard and standard curve was plotted for the estimation of total phenolic contents (Ashraf *et al*., 2015). The standard aluminium chloride method, as suggested by Krishnaiah *et al*. (2009) was used with minor modifications to determine the total flavonoid contents of each extract. To quantify alkaloid content protocol of Harborne (1973) was followed. Tannins content was quantified by using the method of Van Buren *et al*. (1969). Spectrophotometer analysis was carried out at 495 nm. Method of Omar *et al*. (2012) was followed to determine cyanogenic glycoside in *E. royleana*. Saponin content was measured by using the protocol of Bibi *et al*. (2011, 2012).

Evaluation of cytotoxic activity

BSL Assay (Brine shrimp lethality assay) was applied to examine cytotoxicity of extracts of *E. royleana* by following the procedure of Sarah *et al*. (2017). The following formula was used to calculate percentage mortality (% M).

% Mortality = (no. of dead nauplii/ total no. of live nauplii) ×100

LD_{50} values were obtained from the best-fit line plotted percentage lethality verses concentration. Podophyllotoxin was used as positive and DMSO (Dimethyl Sulfoxide) as negative control (Ashraf *et al*., 2015).

Antioxidant activity analysis

The antioxidant potential of crude extract and fraction of *E. royleana* stem were examined using ABTS radical scavenging assay, DPPH method, Phosphomolybdate assay and reducing power assay. The scavenging activity for all methods were evaluated by using the formula below:

Scavenging (%) = Absorbance $_{(control)}$ − Absorbance $_{(sample)}$/ Absorbance $_{(control)}$ × 100

DPPH assay

The antioxidant activity of crude extract and fractions was evaluated as described by Chaves *et al*. (2020). Methanolic dilution of DPPH was used as a blank and ascorbic acid as a standard. A stock solution (5 mg / 8

mL) was made for crude extract and fractions separately. Aliquot (1 mL) of different concentrations (25, 50, 100, 200 & 400 µg/mL) was prepared. Then from each concentration 200 µL, was added in 200 mL DPPH solution. For 30 minutes, kept at room temperature, then absorbance was measured with a spectrophotometer at 517 nm.

ABTS assay

Stock solution with equal volume of (7 mM ABTS solution) and (2.4 Mm potassium persulphate) was kept for 14 hours at 25 °C in the dark. Dilution of mixture was done by adding 1 mL of ABTS solution with 60 mL of methanol. 1 mL of plant extract, fractions and ABTS were allowed to react before measuring absorbance at 734 nm (Arnao *et al*., 2001).

Total antioxidant capacity by phosphomolybdate assay

1 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was mixed with an aliquot of 10 mL of the extract solution. The tubes were kept in a water bath at 95 °C for 90 minutes, then allowed to cool. Absorbance was measured at 695 nm, as a reference, ascorbic acid was utilized to obtain the standard curve (Gupta *et al*., 2020).

Ferric reducing antioxidant power assay

Plant extracts (5 mg) were mixed with 5 mL phosphate buffer (pH 6.6) and 1% potassium ferrocyanide (5 mL), then kept in water bath for 20 minutes. Centrifuged the mixture for 10 minutes at 3000 rpm with 5 mL of Tri-chloro acetic acid (10%) added. Absorbance was measured by spectrophotometer at 700 nm (Orhan and Üstün, 2011).

Mineral analysis

Plant extract (2 g) was mixed with acid solutions (nitric acid, sulfuric acid and perchloric acid 5:1:0.5). Digestion was done on hot plate in a fume hood, after cooling added water to raise the volume up to 100 mL. Filtered the extract, then the elemental analysis was done by using an Atomic Absorption Spectrometer (Paul *et al*., 2014).

Determination of antibacterial activity

Agar well diffusion assay as described by Bibi *et al.* (2011) was followed. Four bacterial strains (*E. coli*, *Klebsiella*, *Xanthomonas* and *Staphylococcus aureus* were used to assess plant antibacterial activity. Streptomycin was used as positive and DMSO as a negative control. The % growth inhibition was estimated using the formula with reference to the standard drug is given below:

$$\%\ Inhibition = \frac{[\text{Diameter (sample zone of inhibition)} - \text{Diameter (control zone of inhibition)}]\ \times 100}{[\text{Diameter (drug zone of inhibition)} - \text{Diameter (control zone of inhibition)}]}$$

Statistical analysis

All experiments were done in triplicate. Results were evaluated statistically by using Microsoft word and Excel software. IC_{50} values were calculated by regression line equation.

Results

GC-MS analysis

The GC-MS profile of the n-hexane fraction is shown in Figure 2, which displays 16 biomolecules peak location. The phytocomponents, their retention time, peak area percentage and molecular weight are shown in

Table 2. These few significant compounds have large peak area percentages, 24-Noroleana-3, 12-diene (1.06%), 11, 14-Eicosadienoic acid (2.46%), Conjugated linoleic acid (3.84%), Olean-12-en-3-ol, acetate (4.53%), (-)-Isolongifolol, acetate (7.71%), n-Hexadecanoic acid (7.80%), Octadecanoic acid (10.57%), Urs-12-en-3-ol, acetate (13.49%) and cis-Vaccenic acid (45.44%).

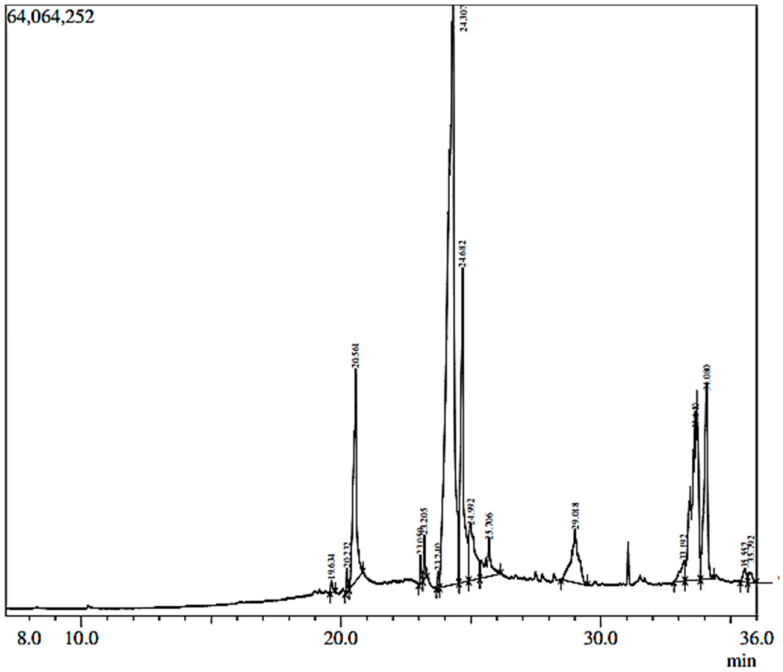

Figure 2. GC-MS analysis of n-hexane fraction of *E. royleana* stem extract

Table 2. List of phytochemicals identified in n-hexane fraction of *E. royleana*

Sr. No.	R/T	Name of compound	Molecular formula	M/W	Peak area %
1	19.634	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	0.26
2	20.232	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	0.34
3	20.561	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	7.8
4	23.059	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294	0.45
5	23.205	9-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	0.73
6	23.74	Octadecanoic acid, methyl ester	$C1_9H_{38}O_2$	298	0.3
7	24.307	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	45.44
8	24.682	Octadecanoic acid	$C_{18}H_{36}O_2$	284	10.57
9	24.992	Conjugated linoleic acid	$C_{18}H_{32}O_2$	280	3.84
10	25.706	11,14-Eicosadienoic acid	$C_{20}H_{36}O_2$	308	2.46
11	29.018	Olean-12-en-3-ol, acetate	$C_{32}H_{52}O_2$	468	4.53
12	33.192	24-Noroleana-3,12-diene	$C_{29}H_{46}$	394	1.06
13	33.64	Urs-12-en-3-ol, acetate	$C_{32}H_{52}O_2$	468	13.49
14	34.08	(-)-Isolongifolol, acetate	$C_{17}H_{28}O_2$	264	7.71
15	33.557	Olean-12-en-3-ol, acetate,	$C_{32}H_{52}O_2$	468	0.57
16	33.792	9,19-Cyclolanostan-3-ol, 24-methylene	$C_{31}H_{52}O$	440	0.43

Phytochemicals screening

The qualitative phytochemical screening of *E. royleana* extracts showed the occurrence of medicinally important compounds (Table 3). Alkaloid, tannins, flavonoids, saponins, steroids, sterols and phenols are present in crude and fractionated extracts (Table 3). Alkaloids were absent from methanol, EA and chloroform fractions (Tabel 3). However, other phytochemicals were strongly observed in crude extract and n-hexane fraction (Tabel 3). The total phenolic content (gallic acid equivalent, µg/mg) in crude extract and n-hexane fraction was 1.886 and 0.855 µg/mg respectively (Tabel 4). The quantity of, flavonoids, tannins and glycol cyanide were detected to be high in crude extract (0.551±0.01 µg/mg, 2.231±0.03 µg/mg and 6.001±0.05 µg/mg respectively) and n-hexane fraction (0.090±0.01 µg/mg, 1.149±0.02 µg/mg and 3.952±0.03 µg/mg respectively) (Tabel 4). Highest saponin content was observed in crude extract than all fractions, this is due to the synergistic effect of plant. In combine form all fractions showed synergism leads to excellent activity of crude extract as compared to all fractions. Minor quantity of alkaloids is observed in crude extract and n-hexane (0.092±0.01 µg/mg and 0.086±0.01 µg/mg respectively) (Tabel 4). Total phenolic content was measured at different absorbance through spectrophotometer, gallic acid was used as standard (Figure 3).

Table 3. Qualitative phytochemical screening

Phytochemicals	Crude extract	Methanol fraction	EA fraction	n-hexane fraction	Chloroform fraction
Alkaloids	+	-	-	+	-
Phenols	++	++	++	++	++
Steroids	++	+	+	++	+
Saponins	++	++	++	++	++
Tannins	++	++	++	++	++
Flavonoids	++	++	++	++	++
Sterols	+	+	+	+	+

Key: (++) = high quantity, (+) = small quantity, (-) = absent.

Table 4. Quantitative phytochemical screening (in µg/mg)

Phytochemicals	Crude extract	Methanol fraction	EA fraction	n-hexane fraction	Chloroform fraction
Alkaloids	0.092±0.01	0.01±0.00	0.001±0.00	0.086±0.01	0.04±0.00
Phenols	1.886±0.02	0.505±0.01	0.848±0.01	0.855±0.01	0.383±0.01
Glycol cyanide	6.001±0.05	3.733±0.03	2.029±0.03	3.952±0.03	0.149±0.00
Saponins	0.884±0.01	0.642±0.01	0.875±0.01	20.852±0.07	0.997±0.02
Tannins	2.231±0.03	0.449±0.01	0.471±0.01	1.149±0.02	0.476±0.01
Flavonoids	0.551±0.01	0.551±0.01	0.090±0.01	0.090±0.01	0.076±0.00

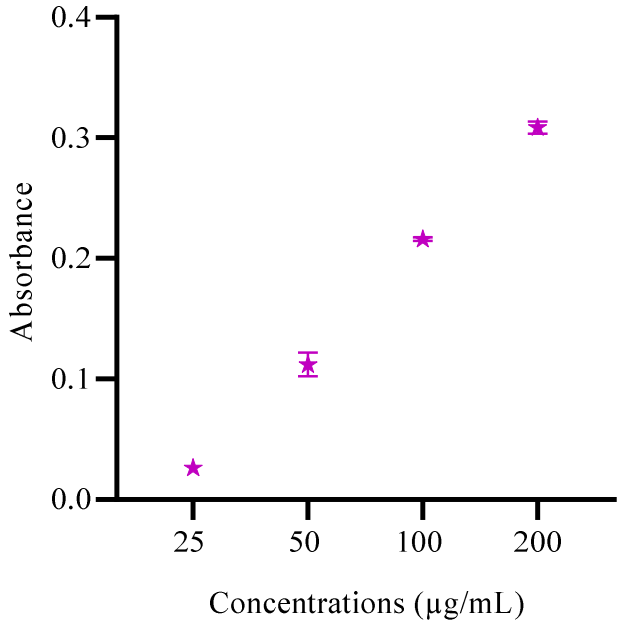

Figure 3. Plant crude and fractionated extract total phenolic content with different absorbance

Cytotoxic activity

Plant crude extract and fractions (methanol, ethyl acetate, n-hexane, and chloroform) were tested for possible cytotoxicity on chosen organism through the brine shrimp lethality (BSL) assay. The results of the BSL Assay of *E. royleana* stem extract and fractions are shown in (Figure 4a). All samples demonstrated maximum mortality at the highest tested concentration (400 µg/mL). At 400 µg/mL, the mortality rate for crude extract, methanol, n-hexane, EA, chloroform, and drug was 80, 50, 70, 60, 50, and 90 %, respectively (Figure 4a). At 100 µg/mL, there was no obvious difference in the cytotoxic activity of the samples (Figure 4a). Crude and n-hexane extract exhibited the highest LD_{50} value 168.4636119 and 220.3002611% respectively (Figure 4b).

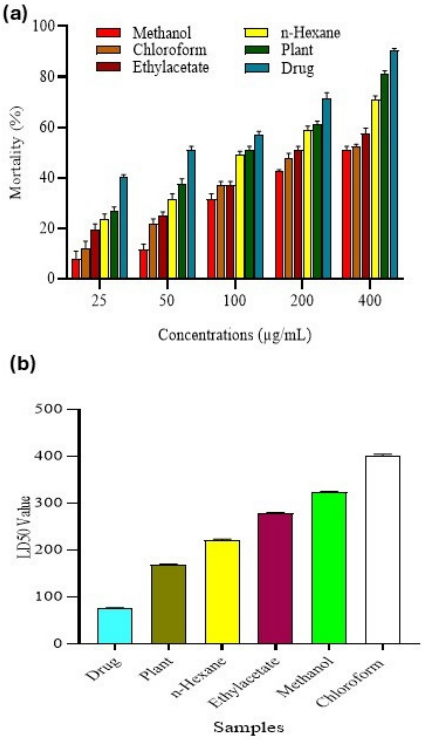

Figure 4. Mortality % of *E. royleana* stem crude extract, all fractions and drug at different concentrations (a) and LD_{50} of plant crude extract, fractions and drug (b)

Antioxidant activity

Against DPPH free radical the highest % scavenging ability of crude extract and EA, chloroform, n-hexane and methanol fraction was 39.4, 32.03, 23.2, 34.8 and 20.2% respectively at 400 µg/mL (Figure 5a). The extracts have concentration-dependent antioxidant capability with various degrees of efficiency. According to this study, an increase in DPPH scavenging ability was observed with an increase in concentration of extracts.

The % scavenging ability of crude and fractionated extracts against ABTS free radical range from 23.3 - 61.2% at 25-400 µg/mL (Figure 5b). Maximum % inhibition activity of fractionated extracts (n-hexane, ethyl acetate, chloroform and methanol) was 50.2, 28.9, 42.5 and 45.9 respectively at 400 µg/mL (Figure 5b). Crude extract showed highest scavenging activity compared to fractionated extract revealed the synergistic effect. Amongst the four fractions n-hexane showed the highest % scavenging ability, showed that nonpolar fractions have more potential to scavenge free radicals. The total antioxidant capacity of crude and fractionated extracts through total antioxidant capacity (TAC) assay is shown in (Figure 5c). At low doses inhibition activity is weak but at higher doses extracts presented significant % scavenging activity. At low concentration (25 µg/mL) the inhibition activity showed by crude and fractionated extracts is ranging from 22-34% (Figure 5c). At maximum dose 400 µg/mL crude and hexane extract exhibit highest free radical scavenging ability (62.1 and 57.4% respectively). Highest ferric free radical scavenging % of crude and fractionated extracts (n-hexane, methanol, ethylacetate and chloroform), at 400 µg/mL was 53.1, 43.5, 31.1, 42.3 and 26.1% respectively are shown in (Figure 5d). At doses higher than 200 µg/mL, crude extract exhibit over 50% inhibition on ferric free radical (Figure 5d). Reference standard ascorbic acid showed significant inhibition activity-65%.

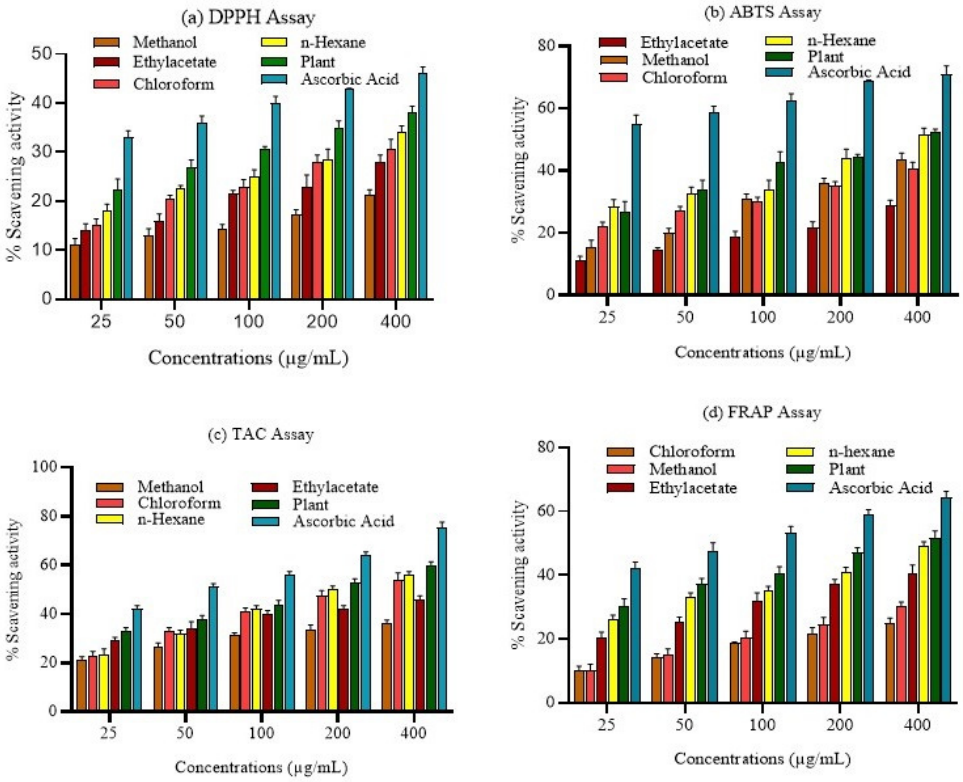

Figure 5. Assessment of antioxidant potential of crude extract and fractions of *E. royleana*. a) DPPH free radical scavenging assay, b) Radical scavenging potential by ABTS assay, c) Determination of total antioxidant capacity by Phosphomolybdate assay and d) Reducing power assay and. Ascorbic acid was used as standard

Mineral analysis

Mineral analysis of crude extract revealed the presence of some minerals in mg/g such as calcium 0.055±0.01, sodium 1.201±0.03, potassium 0.055±0.01, cadmium 0.121±0.01, zinc 0.007±0.00, iron 0.014±0.00, manganese 0.0008±0.00 and chromium 0.136±0.01 µg/mg (Table 5). Amongst all minerals sodium is present in the highest amount and manganese has the lowest value (Table 5).

Tabel 5. Percent concentration of various elements

Sr. No.	Minerals	Quantity (µg/mg)
1	Na	1.201±0.03
2	K	0.055±.01
3	Ca	0.009±0.00
4	Cd	0.121±0.01
5	Zn	0.007±0.00
6	Fe	0.014±0.00
7	Mn	0.0008±0.00
8	Cr	0.136±0.01

Antibacterial activity

Plant crude extract and fractions (methanol, ethyl acetate, n-hexane, and chloroform) were tested for possible antibacterial potential against following bacterial strains (E. *coli, Xanthomonas* spp., *Klebsiella* spp., and *S. aureus*) is presented in (Figure 6). Plant extract and hexane fraction showed highest % inhibition against *S. aureus* that was 60.6 and 50.92% respectively (Figure 6a). Likewise, 48.4 and 54.6% inhibitory activity were shown by n-hexane and crude extract against *Xanthomonas* spp. respectively (Figure 6b). Again, the highest inhibitory activity was shown by crude extract and n-hexane contrary to *Klebsiella* that is 56.1 and 49.4% respectively (Figure 6c). Similarly, crude and hexane extract present highest antibacterial activity against *E. coli* (Figure 6d). Rest of fractions also showed considerable antibacterial potential but lesser that crude and n-hexane fraction.

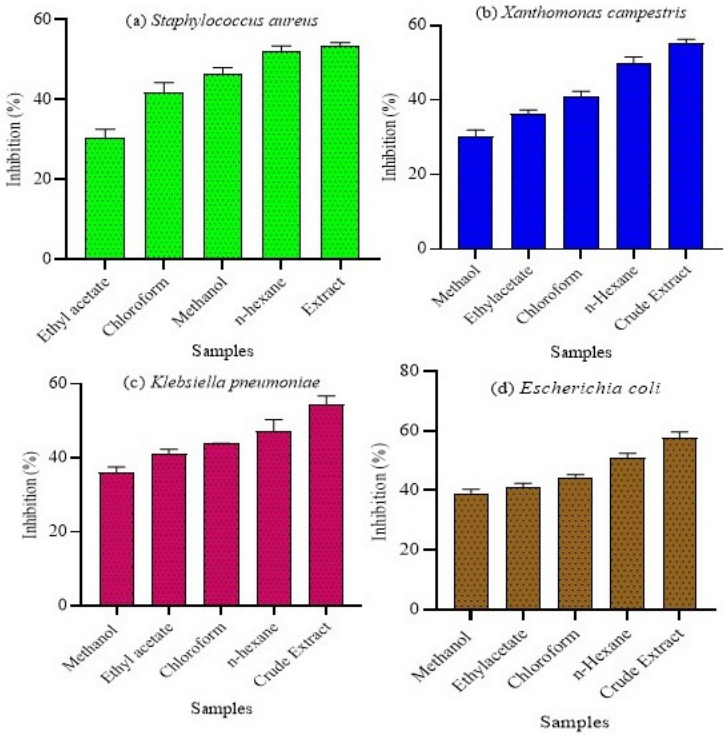

Figure 6. Antibacterial activity of plant extract and fractions (chloroform, methanol, n-hexane and ethyl acetate) against four bacterial strains

Discussion

Previously reported by Al Abboud *et al*. (2023) GC-MS analysis of genus *Euphorbia* revealed n-Hexadecanoic acid and a methyl ester of fatty acid, also known as palmitic acid, is known to have antibacterial activity. Both these compounds are also present in *E. royleana* n-hexane fraction. Olean-18-ene was detected by GC/MS in *E. inarticulata, E. triaculeata*, and *E. fractiflexa.* In *E. royleana* fraction Olean-12-en-3-ol was identified. Previously it was reported by Rautela *et al*. (2020) Octadecanoic acid is identified in *E. hirta* and *E. milli* extracts. Ricinoleic acid, 6-Octadecenoic acid, and (9E, 11E)-Octadecadienoic acid were identified as dominant phytochemicals with the maximal peak area (%) of 15.25%, 36.83%, and 12.37%, respectively (Ullah *et al*., 2023). Ingenol-20-myristate, ingenol-20-palmitate and 12-O-acetyl-8-omethylingol were identified by comparison of their GC-MS data with those in the literature (Wu *et al*., 2023). Existence of secondary

metabolites in plants produces some biological activity in humans and animals, which accounts for their use as herbs (Kennedy and Wightman, 2011). Presence of secondary metabolites in *E. royleana* may be responsible for its pharmacological potential and vital inhibitory action (Paiva *et al*., 2010). Saponins have vital inhibitory effect on inflammation, along with the property of precipitating and coagulating red blood cells (Yadav and Agarwala, 2011). Plant extract contains phenols and flavonoids, both of which have antioxidant properties and biological activity (Ullah *et al*., 2023). The pharmaceutical potential of *E. royleana* against various disorders is associated with high phytochemical contents (Bhattacharya *et al*., 2016). Flavonoids are well known for their vital role in the removal of reactive oxygen species (ROS) as well as suppression of tumor initiation, development, and breakthrough. Tannins and alkaloids have antibacterial, antiviral, anti-HIV, and antiparasitic properties. Terpenoids have therapeutic values against certain viral and bacterial diseases as well as, possess anti-inflammatory and anticancer potential (Nugraha *et al*., 2019). The presence of anthraquinones, saponins, cardiac glycosides, and flavonoids was verified by phytochemical analysis. Studies by Anees-ur-Rehman *et al*. (2022) revealed that methanol extract of the complete *Euphorbia royleana* plant had the highest phenolic concentration. Phenolics provide therapeutic strength to the plants due its properties like anticancer, anti-oxidant and antimutagenic etc. (Ahmed *et al*., 2019). Steroids presence in plant extract showed that plant has great pharmacological importance. The fresh sample has higher steroids content than the dried ones. The majority of phytochemicals showed a similar pattern, except terpenoids, which are rare in both fresh and dried samples. It should be mentioned that steroidal chemicals are significant and a great interest for chemists because of their interactions with substances like sex hormones (Chen *et al*., 2017; Nugraha *et al*., 2019).

The brine shrimp lethality test is a quick, low-cost, and simple bioassay for evaluating plant extract bioactivity, which is typically linked with cytotoxic and anti-tumor properties. It's a preliminary toxicity test in advance of further study on mammalian animal models. Several studies have shown that the brine shrimp assay is a valuable technique for early toxicity evaluations, screening medicinal plants routinely used for a variety of reasons, and monitoring the isolation of a diverse range of biologically active compounds. Cytotoxic activity of plants is might be due to the presence of phytochemicals such as phenols, flavonoids, saponins, alkaloids, and tannins (Bhattacharya *et al*., 2016). Cytotoxic evaluation of crude extract showed good results as compared to fractions which is due to synergistic effect, similar effect was already done by Komape *et al*. (2017). Previous study of Gull *et al*. (2022) and Biswas *et al*. (2013) also showed similar manner. In the present study, an increase in radical scavenging ability was observed with an increase in concentration of extracts. Likewise, research conducted by Ozturk *et al*. (2011) support our study that there was increased scavenging of oxidants with increase in plants concentration. Current research showed the antioxidant potential of the plant extract and fractions in a concentration-dependent manner which is according to the study of Ashraf *et al*. (2015). Scientific investigations have shown that the antioxidant potential of the plant extract is attributed to various aromatic phytochemicals with reduction potential, such as polyphenols, tannins, terpenoids, and flavonoids (Sivaraman *et al*., 2013). The findings that *E. royleana* dichromate and methanol extract having significant potential as an antioxidant agent (Anees-ur-Rehman *et al*., 2022). These findings suggested that the methanolic extract's antioxidant activity was related to a high concentration of phenol and flavonoid compounds (Alavi *et al*., 2022). The presence of hydroxyl groups in phenolic substances confers free radical scavenging potential (Khan *et al*., 2017). Flavonoids inhibit reactive oxygen synthesis by creating a chelate with trace elements involved in free radical formation, scavenging reactive species, and up-regulating and maintaining antioxidant defenses (Aliyazicioglu *et al*., 2016). These findings are consistent with our findings. Meanwhile, Ke *et al*. (2015) verified the antioxidant, anti-inflammation, and anti-aging properties of several flavonoids obtained from plant sources. The current findings showed that the extract has an effective antioxidant potential and iron antioxidant capacity. The reagent Phosphomolybdate can detect chemicals in extracts such as carotenoids, flavonoids, phenols, and tocopherol, which are nucleophilic in nature and hence have chelating potential (Taslimi *et al*., 2018). This dependency of potential on concentration was also reported

by (Hajimehdipoor et al. 2016). These findings are also in agreement with Prasad and Bisht (2011), Shen *et al*. (2017) and Wei *et al*. (2018), who analyzed the mineral composition of aerial parts of *Euphorbia thymifolia* Linn.

Studies revealed that genus euphorbia has considerable amount of variety of minerals including zinc, iron, copper, sodium, potassium and manganese. In biological systems, zinc performs structural, regulatory, and catalytic roles. Almost 300 enzymes are necessary for biological activity and depend on zinc for their catalytic action. The structural role that zinc plays in maintaining the tertiary structure of enzymes is another important function of zinc. Without a doubt, the plant's Zn content may have contributed to its nutritional and therapeutic value, as reported by the plant's traditional consumers (James and Friday, 2010).

Ashraf *et al*. (2015) studies revealed that *E. royleana* stem has antibacterial activity against various bacterial strain. Moreover, *Euphorbia royleana* leaves extract also showed significant antibacterial activity against five bacterial strains including; *Escherichia coli, Streptococcus pneumonia, Salmonella typhi, Klebsiella pneumonia, Proteus vulgaris* (Sivaraj *et al*., 2011). The antimicrobial activity of plant extracts of *E. royleana* might be due to several phytochemicals such as phenols, flavonoids, saponins, alkaloids, and tannins. Phenols affect the function of the cytoplasmic membrane, disturbing the metabolism of energy, and thus affecting the synthesis of nucleic acids (Salehi-Sardoei and Khalili, 2022). Terpenoids and alkaloids interact with enzymes and proteins of the microbial cell membrane causing its disruption to disperse a flux of protons towards the cell exterior which induces cell death or may inhibit enzymes necessary for amino acids biosynthesis (Silva *et al*., 2016; Huang *et al*., 2023). Flavonoids have been shown to inhibit bacterial DNA polymerase, RNA polymerase, reverse transcriptase, and telomerase (Kouadri, 2018; Su *et al*., 2023). The saponins decrease surface tension causing an increase in permeability or leakage of cells, resulting in the discharge of intracellular compounds (Bhattacharya *et al*., 2016; Niu *et al*., 2023; Yang *et al*., 2024).

Conclusions

The current investigation found that the stem extract and fractions of *E. royleana* had a high therapeutic potential as well as antibacterial, antioxidant, and cytotoxic properties. The highest antioxidant and anticancer activity were identified, which could be credited to its high phenolic and flavonoid content. Especially, crude extract and hexane fraction has good antioxidant, antibacterial and cytotoxic activity so it will be used in the preparation of medicines. GC-MS analysis showed that plants have a variety of compounds, leading to its therapeutic potential. Elemental analysis revealed that the plant contained a significant amount of minerals, thus it will be utilized to manufacture cosmetics. Minerals have great nutritional values so this plant will used in the manufacturing of food supplements. Future research will focus on isolating bioactive components from fresh *E. royleana* extract in order to find vital pharmacological agents.

Authors' Contributions

YB designed the study. NN and SI performed the experiments. AQ and SA helped in data curation and analysis of data. AAA and MJA collected literature reviews and helped in writing the original draft of the article. YB and AQ provided technical expertise to improve the article and helped in funding acquisition. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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