

Biological and phytochemical analysis of fractionated extracts of *Ziziphus nummularia* against pathogenic bacteria, oxidative stress and cytotoxicity

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Abstract

In modern medicine, synthetic drugs are predominant, yet they often pose health hazards. So, there is a need to find natural alternatives. *Ziziphus nummularia* (Burm. F.) Wight & Arn., belongs to family Rhamnaceae, is well known for its traditional value such as its fruits have been used as natural appetizer and orally consumed around the world. This study investigated the phytochemical composition and bioactivity of crude and fractionated extracts from the stem bark of *Ziziphus nummularia*. Crude extracts were prepared using cold maceration, followed by partitioning based on polarity into n-hexane, chloroform, and ethyl acetate fractions. These extracts were subjected to phytochemical analysis and evaluated for antioxidant, antibacterial, and cytotoxic activities. The crude methanolic extract exhibited the highest antioxidant activity, with IC₅₀ values of 136.49 µg/mL in DPPH and 78.9 µg/mL in ABTS assays. Antibacterial tests using the agar well diffusion method revealed that the methanolic extract, ethyl acetate, and chloroform fractions demonstrated broad-spectrum antibacterial activity against *E. coli*, *K. pneumoniae*, *S. aureus*, and *S. epidermidis*, with inhibition zones ranging from 0 to 8.43 ± 0.23 mm at 400 mg/mL. The brine shrimp lethality assay indicated significant cytotoxicity in the methanolic, ethyl acetate, and chloroform extracts, compared to the negligible effect of the n-hexane fraction. These findings highlight the potential of *Z. nummularia* as a source of bioactive compounds with therapeutic applications against infectious diseases, toxins, and oxidative stress. Further isolation of pure compounds and animal trials are recommended to explore its potential in drug discovery.

Keywords: antioxidant; antibacterial; brine shrimps; cytotoxicity; phytochemical; *Ziziphus nummularia*

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Introduction

Demand for bioactive constituents from nature increasing because natural substances are important for developing new medicines and discovering drugs. Furthermore, there is increasing utilization of herbal medicines for the treatment of many severe diseases (Nembo *et al.*, 2020). In Covid-19 pandemic, the use of medicinal plants was incredible and therefore needs further investigations (Soukand *et al.*, 2022). Phytomedicine has offered significant antibacterial and anticancer leads. Paclitaxel, podophyllotoxins, and vinblastine are derived directly from plants, etoposide, teniposide, irinotecan, and docetaxel are semi-synthetic derivatives of these anticancer leads that are employed as anticancer medications with various mechanisms of action (Alghamdi *et al.*, 2022). Today, many scientists and pharmacologist focus on concern of novel way for the efficient action of herbal drugs (Jaleel *et al.*, 2020).

Ziziphus nummularia is a wild, small, thorny, branched, shrub found throughout Pakistan, India, China (Khan *et al.*, 2020; Abdallah *et al.*, 2024), Afghanistan, Iraq, Iran, and Egypt with 6-8 feet height (Rauf *et al.*, 2016). This plant is known by different local names such as Jharberi, Bhor (Uddin *et al.*, 2020), Malla (Khalil *et al.*, 2022). It grows mostly in arid and semi-arid regions and has potential to combat drought environment. The classification of *Ziziphus nummularia* is Kingdom of Plants, Tracheophyta Phylum, Magnoliopsida Class (Dicotyledons), Rosales Order, Rhamnaceae Family *Ziziphus* is a genus, a species, and its binomial name is *Ziziphus nummularia* (Burm. Fil.) Wight & Arn (Imran *et al.*, 2023). The stem bear spines, hairy or smooth surface of brown, ash, purplish colored. Leaves are small coin size, ovate, with serrate margin. Flowers are yellowish, emerge on axillary cymes in August-October. Fruits are drupes, brown to orange colored, round shape, sweet sour in taste, ripen in November-December in cold season. Fruit is eaten fresh and dried as well. Seed has hard covered of seed coat (Pandey *et al.*, 2010). The plant is used in the form of paste, decoction, powder, extract or in infusion with other plants extract. Different Plant parts are directly eaten for heart problems and nutritional source, anemia, hypertonic, nervous diseases (Ullah *et al.*, 2023) and used as a folk remedy to aconite poisoning, ligament pain tuberculosis, blood disorder (Table 1) (Beg *et al.*, 2016). In China, the plant extensively used to heal the burns (Kumar *et al.*, 2010). The fruit of *Ziziphus nummularia* is big sources of mineral and contain vitamin C, carbohydrates, which contribute to natural appetizer, cooling, stomachic, astringent, improves digestion, aphrodisiac, laxative and remove biliousness, burning sensations, thirst, vomiting, they cure mucous, tuberculosis and blood diseases (Uddin *et al.*, 2022; Sharma and Chakraborty, 2021; Ji *et al.*, 2023).

This plant is reported to have anti-athelmic (Aggarwal *et al.*, 2018), anti-inflammatory (Ray *et al.*, 2021), anti-proliferative, anti-migratory, anti-invasive, anti-diabetics (Dubey *et al.*, 2017), molluscicidal and phytotoxicity (Shah *et al.*, 2018), hepa-protective (Yadav *et al.*, 2022) activities. The study was carried out to assess the cytotoxic efficacy of fractionated extract of *Ziziphus nummularia* in addition to anti-bacterial and antioxidants. Furthermore, the qualitative and quantitative phytochemical analysis were evaluated by standard protocol. To the best of our knowledge, antioxidant, and cytotoxicity of the *Ziziphus nummularia* stem bark fractionated extracts were not reported in previous literature.

Table 1. Traditional medicinal uses of *Ziziphus nummularia* according to Ayurveda

Plant parts	Used as	Traditional Uses	References
Leaves (pala)	Paste	Reducing obesity, act as antipyretic, chewed for toothache cuts, wound healing, and treatment of strangury	Ullah <i>et al.</i> , 2021; Imran <i>et al.</i> , 2023; Abdallah <i>et al.</i> , 2024
Bark	Decoction	Dysentery and diarrhea, febrifuge and astringent	Sharma and Chakraborty, 2021
Fruit	Raw, Powder, Paste	Natural appetizer, cooling, stomachic, astringent, improves digestion, aphrodisiac, laxative and remove biliousness, burning sensations, thirst, vomiting, they cure mucous, tuberculosis and blood diseases	Uddin <i>et al.</i> , 2022; Sharma and Chakraborty, 2021
Root	Crushed into fine powder Decoction	Ulcers and old wounds, coughs, biliousness and headache, fever	Kumar <i>et al.</i> , 2010; Sharma <i>et al.</i> , 2012
Seed	-	Employs to treat eye diseases	Beg <i>et al.</i> , 2016

Materials and Methods

Collection of Z. nummularia bark & preparation of crude extract

The bark and fruit of *Ziziphus nummularia* were collected in April 2023 from surrounding area of Malakwal, Mandi Bahauddin province Punjab in Pakistan. The plant was first recognized by its native name and then identified by Dr. Rehmat Ullah Qureshi, Department of Botany, PMAS, Arid Agriculture University Rawalpindi, Pakistan. At the Herbarium of University, plant specimen of *Ziziphus nummularia* was kept. Plant material was washed thoroughly with water to remove dust particles. After washing plant parts with double distilled water, the water was removed by keeping it in strainer (Bibi *et al.*, 2018). The plant material was shade dried in the open air. The dry plant matter was grounded to powder with the help of mixer grinder. Plant sample was stored in plastic sealed bag until needed (Bibi *et al.*, 2018). Crude extract was prepared by using cold maceration technique. According to the protocol of Afzal *et al.* (2022) 30 g of plant sample powder were dipped in 300 mL of methanol and placed at room temperature for 7-10 days. Next, the solution was filtered by Whatman No.1 filter paper. The obtained filtrate was evaporated under open air and final thick dried extract known as crude extract (Figure 1).



Figure 1. Preparation of crude extract using *Ziziphus nummularia* stem bark
Fractionation of ZN crude extract

For the preparation of fraction, methods describe by Ahmed *et al.* (2018) and Bibi *et al.* (2012) with few modifications were employed. The quantity of 3 g crude methanolic extract was dissolved in 100 mL of distilled water and was subjected to prepare fraction in the separating funnel. Subsequently fractions were prepared based on solvent-solvent partitioning, all fractions dried in open air. The suspended extract in distilled water, and then partitions were created using n-hexane, chloroform, ethyl acetate, all of which had an increasing degree of polarity. Then, using a rotary evaporator, all fractions were dried and stored at 4 °C. The yield was calculated as a percentage of the weight of the air-dried plant material after the dried extract was weighed. The scheme was described below (Figure 2).

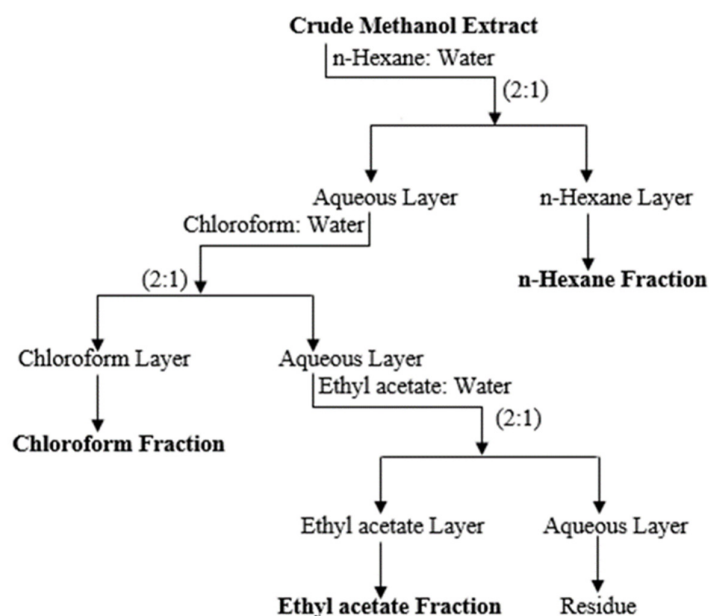


Figure 2. Fraction schemes for preparation of n-Hexane, chloroform and ethyl acetate fractions

Antioxidant activity

ABTS radical scavenging activity

The antioxidant activity of plant extract was determined by the ABTS method described by Zhou *et al.* (2020). A mixture of ABTS 7.4 mM (dissolve 0.38 g of powder into 100 mL of distilled water), Potassium persulfate $K_2S_2O_8$ 6mM (0.086 g dissolved in 100 mL of distilled water) was prepared and placed in darkness for 16 hours at room temperature. Next, the prepared solution was diluted with methanol and measured its absorbance at 734 nm with the value 1-1.5. Different concentrations of plant extract were prepared and measured the absorbance at 734 nm with the help of spectrophotometer. The blank control was distilled water, and ascorbic acid as a standard. Took the final reading by using formula mentioned below.

Reducing power assay

Phosphate buffer (2.5 mL) and 2.5 mL potassium ferro-cyanide were added to the plant sample fraction, and it was allowed to remain inside the water bath at 50 °C for 20 minutes. After centrifuging the solution, trichloroacetic acid (TCA) was added. At 700 nm, absorbance was measured with a spectrophotometer. A positive control was ascorbic acid (Padalia and Chanda, 2021).

Phosphomolybdate assay

To evaluate the antioxidant capacity of plants sample was applied as an assay describe by Meshginfar *et al.* (2018) and Fernández-Poyatos *et al.* (2020). Initially, 4 mL Phosphomolybdate reagent was prepared by

mixing 0.6 M sulphuric acid, 28 mM sodium phosphate, 4 mM ammonium molybdate in a micro centrifuge tube. 4 mL of phosphomolybdate reagent was mixed with the 1 mL of sample plant crude extract and fraction, in falcon tube. For blank, 4 mL of reagent was added in 1 mL of ethanol. All the tubes were wrapped with aluminium foil and allowed to stand for about 10 mins. Tubes were positioned in a water bath for 90 mins at 95 °C temperature. After incubation, tubes were allowed to cool and then measured absorbance at 695 nm by spectrophotometer.

DPPH assay

DPPH radical-scavenging activity of the samples were assessed by using the protocol of Fernández-Poyatos *et al.* (2020). A net volume 0.2 mM DPPH solution was prepared in methanol. The mixture solution was allowed to rest at room temperature. Different concentrations of plant extract were prepared, and equal volume of DPPH solution was mixed in falcon tube. Test samples were kept for 30 minutes of incubation. At 517 nm, the absorbance was determined. Methanol was used as blank and Ascorbic acid were employed positive control. IC₅₀ (extract concentration with 50% DPPH inhibition) was determined using plotting the inhibition (%) vs. the extract concentration. The radical scavenging activity was expressed using a standard calibration curve constructed by plotting percentage inhibition against concentration of positive control (Fernández-Poyatos *et al.*, 2020).

Antibacterial activity

Protocol of Ishaque *et al.* (2021) was used to determine the anti-bacterial activity of plant extract fractions with some modification. The Agar well diffusion method is considered as appropriate method for testing antibacterial activity of plants extracts.

Bacterial strain and control

The antibacterial activity of plant extracts alone and its fraction was tested on a panel of two Gram-negative and two Gram-positive bacteria. Four distinct bacterial strains were tested against various fraction of *Ziziphus nummularia*, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumonia*, and *Escherichia coli*. A well-known antibiotic called streptomycin and the chemical DMSO were utilized as positive and negative controls, respectively.

Agar well diffusion method

Nutrient medium

For preparation of microbial culture media, 28 g agar powder was added in 1 liter of distilled water to prepared nutrient agar medium.

Preparation of bacterial culture

Bacterial strains were grown in nutrient broth at 37 °C for 24-48 hours. This culture was kept alive for the duration of the investigation in a liquid broth media at 4 °C. To prepare inoculum, liquid broth media was taken in flask and carefully inserted a colony of bacterial strain in it. After addition, put this flask on rotatory shaker at 150 rpm for 2 hours. Then, kept in incubator for 24 hours for complete processing.

Procedure

First, the instruments and equipment used in experiment were autoclaved. The media was autoclaved, adjusted to the pH at 7.0, and allowed to cool. Media was poured into autoclaved labeled petri plates. This media was seeded with bacterial suspension and allowed to solidify. Agar plates were pricked with 8 mm sterile cork borer to make the wells. Subsequently, 400 µL test sample was added in respective well with the help of micropipette. 100 µL of liquid nutrient agar medium were added in each well to seal them. streptomycin (2 mg/mL) in Dimethyl Sulfoxide (DMSO) and pure DMSO were considered as positive and negative control

respectively. Petri-plates were wrapped with parafilm tap and then placed in incubator at 37 °C for 24 h. Vernier caliper was used to measure the diameter of inhibition zone. To avoid any mistake, experiments were conducted in triplicates. The mean was computed with standard deviation. Percentage inhibition was calculated by using the following formula mentioned below.

Cytotoxicity

Brine shrimp's lethality assay

The Brine Shrimp Lethality Test (BSLT) method has been utilized as a standard bioassay for identifying the bioactivity of plant extract. This approach is used to determine the toxicity of active constituents in plant extract. This is a plain, cost-effective, and attractive method, *Artemia salina* is used as model test organism because of provide analogous response to that of the mammalian organism.

Procedure

Preparation of sea salt

The quantity of 38 g sea salt was dissolved in one liter (1000 mL) of deionized water then filter to obtained pure solution.

Hatching of brine shrimps

As test organisms, *Artemia salina* eggs were utilized. We took some seawater within the small container, sealed the one side where eggs shrimp were moved to. Constant supply of oxygen was rendered and allowed for 2 days for hatching. Subsequently, hatched shrimps were moved toward illuminated side. So, all those nauplii were collected.

Brine shrimp lethality assay

Almost 10 nauplii was taken by micropipette and added in test tube containing seawater. Now the resultant solution was known as brine solution. Various concentrations (25 µL/1 mL, 50 µL/1 mL, 100 µL/1 mL, 200 µL/1 mL, 400 µL/1 mL) of test samples were mixed in brine solution and place under light, for 24 hours at room temperature. After 24 hours' time, number of live nauplii were counted and find the lethality percentage by using following formula.

$$\text{Lethality \% of brine shrimp} = \frac{\text{no. of dead nauplii}}{\text{total no of nauplii}} \times 100$$

As a negative control, dimethyl sulfoxide (DMSO) was utilized (Indriaty *et al.*, 2022) and Vincristine as a positive control. Triplicate experiment was applied for precise results.

Phytochemical analysis

Primarily, qualitative, and then quantitative estimation was carried out by using standard protocol for the analysis of phytochemical. Phytochemical evaluation of fractionated extract of *Ziziphus nummularia* were performed by using standard procedures to find out the active compounds present in the extracts. Tests for alkaloids, flavonoids, tannins, and phenols were performed in subsequent methods developed by (Nigussie *et al.*, 2021).

Qualitative analysis

For estimation of alkaloids test, crude extracts and fractions were tested for the presence of alkaloids using Wagner's reagent. A portion of the extract was treated with Wagner's reagent, allowing it to stand for three to five drops. Wait until the appearance of a reddish-brown precipitate (Anusmitha *et al.*, 2022). For the estimation of flavonoids, the protocol of Des and Gezici, (2018) was followed with minor modifications. In the test tube, 10 mL of ethyl acetate was added in extract fractions and placed in water bath for 3 minutes. The

obtain filtrate followed by filtration was added in dilutes ammonium solution and shake well. The emergence of yellow colour was confirmed by the flavonoid's presence. About 0.5 g of all prepared fraction of plant extract was treated with some drops of 2% of ferric chloride (FeCl_3). As a result, if black, blue, green colour appeared it indicates the presence of total phenol in extract (Des and Gezici, 2018). The test was carried out according to the method put forward by Sharaf *et al.* (2022) with slight modifications. In plant extract, 10% NaCl and 1% gelatin solution was added. Consequently, formation of weight precipitate confirmed the tannins presence. A test tube containing a crude plant extract and 5.0 mL of distilled water was forcefully stirred. A couple of drops of olive oil were vigorously mixed into the foaming, and the look of the foam indicated the existence of saponins (Gul *et al.*, 2017).

Quantitative analysis

The quantification of Alkaloids, Tannins, phenolic constituents and total flavonoid contents in *Ziziphus nummularia* crude extract and fractions is a crucial effort, providing valuable insights into their potential bioactivity and medicinal properties. The total phenolic content was assessed by using the Folin-Ciocalteu reagent method, as outlined by Mohammed *et al.* (2021). Gallic acid was used as standard and at 760 nm absorbance of spectrophotometer was noted (Mohammed *et al.*, 2021). While the total flavonoids content was measured by using aluminum chloride method, with slight modifications per Oikeh *et al.* (2020). The absorbance was determined at 415 nm. The results were given in milligrams of quercetin equivalents. The total tannins content was determined by using Ferric chloride method with some minor modification (Zahara *et al.*, 2019). Absorbance of solution was measured by spectrophotometer at 495 nm. Tannin acid was used as standard chemical. Alkaloid content was determined by using the methodology put forward by Ajanal *et al.* (2012). For the total alkaloids analysis, Atropine was used as standard chemical, and at 478 nm absorbance was noted. All analysis was performed in triplicates, and the results are presented as mean values \pm SEM.

Results

Antioxidant

The free radical scavenging potential of *Ziziphus nummularia* crude methanolic extract and its fractions was evaluated using several antioxidant assays, including DPPH, ABTS, Reducing Power Assay, and Phosphomolybdate Antioxidant Assay. The results, displayed in Figure 3 (a, b, c, and d), illustrate the percentage of free radical scavenging potential of test solutions and standards at various concentrations. Specifically, Figure 3c demonstrates the outcomes of the Reducing Power Assay, where the absorption of light directly corresponds to the free radical scavenging potential at a given concentration. For instance, the IC_{50} values of *Ziziphus nummularia* crude methanolic extract were found to be 136.49 $\mu\text{g}/\text{mL}$, 78.9 $\mu\text{g}/\text{mL}$ for DPPH, ABTS, respectively. The overall antioxidant activity of the samples employed in this investigation was lower than other data, such as the DPPH radical scavenging activity of these samples, which showed higher activity. All samples showed radical scavenging activity in a dose-dependent manner. The finding suggests that crude extracts of *Ziziphus nummularia* bark and all its three fractions studied herein exhibit potent DPPH radical scavenging properties (Figure 3). The scavenging capacity rises with concentration in the range of 25-400 $\mu\text{g}/\text{mL}$, with crude methanolic extract having the highest scavenging power. Therefore, it is highly important and valuable to use the crude methanolic extract as a natural antioxidant in the food sector.

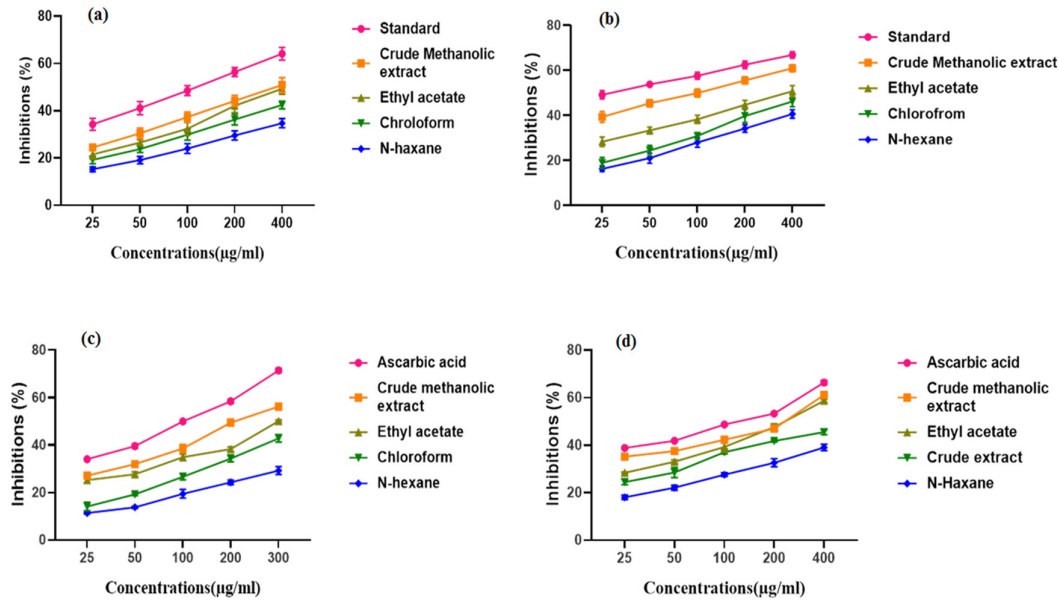


Figure 3. The total antioxidant capacity of the crude extract and fractions of *Ziziphus nummularia* Different concentrations of all extract were applied: (a) DPPH (b) ABTS (25-400 µg/mL) (c) FRAP (d) percentage of phosphomolybdenum activity

Antibacterial assay

The antibacterial activity of extracts was determined using the agar well diffusion method. In this method, wells served as reservoirs for both sample and standard dilutions. Sample dilutions were brought into contact with the inoculated medium, and after 18 to 24 hours of incubation, the diameter of clear zones of inhibition around the well was measured using a vernier caliper. If no activity was observed for the sample, no zone of inhibition would develop. Extracts at a concentration of 100-400 mg/ml were tested for their antimicrobial activity against two common human pathogenic bacteria. The methanol extract of all three-extract demonstrated moderate to good antimicrobial activity against the test organisms, while the n-hexane showed no remarkable activity. However, the ethyl acetate fraction from crude extract exhibited significant activity against the organisms. Conversely, other fractions from crude extract did not display such effects (Table 2). The zone of inhibition for the fractions sampled in N-hexane, ethyl acetate, and chloroform was quite selective. The recorded diameter is between 0 and 8.43 ± 0.23 mm, and it shows that bark crude extract and fractions prevented the growth of all (100%) of the tested bacteria. Our initial research revealed that all crude, chloroform, n-hexane, and ethyl acetate extracts of *Ziziphus nummularia* were effective against localized human pathogens such *Escherichia coli* and *Staphylococcus aureus*. The crude methanolic extract of *Ziziphus nummularia* bark shown substantial antibacterial activity against clinical isolates of bacteria that were multi-drug resistant (Figure 4). At maximum concentration of dose of 400 mg/mL, all the extracts and fractions showed effective inhibition. However, at minimum concentration (100-300 mg/mL) negligible result report obtained. In the case of all bacterial strains crude methanolic extract and ethyl acetate were more effective than chloroform and N-hexane. Nevertheless, all the bacterial strains displayed less resistance against crude methanolic extract.

Table 2. The inhibition zone observed by the application crude extract and different fraction of *Ziziphus nummularia*

Test organisms	Cefotaxime (mm)	Crude methanolic extract (mm)	Ethyl acetate (mm)	Chloroform (mm)	n- hexane (mm)
<i>Staphylococcus aureus</i>	13.8 ± 0.16	8.43 ± 0.23	6.5 ± 0.31	4.5 ± 0.11	1.4 ± 0.23
<i>Staphylococcus epidermidis</i>	12.7 ± 0.37	7.6 ± 0.42	5 ± 0.28	3.46 ± 0.29	2.2
<i>Escherichia coli</i>	12.26 ± 0.31	5.2 ± 0.14	3.4 ± 0.05	2.4 ± 0.21	-
<i>Klebsiella pneumonia</i>	12.4 ± 0.21	5.3 ± 0.2	3.2 ± 0.2	2.9 ± 0.2	1.2 ± 0.14

Zone of inhibition (mm) and values are expressed in mean ± standard error of the mean. (Mean ± S.E)

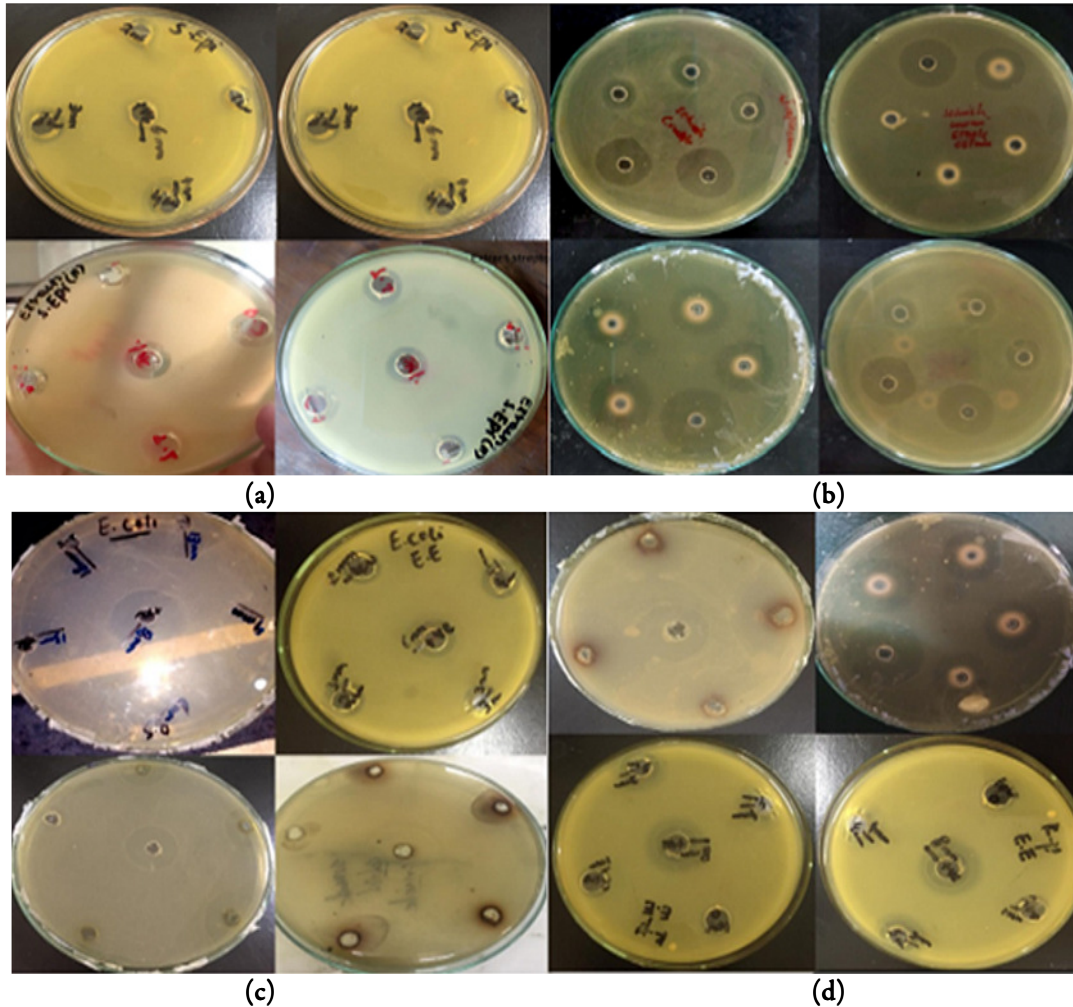


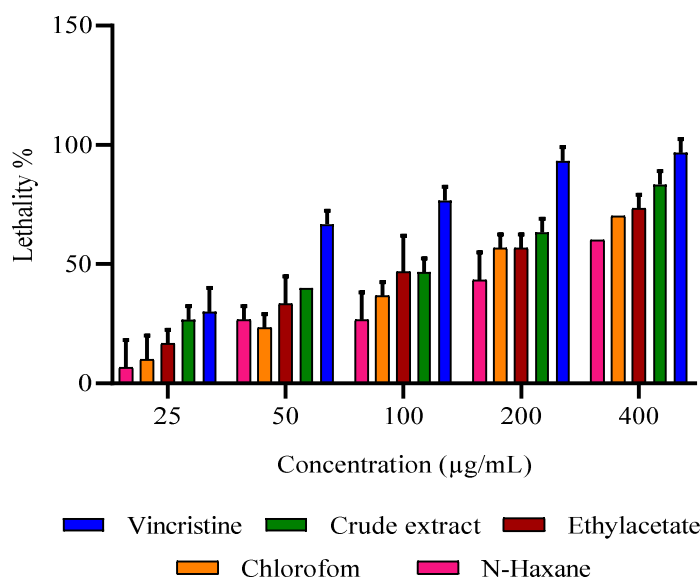
Figure 4. The antibacterial activity of different extract of *Ziziphus nummularia* stem bark (a) The inhibition zone (mm) of crude methanolic extract, N-hexane, chloroform, ethyl acetate fraction against *Staphylococcus epidermidis* at concentration of 100-400 mg. Also, the negative control, 10% v/v DMSO. (b) The inhibition zone (mm) of crude methanolic extract, N-hexane, chloroform, ethyl acetate fractions against *Staphylococcus aureus* (SA). (c) The inhibition zone (mm) of crude methanolic extract, N-hexane, chloroform, ethyl acetate fractions against *Escherichia coli*. (d) The inhibition zone (mm) of crude methanolic extract, N-hexane, chloroform, ethyl acetate fractions against *Klebsiella pneumoniae*.

Cytotoxicity

The cytotoxic activity of a large variety of plant-derived extracts was originally determined throughout the medication development process prior they were subsequently evaluated on cancer cell lines or higher animals. Numerous studies have demonstrated a positive link between the BSLT (Brine Shrimp Lethality Test) and several tumor cell lines. If the plant extract's LC₅₀ value was below 1000 µg/mL (Table 3), it was thought to be poisonous to brine shrimps. The level of toxicity will reveal an extract's anticancer effectiveness (Elsyana *et al.*, 2016). In current investigation, the cytotoxicity of fractionated extract of *Ziziphus nummularia* was evaluated using the brine shrimp lethality test. Crude, n-hexane, chloroform, ethyl acetate fractions at five different range of concentrations 25-400 µg/mL were assessed against 10 nauplii in each vial. Results were obtained based on percentage mortality rate of larvae. The extract showed result on concentration dependent, at higher concentration of 200 and 400 µg/mL, mortality rate became high. Maximum lethality was shown by crude methanolic fraction after standard with 90% mortality at 400 µg/mL. While at lowest concentration 25 µg/mL, the observed death rate was 30%. In case of Ethyl acetate, maximum lethality rate was 80% at 400 µg/mL and minimum death was 20% at 25 µg/mL. Following N-hexane, chloroform had 10% mortality at low concentration and 70% at higher concentration. N-hexane showed less pronounced result with (5-60%) mortality at 25-400 µg/mL (Figure 5). In this study, the LC₅₀ value of Crude extract was very close to the standard value. It means that Crude extract showed higher toxicity after standard (vincristine).

Table 3. LC₅₀ value of *Ziziphus nummularia* crude extract and fractions cytotoxicity

Sample	LC ₅₀ µg/mL
Vincristine	33.01 ± 0.02
Crude extract	74.21 ± 0.05
Ethyl acetate	158.42 ± 0.07
Chloroform	220.9 ± 0.09
N-Hexane	301.8 ± 1.04

**Figure 5.** Cytotoxicity activity of crude extract, and various fraction of *Ziziphus nummularia* stem bark

Preliminary phytochemical screening

The preliminary qualitative phytochemical screening of *Ziziphus nummularia* stem bark crude extract and fraction revealed the presence of flavonoids, phenols, alkaloids, tannins, saponins, terpenoids (Table 4). A greater number of phytochemicals are present in all these extracts and fractions. Phenols were highly positive in ethyl acetate and crude extract. While saponins are very positive in crude extract and n-hexane. Tannins and alkaloids are present in extract in same proportion. The concentration of alkaloids, flavonoids, phenols, tannins, of crude extract and all fractions are shown (Table 5). The quantity of phenols, flavonoids, tannins and alkaloids in crude extract and fractions were all significantly higher, suggesting that these bioactive compounds had been largely enriched in stem bark of *Ziziphus nummularia*. The crude methanolic extract of *Ziziphus nummularia* was found to have higher phenolic content of 39.54 ± 0.7 mg/g of gallic acid equivalents while have higher flavonoid content estimated was 112.44 ± 1.3 mg/g of quercetin equivalents. The n-hexane extract has found less quantity of calculated phytochemicals (Table 5). These established techniques, involving spectrophotometric analysis at specific wavelengths, allow for the precise measurement of absorbance, correlating directly with the concentration of phenolic, tannins, alkaloids flavonoids. Such quantification not only sheds light on the antioxidant potential of *Ziziphus nummularia* but also offers implications for its traditional medicinal use and future therapeutic applications. Further exploration into the specific constituents and their biological activities, alongside investigations into bioavailability and in vivo metabolism, promises to deepen our understanding and unlock the full therapeutic potential of *Ziziphus nummularia*.

Table 4. Qualitative phytochemical estimations of *Ziziphus nummularia* bark extracts

Phytochemicals	Test performed	Crude extract	Ethyl acetate	Chloroform	N-hexane
Alkaloids	Wagner test	+	+	+	+
Tannins	Folin-Ciocalteu test	+	+	+	+
Flavonoids	Ammonium test	+	++	+	+
Phenols	Ferric chloride	++	++	+	+
Saponins	Foam test	++	+	+	+

Key (++) = high quantity while (+) = less quantity, indicate presence of the tested phytochemical.

Table 5. Quantitative phytochemical analyses of *Ziziphus nummularia* crude and fractionated extract

Phytochemicals	Crude extract	Ethyl acetate	Chloroform	N-hexane
Alkaloids (mg/g)	17.14 ± 0.3	11.91 ± 1.3	5.67 ± 1.4	1.96 ± 1.2
Flavonoids (mg/g)	112.44 ± 1.3	27.91 ± 1.7	90.37 ± 1.5	28.87 ± 1.6
Tannins (mg/g)	1.39 ± 0.1	1.28 ± 0.4	1.29 ± 0.2	1.129 ± 1.1
Phenols (mg/g)	39.54 ± 0.7	23.67 ± 1.3	17.01 ± 0.4	6.93 ± 1.3

Values are expressed in mean \pm standard error of the mean.

Discussion

Reactive oxygen species (ROS) play crucial roles as signaling molecules and have been reported to have pro-tumorigenic as well as anti-tumorigenic effects (Huang *et al.*, 2021). At low concentrations, ROS performs essential signaling roles, contributing to cellular equilibrium maintenance. Conversely, elevated ROS levels can inflict damage upon cellular components such as lipids, proteins, and genomic DNA, potentially initiating tumorigenesis (Figure 6). Furthermore, ROS has been associated with tumor invasion and metastasis. Interestingly, increased levels of reactive oxygen species (ROS) can paradoxically hinder tumor advancement by inhibiting the proliferation of cancer cells and inducing their programmed death. It's noteworthy that chemotherapy and radiotherapy predominantly exert their anticancer effects by boosting intracellular ROS

levels (Khan *et al.*, 2021). Our findings showed that the stem bark's antioxidant capacity measured by the ABTS assay ($IC_{50} = 78.969 \mu\text{g/mL}$) was higher than that measured by the DPPH assay ($IC_{50} = 136.49 \mu\text{g/mL}$) for the crude extract. The antioxidant activity of $IC_{50} 78.9 \mu\text{g/mL}$ in crude methanolic extract was determined using the total free radical scavenging ABTS test. One could suggest that most plant components exhibit stronger antioxidant action against ABTS radicals than DPPH radicals. This is because of the ABTS assay's increased sensitivity in detecting antioxidant activity, which accelerates the kinetic reaction. As a result, enhances antioxidant activity (Wei *et al.*, 2018; Akbari *et al.*, 2019).

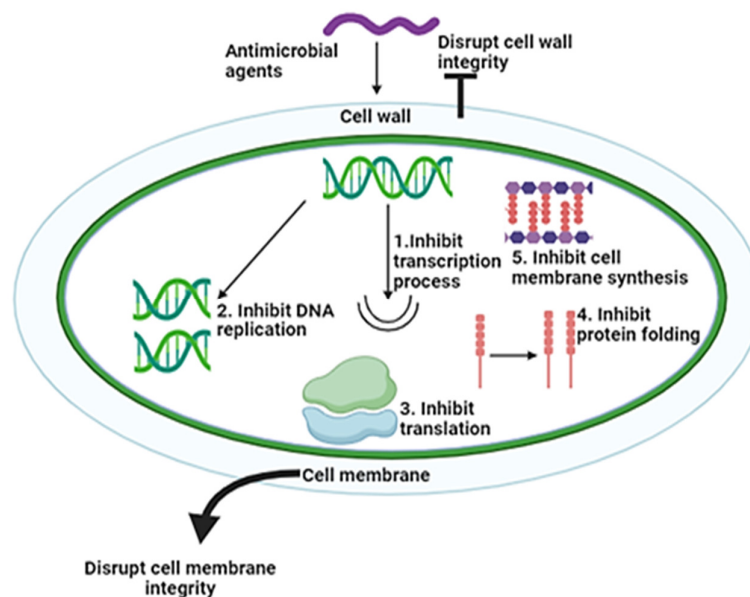


Figure 6. Mechanism of action antimicrobial agents

Nevertheless, these findings were consistent with prior research that revealed stronger antioxidant properties in extract (Beg *et al.*, 2016). The IC_{50} of CME and fractions of *Z. nummularia* were compared with standard antioxidant compound (ascorbic acid). The order of IC_{50} for antioxidant assay were $nHF > CHF > EAF > CME > \text{ascorbic acid}$. These findings are in agreement with previously published data regarding antioxidant activities of *Pseudocaryopteris foetida* leaves (Lou *et al.*, 2018; Afzal *et al.*, 2022). Similar finding was observed for *Ziziphus mauritiana* plant extract's that possesses a sizable amount of free radical scavenging activity with its IC_{50} value was 0.875 g/mL . Therefore, *Ziziphus mauritiana* Lam. leaves' crude methanolic extract may be used to treat a variety of human disorders (Cao *et al.*, 2016; Ghasham *et al.*, 2017). Similarly, previous research has reported that stem ethanol extract and hexane fraction of *Carpolobia lutea* had the highest inhibitory activity was $IC_{50} = 140 \text{ g/mL}$. Study concluded that stem has the highest total phenolic content that shows highest antioxidant power (Nwidi *et al.*, 2017; Hu *et al.*, 2022). In our study, the total phenolics and flavonoid content of *Z. nummularia* stem bark was measured using spectrophotometric method. The total flavonoid content of was 112.44 mg/g and total phenols of 39.44 mg/g for crude extract. These results support the good antioxidant capacity of stem bark extract and fraction. High content of phenolic and flavonoid compounds means higher antioxidant activity of the plants (Noreen *et al.*, 2017; Yan *et al.*, 2024).

The inhibitory zones of plant extracts in this investigation were found to be narrower than those of antibiotics. *Z. nummularia*'s crude extract and fraction, which exhibited an antibacterial impact on *E. coli* bacteria, had different effects on other examined bacteria. Numerous factors are known to influence

antibacterial activity. At a concentration of 400 mg/mL, the highest zone of inhibition against the tested strains was observed with the methanol crude extract, followed by the ethyl acetate and chloroform extracts (Table 2) n-hexane extract was less effective. The efficiency of the extracts clearly relies on the type of solvent employed, even if the exact mechanism by which these plant elements work is still not entirely understood but general mechanism is illustrated in Figure 7. Sharma *et al.* (2012) highlighted that most of the antibiotic chemicals found in plants are allegedly saturated or aromatic organic molecules that are easily soluble in organic solvents. All the strains that were tested respond effectively to the positive control cefotaxime.

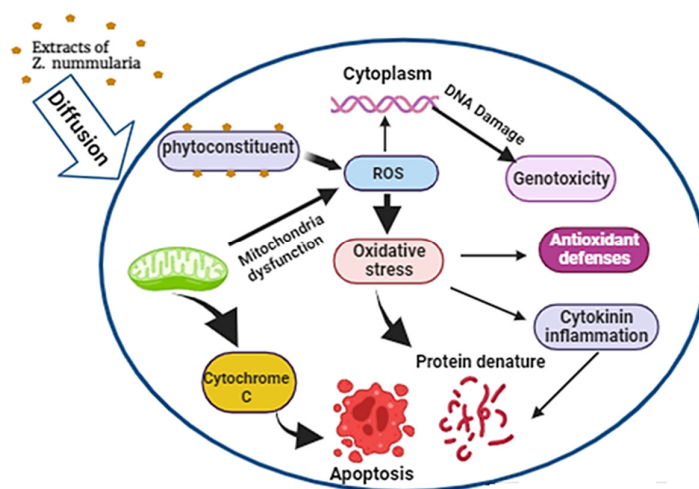


Figure 7. Oxidative stress is caused by an abundance of active oxygen species and can cause several biochemical and physiological diseases, including metabolic dysfunction and cell death. Illustration also proposed uptake process and mechanism of toxicity induced by extracts *Ziziphus nummularia* in cell

Some allied substances such as ascorbic acid, thiamine, riboflavin and bioflavonoids and pectin-A present in the *Ziziphus* species exhibited significant anti-bacterial. which makes it to be effective in curing or preventing various diseases (Beg *et al.*, 2016; Song *et al.*, 2023). Recent study demonstrated that cardiovascular glycosides, coumarins, flavonoids, tannins, phenols, phytosterols, saponins, and terpenoids are among the bioactive components of the crude extract of *Z. mauritiana* that can be detected as responsible for these biological activity (Ghasham *et al.*, 2017; Huang *et al.*, 2023). Another study reported that *S. aureus* is susceptible to five plant extracts. The outer membrane and cell wall composition of *S. aureus* may contribute to its high sensitivity. According to our findings, gram-positive bacteria are typically extra vulnerable to the extracts of spices and herbs. This was in line with earlier investigations on various herbs and spices (Thirumurugan *et al.*, 2010; Niu *et al.*, 2023). Therefore, we believe that the bacterial inhibition may differ depending on the plant extract, the extraction solvent, and the organism being examined. Khalil *et al.* (2022) reported that crude extracts and fraction prepared from *Z. nummularia* fruit possess an effective antibacterial property against *E. coli*, *S. aureus*, *Pseudomonas*, *Streptococcus pyogenes* (Khalil *et al.*, 2022). Another study is agreement with our finding that among *Z. mauritiana* leaf extracts, the methanol extract showed superior antimicrobial efficacy, with *Escherichia coli* displaying the highest resistance (Ashraf *et al.*, 2015). Our findings suggest that bacterial inhibition varies based on plant extract, solvent, and organism. Extracts with antibacterial activity consistently contained alkaloids, saponins, and flavonoids, indicating these metabolites confer antibacterial properties. However, the absence of activity in certain extracts doesn't imply a lack of bioactive

compounds; rather, it may result from lower quantities by other substances (Kurek *et al.*, 2022; Huang and Zhou, 2023; Mao *et al.*, 2023).

Crude methanolic extract showed maximum mortality as compared to other extracts, with LC₅₀ value of 74.21 µg/mL. Ethyl acetate extract showed maximum death rate at 500 µg/ml with LC₅₀ value of 158.42 µg/mL. Hence, concluded that crude methanolic extract was more toxic than chloroform and n-Hexane (Table 3). Our results reported that cytotoxicity is dose-dependent. Same outcomes were achieved by examined Brine shrimp lethality assay of Rhizophoraceae methanol extract (Indriaty *et al.*, 2022). Another finding concluded that *C. decandra* and *R. mucronata* were the most toxic plant extracts for the bark. (Indriaty *et al.*, 2022). With a LC₅₀ of 10.93 ppm, another investigation demonstrated that stem bark extract of *Rhinachantus nasutus* extract had a potent cytotoxic impact (Irawan *et al.*, 2023). Our result was also in agreement with previous data on the anti-plasmodium activity of *Z. nummularia* stem bark extracts (Aggarwal *et al.*, 2020).

Consequently, we investigated the phytochemical composition of ZN crude extract, fraction and were able to identify major chemicals that may be responsible for ZNE antioxidant and antibacterial effects. Qualitative phytochemical analysis showed that crude extract and Ethyl acetate fraction had high alkaloids, TFC, TPC and Tannins values. This indicated that plants are enriched in phenolic metabolites and flavonoids, in agreement with previous reports (Sonia and Singh, 2019). A similar result to our study was reported by another study on *Ziziphus spina-christi* put forward by Ads *et al.* (2017). GC-MS revealed that *Ziziphus spina-christi* crude extract contains tannins, flavonoids, and alkaloids (Ads *et al.*, 2017). This finding is relevant, especially that therapeutic and antioxidant properties of plant extracts are mostly attributed to flavonoids and phenols (Abdullah *et al.*, 2024). Phenols are reported to have antioxidant, antidiabetic, cardioprotective, neuroprotective effects. They are also anticancer agents that can inhibit cell cycle progression, induce apoptosis, inhibit metastasis.

In the current study preliminary phytochemical constituents of *Z. nummularia* along with cytotoxic, antibacterial and antioxidant potential of this plant species were determined. The preliminary qualitative phytochemical analysis revealed the presence of valuable different classes of phytochemicals in stem bark of *Z. nummularia* including Alkaloids, phenols, tannins, saponins, flavonoids. The presence of these diverse categories of bioactive compounds in *Z. nummularia* indicated the medicinal potential of plant. Consequently, future research employing various extraction solvents, additional bacteria, and various plant sections can precisely explain the antibacterial activity of the examined plants. As a result, medicines, nutraceuticals, and food supplements are incorporating medicinal plants.

Conclusions

Ziziphus nummularia is a spiny shrub that primarily grows in semi-arid and arid regions. Because of its medicinal characteristics, it is frequently employed in traditional medicine. The crude methanolic bark stem extract (CME) of this plant species and its fractions, including the n-hexane fraction (NHF), chloroform fraction (CHF), and ethyl acetate fraction (EAF), were examined for cytotoxic, antibacterial, and antioxidant activity as well as phytochemical analyses in the current study. CME and other fractions showed positive results in each of these assays. The range of IC₅₀ values for antioxidant assay was 78.9 with crude extract exhibiting the greatest antioxidant potential. The cytotoxic potential was evaluated using the Brine Shrimp Lethality Test and IC₅₀ values ranging from 0.18-1.4 mg/mL. At the concentration of 300-400 g/mL all the extract and fractions showed high antibacterial efficacy. In comparison to all extracts, crude extracts demonstrated stronger antibacterial activity. The phytochemical examination of crude methanolic extract and fractions also demonstrated the presence of alkaloid compounds, flavonoids, phenols, tannins and saponins, in various amounts. The current study gives researchers the fundamentals they need to continue researching the plant to confirm its other medical qualities as well as the bioactive compounds that are specifically responsible for its

unique action. As a result, the plant *Ziziphus nummularia* might play a part in the development of herbal medicine.

Authors' Contributions

YB and SN designed the study. SI performed the experiments. AQ and RS helped in data curation and analysis of data. SAH 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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