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Antioxidant, Anti-inflammatory and Antibacterial Potential of Lyophilized Powder of *Ziziphus rugosa* Lam. Leaf Extract (ZLE)

Running Title: Antioxidant Anti-Inflammatory and Antibacterial Potential of ZLE

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Abstract In the present investigation, an attempt was made to determine the therapeutic potential of ethnomedicinal plant *Ziziphus rugosa* Lam. leaf extract (ZLE) as an antioxidant, anti-inflammatory and antibacterial drug. Analytically, it was noticed that, the lyophilized powder has a significant quantity of total phenols (122 mg/g ZLE powder), flavonoids 157.19 mg/g ZLE powder), and flavonol (214.93 mg/g ZLE powder) contents. The antioxidant assays revealed a remarkable capacity to scavenge free radicals. For 100 µg of ZLE powder, the assay value showed a scavenging potency at levels of 73.01% for DPPH(1,1-diphenyl-2-picrylhydrazyl) and 43.80% for NO_X (Nitric Oxide), assays respectively. The ability of the ZLE for H_2O_2 scavenging was assessed using peroxidase assay. 100µg of ZLE powder inhibited albumin denaturation at a level of 27.64% and proteinases by 39.47%. The inhibition by standard drug aspirin (100µg) on albumin denaturation and proteinases was 84.70% and 51.71% respectively. ZLE lyophilized powder was tested for its ability to inhibit the growth of three common wound infecting bacteria- *Pseudomonas aeruginosa, Klebsiella pneumoniae and Staphylococcus aureus.* The inhibition zones formed were compared to the inhibition zones formed by the standard drug cefotaxime (30 mcg/disc) and the Minimum Inhibitory Concentration (MIC) values of ZLE against test organisms were determined.

Keywords ZLE, Polyphenolics, Antioxidant Activity, Anti-Inflammatory, Free Radical Scavenging, Albumin Denaturation, Proteinases, Minimum Inhibitory Concentration (MIC)

1. Introduction

Medicinal plants are the source of numerous bioactive compounds many of which have the potential to be developed into powerful drugs. Isolation and purification of these bioactive compounds by screening and exploring traditional medicinal plants provides a base for further pharmacological studies [1]. Several active principles of many medicinal plants have been identified and presented as important drugs in modern medical systems with the aid of novel phytochemical techniques.

During the metabolic process, the body generates a huge number of reactive oxygen species (ROS) such as superoxide anions, hydroxy radicals, and hydrogen

peroxide. This ROS covalently attaches to several cell organelles and contributes to disorders such as cancer, inflammation, arthritis, ageing, and cardiac issues [2]. Antioxidants act as a crucial line of defense against the toxicity brought on by free radicals by preventing the damage produced by these entities. When the system's antioxidant capacity is exceeded by the generation of reactive oxygen species (ROS), oxidative stress occurs in the cell system. The process of aging and the development of many degenerative diseases are all significantly influenced by this phenomenon. An increase in antioxidant status aids in reducing oxidative damage, which in turn serves to delay or reduce the chance of contracting numerous chronic, free-radical-induced disorders [3,4]. Being a crucial component of an immune response, inflammation promotes proper wound healing and serves as one of the main barriers against pathogenic wound invasion [5]. Bacterial colonisation causes the majority of wound infections, which can come from the skin's regular flora, bacteria from body parts, or environment. The most prevalent bacteria that cause infections are *Staphylococcus aureus* and other *Staphylococci.* Infected wound complications can range from local to systemic. Stalled wound healing, which leaves a wound that will not heal, is the most serious local complication of an infected wound. Septicemia, osteomyelitis, or cellulitis are examples of systemic complications. A bacterial infection of the superficial or subcutaneous layers of skin is called cellulitis [6].

Ziziphus species are members of the Rhamnaceae family, which is commonly used to treat illnesses in traditional medicine such as gastrointestinal issues, diabetes, fever, obesity, skin infections, wounds, ulcers, urinary issues, bronchitis, diarrhea, insomnia, and anemia [7]. *Ziziphus rugosa* Lam. is a woody climber and spiny shrub with subcordate elliptic leaves, and reddish wood. The fruit is a tiny drupe that is glabrous and turns white when ripe, while the flowers grow in panicles. Various kinds of herbal formulations are prepared using its stem, bark and root. The bark is anti-diarrheal and shows astringent properties. Menorrhagia medications often include flowers. Fruit and stem are hypotensive [8]. The plant shows antihelmintic [9], antibacterial [10], cytotoxic [11], α-glucosidase inhibitory [12], analgesic, CNS depressant [13], antifungal [14], and anti-diabetic [15] properties. Triterpenoid compounds like lupeol, betulinic aldehyde, betulin, betulinic acid, alphitolic acid, zizyberenalic acid, euscaphic acid, and -sitosterol, one coumarin- scopoletin, and four flavonoids- kaempferol, afzelin, quercitrin, and catechin were isolated from the plant [16].

It is well-accepted that the amount of total phenolics and flavonoids is responsible for the antioxidant potential of plant extracts [17]. Phenolics exhibit a wide range of biological actions, including free radical scavenging, antibacterial and reducing inflammation. Furthermore, phenolic chemicals are important in the prevention of cancer and heart disease [18]. Evaluating the antioxidant, anti-inflammatory and antibacterial potential of this ethnomedicinal plant could form the basis for in-depth scientific work on its therapeutic potential.

2. Materials and Methods

2.1. Preparation of *Z. rugosa* Leaf Extracts (ZLE)

One kilogram of fresh mature *Z. rugosa* leaf was washed in tap water, followed by rinsing in sterile water. It was then surface sterilized by grinding with an equal amount of RO water in a waring blender. For effective separation of remaining particles, the extract was next filtered through a double layered cheesecloth and centrifuged (Remi, India) at 10,000 rpm for 10 min. Freeze-drying was used to lyophilize the supernatant. Soxhlet extraction was carried out using methanol for the lyophilized powdered plant material. The obtained extracts were concentrated using a rotary vacuum evaporation technique (40 ℃) before being air-dried. For analysis, methanolic extracts were employed.

2.2. Estimation of Polyphenolic Components

The ZLE was assessed for the existence of total phenols, flavonoids and flavonols by the phytochemical analysis using typical standard methods [19-21].

2.3. Phenol Estimation

100 µl of the leaf sample (1g/10ml) was used for the test reaction. The mixture was combined with 2 ml of 20% Na_2CO_3 and 0.5 ml of the Folin-Ciocalteu reagent. A blank containing 3 ml of 80% aqueous methanol and 0.5 ml of the Folin-Ciocalteu reagent was used to measure the absorbance of the clear light blue solution at 650 nm. A standard graph of phenol was constructed with a mg/ml solution of pyrocatechol. The total phenol/g ZLE powder was calculated from the standard graph of pyrocatechol [19].

2.4. Estimation of Flavonoids

A 2% $AlCl_3$ ethanol solution was added to 1.0 ml of the sample's methanolic extract (1 g/10 ml). The absorbance at 420 nm was measured after 60 min of room temperature incubation. Flavonoids were detected by a yellow colour. The total flavonoid/g ZLE powder was estimated using standard values of Rutin (10 mg/ml) [20].

2.5. Estimation of Flavonol

An aliquot of 100 µl of (2 mg/ml in 80% aqueous methanol) sample was used for the estimation which was done following the procedure [21]. To each reaction mixture, 2 ml of $AlCl_3$ (20 mg/ml) and 6 ml of sodium acetate (50 mg/ml), were added and incubated at room

temperature for 120 min. The reaction mixture was read against the blank at 440 nm. A standard graph of flavonol was constructed with Rutin (10 mg/ml) in 80% aqueous methanol. The total flavonol/g lyophilized powder was calculated from the standard graph.

2.6. Free Radical Scavenging Assays

For the research, the ZLE freeze-dried powder was dissolved (mg/ml) in 80% aqueous methanol.

2.7. 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Assay

The reaction mixture included 1.5 ml of DPPH reagent (1.0 mM), 1.5 ml of 80% aqueous methanol, and 100 µl methanolic extract of the sample (100 µg). At 517 nm, the absorbance was measured after 30 mins of incubation in dark. According to the OD value, the scavenging potency was calculated [22].

$$DPPH\ \ scavenging\ \ activity(\%) = \frac{Abs\ \ Control - Abs\ \ Sample}{Abs\ \ Control} \times 100$$

2.8. Nitric Oxide (NO_X) Scavenging Activity

Test reactions contained aliquots of 100 µl sample (100 µg), added 1 ml of 100 mM SNP (sodium nitroprusside) and incubated at 25 ℃ for 120 min. Finally, 1 ml of Griess' reagent (sulphanilamide (1%) in H_3PO_4 (2%) and N-1- Naphthyl ethylenediamine dihydrochloride (0.1%)) as added. The produced chromophores absorbance was read immediately at 540 nm against an aqueous blank. Control reactions included all reagents and 100 µl of water instead of samples [22].

$$NO\ \ Scavenging\ \ activity(\%) = \frac{Abs\ \ Control - Abs\ \ Sample}{Abs\ \ Control} \times 100$$

2.9. Isolation of Peroxidase Enzyme

Fresh tissue samples were collected and purified with distilled water. 1 g of tissue was weighed, and extracted using 10 ml of 0.1 M PO_4 buffer (pH 6). Homogenates were filtered through double layered cheese cloth and centrifuged at 12,000 rpm for 20 min at 40 ℃. The supernatant was collected and made up to 10 ml with 0.1 M PO_4 buffer. This was used for peroxidase assay and quantifying protein.

2.10. Assay of Peroxidase:

The reaction mixture contained 2 ml of 0.1 M PO_4 buffer (pH 7), 40 µl of enzyme extract, 1 ml of 20 mM guaiacol and 50 µl of 10 mM H_2O_2. The rise in absorbance was measured at 470 nm for 5 min compared to a control containing all reagents except guaiacol.

$$Enzyme\ \ activity = \frac{Difference\ \ in\ \ absorbance}{Volume\ \ of\ \ enzyme\ \ pipetted} \times \frac{Total\ \ volume\ \ of\ \ enzyme}{Weight\ \ of\ \ tissue}$$

2.11. Estimation of Total Protein by Lowry:

Lowry method was used to quantify protein in the sample [23]. A blank reaction containing all reagents except the protein source used. The above reactions were done using different concentrations (mg/ml) of Bovine Serum Albumin (0.1 mg- 0.5 mg) to generate a standard plot and the concentration was calculated.

2.12. Determination of Specific Activity of Peroxidase

Specific activity of an enzyme can be defined as the enzyme activity per milligram of total protein. It is the number of micromoles of product formed by an enzyme at a specific time under given conditions per milligram of total protein.

The specific activity was calculated with the formula:

$$Specefic\ \ activity = \frac{Total\ \ Enzyme\ \ Activity}{Total\ \ Protein}$$

2.13. Anti-Inflammatory Analysis-Aqueous Extract Preparation

One gram of lyophilized *Z. rugosa* powder was dissolved in 10 ml of distilled water and filtered. After dilution to the required concentration, it was used in assays.

2.14. Inhibition of Albumin Denaturation

The potential of ZLE to reduce inflammation was studied by evaluating the rate of inhibition of albumin denaturation using a modified method [24]. To 0.5 ml test extract (200 µg/ml), 4.5 ml of 5% BSA (Bovine Serum Albumin) solution was added. After adjusting the pH to 6.3 using HCl (1N), it was kept for 20 min incubation at 37 ℃ and then heated at 57 ℃ for 30 min. Once the sample had cooled, 2.5 ml of PO_4 buffered saline (pH 6.3) was added, and turbidity was assessed at 660 nm. Aspirin (250 µg/ml) was taken as standard.

$$\%\ inhibition\ \ of\ \ albumin\ \ denaturation = \frac{Abs\ \ Control - Abs\ \ Sample}{Abs\ \ Control} \times 100$$

2.15. Proteinase Inhibition Activity

The reaction was carried out using 0.06 mg trypsin, 2 ml 25 mM Tris-HCl buffer (pH 7.4), and 1 ml test sample (100 µg/ml). The standard reaction included all of the above chemicals except the test sample, which was replaced with aspirin at a concentration of 250 µg/ml. After 5 min of incubation at 37 ℃, 1 ml of 0.8% (w/v) casein was added. This was followed by incubation for another 20 min. To stop the process, 2 ml of perchloric acid (70%) was added. The absorbance of the supernatant at 210 nm was measured in comparison to the control buffer [22].

$$\%\quad inhibition\ \ on\ \ Proteinases = \frac{Abs\ \ Control - Abs\ \ Sample}{Abs\ \ Control} \times 100$$

2.16. Antibacterial Studies-Selection of Bacterial Strains

Three common wound infecting bacterial strains were obtained from Microbial Type Culture Collection (MTCC), Chandigarh. Two Gram-negative and one Gram-positive bacterial strains were used for the investigation as listed below.

Bacterial Strain	MTCC Code
Pseudomonas aeruginosa (Gram-negative)	7453
Klebsiella pneumoniae (Gram-negative)	4030
Staphylococcus aureus (Gram-positive)	3160

2.17. Maintenance of Bacterial Strains

Bacteria were smeared on a nutrient agar slant and incubated for 24 hours at 37 ℃, and then stored a 4 ℃ to maintain the stock culture. They are then subcultured in a nutrient broth. The turbidity of the bacterial suspension is compared to that of the McFarland Standard to obtain a bacterial suspension of approximately 1.5×10^8 CFU/ml [25].

2.18. Antibacterial Screening- Disc Diffusion Method

Plates were prepared by pouring 20 ml of Mueller-Hinton (MH) agar medium (Hi-media chemicals, Mumbai, India) into sterilized petri dishes and allowed to solidify. The 24-hour old culture suspension of selected gram-positive and gram-negative bacteria from the nutrient broth was poured onto the dish to form a homogeneous top layer. Lyophilized ZLE powder was dissolved in distilled water to obtain a concentration of 10 mg/ml. A 6 mm diameter sterile filter paper disk (Hi-media chemicals, Mumbai, India) was loaded with 10 µl of the extract and dried. Discs were placed on the medium surface under sterile conditions. Test reactions were performed in triplicate with standard discs containing 30 µg cefotaxime/disk (Hi-media chemicals, Mumbai, India). Discs containing distilled water served as controls. Incubated the plates at 37 ℃ for 24 hours and then evaluated. The diameter of the developed inhibitory zones was measured in order to determine the antibacterial activity, and the mean values and standard deviations of these diameters were determined [26].

2.19. Minimum Inhibitory Concentration (MIC)

The two-fold serial broth microdilution technique was used to determine the MIC of ZLE. A stock solution containing 10mg/ml of the compound was prepared in water. A portion of this solution was serially diluted with nutrient broth to achieve concentrations ranging from 5mg/ml to 0.0024 mg/ml. At 37 ℃, the bacteria inoculums were cultured in 5 ml of nutritional broth. The final inoculums were approximately 1.5×10^8 CFU/ml. To each dilution 50 µl of microbial culture was added followed by mixing and incubation for 24 hours. The lowest concentration of the substance that, following a 24-hour incubation period at 37 ℃, inhibited the test microorganisms from multiplying (zero absorbance, i.e., no turbidity) was referred to as the MIC [27].

2.20. Statistical Analysis

Each experiment was carried out three times. The arithmetic means and Standard deviation of the entire set of data were calculated statistically. The findings are shown as Mean ± Standard deviation (SD). Using SPSS software's one-way analysis of variance (ANOVA) function, the significance of the difference was analysed. $P<0.05$ was considered significant.

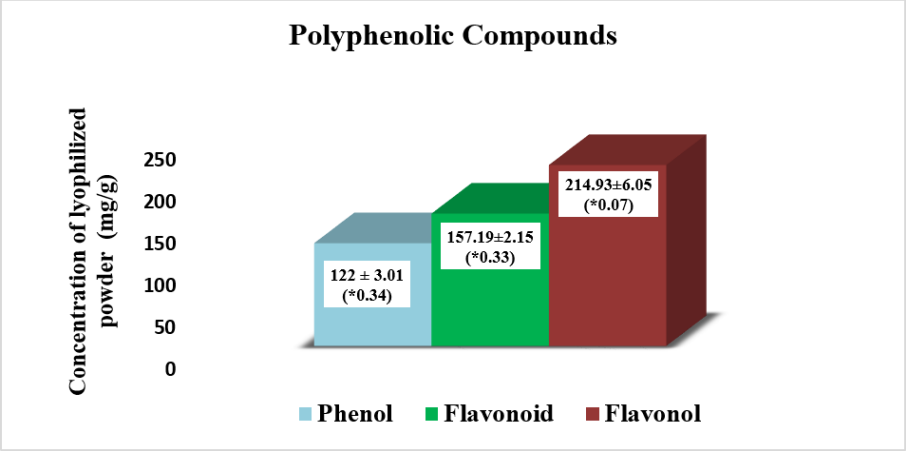
3. Results

3.1. Total Phenolic Compounds

The majority of the antioxidant activity in plants is contributed by the largest group of phytochemicals, called phenolics. The total amount of phenol, flavonoid, and flavonol components in ZLE was evaluated and was found to be 122, 157.19, and 214.93 mg/g of lyophilized powder respectively. The presence of these chemicals suggests that they could be used as an antioxidant (Figure 1).

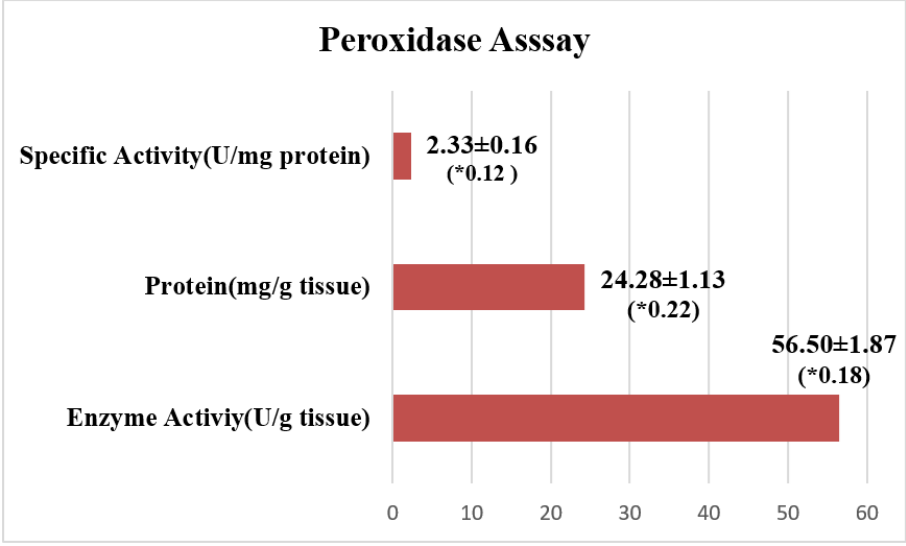
3.2. Antioxidant Activity

DPPH and NO_X free radical scavenging assays were used to measure the antioxidant potential of ZLE. For 100 g of ZLE powder, the assay value demonstrated scavenging potency of 73.01% and 43.80% for DPPH and NO_X respectively (Table 2). Thus, *Z. rugosa* leaf has a strong DPPH radical inhibition capacity with a minimal amount of sample (Table 1).



Data are presented as mean ± SD. P value is denoted by *

Figure 1. Estimation of polyphenolic components in ZLE lyophilized powder



Data are presented as mean ± SD. P value is denoted by *

Figure 2. Activity of peroxidase enzyme in *Z. rugosa* Lam. leaves

Table 1. Free radical scavenging potential of ZLE lyophilized powder

Assay	Scavenging Potential (%) (100 µg ZLE Lyophilized Powder)
1,1-diphenyl-2 picrylhydrazyl (DPPH)	73.01 ±1.09 (*0.10)
Nitric oxide (NO_X)	43.80 ±2.05 (*0.22)

Data are presented as mean ± SD. P value is denoted by *

Table 2. Anti-inflammatory potential of ZLE lyophilized powder

Assay	Inhibition (%) (Aspirin 100 µg)	Inhibition (%) (Sample 100 µg)
Albumin denaturation	84.70 ±3.42 (*0.13)	27.64 ±0.92 (*0.09)
Proteinase activity	51.71 ±3.01 (*0.31)	39.47 ±0.96 (*0.16)

Data are presented as mean ± SD. P value is denoted by *

3.3. Peroxidase Assay

Plant peroxidases perform diverse functions in plants including Reactive Oxygen Species (ROS) metabolism, and wound healing. They support the plant's ability to fight inflammation and scavenge free radicals. 56.50 U/g Peroxidase enzyme activity clearly indicates the capability of dissociating the H_2O_2 from the cell as scavenging (Figure 2).

3.4. Anti-Inflammatory Effect

Using the assays for inhibition of albumin denaturation and proteinases, ZLE's ability to reduce inflammation was evaluated. Protein denaturation is associated with the inflammatory response and contributes to a variety of inflammatory illnesses. The inhibitory effect of ZLE on denaturation of albumin was found to be 27.64%, and the standard drug aspirin showed an inhibitory potential of 84.70%. Proteinases are one of the key enzymes of protein denaturation. The inhibition of proteinases points towards the anti-inflammatory potential of the plant. The percentage of inhibition was 39.47% and 51.71% for the sample and standard drug aspirin respectively (Table 2).

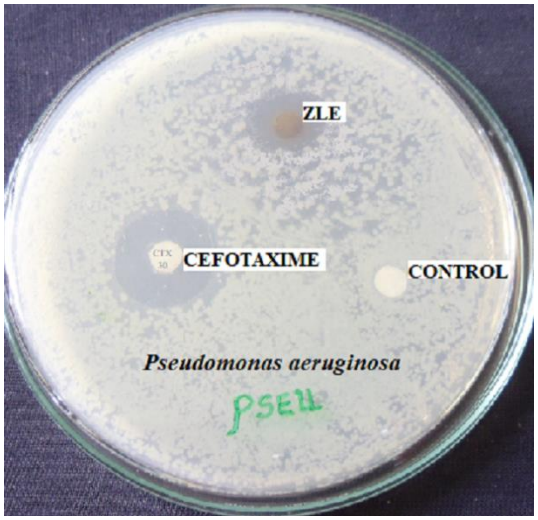
3.5. Antibacterial Studies

The potential of ZLE lyophilized powder for inhibiting the growth of three common wound infecting bacteria was evaluated. *Pseudomonas aeruginosa* showed the highest sensitivity whereas *Klebsiella pneumoniae* was most resistant towards ZLE. The inhibition zones developed were compared to the zones developed by the standard drug cefotaxime. The MIC values of ZLE were 1.6 ±0.88 mg/ml, 4.16 ±0.96 mg/ml, 2.08 ±1.24 mg/ml respectively for *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus* respectively (Table 3, Figure 3).

Table 3. Antibacterial potential of ZLE lyophilized powder

Bacterial Strain	Inhibition Zone diameter(mm)		MIC of ZLE (mg/ml)
	Cefotaxime (30 mcg/disc)	ZLE	
Pseudomonas aeruginosa	21.3 ±0.98 (*0.14)	15.3 ±1.85 (*0.07)	1.6 ±0.88 (*0.09)
Klebsiella pneumoniae	18.2 ±1.12 (*0.15)	12.2 ±0.85 (*0.17)	4.16 ±0.96 (*0.19)
Staphylococcus aureus	17.6 ±1.45 (*0.28)	13.1 ±0.75 (*0.26)	2.08 ±1.24 (*0.15)

Data are presented as mean ± SD. P value is denoted by *



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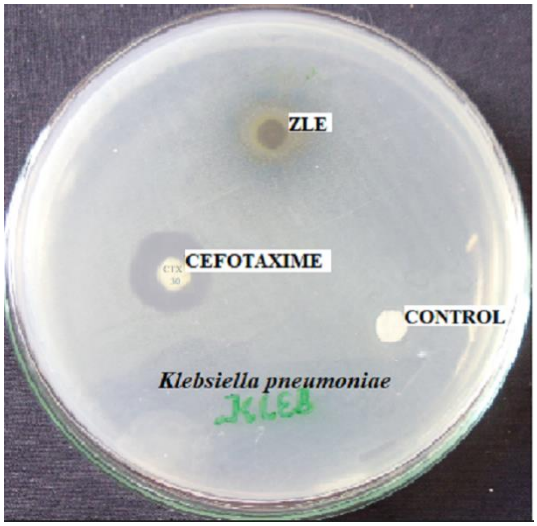

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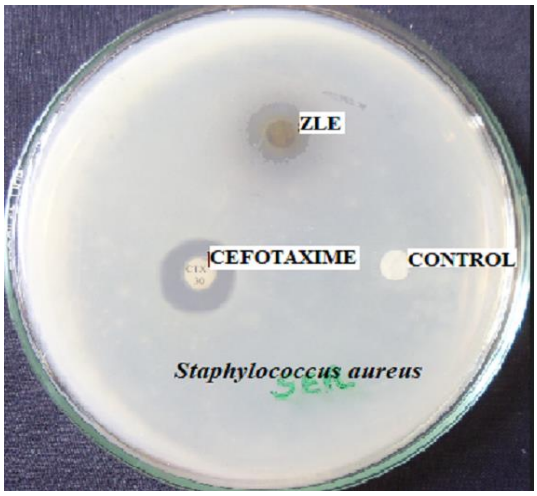

(c)

Figure 3. Antibacterial disc diffusion studies: A- Pseudomonas aeruginosa B-Klebsiella pneumoniae C- Staphylococcus aureus

4. Discussion

Phytochemical screening of *Z. rugosa* leaves revealed the presence of phenolics, flavonoids and flavanols. There hasn't been any research found in the literature to date that would support the existence of these phytoconstituents. The composition of the methanolic extract comprises a significant amount of phenolic chemicals, according to High Performance Thin Layer Chromatographic examination [28]. The relationship between the total amount of phenolic compounds, flavonoids, and flavanols in the ZLE and their antioxidant activity may be better understood through the quantitative analysis of the primary chemicals present in the extract.

The ZLE contained a notable amount of polyphenolic compounds which contribute towards the pharmacological actions of the plant. Plant derived antioxidants can inhibit the production of free radicals and reduce illnesses induced by the accumulation of highly reactive free radicals. The various classes of phenolic compounds found in medicinal plants contribute to various biological actions like wound healing, free radical scavenging, cardio protective agents, immune system stimulants and decreasing inflammation [29]. Phenolic compound inhibits pro-inflammatory mediators by inhibiting their activity or gene expression including cyclooxygenase (COX) [30]. Furthermore, several phenolic compounds have the ability to upregulate or downregulate transcriptional factors in inflammatory and antioxidant pathways [31]. Phenolic compounds have been shown in vitro to be superior antioxidants to ascorbic acid [32]. Polyphenols are highly reactive as electron or hydrogen donors and can chelate metal ions and hence are considered to be potent antioxidants [33]. Flavonoids, one of the most prevalent polyphenolic chemicals in plants, are recognized for their effective radical scavenging capabilities due to their OH group at diverse locations. Cells employ flavonoids to defend themselves from the damaging effects of reactive oxygen species. Flavonoids directly scavenge reactive oxygen species (ROS), stimulate antioxidant enzymes, decrease α-tocopheryl radicals, mitigate oxidative stress brought on by nitric oxide, and chelate metals thereby protecting cells from free radical damages [34]. The presence of these potent phytoconstituents proved conclusively that ZLE leaves have strong antioxidant activities and can be used as a source for future pharmacological activity research.

The ZLE exhibited significant DPPH and NO_X scavenging potential which reveals the antioxidant capacity of the plant. The primary cause of many degenerative diseases is free radicals and oxidative stress [35]. An accumulation of precursors to ROS and/or a decline in the effectiveness of scavenger and inhibitory mechanisms can both contribute to increased oxidative stress. The oxidative stress might then be intensified and spread by an autocatalytic cycle of metabolic stress, tissue damage, and cell death. This would lead to a rise in the production of free radicals and reduced inhibitory and scavenger mechanisms [36]. Due to their potent preventive and therapeutic actions, plant derived antioxidants are of great importance in the pharmacological industry. The high percentage of DPPH radical inhibition and Peroxidase enzyme activities indicate the antioxidant property of the plant.

The capacity of ZLE to prevent albumin denaturation supports its potential to reduce inflammation. Proteinases are enzymes that can break down other proteins as well as hydrolyze peptide bonds. They have the ability to induce inflammation by altering the activity and expression of a variety of pro-inflammatory cytokines, chemokines, and other immune components [37]. The beneficial effect of leaf extract on proteinase inhibition demonstrates its capacity to prevent protein denaturation, which is critical for infection suppression. Other intrinsic features of proteinase inhibitors, such as control of expression of cytokines, signal transmission, and tissue remodelling, often contribute to the stoppage of the inflammatory process [38].

The ZLE exhibited a significant inhibitory effect against the wound infecting bacteria. Although the mechanisms by which phenolic compounds inhibit bacteria are not entirely understood, it is known that these compounds are involved in numerous cellular sites of action [39]. These compounds alter the cell membrane permeability and the metabolic functions inside the cell may be affected due to the binding of hydrogen of phenolic compounds to enzymes. Modifications in cell wall rigidity and integrity may also occur due to interactions with cell membranes [40].

5. Conclusions

The presence of free radicals delays the process of wound healing. Reducing the inflammation is another pre-

requisite for proper and faster healing of injuries. The present study demonstrated that *Z. rugosa* possesses valuable antioxidant, anti-inflammatory and antibacterial potential and can be used as a medicine for treating wounds. Further in-depth research work is ongoing at the cellular and molecular level for evaluating the wound healing properties of *Z. rugosa*.

Conflict of Interest Statement

The authors declare no conflict of interest.

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