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Investigating the Antioxidant and Antimicrobial Efficacy of *Enicostemma axillare* (Poir. ex Lam.) A. Raynal, Leaves: A Potent Natural Remedy

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ABSTRACT

Enicostema axillare (Lam.) A. Raynal, often known as Indian whitehead, belongs to the Gentianaceae family and is well-known for its traditional medicinal usage in India and around the world. The antioxidant and antibacterial activities of methanolic *E. axillare* leaf extracts were investigated in vitro. The antioxidant activity was measured using ABTS radical and nitric oxide (NO) scavenging tests, with ascorbic acid as the standard. The ABTS experiment showed concentration-dependent antioxidant activity, with an IC_{50} value of 65.41 µg/mL, less potent than ascorbic acid (IC_{50}: 1.32 µg/mL). NO scavenging activity was substantial, with IC_{50} values ranging from 67.24 ± 1.82 µg/mL to 69.84 ± 1.75 µg/mL. However, ascorbic acid performed better (IC_{50}: 24.28 ± 1.29 µg/mL to 84.93 ± 3.37 µg/mL). Antibacterial activity was tested using the disc diffusion method, and it demonstrated superior efficacy against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) with inhibition zones of 10-16 mm, compared to Gram-positive strains (*S.* aureus and *S. pneumoniae*). In contrast, the common antibiotic ampicillin had much higher antibacterial activity. Antifungal activity, as measured by the well diffusion method, showed low efficacy for *E. axillare* (inhibition zones: 0.8-10 mm) compared to fluconazole (12-25 mm). These findings emphasize *E. axillare's* potential as a natural source of antioxidants and antibacterial agents, necessitating additional optimization and research for improved therapeutic uses.

Keywords: *Antioxidant, Anti-microbial, MIC, Enicostemma axillare and Gentianaceae.*

INTRODUCTION

Plant antioxidants are a natural reservoir of bioactive compounds that play crucial roles in both plant resilience and human health. These antioxidants enable plants to acclimate and adapt to environmental challenges, acting as a defense mechanism against oxidative damage. As sessile organisms, plants are constantly exposed to a multitude of stressors from natural and anthropogenic sources, including fluctuations in temperature, water scarcity or excess, nutrient deficiencies, soil degradation, pest infestations, and habitat disturbances. Abiotic factors such as pollution, extreme temperature variations (heat or cold), irregular water availability (droughts or flooding), changes in light intensity, and exposure to radiation disrupt the equilibrium between the generation and scavenging of reactive oxygen species (ROS). This imbalance leads to oxidative stress, a condition that adversely affects cellular structures, metabolic pathways, and overall plant growth and development. Furthermore, oxidative stress induces a cascade of biochemical and physiological responses in plants, activating their antioxidant defense systems to mitigate ROS damage and restore homeostasis.

These plant-derived antioxidants, such as flavonoids, phenolic acids, carotenoids, and tocopherols, have garnered significant attention for their potential health benefits in humans. They exhibit anti-inflammatory, anticancer, and cardioprotective properties, among other therapeutic effects. Additionally, their ability to counteract oxidative stress underscores their importance in addressing chronic diseases linked to oxidative damage. [1-4], with plant extracts and their active compounds forming the cornerstone of these treatments [5]. When the body is under stress, it generates an excess of reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals, and superoxide anion radicals. This overproduction can overwhelm the body's antioxidant defense systems, which include enzymatic antioxidants like catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD), as well as non-enzymatic antioxidants such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, carotenoids, and flavonoids. This imbalance causes harm to cells. [6-10] and health problems [11-12]. The deficiency of antioxidants, which neutralize reactive free radicals, promotes the onset of degenerative illnesses. [13], including cardiovascular diseases, cancers [14], neurodegenerative diseases, Alzheimer's disease [15] and inflammatory diseases [16]. One solution to this problem is to supplement the diet with antioxidant compounds that are contained in natural plant sources [17]. These natural plant antioxidants can so function as a form of preventive medication.

Plants have served as the foundation of traditional medicine globally for millennia and persist in offering novel treatments to humanity; hence, much effort has been directed into employing experimental techniques to isolate natural antioxidants from plants [18-19]. Plants may possess a diverse array of free radical scavenging molecules, including phenolic compounds (such as phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, and tannins), nitrogenous compounds (alkaloids, amines), vitamins, terpenoids (including carotenoids), and various other metabolites, all of which exhibit significant antioxidant activity [20-21]. There is a growing interest in naturally occurring antioxidants for application in meals as substitutes for manufactured antioxidants. The therapeutic effects of phytochemicals are attributed to their physiologically active polyphenol constituents, including phenolic acids and flavonoids, which exhibit significant antioxidant properties. Previous literature indicates that numerous antioxidant chemicals exhibit varying degrees of anti-inflammatory, cardioprotective, antitumor, anticarcinogenic, hepatoprotective, and antibacterial properties [22-26] Free radicals are neutralized by essential chemical substances known as antioxidants, which mitigate and prevent their detrimental effects on normal bodily cells. Artificially manufactured antioxidants, such as butylated hydroxytoluene and butylated hydroxylanisole, which are commercially accessible, exhibit lower stability and significant toxicity, whereas natural antioxidants are harmless and have few adverse effects. For safety concerns, antioxidants have been predominantly sourced from natural resources [27]. Fruits, vegetables, seeds, herbs, sprouts, edible mushrooms, and cereals serve as natural food sources that efficiently provide antioxidants to mitigate the adverse effects and damage caused by free radicals [28].

Pathogenic microbes have posed a threat to humanity since its inception, significantly contributing to human illness and mortality. Prior to the discovery of the first genuine antibiotic—penicillin—in 1928 and sulfa medicines in the 1930s, the only methods for combating infectious infections, aside from poisonous arsenic, were various plant extracts, which produced inconsistent outcomes.

[29-31]. For the past 60 years, antibiotics have been pivotal in treating infectious diseases caused by bacteria and fungus; yet, the prevalence of hazardous, antibiotic-resistant bacteria has been reported to rise significantly in recent decades. Drug resistance can arise from various mechanisms; therefore, addressing this issue is a complex challenge. [30]. The emergence of antibiotic resistance is attributed to the reckless, inappropriate, or excessive use of antibiotics in sectors such as medicine, veterinary practice, and particularly agriculture. [32; 33-34], leading some to claim that a post-antibiotic era is eminent [35]. According to the WHO, the primary causes of mortality in low- and middle-income countries include respiratory infections, tuberculosis, and diarrheal diseases, underscoring the critical need for effective antimicrobial strategies to combat these pervasive threats [36]. Numerous infectious diseases exist, with certain ones attributable to bacteria, including tuberculosis, typhoid, pneumonia, cholera, and gonorrhea. The rise in microbial drug resistance, along with a deficiency of newly developed antibacterial agents, is contributing to an increase in bacterial infections [37].

The quest for novel antibacterials to address infections and resistance issues has been a primary focus for the pharmaceutical sector and academic institutions.

Plant secondary metabolites are regarded as a promising source of novel antibacterial agents. [38]. According to estimates, each plant species has between 500 and 800 distinct secondary metabolites. Secondary metabolites such alkaloids, coumarins, isoflavonoids, quinones, tannins, and terpenes that have strong antibacterial properties are accumulated by plants. It is well recognized that secondary metabolites from plants can impact microbial cells in a number of ways, including by rupturing cell membranes, stopping bacterial transcription and replication, and preventing cell division [39-40]. Knowing this makes it worthwhile to look for antibacterial compounds derived from plants.

Enicostema axillare (Lam.) A. Raynal is glabrous medicinal herb belonging from Gentianaceae. This plant has garnered significant attention in traditional medicine due to its diverse therapeutic properties and bioactive compounds [41-42]. *Enicostema axillare* thrives in a variety of habitats, including wastelands, riverbanks, grasslands, and coastal regions near saltwater lakes. It is particularly well-suited to extremely acidic environments [43]. The plant's native distribution spans the tropics of Southeast Asia, Malaysia, and Africa, including the Lesser Sunda Islands, and it is commonly found throughout India. This herb plays a significant role in traditional human healthcare, with its various parts—particularly the leaves and roots—being widely used to treat numerous ailments such as malaria, skin diseases, leprosy, diabetes, and more.

The leaves of *E. axillare* are particularly valued for their hypoglycemic, antioxidant, hepatoprotective, and hepatomodulatory properties, and they have been reported to aid in reducing obesity [44]. The plant's medicinal components are considered highly effective due to their low toxicity, eco-friendliness, pleasant taste, long shelf life, and absence of adverse side effects. Additionally, *E. axillare* is a rich source of essential nutrients, including iron, potassium, sodium, calcium, magnesium, silica, chloride, sulfate, phosphate, and vitamins B and C. These attributes highlight its potential as a natural therapeutic agent for various health conditions [45].

Uses in folklore medicine

Enicostema axillare has been traditionally used in India is a remedy for appetite loss. *E. littorale* (a closely related species) is often combined with other herbs to treat diabetes. This plant is particularly effective in managing type 2 diabetes, as it helps lower blood glucose levels, increases serum insulin levels, and significantly improves kidney function, lipid profiles, blood pressure (systolic and diastolic), and pulse rate. Its anti-inflammatory properties and tumor-inhibiting activity have also been demonstrated in animal studies [46]. Notably, the secondary metabolite swertiamarin found in *E. axillare* exhibits (CNS) depressant effects in rats, highlighting its therapeutic potential for neurological disorders [47].

The plant has also shown promise in enhancing glucose-dependent insulin release, as observed by Nampalliwar and Godatwar, making it a valuable resource for diabetes management [48. Traditional healers have long utilized the hot aqueous extract of *E. axillare* for treating conditions like malaria and dyspepsia [49]. In folk medicine, the plant has been employed to address ailments such as rheumatism, stomach ulcers, diabetes mellitus, swelling, itching, and insect poisoning. Recent studies have further validated its medicinal value, revealing its antibacterial, hepatoprotective, anti-nociceptive, hypolipidemic, and antioxidant properties. Additionally, *E. axillare* has been used as an anticancer agent, a treatment for veterinary conditions, and a remedy for leukoderma [51-57].

These diverse applications underscore its importance as a multifaceted medicinal plant with significant therapeutic potential.

MATERIALS AND METHODS

Plant Material Collection

In the rainy season months of July and August 2023, healthy, fresh plant materials were collected for research purposes, from many different of locations in the Eturnagaram Wildlife Sanctuary, located in the Mulugu District of Telangana, India, at latitude 18°20′28 N and longitude 80°19′48 E. The herbarium specimen had been made and submitted to the Herbarium, Hyderabadensis Department of Botany, Osmania University, Hyderabad, Telangana, India. The submitted plant species was identified and authenticated by the Botanical Survey of India, Deccan Regional Centre, Hyderabad, Telangana, with detailed taxonomic study and given the herbarium voucher number (Voucher Number- BSI/DRC/2023-24/Identification/403).

Drying of Plant Material

Freshly picked leaves were thoroughly cleaned and then cut into small pieces using sharp tools. The dimensions of the pieces were standardized to approximately 0.5 x 1.5 x 1 x 0.2 x 3 cm. The leaves were spread on blotting paper and placed in the shade for 10 days to allow air-drying. Subsequently, the leaves were dried for one hour in an oven set at 40°C to ensure proper dehydration. These prepared samples were then used for anatomical studies and extraction processes.

ABTS Radical Scavenging Assay of *E. axillare*

The ABTS radical-scavenging assay of the methanolic leaf extract of *Enicostema axillare* was evaluated following the method described by [58-59]. The ABTS.+ cation radical was generated by mixing 5 ml of a 14 mM ABTS solution with 5 ml of a 4.9 mM potassium persulfate ($K_2S_2O_8$) solution. This mixture was stored in the dark at room temperature for 16 hours to ensure complete reaction. Prior to the assay, the resulting ABTS.+ solution was diluted with ethanol to achieve an absorbance of 0.700 ± 0.020 at 734 nm.

For the assay, 1 ml of the ABTS.+ solution was homogenized with various concentrations of the *E. axillare* methanolic leaf extract, and the absorbance was measured at 734 nm after at least 6 minutes of reaction time. Ethanol blanks were included in each assay for calibration. For comparison, a standard reaction mixture was prepared by mixing 950 µl of the ABTS.+ solution with 50 µl of butylated hydroxytoluene (BHT). The ABTS radical-scavenging ability was expressed as the IC_{50} value (µg/ml), which indicates the concentration required to inhibit 50% of the ABTS radical activity.

The percentage of ABTS radical inhibition was calculated using the following formula:

ABTS scavenging activity (%) = $[(A_0 - A_1) / A_0] \times 100$

Where:

- A_0 absorbance of the control
- A_1 absorbance of the sample

This method provides a reliable measure of the antioxidant capacity of the *E. axillare* methanolic leaf extract.

Nitric Oxide Scavenging Activity of *E. axillare* [60-61]

The nitric oxide (NO) scavenging activity of the *Enicostema axillare* methanolic leaf extract was assessed based on its ability to inhibit the formation of nitrite ions, which are generated when sodium nitroprusside (SNP) in aqueous solution reacts with oxygen at physiological pH to produce nitric oxide.

This reaction can be monitored using the Griess-Iliosvosy reaction, which forms a pink-colored chromophore when nitrite ions are present. In the presence of NO scavengers, such as the *E. axillare* extract, competition with oxygen reduces the production of nitric oxide, leading to a decrease in nitrite formation and hence, a reduced chromophore intensity.

For the assay, the SNP solution was incubated with various concentrations of the methanolic leaf extract of *E. axillare*. The resulting reaction mixtures were measured for absorbance at 540 nm against their respective blank solutions. The degree of inhibition of nitric oxide production was calculated as the percentage inhibition of the control reaction.

The NO scavenging activity was calculated using the following formula:

NO scavenging activity (%) = $[(A_0 - A_1) / A_0] \times 100$

Where:

- A_0 absorbance of the control
- A_1 absorbance of the sample

This method provides an effective way to quantify the potential of *E. axillare* as a nitric oxide scavenger, indicating its possible therapeutic role in reducing oxidative stress and related inflammatory conditions.

Antimicrobial activity of *E. axillare* [62-63]

Bacterial Strains

The bacterial strains used included both Gram-positive and Gram-negative bacteria, which were sourced from the American Type Culture Collection (ATCC). The Gram-positive strains used were *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pneumoniae* (ATCC 33400), while the Gram-negative strains were *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). These strains were utilized to evaluate the antimicrobial activity of the *Enicostema axillare* methanolic leaf extract against a range of pathogenic bacteria.

Media Preparation for Anti-Bacterial Assay

A) Nutrient Agar Media

The Nutrient Agar used in this study was procured commercially. For the preparation, 28.0 grams of the nutrient agar powder was weighed and dissolved in 1000 mL of distilled water. The solution was mixed thoroughly to ensure uniform distribution of the agar.

The dissolved nutrient agar was then sterilized by autoclaving at 121°C for 15 minutes to eliminate any microbial contaminants. After sterilization, the media was allowed to cool to approximately 45°C before being poured into petri dishes for plate preparation. These prepared plates were used to assess the antibacterial activity of the *Enicostema axillare*methanolic leaf extract against the bacterial strains.

B) Nutrient Broth

The Nutrient Broth used in this study was procured commercially. To prepare the broth, 1.3 grams of nutrient broth powder was weighed and dissolved in 100 mL of distilled water. The solution was thoroughly mixed to ensure uniform distribution of the ingredients.

The dissolved nutrient broth was then sterilized by autoclaving at 121°C for 15 minutes. Once sterilized, the broth was allowed to cool and was used for the preparation of bacterial inoculum.

C) Preparation of Stock Solution

The stock culture of each bacterial organism was prepared by aseptically transferring a small portion of each organism to two separate nutrient agar slants.

One slant was designated as the stock culture, and the other was used as the working culture. The cultures were stored at 4°C for long-term storage as stock cultures. Additionally, glycerol stocks were prepared and stored at -20°C for preservation.

D) Inoculum Preparation

The selected bacterial pathogens were inoculated into nutrient broth and incubated at 37°C for 24 hours. After incubation, the bacterial suspensions were adjusted to a concentration of approximately 10^5 CFU/mL, which was confirmed using standard microbial counting methods. These inoculum suspensions were then used to test the antimicrobial activity of the *Enicostema axillare* methanolic leaf extract.

Antibacterial Activity of *Enicostema axillare* Methanolic Leaf Extract

The antibacterial activity of *Enicostema axillare* methanolic leaf extract was assessed using the agar well-diffusion method against bacterial pathogens *Staphylococcus aureus*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, and *E. coli*. Four different concentrations of the extract (25, 50, 75, and 100 µL) were tested, and the plates were incubated at 37°C for 18-24 hours. After incubation, the inhibition zones were measured in millimeters, and the activity index was calculated to determine the effectiveness of the extract. The readings were taken in three fixed directions, and the average values were recorded for each concentration, demonstrating the antimicrobial potential of the *E. axillare* leaf extract.

Minimum Inhibitory Concentration (MIC) of *Enicostema axillare* (E. axillare)

The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial compound that prevents visible growth of microorganisms after an overnight incubation period. The MIC of *E. axillare* extracts was evaluated using the following procedure:

Compound Preparation: Compounds were accurately weighed (1 mg) and dissolved in methanol to achieve a final stock concentration of 1 mg/ml. For comparison, the standard antibiotic ampicillin was prepared in the same manner.

Culture Preparation: A loop of the bacterial culture was inoculated into 3 ml of nutrient broth and incubated at 37°C for 18-24 hours in a shaking incubator to allow growth.

Inoculum Preparation: From the overnight-grown culture, 20 µl was transferred into 1.5 ml of fresh nutrient broth. Different concentrations of the *E. axillare* compound were added to the inoculated broth. The mixture was incubated overnight at 37°C to assess antimicrobial activity.

Anti-Fungal Activity of *E. axillare*

Fungal Strains: The antifungal activity of *E. axillare* was tested against *Candida albicans* (MTCC 183) and *Aspergillus niger* (MTCC 2584), which were obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh.

Media Preparation for Antifungal Activity:

1. Sabouraud Dextrose Agar (SDA): SDA was procured commercially, and 32.5 g of the powder was dissolved in 500 ml of distilled water. The mixture was sterilized by autoclaving at 121°C for 15 minutes.

The prepared SDA was used for plate preparation to study the antifungal activity.

Antifungal Activity Testing: The antifungal activity of *E. axillare* was evaluated using the well-diffusion method. For this, SDA plates were first inoculated with *Candida albicans* and *Aspergillus niger* using the spread plate technique. Wells were made on the agar surface, and the methanolic extract of *E. axillare* leaves was added into the wells. The plates were incubated at 37°C ± 2°C for 48 hours.

After the incubation period, the plates were examined for the formation of inhibition zones around the wells. The diameter of the inhibition zone was measured in millimeters (mm) to determine the antifungal activity of the extract.

Observation and Results

Morphological description of *Enicostema axillare (Lam.) A.Raynal*

Erect herb to 40 - 50 cm, stem 4 - angular; tender parts glaucous. Leaves lanceolate- oblong, 3-4.5 x 0.5-1 cm., base cuneate, apex acute; petiole to 0.5 cm. Flowers 5. merous, in axillary, sessile, ebracteate fascicles. Calyx cupular, lobes 5, oblong, unequal, herbaceous, acute, recurved with age. Corolla white, 2 mm., across, salver form; lobes 5, equal, twisted, 7 mm., obtuse. Stamens 5, all equal and fertile, included; filaments 1.2 mm., with 2-fid scales at base; anthers 1.2 mm., apiculate, dehiscence longitudinal. Ovary globose, 1.5 x 1 mm., 1- locular; bifurcate placentae, styles 1.2 mm; stigma capitate or obscurely 2-lobed. Capsule 3.5 x 3 mm., septicidally 2 - valved; seeds circular, minute, reticulate (Fig1A-E).

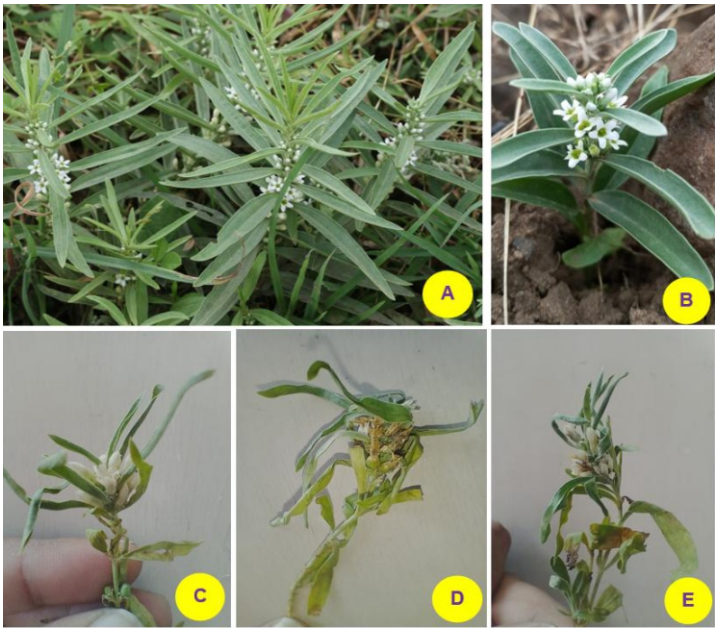

Fig1A-E: Habitat of Enicostema axillare

Antioxidant activity of *E. axillare* (ABTS method)

The antioxidant efficacy of the methanol leaf extract of *E. axillare* was assessed in vitro via the ABTS radical scavenging assay, employing ascorbic acid as the reference antioxidant. The findings, displayed in both tabular and graphical representations, demonstrate a concentration-dependent enhancement in the percentage of ABTS radical inhibition for both *E. axillare* and ascorbic acid. The inhibition percentage for *E. axillare* varied from 6.38 ± 0.73% at the minimum dose of 5 µg/ml to 69.04 ± 2.31% at the maximum concentration of 100 µg/ml.

The IC_{50} value, denoting the dose necessary for 50% inhibition, was determined to be 65.41 ± 1.487 µg/ml, signifying considerable antioxidant capacity. Ascorbic acid exhibited markedly superior radical scavenging action, with percentage inhibition values spanning from 26.28% at 5 µg/ml to 86.35% at 100 µg/ml. The IC_{50} value was notably low at 1.32 µg/ml, demonstrating its exceptional potency as an antioxidant. The bar graphs for both samples further demonstrate this tendency, exhibiting more pronounced rises in % inhibition for ascorbic acid throughout the concentration range. The data indicate that although *E. axillare* demonstrates significant antioxidant activity attributed to its bioactive components, its efficacy is markedly inferior to that of ascorbic acid. This may be due to a reduced concentration of active radical-scavenging phytochemicals in the extract relative to the pure standard. Nonetheless, the modest antioxidant activity of *E. axillare* suggests its potential as a natural source of antioxidants, warranting further exploration for therapeutic uses, especially in addressing oxidative stress-related illnesses. Nonetheless, its efficacy may necessitate elevated dosages or synergistic combinations with other antioxidants to attain results comparable to those of ascorbic acid (Table 1,2 & Fig 2,3).

Table 1: ABTS Radical Scavenging Activity of Ascorbic acid

Test Compound	Concentration (µg/ml)	% Inhibition	IC_{50} (ug/ml)
Ascorbic acid	5	26.28	1.32
	10	43.16	1.74
	25	57.83	2.08
	50	64.27	2.31
	75	72.48	2.72
	100	86.35	3.27

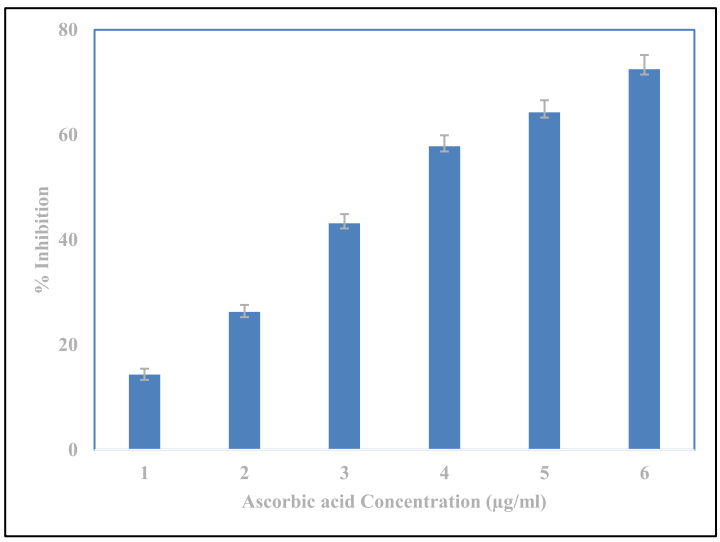

Fig 2: ABTS Radical Scavenging Activity of Ascorbic acid

Table 2: ABTS Radical Scavenging Activity of E. axillare

Test Compound	Concentration (µg/ml)	% Inhibition	IC_{50} (ug/ml)
E. axillare **methanolic leaf extract**	5	6.38±0.73	65.41±1.487
	10	13.64±1.22	
	25	26.31±1.47	
	50	43.65±1.64	
	75	57.88±2.19	
	100	69.04±2.31	

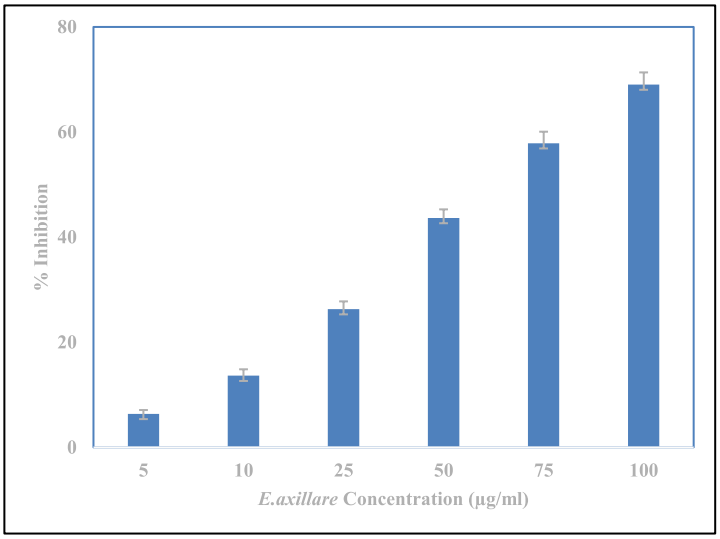

Fig 3: ABTS Radical Scavenging Activity of E. axillare

Antioxidant activity of *E. axillare* (NO method)

The examination of the nitric oxide scavenging activity of *E. axillare* methanolic leaf extract in comparison to ascorbic acid as a reference demonstrates a concentration-dependent antioxidant effect. The *E. axillare* methanolic leaf extract exhibited significant scavenging activity, with an IC_{50} value of 69.84 ± 1.75 µg/mL. At concentrations ranging from 5 µg/mL to 100 µg/mL, the percent inhibition varied from 4.81 ± 0.52% to 67.24 ± 1.82%, demonstrating an enhanced scavenging capacity with increasing concentrations. Conversely, ascorbic acid, functioning as a benchmark antioxidant, demonstrated significantly more action with an IC50 value of 31.24 ± 1.174 µg/mL. The inhibition percentages for ascorbic acid ranged from 24.28 ± 1.29% at 5 µg/mL to 84.93 ± 3.37% at 100 µg/mL. The graphical representation highlights the greater nitric oxide scavenging ability of ascorbic acid in comparison to *E. axillare* methanolic leaf extract. The results indicate that although *E. axillare* exhibits potential antioxidant action, it is less effective than the usual ascorbic acid. The in vitro nitric oxide technique underscores the therapeutic potential of *E. axillare* methanolic leaf extract, especially in the control of oxidative stress, with ascorbic acid acting as a standard for assessing antioxidant activity (Table 3,4 & Fig 4,5).

Table:3. NO Scavenging Activity of Ascorbic acid

Test Compound	Concentration (µg/ml)	% Inhibition	IC_{50} (ug/ml)
Ascorbic acid	5	24.28±1.29	31.24±1.174
	10	41.32±1.83	
	25	56.84±2.26	
	50	63.52±2.64	
	75	70.61±2.93	
	100	84.93±3.37	

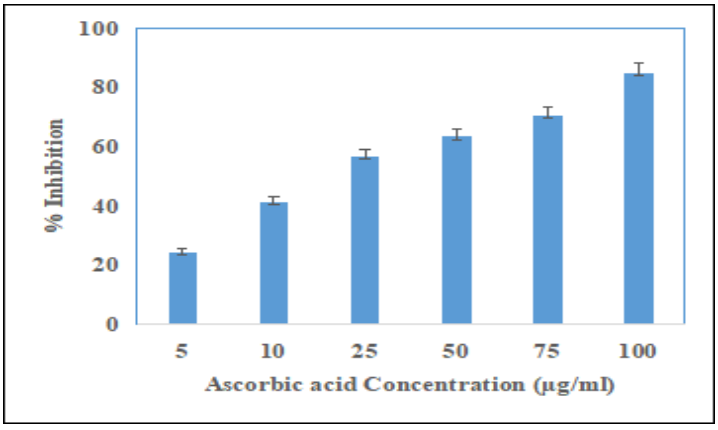

Fig: 4 NO Scavenging Activity of Ascorbic acid

Table: 4 NO Scavenging Activity of E. axillare

Test Compound	Concentration (µg/ml)	% Inhibition	IC_{50} (ug/ml)
EA	5	4.81±0.52	69.84±1.753
	10	9.36±0.87	
	25	22.59±1.21	
	50	41.18±1.49	
	75	53.66±1.53	
	100	67.24±1.82	

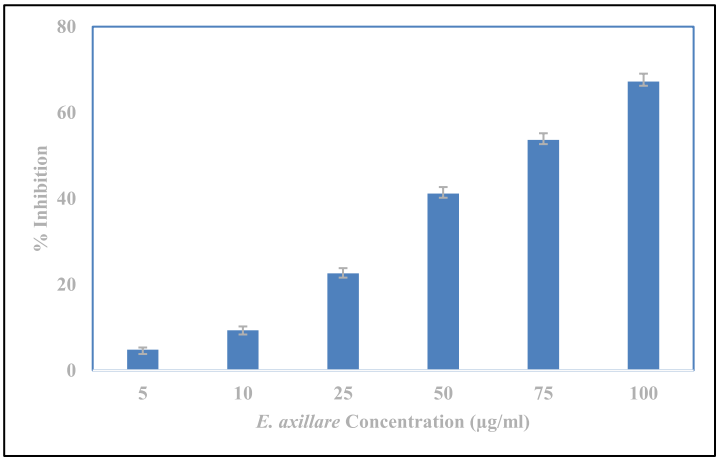

Fig: 5 NO Scavenging Activity of E. axillare

Anti-bacterial activity of *E. axillare*

E. axillare methanolic leaf extract in order to ascertain its effectiveness against both Gram-positive and Gram-negative bacteria. To assess the extract's relative potency, the well-known antibiotic ampicillin was utilized as a benchmark. An extensive evaluation of the extract's antibacterial spectrum was provided by the bacterial strains that were examined, which included two Gram-positive species (*S. aureus* and *S. pneumoniae*) and two Gram-negative species (*P. aeruginosa* and *E. coli*). Higher zones indicate more antibacterial efficiency. The methanolic leaf extract was made in concentrations of 25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL.

The zones of inhibition for *S. aureus* gradually increased from 0.4 mm at the lowest concentration (25 µg/mL) to 1.2 mm at the highest dosage (100 µg/mL), indicating a modest but concentration-dependent action against this Gram-positive strain. On the other hand, the extract's antibacterial activity against the Gram-negative bacterium *E. coli* was significantly stronger, as evidenced by the much broader zones of inhibition that ranged from 13 mm at 25 µg/mL to 16 mm at 100 µg/mL. Similar to this, *P. aeruginosa* displayed inhibition zones that ranged from 10 mm at 25 µg/mL to 15 mm at 100 µg/mL, but *S. pneumoniae* shown intermediate sensitivity, with inhibition zones growing from 6.4 mm at 25 µg/mL to 14 mm at 100 µg/mL., and There was no inhibition zone found for *Staphylococcus aureus* at any concentration.

As anticipated, the common antibiotic ampicillin showed much stronger antibacterial activity against every strain that was tested. The inhibitory zones that ampicillin generated for *S. aureus* varied from 16 mm at the lowest concentration to 25 mm at the highest. The inhibition zones ranged from 11 to 16 mm against *E. coli*, demonstrating strong action against these Gram-negative bacteria. Additionally, when ampicillin was used instead of the extract, *S. pneumoniae* and *P. aeruginosa* displayed wider zones of inhibition, the methanolic leaf extract of *E. axillare* had strong antibacterial action, especially against Gram-negative bacteria like *E. coli* and *P. aeruginosa*. However, its potency was much lower than that of ampicillin, a common antibiotic. According to the results, *E. axillare* may be a good source of natural antibacterial agents, especially for Gram-negative bacterial infections. However, in order to increase its effectiveness, more active ingredient optimization and purification could be needed (Table 3& Fig 6,7).

Table:5. Anti-bacterial activity of E. axillare against pathogenic bacteria

Sr. No	Bacterial Strain	Ampicillin				*E. axillare* methanolic leaf extract			
		Concentration (µg) / Zone of Inhibition (mm)							
		25	50	75	100	25	50	75	100
1.	*Staphylococcus aureus*	16	20	22	25	0	0	0	0
2.	*E. coli*	11	13	14	16	13	14	14	16
3.	*Streptococcus pneumonia*					6.4	11	12	14
4.	*Pseudomonas aeruginosa*					10	11	13	15

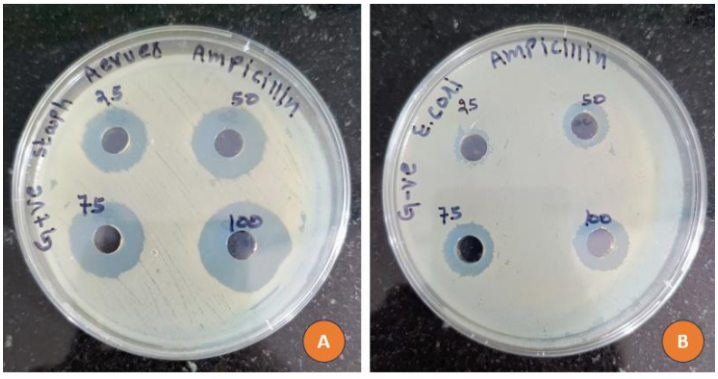

Fig:6. Antibacterial Activity of Standard Ampicillin (A) S.aureus (B) E. coli

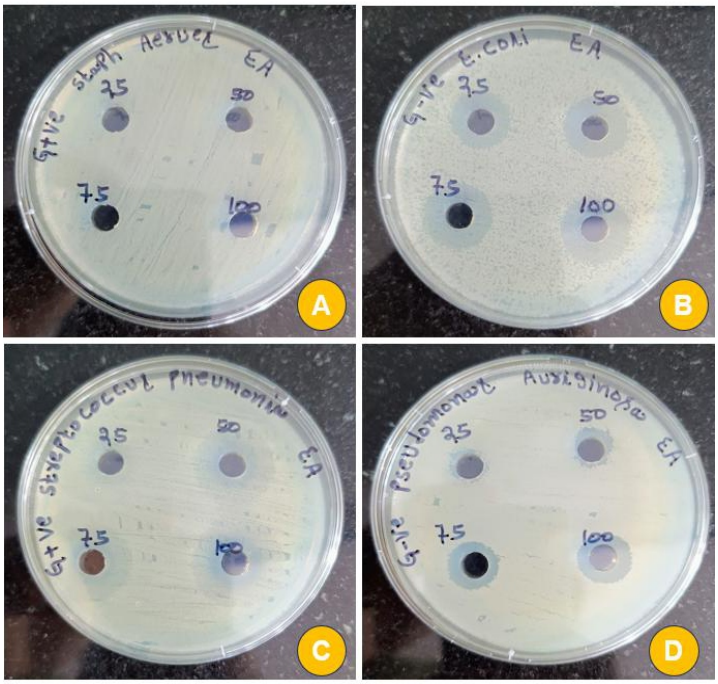

Fig:7. Antibacterial activity of E. axillare leaf methanolic extract (A) S. aureus (B) E. coli (C) S. pneumonia (D) P. eruginosa

Minimum Inhibitory Concentration (MIC) of *E. axillare*

The Minimum Inhibitory Concentration (MIC) analysis of the methanolic leaf extract of *E. axillare* offers essential information into its antibacterial efficacy. The Minimum Inhibitory Concentration (MIC), a standard metric for determining the lowest concentration of an antimicrobial agent necessary to prevent observable growth of a microbe following overnight incubation, was employed to assess the efficacy of the extract. The research encompassed rigorous preparation and testing methods to guarantee precision and dependability. The methanolic extract was made by dissolving 1 mg of the leaf extract in methanol, resulting in a stock concentration of 1 mg/ml. A comparable preparation technique was employed for the conventional antibiotic, ampicillin, which acted as a reference for comparison. Bacterial cultures were established by inoculating a loopful of microorganisms into 3 ml of nutrient broth and incubated at 37°C overnight in a shaking incubator.

Subsequently, 20 µl of the overnight-cultivated bacterial culture was inoculated into 1.5 ml of nutrient broth, to which different quantities of the extract were introduced. The mixes were subsequently incubated at 37°C for 24 hours to assess the growth-inhibitory effects of the extract at varying doses.

The findings demonstrated intriguing activity patterns for both the methanolic leaf extract and the conventional antibiotic. Ampicillin had inhibitory effects against *S. aureus* and *E. coli* at a dose of 200 µg/ml, as indicated by a substantial decrease in bacterial growth. Bacterial growth was quantified at optical density values of 0.095 for *S. aureus* and 0.113 for *E. coli* at this concentration, demonstrating significant activity. The methanolic extract of *E. axillare* exhibited a more selective antibacterial profile. The highest tested concentration of 200 µg/ml demonstrated the greatest efficacy, especially against *E. coli*, *S. pneumoniae*, and *P. aeruginosa*, as evidenced by optical density readings of 0.183, 0.239, and 0.245, respectively, indicating decreased bacterial growth. Nevertheless, the extract had minimal efficacy against *S. aureus*, with consistent growth observed at all tested concentrations, indicating the bacterium's tolerance to the extract's antibiotic constituents.

An in-depth examination of the concentration-dependent activity revealed that the methanolic extract exhibited a progressive decline in bacterial growth for certain strains with increasing concentrations, although its overall effectiveness was attained solely at the elevated dosage of 200 µg/ml. This contrasts with the more pronounced effects noted for ampicillin at reduced dosages, highlighting the extract's potential as a supplementary or alternative antimicrobial agent rather than a primary treatment choice. The variations in activity may be ascribed to the phytochemical content of the extract, which potentially harbours bioactive chemicals that specifically target microbial processes, hence demonstrating selective efficacy. The research highlights the potential of *E. axillare* as a source of natural antibacterial compounds, especially against gram-negative bacteria such as *E. coli* and *P. aeruginosa*. However, the restricted efficacy against *S. aureus* indicates that its activity spectrum is strain-specific, necessitating additional investigation to isolate and identify the active phytochemicals responsible for its antibacterial properties. Furthermore, investigating potential synergistic interactions with traditional antibiotics may improve its effectiveness and expand its use.

The methanolic leaf extract of *E. axillare* exhibited notable antimicrobial efficacy at elevated concentrations, especially against *E. coli*, *S. pneumoniae*, and *P. aeruginosa*, indicating its potential for the formulation of plant-derived antimicrobial treatments. Nonetheless, its comparatively diminished potency comparison to conventional antibiotics such as ampicillin underscores the necessity for additional optimization and investigation of its active constituents. These findings provide significant data to the expanding study on natural antimicrobials and their potential implications in combating antibiotic resistance and offering sustainable therapeutic alternatives (Table 6& Fig 8,9).

Table:6. MIC determination for E. axillare methanolic leaf extract in comparison with Ampicillin.

S. No	Bacteria	Concentration (µg/ml) Ampicillin						Concentration (µg/ml) *E. axillare* methanolic leaf extract					
		5	10	25	50	100	200	5	10	25	50	100	200
1	*S.aureus*	0.337	0.300	0.257	0.253	0.192	0.095	0.422	0.416	0.391	0.373	0.384	0.395
2	*E. coli*	0.295	0.282	0.278	0.283	0.203	0.113	0.372	0.359	0.327	0.254	0.215	0.183
3.	*S.pneumoniae*							0.428	0.411	0.381	0.357	0.262	0.239
4.	*P.aeruginosa*							0.405	0.383	0.364	0.322	0.286	0.245

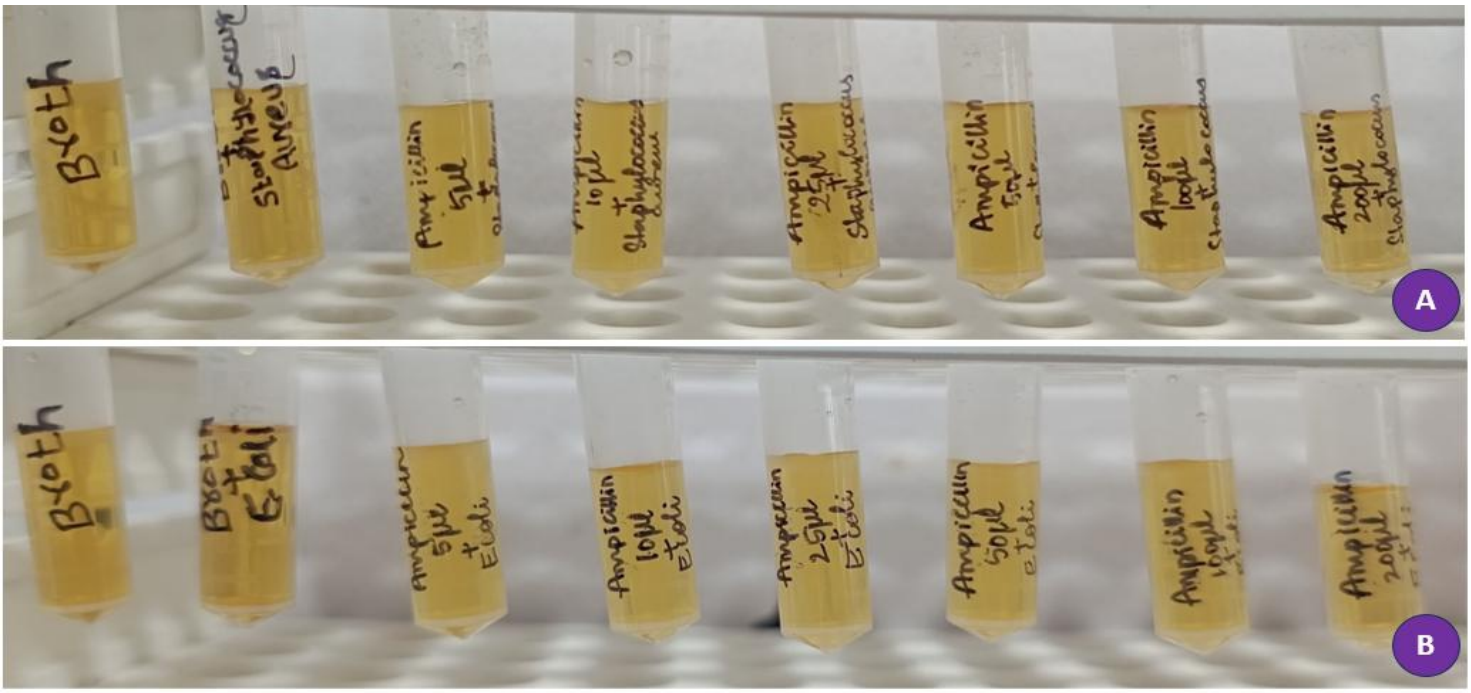

Fig: 8 AMIC activity of Ampicillin on S. aureus: B. MIC activity of Ampicillin on E.coli

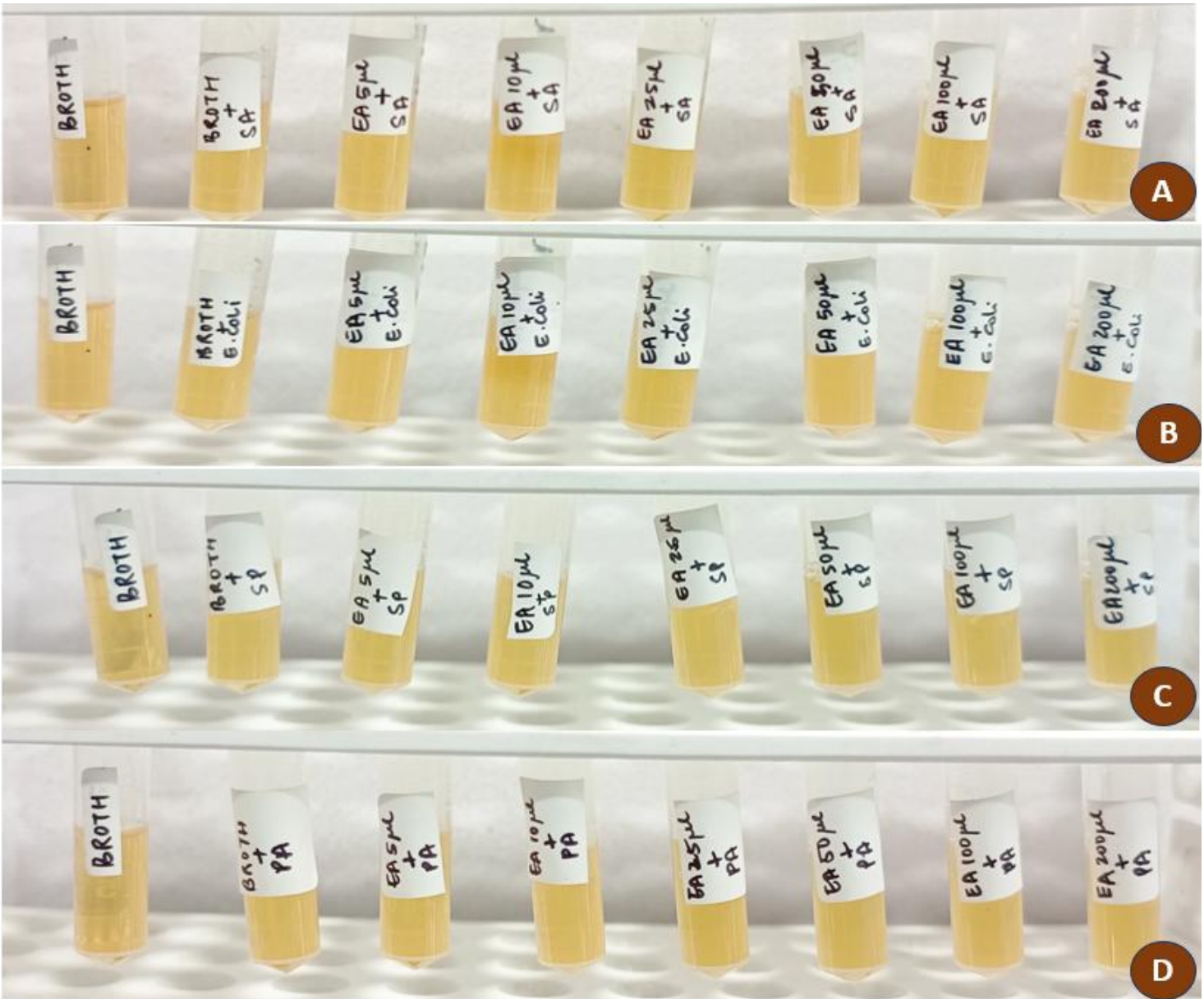

Fig:9A. MIC activity of E. axillare on S. aureus; B. MIC activity of E. axillare on E. coli C. MIC activity of E. axillare on S.pneumoniae; D. MIC activity of E. axillare on P.aeruginosa

Anti-fungal activity of *E. axillare*

The antifungal efficacy of *E. axillare* methanolic leaf extract was assessed against *Candida albicans* and *Aspergillus niger*, and compared to fluconazole, a commonly utilized standard antifungal medication. The results indicate a distinct disparity in the effectiveness of the two treatments. Fluconazole exhibited markedly superior antifungal efficacy at all evaluated concentrations (25 µg, 50 µg, 75 µg, and 100 µg), with the inhibition zone for C. *albicans* varying from 13 mm at 25 µg to 22 mm at 100 µg, and for *A. niger* from 12 mm at 25 µg to 25 mm at 100 µg. The results demonstrate a constant rise in efficacy with higher concentrations of fluconazole, highlighting its reliable and powerful antifungal characteristics. Conversely, the methanolic leaf extract of *E. axillare* shown significantly reduced antifungal activity. The zone of inhibition against *C. albicans* measured 0.8 mm at 25 µg and rose to 10 mm at 100 µg, demonstrating limited activity even at elevated dosages. For *A. niger*, the zone of inhibition varied from 1.6 mm at 25 µg to 9 mm at 100 µg, indicating that the extract is less efficacious against this strain than fluconazole. Fluconazole exhibited superior and consistent antifungal efficacy against both strains, with *A. niger* displaying somewhat greater susceptibility. In contrast, the *E. axillare* extract exhibited negligible action, particularly at lower doses, with substantial enhancements at elevated concentrations, especially against *C.albicans*. These findings underscore the necessity for more study to isolate and concentrate the active components in the *E. axillare* extract to augment its antifungal efficacy and investigate its potential as an alternative or adjunct antifungal medication (Table 7& Fig 10).

Table: 7. Anti-fungal activity of E. axillare methanolic extract against pathogenic fungal strains

S. No	Fungal Strain	Concentration (µg/ml) Fluconazole				Concentration (µg/ml) *E. axillare* methanolic extract			
		25	50	75	100	25	50	75	100
1	*Candida albicans*	13	16	18	22	0.8	3.2	8	10
2	*Aspergillus niger*	12	16	21	25	1.6	2	2.4	9

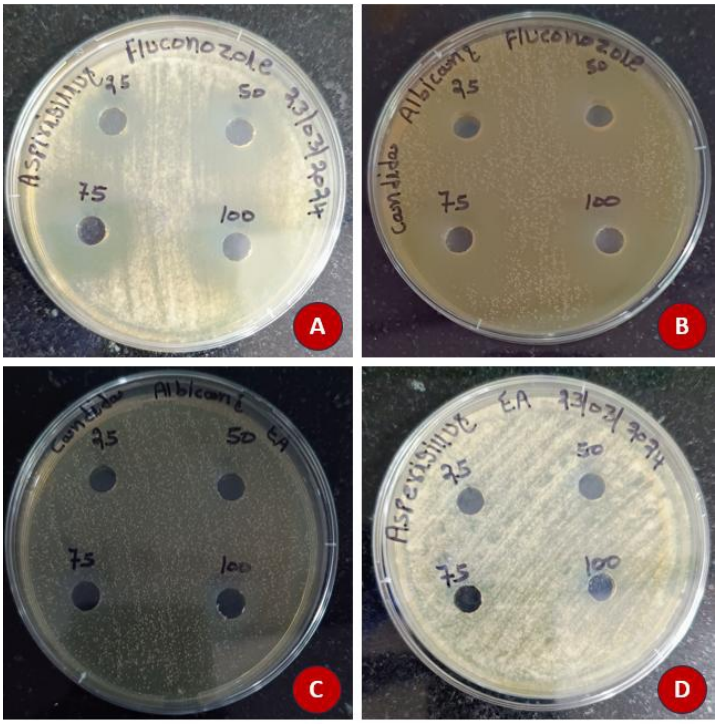

Fig:10A-D. Anti-fungal Activity of Fluconozole A.C.albicans B. A. niger Anti-fungal Activity of E. axillare C. C. albicans D. A. niger

Discussions

Present studies traditional use of the entire *E. axillare* plant involves applying a mixture of the leaf paste and ghee to wounds to stop pus from forming. It can also be used to heal cattle bone fractures, and the leaves have antidiabetic properties. The chloroform extract of *Enicostema axillare* exhibited significant antioxidant activity, as demonstrated by its ability to scavenge various free radicals and reduce lipid peroxidation. The IC_{50} values for the inhibition of nitric oxide, hydrogen peroxide, hydroxyl radicals, and lipid peroxidation were found to be 60.66 µg/ml, 16.99 µg/ml, 25.06 µg/ml, and 94.66 µg/ml, respectively. [41-43, 49;66-67, 68]. The antioxidant potential of *E. axillare* methanolic leaf extract was evaluated using ABTS radical scavenging and Nitric Oxide (NO) scavenging activity assays, with ascorbic acid serving as the standard. In the ABTS assay, ascorbic acid demonstrated an IC_{50} value of 1.32 µg/mL at a concentration of 5 µg/mL and 3.27 µg/mL at 100 µg/mL.

The *E. axillare* methanolic leaf extract exhibited an IC_{50} value of 65.41 ± 1.487 µg/mL. Similarly, in the NO scavenging activity assay, ascorbic acid achieved an IC_{50} value of 31.24 ± 1.174 µg/mL, while *E. axillare* methanolic leaf extract displayed an IC_{50} value of 69.84 ± 1.753 µg/mL. These findings suggest moderate antioxidant activity of *E. axillare* methanolic leaf extract in comparison to the standard ascorbic acid.

According to Antony Rose *et al.* (2016) and Anita Jain *et al.* (2010), the antibacterial activity of *Enicostema axillare* leaf extracts was evaluated against five pathogenic bacterial strains. The study observed a dose-dependent antibacterial effect, where higher concentrations (10 mg/ml) exhibited more significant activity compared to lower concentrations (5 mg/ml) against all tested microorganisms. The diameter of the inhibition zones ranged from 10 mm to 16 mm, indicating the potential of *E. axillare* leaf extracts as an effective natural antimicrobial agent [68-69]. Present study confirms the methanolic leaf extract of *E. axillare* showed concentration-dependent antibacterial activity, with stronger effects against Gram-negative bacteria (*E. coli*: 13–16 mm; *P. aeruginosa*: 10–15 mm) compared to Gram-positive strains (*S. aureus*: no inhibition; *S. pneumoniae*: 6.4–14 mm). In contrast, ampicillin exhibited significantly higher activity across all tested strains (*S. aureus*: 16–25 mm; *E. coli*: 11–16 mm; *S. pneumoniae* and *P. aeruginosa*: wider zones). While *E. axillare* demonstrates potential as a natural antibacterial agent, further optimization is needed to enhance its efficacy. The E. axillare methanolic leaf extract's MIC analysis demonstrated selective antibacterial activity, with 200 µg/mL being effective against *E. coli* (0.183*), S. pneumoniae* (0.239), and *P. aeruginosa* (0.245), but having negligible effects on S. aureus. At lower concentrations, however, ampicillin showed greater action. These findings point to the necessity for more optimization and the isolation of active compounds while also suggesting the potential of *E. axillare* as a natural antibacterial agent, especially for Gram-negative bacteria. The methanolic leaf extract of *E. axillare* exhibited limited antifungal activity, with inhibition zones of 10 mm for *Candida albicans* and 9 mm for *Aspergillus niger* at 100 µg, compared to fluconazole's superior efficacy (22 mm for *C. albicans* and 25 mm for *A. niger* at the same concentration). Fluconazole demonstrated consistent and stronger antifungal effects across all concentrations.

Conclusions

The study emphasizes the potential of *Enicostema axillare* methanolic leaf extract as a source of natural antioxidants and antibacterial compounds. The antioxidant properties, evidenced by ABTS and NO scavenging experiments, suggest moderate efficacy with concentration-dependent activity. Nonetheless, its efficacy is subpar compared to ascorbic acid, indicating a requirement for increased dosages or combinatory approaches to equate with conventional antioxidants.

The antibacterial assessment demonstrated selective and concentration-dependent effectiveness, especially against Gram-negative bacteria like *E. coli* and *P. aeruginosa*. This selective action indicates the existence of bioactive chemicals that target certain bacterial strains. The extract shown minimal effects on *S. aureus*, highlighting its restricted spectrum of activity in comparison to conventional antibiotics such as ampicillin, which displayed enhanced and reliable antibacterial efficacy.

The extract had limited antifungal activity against *Candida albicans* and *Aspergillus niger*, showing notable differences in efficacy when compared to fluconazole. The restricted antifungal efficacy indicates a necessity for additional refining and increased active component concentration to augment its potential as an antifungal drug.

The results identify *E. axillare* as a viable candidate for the creation of natural antimicrobials and antioxidants. Nonetheless, its diminished efficacy relative to traditional pharmaceuticals highlights the need for additional optimization, extraction of active phytochemicals, and investigation of synergistic combinations with current treatments. This method may improve its therapeutic uses, especially in mitigating oxidative stress and treating Gram-negative bacterial infections.

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References

1. Meena, K. K., Sorty, A. M., Bitla, U. M., Choudhary, K., Gupta, P., Pareek, A., ... & Minhas, P. S. (2017). Abiotic stress responses and microbe-mediated mitigation in plants: the omics strategies. *Frontiers in plant science*, *8*, 172.

2. García-Sánchez, F., Simón-Grao, S., Martínez-Nicolás, J. J., Alfosea-Simón, M., Liu, C., Chatzissavvidis, C., ... & Cámara-Zapata, J. M. (2020). Multiple stresses occurring with boron toxicity and deficiency in plants. *Journal of hazardous Materials*, *397*, 122713.

3. Gou, L., Zhuo, C., Lu, S., & Guo, Z. (2020). A Universal Stress Protein from Medicago falcata (MfUSP1) confers multiple stress tolerance by regulating antioxidant defense and proline accumulation. *Environmental and Experimental Botany*, *178*, 104168.

4. Nadarajah, K. K. (2020). ROS homeostasis in abiotic stress tolerance in plants. *International journal of molecular sciences*, *21*(15), 5208.

5. Winston, C., & Beck, L. (1999). Phytochemicals: health protective effects. *Canadian Journal of Dietetic Practice and Research*, *60*(2), 78.

6. Aruoma, O. I. (1998). Free radicals, oxidative stress, and antioxidants in human health and disease. *Journal of the American oil chemists' society*, *75*(2), 199-212.

7. Lefer, D. J., & Granger, D. N. (2000). Oxidative stress and cardiac disease. *The American journal of medicine*, *109*(4), 315-323.

8. Smith, M. A., Rottkamp, C. A., Nunomura, A., Raina, A. K., & Perry, G. (2000). Oxidative stress in Alzheimer's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1502*(1), 139-144.

9. Bhatia, S., Shukla, R., Madhu, S. V., Gambhir, J. K., & Prabhu, K. M. (2003). Antioxidant status, lipid peroxidation and nitric oxide end products in patients of type 2 diabetes mellitus with nephropathy. *Clinical biochemistry*, *36*(7), 557-562.

10. Peuchant, E., Brun, J. L., Rigalleau, V., Dubourg, L., Thomas, M. J., Daniel, J. Y., ... & Gin, H. (2004). Oxidative and antioxidative status in pregnant women with either gestational or type 1 diabetes. *Clinical biochemistry*, *37*(4), 293-298.

11. Steer, C. J., Jackson, P. R., Hornbeak, H., McKay, C. K., Sriramarao, P., & Murtaugh, M. P. (2017). Team science and the physician–scientist in the age of grand health challenges. *Annals of the New York Academy of Sciences*, *1404*(1), 3-16.

12. Uchida, R. (2000). Essential nutrients for plant growth: nutrient functions and deficiency symptoms. *Plant nutrient management in Hawaii's soils*, *4*, 31-55.

13. Shahidi, F., Janitha, P. K., & Wanasundara, P. D. (1992). Phenolic antioxidants. *Critical reviews in food science & nutrition*, *32*(1), 67-103.

14. Gerber, M., Boutron-Ruault, M. C., Hercberg, S., Riboli, E., Scalbert, A., & Siess, M. H. (2002). Food and cancer: state of the art about the protective effect of fruits and vegetables. *Bulletin du cancer*, *89*(3), 293-312.

15. Amodio, R., De Ruvo, C., Di Matteo, V., Poggi, A., Di Santo, A., Martelli, N., ... & Esposito, E. (2003). Caffeic acid phenethyl ester blocks apoptosis induced by low potassium in cerebellar granule cells. *International journal of developmental neuroscience*, *21*(7), 379-389.

16. Sreejayan, N., & Rao, M. N. (1996). Free radical scavenging activity of curcuminoids. *Arzneimittel-forschung*, *46*(2), 169-171.

17. Knekt, P., Järvinen, R., Seppänen, R., Pukkala, E., & Aromaa, A. (1996). Intake of dairy products and the risk of breast cancer. *British Journal of Cancer*, *73*(5), 687-691.

18. Scartezzini, P., & Speroni, E. (2000). Review on some plants of Indian traditional medicine with antioxidant activity. *Journal of ethnopharmacology*, *71*(1-2), 23-43.

19. Matkowski, A., Tasarz, P., & Szypuła, E. (2008). Antioxidant activity of herb extracts from five medicinal plants from Lamiaceae, subfamily Lamioideae. *Journal of Medicinal Plants Research*, *2*(11), 321-330.

20. Vaz JA, Barros L, Martins A, Morais JS, Vasconcelos MH, and Ferreira IC. (2011). Phenolic profile of seventeen Portuguese wild mushrooms, *LWT-Food Science and Technology*, 44(1), 343-346.

21. Masoko P and Elof JN. (2007). Screening of Twenty-Four South African *Combretum and* Six *Terminalia* Species (Combretaceae) for Antioxidant Activities, *African Journal of Traditional, Complementary and Alternative Medicine*. 4(2), , 231–23.

22. Lizcano LJ, Viloria-Bernal M, Vicente F, Berrueta LA, Gallo B, Martínez-Cañamero M, and Ruiz-Sanz JI.(2012). Lipid oxidation inhibitory effects and phenolic composition of aqueous extracts from medicinal plants of *Colombian amazonia*, *International journal of molecular sciences*, 13(5), , 5454-5467.

23. Muanda FN, Bouayed J, Djilani A, Yao C, Soulimani R, and Dicko A. (2011). Chemical composition and, cellular evaluation of the antioxidant activity of *Desmodium adscendens* leaves. *Evidence-Based Complementary and Alternative Medicine*, Volume, Article ID 620862, 9 pages.

24. Macone A, Fontana M, Barba M, Botta B, Nardini M, Ghirga F, and Matarese RM. (2011). Antioxidant properties of aminoethyl cysteine ketimine decarboxylated dimer: a review. *International journal of molecular sciences*, 12(5), 3072-3084.

25. Halilu ME, Balogun M, Lall N, and Abubakar MS. (2013). Studies of In vitro Antioxidant and Cytotoxic Activities of Extracts and Isolated Compounds from *Parinari curatellifolia* (Chrysobalanaceae). *Journal of Natural Sciences Research*, 3(13), 149-154.

26. Mahajan RT, and Gajare SM (2012). Manifestation of erectile dysfunction with adaptogenic antioxidant aphrodisiac plants, *Int J Pharm Biomed Res*, 3(1), 52-68.

27. Knight, J. A. (1998). Free radicals: their history and current status in aging and disease. *Annals of Clinical & Laboratory Science*, *28*(6), 331-346.

28. Kaur, C., & Kapoor, H. C. (2001). Antioxidants in fruits and vegetables–the millennium's health. *International journal of food science & technology*, *36*(7), 703-725.

29. Dar, K. B., Bhat, A. H., AmIN, S., ANeeS, S. U. H. A. I. L., Masood, A., Zargar, M. I., & Ganie, S. A. (2016). Efficacy of aqueous and methanolic extracts of Rheum spiciformis against pathogenic bacterial and fungal strains. *Journal of clinical and diagnostic research: JCDR*, *10*(9), Bc18.

30. Saleem, M., Nazir, M., Ali, M. S., Hussain, H., Lee, Y. S., Riaz, N., & Jabbar, A. (2010). Antimicrobial natural products: an update on future antibiotic drug candidates. *Natural product reports*, *27*(2), 238-254.

31. Miert, A. V. (1994). The sulfonamide-diaminopyrimidine story. *Journal of veterinary pharmacology and therapeutics*, *17*(4), 309-316.

32. Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of natural products*, *63*(7), 1035-1042.

33. Croft, A. C., D'Antoni, A. V., & Terzulli, S. L. (2007). Update on the antibacterial resistance crisis. *Medical science monitor*, *13*(6), RA103-RA118.

34. Shah, P. M. (2005). The need for new therapeutic agents: what is in the pipeline?. *Clinical Microbiology and infection*, *11*, 36-42.

35. Appelbaum, P. C. (2012). 2012 and beyond: potential for the start of a second pre-antibiotic era?. *Journal of Antimicrobial Chemotherapy*, *67*(9), 2062-2068.

36. WHO. (2020). Noncommunicable diseases progress monitor. Geneva, Switzerland: WHO; 2020.

37. Prestinaci F, Pezzotti P, Pantosti A. (2015). Antimicrobial resistance: a global multifaceted phenomenon. *Pathog Glob Health*.;109(07): 309 18.

38. Gorlenko CL, Kiselev HY, Budanova EV, Zamyatnin AA, Ikryannikova LN. (2020). Plant secondary metabolites in the battle of drugs and drug-resistant bacteria: new heroes or worse clones of antibiotics. *Antibiotics*. Apr 10;9(4):170.

39. Gupta PD, Birdi TJ. (2017). Development of botanicals to combat antibiotic resistance. *J Ayurveda Integr Med*. Oct–Dec;8(4):266–75.

40. Radulovi NS, Blagojevi PD, Stojanovi-Radi ZZ, Stojanovi NM. (2013). Antimicrobial plant metabolites: structural diversity and mechanism of action. *Curr Med Chem*.; 20:932–52.

41. Abirami, P. Gomathinayagam, M Panneerselvam R. (2012), Preliminary study on the antimicrobial activity of Enicostemma littorale using different solventsAsian Pac. J. Trop. Med., 5 (7) pp. 552-555.

42. Ahamad, J. Ali Alkefai, N.H. Amin, S. Mir S.R. (2020). Standardized extract from Enicostemma littorale ameliorates post-prandial hyperglycaemia in normal and diabetic ratsJ. Biol. Act. Prod. Nat., 10 (1) pp. 34-43.

43. Gite, V. N., Pokharkar, R. D., Chopade, V. V., & Takate, S. B. (2010). Hepato-protective activity of Enicostemma axillare in paracetamol induced hepato-toxicity in albino rats.

44. Murali, B., Upadhyaya, U. M., & Goyal, R. K. (2002). Effect of chronic treatment with Enicostemma littorale in non-insulin-dependent diabetic (NIDDM) rats. *Journal of ethnopharmacology*, *81*(2), 199-204.

45. Sathishkumar, R., Lakshmi, P. T. V., & Annamalai, A. (2009). Effect of drying treatment on the content of antioxidants in *Enicostemma littorale* Blume. *Research Journal of Medicinal Plant,* Vol. 3, No. 3, 93-101 ref. 28.

46. Sankaranarayanan, S., Bama, P., Ramachandran, J., Kalaichelvan, P. T., Deccaraman, M., Vijayalakshimi, M., ... & Sathya Bama, S. (2010). Ethnobotanical study of medicinal plants used by traditional users in Villupuram district of Tamil Nadu, India. *J Med Plants Res*, *4*(12), 1089-1101.

47. Garad, M. C., Upadhya, M. A., Kokare, D. M., & Itankar, P. R. (2012). Aerial parts of *Enicostemma littorale* Blume serve as antipyretic and antacid: in vivo and in vitro evaluations. *screening*, *13*, 16.

48. Upadhyay, U. M., & Goyal, R. K. (2004). Efficacy of *Enicostemma littorale* in type 2 diabetic patients. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *18*(3), 233-235.

49. Leelaprakash, G., & Dass, S. M. (2011). Invitro anti-inflammatory activity of methanol extract of *Enicostemma axillare*. *International Journal of Drug Development and Research*, *3*(3), 189-196.

50. Laxman, S., Bala, P., Yusuf, K., & Nancy, P. (2010). Pharmacognostical standardization of *Enicostemma littorale* Blume. *Pharmacognosy Journal*, *2*(16), 15-23.

51. Kavimani, S., & Manisenthlkumar, K. T. (2000). Effect of methanolic extract of *Enicostemma littorale* on Dalton's ascitic lymphoma. *Journal of ethnopharmacology*, *71*(1-2), 349-352.

52. Nampalliwar, A. R., & Pawankumar Godatwar, P. G. (2012). Antidiabetic activity of Enicostemma littorale (Mamajjaka) leaf extracts in streptozotocin-induced diabetic rats.

53. Thirumalai T, Therasa V, Elumalai EK, David E.(2011). Hypolipidaemic and antioxidant effect of *Enicostemma littorale* Blume. *Asian Pac J Trop Biomed*.; 1 (5): 381-385.

54. Oliveira MM, Ugarte D, Zanchet D, Zarbin AJ. (2005). Influence of synthetic parameters on the size, structure, and stability of dodecanethiol-stabilized silver nanoparticles. *J Colloid Interface Sci*. 292 (2): 429 435.

55. Jaishree V, Badami S. (2010). Antioxidant and hepatoprotective effect of swertiamarin from *Enicostema axillare* against D-galactosamine induced acute liver damage in rats. *J Ethnopharmacol;* 130 (1): 103-106.

56. Jaishree V, Badami S, Kumar MR, Tamizhmani T. (2009). Antinociceptive activity of swertiamarin isolated from *Enicostema axillare*. *Phytomed*; 16 (2): 227-232.

57. Deore SL, Khadabadi SS, Bhagure L, Ghorpade DS.(2008). In vitro antimicrobial and antioxidant studies on *Enicostema axillare* (Lam.) Raynal. leaves. *Nat Prod Rad*; 7 (5): 409-412.

58. Egu, S. A., Ali, I., Khan, K. M., Chigurupati, S., Qureshi, U., Salar, U., ... & Taha, M. (2024). Rhodanine-benzamides as potential hits for α-amylase enzyme inhibitors and radical (DPPH and ABTS) scavengers. *Molecular Diversity*, 1-19.

59. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine*, *26*(9-10), 1231-1237.

60. Nah, Y., Sobha, S., Saravanakumar, G., Kang, B. Y., Yoon, J. B., & Kim, W. J. (2024). Nitric oxide-scavenging hyaluronic acid nanoparticles for osteoarthritis treatment. *Biomaterials Science*, *12*(6), 1477-1489.

61. Thangaraj, P., John, P. M., Kondratyuk, T. P., Park, E. J., Rajan, M., & Kotchoni, S. O. (2024). Inhibition of Nitric Oxide and Free Radical Production by Leaf and Stem Extracts of Psychotria nilgiriensis. In *Bioactive Compounds from Medicinal Plants for Cancer Therapy and Chemoprevention* (pp. 103-119). Bentham Science Publishers.

62. Meri Amerikova, Ivanka Pencheva El-Tibi, Vania Maslarska, Stanislav Bozhanov and Konstantin Tachkov. (2019). Antimicrobial activity, mechanism of action, and methods for stabilization of defensins as new therapeutic agents: *Biotechnology & Biotechnological Equipment.* 33:1, 671-682, DOI: 10.1080/13102818.2019.1611385:

63. Sumaiya Naeema Hawar, Zainab K. Taha, Atyaf Saied Hamied, Hanady S. Al-Shmgani, Ghassan M, Sulaiman and Sobhy E. Elsilk. (2023). Antifungal Activity of Bioactive Compounds Produced by the Endophytic Fungus Paecilomyces sp. (JN227071.1) against Rhizoctonia solani: *International Journal of Biomaterials* PB - Hindawi.. 1687-8787 https://doi.org/10.1155/2023/2411555:

64. Kowalska-Krochmal, B., & Dudek-Wicher, R. (2021). The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. *Pathogens*, *10*(2), 165.

65. Rajput S, Kumar D and Agrawal V. (2020). Green synthesis of silver nanoparticles using Indian Belladonna extract and their potential antioxidant, anti-inflammatory, anticancer and larvicidal activities: *Plant Cell Rep*. 39(7):921–939.

66. Nampalliwar, A. R., & Pawankumar Godatwar, P. G. (2012). Antidiabetic activity of Enicostemma littorale (Mamajjaka) leaf extracts in streptozotocin-induced diabetic rats.

67. Vaijanathappa, J., Badami, S., & Bhojraj, S. (2008). In vitro antioxidant activity of Enicostemma axillare. *Journal of health science*, *54*(5), 524-528.

68. Antony Rose Immaculate C., Umarani V., Sankaranarayanan S., Bama P. and Ramachandran J. (2016). Antioxidant, antibacterial and cytotoxicity studies from flavonoid rich fraction of *Enicostemma axillare* (LAM.) raynal leaves. *African Journal of Pharmacy and Pharmacology.* Vol. 10(43), pp. 916-925, 22 November.

69. Anita Jain, Manju Choudhary and S.S. Katewa. (2010). Antimicrobial Activity of *Enicostemma axillare* (Lam.) Raynal. *J Pure Appl Microbiol.* 2010;4(1):373-377.