

Phytochemical Screening and Evaluation of Antibacterial, Anticandidal, and Sporicidal Properties of *Euphorbia tirucalli* Extract in Terengganu, Malaysia

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ABSTRACT

Euphorbia tirucalli, commonly known as the pencil cactus or milk bush plant, is used as an alternative medicine. The current study evaluated the phytochemical contents, antibacterial, anticandidal, and antisporicidal potential of the *E. tirucalli* methanolic extract. The antibacterial and anticandidal activity of *E. tirucalli* methanolic extract was determined by performing a disc diffusion assay, MIC, MBC, and MCC. The sporicidal activity was tested at different concentrations of extract and exposure times. Phytochemical analyses revealed the presence of alkaloids, saponins, cardiac glycosides, terpenoids, and tannins in *E. tirucalli* methanolic extract. Results demonstrated inhibition zones of the extract against Gram-positive bacteria were in range of 22.00-7.00 mm. Meanwhile, inhibition zones of the extract against Gram-negative bacteria were in range of 13.00-7.00 mm. All bacteria were inhibited with MIC values at 1.56-25.0 mg/ml and can be completely killed with MBC values of 20-200 mg/ml. Inhibition zones of *E. tirucalli* methanolic extract against *Candida* spp. were in the range of 20.00-8.00 mm. All *Candida* spp. were inhibited with MIC values at 1.56-100.00 mg/ml and MBC values of 100-300 mg/ml. All concentrations of the extract inhibited all *Bacillus* spp. spores at different exposure times. In conclusion, the methanolic extract of *E. tirucalli* exhibits antibacterial, anticandidal, and sporicidal activities. The findings indicated that the methanolic extract of *E. tirucalli* has good potential for prospective nature-based antimicrobial agents.

1. Introduction

Euphorbia tirucalli belongs to the Euphorbiaceae family and encompasses over 8,000 species. According to the online agroforestry database, its common names in different languages include English (finger euphorbia, milk bush, pencil tree); Malay (tulang-tulang, tentulang); Hindi (sehund, thuhar, konpalsehnd); Thai (khia thian, khia cheen) (Julius and Patrick 2011; Azanaw and Ketema 2022). Plants belonging to the genus *Euphorbia* have been used in folk medicine to treat various conditions throughout ancient history (Binckley and Zahra 2023). Species such as *E. tirucalli* (pencil cactus), *Euphorbia milii* (crown of thorns), and *Euphorbia pulcherrima* (Poinsettia) are commonly used as

decorative houseplants and in landscaping (Forrester *et al.* 2020). *E. tirucalli* is also used in essential traditional medicines. The roots of the plants are used as antimicrobial, nephroprotective, anti-arthritic, purgative, carminative, and antileprosy (Tienda-Vázquez *et al.* 2022).

Growing worries from the medical and scientific communities concerning the rapid development of antibiotic-resistant bacteria (ARB), especially drug-resistant bacteria, have sparked this interest. Even the most advanced antibiotics are occasionally ineffective against bacteria. Massive use and misuse of antibiotics have accelerated the development of ARBs and antibiotic resistance genes in the environment, thereby increasing the risk of transmission of environmentally resistant bacteria to humans (Serwecińska 2020). Bacterial contamination can occasionally result in the development of endospores. Since spores are more

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resistant to antimicrobial treatment than vegetative cells, alternate strategies for preventing bacterial endospore contamination are urgently needed in various sectors and applications. Recently, the pharmaceutical industry has been more interested in using natural ingredients to create novel and more potent medications. The biological potential of phytochemical compounds, particularly their antiviral, antibacterial, and anti-diabetic properties against human diseases has been shown in several studies (Heya *et al.* 2022). Plants are an essential source for the evolution of pharmaceuticals. The genus *Euphorbia* is one of the most popular in traditional medicine across many parts of the world among the extensive range of plants with pharmacological potential. The scientific community has become interested in reports of its antibacterial properties. According to ethno-medical reports, *E. tirucalli* may be able to treat a number of illnesses, including rheumatism, edema, asthma, coughing, and skin issues. Its latex is used to treat various ailments, including cough, toothache, haemorrhoids, warts, coughing, and even snake bites (Sultan *et al.* 2016; Jakhar and Dahiya 2017).

E. tirucalli was chosen because research on the plant species showed it had antioxidant, hepatoprotective, larvicidal, antibacterial, and anticancer properties. As a result, the plant has become the centre of biochemical studies that have already isolated several substances. Numerous investigations have demonstrated that *E. tirucalli* is a good source of therapeutic substances, including triterpenes euphol, tirucallol, phytosterols, triterpenes, diterpenes, polyphenols, and tannins (Vuong *et al.* 2014; Munro *et al.* 2015; Yusuf *et al.* 2020). *E. tirucalli* extract contains euphol, a tetracyclic triterpene alcohol and a primary component with anti-inflammatory, antiviral, and analgesic effects (Salehi *et al.* 2019). Euphol prevents human immunodeficiency virus (HIV) reverse transcriptase. Recently, it has been proposed that euphol has anticancer properties. Studies conducted *in vitro* on stomach and breast cancer cells revealed that euphol reduces cell survival (Silva *et al.* 2019). A number of studies have found that the crude extract and fractions from *E. tirucalli* stem possessed broad-spectrum antimicrobial activity, showing more studies using Gram-positive and Gram-negative bacteria, *Candida* spp., and vegetative and spore cells of *Bacillus* spp. can be carried to learn its anti-

bacterial activities which to benefit natural-based antimicrobial agent and food industry (Le *et al.* 2021).

The present study has been designed to evaluate the *in vitro* antibacterial, anticandidal and sporicidal roles of *E. tirucalli* methanolic extract activity against human pathogens. Research related to the antimicrobial activity of *E. tirucalli* extract has been widely carried out, but the plant substances used in this present study were collected from Terengganu, an East Coast Region of Peninsular Malaysia (De Araújo *et al.* 2014; Le *et al.* 2021; Heya *et al.* 2022). Consequently, this study was conducted as in an effort to add new uses of these plants in Malaysia which have not been fully explored until today. In the recent study, phytochemical components of *E. tirucalli* stem methanolic extract were determined using standard methods. The antibacterial activity of *E. tirucalli* stem methanolic extract against Gram-positive bacteria (*Streptococcus epidermidis*, *Streptococcus pyogenes*, *Propionibacterium acne*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Bacillus cereus*, *B. pumilus*, *B. subtilis*, and *B. megaterium*) and Gram-negative (*Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella Typhi*) were determined by performing disc diffusion assay or Kirby-Bauer test, minimum inhibition concentration and minimum bacterial concentration. The anticandidal activity of *E. tirucalli* stem methanolic extract against *Candida* spp. (*Candida albicans*, *C. tropicalis*, and *C. glabrata*) by performing a disc diffusion assay or Kirby-Bauer test, minimum inhibition concentration, and minimum candidal concentration. Then, the sporicidal activity was tested at different concentrations of *E. tirucalli* stem methanolic extract and exposure times against *Bacillus* spp. spores.

2. Materials and Methods

2.1. Source of the Plant Material

Stem parts of the *E. tirucalli* were collected from Kuala Terengganu, Terengganu, Malaysia. The plant was authenticated by a skilled botanist from Universiti Sultan Zainal Abidin.

2.2. Preparation of Plant Extract

Stem of *E. tirucalli* (2.8 kg) was divided into tiny fragments. The plants were rinsed with tap water. After washing, the plants were further oven-dried for

two days at 60°C. The plants were ground using an electric blender after they had been dried. The dried powder of stem parts of the *E. tirucalli* (380 g) was macerated in 80% methanol for 48 hours at ambient temperature. The filtrate was gathered by employing filter paper (Whatman No. 1, USA) and condensed utilizing a rotary evaporator at a temperature of 60°C in a vacuum condition. Subsequently, the extract was measured and stored at 4°C until further studies were conducted (Abd Wahab and Abd Rahman 2022).

2.3. Test Organism

The bacterial species used as test organisms were *Propionibacterium acne*, *Streptococcus pyogenes* (ATCC 12344), *B. subtilis* ATCC6633, *Bacillus pumilus* ATCC14884, *B. megaterium* ATCC14581, *B. cereus* ATCC33019, *Streptococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 11632), clinical isolate methicillin-resistant *S. aureus* (MRSA), *Klebsiella pneumoniae* (ATCC 10031), *Escherichia coli* (ATCC 10536), and clinical isolate *Salmonella* Typhi. The candida species used as test organisms were *Candida glabrata* (ATCC 2001), *Candida albicans* (ATCC 10231), and *Candida tropicalis* (ATCC 13803). All the stock cultures were obtained from the Microbiology Laboratory, Faculty of Medicine, Universiti Sultan Zainal Abidin, except for *Bacillus* species, which were obtained from the Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia.

2.4. Phytochemical Screening

2.4.1. Flavonoids

Two ml of 25% of dilute ammonia was added to 10 mg/ml of extract. Followed by 500 µL of concentrated H₂SO₄ was added. Flavonoids are present when a yellow coloration appears (Abd Wahab *et al.* 2019).

2.4.2. Alkaloids

10 mg/ml of the extract was diluted for several minutes in 2 ml of 25% ammonia. To extract the alkaloidal base, 5 ml of chloroform was then added and gently shaken. It was followed by the addition of Mayer's reagent. The formation of cream with Mayer's reagent was observed, indicating the presence of alkaloids (Abd Wahab and Ja'afar 2021).

2.4.3. Saponins

One ml of distilled water was used to dissolve 10 mg/ml of extract. The mixture was then mixed

for 5-15 minutes. Saponins can be detected by the appearance of a soap-like foam layer (Abd Wahab and Abd Rahman 2023).

2.4.4. Cardiac Glycosides

50 mg/ml of the extract was diluted in 2 ml of chloroform. Following that, one ml of concentrated H₂SO₄ was added. Deoxysugar is indicated by a brown ring appearance (Abd Wahab and Abd Rahman 2023).

2.4.5. Steroids

10 mg/ml extract was mixed with two mL of chloroform and two ml of concentrated H₂SO₄ and thoroughly shaken. The layer of chloroform appeared red, demonstrating that sterols and steroids are present (Abd Wahab and Ja'afar 2021).

2.4.6. Terpenoids

10 mg/ml of the extract was mixed with two ml of chloroform. Three ml of concentrated H₂SO₄ were then slowly added to create a layer. The presence of terpenoids is indicated by the interface's reddish-brown coloration (Abd Wahab and Ja'afar 2021).

2.4.7. Test for Tannins

50 mg/ml of extract was mixed with one ml of 0.1% Ferric Chloride. Tannins are present when dark green or blue-green coloration forms (Abd Wahab and Ja'afar 2021).

2.5. Antibacterial Assay

2.5.1. Disc Diffusion Assay

The disc diffusion method is used to measure the amount of inhibition of the bacteria by *E. tirucalli* methanolic extracts.

The Mueller-Hinton agar (MHA) plates were inoculated with a bacteria species with 0.5 McFarland standards. 20 µL of each extract with concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 mg/ml is impregnated into sterile, blank discs 6mm in diameter and placed on the agar surface. A positive control of chloramphenicol (30 µg/ml) and a negative control of 10% methanol were employed. The MHA plates were placed in an aerobic environment and incubated at 37°C for 24 hours. The experiment was replicated three times. Following that, the size of the inhibition zone around the extract discs, positive control, and negative control were assessed, and the results were documented (Abd Wahab and Abd Rahman 2023).

2.5.2. Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MIC was performed in the 96 well microplate. The plant extract underwent two-fold serial dilutions to reach a final volume of 100 μ L, with final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 mg/ml. Then, 100 μ L of stock solutions of *S. aureus*, *S. pyogenes*, *S. epidermidis*, *P. acne*, MRSA, *B. pumilus*, *B. subtilis*, *B. megaterium*, *B. cereus*, *E. coli*, *S. Typhi* and *K. pneumoniae* was added to each well to reach a final volume of 200 μ L. Methanol-containing wells served as the negative control, whereas wells containing MHB and bacterial inoculum were used as the viability control. Wells with only MHB served as the sterility control. The experiments were conducted in triplicate and placed in an incubator at 37°C for 24 hours. 50 μ L of MTT assay were added into each well and then incubated for 2 hours. The yellow colour of the solution in the well indicates no visible growth, while the purple colour solution indicates the visible growth of tested bacteria (Abd Wahab and Abd Rahman 2023). After the MIC is determined within 24 hours, the sub-culturing process is promptly conducted to determine the minimum bactericidal concentration (MBC) suspension from each MIC well onto the MHA plate. The MBC was calculated by combining 50 μ L of the suspensions obtained from the wells that exhibited no growth after the incubation period in MIC assays with 150 μ L of fresh broth. These suspensions were reincubated at 37°C for 48 hours. The suspension was seeded on NA and incubated at 37°C for 24 hours in aerobic conditions (More *et al.* 2008). No growth was seen on NA, which indicated an MBC endpoint (Azizan *et al.* 2020).

2.6. Anticandidal Assay

2.6.1. Disc Diffusion Assay

The anticandidal assay of the plant extracts was evaluated by employing an agar well diffusion method. *Candida albicans*, *C. glabrata*, and *C. tropicalis*, were selected for anticandidal screening. The sterilized Sabouraud Dextrose Agar (SDA) plates were inoculated with a *Candida* spp. with 0.5 McFarland standards. 20 μ L of each extract with concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 mg/ml was impregnated into sterile, blank discs 6 mm in diameter and placed on the agar surface. The control experiment was carried out by using chloramphenicol at the concentration of 30 μ g/ml as the standard drug (Joseph *et al.* 2015). The

MHA plates were incubated at 28°C for 24 hours. The experiment was replicated three times. The efficacy of the *Candida* spp. on the methanolic extracts of *E. tirucalli* was determined by measuring the sizes of inhibitory zones (Nordin *et al.* 2013).

2.6.2. Minimal Inhibition Concentration (MIC) and Minimal Candidacidal Concentration (MCC)

The broth dilution method was used to determine the minimal inhibitory concentrations (MICs) of the methanolic extracts of *E. tirucalli*, which is a reference drug against *Candida* strains. MIC values were determined after incubation at 48 h at 35°C. Visual determination of MIC endpoints was based on the lowest concentration that produced a 100% inhibition for *E. tirucalli* methanolic extract. MIC end points for *E. tirucalli* methanolic extract were visually determined by identifying the lowest concentration that resulted in 100% inhibition. MCC was determined by transferring 10 μ L volumes from wells showing no visible growth in SDA and then incubating them at 35°C for 48 hours. The minimum inhibitory concentration of the plant extract that hinders the growth of the *Candida* spp. is defined as the MFC (Yassin *et al.* 2020).

2.7. Sporocidal Assay

The sporocidal activity of *E. tirucalli* stem methanolic extract was carried out with the four *Bacillus* spp. (*B. cereus*, *B. subtilis*, *B. pumilus*, and *B. megaterium*), where the spore suspensions were all thawed and then diluted in a 1% PBS solution in order to create spores within the range of 10^6 - 10^7 spores/ml. 100 μ L of each extract concentration are incubated in a 37°C water bath for 0, 1, 2, 3, and 4 hours. After incubation, the solution was centrifuged at 12,000 x g at 4°C for 5 minutes and rinsed two times with 0.9 ml of 1% PBS solution (pH 7). This is to ensure that the spores are bacteria-free and that the spores are not affected by the residue of vegetative cells, and the pellets are suspended in 100 ml of 1% PBS solution. Serial dilution was performed using 1% PBS solution (10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4}), spread 100 μ L suspensions on nutrient plate agar, and incubated at 37°C for 24 h until the colonies were formed on the plates.

The number of colonies formed was counted, and subsequently, the average of colony-forming units (CFU/ml) was calculated. The differences between

the control (0%) and the examined concentrations were derived by subtracting the log 10 CFU/ml. The reduction of spores in CFU was expressed as sporicidal activity (Rukayadi *et al.* 2009). The positive control is chlorhexidine (10%), and the negative control is methanol (10%).

2.8. Statistical Analysis

The experiments conducted in this research were carried out three times to ensure accuracy and reliability. The data from each group was analyzed and presented as the mean ± standard deviation (SD) calculated using Microsoft Excel 2019. A statistical significance of 95% was observed between the treatments through the implementation of Tukey's test, with a p-value less than 0.05.

3. Results

3.1. Phytochemical Analysis

The phytochemical analysis of methanolic extract of *E. tirucalli* showed the existence of alkaloids, saponins, cardiac glycosides, terpenoids, and tannins. However, the plant extracts are negative for flavonoids and steroids, as given in Table 1.

3.2. Antibacterial Activity

Varying antimicrobial activities were shown by the methanolic stem extract of *E. tirucalli* against tested bacteria species (Table 2). Based on Sanam *et al.* (2022), the classification of antibacterial strength activity is based on the diameter of the inhibition zone: weak (5 mm), moderate (5–10 mm), strong (10–20 mm), and very strong (20–30 mm). Therefore, the findings showed all of the tested bacteria showed sensitivity against 80% methanolic stem extract of *E. tirucalli* at concentrations of 25–200 mg/ml. The findings showed four out of twelve bacteria tested were strongly inhibited by the extract at the concentration of 12.5mg/ml. Meanwhile, three over twelve tested bacteria were strongly inhibited by the extract at the concentration of 6.25 mg/ml, and only *S. aureus* was strongly inhibited by the extract at the concentration of 3.12 mg/ml. Lastly, all of the tested bacteria were moderately inhibited by the extract at the concentration of 1.56 mg/ml. The methanolic stem extract of *E. tirucalli* displayed concentration-dependent antibacterial activity against Gram-positive bacteria, Gram-negative bacteria, and *Candida* strains. The antimicrobial activities of *E. tirucalli* stem methanolic extract were comparable

Table 1. Phytochemical content of methanolic extract of *E. tirucalli*

Phytochemical tests						
Flavonoids	Alkaloids	Saponins	Cardiac glycosides	Steroids	Terpenoids	Tannins
-	+	+	+	-	+	+

Indicator: - : absence, +: presence

Table 2. Zone of inhibition (mm) for disc diffusion method of methanolic extract of *E. tirucalli* against selected bacteria. The result is expressed in mean ± SD

Tested bacteria species/extract concentration (mg/ml)	Inhibition zone (mm)								Methanol 10% (negative control)	Chloramphenicol 30 µg/ml (positive control)
	200	100	50	25	12.5	6.25	3.12	1.56		
<i>S. aureus</i>	22±0.5	20±0.5	18±0.5	16±0.5	15±0.5	14±0.5	10±0.5	8±0.5	6±0.0	26±0.5
<i>S. epidermidis</i>	16±0.5	14±0.5	13±0.5	12±0.5	11±0.5	10±0.5	9±0.5	8±0.5	6±0.0	22±0.5
<i>S. pyogenes</i>	16±0.5	15±0.5	14±0.5	12±0.5	9±0.5	8±0.5	7±0.5	7±0.5	6±0.0	22±0.5
<i>P. acne</i>	14±0.5	13±0.5	12±0.5	11±0.5	10±0.5	8±0.5	7±0.5	7±0.5	6±0.0	20±0.5
MRSA	13±0.5	11±0.5	10±0.5	8±0.5	7±0.5	6±0.0	6±0.0	6±0.0	6±0.0	19±0.5
<i>B. pumilus</i>	15±0.5	13±0.5	12±0.5	10±0.5	8±0.5	6±0.0	6±0.0	6±0.0	6±0.0	28±0.5
<i>B. subtilis</i>	19±0.5	18±0.5	17±0.5	14±0.5	11±0.5	10±0.5	8±0.5	6±0.0	6±0.0	28±0.5
<i>B. megaterium</i>	15±0.5	14±0.5	12±0.5	10±0.5	7±0.5	6±0.0	6±0.0	6±0.0	6±0.0	27±0.5
<i>B. cereus</i>	12±0.5	10±0.5	8±0.5	7±0.5	6±0.0	6±0.0	6±0.0	6±0.0	6±0.0	25±0.5
<i>E. coli</i>	13±0.5	12±0.5	10±0.5	8±0.5	7±0.5	6±0.0	6±0.0	6±0.0	6±0.0	19±0.5
<i>K. pneumoniae</i>	12±1.0	10±1.0	9±1.0	7±1.0	6±0.0	6±0.0	6±0.0	6±0.0	6±0.0	17±0.5
<i>S. Typhi</i>	12±1.0	9±1.0	8±1.0	7±1.0	6±0.0	6±0.0	6±0.0	6±0.0	6±0.0	17±0.5

*Data are means of three replicates (n = 3) ± standard error

with that of chloramphenicol (30 µg), the standard antibiotic (Table 2), whilst the negative control (methanol 10%) showed no inhibitory activity.

3.3. MIC and MBC

Due to the significant antibacterial effect in disk diffusion method against the tested bacterial strains, the MIC was determined in the *E. tirucalli* stem methanolic extract. MIC values for *E. tirucalli* stem methanolic extract ranged from 1.56 to 50 mg/ml against tested bacterial species (Table 3). MBC was done to determine the lowest concentration of the methanolic extract of *E. tirucalli* stem to kill the tested bacterial species. MBC values for methanolic extract of *E. tirucalli* stem ranged from 25 to 200 mg/ml against tested bacterial strains. These results indicated the potency of *E. tirucalli* stem methanolic extract as an antibacterial agent. Bioactive compounds may be

Table 3. MIC and MBC of *E. tirucalli* methanolic extract against tested bacteria species

Tested bacteria species	MIC (mg/ml)	MBC (mg/ml)
<i>S. aureus</i>	1.56	25
<i>S. epidermidis</i>	1.56	50
<i>S. pyogenes</i>	1.56	50
<i>P. acne</i>	1.56	25
MRSA	12.5	200
<i>B. pumilus</i>	12.5	100
<i>B. subtilis</i>	3.12	50
<i>B. megaterium</i>	12.5	100
<i>B. cereus</i>	25.0	200
<i>E. coli</i>	12.5	100
<i>K. pneumoniae</i>	25.0	200
<i>S. Typhi</i>	25.0	100

*Data are means of three replicates (n = 3)

Table 4. Zone of inhibition (mm) for disc diffusion method of methanolic extract of *E. tirucalli* against selected *Candida* spp. The result is expressed in mean ± SD

Tested <i>Candida</i> spp./extract concentration (mg/ml)	Inhibition zone (mm)								Methanol 10% (negative control)	Chloramphenicol 30 µg/ml (positive control)
	200	100	50	25	12.5	6.25	3.12	1.56		
<i>C. albicans</i>	20.0±0.5	18.0±0.5	16.0±0.5	14.0±0.5	13.0±0.5	10.0±0.5	9.0±0.5	8.0±0.5	6.0±0.0	24±0.5
<i>C. tropicalis</i>	18.0±0.5	16.0±0.5	14.0±0.5	12.0±0.5	10.0±0.5	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	22±0.5
<i>C. glabrata</i>	12.0±0.5	10.0±0.5	6.0±0.5	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	18±0.5

*Data are means of three replicates (n = 3) ± standard error

responsible for the high antibacterial potency of the extracts.

3.4. Anticandidal Assay

The inhibition zone of *E. tirucalli* methanolic stem extract against three *Candida* spp. is shown in Table 4. The inhibition zones were between 8 to 20 mm compared with the positive control (chloramphenicol). The extract exhibited significantly high inhibitory activity against the tested *Candida* spp. while the negative control (10% methanol) exhibited no inhibitory activity against the *Candida* spp. Methanolic extract of *E. tirucalli* stem was effective against *C. albicans* compared to the other two *Candida* spp. Interestingly, *C. albicans* employed in this study were susceptible to all the extract concentrations used, of which the inhibitory activity is concentration dependent, followed by *C. tropicalis* and *C. glabrata*.

3.5. MIC and MCC

MIC values for methanolic extract of *E. tirucalli* stem ranged from 1.56 to 100 mg/ml against tested *Candida* spp. Meanwhile, MCC values for methanolic extract of *E. tirucalli* stem ranged from 100 to 300 mg/ml against tested *Candida* spp. (Table 5).

3.6. Sporicidal Assay

The sporicidal activity of varying concentrations of methanolic extract of *E. tirucalli* stem (1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 mg/ml) against *B. pumilus*, *B. subtilis*, *B. megaterium*, and *B. cereus* are shown in Figure 1, 2, 3 and 4 respectively. There is a slight reduction of spore percentage from 0, 1, 2, 3, and 4 hours. Chlorhexidine was used as the positive control, while DMSO as the negative control.

Table 5. MIC and MCC of *E. tirucalli* methanolic extract against tested *Candida* spp.

Tested <i>Candida</i> spp.	MIC (mg/ml)	MCC (mg/ml)
<i>C. albicans</i>	1.56	100
<i>C. tropicalis</i>	12.5	200
<i>C. glabrata</i>	100	300

*Data are means of three replicates (n = 3)

4. Discussion

During their life cycle, plants are exposed to many stresses, such as drought, pests, and diseases. By coping with these challenges, they generate secondary metabolites that support stress management but are not essential to the plant's

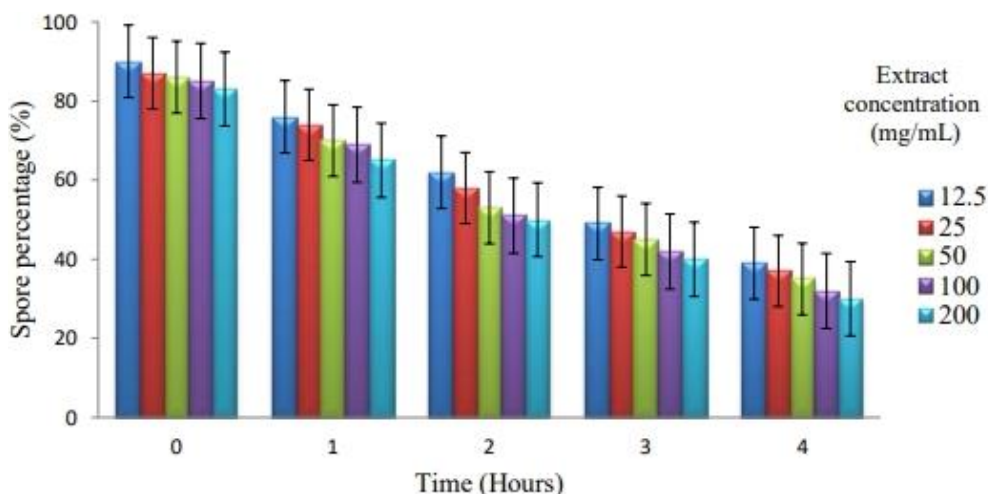


Figure 1. The sporicidal activity of methanolic extract of *E. tirucalli* stem against the spore of *B. pumilus*. The results are presented as the mean \pm standard deviation. Significant differences in means (n = 3) ($p < 0.05$)

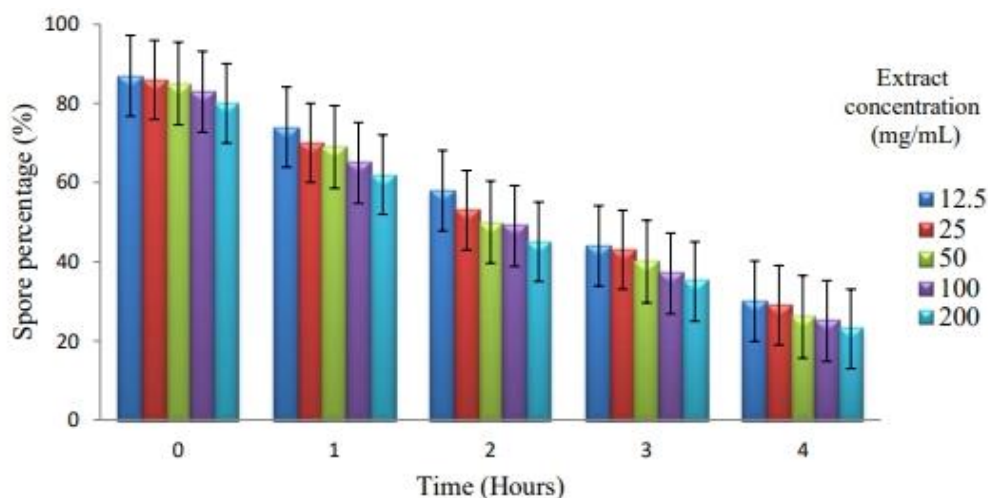


Figure 2. The sporicidal activity of methanolic extract of *E. tirucalli* stem against the spore of *B. subtilis*. The results are presented as the mean \pm standard deviation. Significant differences in means (n = 3) ($p < 0.05$)

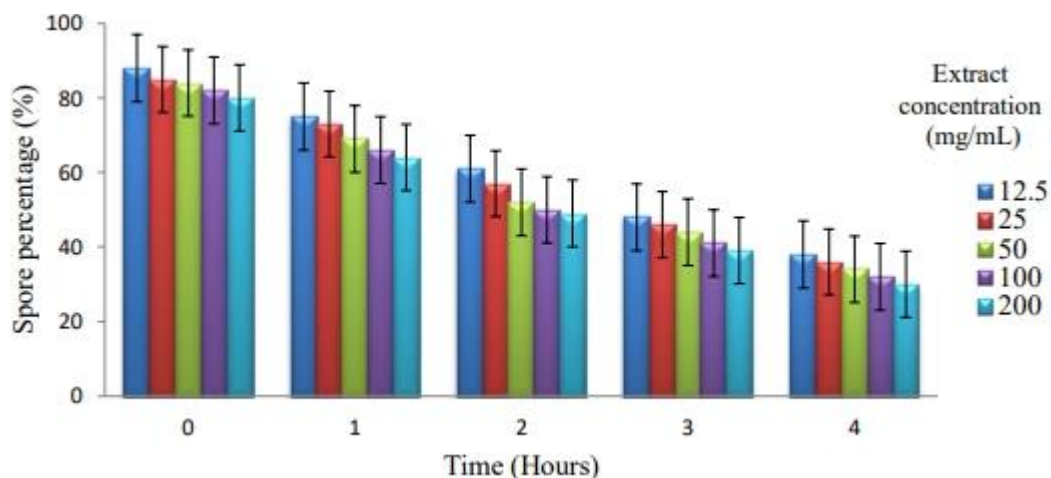


Figure 3. The sporicidal activity of methanolic extract of *E. tirucalli* stem against the spore of *B. megaterium*. The results are presented as the mean \pm standard deviation. Significant differences in means ($n = 3$) ($p < 0.05$)

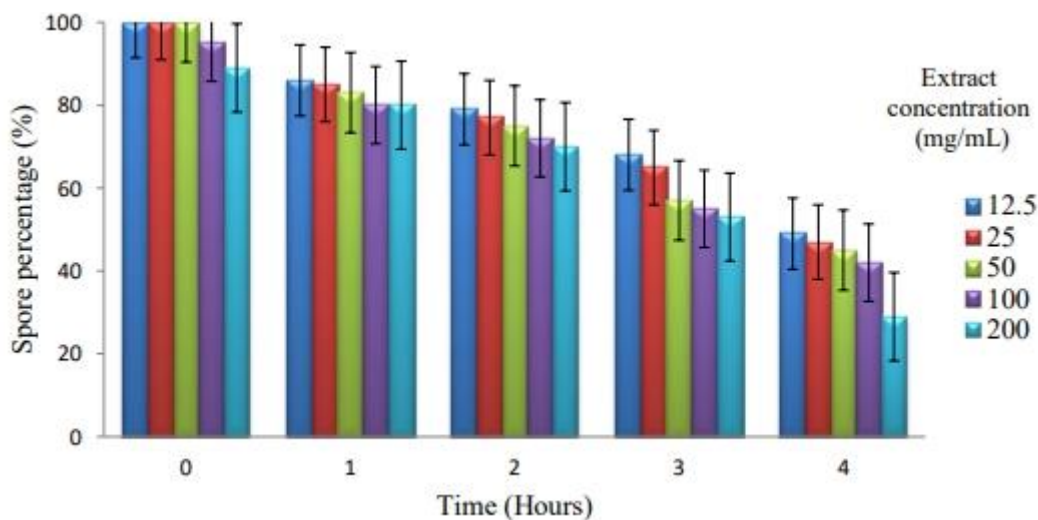


Figure 4. The sporicidal activity of methanolic extract of *E. tirucalli* stem against the spore of *B. cereus*. The results are presented as the mean \pm standard deviation. Significant differences in means ($n = 3$) ($p < 0.05$)

metabolic processes. Several secondary metabolites have antibacterial and therapeutic characteristics (Muthu *et al.* 2006; Mrid *et al.* 2021). Previous studies have shown that phytoconstituents from biologically active compounds have a synergistic effect on drug-resistant microorganisms. These agents can be tested for their safety and efficacy to discover their therapeutic potential in modern infectious disease medicine. In qualitative phytochemical analyses of crude plant extracts of *E. tirucalli* stem, identifying active components in medicinal plants plays a strategic role. The phytochemicals observed in this study include alkaloids, saponins, terpenoids, cardiac glycosides, and tannins. Detection of all these phytochemicals was consistent with previous

data from Sultan *et al.* (2016) and Sugumar *et al.* (2010). However, the presence of flavonoids and steroids and the absence of saponins contrast with the results reported by Sultan *et al.* (2016). This variability may be due to geographic differences in plant material (Larayetani *et al.* 2019). Screening differences may also be related to organic solvent potency and different phytochemical screening protocols (Umar *et al.* 2020).

Alkaloids are one of the main classes of secondary metabolites with important biological properties such as analgesics, muscle relaxants, and antioxidants in traditional and modern medicine (Casciaro *et al.* 2020). It should be noted that their unique biological activity allows the formation

of hydrogen bonds with enzymes, receptors, and proteins since the nitrogen atom accepts protons, and one or more protons donate hydrogen atoms in amines (Jaafar *et al.* 2021). The presence of alkaloids in the methanolic extract of *E. tirucalli* may impact the extract's ability to fight bacteria by depolarizing the cell wall, intercalating into bacterial DNA, and inhibiting mRNA transcription (Han *et al.* 2022). Another study conducted indicates that the plant alkaloid is highly effective against several phytopathogenic and saprophytic fungi. The alkaloid was also active against pigmented and non-pigmented spores of several fungi (Sahni *et al.* 2005). Saponins are one of the broadest and various natural goods derived from plants. Saponins are a plant's defense mechanism against pathogens, herbivores, and an allelopathic agent when interacting with other plants. Saponin can disrupt cell structure interfere with enzyme synthesis and cell permeability, inhibit efflux pumps, and affect energy formation (Odilia *et al.* 2022). Terpenoids are the most essential and structurally diverse class of secondary metabolites derived from plants. Previous investigations on plant methanol extractions have shown that terpenoids may explain the therapeutic value of plant extracts (Truong *et al.* 2019). The presence of numerous tannins in *E. tirucalli* explains the antibacterial action. Tannins are regarded as surfactants and can inhibit microbial adhesions. They also consist of polysaccharide membranes. Numerous plant genetic sources' active components with antibacterial properties have been investigated. The antimicrobial properties of *E. tirucalli* can be related to the different chemical compounds present, which also exhibit antimicrobial activity due to their single or combined action (Altamimi *et al.* 2019). Cardiac glycosides are a diverse family of naturally occurring compounds widely recognized for their ability to bind and inhibit the sodium pump (Reddy *et al.* 2007). These bioactive compounds are naturally present in most extracts and have been shown to have bactericidal or fungicidal properties against the human pathogens studied (Kebede *et al.* 2021).

The extraction solvent affects the extracts' yield and biological activity. Raw plant materials must undergo phytochemical processing to preserve antibacterial activity and maximize constituent concentration. Several solvents, including distilled water, acetone, methanol, and an aqueous mixture

of ethanol and ethanol, are frequently used in plant extraction (Abubakar and Haque 2020). The type of plant, the part of the plant that needs to be extracted, the chemical composition of the bioactive elements, and the solvent's accessibility influence the decision on the type of solvent to be used. Polar solvents such as methanol, ethanol, and water extract polar compounds. In contrast, nonpolar solvents such as hexane and dichloromethane extract nonpolar compounds. In this study, the solvent of extractions used is 80% methanol, a polar solvent soluble in water and can extract polar secondary metabolites (Davinelli and Scapagnini 2022). The benefits of methanol include self-preserving when the concentration is above 20% and only a little heat is needed to concentrate the extract, which is not toxic at low concentrations. The more polar extract (methanolic) was found to be more active than other extracts, so it may be concluded that bioactive antimicrobial compounds from *E. tirucalli* are polar, and a high amount was extracted with methanol. These findings are in agreement with previous reports, that most of the antimicrobial compounds are extracted with methanol (Yi *et al.* 2017; Jayalakshmi *et al.* 2021). Acetone, water, and ethyl acetate extracts of some plants also showed medium antimicrobial activity (Kebede and Shibeshi 2022). The relative amount of phytochemical substances from plant extraction depends on the solubility of the phytochemical in the solvent used for extraction (Altamimi *et al.* 2019).

The results obtained from this study demonstrated that methanolic extract of *E. tirucalli* stem concentrations showed varying levels of antibacterial activity (Table 2). According to Khanal *et al.* (2022), extracts having activities where MIC values are below 8 mg/ml are considered to possess some antimicrobial activity and natural products with MIC values less than 1 mg/ml are considered noteworthy. According to the results, Gram-positive bacteria (*S. aureus*, MRSA, *S. epidermidis*, *P. acne*, *S. pyogenes*, *B. pumilus*, *B. subtilis*, *B. megaterium* and *B. cereus*) were more susceptible to the extract tested than the Gram-negative bacteria (*E. coli*, *K. pneumoniae*, and *S. Typhi*). When comparing all tested plant extracts, the methanolic extract of *E. tirucalli* stems exhibited the best MIC value for Gram-positive bacteria to inhibit *S. aureus*, *S. epidermidis*, *P. acne*, and *S. pyogenes* at 1.25 mg/ml. For Gram-negative bacteria, the extract was

the most successful in inhibiting *E. coli* at 12.5 mg/ml. *B. subtilis* is effective compare to *B. pumilus*, *B. megaterium* and *B. cereus* with 3.12 mg/ml MIC value. The result is consistent with the previous study (Mali and Panchal 2017; Mishra and Parida 2020). For all bacterial species, the MBC values were more significant than the MIC values, showing that the crude plant extracts were bacteriostatic at low doses and bactericidal at high concentrations (Naynika *et al.* 2023). From the result, *S. aureus* and *P. acne* required the lowest bactericidal concentration with only 25 mg/ml. Doncheva *et al.* (2020) have recently shown that alkaloid for antibacterial activity effectively treats various infectious diseases that exhibit multi drug resistant features. The presence of alkaloids in the methanolic extract of *E. tirucalli* may impact the extract's ability to fight bacteria. According to Dasgupta and Acharya (2019), terpenoids have a variety of therapeutic benefits, including immunomodulatory, antiviral, antihyperglycemic, antifungal, antiparasitic, anti-inflammatory, antibacterial, and antioxidant activities. Terpenoids may impact the methanolic extract of *E. tirucalli* antibacterial activity. In a different research, oxidative phosphorylation and oxygen uptake—two crucial processes for microbial viability were successfully suppressed by terpenoids (Yang *et al.* 2020). The cardiac glycoside compounds are naturally present in most extracts and have been shown to have bactericidal or fungicidal properties against the human pathogens studied (Kebede *et al.* 2021).

The antibacterial screening disc diffusion method was used to determine the inhibitory potentials of methanolic extract of *E. tirucalli* stem with different solvents for extraction. The presence of clear zones of inhibition around active extracts established positive results. The results obtained were compared with that of the zone of inhibition produced by standard antibiotic discs. Out of the twelve bacterial species analyzed for antibacterial effect, *S. aureus* is the most susceptible as it creates the largest zone of inhibition. Their various susceptibilities may result from variations in the bacterial organism's primary resistance or the physicochemical action of phytochemicals found in plant components. From the results, it can be concluded that the extract is more effective on Gram-positive bacteria compared to Gram-negative bacteria. As reported by Upadhyay *et al.* (2010), Gram-negative bacteria are more

resistant to extracts than Gram-negative bacteria. The lipopolysaccharide layer, together with proteins and phospholipids, is an essential part of the outer surface of Gram-negative bacteria. Therefore, it slows the evaluation of most phytochemicals towards the peptidoglycan layer (Breijyeh *et al.* 2020). As a result, plant extracts having antimicrobial activity do not have the same adverse impacts on Gram-negative organisms.

Table 5 indicates that the MIC value for *C. albicans* is 1.56 mg/ml. 12.5 mg/ml for *C. tropicalis* and 100 mg/ml for *C. glabrata*. Based on the result, *C. albicans* was the most susceptible *Candida* spp. The result agrees with previous studies conducted by Mishra and Parida (2020). The effect of anticandidal activity of eight different concentrations (200, 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml) for a methanolic extract of *E. tirucalli* stem was tested against three *Candida* spp. as shown in Table 4. All the test solution concentrations inhibited the *Candida* spp. with varying sensitivity. *C. glabrata* was defiantly compared to *C. albicans* and *C. tropicalis*. A decrease in anticandidal activity could imply that the active compounds became unstable during incubation or that the *Candida* species became resistant to the extracts. The high concentration had a high inhibition zone compared to the low concentration, which is an indication that at a high concentration, the anticandidal activity is more pronounced than at a lower concentration. The highest inhibition zone revealed by chloramphenicol used as control (positive control) compared to the plant extracts could be due to its effectiveness as an anticandidal medicine. From the result, it is shown that the methanolic extract of *E. tirucalli* stem possesses antifungal effect. The results of the present study indicate that the plant alkaloid is highly effective against several phytopathogenic and saprophytic fungi. The alkaloid was also active against pigmented and non-pigmented spores of several fungi (Sahni *et al.* 2005). A study conducted by Wang *et al.* (2023) shows that the alkaloid can affect the surface membrane of fungal cells preventing infection.

Based on Figures 1 to 4, methanolic extract of *E. tirucalli* stem was used to treat the spore of *B. pumilus*, *B. subtilis*, *B. megaterium* and *B. cereus* at different concentrations (200, 100, 50, 25 and 12.5 mg/ml) and exposure time (0, 1, 2, 3, and 4). However, a higher concentration was needed to kill the spore. The different incubation times of 0, 1, 2,

3 and 4 hours were selected to observe the optimal reduction time. A slight reduction of *Bacillus* spp. Spores percentage can be seen from 0 hours to 1 hour. *B. pumilus*, *B. subtilis*, *B. megaterium* and *B. cereus* show a significant reduction from 0 hours to 4 hours. The methanolic extract of *E. tirucalli* stem is more effective in inhibiting the spore of *B. subtilis* with the reduction from 80% to 23% at the concentration of 200 mg/ml and least potent in inhibiting the spore of *B. cereus* where the spore percentage reduces from 100% to 40% at the concentration of 200 mg/ml. The reduction of spore percentage of *B. pumilus*, *B. subtilis*, *B. megaterium* and *B. cereus* at the concentration of 200 mg/ml for 4 hours from 83% to 30%, 80% to 23%, 80% to 29% and 89% to 40% respectively. Simple analyses are challenging due to variations in the tested bacteria and the used concentrations. Reports of sporicidal compounds isolated from plants are still uncommon. The sporicidal properties of medicinal plants are related to the phytochemical components present. An isolated from the roots of licorice (*Glycyrrhiza inflata*) has antibacterial activity against vegetative cells of *B. subtilis*, but did not inhibit the germination *B. subtilis* spores (Tsukiyama *et al.* 2002). Several studies have previously discovered and documented the bioactive components of *E. tirucalli* extract that exhibit antibacterial properties. Consequently, they could also be responsible for the sporicidal inhibition of the *Bacillus* spp. spores in the current study.

In conclusion, the study has demonstrated that the methanolic extract of *E. tirucalli* stem exhibits significant antibacterial, anticandidal, and antispore activity. Based on the results obtained, it is concluded that the stem methanol extract can be used to inhibit microbial infection. The study also reveals various phytochemical constituents like alkaloids, saponins, terpenoids, tannins, and cardiac glycosides in the crude extracts. Further work on the types of phytoconstituents is needed to identify bioactive components that might be relevant for pharmacological effects and drug design. *In vitro* and *in vivo* toxicity tests testing are crucial to ensure that the plant extract is safe for use and does not pose any significant health risks to humans or the environment.

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