Research article

Phytochemical investigation and antimicrobial Screening of *Euphorbia Tithymaloides* leaves

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ABSTRACT

Euphorbia tithymaloides leaves were collected and extraction was done in water, methanol, and pet. ether (SA, SM and SP). All extracts were subjected to phytochemical investigation and TLC analysis. Results shows various phytoconstituents were present in the crude extract SA, SM and SP. Also, TLC analysis showed that crude methanolic extracts (SM) has more number of phytoconstituents. Results showed that all crude extracts have presence of constituents generally those that are well known for their antimicrobial activity like tannins, phenolic and glycosides. Thus, the extracts were subjected to antimicrobial screening. Results shows that the MIC value of crude extracts (SA, SM and SP) is less than the MIC value of standard drug Levofloxacin. Hence it is concluded that the extracts do not show desirable degree of antibacterial activity as compared to standard drug. Further research aims to extract and evaluate other *Euphorbia species for their* antimicrobial potential. As a result, it might be useful for future research as an active antimicrobial.

Keywords: Antibacterial Activity, *E. tithymaloides*, extraction, TLC, MIC

INTRODUCTION

Developing novel antibacterial medicines has become necessary due to the emergence of bacterial resistance to currently existing antibiotics[1]. Most post-operative surgical site infections, toxic shock syndrome, endocarditis, osteomyelitis, and food poisoning are caused by gram-positive bacteria like *Staphylococcus aureus*[2]. These antimicrobial compounds are assumed to be of natural origin, have little environmental effect, and may be employed as biological agents[3]. Considering the growing demand for natural ingredients that may be used as food additives, components of functional foods, preventative measures against powdery mildew, and nutraceuticals, as well as for other applications, some medicinal herbs, however, have not found broader applications and are sometimes referred to as "forgotten plants."[4] By evaluating the "lost plants'" applicability and advantages using current scientific analytical techniques, it is feasible to modify their status. Although several new antibiotics have been created by the pharmaceutical industry over the last three decades, microorganisms have evolved resistance to these drugs Bacteria have the potential for genetic acquisition and dissemination of chemotherapeutic drug resistance[5]. The succulent tropical and subtropical shrub *Euphorbia tithymaloide*, also known as *Pedilanthus tithymaloides* commonly called Naagdon, belongs to the family Euphorbiaceous[6]. Evidences from folk medicines show that Euphorbia tithymaloides plant is used for its antibacterial and wound healing potential[7]. Hence it was thought to evaluate it experimentally. The research work aims to find out such phytochemicals having antimicrobial activity.

METHODS AND MATERIALS

Collection of Plant Leaves: The leaves of *Pedilanthus tithymaloides* were collected from, Agra district, Uttar Pradesh (India) during July, 2022. The herbarium specimen of the plant is preserved and deposited in the Raw Material Herbarium and Museum, Delhi. Collected leaves

were washed with sterile water to remove any dirt or filthy particles present on the surface. They were then further subjected to rinsing with distilled water for five times and were then subsequently air shaded dried.

Morphological Analysis of leaves: The fresh leaves of plant were taken and washed with water and checked for its morphological characteristics.

Powder Microscopy of dried leaf: The dried leaves powder of *E. tithymaloides* was taken to prepare a slide and a drop of safranin was poured on it and then it was covered with a cover slip and watched in the microscope.

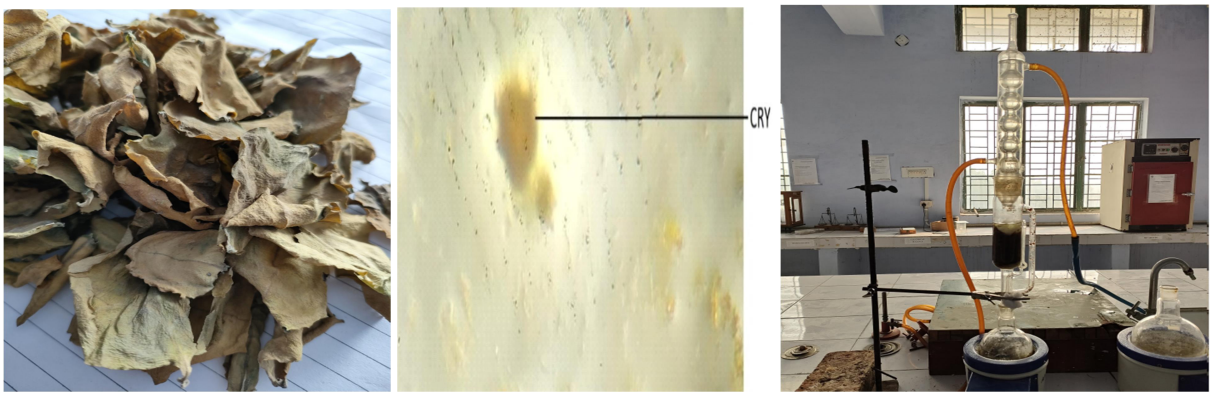

Figure 1: (a) Dry Leaves of *E. tithymaloides*; (b) CRY-Crystal of Calcium oxalate; (c) Soxhlet apparatus

Preparation of Aqueous, Methanolic and Pet. ether extract of *E. tithymaloides* by Soxhletion

Preparation of Methanolic Extract: 15g of air-dried powdered dried leaves of *Euphorbia tithymaloides* were weighed and taken in Soxhlet apparatus and extracted with 250 ml 95% of methanol for 12-14 hours at a temperature not exceeding the boiling point of solvent. Then the extract was filtered and allowed to evaporate by Rotary Evaporator.

Aqueous method: The dried leaves of *Euphorbia tithymaloides* were taken in pestle and mortar, coarsely powdered and passed through 40# mesh. 15g of powdered sample was taken into 100ml of conical flask and 50 ml of water was added; the conical flask was covered and left for 3 days with occasional stirring. Whatman No. 1 was taken for filtration process of the crude extract. The filtrate was then collected in a clean beaker. The extract was obtained on evaporating the solvent by rotary evaporator. The semi-solid extract was obtained and weighed to determine the yield and kept in refrigerator unit was subjected to antibacterial evaluation.

Phytochemical analysis of prepared extract (SM, SP and SA)

For phytochemical characterization of significant photoactive compounds, all the prepared extracts were exposed to a variety of qualitative assays. HI media and Merck in India provided

the various chemicals utilized in the phytochemical study. Qualitative tests were conducted to determine the existence of flavonoids, polysterols, tannins, saponins, alkaloids, phenolics, and carbohydrates **(Table 1)**.

Table 1: Phytochemical analyses of prepared extract (SM, SP and SA)

S. No	Test	Extraction Process
1	**Benedict's test for Carbohydrates**	Few ml aqueous solution of crude extract (5ml) was taken, in which few drops of Benedict's reagent was added and gently heated. Orange color ppt indicates the presence of Carbohydrate.
2	**Fehlings Test-**	The 2 reagents of Fehling solution A and B were taken, take 2 ml of both reagent were taken in a test tube and little amount of crude extract were added in it. Brick red color presence indicates the presence of carbohydrate (reducing carbohydrates).
3	**Salkowski's Test**	Alcoholic extract of drug were taken and few drops of chloroform and concentrated sulphuric acid were added from the side wall of test tube, appearance of yellow color ring at junction of both liquids which turn into red after few time indicates the presence of Sterol (Glycoside).
4	**Saponins Test**	A little amount of crude extract was taken in a test tube and added few ml of water and then shaken, if form formed in the test tube, it shows the presence of Saponins.
5	**Ninhydrin test**	2 ml of crude extract was taken with 2% Ninhydrin solution and heated on Bunsen burner; violet color shows presence of Amino acid.
6	**Test of Ammonium for flavonoids**	The crude extract was dissolved in water and taken in a test tube and few drops of dilute NaOH solution was added by which we got an intense yellow color in the test tube, and then few drop of dilute acid was added. The yellow color vanishes and become colorless which indicate the presence of Flavonoids.
7	**Steroid Test**	1mg of crude extract was taken in a test tube and 10ml chloroform was added in it to dissolved it, then equal amount of concentrated sulphuric acid (H 2 SO 4) was added sidewise, the upper layer turns into red and H 2 SO 4 shows yellow color with green fluorescence which shows the presence of Steroids.
8	**Perform a protein Millon's test**	In this test crude extract is combined with 2ml Millon's reagent, it forms a white precipitate, which becomes red when heated gently, indicating the presence of protein.
9	**Millon's Test**	Crude extract was taken in a test tube and 2ml of Millon's Reagent was mixed, it forms white ppt, which on heating turns red which indicates the presence of amino acids or Protein.
10	**Test for Phenols**	In 5 mL of pure water, the extract (500 mg) was dissolved. Some few drops of neutral 5% FeCl3 were added to this. The presence of phenolic compounds is represented by a dark green color.

11	**Iodine test**	2ml of crude extract solution in the test tube were taken and added few drops of iodine solution in the test tube. Blue black color shows the presence of starch.
12	**Dragendorff's test for Alkaloids**	A little amount of crude extracts was added in a test tube and few drops of HCl was added, then Dragondroff's reagent was added. Orange-brown color ppt indicates the presence of Alkaloids.
13	**Ferric Chloride Test**	Little amount of crude extract in the test tube was taken and 1% FeCl 3 was added to give Blue, Green or Red-Brown Color..
14	**Test for tannins using Lead acetate**	A little amount of the crude extract was separately extracted in water, as well as the occurrence of tannins was determined by introducing a solution of lead acetate 10%. The occurrence of tannins is shown by the production of white precipitate.
15	**Liebermann's test for glycosides**	2 mL of acetic acid and chloroform were added to crude extract. Ice was used to chill the decoction. H_2SO_4 was added in a concentrated form. A color shift from violet to blue to green indicating the presence of steroidal nucleus, i.e., glycone part of glycoside.

Thin Layer Chromatography

To prepare the TLC plates, Silica gel 'G' was used. First, thirty grams of silica gel were weighed and then a homogenous suspension was made with sixty milliliters of distilled water for two minutes. This suspension was then distributed over the plate, which was then air dried until the transparency of the layer was no longer visible. After drying in a hot air oven at 110 degrees Celsius for thirty minutes, the plates were kept in an airtight container in a dry environment until they were needed. To prepare the samples, the crude extracts of methanol, water and pet. ether was first diluted with their respective solvents and then applied to the origins of a TLC plate 2 centimetres above the plate's bottom using capillary tubes. Typical application volumes ranged from 1 to 10 microliters. For the TLC plates were developed in various solvent systems namely methanol – acetone (1:1), methanol – ethyl acetate (1:1), ethyl acetate – pet. ether (1:1) and methanol –acetone – pet. Ether (2:1:2). the TLC were developed Iodine chambers and number of spots there Rf value reported.

Evaluation of Antimicrobial Activity

Maintenance of culture: *E. coli, S. aureus, P. aeruginosa, K. pneumoniae, A. baumanni* was isolated from various hospital in suitable media. Then antimicrobial activity was evaluated by using disc plate method. The stock culture was subculture before 24 hours in a suitable nutrient media prior to use for bioassay.

Inoculation: Nutrient agar media was selected as the nutrient media was prepared. It was autoclaved for the sterilization of the prepared nutrient agar for 30 minutes. 15ml of nutrient agar was poured into petri plates. After the media solidified, it was inoculated with 200-250µl of *E. coli*(ATCC 25922), A. *baumanii(*BAA 1605), *S. aureus*(ATCC 29213), *P. aeruginosa*(ATCC

27853) and *K. pnemoniae*(BAA 1705) They were spread uniformly over the agar plate with the help of the cotton swabs.

Disc Diffusion Method: Disc diffusion method also known as Petri plate was used for evaluation of the antibacterial activity of the leave extract *E. tithymaloides*. The disc was dipped in the different extract of different concentration solution and were used for the antibacterial Bioassay. Standard antibiotics disc was used as a positive control, both the disc was placed carefully with adequate spacing between each other. The all-petri plates were put in BOD Incubator for incubation at 37°C For 24 hours. The radius of ZOI was measured with a ruler and the antibacterial activity compared with the standard antibiotics disc was measured.

Determination of MIC of Extract by (ESKAPE): As per the European Committee for antimicrobial Susceptibility Testing (EUCAST)/CLASI, the broth dilution method carried out in a serial of different dilution were used for determining the minimum inhibitory concentration (MIC). Muller-Hinton cation supplement broth II were used in medium for sterility control 100µL. Serial dilution of the crude plant extract was prepared and 2ml aliquots of various concentration of the solution was added in 18ml of pre-sterilized molten Agar media for bacteria, at 40°C to give final concentration of 1.0, 2.5, 5.0, 10.0 and 20.0 µ/L. Then the medium was poured into clean and sterile Petri dishes, allowed to set for few minutes. The inoculation was performed in such a way so that it could contain $5x10^5$CFU/ml. After that the plates were incubated at 37°C for 18-24 hours for bacterial growth after which they all were ready for examined for the presence or absence of bacterial growth. The MIC was taken as lowest concentration of test bacteria or micro-organism.

RESULT & DISCUSSION

Morphological and Microscopic Analysis of Leaves: Microscopic study of leaf specimen was done by taking a transverse section (TS) along small portion of lamina through midrib, and thin section was double stained with haematoxylin and safranin and observed under compound microscope and photographs were taken.

Table 2: Microscopy of leaf

Character	Observation
Leaf condition	Fresh
Leaf Size	Length: 9cm Width: 5cm
Leaf Shape	Ovate to Cordate
Leaf Margin	Entire
Leaf Color	Upper Surface: Dark Green; Lower Surface: Light Green
Odor	Slightly Aromatic
Taste	Mucilaginous
Leaf Surface	Spongy
Extra Features	Thick Leaf with Cuticle Present

Quantitative Microscopy Analysis of Leaf: Upper surface and lower Surface of leaf were examined with the help of microscope and eye piece.

Table 3:Quantitative Microscopy of leaf

Leaf Constant	Value (Upper Surface)	(Lower Surface)
Stomatal Number	12.18	23.45
Stomatal Index	4.32	8.21

Percentage Yield of *Euphorbia tithymaloides* extract

Dried germinated seeds of H. Vulgar were extracted by water, methanol and petroleum ether. The yields of the extracts were determined.

Percentage Yield = Weight of extract / Weight of Sample x 100

- The amount of Aqueous Extract from 15g of dried leaves was calculated to be 2.893 gm (19.3%) in semisolid muclilageneous form.
- The amount of Methanolic extract from 15g of dried leaves with Soxhlet was calculated to be 3.128gm (21%) as a dark green powder.
- The amount of Petroleum Ether extract from 15g of dried leaves with Soxhlet was calculated to be 2.973gm (19.82%) as greenish brown powder.

Phytochemical Investigation

Table 3: The quantitative investigation of phytochemical components in various extracts of *Euphorbia tithymaloides* yielded the following results

S. No.	Constituents	Aqueous extract	Methanol extract	Petroleum extract
1	Steroids	-	+	-
2	Flavonoids	+	+	-
3	Saponins	+	+	+
4	Glycosides	-	+	-
5	Phenols	+	+	-
6	Tannins	+	-	+
7	Alkaloids	+	+	-
8	Triterpenes	-	+	+
9	Carbohydrates	-	+	+
10	Protein	-	+	-

The presence (+) and absence (-) of phytochemical constituents

TLC analysis

The TLC plate were dried in hot air oven at 110°C for 30 minutes, kept in dry and clean place and used whenever required. Samples of extract were prepared by diluting with required solvent

and then applied usually 1-10µl on the TLC plate 2cm above the bottom with the help of capillary tube carefully. For development of the chromatogram after the application of sample on TLC plate, the plate was kept in the TLC chamber containing mobile phase (solvent saturated), and allowed to run the mobile phase in the plate adsorbent. A typical application volume range which was from 1 to 10 ml. Spots were identified by iodine vapours. TLC plates were developed in various solvent system like methanol-acetone (1:1), Ethyl acetate-Pet ether (1:1) and methanol-acetone-pet ether (2:1:2). The Rf value of all the spots were determined and reported.

Table 4: R_f value of Hordeum vulgare extract

extracts	TLC analysis
SA	2 spots at R_f -0.58 and 0.71
SM	3 spots at R_f 0.32, 0.55 and 0.60
SP	2 spot at R_f 0.4 and 0.42

Solvent system: methanol – acetone – pet. Ether (2:1:2), developing agent iodine vapours

TLC analysis shows that maximum numbers of phytochemicals were extracted in methanolic extract. Pet. Ether extract did not separate well in any of the solvent system used and aqueous extract showed a smaller number of phytoconstituents extracted.

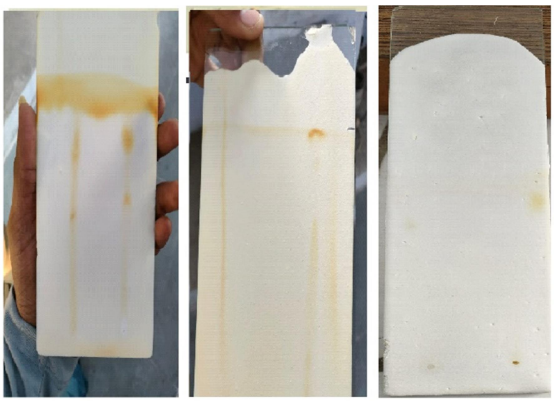

Figure 2: Analysis of SA, SM, SP

Antimicrobial activity

Antimicrobial activity was performed from biological assay division (CDRI-Lucknow)

MIC of the extracts: As per the European Committee for antimicrobial Susceptibility Testing (EUCAST)/CLASI, the broth dilution method carried out in a serial of different dilution were used for determining the minimum inhibitory concentration (MIC). Muller-Hinton cation supplement broth II were used in medium for sterility control 100µL. Serial dilution of the crude plant extract was prepared and 2ml aliquots of various concentration of the solution was added in 18ml of pre-sterilized molten Agar media for bacteria, at 40°C to give final concentration of 1.0, 2.5, 5.0, 10.0 and 20.0 µ/L. Then the medium was poured into clean and sterile Petri dishes, allowed to set for few minutes. The inoculation was performed in such a way so that it could

contain 5x10 5 CFU/ml. After that the plates were incubated at 37°C for 18-24 hours for bacterial growth after which they all were ready for examined for the presence or absence of bacterial growth. The MIC was taken as lowest concentration of test bacteria or micro-organism.

Table 5: The MIC values of different bacterial strains against the 3 extracts

S. No.	Bacterial strains	Aqueous extract mg/ml)	Methanol extract (mg/ml)	Pet. Ether	Levofloxacin
1	E. coli ATCC 25922	>64	>64	>64	0.0078
2	S. aureus ATCC 29213	>64	>64	>64	0.0625
3	*K. pnemoniae BAA 1705*	>64	>64	>64	64
4	A. baumanii BAA 1605	>64	>64	>64	4
5	*P.aeruginosa ATCC 27853*	>64	>64	>64	0.5

(Note: Sample SA, SM and SP were not completely soluble in DMSO @ 200mg/ml conc.)

DISCUSSION

As the biggest genus in the *family Euphorbiaceae,* with almost 2,000 recognized species, Euphorbia is also one of the most diverse. Several studies have shown the antibacterial and antioxidant activities of many Euphorbia spp., lending credence to the traditional use of these plants in the treatment of a wide range of ailments. All the crude extracts showed MIC value >64 mg /ml which was not equal to or more than the MIC value of standard drug used. Hence it was concluded that the crude extracts (SA, SM and SP) prepared did not show required degree of antimicrobial action.

CONCLUSION

The findings demonstrated that the plant investigated had medicinally essential components. There is a lot of evidence from previous research that the discovered phytochemicals are bioactive. Several studies have proved that these phytochemicals provide physiological and pharmacological qualities to the plants examined in the medication of many diseases. As a result, extracts from these plants might be considered a promising source of therapeutics.

In light of the above, it was possible that *Euphorbia tithymaloides* shows a strong antibacterial impact in laboratory experiments. Additional effort should be done to separate, purify, and characterize the active ingredients responsible for activity of these plants, as well as further work to isolate, purify and describe the active compounds responsible for activity of these plants. As decided, *Euphorbia tithymaloides* leaves were collected and extraction was done in water, methanol and pet. ether (SA, SM and SP). All extracts were subjected to phytochemical investigation and TLC analysis.

Results shows various phytoconstituents were present in the crude extract SA, SM and SP Also TLC analysis showed that crude methanolic extracts (SM) has more number of

phytoconstituents. Results showed that all crude extracts have presence of constituents generally those that are well known for their antimicrobial activity like tannins, phenolic and glycosides. Thus the extracts were subjected to antimicrobial screening, against their antibacterial ESKSAP pathogen panel. Results shows that the MIC value of crude extracts (SA, SM and SP) is less than the MIC value of standard drug Levofloxacin. Hence it is concluded that the extracts SA, SM and SP does not show desirable degree of antibacterial activity as compared to standard drug. Because satisfactory antimicrobial activity was not observed, chemical constituent separation by column chromatography was not performed. It was decided to subject the extracts for wound healing activity, as per other folklore claims. Further research aims to extract and evaluate other Euphorbia species for their antimicrobial potential. As a result, it might be useful for future research as an active antimicrobial.

CONFLICTS OF INTERESTS

There are no conflicts of interests.

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