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RESEARCH ARTICLE

Exploring the Potential of *Amaranthus tricolor* (L.) in Combating Multidrug-resistant Uropathogens

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Abstract

Background: Urinary Tract Infections (UTIs) rank among the most frequently occurring bacterial infections and can impact people of all ages and genders. Multidrug Resistance (MDR) among uropathogens has emerged as a global health crisis. The World Health Organisation (WHO) states that Antimicrobial Resistance (AMR) poses a serious threat to the successful treatment of infections, leading to increased mortality and healthcare expenditures. **Aim:** This study investigates the antibacterial potential of *Amaranthus tricolor* (L.) leaves phytoconstituents against multidrug-resistant uropathogens. **Method:** The methanolic leaf extract of the plant (ATME) was prepared by a cold maceration process and subsequently screened for phytoconstituents. The ATME extract was partitioned using an equal volume of chloroform and water. Upon evaporation and purification with acetonitrile, the aqueous fraction yielded a colourless compound, designated as SOWIS-P. Meanwhile, the chloroform fraction underwent further partitioning using N-hexane: ethyl acetate (6:4 v/v) as the mobile phase, guided by HPTLC analysis. Bio-autography identified the third fraction as the most significant, and it was labelled as SOWIS-III. The antibiotic resistance profile of pathogens isolated from urine samples was evaluated using the Resazurin Microtiter Plate Assay (REMA). **Results:** The structural analysis of the isolated compound SOWIS-III revealed it to be a flavonol glycoside, 24-methylene cycloartanol, while SOWIS-P was identified as a gallic acid derivative, methyl 3,4,5-trihydroxybenzoate. The isolated pathogens exhibited substantial resistance to ampicillin (81.4%), moderate resistance to azithromycin and doxycycline (74.6%) as well as nitrofurantoin (74.4%), and lower resistance to gentamicin (67.7%).

Major Findings: The compounds demonstrated potent broad-spectrum antibacterial activity, effectively inhibiting the tested multidrug-resistant UTI pathogens at concentrations ranging from 6.25 µg/ml to 12.5 µg/ml.

Keywords: *A. tricolor* (L.), Antibacterial Activity, Multidrug-resistant Uropathogens, Phytoconstituent

1. Introduction

In recent years, infections caused by MDR uropathogens have increasingly become common in both healthcare settings and community environments. This rise in prevalence significantly influences treatment protocols and clinical outcomes, establishing conditions such as Urinary Tract Infection (UTI) as one of the most pressing concerns for public health in nations that are both developed and developing. The rapid emergence and widespread dissemination of MDR genes in uropathogens occur through a variety of complex and multifaceted mechanisms. These include, but are not limited to, the reduced penetration of antibiotics due to the blocking of drug entry into the bacterial cell, the action of multidrug efflux pumps, and the reduction of antibiotic affinity for their target sites because of mutational changes or replacements, and the bypassing of target enzymes through alternative biosynthetic pathways. Additionally, bacteria have developed antibiotic-destroying enzymes or possess the ability to quench or reactivate through their metabolic products, all of which inhibit key processes such as cell wall and protein synthesis. These are merely a few

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examples of the numerous strategies that bacterial uropathogens have evolved to combat the effects of man-made antibiotics. In summary, uropathogens demonstrate a remarkable ability to adapt to high-resistance mechanisms, which leads to frequent treatment failures and complicates therapeutic efforts significantly[1-7].

The emergence and proliferation of multidrug-resistant uropathogens present a considerable and significant challenge to public health systems around the world, leading to not only increased morbidity but also heightened healthcare costs that burden both patients and health services[8-10].

In therapeutic reality, multidrug-resistant derivatives of various gram-negative pathogens, such as *Pseudomonas*, *Escherichia coli*, and *Klebsiella* spp., are quite common. For instance, uropathogenic *E. coli*, a frequent causative bacterial pathogen of urinary tract infections, has built up resistance to the commonly employed antibiotic. Hitherto, approximately a quarter of *E. coli* strains are resistant to this therapeutic agent. The spectrum of antibiotic resistance in *E. coli* is also continuously altering; for example, ciprofloxacin resistance in *E. coli* increases by 5% per year. Early detection, successful management of urinary tract infections caused by multidrug-resistant strains, and measures for the interruption of their transmission are of paramount importance due to dismal implications, including increased morbidity, high societal economic burden, and therapeutic failure due to the ineffectiveness of available sanitising agents. Innovative approaches to counteract these uropathogens in a pre-existing scenario of limited or no antibiotics are necessary[11-18].

Recent and comprehensive statistical information reveals a significant and notable increase in the number of infections that are caused by multidrug-resistant uropathogens. This situation highlights the urgent necessity for the development of innovative and effective treatment strategies that can address and combat these particularly difficult infections that present a major challenge to public health[19,20].

It is crucial and quite timely to actively seek out new alternatives that exhibit the least resistance or ideally no resistance at all. Both historical and current trends increasingly illustrate that plant-based therapies could indeed serve as a viable alternative to traditional synthetic antibiotics. Particularly among communities living in remote or rural areas, there is a notable reliance on various plant extracts and their derivatives, which are utilised not only in oral forms but also in topical applications to alleviate and decrease the pain commonly associated with UTIs. At the same time, scientific research and evidence have also distinctly revealed that bioactive phytochemicals may offer new insights into effective infection management strategies. For instance, *Amaranthus tricolor*, recognised as an ancient crop, has been valued for both its nutritional and medicinal properties. Researchers have found that nearly 50 distinct compounds have been identified and reported, demonstrating a range of activities, including antioxidant, antibacterial, anti-candidal, antifungal, and anti-inflammatory properties. Surprisingly, it is worth noting that very few plants from India have been thoroughly reported in the context of UTI-related findings. Therefore, we have embarked on a study utilising *Amaranthus tricolor*, exploring it from a new perspective to address infections that arise due to MDR, focusing on *in vitro* investigations[21-26].

2. Materials and Methods

2.1 Plant Material

The plant *Amaranthus tricolor* (L) from the Amaranthaceae family was gathered from agricultural fields in and around Guntur, Andhra Pradesh, India and its identification was authenticated by the Botanical Survey of India (BSI), Coimbatore, Tamil Nadu, India.

2.1.1 Antibiotic Discs

Nitrofurantoin 300 µg (HiMedia)

2.1.2 Bacteriological Media

The nutrient broth, nutrient agar, Muller Hinton agar, and CLED agar media used in the study were procured from HiMedia, Mumbai.

2.1.3 Bacterial Isolates

The Gram-positive and Gram-negative bacteria used in the study were obtained from the Microbiology Laboratory in Guntur, Andhra Pradesh. The study focused on bacteria responsible for urinary tract infections, including *Staphylococcus aureus, Staphylococcus saprophyticus, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris* and *Proteus mirabilis.*

2.2 Extraction of Phytoconstituents

Fresh, healthy leaves were dried in the shade and subsequently ground into a coarse powder. The powdered plant material from *A. tricolor* (L) was subjected to methanol extraction using the cold maceration technique. The resulting extract was labelled as ATME, and it was stored in a desiccator for future analysis.

2.2.1 Preliminary Phytochemical Screening

The prepared extracts were analysed for their phytochemical constituents using established standard methods[27,28].

2.2.2 Evaluation of Antibacterial Activity of Crude Extracts

The susceptibility of microorganisms was assessed by inoculating specific organisms (1×10^8 CFU/mL) onto Mueller-Hinton agar medium. Using a sterile borer, 6 mm diameter wells were punched at equal distances on the agar plate. Each well was filled with 500, 750, and 1000 µg/mL of ATME, respectively. The plates were incubated upside down at 37°C for 24 hours, and the antibacterial effectiveness was evaluated in comparison with nitrofurantoin (300 µg/disc).

2.2.3 Determination of Minimum Inhibitory Concentration (MIC) by Micro-Broth Dilution

The Minimum Inhibitory Concentration (MIC) of the extract was assessed against the bacterial strain using a micro-broth dilution assay with resazurin as a growth indicator. The initial well in the dilution series received 100 µL of the extract solution (10% w/v). All subsequent wells were pre-filled with 50 µL of sterile nutrient broth. A two-fold serial dilution was performed by transferring 50 µL of the solution from the initial well to the next well containing broth. This mixing and transfer process was repeated stepwise down the plate. After each transfer, the well contained 50 µL of liquid. Each well in the dilution series then received 10 µL of a standardized bacterial suspension (approximately 1×10^8 CFU/mL) and 30 µL of resazurin solution (0.02%). The plate was sealed and incubated at 37°C for 24 hours. Following incubation, wells were visually inspected for a colour shift from purple to pink, indicating bacterial growth (positive response). The MIC value was defined as the lowest extract concentration in the dilution series that prevented this colour change, signifying no bacterial growth[29-31].

2.2.4 Fractionation of Phytoconstituents from ATME

2 grams of ATME (A) were dissolved in 100 mL of chloroform. This solution was transferred to a separating funnel and partitioned against an equal volume (100 mL) of water. The funnel was shaken vigorously to facilitate separation. The resulting distinct chloroform and aqueous layers were then carefully collected into separate china dishes. The chloroform fraction was subjected to evaporation. The dried residues were stored in a desiccator pending further analysis. Evaporation of the aqueous fraction yielded a crystalline material. This crude crystalline compound was purified by repeated washing with a mixture of methanol and acetonitrile. During each wash step, the solvents dissolved colored impurities, and the supernatant was carefully removed after dissolution. This iterative process was continued until a pure, colourless crystalline compound was obtained. The purified colourless compound was designated as SOWIS-P.

The chloroform fraction was evaporated and used for further phytoconstituents separation. It was subjected to High-Performance Thin-Layer Chromatography (HPTLC) to optimise the mobile phase, which was determined to be N-hexane: ethyl acetate (6:4 v/v). Phytoconstituents were separated by column chromatography, and the third fraction was selected based on bioautography results. This fraction was isolated and labelled as SOWIS-III.

2.2.5 Fingerprint Analysis of Chloroform Fraction of ATME by HPTLC: Instrumentation and Chromatographic Conditions

A 10 µL aliquot of the sample solution was applied onto a pre-coated silica gel 60 F254 aluminium-backed TLC plate (5 cm × 10 cm; layer thickness 250 µm; E. MERCK, Darmstadt, Germany). Application was performed using a 100 µL syringe (Hamilton, Bonaduz, Switzerland) and a CAMAG Linomat 5 automatic sample applicator (Muttenz, Switzerland). The prepared plate was developed in a twin-trough glass chamber (10 cm × 10 cm; CAMAG, Muttenz, Switzerland). The

mobile phase, consisting of *n*-hexane: ethyl acetate (6:4, v/v), was used. Before development, the chamber was saturated with the mobile phase vapour for 15 minutes. The chromatogram was then developed over a distance of 8 cm, which required approximately 15 minutes. After development, the plate was dried using a stream of warm air from a hair dryer. The dried plate was scanned densitometrically using a CAMAG TLC scanner. Scanning was performed at a wavelength of 350 nm. Instrument control and data acquisition were managed using CAMAG WINCATS software (version 1.4.2).

2.2.6 *Bioautography Screening for Antibacterial Analysis*

The fractions of the extract that exhibited strong antimicrobial activity were identified using contact bioautography. After development, the TLC plates were air-dried until all solvent traces were completely removed. The dried chromatogram was carefully placed, developed side down, onto the surface of a freshly prepared agar medium pre-inoculated with the test microorganism. The plate remained in contact with the agar for 3-4 hours to allow bioactive compounds to diffuse from the TLC adsorbent into the underlying agar layer. After the diffusion period, the TLC plate was gently removed. The agar plate was then incubated at 37°C for 24 hours to permit microbial growth. Post-incubation, clear zones of inhibition (areas lacking microbial growth) became visible on the agar surface. These zones corresponded precisely to the locations on the original TLC plate where antimicrobial compounds had been deposited.

2.2.7 *Isolation of Phytoconstituents*

The separation and isolation of the chloroform fraction of ATME were carried out using column chromatography. According to the HPTLC analysis, compounds present in the $CHCl_3$ fraction of ATME were identified. The column was packed using the wet packing mount method, and its porosity was enhanced by mixing the extract with silica gel G (Mesh 60-120). The phytoconstituents were separated and isolated using the mobile phase n-hexane: ethyl acetate (6:4 v/v). Similar fractions were pooled together and further purified using preparative TLC, with confirmation by HPLC.

2.2.8 *Structural Interpretation of Isolated Compounds*

The bioautography results indicated that fractions I, III, V, VI, and VIII may exhibit antibacterial activity. Among these, fraction III (SOWIS-III) and the compound from the aqueous fraction (SOWIS-P) were selected for further investigation, including structural analysis, *in-silico* studies, and evaluation of biological activities. The structures of these fractions were determined through NMR and mass spectrometry studies.

2.2.9 *Detection of Drug Resistance of UTI pathogens by Resazurin Micro-titre Plate Assay (REMA)*

The drug resistance profile of UTI pathogens was studied by Resazurin Micro-titre plate Assay (REMA). The antibiotics included in the study are presented in Table 1, and clinical isolates of uropathogens are listed in Table 2.

A 100 µL solution of the test antibiotic (1% w/v) was added to the wells in the first row, while the other wells were filled with 50 µL of sterile nutrient broth. Serial dilutions of the antibiotic solution were performed by transferring 50 µL from one well to the next, ensuring each well contained 50 µL. Subsequently, 10 µL of the respective bacterial suspension and 30 µL of resazurin solution (0.02%) were added to each well.

The experiment included a positive control, consisting of a column with all components except the antibiotic, and a negative control, which contained sterile nutrient broth without the antibiotic and

Table 1. List of antibiotics used in the study

S. No.	Class of Antibiotic	Examples
1	Beta-lactam	Ampicillin (AP), Amoxicillin-Clavulanic acid (Amoxyclav) (AC)
2	Cephalosporins	Cephalexin (CF), Cefadroxil (CD)
3	Fluoroquinolones	Ciprofloxacin (CP), Levofloxacin (LF)
4	Tetracyclines	Tetracycline (TC), Doxycycline (DC)
5	Sulfonamides	Co-trimoxazole (CT)
6	Aminoglycosides	Amikacin (AK), Gentamicin (GT)
7	Macrolides	Erythromycin (ET), Azithromycin (AT)
8	Nitrofurans	Nitrofurantoin (NF)

Table 2. List of clinical isolates of bacteria procured for the study

S. No	Name of the Microorganism	No. of isolates
1	*Staphylococcus aureus*	13
2	*Staphylococcus saprophyticus*	14
3	*Enterococcus faecalis*	13
4	*Escherichia coli*	19
5	*Klebsiella pneumoniae*	18
6	*Pseudomonas aeruginosa*	19
7	*Proteus vulgaris*	11
8	*Proteus mirabilis*	12
	Total	119

bacterial suspension. The plates were covered and incubated at 37°C for 24 hours. After incubation, the colour change was observed: a shift from blue to pink indicated a positive response. Resazurin, which is blue in its oxidised state, turns pink when reduced by viable cells. The lowest concentration at which the colour change was noted was recorded as the inhibitory concentration of the antibiotic for the specific bacterial strain, representing the drug resistance concentration exhibited by the microbial strain[32].

3. Results

3.1 Fingerprint Analysis of Chloroform Fraction of ATME by HPTLC

The HPTLC fingerprint analysis of the chloroform fraction of ATME revealed the presence of 11 compounds. Their separation was facilitated using n-hexane: ethyl acetate (6:4 v/v) as the mobile phase.

3.2 Bioautography Screening for Antibacterial Analysis

The contact bioautography method was employed to identify antibacterial compounds. Fractions I, III, V, VI, and VIII, with Rf values of 0.07, 0.18, 0.29, 0.48, and 0.67, respectively, showed bacterial growth inhibition. Among these, the third fraction (SOWIS-III) was selected for further study due to its higher bacterial inhibition rate (Figure 2).

3.3 Isolation of Phytoconstituents

The chloroform fraction of ATME was further separated using column chromatography with

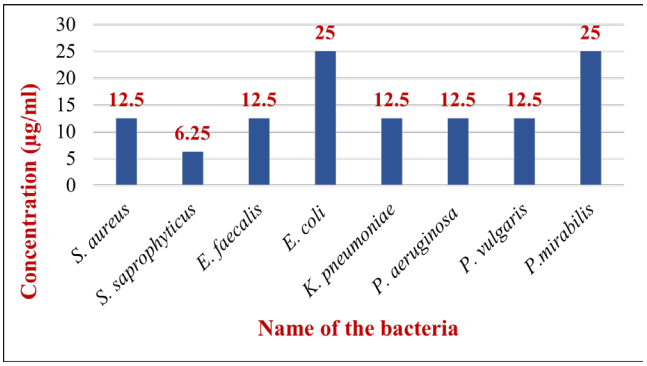


Figure 1. Minimum Inhibitory Concentration of ATME against clinical isolates of UTI pathogens (µg/mL).

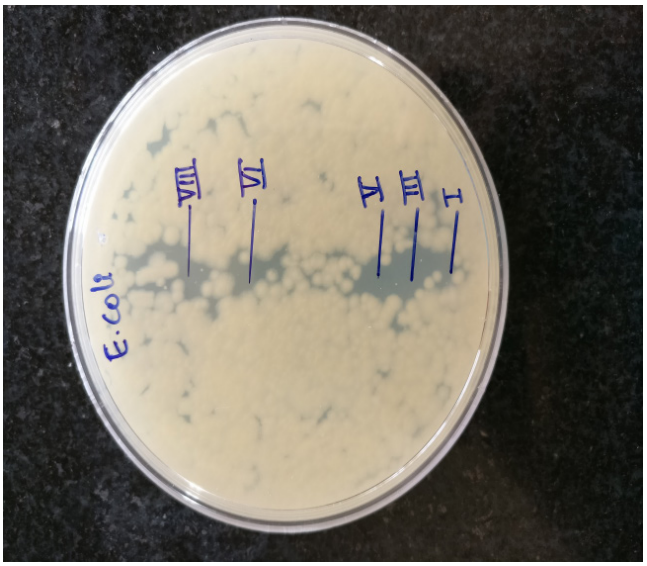


Figure 2. Bioautography screening for antibacterial analysis.

n-hexane: ethyl acetate (6:4 v/v) as the mobile phase, yielding 11 fractions. Based on bioautography screening results, the third fraction (SOWIS-III) was collected and purified using preparative TLC. The purity of SOWIS-III and the aqueous fraction compound (SOWIS-P) was confirmed through HPLC analysis.

3.4 Structural Interpretation of Isolated Compounds

Based on structural interpretation, SOWIS-III was identified as a flavonol glycoside and 24-methylene cycloartanol, while SOWIS-P was characterised as a gallic acid derivative, specifically methyl 3,4,5-trihydroxybenzoate.

4. Discussion

The preliminary phytochemical analysis of ATME indicated the presence of various compounds. The extract was found to contain carbohydrates, amino acids, proteins, alkaloids, glycosides, steroids, flavonoids, tannins, and phenolic compounds, as detailed in Table 3.

In the present study, crude methanolic leaf extract of *Amaranthus tricolor* (ATME) was tested to evaluate the *in vitro* effect against MDR strains of uropathogens. High antimicrobial potential was seen against all the examined strains of uropathogens, and the results are presented in Table 4. It was concluded that the ATME possess excellent soluble compounds for the maximum exudate yield, as well as strong antibacterial activity against resistant uropathogens. The MIC of ATME ranged between 6.25 and 25 mg/mL, as shown in Figure 1.

The presence of quantitatively high secondary metabolites and a significant antibacterial efficacy of ATME made to further study fractionation, isolation, structural elucidation, *in-silico* analysis and biological activities evaluation[33]. The structural analysis of the isolated compound SOWIS-III identified it as a flavonol glycoside, 24-methylene cycloartanol (Figure 3). The structural analysis of SOWIS-P revealed it to be a gallic acid derivative, methyl 3,4,5-trihydroxybenzoate (Figure 4).

The antibiotic resistance profile of urinary tract pathogens studied by Resazurin micro-titre plate assay showed high resistance rates among all the tested

Table 3. Preliminary phytochemical screening of ATME

NAME OF THE TEST	ATME
Alkaloids	+
Glycosides	+
Aminoacids	+
Proteins	+
Carbohydrates	+
Steroids	+
Flavonoids	+
Tannins & Phenolic compounds	+

"+" indicates positive, "-" indicates negative

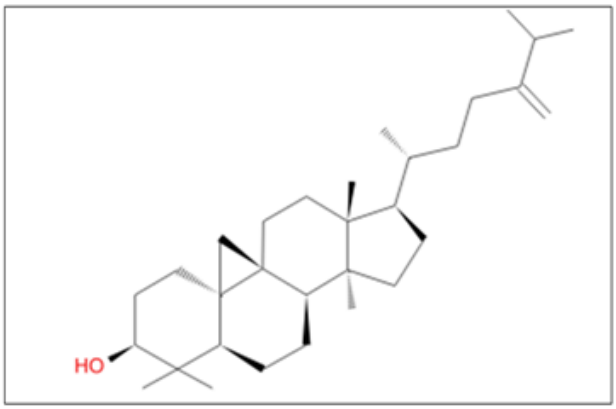


Figure 3. Structure of 24-Methylene cycloartenol.

Table 4. Antibacterial screening of ATME, Nitrofurantoin and DMSO against clinical isolates of UTI pathogens

Name of the organism	Diameter of Zone of inhibition in mm				
	500 µg	750 µg	1000 µg	Nitrofurantoin 300 µg	DMSO
S. aureus	15.66±0.57	19.33±0.57	22.33±1.52	14.66±0.57	-
S. saprophyticus	17.66±0.57	21.0±1.0	25.0±1.0	13.66±0.57	-
E. faecalis	11.33±0.57	13.66±0.57	16.66±1.15	13.33±0.57	-
E. coli	9.33±0.57	12.66±1.52	14.33±0.57	12.66±0.57	-
K. pneumoniae	8.66±0.57	12.66±0.57	16.66±0.57	13.66±0.57	-
P. aeruginosa	16.33±0.57	20.0±1.0	21.33±0.57	12.33±0.57	-
P. vulgaris	10.66±0.57	13.66±0.57	15.33±0.57	14.33±0.57	-
P. mirabilis	12.33±0.57	15.66±1.52	18.33±0.57	13.33±0.57	-

Results are mean ± SD of triplicate analysis

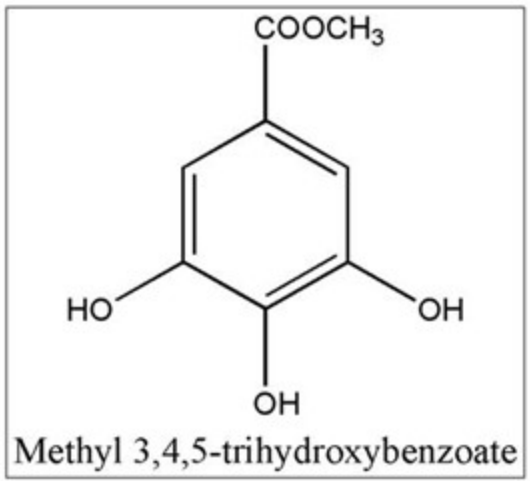


Figure 4. Structure of Methyl 3,4,5-trihydroxybenzoate.

strains were detected with ampicillin, amoxyclav and cephalexin where all the strains were inhibited at 25 µg/mL concentrations and with gentamicin and ciprofloxacin in a range of 3.12 to 12.5 µg/mL concentrations followed with other tested antibiotics in a range of 6.25 to 25 µg/mL. The study identified that ampicillin, amoxyclav and cephalexin are less effective than gentamicin and ciprofloxacin.

The isolates showed high resistance towards ampicillin (81.4%), moderate resistance to azithromycin and doxycycline (74.6%) and nitrofurantoin (74.4%), and less resistance to gentamicin (67.7%) as illustrated in Figure 5. A similar resistance pattern was reported from different parts of India

The antibacterial activity of two isolated compounds from ATME, 24-methylene cycloartanol and methyl 3,4,5-trihydroxybenzoate, was evaluated against MDR strains of uropathogens such as *S. aureus*, *S. saprophyticus*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. vulgaris*, and *P. mirabilis*. The MIC of the compounds was determined using the micro-broth dilution method.

The compound 24-methylene cycloartanol inhibited the growth of *E. faecalis*, *E. coli*, *K. pneumoniae*, and *P. mirabilis* at a very low concentration of 6.25 µg/ml. The growth of *S. aureus*, *S. saprophyticus*, *P. aeruginosa*, and *P. vulgaris* was inhibited at 12.5 µg/ml.

The compound methyl 3,4,5-trihydroxybenzoate demonstrated significant antibacterial activity. It inhibited the growth of *E. faecalis*, *K. pneumoniae*, and *P. mirabilis* at a concentration of 6.25 µg/ml, while the growth of *S. aureus*, *S. saprophyticus*, *E. coli*, *P. aeruginosa*, and *P. vulgaris* was inhibited at 12.5 µg/ml. Both compounds exhibited potent broad-spectrum antibacterial activity, as shown in Figure 6.

The compound methyl 3,4,5-trihydroxybenzoate, isolated from *Galla rhois*, has been reported to exhibit inhibitory effects on periodontopathic bacteria and the formation of *Streptococcus mutans* biofilms, with a MIC of 1 mg/mL. Therefore, it holds potential for use in preventing oral biofilms[34].

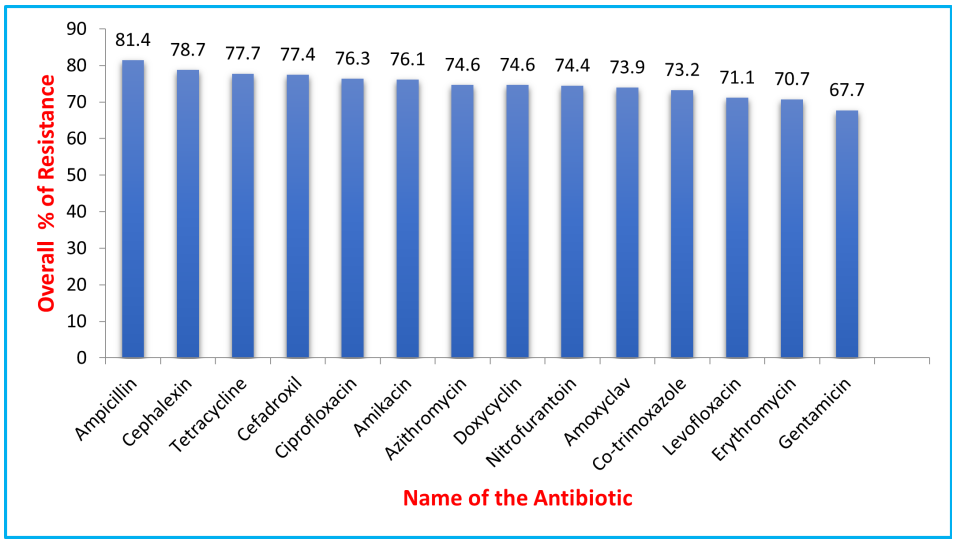


Figure 5. Overall % of antibiotic resistance patterns of clinical isolates of UTI pathogens.

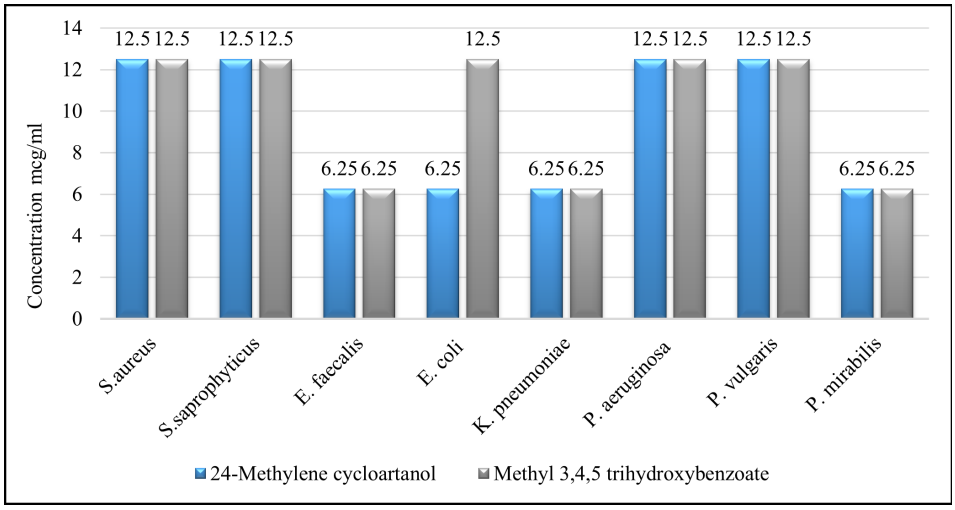


Figure 6. Antibacterial Screening of 24-Methylene cycloartanol and Methyl 3,4,5 trihydroxybenzoate against clinical isolates of UTI pathogens (µg/mL).

5. Conclusion

There have been multiple preliminary antimicrobial studies conducted, which demonstrated that *Amaranthus tricolor* can prevent the growth as well as biofilm formation of multidrug-resistant uropathogens. In this regard, compounds such as flavonoids, tannins, saponins, phenolic acids, and alkaloids have been identified as the potential bioactive compounds responsible for its antimicrobial activity. The antibacterial potential of *A. tricolor* is attributed to various bioactive compounds present in the extract, which function by disrupting the cell wall, enzymes, transcription, and translation processes. The interaction between the *A. tricolor* bioactive compounds and bacterial cells might retard growth or eliminate cell replication, leading to reduced cell density. Hence, a better understanding of the mechanism of action is necessary for the development of good combination antibiotics using antibiotic resistance-reversing agents.

Plant-based drugs carry few adverse effects, but a few cases of hepatotoxicity as a consequence of the use of many *A. tricolor*-containing products have been reported clinically. As with several anti-infective agents, the development of resistance by uropathogens to plants is likely. Additionally, allergic reactions are possible when any organic product, including *A. tricolor*, is used. Fungal contamination is likely in medicinal plants, which is strongly related to the time and conditions of harvesting, drying, and storage. Therefore, the production process should be carefully monitored. Although approximately 80% of the Asian and African populations currently use plant resources to manage various types of infections due to their better bulk availability, lower side effects, and higher effectiveness, a regulatory framework is needed to ensure the safety, quality, and efficacy of herbal medicines. Plant-based treatments are complex, as they are typically used as complementary therapies in conjunction with conventional medications and may work with or interfere with standard medications.

It is clear that *Amaranthus tricolor*, a safe and well-known leafy vegetable, has the potential to act as a cheap and natural remedy for combating a widespread uropathogen. Several further investigations should be conducted to evaluate its clinical applications. At first, further trials are needed to assess reactions (efficacy, toxicity), and medicinal products should be carried out *in vivo* studies using a suitable animal model they assess toxicity. An example of this study is the methanol leaf extract, which can be included in a capsule or liquid form[35,36]. It was recommended that *A. tricolor* undergo further clinical trials and expert consultations to ensure reliable and consistent medical applications.

6. Acknowledgement

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to the authors and publishers of the referenced articles, whose work formed the foundation for the literature reviewed and discussed in this manuscript.

7. Author Contribution

The author independently conceived conceived the study, carried out the research, performed the data analysis, and drafted the manuscript.

8. References

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