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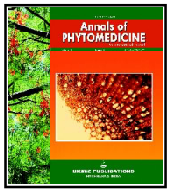

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Isolation of multidrug resistant bacterial pathogens and screening against *Coleus amboinicus* L. extracts

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Abstract

World health organisation (WHO) has declared antimicrobial resistance as a global crisis. Antimicrobial resistance is an emerging issue following the COVID-19. The development of novel antibiotics has become necessary for the treatment of MDR infections. Plants serve as a potential source for developing novel drugs, and herbal-based drugs are often safe. Therefore, the present study focuses on evaluating the anti-MDR bacterial activity of *Coleus amboinicus* L. against multidrug-resistant *Escherichia coli* and *Staphylococcus aureus*. The clinical pathogens were collected and an antibiotic susceptibility test was performed using standard antibiotics to evaluate the resistant pattern of the pathogens. Bioactive compounds of the *C. amboinicus* leaves were extracted using methanol as solvent. DPPH radical scavenging activity and ferric reducing antioxidant power assay were performed. The antibacterial activity and minimum inhibitory concentration (MIC) of the leaf extract against isolated MDR pathogens were investigated. Crude plant extract showed better antioxidant values, 69.31 ± 0.69% of DPPH scavenging and 78.35 ± 0.32% of reducing power was observed on 1000 µg of extract. 500 mg/ml and 1000 mg/ml showed higher zones against both the test organisms. MIC of *E. coli* and *S. aureus* was found to be 15.6 mg and 7.8 mg. From the analysis, bioactive compounds from *C. amboinicus* showed evident antibacterial activity against MDR pathogens and, therefore can be used for the development of antimicrobial drugs for the treatment of MDR infections.

1. Introduction

Antimicrobial resistance refers to a microorganism's capacity to withstand the effects of antibiotics. Resistant microbes are more difficult to treat, necessitating the use of alternative medications or higher antimicrobial doses. Resistance develops by one of three mechanisms: inherent resistance in some bacteria, genetic mutation, or the acquisition of resistance from another species. Antibiotic resistance is not completely understood worldwide, although it is more prevalent in poorer nations with weakened healthcare systems (Elbossaty, 2017).

Superbugs are strains of bacteria that have changed (or mutated) and developed resistance after coming into contact with fourth-generation antibiotics. Methicillin-resistant *Staphylococcus aureus* (MRSA) is probably the best-known superbug that was first observed in 1960. Infection with a superbug might cause no symptoms in some persons. Healthy people can infect vulnerable people without even realising it, if they carry germs without showing symptoms. When symptoms of superbug infections do appear, they differ greatly depending on which organism is attacking. Even young and healthy people can contract a superbug infection. If, the person's immune system has been compromised by a chronic disease or cancer treatment, they will be more susceptible to infection (Read, 2020; Samanjit Kaur and Sneha Hariharan, 2020).

Multidrug resistant (MDR which show resistance against three or more antibiotics) strains of bacterial pathogens is the major challenge the scienticfic world face in this century (Vidya Seshadri, 2021) and they raise medical expenses, lengthens hospital stays, and raises fatality rates. They have begun to wreak havoc on human health in recent years, taking longer to recover and increasing medical expenditures and death. Antimicrobial resistance is expected to kill 10 million people worldwide per year by 2050. Drug-resistant infections claim the lives of at least 700,000 individuals each year. According to the Centres for Disease Control and Prevention (CDC) 2019 reports, more than 2.8 million drug resistant illnesses occur in the United States each year, with more than 35,000 of those being fatal (Wieczorek and Osek, 2011).

The vast majority of *E. coli* strains are harmless, and they are an important part of a healthy human digestive system. On the other hand, some of the *E. coli* strains can cause diseases like diarrhoea or illness outside of the intestine. Diarrhoea-causing *E. coli* strains can be spread through contaminated water or food, as well as contact with animals or people. There are little research on the characterisation of *E. coli* strains that cause bacteremia. Multidrug resistance in *E. coli* is also becoming a global problem, focusing on *E. coli* sequence type (ST) 131, which is increasingly being reported in UTIs (Elbehiry *et al*., 2021).

MRSA (Methicillin-resistant *Staphylococcus aureus*) is a versatile infection that may cause many human disorders. According to data

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from the National Nosocomial Infections Surveillance System, the frequency of nosocomial MRSA infections is gradually growing in the United States. These infections account for more than 60% of critical care unit hospitalisations. Several antimicrobial medicines, including second and third-line medications, have acquired resistance in *S. aureus*. It is also a common cause of food poisoning over the world. While *S. aureus* colonisation does not damage the host, it is a risk factor for developing later symptomatic infections (Panjla *et al*., 2019).

In the face of rising resistance to older antimicrobials and increasingly difficult bacterial infection management due to the increased incidence of multidrug-resistant (MDR) pathogens, the need for innovative antimicrobials has recently been stronger. There is a constant demand for new antibacterial drugs year after year. There is an increase in mortality, particularly for illnesses with few treatment options, such as gram-negative bacteria, which the World Health Organization has designated as "priority pathogens" and a worldwide hazard. As a result, we require innovative antibiotics to treat infections and lower the fatality rate (Rolain *et al*., 2016).

To tackle the issue raised by the MDR strains of bacterial pathogens, unfortunately, there are no new antibiotics with potential activities and thus, the scientists turned to natural bioactive compounds including the secondary metabolites produced in plants in an effort to extract them and to use them in various medical application, provided with the supportive informations handed over through generations (Saravanakumari *et al*., 2020; Gayathiri *et al.,* 2020). Herbal drugs are promising for creating effective and novel pharmaceuticals because plants are a rich source of novel phytocompounds. India is projected to have around 47,500 plant species, accounting for more than 11.4% of the world's total plant species. Approximately, 28% of the plants found in India are indigenous to the country. These medicinal herbs have various biological activities, including antibacterial, antidiabetic, antimicrobial, antiulcer, analgesic, and antiamnesic capabilities, among others. Medicinal herbs are commonly utilised and thought to be safe and less expensive than chemically manufactured medications (Umamaheswari *et al*., 2021).

C. amboinicus, also known as *Plectranthus amboinicus* in the family Lamiaceae is a semi-succulent perennial plant with a spicy oregano-like flavour and odour. It is utilised as a spice, as well as a decorative and medicinal plant. *C. amboinicus* can reach a height of 1 m (3.3 ft). The stem is fleshy, measuring 30-90 cm (12-35 in), and is coated with either long inflexible hairs (hispidly villous) or soft, short, and upright hairs (tomentose). The stems of old trees are smooth (glabrescent). This plant has been used in ancient traditional system for treatment of various ailments. As there were several studies on antibacterial potential of *C. amboinicus,* no reports available on evaluating the anti-MDR bacterial activity. Therefore, the present study focuses on evaluating the anti-MDR bacterial activity of *C. amboinicus* against MDR *Escherichia coli* and *Staphylococcus aureus*. DPPH radical scavenging activity and ferric reducing antioxidant power assay of the extracts were performed.

2. Materials and Methods

2.1 Isolation and identification of pathogens

Clinical isolates of pathogenic bacteriawhich were isolated from the in-patients of a tertiary hospital in Erode, Tamil Nadu were cultured on nutrient media (Himedia). The pathogens were identified by the selective media growth on eosin methylene blue (EMB) agar and mannitol salt agar (MSA). The green metallic sheen on EMB and yellow colonies on MSA indicate *Escherichia coli* and *Staphylococcus aureus.* The strains were isolated and used for additional studies.

2.2 Antibiotic susceptibility test (ABST)

Disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol) is done to test the antibiotic susceptibility of pathogens on Muller-Hinton agar. The isolated *S. aureus* and *E. coli* were separately tested against five antibiotics. Disk diffusion method (Microbiology Systems, Becton Dickinson, USA, MD) as defined in the (NCCLS 2000) National Committee For Clinical Laboratory Standards were used to determine the results. The tested antibiotics and concentration ranges are methicillin (5 mcg), ampicillin (25 mcg), tetracycline (30 mcg), amoxicillin (30 mcg) and streptomycin (30 mcg). The resistant pattern was identified by measuring the zone size and compared by the zone interpretation chart (as per CLSI).

2.3 Collection and processing of plant

C. amboinicus leaves (Figure 1, collected from Mannarkkad, Kerala, identified and authenticated with the number PIDA-3/2021 by Dr. Shamna, Deseeya Ayurvedic Factory, Calicut, Kerala) were washed with distilled water and dried in the shade at room temperature. The dried leaves were grounded to powders and stored in a sterile container for extraction.

Figure 1: ***Coleus amboinicus*** **used in the study.**

2.4 Extraction of bioactive compounds using Soxhlet apparatus

Powders of *C. amboinicus* leaves were packed and positioned in a Soxhlet apparatus. Solvent solution of methanol is filled in the extractor, and the temperature of 60ºC was set and left for 6 h. The extracts were procured and the solvents were vaporised. The dried extracts were procured and kept in sterile containers.

2.5 Antioxidant activity of the plant extracts

2.5.1 DPPH radical scavenging assay (Szerlauth *et al*., 2019)

By utilising the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), as per the procedure described by Blois (1958), the antioxidant activity of the methanolic *C. amboinicus* extracts was

assessed in terms of ability to donate hydrogen or scavenging radicals. Various concentrations (100 µg-1000 µg) of the extracts were taken and the total volume was adjusted to 100 µl with methanol. About 3 ml of methanol solution of DPPH (2.4 mg in 100 ml) was mixed and allowed to rest for 30 min at 27°C. The absorbance (OD values) was calculated at 517 nm. Percentage radical scavenging activity of the sample was measured as follows:

% DPPH radical scavenging activity = (control OD-sample OD / control OD) × 100.

The assay was executed in triplicates and the outcomes were determined in mean ± SD.

2.5.2 Ferric reducing antioxidant power (FRAP) assay (Naji *et al.*, 2020)

The ferric reducing antioxidant power assay was utilised conferring to the method described by Strain and Benzie (1996) to evaluate the reducing ability of the plant extract. The FRAP reagent containing 2.5 ml of 20 mM $FeCl_3.6H_2O$,2.5 ml of a 10 mMTPTZ solution in 40 mMHCl, and 25 ml of 300 mM acetate buffer (pH 3.6) was prepared and kept at 37°C. 3 ml FRAP reagent was introduced with 60 µl of the sample. The reaction mixture was set to incubation for 30 min at 37°C and the absorbance (OD values) was calculated at 610 nm. $FeSO_4$ at different concentrations were used for calibration.

2.6 Antibacterial activity using well diffusion method

Antibacterial efficacy of the plant extract was evaluated against the isolated clinical pathogens *S. aureus and E. coli*. Nutrient agar (nutrient agar composition for 100 ml: sodium chloride: 0.5 g, peptone: 0.5 g; yeast extract: agar 1.5 g; 0.5 g, beef extract: 0.3 g, total pH: 7.0 ± 0.2) was prepared and sterilized, and dispensed into plates. Overnight cultures of test pathogens were used and the culture inoculum of each test organism was streaked with the sterile cotton swab by rotating the plate at a 60º angle for every streaking. To bore wells on the agar surface, a 6 mm well borer was used. To each well, 100 µl of the samples were added and the plates were kept in an incubator at 37ºC for 48 h. The antibacterial efficacy was identified based on the zone of inhibition around the wells in all the nutrient agar plates having test pathogens. The clear zones were observed and measured in millimetres (mm).

2.7 Evaluating minimum inhibitory concentration (MIC) of the plant extracts

To find out minimum inhibitory concentration (MIC), a slight modification of the dilution technique was performed. 1 ml of plant extracts was diluted into various concentrations (1.95 mg/ml, 3.9 mg/ml, 7.8 mg/ml, 15.6 mg/ml, 31.5 mg/ml, 62.5 mg/ml, 125 mg/ml and 250 mg/ml, in 1 ml of sterile nutrient broth in test tubes. A 100 µl of *E. coli* culture at 0.5 Mc Farland standard (Eucast, 2003) was inoculated to the tubes. Correspondingly, this was repeated for *S. aureus*. The tubes were incubated at 37°C for 24 h and observed for growth or turbidity by using an unaided eye (CLSI, 2012).

3. Results

3.1 Antibiotic sensitivity test (Al Laham and Al Fadel, 2014)

The antibiotic sensitivity test is carried out to identify the resistant pattern of the isolated clinical pathogens. The sensitivity/resistance of the pathogens was tested against five broad-spectrum antibiotics. Figure 2 and Figure 3 show the resistance pattern of the pathogens against the antibiotics. The isolated clinical pathogen was resistant to four antibiotics (methicillin, ampicillin, tetracycline and amoxicillin) and intermediate to the streptomycin (Table 1).

Table 1: Antibiotic sensitivity test

S. No	Pathogens	Antibiotics	Inhibitory zones (mm)	Interpretation
1	*Escherichia coli*	Methicillin	-	R
		Ampicillin	-	R
		Tetracycline	-	R
		Amoxicillin	-	R
		Streptomycin	12	I
2	*Staphylococcus aureus*	Methicillin	-	R
		Ampicillin	-	R
		Tetracycline	-	R
		Amoxicillin	-	R
		Streptomycin	14	I

*S-sensitive; I-intermediate; R-resistant

3.2 Antioxidant activity of the plant extracts

3.2.1 DPPH radical scavenging activity

DPPH radical scavenging activity of *C. amboinicus* extracts was evaluated using ascorbic acid as standard. Five concentrations of the plant extracts were used (100 µg, 250 µg, 500 µg, 750 µg and 1000 µg). 100 µg of plant extracts showed 13.68 ± 2.35% of DPPH scavenging, 26.13 ± 0.78% for 250 µg, 43.77 ± 1.54% for 500 µg, 65.21 ± 0.88% for 750 µg and 69.31 ± 0.69% for 1000 µg. Similarly, ascorbic acid at showed 44.06 ± 0.48%, 62.54 ± 0.35%, 75.43 ± 0.89%, 81.25 ± 0.5% and 88.73 ± 1.12% of DPPH scavenging for 100 µg, 250 µg, 500 µg, 750 µg and 1000 µg. Figure 4 shows the graphical representation of the DPPH radical scavenging activity of plant extract.

3.2.2 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power of the plant extract was evaluated using 5 different concentrations. 100 µg, 250 µg, 500 µg,

750 µg and 1000 µg of plant extracts showed 26.13 ± 0.97%, 58.29 ± 0.41%, 64.82 ± 0.58%, 70.36 ± 1.27% and 78.35 ± 0.32% of reducing power. Whereas, standard ascorbic acid showed 51.27 ± 1.38%, 61.46 ± 1.57%, 66.91 ± 0.77%, 89.47 ± 0.24% and 97.13 ± 0.96% of reducing power. Figure 5 shows the graphical representation of reducing power of the plant extract.

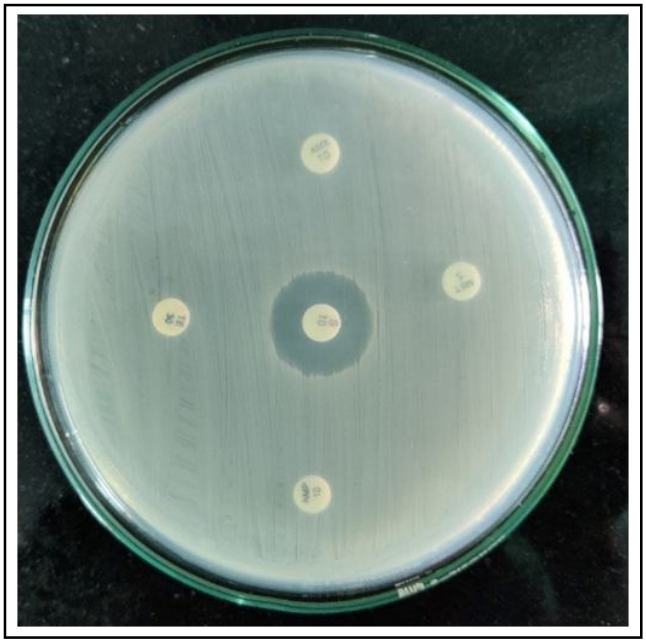

Figure 2: Antibiotic susceptibility of the *Escherichia coli.*

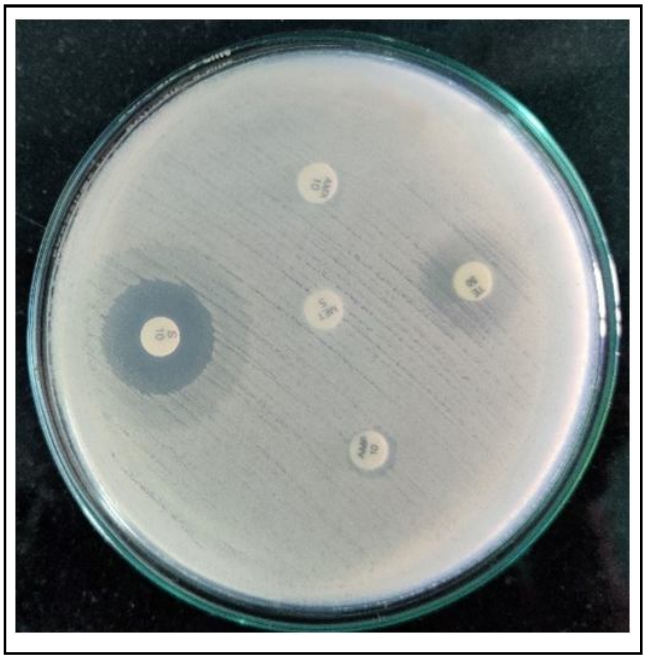

Figure 3: Antibiotic susceptibility of the *Staphylococcus aureus.*

3.3 Antibacterial analysis

Antibacterial activity of the *C. amboinicus* methanolic extracts were evaluated using the well diffusion method. Four concentrations, *i.e.*, 125 mg/ml, 250 mg/ml, 500 mg/ml and 1000 mg/ml were used against the isolated multidrug resistant pathogens. 500 mg/ml and 1000 mg/ml showed higher zones against both the test organisms. Figure 6 and Figure 7 show the antibacterial activity of the *C. amboinicus* extract against MDR *E. coli* and *S. aureus* using well diffusion method (Table 2 and Figure 8).

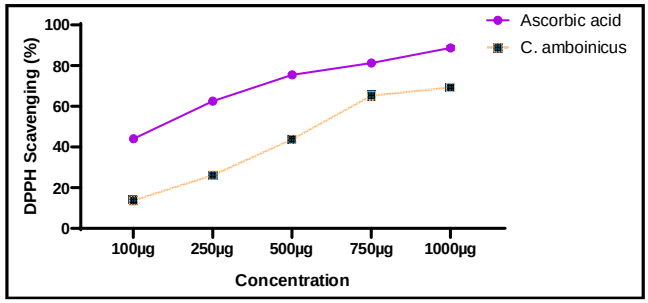

Figure 4: DPPH radical scavenging activity of *C. amboinicus* extracts.

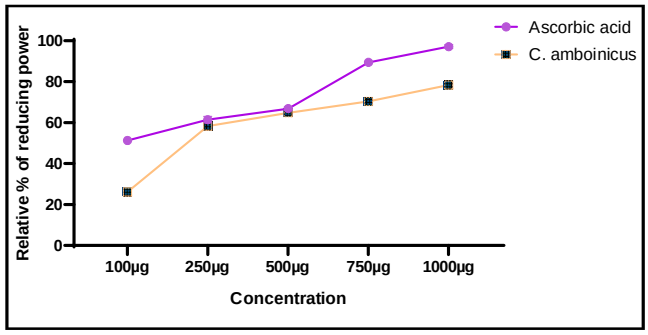

Figure 5: Ferric reducing antioxidant power of *C. amboinicus* extracts.

Figure 6: Antibacterial activity of *C. amboinicus* extracts against *E. coli* using well diffusion (extract concentration from top, clockwise: 0.125 g, 1 g, 0.5 g and 0.25 g).

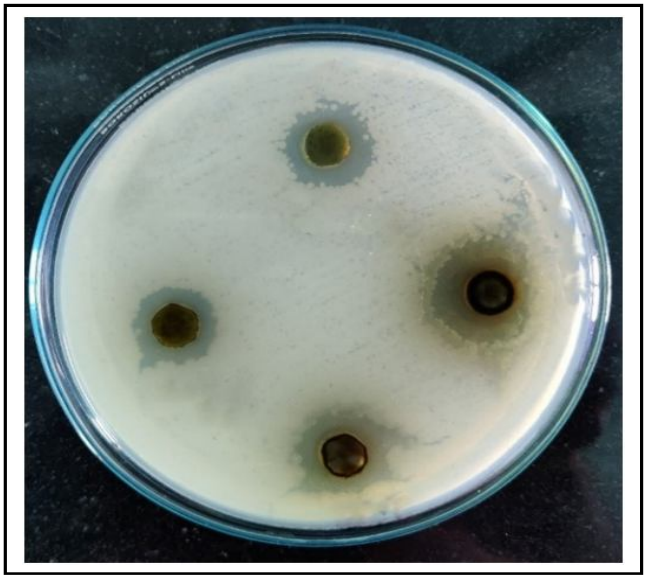

Figure 7: Antibacterial activity of *C. amboinicus* extracts against *Staphylococcus aureus* using well diffusion (extract concentration - from top, clockwise: 0.125 g, 1 g, 0.5 g and 0.25 g).

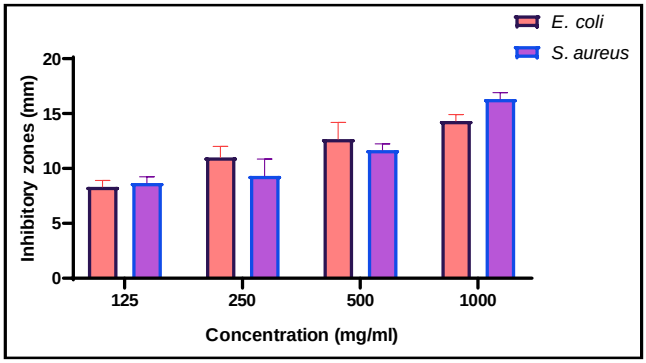

Figure 8: Antibacterial analysis of *C. amboinicus* extracts against MDR pathogens.

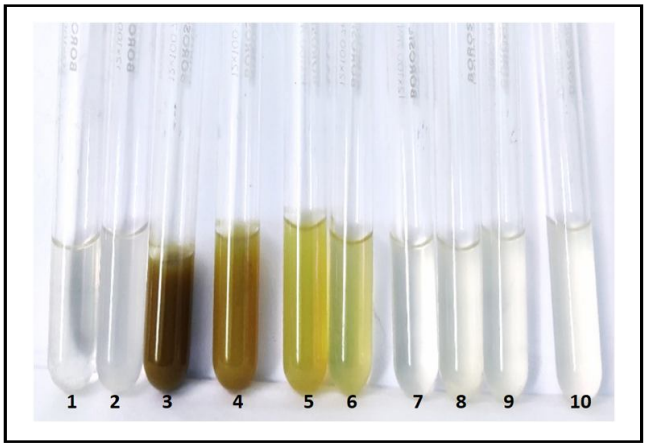

Figure 9: MIC evaluation *C. amboinicus* extracts against *Escherichia coli* (tubes from left to right: 1: positive control, 2: negative control, 3: 250 mg, 4: 125 mg, 5: 62.5 mg, 6: 31.5 mg, 7: 15.6 mg, 8: 7.8 mg, 9: 3.9 mg and 10: 1.95 mg).

Table 2: Antibacterial activity of the *C. amboinicus* methanolic extracts using the well diffusion method

S. No.	Concentration	Inhibitory zones (mm)	
		E. coli	*S. aureus*
1	125 mg/ml	8.33 ± 0.57	8.66 ± 0.57
2	250 mg/ml	11 ± 1	9.33 ± 1.52
3	500 mg/ml	12.66 ± 1.52	11.66 ± 0.57
4	1000 mg/ml	14.33 ± 0.57	16.33 ± 0.57

3.4 Minimum inhibitory concentration (MIC) analysis (Chikezie, 2017)

The minimum concentration of the plant extract that shows inhibition against the test pathogens were evaluated using the turbidity method. About 8 concentrations of the plant extracts were used. The concentration showing no growth of the pathogen was identified by comparing with the positive control. Figure 9 shows the MIC determination against *E. coli* and Figure 10 shows the MIC determination against *S. aureus*. From the analysis, MIC of *E. coli* and *S. aureus* was found to be 15.6 mg and 7.8 mg, respectively.

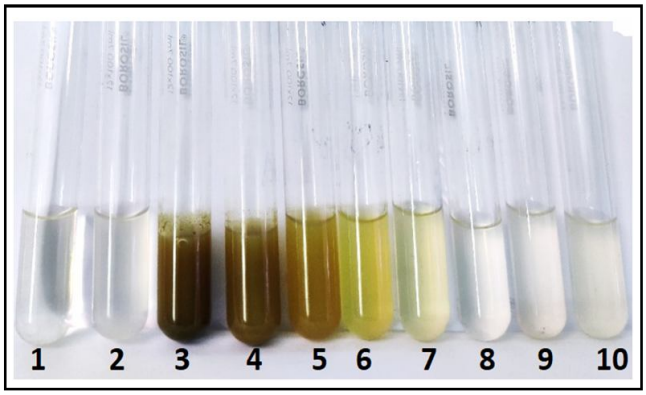

Figure 10: MIC evaluation *C. amboinicus* extracts against *Staphylococcus aureus* (tubes from left to right: 1: positive control, 2: negative control, 3: 250 mg, 4: 125 mg, 5: 62.5 mg, 6: 31.5 mg, 7: 15.6 mg, 8: 7.8 mg, 9: 3.9 mg and 10: 1.95 mg).

4. Discussion

World health organisation (WHO) has announced antimicrobial resistance and a global crisis. Antimicrobial resistance is an emerging issue following the COVID-19. Development of novel antibiotics has become necessary for treatment of MDR infections. Plants serves as a potential source for development of novel drugs and herbal based drugs are often safe. Therefore, the present study focuses on evaluating the anti-MDR bacterial activity of the *C. amboinicus.*

C. amboinicus is native to Southern and Eastern Africa, ranging from South Africa (KwaZulu-Natal) and Eswatini to Angola and Mozambique, and north to Kenya, Tanzania, and India, though it is largely grown and naturalised elsewhere in the tropics, where it can be found in coastal or bushwoodland, on rocky slopes and loamy or sandy flats at low elevations. *C. amboinicus* is a medicinal plant that has been used for centuries to cure a variety of ailments including throat infections, cough and fever, diarrhoea, nasal congestion, and digestive issues. Endophytic fungi that produce antibacterial

compounds were found in the plant. It is commonly used to treat colds and also it aids in the prevention of foul breath.

C. amboinicus leaves were collected and dried. Bioactive compounds were extracted using methnol as solvent. Leaf extracts showed significant inhibitory zones against multidrug resistant bacterial pathogens. Menéndez and Pavón (1999) found that an ethanolic extract of *C. amboinicus* has bacteriolytic activity against *S. aureus* and intestinal infections. The antibacterial action of *C. amboinicu* ssential oil, as reported by Judith *et al*. (2009) is most likely owing to the presence of carvacrol, which accounts for 65.2% of the oil and is well recognised as an antibacterial and insecticidal. Only ethanolic extracts demonstrated antibacterial action against all strains, whereas aqueous extracts showed no activity at all. According to Nivya *et al*., (2014) ethanol extracts from the leaves of *C. amboinicus* showed antibacterial efficacy against *Bacillus cereus, Bacillus subtilis, S. aureus, Shigella flexneri* and *Salmonella paratyphi. A*. These findings well correlates with the present study.

Furthermore, essential oil derived from the leaf of *C. amboinicus* was found to have antifungal action against two fungus species, *Candida albicans* and *Aspergillus niger* (Anjali *et al*., 2014). The highest zone of inhibition was identified against *Candida albicans*. Vizoso *et al*. (1999) showed dose-dependent cytotoxic and genotoxic effects against *Aspergillus nidulans*, indicating that the essential oil of *C. amboinicus* has antifungal potential. Farida *et al*. (2020) evaluated the cytotoxicity of *C. amboinicus* against colon cancer, finding that the extract had substantial anticolon cancer action against WiDr cells when compared to 5-flurouracil, a conventional medication. According to Puji *et al*. (2021), *C. amboinicus* demonstra-ted modest cytotoxicity against T47D cells and was not selective towards normal cells and so has the potential to be used as a lead chemical therapeutic agents.

5. Conclusion

Anti-multidrug resistant bacterial activity of *C. amboinicus* against multidrug resistant *Escherichia coli* and *Staphylococcus aureus* was investigated in the present study. The clinical pathogens were collected and antibiotic susceptibility test was performed using standard antibiotics to evaluate the resistant pattern of the pathogens. Bioactive compounds of the *C. amboinicus* leaves were extracted using methanol as solvent. The antibacterial activity and minimum inhibitory concentration (MIC) of the leaf extract against isolated MDR pathogens was investigated. From the analysis, bioactive compounds from *C. amboinicus* showed evident antibacterial activity against MDR pathogens. Apart from the antibacterial analysis other biological properties of the plant extract, *i.e.*, antioxidant activity was performed. The plant extract showed significant antioxidant values on DPPH radical scavenging and reducing power. Further studies are required to purify the bioactive compounds responsible for the antibacterial action and such compounds can be used for novel antibiotics for treatment of MDR infections.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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