OPEN ACCESS

**Saudi Journal of Biomedical Research**
Abbreviated Key Title: Saudi J Biomed Res
ISSN 2518-3214 (Print) |ISSN 2518-3222 (Online)
Scholars Middle East Publishers, Dubai, United Arab Emirates
Journal homepage: https://saudijournals.com

**Original Research Article**

# Antimicrobial Activity of *Madhuca longifolia* leaf Extract against Multi Drug Resistant Bacteria and its Synergistic Effects

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**DOI:** 10.36348/sjbr.2023.v08i12.001 | **Received:** 18.11.2023 | **Accepted:** 26.12.2023 | **Published:** 27.12.2023

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## Abstract

*Madhuca longifolia*, commonly known as Mahua, is a revered tree indigenous to the Indian subcontinent, celebrated for its versatile uses in traditional medicine and cultural practices. In this study, the antibiotic effects of ethanolic extract of *Madhuca longifolia* leaves was evaluated against six MDR and ATCC bacterial strains. The results showed that the leaf extract of *Madhuca longifolia* exhibited significant antimicrobial activity against all tested MDR bacterial strains. The plant has enormous bioactive compounds, which can be potentially responsible for the observed antimicrobial activities. Also, the synergistic effect of the leaf extract gave promising results when applied along with Amikacin and Ceftazidime-Avibactam. The acquired results suggest that *Madhuca longifolia* may be a potential source of natural antibacterial agents that can be used in the development of new drugs to treat resistant bacterial infections. Further studies are required to identify and isolate the active ingredients responsible for the observed activity and to evaluate their efficacy and safety in vivo.

**Keywords:** *Madhuca longifolia*, Antimicrobial Resistance, Synergy, Antibiotics.



## INTRODUCTION

In the relentless struggle against infectious diseases, the alarming escalation of multi-drug resistant (MDR) bacteria has become a major global health concern (WHO, 2017). MDR bacteria, or multi-drug resistant bacteria, refer to strains of bacteria that have developed resistance to multiple classes of antimicrobial drugs, commonly including antibiotics. These bacteria have acquired genetic mutations or mechanisms that allow them to survive exposure to a range of drugs that are designed to inhibit or kill them. As a result, the effectiveness of traditional antibiotic treatments becomes compromised, making infections caused by MDR bacteria more challenging to manage and treat. Their capacity to resist the therapeutic effects of multiple antimicrobial agents, including antibiotics, thereby limiting the efficacy of conventional treatment modalities (WHO, 2017) is causing the biggest threat to public health leading to increased morbidity, mortality, and healthcare costs (CDC, 2019).The development of multi-drug resistance is a complex process driven by various factors, primarily the selective pressure exerted by the widespread and often inappropriate use of antibiotics in both human and veterinary medicine (Ventola *et al*., 2015). The overuse of these life-saving drugs has led to the evolution of bacterial strains equipped with sophisticated mechanisms, such as the acquisition of resistance genes through horizontal gene transfer and the activation of intrinsic defense mechanisms (Blair *et al*., 2015). The dire need for innovative solutions to combat MDR bacteria has prompted an exploration of alternative therapeutic avenues, with natural products emerging as a promising source of novel antimicrobial compounds.

One such botanical candidate, *Madhuca longifolia* commonly known as Mahua or Indian Butter Tree, has been historically acknowledged for its medicinal properties (Pareek *et al*., 2011). With a legacy deeply rooted in indigenous healing practices, *Madhuca longifolia* emerges as a promising candidate for the investigation of novel therapeutic agents, particularly in the context of antimicrobial activities against challenging

microbial adversaries like multi-drug resistant bacteria. Indigenous to the Indian subcontinent, *Madhuca longifolia* has been utilized in traditional medicine for centuries, with its leaves recognized for their diverse pharmacological potential (Jain *et al*., 2010). Recent studies have highlighted the complex phytochemical profile of *Madhuca longifolia* leaves, which includes alkaloids, flavonoids, tannins, and other bioactive constituents. Such constituents are of particular interest due to their documented antimicrobial activities against various pathogens (Gupta *et al*., 2017, Cowan, M. M., 1999).

This research aims to investigate the antimicrobial efficacy of *Madhuca longifolia* leaf extract specifically against MDR bacteria, addressing a critical gap in our current understanding of alternative therapies. By unraveling the molecular interactions between the bioactive compounds in *Madhuca longifolia* and MDR bacterial strains, we seek to contribute valuable insights that may inform the development of novel antimicrobial agents derived from natural sources. The urgency of this investigation is underscored by the pressing global need for effective strategies to combat the rise of MDR bacteria and the potential of botanical remedies to offer sustainable and innovative solutions (Cushnie *et al*., 2011).

In this paper, we present the results of our comprehensive study on the antimicrobial activity of *Madhuca longifolia* leaf extract against MDR bacteria, providing a foundation for future research and the development of new therapeutic approaches in the battle against drug-resistant infections. Also, we will show the synergistic effect of the extract with already existing antibiotics in the market and how it can enhance the efficiency of those antibiotics against MDR bacterial strains.

## EXPERIMENTAL PROCEDURES

### MATERIALS

*Madhuca longifolia* leaves were collected from Kankrajhore Forest (Latitude *is 22.70701 and Longitude is 86.67468) on 11th November 2023* and thoroughly washed with distilled water. The clean leaf was then dried for a bit and was cut into very small pieces leaving behind the vein of the leaf which was discarded. The 6 bacterial strains namely ATCC *Escherichia coli*, MDR *Escherichia coli*, MDR *Klebsiella pneumoniae*, MDR *Pseudomonas aeruginosa*, ATCC *Staphylococcus aureus* and MRSA were collected from the repository of the Department of Microbiology and Molecular Biology at Peerless Hospitex Hospital and Research Centre Ltd. 70% ethanol was used for the entire working setup. Antibiotic disks of Amikacin (AK, 30 µg) and Ceftazidime-Avibactam (CAZ, 10/4 µg) were taken from the departmental stock.

### PREPARATION OF EXTRACT

1 gram of the crushed leaf was suspended in a small falcon tube and submerged in 5ml (70%) Ethanol. It was vortexed and left for 48 hours at room temperature. After 48 hours, it was centrifuged and discarded the leaf pieces and only the green alcohol part was taken for the experiment. Figure 1 shows details about the plants and extract prepared.

**Fig 1: The leaves of *Madhuca longifolia*, its small cut pieces without the vein and the extract**

### AGAR AND BROTH PREPARATION

4.2 gram of Mueller Hinton Broth powder was dissolved in 200ml of distilled water (Standard rate: 21 gram in 1 litre of water) in a conical flask and autoclaved at 121º Celsius for 15min to sterilize it.

19 gram of Mueller Hinton Agar Powder was dissolved in 500ml of distilled water (Standard rate: 38 gram in 1 litre of water) in a conical flask and autoclaved at 121° Celsius for 15min to sterilize it. It was poured into sterilized petri plates.

**MacFarland 0.5 suspension**

Accurate measurement of microbial density is fundamental in microbiological research, diagnostics, and industrial applications. The MacFarland standard, particularly the MacFarland 0.5 suspension, serves as a crucial reference point for bacterial density standardization. The meticulous steps involved in preparing a MacFarland 0.5 suspension, ensuring precision and reproducibility in microbiological experiments were as follows:

**Selection of Microorganisms:** After selecting, the specific bacterial strains (in this experiment 6) relevant to this study and after confirmation that the strain was pure and well characterized (O'Neill, J. 2016) they were used in this experiment.

**Subculture Preparation:** The pure culture of the chosen microorganisms were inoculated in lawn cultures onto a suitable agar medium. After incubation under optimal conditions until well-defined colonies are formed (Garrity, G. M *et al.*, 2005) it was used in the experiment.

**Suspension Preparation:** Several isolated colonies were aseptically transferred into a tube containing a sterile saline solution (0.9% NaCl). The colonies were gently emulsified in the saline solution to create a homogenous bacterial suspension (Willey *et al.*, 2019).

**Densitometer Calibration:** It was **c**alibrated in a spectrophotometer or a densitometer to the MacFarland standard and adjusted the instrument to the turbidity level equivalent to a 0.5 McFarland standard (Wayne, PA 2018, McFarland, J., 1907). Finally, each bacterial strain was emulsified in sterile 0.9% NaCl (w/v) to obtain $10^8$ CFU (0.5 McFarland scale) per ml as inoculum for MIC measurements (Onawunmi, 1989).

## METHODS

**Minimum inhibitory concentration (MIC) assay**

Antibacterial activity was determined followed by minimum inhibitory concentration (MIC). 100 µl of plant extract was poured into each microtiter well, and then 100 µl of extract was mixed with it in the first well. The 100 µl was then added to the next well along with 100 µl of Mueller-Hinton broth and the process continued until the 8th well. As a result, the concentration of the extract in the first well was halved, and the extract was serially diluted 2-fold up to the 8th well. Then, 10 µL of different bacterial suspensions were added to each well in separate columns. On the other hand, a control series without added plant extract was selected for each strain. Only MH broth, bacterial suspension, and ethanol were present in serial dilutions. The optical density at 620 nm was then measured and used as the baseline absorbance value. After the optical density measurements were completed, the plates were incubated at 37°C for 24 h. After 24 hours, the optical density at 620 nm was measured again. The optical density values of the wells in the initial measurements were then subtracted from the final measurements. MIC values were determined by determining the lowest concentration of extract that gave an optical density value lower than that of the control wells. Figure 2 shows a picture of the microtiter plate used.

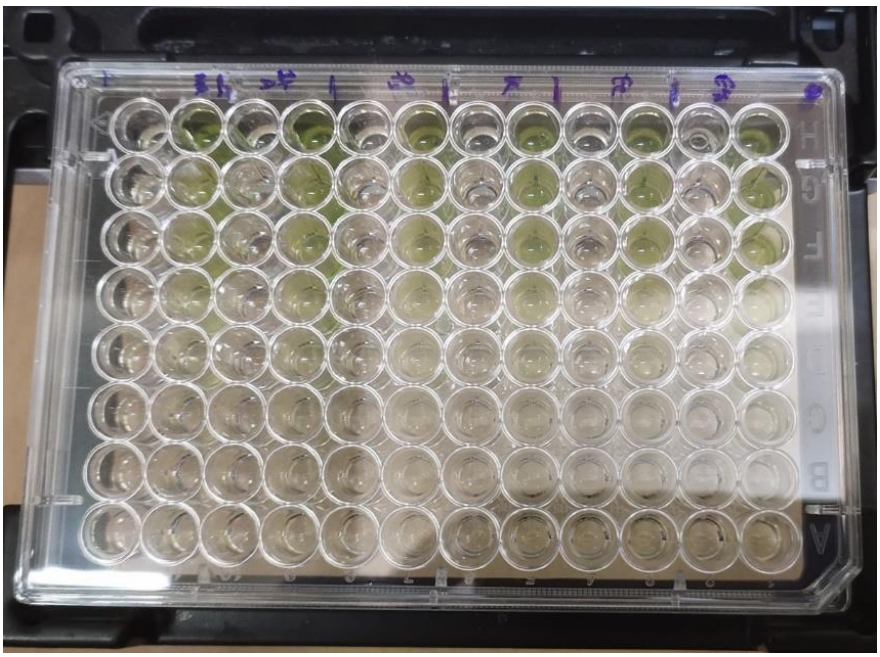

**Fig 2: Microtiter plate for Minimum Inhibitory Concentration (MIC) assay**

**SYNERGY TEST**

Furthermore, a synergy test was performed on MDR *Pseudomonas aeruginosa* using disk diffusion method. The antibiotic disks that were chosen for this experiment were Amikacin (AK) and Ceftazidime Avibactam (CAZ). A single MH agar petri plate was taken and streaked with MDR *Pseudomonas aeruginosa*. The plate was then divided into half with a marker in the middle, and on one side a small circular piece of whatman filter paper dipped and soaked in 70% ethanol was placed followed by two small circular pieces of whatman filter paper dipped and soaked in the madhuca extract along with single disc of AK and a disc of AK along with a small circular pieces of whatman filter paper dipped and soaked in the madhuca extract. Similarly, on the other side of the plate, same process was repeated, just in place of AK, we used CAZ. The plates were then kept for overnight incubation at $37^0$ Celsius. Figure 9 shows the disk diffusion plate of synergy test.

## RESULTS

The analysis of the generated results showed *Madhuca longifolia* ethanolic extract was effective against all 6 bacterial strains that were chosen for this study. Out of the 6 bacterial strains, the ATCC *Escherichia coli*, MDR *Escherichia coli* and the MDR *Klebsiella pneumoniae* have shown a MIC value of 1.56 mg/ml each, whereas MDR *Pseudomonas aeruginosa*, ATCC *Staphylococcus aureus and* MRSA have shown a MIC value of 0.78 mg/ml each. All results are shown in Figure 4-9.

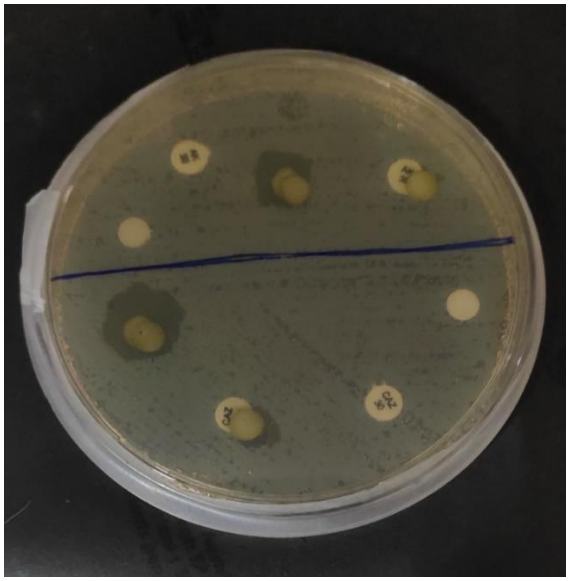

**Fig 3: Synergy test using CAZ and AK with extract soaked in discs**

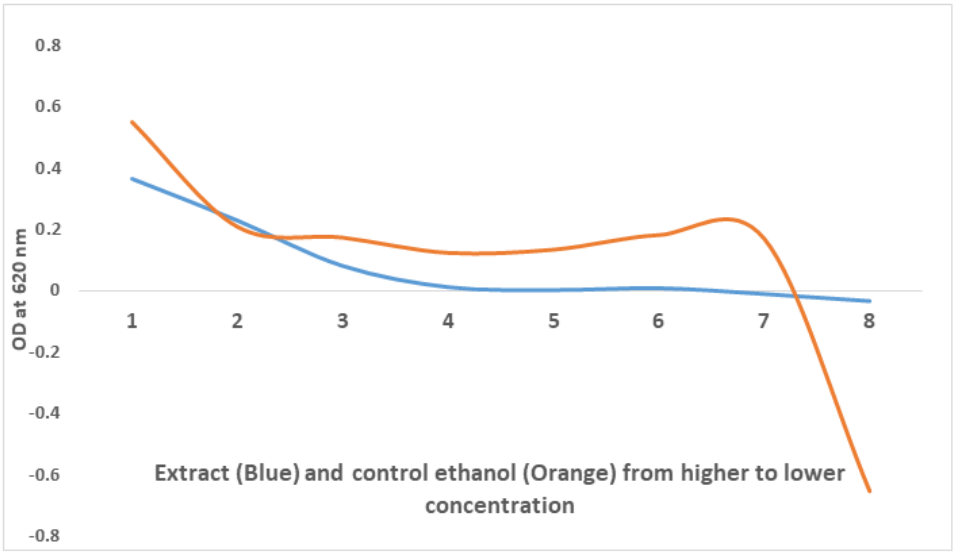


**Fig 4: Antimicrobial action of *Madhuca longifolia* leaf extract against ATCC *Escherichia coli*. The MIC value is 1.56 mg/ml. Initial higher O.D. values were due to the colour of the extract**

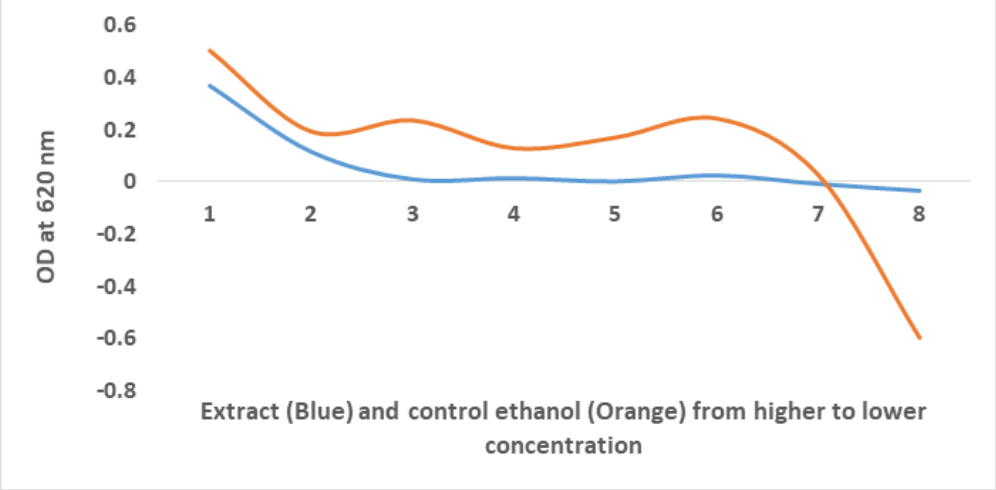


**Fig 5: Antimicrobial action of *Madhuca longifolia*leaf extract against MDR *Escherichia coli*. The MIC value is 1.56 mg/ml. Initial higher O.D. values were due to the colour of the extract**

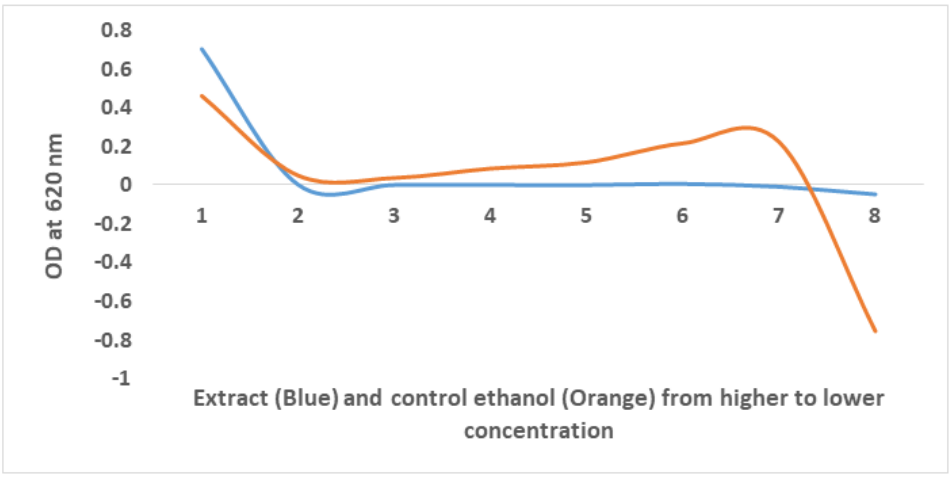


**Fig 6: Antimicrobial action of *Madhuca longifolia* leaf extract against MDR *Klebsiella pneumoniae*. The MIC value is 1.56 mg/ml. Initial higher O.D. values were due to the colour of the extract**

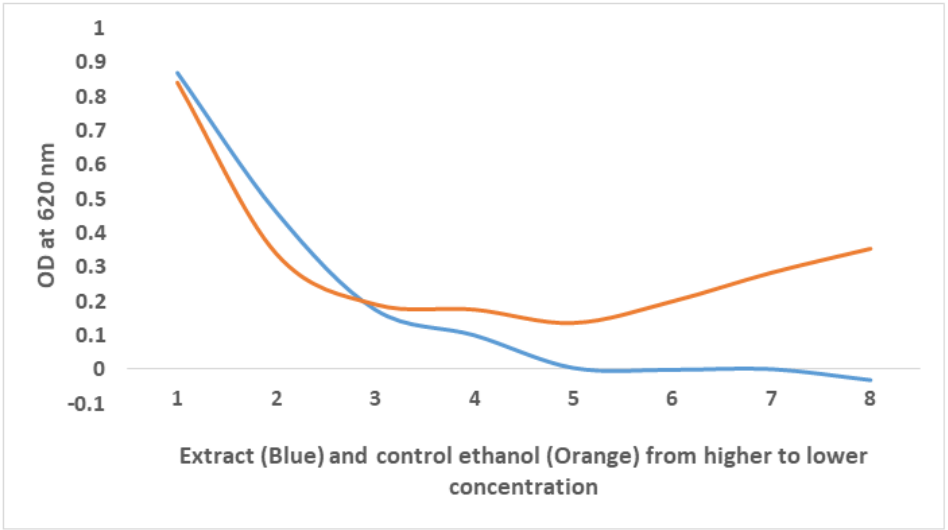


**Fig 7: Antimicrobial action of *Madhuca longifolia* leaf extract against MDR *Pseudomonas aeruginosa*. The MIC value is 0.78 mg/ml. Initial higher O.D. values were due to the colour of the extract**

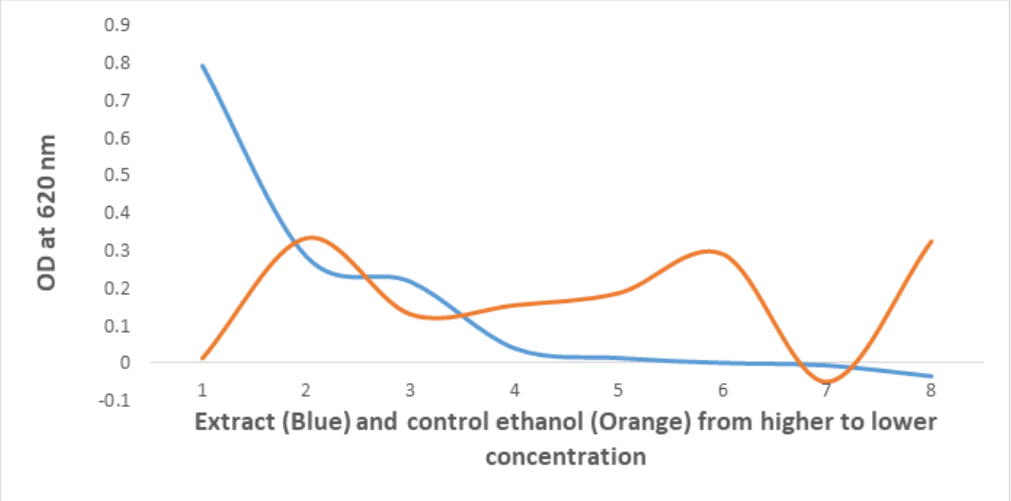


**Fig 8: Antimicrobial action of *Madhuca longifolia* leaf extract against ATCC *Staphylococcus aureus*. The MIC value is 0.78 mg/ml. Initial higher O.D. values were due to the colour of the extract**

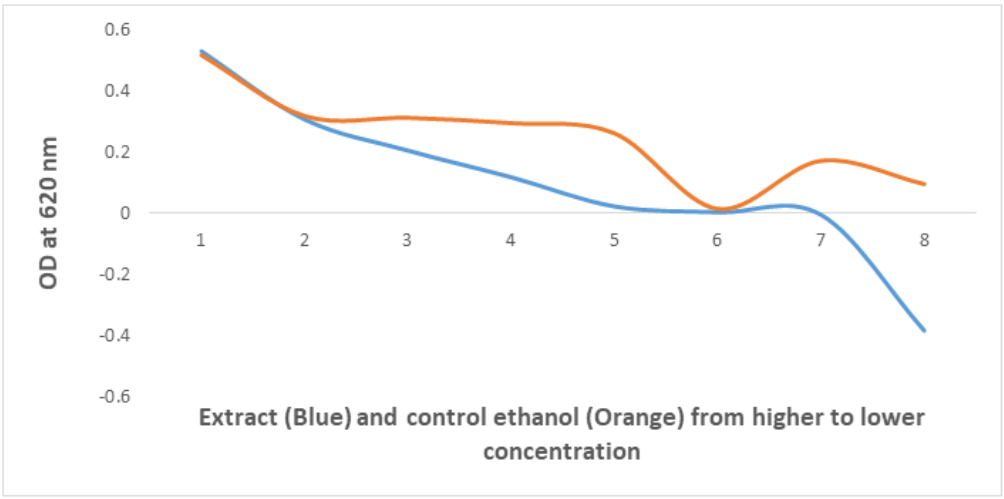


**Fig 9: Antimicrobial action of *Madhuca longifolia* leaf extract against MRSA. The MIC value is 0.78 mg/ml. Initial higher O.D. values were due to the colour of the extract**

The synergy test results showed a zone of 14mm in double disc soaked in the extract on the side of CAZ, a zone of 5mm where extract soaked disc was placed with a CAZ disc, and a zone of 12mm in double disc soaked in the extract on the side of AK. Figure 3 shows the disc diffusion plate of the synergy test.

## DISCUSSION

Even in ancient times, people used plants to treat various infections and infectious diseases. Scientific research is being conducted to demonstrate the therapeutic effects of numerous medicinal plants. Today, in many countries, medicinal plants are used to treat various infectious diseases. The rise in drug-resistant bacteria and the emergence of new and dangerous bacterial strains is currently sparking global interest in medicinal plants as therapeutic agents. After testing a large number of plants against different bacterial strains in vitro, it has been observed that extracts and pure ingredients of many medicinal plants are particularly effective against bacterial strains. The results of the study conducted by our group revealed that *Madhuca longifolia* leaf extract exhibited antibacterial activity against the tested pathogenic strains that are resistant to antibiotics and can even be used in a synergistic manner with existing antibiotics to increase their efficiency.

The effectiveness of the active ingredients that are present in the plant extract inhibits the growth of these tested pathogenic strains within the microtiter wells. Some bacterial strains may have resistance mechanisms such as for example, reducing intracellular drug accumulation, altering target sites, inactivating enzymes, etc. No inhibition was observed in the control, indicating that the solvent cannot act as an antimicrobial agent. Almost all tests showed that the crude ethanol extract showed better inhibition against all bacterial strains tested, suggesting that the active components of the plant material can be extracted with ethanol. Successful isolation, purification, and characterization of

bioactive compounds requires further research using chromatographic and spectroscopic methods. The different components that are present in the plant extracts of *Madhuca longifolia* are being separated using spectroscopic methods and carbohydrates, flavonoids, glycosides, triterpenoids, phenolic compounds and tannins were found (Sarma *et al*., 2013). Quercetin is a flavone found in the leaves of *Madhuca longifolia*. It has antibacterial, antiviral, anti-carcinogenic, and anti-inflammatory properties (Umadevi *et al*., 2015).

Lastly, the gene associated with that particular compound present in the plant that is responsible for antimicrobial effect can be extracted and introduced into bacterial system as a recombinant DNA for bulk production of that compound and sustainable biotechnology. This can give rise to a new class of antibiotics (Nguyen TLA *et al*., 2022).

## CONCLUSION

In conclusion, *Madhuca longifolia* leaf extract has shown strong antibacterial effects in various studies. The presence of secondary metabolites such as tannins, flavonoids, alkaloids, and phenols or Quercetin in the extract is believed to be responsible for its antibacterial activity. This extract has been shown to be effective against many pathogenic bacteria and fungi, including multidrug-resistant strains. The results of these studies suggest that *Madhuca longifolia* may be a promising source of natural antibacterial agents for the development of new drugs and treatments. However, further research is needed to identify the specific compounds responsible for the extract's activity and assess its safety in terms of toxicity, side effects and efficacy in clinical practice.

**Conflict of Interest:** The author declares no conflict of interest.

**Author's Contribution**

Dr. Satadal Das designed the study procedure and collected the plant sample. Mr. Swapnil Chakraborty prepared the plant extract and carried out the experiment under the supervision of Mr. Arup Kumar Dawn with proper guidance. Mr. Swapnil Chakraborty analysed the data and wrote the manuscript. Dr. Satadal Das and other Authors reviewed and edited the manuscript.

**Funding Source:** This study was not supported by any funding.

## ACKNOWLEDGEMENT

We hereby acknowledge the Managing Director, Peerless Hospitex Hospital & Research Centre Limited, Kolkata for providing me with the opportunity to pursue this research work in this esteemed institute.

**ABBREVIATIONS**

ATCC - American Type Culture Collection
MDR - Multi Drug Resistant
OD - Optical Density
MH - Mueller Hinton
MRSA - Methicillin Resistant *Staphylococcus aureus*
MIC - Minimum Inhibitory Concentration
AK – Amikacin
CAZ – Ceftazidime-Avibactam

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