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COMBATTING COLISTIN RESISTANCE: FLAVONOIDS FROM NAGARMOTHA (*CYPERUS ROTUNDUS*) AGAINST BACTERIA ISOLATED FROM THE NARMADA RIVER

JUHI SHARMA[1], SHREYA SHUKLA[1]*, ANJANA SHARMA[2] AND PUSHPENDRA SINGH BUNDELA[3]

[1]School of Life and Basic Sciences, Jaipur National University, Jaipur (Raj) India
[2]Member Academics, CG private University Regulatory Commission, Raipur, India
[3]Regional Director, Pollution Control Board, Rewa (MP), India

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Key words :

Abstract– Narmada, a holy river of central India, was studied for the presence of colistin-resistant Gram-negative bacteria, through its course in Madhya Pradesh, and possible uses of ethanolic extract of Nagarmotha (*Cyperus rotundus*) were investigated. Out of 250 water samples, 83% showed presence of Gram-negative bacteria, including pathogenic forms, a higher percentage of which were resistant to colistin. The ethanolic extract of *C. rotundus* (*Nagarmotha*) was effective against more than 90% isolates, except *Shigella sp*. The flavonoid rich ethanolic extract of *C. rotundus* showed presence of flavonoids i.e., quinic acid, gallic acid, kaempferol, luteolin and quercetin, and some terpenoids, *i.e*., ursolic acid, acetic acid and rhamnetin when chromatographed via GC-MS. *Cyperus rotundus* extract showed potential as an adjunct to colistin therapy, particularly in combating colistin-resistant bacteria.

INTRODUCTION

Fresh surface water is a vital resource that significantly impacts human health, well-being, and economic stability. It plays a crucial role in food production, safety, and security. The link between water pollution and negative effects on human health, particularly concerning food safety, is well-established (Bergen, *et al.,* 2006; Gupta *et al*., 2020).

In India, the Narmada River is a critical water source, particularly in Madhya Pradesh, where it originates and flows through a significant portion of the state. Revered as one of Hinduism's seven most sacred rivers, the Narmada is a center for religious pilgrimages and serves as a key water resource for millions, supporting drinking water needs, agriculture, and daily life.

However, contamination of this vital river has raised concerns about public health. One of the major global public health threats linked to water contamination is antibiotic resistance, specifically the rise of multidrug-resistant (MDR) Gram-negative bacteria (MDR-GNB). The lack of new antibiotics to combat MDR-GNBs has led to a renewed interest in older antibiotics, such as polymyxins, which were once considered too toxic for clinical use. Currently, polymyxin E (colistin) and polymyxin B are still in use for treating resistant infections (El-Khatib and Basyony, 2024).

However, resistance to colistin has also emerged rapidly, with various resistance mechanisms being identified, including endogenous, mutational, and transmissible pathways (Mohamed Abd El-Gawad El-Sayed *et al*., 2020). This development has heightened the urgency for finding alternative treatments, where use of plant derivatives being the front runners.

In light of the growing problem of antibiotic resistance, plant-based treatments have garnered attention as a promising alternative. These treatments are often cost-effective and carry fewer side effects compared to conventional antibiotics. Research into plant-based antibacterial agents is becoming increasingly important as the rise of resistant pathogens continues. Additionally, some plant compounds have been shown to enhance the

effectiveness of traditional antibiotics through synergistic interactions, making them valuable in combating resistant bacteria (Grimbs *et al*., 2017).

This study aims to evaluate the effectiveness of various medicinal plants in combating Colistin-resistant pathogenic bacteria isolated from the Narmada water throughout Madhya Pradesh. The findings could contribute to the development of alternative therapeutic strategies to combat antibiotic-resistant infections, particularly in regions where contaminated water sources significantly impact public health. By exploring plant-based alternatives, this research hopes to offer sustainable solutions for addressing the growing concern of antibiotic resistance and its impact on global health.

MATERIALS AND METHODS

Collection of water samples from Narmada River (M.P. region)

For the present study, the water samples were collected from major ghats (known for the human activities including drinking of the river water during rituals) of Narmada River. Water samples were collected from the origin of Narmada River; Amarkantak and following its course through Dindori, Mandla, Jabalpur, Narsinghpur, Narmadapuram (Hoshangabad), Omkareshwar (Khandwa), Maheshwar, Harda, Dewas, Khargone, and Raisen.

Isolation and identification of bacteria

The water samples (1 ml) were plated on plate count agar (HiMedia, India) and incubated for 48 h at 37 ±1 °C. Bacterial colonies appearing on to the plates were identified through a combination of macroscopic and microscopic examination, characters on the specified medium, as well as the biochemical testing using standard methods.

Colistin Resistance Test

The colistin resistance of the isolated bacteria was done using the disc diffusion method (Bauer, *et al*., 1966). The isolated test bacterium swabbed thoroughly on to Müller Hinton agar plates (HiMedia, India) were allowed to grow with a colistin disc (50 µg, HiMedia, India) placed at the center of the plate. The plates were incubated at 37 ± 1°C for 48 hours. After incubation, the absence of the clearing zone (zone of inhibition) around the colistin disc represented bacterial resistance towards colistin.

Collection of *Cyperus rotundus* plants

Whole plants of *C. rotundus* (*Cyperus rotundus*) were collected from the Medicinal Plants Nursery of Jawaharlal Nehru Krishi Vishwavidyalaya (JNKVV), Jabalpur (MP). The identity of these plants was confirmed by the competent authority of the JNKVV. Plants were shade dried, powdered to pass 100 µM sieve and stored in air tight containers.

Extraction of phytochemicals

For the extraction process, 10 g of the dried plant powder was subjected to ethanol extraction using a Soxhlet extractor for a period of 15 h. The resultant extract was concentrated to achieve a final concentration of 500 mg ml^{-1} in terms of plant dry weight.

Antimicrobial activity of ethanolic extract of *C. rotundus*

The antimicrobial potential of *C. rotundus* extract was assessed against colistin resistant bacteria isolated from Narmada River. The antibacterial activity of the extracts was determined using the agar well diffusion method (Chavez-Esquivel *et al*., 2021).

The colistin resistant test bacterium was swabbed thoroughly on to Müller Hinton agar plates (HiMedia, India) and 50 µl of the plant extract was added to the 6 mm wells punched on to the plate. The plates were incubated at 37 ± 1°C for 48 hours. After incubation, the results were evaluated by measuring the diameter of the clearing zone around the well. The size of the clearing zone indicates the antibacterial effectiveness of the plant extract, with larger zones indicating greater antimicrobial activity towards colistin resistant bacteria.

Quantitative screening of phytochemicals in *C. rotundus* extract

Alkaloids

To quantify alkaloids, 5 g plant powder was mixed with 200 ml of 10% diethyl ether in ethanol for 4 hours, filtered, and concentrated. NH OH was added to precipitate alkaloids, which were then dried, re-weighed, and calculated (Awasthi *et al*., 2015).

Flavonoids

Flavonoids were extracted by repeatedly treating 10 g plant sample with 100 ml of 80% methanol, evaporating the filtrate, and calculating the dry matter weight (Awasthi *et al*., 2015).

Terpenoids

Ten grams of plant powder were soaked in alcohol for 24 hours, filtered, and extracted thrice with hexane. The pooled extracts were dried to constant weight, representing total terpenoids (Awasthi *et al*., 2015).

Saponins

For saponin quantification, 20 g plant sample was heated with 100 ml 20% ethanol, filtered, and re-extracted. The extracts were purified with diethyl ether, then n-butanol, evaporated, and dried. The saponin content was determined by calculating the weight difference (Awasthi *et al*., 2015).

Identification of Active Molecules from Plant Extract

Given that flavonoids were found to be abundant in the ethanolic extract of *Nagarmotha* (*Cyperus rotundus*), the flavonoid-enriched fraction of the extract was subjected to detailed analysis using Gas Chromatography-Mass Spectrometry (GC-MS) to identify the active molecules responsible for the observed bioactivity.

GC-MS analysis of the flavonoid-rich fraction was conducted using a Shimadzu TQ8040 Triple Quadrupole Gas Chromatograph-Mass Spectrometer System, equipped with a TG-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 ìm thickness, Thermo Scientific, USA). Helium gas was used as the carrier at 1.0 ml min^{-1}. The sample (1 µl) was injected through heated injected port (280°C) with split ratio of 20:1. The oven temperature was programmed to start at 60 °C (held for 2 minutes), gradually increasing to 280 °C at a rate of 3 °C per minute. The mass spectrometer utilized ionizing energy of 70 eV to ionize the sample and mass spectra were collected in the 40–550 amu range for further analysis (Abo-Altemen *et al*., 2019).

To identify the phytochemicals, the obtained mass spectra were compared against standards available in mass spectral libraries using the mzCloud™ Mass database software. Only compounds that matched more than 95% from the database entries were reported as identified compounds in the study.

RESULTS

Isolation and enumeration of bacteria from water samples

In total, 250 samples were collected from various sites of Narmada River, covering all the test sites at least three times. The samples were cultured onto the plate count agar, and the colonies appearing on the plates were further identified. Overall, out of 250 samples, 11% samples were sterile (no bacterial colonies observed on plate), and 6% samples had prevalence of Gram-positive bacteria. Most (83%) of the water samples were found to harbor Gram negative bacteria (Fig. 1).

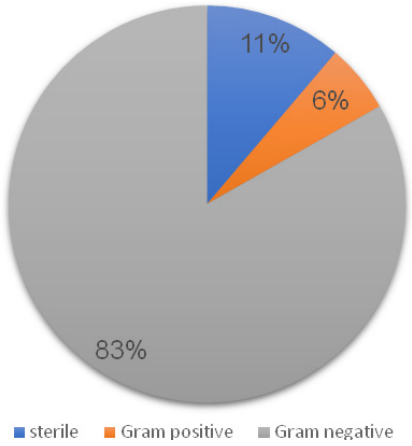

Fig. 1. Bacterial bioburden of Narmada River

The bacteria that could be successfully isolated and identified included *Escherichia coli, Klebsiella sp., Proteus sp., Pseudomonas sp., Salmonella sp., Shigella sp., Enterobacter sp.* and *Vibrio sp.*Among the Gram-negative bacteria, *E. coli* and *Klebsiella sp*. were most abundant with 15% presence in each case, followed by *Shigella sp.* (14%), *Enterobacter sp.* and *Salmonella sp.* (13% each). *Vibrio sp.* was also recorded in 8% of the samples (Fig. 2).

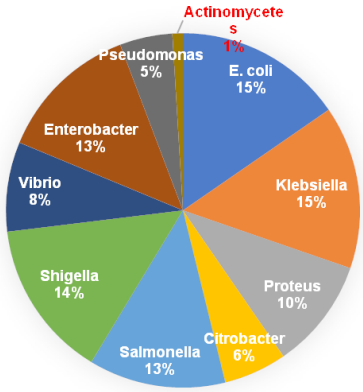

Fig. 2. Prevalence of different Gram-negative bacteria isolated from Narmada River

All the isolates of Gram-negative bacteria were simultaneously tested for their sensitivity (or

resistance) towards colistin discs. In total, 40 isolates showed resistance towards colistin *in vitro*. When the results were examined carefully, it was observed that *Proteus sp*. showed resistance in 47.6% of the isolates, followed by *E. coli* which has 43.75% resistance among isolates. *Pseudomonas aeruginosa* showed resistance in 3, out 10 isolates showing 30% resistance. Importantly, the rate of colistin resistance was very low in *Klebsiella sp*., *Salmonella sp*., and *Shigella sp*., and nil in *Vibrio sp*. (Fig. 3).

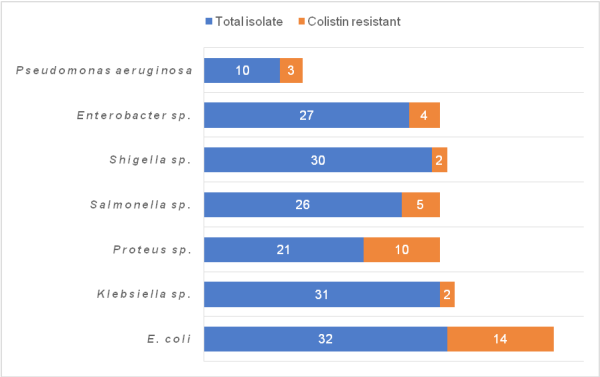

Fig. 3. Pattern of colistin resistance by the Gram-negative bacteria isolated from Narmada River

Screening of *C. rotundus* extract against colistin resistance bacteria

The plant extracts were screened for their antimicrobial activity against the 40 isolates which have shown the resistance towards colistin. The results showed that against the *E. coli* isolates, ethanolic extracts of *C. rotundus* was able to produce a zone of inhibition against 11 out of 14 isolates. Against colistin resistant *Proteus sp*. isolates, *C. rotundus* extracts produced antibacterial activity against 8 of 10 isolates of colistin resistant *Proteus sp*., the zone of inhibition ranged between 8-17 mm. Against the colistin resistant *Salmonella sp*., *C. rotundus* extract produced a zone size in the range of 15-20 mm, while against the colistin resistant *Enterobacter sp*., where in all 4 cases, antibacterial activity was recorded with the zone size of 15-16 mm. Against colistin resistant *Pseudomonas aeruginosa*, and *Klebsiella sp*., *C. rotundus*, showed good antibacterial activity as well. However, *C. rotundus* extract was not effective against any of the colistin resistant *Shigella sp*. isolated from Narmada River. Overall, *C. rotundus* extract produced a zone of inhibition in 80% of the test organisms. This extract was used for further identification of plant lead molecule active against colistin resistant bacteria.

Quantitative phytochemical estimation of *C. rotundus* extract

Before starting the identification protocol, the ethanolic extract of *C. rotundus* was subjected to the quantitative estimation of major secondary metabolites. *C. rotundus* extract showed alkaloids as 22.01±3.4 mg g^{-1}, flavonoids as 37.75 ± 1.4 mg g^{-1}, Saponins as 7.82 ± 1.2 mg g^{-1} and terpenoids as 2.18 ± 0.07 mg g^{-1} (Fig. 4).

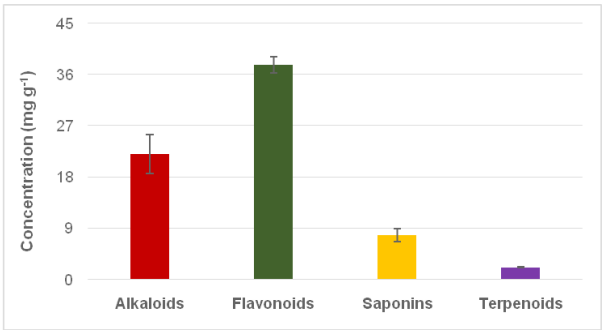

Fig. 4. Estimation of secondary metabolites from ethanolic extract of *C. rotundus*

When subjected to Gas Chromatography Mass Spectroscopy (GC-MS) analysis, the flavonoid rich fraction showed presence of flavonoids i.e., Quinic acid, Gallic acid, Hydrocinnamic acid, Quercetin-3â-D-glucoside, Kaempferol, Luteolin and Quercetin. Further, some of the terpenoids, known for the antimicrobial activity were also detected in the ethanolic extract of *C. rotundus i.e*., Ursolic acid, Asiatic acid and Rhamnetin (Fig. 5).

DISCUSSION

Our study revealed that Narmada water is heavily contaminated with pathogenic Gram-negative bacteria, with higher bacterial loads reported in previous research. Various studies have reported elevated coliform counts and pathogenic bacteria like *Pseudomonas*, *Salmonella*, and *Shigella* in Narmada River water (Soni *et al*., 2013; Sharma, 2018). In our study, 40 Gram-negative isolates were resistant to colistin, but Nagarmotha (*Cyperus rotundus*) ethanolic extract showed strong antibacterial activity against these resistant strains. Previous studies have documented *C. rotundus*'s antibacterial effects, confirming its effectiveness against Gram-negative bacteria, including *Proteus sp*. and *E. coli* (Sharma, 2018; Agbo *et al*., 2021).

Our analysis revealed that the ethanolic extract of *C. rotundus* contained major flavonoids such as

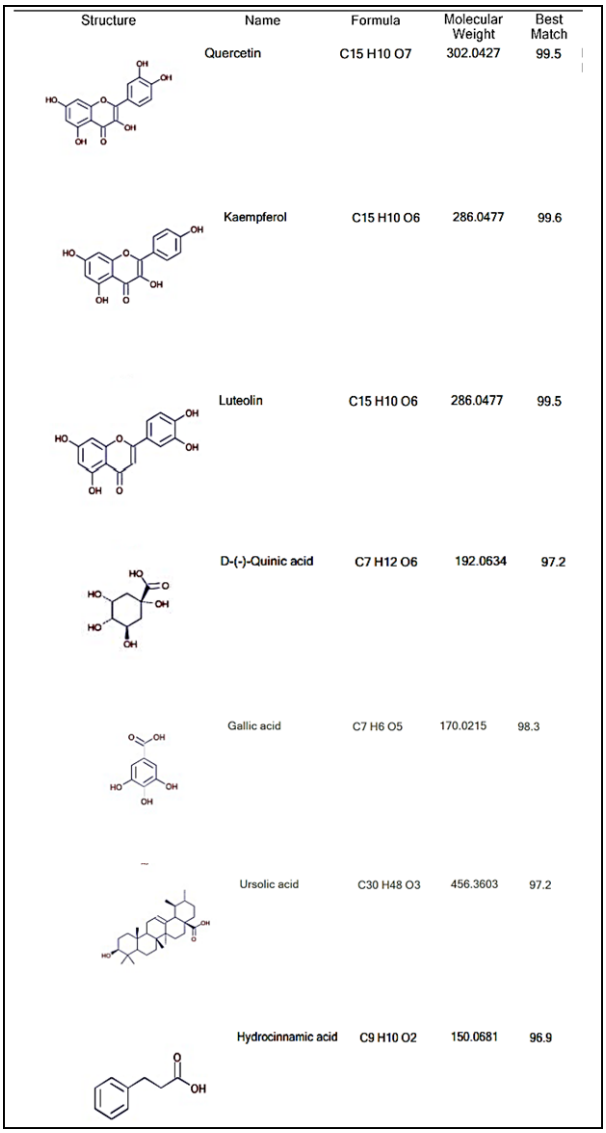

Fig. 5. Major Phytochemicals identified using mzCloud™ database

Quinic acid, Gallic acid, Hydrocinnamic acid, Quercetin-3â-D-glucoside, Kaempferol, Luteolin, and Quercetin, along with antimicrobial terpenoids like Ursolic acid, Asiatic acid, and Rhamnetin. These findings support Taheri *et al*., 2021, who identified quercetin and chlorogenic acid in *C. rotundus* rhizomes. The antibacterial properties of flavonoids, particularly quercetin and kaempferol, are well-documented and contribute to broad-spectrum activity against pathogens.

Cyperus rotundus (*Nagarmotha*) extract holds significant promise as an adjunct to colistin therapy, particularly in combating colistin-resistant bacteria. Its broad-spectrum antibacterial activity, ability to disrupt bacterial membranes, and potential to inhibit resistance mechanisms such as efflux pumps and lipid polysaccharide modification make it a valuable candidate in the fight against multidrug-resistant infections. The synergistic effects of *C. rotundus* with colistin could provide a novel therapeutic strategy to restore the efficacy of colistin and reduce the clinical impact of colistin resistance. However, further research and clinical trials are necessary to confirm the safety, efficacy, and underlying mechanisms of *C. rotundus* extract in combination with colistin.

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Conflict of Interest – None

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