Environment and Ecology 43 (3) : 567—575, July—September 2025
Article DOI: https://doi.org/10.60151/envec/NDUE2088
ISSN: 0970-0420 (Print), ISSN: 3049-3625 (Online)

Assessment of the Potential Efficacy of Herbal Formulations in Combating Carbapenem Resistance - An Emerging Environmental Menace

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Received 7 April 2025, Accepted 7 June 2025, Published on 2 July 2025

ABSTRACT

In recent years, carbapenem-resistant pathogens have emerged as a critical health concern worldwide. Improper antibiotic use has been a prime driver for the proliferation of multidrug-resistant bacterial strains. Medicinal plants possess bioactive compounds with antibacterial properties that could serve as novel therapeutic agents against resistant strains. The present study explores the therapeutic efficacy of four herbal formulations, Neem oil (NO), Olive oil (OO), Jatyadi oil (JO), and Rasamanikya as alternative medicine to combat carbapenem-resistant pathogens. The comparative study evaluated the herbal formulations with known antimicrobial activity, analyzing their efficacy, mechanisms of action and potential synergies when used in combination with conventional antibiotics. The environmental impact of carbapenem resistance has been highlighted, emphasizing the need for eco-friendly and biodegradable antimicrobial solutions. It is suggested that the potential of these promising herbal alternatives evaluated in the present study would contribute to the development of safer, sustainable, cost-effective antimicrobial approaches, addressing both public health and environmental concerns.

Keywords Carbapenem resistance, Alternative medicine, MDR pathogens, Environmental menace, Eco-friendly therapeutics.

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INTRODUCTION

In recent years, Antibiotic resistance (AR) has been associated with perceptibly higher mortality, longer

hospital admissions and higher expenses, consequently aggravating global public health problems (Huijbers *et al.* 2015). Prevalence of high AR has been identified in community-acquired illnesses (CAI) and diseases linked to healthcare in a global report that was released by the World Health Organization (WHO) in 2024. Since carbapenems are effective antibiotics of last resort that work well against infections that are resistant to several drugs, Carbapenem-Resistant Enterobacteriaceae (CRE) is reported to be responsible for very serious antibiotic-resistant (AR) related illnesses (Van Duin and Doi 2017, Iovleva and Doi 2017). As a result, the rise and dissemination of carbapenem-resistant enterobacteriaceae (CRE) among antibiotic-resistant bacteria pose global public health problems. Unfortunately, CRE surveys in clinical settings have significantly advanced, but meagre published research on environmental components of the ecosystem worldwide is available. Piedra-Carrasco *et al.* (2017) observed the urban river ecology of Spain to harbor CRE with imipenemase (IMI-2) and Verona integron-encoded metallo-β-lactamase (VIM-1) and Klebsiella pneumoniae carbapenemase (KPC). Portuguese river water samples have been shown to contain KPC-producing Enterobacteriaceae (Dantas Palmeira *et al.* 2022). In India *A. baumannii* has been reported to harbour a high carbapenem resistance of 71%. As a result, the ultimate antibiotic drug Colistin use has become imperative. Thus carbapenemase resistance at present is regarded as a major threat to food animals and subsequently an environmental imbalance via dissemination of AMR (antimicrobial resistance) (Gandra *et al.* 2020, Das *et al.* 2023). A dearth of research on CRE-related environmental contamination in both industrialized and developing nations is evident from the literature surveyed. Kumarasamy *et al.* (2010), reported that the bla NDM-1 gene initially discovered in clinical settings is now prevalent in environmental samples such as water, soil, and sediment in India. In central India, the prevalence of *E. coli* strains and the drivers influencing their resistance were studied in hospital effluents. The findings of Chandran *et al.* (2014) demonstrated that antimicrobial genes (ARGs), such as CTXM, OXA-48, and TEM, coexisted with the presence of NDM-1 in one isolate. It is thus evident that the hospital waste in India is an important source of CRE from aquaculture environment. The findings endorse the contention of Haller *et al*. (2018), who proposed hospital effluents as an important source of CRE. Of significance is the fact that in Northern India sewage samples released from hospitals were found to contain *E. coli,* which was observed to harbour the blaNDM gene. Lubbert *et al.* (2017) reported the prevalence of ESBL and CRE harbouring blaNDM and blaKPC in the environmental samples that were polluted by antimicrobials produced from pharmaceutical industries in South India. In recent years, an alarming increase in carbapenem resistance across all environmental sectors, including food animals and companion animals, has been witnessed (Banerjee *et al.* 2024). Considering the enhanced occurrence of carbapenem-resistant ARGs in various foods, companion animals, and aquatic matrices, it becomes imperative to search for alternative medicinal plants. Investigations by various researchers have reported the presence of bioactive compounds such as alkaloids, coumarins, terpenoids, flavonoids, phenolics, tannins, polyacetylene essential oils, lectins, polypeptides, and are present in many medicinal plants (El-Saadony *et al.* 2025). These components have shown antimicrobial efficacy either singly or in combination with standard therapeutics to tackle resistance in the form of MBLs (Metallo beta lactamases including carbapenemases) (Angelini 2024). The present study evaluated four herbal formulations, viz Neem oil (NO), Jatyadi oil (JO), Ozonated Olive oil (OO), and Rasamanikya (RM), for their inhibitory properties against carbapenemase production from microorganisms isolated from the different animals and aquatic matrices through *in vitro* assessment methods.

MATERIALS AND METHODS

Preparation of the herbal formulations

Neem oil

A. indica leaves were ground into a paste to make the decoction. The produced decoction was filtered. Using a mortar and pestle, neem leaves were separately mashed into a paste, which was then used to make little balls. Decoction was combined with sesame oil in a dry vessel, then balls were added one after the other. The mixture was brought to a gentle boil. The prepared oil was strained. It was then used for

different experiments.

Jatyadi oil

The leaves of *Jasminum officinale*, *Azadirachta indica*, *Trichosanthus dioca*, *Pongamia pinnata* and seeds, *Glycyrrhiza glabra* root, *Saussurea lappa* stem, *Curcuma longa* rhizome, *Berberis aristata* stem, *Picrorhiza kurroa* roots, *Rubia cordifolia* stem, *Prunus cerasoides* flower, *Symplocos racemosa* bark, *Terminalia chebula* fruit, *Nelumbo nucifera* flower, *Hemidesmus indicus* root were used in measured quantities with copper sulfate and bee wax to make a paste. Tiny balls were prepared using the paste. In a dry container, 200 g of sesame oil was heated to remove any moisture, and 800 mL of water was added in sequence. The mixture was allowed to boil gently until the oil became moisture-free.

Ozonated olive oil

The OO was used to purge ozone to create the ozonated oil. In a cold ozonator, atmospheric oxygen was converted to ozone. After achieving the targeted peroxide value, the procedure was resumed and ozonation was terminated. The peroxide value is the amount that represents the amount of peroxide in 1000 g of the chemical, expressed in milliequivalents of active oxygen. A 500 mEq/1000 g was the peroxide value of the ozonated OO utilized in this investigation. Iodometric titration was used to solubilize 0.1 g of oil in a (3:2) mixture of glacial acetic acid and chloroform for determining the peroxide value. The value of peroxide was computed using the following formula: Peroxide value = (sample − blank) × $Na_2S_2O_3$ normalcy ×1000/weight of the sample.

Rasamanikya

Raw haritala (orpiment) was purchased from a market in Kolkata, West Bengal, and was verified and processed in the laboratory of the Pharmacy Department of JB Roy State Ayurvedic Medical College & Hospital in Kolkata, West Bengal, India. The voucher sample was processed at the institute's pharmacy department. The raw orpiment was chopped into tiny bits. The parts were removed in a canvas bag. The package was placed in a vessel and steeped for three hours in lime water. The fragments of orpiment were dried, ground into a coarse powder and placed between two mica sheets and melted at 400 degrees Celsius. The arrangement was removed from the heating unit once it had completely melted. When it cooled, it solidified. The material was collected from the mica leaves and was made into powder form. The powder was stored in a glass bottle and was used for the experimental study.

Antimicrobial efficacy

Bacterial isolates used

Ten (10) NDM+ isolates, screened from fecal and water samples collected from two environmental sectors, including food animals and community ponds maintained in the repository of IVRI (Indian Veterinary Research Institute, ERS, Kolkata), were used in this study.

Disc diffusion and combined disc diffusion assay

The experiments used Mueller–Hinton agar (Himedia, India) with the agar layer about 5 mm thick. Approximately 100µl of 0.5 McFarland standard suspensions of all 10 isolates of the MBL strain cultured overnight were spread over the plates. For evaluating the antimicrobial activity of the trial drugs, standard sterile paper discs (diameter of 6 mm and thickness 0.5 mm) were each soaked with 10, 15, 20 and 30 µL of the herbal oil formulations (NO, JO, OO) and Rasamanikya (HNMD) respectively and then were seeded onto the agar media. These plates were parafilm sealed and incubated for 24 h at 37 °C. The microbial growth inhibition zones (in mm) were measured using a ruler. Each experimental setting had the standard antibiotic, IPM (Imipenem), seeded in between for a comparative zone of inhibition analysis (Brozyna *et al.* 2021). The four tested herbal formulations' potency for modifying resistance against resistant bacteria was ascertained by combining the drug concentrations mentioned above (10, 15, 20 and 30 µL) with the antibiotic discs against the resistant bacteria.

Assessment of anti-carbapenemase activity (Carbapenem inhibition method (CIM) and carbape-

nem inactivation percentage)

The anti-carbapenemase activity of the tested formulations was assessed using the carbapenem inhibition method. An inoculation loop of 10 µL culture from a Mueller-Hinton agar plate was mixed with 400 µL water to prepare a suspension. A susceptibility-testing disk containing 10 µg imipenem was immersed in the suspension along with 30 µL concentrations of the four herbal drug formulations for incubation at 35°C for two hours. On completion of incubation using an inoculation loop, the disk was removed from the suspension and subsequently placed on a Mueller-Hinton agar plate inoculated with 100 µL of 0.5 McFarland standard susceptible *E. coli* indicator strain (ATCC 29522) and incubated at 35°C. The carbapenemase activity was monitored, and if positive activity was observed, the imipenem susceptible disk was inactivated, allowing uninhibited growth of the susceptible indicator strain. Disks incubated in suspensions that did not contain carbapenemases yielded a clear inhibition zone (Van der Zwaluw *et al.* 2015). The carbapenem inactivation percentage was also calculated using the formula:

Zone diameter of IPM (Without inactivation) – Zone diameter of IPM (Inactivated)……..(i)

Zone diameter of Blank – Zone diameter of IPM (Inactivated)……(ii)

(ii) ÷ (i) × 100…….(1)

Zone diameter of herbal formulation + IPM – Zone diameter of IPM inactivated……(iii)

(iii) ÷ (i) × 100…….(2)

Carbapenem inactivation % = (2) – (1)

RESULTS AND DISCUSSION

Disc diffusion and combined disc diffusion test

The tests conducted at a concentration of 10, 15, 20 and 30 µL of Neem oil (NO), Olive oil (OO), Jatyadi oil (JO) and Rasamanikya (RM) against ten MBL bacterial isolates singly and in combination with IPM (Imipenem) exhibited variable zone of inhibition as depicted in Table 1 and Figs. 1a-h. It indicates that at concentrations of 20 µL and 30 µL. NO did not show any visible zone of inhibition against the ten MBL isolates (Table 1 and Fig.1a). NO in combination with IPM produced a zone diameter ranging between (10-12) mm for the 30 µL concentration used (Table 1 and Fig. 1b). OO and JO did not produce any zone of inhibition either singly or in combination with IPM (Table 1 and Figs. 1c-f). RM alone produced zone diameters ranging between (8-10) mm for 15 µL concentration and (10-12) mm for 30 µL concentration (Table 1 and Fig. 1g) and in combination with IPM produced zone diameters ranging between (10-11) mm for 10 µL concentration, (11-13) mm for 15 µL concentration, (16-18) mm for 20 µL concentration, and (23-25) mm for 30 µL concentration against all the 10 MBL isolates taken in this experiment (Table 1 and Fig. 1h).

It is thus evident from the Graphical depiction (Fig. 2) that RM produced greater inhibition zone diameters against the tested MBL isolates, single and when combined with the standard drug IPM. The zone diameters obtained in the present investigation, which exhibit synergistic action, conform to the findings of (Sharaf *et al.* 2021, Neglo *et al.* 2022), who reported similar synergistic effects when plant extracts were combined with specific antibiotics, leading to an increase of zone diameter up to 5 mm. Eze *et al.* (2013) in their study also revealed that the antimicrobial efficacy of most of the test antibiotics studied, when combined with extracts of *A. africana* against resistant *E. coli* was very high. Similar activity enhancement of the test antibiotics ciprofloxacin, norfloxacin, and chloramphenicol was observed against antibiotic-resistant *P. aeruginosa* when combined with extracts of *A. africana.*

Carbapenem inhibition method

A comparative assessment of the results shows that RM is the most effective tested drug against carbapenemase, producing the highest zone diameters ranging from 29 to 31 mm, respectively, followed by NO, JO and OO (Table 2 and Figs. 3a-e). A similar trend was also observed in the carbapenem inactivation percentage, where RM displayed the highest inactivation percentage (Table 2). Concurrently, a comparative

Table 1. Disc diffusion and Combined disc diffusion zone of inhibitions produced by NO, OO, JO, RM singly and in combination with IPM against MBL isolates.

Isolate name	ZOI (in mm)	Neem oil concentration (µL)				Neem oil + IPM				Olive oil concentration (µL)				Olive oil + IPM			
		10 µL	15 µL	20 µL	30 µL	10 µL	15 µL	20 µL	30 µL	10 µL	15 µL	20 µL	30 µL	10 µL	15 µL	20 µL	30 µL
MB 1		6	6	6	6	6	6	6	12	6	6	6	6	6	6	6	15
MB 2		6	6	6	6	6	6	6	11	6	6	6	6	6	6	6	14
MB 3		6	6	6	6	6	6	6	12	6	6	6	6	6	6	6	14
MB 4	Zone of	6	6	6	6	6	6	6	11	6	6	6	6	6	6	6	15
MB 5	Inhibition	6	6	6	6	6	6	6	11	6	6	6	6	6	6	6	14
MB 6	(in mm)	6	6	6	6	6	6	6	10	6	6	6	6	6	6	6	14
MB 7		6	6	6	6	6	6	6	11	6	6	6	6	6	6	6	14
MB 8		6	6	6	6	6	6	6	10	6	6	6	6	6	6	6	14
MB 9		6	6	6	6	6	6	6	10	6	6	6	6	6	6	6	14
MB 10		6	6	6	6	6	6	6	10	6	6	6	6	6	6	6	14

Table 1. Continued.

Isolate name	ZOI (in mm)	Jatyadi oil concentration (µL)				Jatyadi oil + IPM				Rasamanikya concentration (µL)				Rasamanikya + IPM			
		10 µL	15 µL	20 µL	30 µL	10 µL	15 µL	20 µL	30 µL	10 µL	15 µL	20 µL	30 µL	10 µL	15 µL	20 µL	30 µL
MB 1		6	6	6	6	6	6	6	7	6	6	9	12	10	12	17	24
MB 2		6	6	6	6	6	6	6	7	6	6	8	11	11	11	16	24
MB 3		6	6	6	6	6	6	6	7	6	6	9	12	10	13	18	23
MB 4	Zone of	6	6	6	6	6	6	6	7	6	6	10	11	9	12	17	24
MB 5	Inhibition	6	6	6	6	6	6	6	7	6	6	8	12	9	12	16	25
MB 6	(in mm)	6	6	6	6	6	6	6	7	6	6	8	10	10	13	17	25
MB 7		6	6	6	6	6	6	6	7	6	6	10	11	9	11	16	23
MB 8		6	6	6	6	6	6	6	7	6	6	9	10	11	12	17	24
MB 9		6	6	6	6	6	6	6	7	6	6	9	11	10	11	16	23
MB 10		6	6	6	6	6	6	6	6	6	6	8	12	9	11	16	24

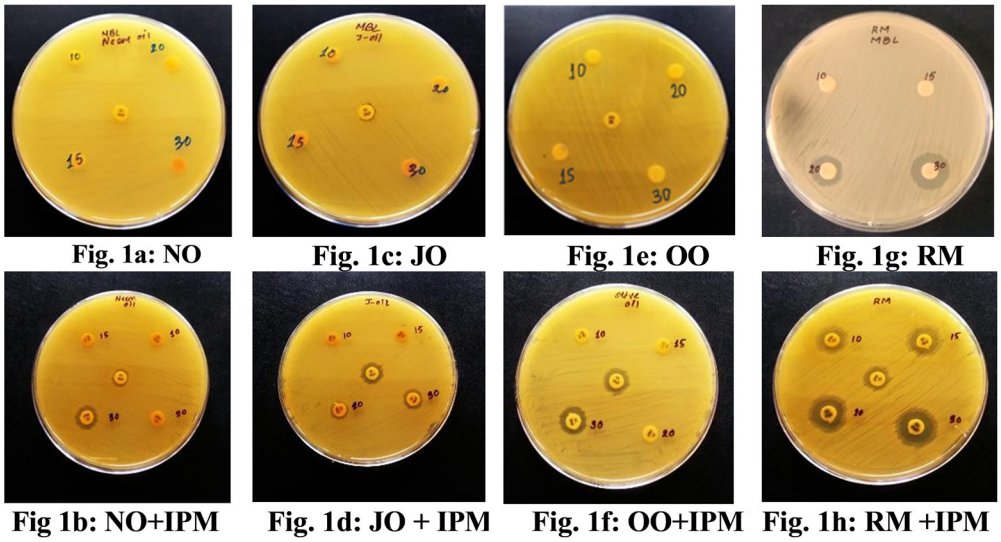


Fig. 1a: NO **Fig. 1c: JO** **Fig. 1e: OO** **Fig. 1g: RM**

Fig 1b: NO+IPM **Fig. 1d: JO + IPM** **Fig. 1f: OO+IPM** **Fig. 1h: RM +IPM**

Figs. 1a-h. Zone diameters exhibited on MH agar plates by NO, JO, OO, RM against MBL isolates singly and in combination with IPM.

Table 2. Zone diameters and inactivation percentage of trial drugs against carbapenemase activity.

Isolates	ZOI (in mm)	Concentration	NO + IPM	OO + IPM	JO + IPM	RM + IPM	Inactivated IPM
MB – 1	Zone of inhi-		23	18	20	30	6
MB – 2	bition (in mm)		21	19	20	28	6
MB – 3			23	17	19	29	6
MB – 4		30 µL	21	18	19	31	6
MB – 5			20	18	19	29	6
MB – 6			21	17	18	29	6
MB – 7			20	16	17	29	6
MB – 8			21	16	16	28	6
MB – 9			17	17	16	29	6
MB –10			20	17	17	29	6

Table 2. Continued.

Isolates	ZOI (in mm)	Concentration	Blank (without drugs)	Carbapenem inactivation percentage			
				IPM+OO	IPM+JO	IPM+NO	IPM+RM
MB – 1	Zone of inhi-		18	0	10	26	63
MB – 2	bition (in mm)		19	0	8	14	50
MB – 3			18	0	6	28	61
MB – 4			18	0	10	15	65
MB – 5		30 µL	18	0	4	9	52
MB – 6			18	0	0	15	58
MB – 7			18	0	0	15	58
MB – 8			18	0	0	10	56
MB – 9			19	0	0	15	58
MB – 10			19	0	0	15	58

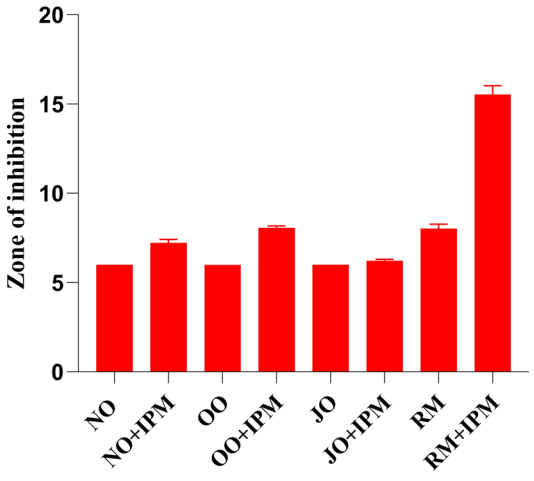
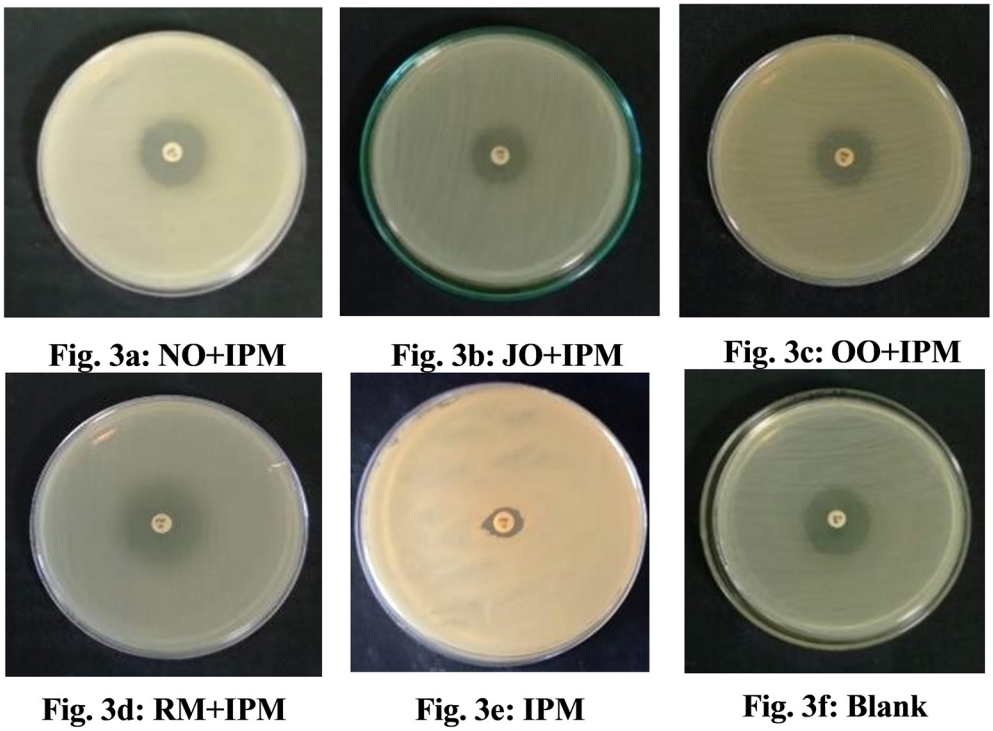


Fig. 2. Graphical depiction of the single and combined zone diameters of NO, OO, JO and RM with IPM against MBL isolates.

evaluation of the inhibition zone diameter for a combination of NO, JO, OO and RM with IPM shows RM to have the highest inhibition zone diameter.

Graphical depictions (Fig. 4) show RM in combination with IPM has the maximum inactivation percentage against MBL isolates. There is meager work conducted on investigating antibacterial properties against carbapenemase-producing bacteria. Carbapenemase-producing enterobacteriaceae detection using the CIM method has been effectively used by Tamma and Simner (2018). It is suggested that further investigation be conducted to utilize the CIM method for the simultaneous detection of carbapenemase activity in a microbe, as well as the presence of anti-carbapenemase activity in antimicrobials. This approach would give a better understanding of the target host and drug interaction mechanism, including carbapenemase production and its subsequent inhibition.



Figs. 3a-f. Figures 3a-e illustrate the zone diameters compared to the blank shown in Fig. 3f by the carbapenem inhibition method (CIM).

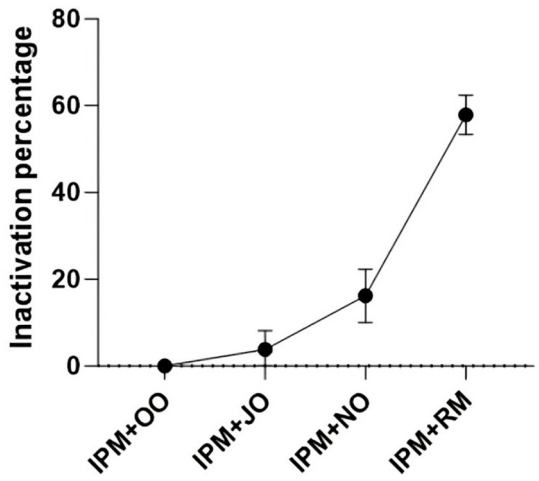


Fig. 4. Graphical depiction of carbapenem inactivation percentage.

CONCLUSION

India is projected to turn into a global hotspot of AMR within a decade. The urgent need is felt to reduce the AMR burden, especially in animals. The national action plan of India envisages optimizing antimicrobial usage and developing suitable alternatives to antimicrobials. The results of the present study displayed only RM to have inactivation activity against carbapenemase. NO showed intermediate carbapenemase inactivating capacity. OO and JO did not have any anti-carbapenemase property. These formulations are therefore suggested for use by clinicians in animals with further elaborate clinical trials. These could possibly be a cheap alternative to the carbapenem group of antibiotics.

ACKNOWLEDGMENT

The authors would like to express their sincere gratitude to the West Bengal University of Animal and Fishery Sciences, the Department of Veterinary Biochemistry, and the Eastern Regional Station of ICAR-IVRI, Kolkata, India. They also extend their heartfelt thanks to the study participants and all those who contributed to the success of this research.

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