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Original Research Article

L-Asparagine Mediation: A New Initiative to Breaking the Microbial Drug Resistance

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Abstract: Due to increasing drug resistance bacterial isolates in hospitals, morbidity and mortality are gradually increasing throughout the globe. Newer antibiotics are also not coming up as most drug manufacturing industries are reluctant to manufacture antibiotics due to low profit. In this context, we have to determine the antimicrobial activities of other common chemical agents. Thus in this experiment, we have studied L-asparagine- an amino acid with various biological activities to find out its antimicrobial activity if any. We were particularly interested in MDR bacteria in this study. Clinically isolated bacteria from different samples were identified by the automated Vitek automated system and stocked in the laboratory for our study. All isolates were used in lawn culture to find out the sensitivity of different bacteria to asparagine. In our experiment, we aimed to find out which specific concentration of L-Asparagine amino acid shows the most potent antibacterial effect on these bacterial species. In general, growths of all tested bacteria found inhibited at a very low concentration of 39 µg/ml. This uniform result against all resistant bacteria tested in this experiment clearly indicates its future utility against all extremely drug resistant bacteria.

Keywords: L-Asparagine; Gram Negative Bacteria; Gram-Positive Bacteria, Antimicrobial action.

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INTRODUCTION

L- Asparagine, our main agent of concern is a basic, non-essential amino acid. For this study, we are interested in the L- isomer of Asparagine which rotates, plane-polarized light in the anti-clockwise direction. It has a molecular weight of 150.13 g/mol and a molecular formula of $C_4H_8N_2O_3$. It exists as a white crystalline solid. Its basic nature can be owed to the fact that it bears two amino groups and a single carboxylic acid group and is synthesized by amino acid precursor aspartic acid and ammonia through the enzyme asparagine synthetase.

In a recent study of 2021, using L-Asparagine as a nitrogen source and 0.009% Phenol red dye, water samples from Thumba Arattuvazhi of Kerala were screened and tested for the presence of L- Asparaginase. It was perceived that a specific organism *Bacillus altitudinis KB1 strain* produced asparaginase which could be used for further biotechnological applications [1].

In cases involving hematologic malignancies like acute lymphoblastic leukaemia [2] as seen in young adults, specific doses of L-asparaginase acquired from *Escherichia coli* have been successful in providing first-line therapy. Although when some patients developed a hypersensitivity reaction against it, they were provided with L-asparaginase extracted from *Erwinia* since it exhibited zero cross-reactivity [3]. With adequate dosing and proper drug monitoring, survival rates in children suffering from this malignancy increased up to 90% in the 1960s.

A specific type of Asparagine known as Legumain, or Asparagine endopeptidase (AEP) [4] was also discovered to play an important role in curing cancers because of its substrate-cleavage ability.

An experimental study in 2022 proved that N and N-dibenzylasparagine had anti-cancer properties when experimented with colon cancer Caco-2 cells [5] and normal NCM-460 cell lines, respectively. A significant decrease in colon cancer cell proliferation was observed without any detectable toxic entities in normal cells.

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To further improve the anti-microbial effect of L-Asparagine, different modifications were made increasing the shelf life, bioavailability, best dosage, adverse effects on different races: ethnicity groups and genders, and serum stability of the amino acid. Introduction of N-Bromosuccinimide to L-Asparagine has shown greater stability [6] and prolonged half-life *in vitro* to proteolytic digestion as compared to unmodified L-Asparagine enzyme. The antiviral capability of L-Asparagine has been made possible by inhibiting attachments, penetrating viral replication, and blocking absorption.

Recent research has proved its efficiency against the Coxsackie B3 virus a pathogenic enterovirus which triggers illness in the gastrointestinal tract, myocarditis, and pericarditis.

For a certain bacterial wall disruption, various penicillin-binding proteins (PBPs) are used which mediate bacterial cell wall synthesis through peptidoglycan synthesis. It is observed that when PBP interacts with L-Asparagine [6], it is presumed that the microbial action of the amidohydrolase enzyme is because it degrades the bacterial cell wall. The functioning is similar to the antimicrobial enzyme lysozyme which hydrolyses the glycosidic bond linking NAM (N-acetylmuramic acid) and NAG(N-acetylglucosamine) present in the bacterial cell wall. It causes disturbance in the cell wall integrity thus causing cell death.

Different biopolymeric nanoparticles act as the carriers for L- Asparagine [6] which facilitates the ease of drug delivery through oral, nasal, intravenous, ocular, and various other routes. These biopolymers act as proficient drug carriers which have the advantage of slow release of drugs at the required site of action.

Biopolymers in association with L-Asparagine promote the sustained and controlled release of L-Asparagine and targeted drug delivery of the enzyme at the site of infection. Different biopolymers require different modifications like- specific organ targeting, enhanced solubility, enhanced cell permeation, proper drug delivery system, biocompatibility, non-toxicity, excellent mobility, and biodegradability. For proper thermal and physical stability, L-Asparagine is encapsulated inside these nanoparticles. Chitosan acts as both an effective drug-delivering biopolymer along with an effective antimicrobial agent. Different biopolymers like Silk Sericin, Silk fibroin, poly-d, l-lactide-co-glycolide, chitosan triphosphate, and PEG are used as biopolymers along with L-Asparagine for proper drug delivery systems.

It has also been found through previous studies that L-asparagine when linked to a Schiff's base [7] has shown potent antimicrobial and anti-mutagenic effects. It has also been found through another study that the accumulation of Asparagine is critical for the intracellular replication of certain pathogenic species.

In this experiment, we have studied the anti-microbial effect of this unusual amino acid on various gram-negative and gram-positive bacteria.

MATERIALS AND METHODS

BACTERIAL STRAINS:

The source of our MDR isolates was sub-cultures from stock cultures on Petri plates, containing the growth of the desired microbe, on a suitable media. *Escherichia coli* and some of the *Klebsiella pneumoniae, Enterococcus faecalis,* and *Pseudomonas aeruginosa* isolates were obtained from UTI Agar, (all units in gL-1; Peptone 15g, Chromogenic mixture 26.8g, Agar 15g, Final pH adjusted to 6.8±0.2), which harboured the growth of microbes isolated from urine. *Klebsiella pneumoniae* isolates were also obtained from cultures on Blood Agar (all units in gL-1; Peptone 10g, Tryptone 10g, Sodium Chloride 5g, Agar 15g, 5% sterile mammalian blood, pH adjusted to 7.3±0.2), and MacConkey Agar (all units in gL-1; Peptone 17g, Proteose peptone 3g, Lactose monohydrate10g, Bile salts 1.5g, Sodium chloride 5g, Neutral red 0.03g, Crystal Violet 0.001g, Agar 13.5g, pH adjusted to 7.1±0.2), in addition to UTI Agar. *Staphylococcus aureus* isolates were also obtained from Blood Agar plate cultures. *Pseudomonas aeruginosa* isolates were obtained from UTI Agar and MacConkey Agar plate cultures. *Shigella flexneri* and *Shigella sonnei* isolates were obtained from Glycerol Stock reactivated directly on Mueller Hinton Agar.

PROCEDURE

The Isolates were identified using the VITEK® automated system, which is also normally used to determine the antibiotic susceptibility patterns of these hospital isolates. Post identification, the isolates were sub-cultured on Mueller-Hinton Agar plates (all units in gL^{-1}; Acid hydrolysate of casein 17.5g, Agar 17.0g, Beef extract 2.0g, Starch 1.5g, adjusted to pH 7.3±0.2). Upon growth, isolated colonies were obtained, and isolates were made into 0.5 McF standard opacity bacterial suspensions using Normal Saline (NS) with DensiCHEK®. Normal Saline (NS) is used to maintain the tonicity of the medium and to prevent the lysis of these bacterial cells. Suspensions were taken in a fixed quantity (10 µL) and added to the wells of a microtitre plate. Before the addition of bacterial suspensions, on these wells, L-Asparagine was serially diluted with 100 µL of Normal Saline, starting with 100 µL of L-Asparagine (10 mg/dL), and continuing, till an eight-fold dilution was reached in the last well. The Optical Density of these wells containing an equal amount of bacterial suspension was measured at 620 nm wavelength and subsequently incubated overnight in a 37°C Incubator. The next day, the Optical Density was measured again. The change in the O.D. value obtained could conclude the potency of the amino acid, for each specific dilution.

The dose with the maximum difference in magnitude is the most potent for that isolate.

RESULTS

The results of the action of asparagine are depicted in Graphs 1-5. In *E. coli* the antimicrobial action was consistent at lower concentrations (39 µg/mL) probably due to optimum enzyme-substrate activity. Other Gram-negative bacteria which were studied showed mostly negative growth patterns in almost all concentrations of L-Asparagine used in this study. Gram-positive bacteria like *Staphylococcus aureus* which was studied showed consistent negative growth patterns in lower concentrations.

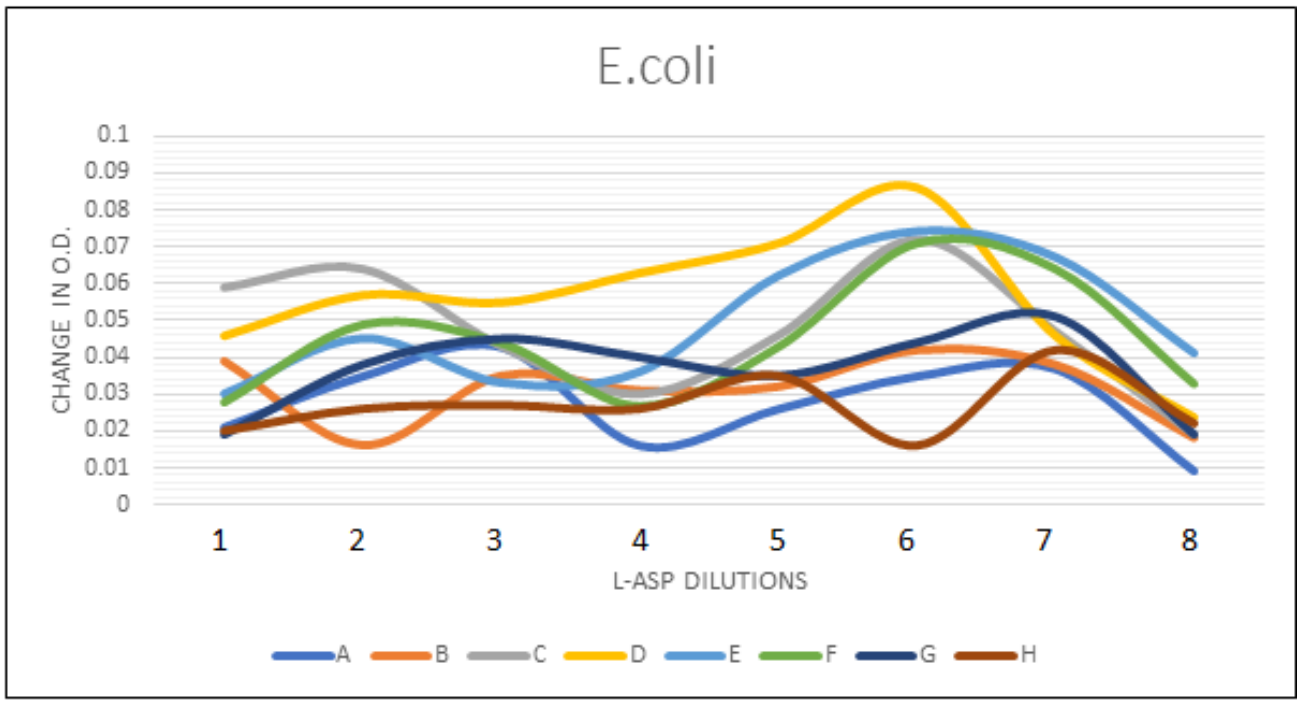

Fig 1: Growth patterns of *Escherichia coli* in different concentrations of L-Asparagine. 1-5000µg/mL, 2-2500 µg/mL, 3-1250 µg/mL, 4-625 µg/mL, 5-312.5 µg/mL, 6-156.25 µg/mL, 7-78.125 µg/mL, 8-39.0625 µg/mL. A-H indicates different strains of *E. coli*, all were urine isolates

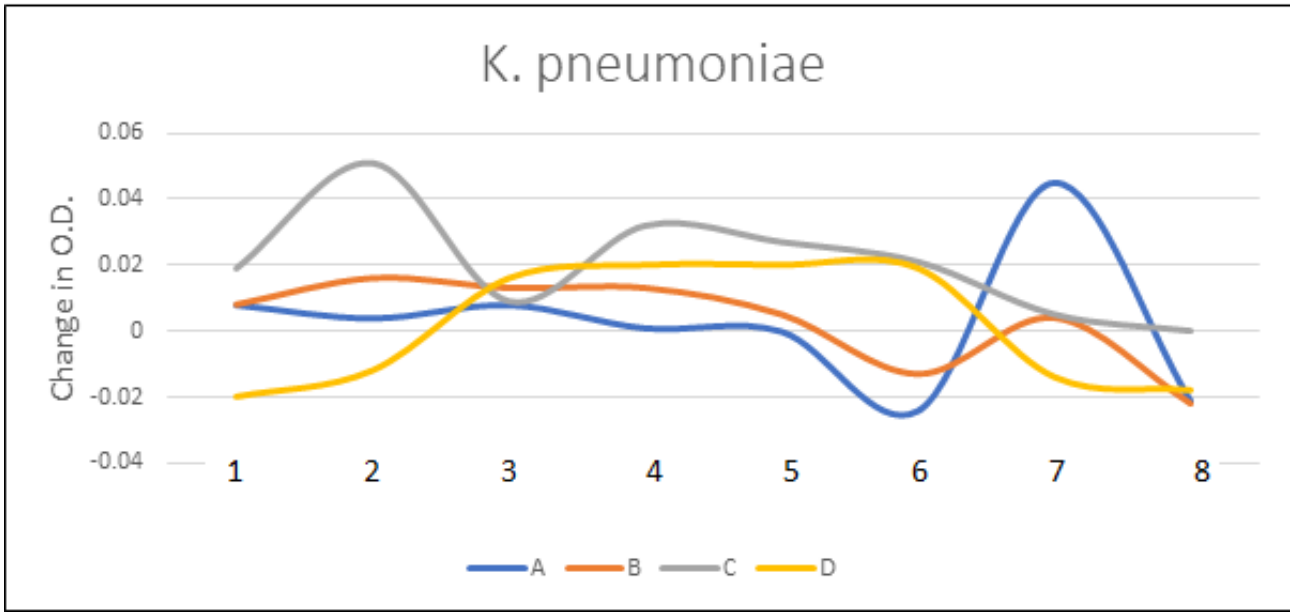

Fig 2: Growth patterns of *Klebsiella pneumoniae* in different concentrations of L-Asparagine. 1-5000µg/mL, 2-2500 µg/mL, 3-1250 µg/mL, 4-625 µg/mL, 5-312.5 µg/mL, 6-156.25 µg/mL, 7-78.125 µg/mL, 8-39.0625 µg/mL. A-D indicates different strains of *K. pneumoniae*, all were wound swab isolates

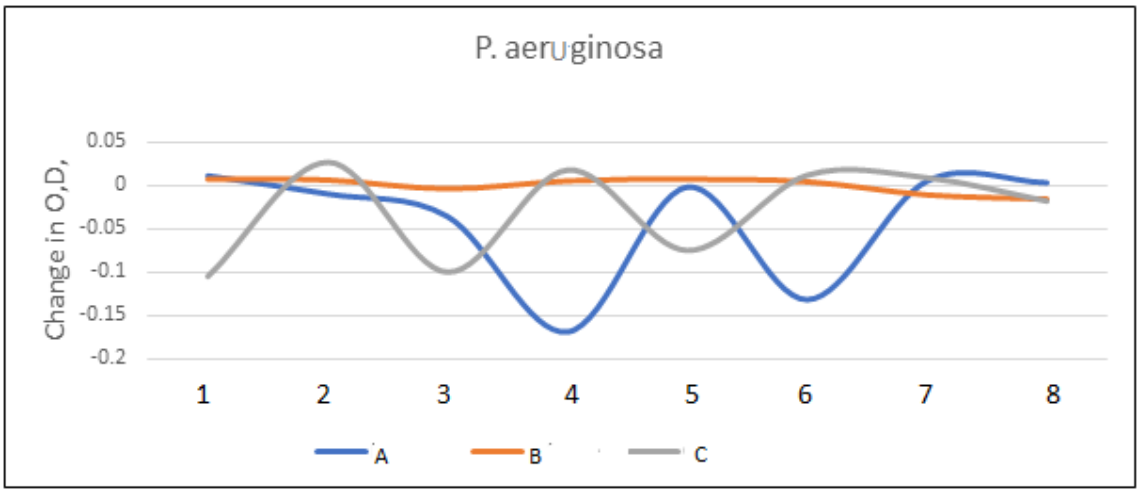

Fig 3: Growth patterns of *Pseudomonas aeruginosa* in different concentrations of L-Asparagine. 1-5000µg/mL, 2-2500 µg/mL, 3-1250 µg/mL, 4-625 µg/mL, 5-312.5 µg/mL, 6-156.25 µg/mL, 7-78.125 µg/mL, 8-39.0625 µg/mL. A-C indicates different strains of *P. aeruginosa*; Strains A and B were urine isolates, strain C was wound swab isolate

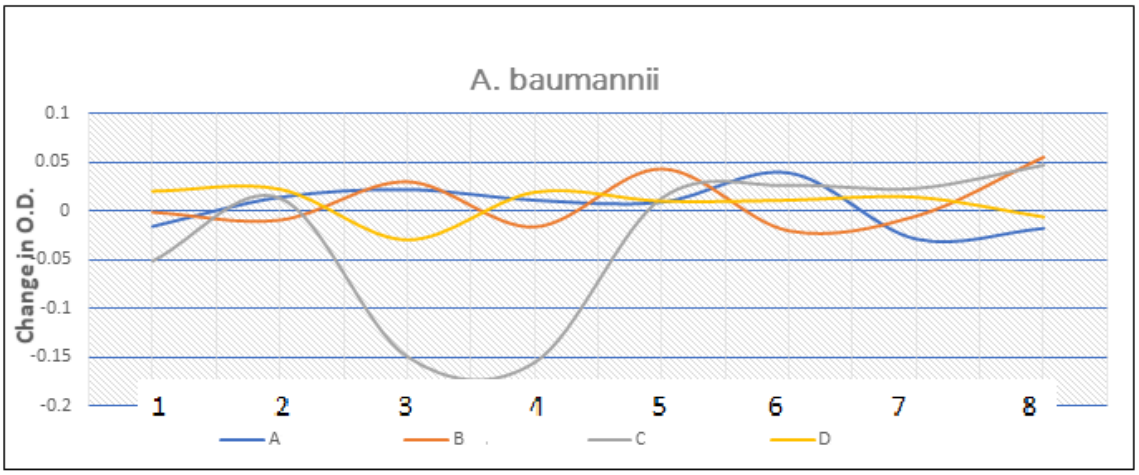

Fig 4: Growth patterns of *Acinetobacter baumannii* in different concentrations of L-Asparagine. 1-5000µg/mL, 2-2500 µg/mL, 3-1250 µg/mL, 4-625 µg/mL, 5-312.5 µg/mL, 6-156.25 µg/mL, 7-78.125 µg/mL, 8-39.0625 µg/mL. A-D indicates different strains of *A. baumannii*; Strains A-D were ET suction isolates

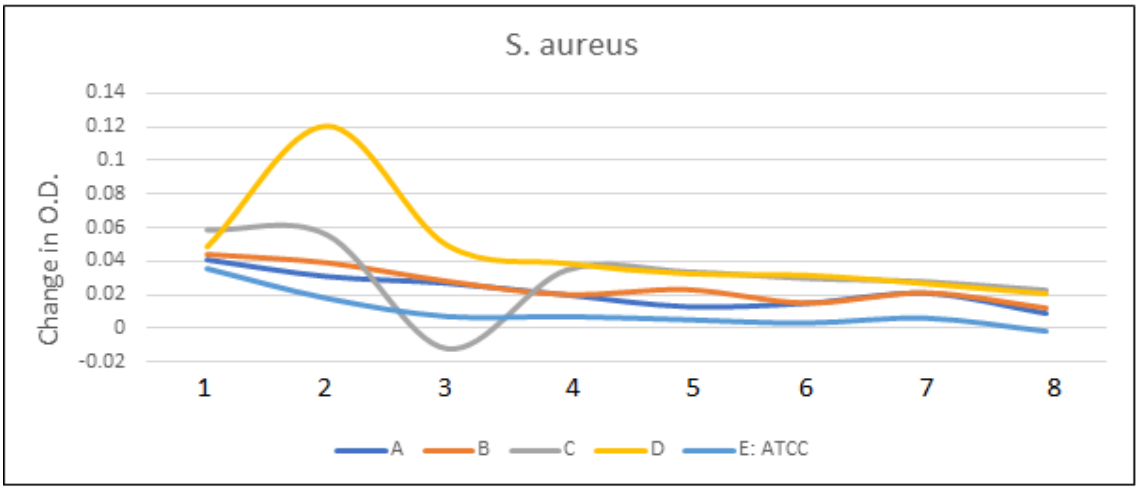

Fig 5: Growth patterns of *Staphylococcus aureus* in different concentrations of L-Asparagine. 1-5000µg/mL, 2-2500 µg/mL, 3-1250 µg/mL, 4-625 µg/mL, 5-312.5 µg/mL, 6-156.25 µg/mL, 7-78.125 µg/mL, 8-39.0625 µg/mL. A-E indicates different strains of *S. aureus*; Strain A was pus isolate, Strain B was ear discharge isolate, strain C was wound swab isolate and strain D was pus isolate, and strain E was ATCC 29213 used for quality control, and this was not MDR strain

DISCUSSION

In this study, we observed antibacterial activities of L-Asparagine on Gram-negative bacteria like *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aerurinosa, Acinetobacter baumannii* at a very low concentration (39µg/mL). A similar finding was also found with Gram-positive bacteria like *Staphylococcus aureus*.

Amino acids are essential agents for cellular protein synthesis. They also provide oxaloacetate to run the citric acid cycle and play an important role as precursors of nucleotide metabolites and precursors of non-essential amino acids [8]. In the human body supply of asparagine is very limited as well as highly regulated, thus bacteria commonly causing infection in human beings are not accustomed to utilizing asparagine, on the contrary, many of them produce asparaginase which breaks the asparagine. If we look into the biosynthesis of asparagine, oxaloacetate transamination generates aspartate, when asparagine synthetase act in the reaction to produce asparagine and glutamate. The source of the amino group is only glutamine. Thus without glutamine, there is no formation of asparagine. It is also important to note that asparagine is synthesized at the end part of the citric acid cycle from oxaloacetate limiting its supply [9]. The enzyme asparaginase can hydrolyze asparagine to aspartate which only occurs in lower animals as well as in microbes [10]. A negative feedback mechanism of glutamine suppresses asparagine synthetase activity restricting the synthesis of asparagine. However, increases in asparagine can increase cancer growth and decreasing biosynthesis of asparagine by genetic and chemical means decreases cancer growth [8, 11, 12].

Although there is no direct study on the antimicrobial potency of L-Asparagine one important study was done by Wagatsuma *et al*., [13], where the Authors demonstrated some asparagines derivatives of amino benzyl penicillin showed excellent antimicrobial properties. One compound N4-methyl-D-asparaginyl amoxicillin demonstrated the most potent antimicrobial activity against Gram-positive and Gram-negative bacteria. Again D-isomers in the asparagines moiety of the derived compounds showed more activity against Gram-negative bacteria like *Escherichia coli* and *Pseudomonas aeruginosa*.

Thus antibacterial activities showed by L-asparagine appear to be due to aspartate formation by bacterial asparaginase, and consistent activities at relatively lower concentrations were possibly due to optimum enzyme substrate concentration at these concentration levels.

CONCLUSION

L-Asparaginase is a drug in use for many years back in the medicinal world helping to cure severe cancerous malignancies like leukaemia, colon cancer and many more. Its recent advances have proved importance of L-Asparagine as an anti-microbial drug and how it is effective against a wide range of diseases caused by bacteria, fungi, viruses and many more.

Though research and various clinical tests are still under consideration, this drug has proved itself to be an effective anti-microbial lead in the diverse pharmaceutical market where there is a need for a proper, efficient new antimicrobial.

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Conflict of Interest: The authors declare no conflict of interest.

AUTHOR'S CONTRIBUTION

Sagnik Majumdar: Collected the data, performed the analyses, wrote the paper; Anwesha Dutta Chaudhuri: Collected the data, performed the analyses, wrote the paper; Satadal Das: Conceived research design and determined the overall modelling of the study, edited the paper Bhaskar Narayan Chaudhuri and Partha Guchhait: Edited the manuscript.

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