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Original Article

Phytochemical, Antimicrobial, and Anti-inflammatory Studies of *Zingiber neesanum* (Graham) Ramam

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ABSTRACT

Background: *Zingiber neesanum*, which belongs to the Zingiberaceae family, was the subject of the current investigation. Besides being a significant medicinal plant species, it is an aromatic stimulant. **Objectives:** Examining the phytochemical content, antibacterial and anti-inflammatory activity of plant extracts is the primary goal of this study. **Materials and Methods:** Phytochemical extraction was performed by ultrasonic-assisted extraction of powdered plant rhizome. The antimicrobial potential was assessed by well diffusion method. The anti-inflammatory activity of the extracts was carried out by the *in vitro* Sheep Red Blood Cell (SRBC) membrane stabilization assay, which includes heat-induced hemolysis and hypotonicity-induced hemolysis**. Results:** Important compounds like alkaloids, saponins, reducing sugars, steroids, and terpenoids were found in the rhizome after a preliminary phytochemical examination of the plant in ethanolic and hexane extracts. The antimicrobial screening was done against four different pathogenic bacterial strains. The antimicrobial study revealed that the maximum average zone of inhibitions in the ethanolic extract was observed against *Staphylococcus aureus* and moderate activity for *Escherichia coli* and *Bacillus cereus* respectively. Hexane extract was found to be effective against Gram-positive bacteria, *B. cereus* and *S. aureus*. **Conclusion:** The plant extracts demonstrated anti-inflammatory property by membrane stabilization effect by inhibiting both hypotonicity-induced erythrocyte membrane lysis and heat-induced hemolysis.

Keywords: Anti-inflammatory, Antimicrobial Properties, Phytochemistry, Traditional Medicine, Ultrasonication, *Zingiber*.

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INTRODUCTION

Bioactive phytochemicals like alkaloids, phenolic compound such as tannins and flavonoids, steroids, glycosides, volatile oils and terpenoids are found in extracts of various plant parts and can combat illness. Phytochemicals that are derived from plants may also contain anti-inflammatory agents (Gonfa *et al*., 2023). The antibacterial properties of phenolic phytochemicals derived from plants are crucial. Antimicrobial drugs interfere with enzyme function and DNA and RNA replication by breaking down the protein components of the cell wall (Nortjie *et al*., 2022). There are different kinds of inflammatory diseases and Non-Steroidal Anti-Inflammatory Medicines (NSAIDs), are widely used to treat it. All inflammatory disorders have not, however, responded well to these medications. Furthermore, negative side effects like ulcers and bleeding are frequently linked with using such medications (Sharma *et al*., 2018). The Indo-Malaysian region of Asia is home to the 52 genera and 1400 species that make up the Zingiberaceae group. The north eastern and peninsular regions of India are native to 178 species and 22 genera (Zou *et al*., 2022).

The Zingiberaceae family members are employed in cosmetics, medications, dyes, fragrances, and other commercial applications (Nair, 2013). Various chemical components have been described for developing products from economically significant genera, such as *Curcuma, Zingiber, and Alpinia* (Sharifi-Rad *et al*., 2017). Plants belong to the Zingiberaceae family primarily consists of edible and medicinal plants, among which, Zingiber is the third most prominent genus (Deng *et al*., 2022). *Zingiber neesanum* is a perennial herb with a thick rhizome that appears yellow in transverse section (Aswati *et al*., 2019). It is endemic to the Western Ghats, a biodiversity hotspot on the Indian peninsula, and flowers from July to September (Judin, 2016). Flavonoids, emetine, alkaloids, quinine, berberine, terpenoids are the anti-infective substances found in plants that continue to be useful to fight against microbial diseases (Destryana *et al*., 2024).

Bioactive compounds of *Zingiber officinale* shows a variety of biological activities including antimicrobial, antioxidant, antiarthritic, antitumor, anti-inflammatory, antithrombotic, and hypoglycemic effects. *Zingiber officinale* reported it contains

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bioactive compounds such as 6-gingerol, 6-shogoal, and 6-paradol. The compounds like [6]-dehydrogingerdione, [6]-shogaol, [10]-gingerol, and [6]-gingerol) derived from ginger shows antibacterial properties against drug-resistant *Acinetobacter baumannii* with an MIC value of 0.132-0.347 mg/mL (Harun *et al*., 2023). Sabulal *et al*., have extensively studied the plants belongs to this genus and reported the biological activities such as anti-inflammatory, anticancer and antimicrobial effects against *Staphylococcus aureus* and *Candida albicans* (Sabulal *et al*., 2006). Thus, the present study was designed to evaluate the phyto-pharmacological properties of *Z. neesanum*.

MATERIALS AND METHODS

Collection of Plant Materials

The rhizome of *Zingiber neesanum* was collected during Sep-Oct 2023 from Vellarimala, Chooralmala, Wayanad District, Kerala (Lat 11.480778333333335 Long 76.16767). Collected samples were authenticated by expert consultation, and the herbarium was deposited at CMS College Kottayam (Voucher No. CMS 2889).

Preparation of Extracts

Extraction was performed by ultrasonic-assisted extraction of powdered plant rhizome (10 g) in 200 mL for 30 min. using hexane and ethanol successively. Ultrasonic-assisted extraction uses ultrasonic sound waves that pass through the solvent, producing energy by enhancing the diffusion of the solvent into the sample array (Nortjie *et al*., 2022). Based on the acoustic cavitation ultrasound concept, the UAE technique creates a molecular disruption in the medium by a series of compression and rarefaction waves. This process can potentially harm the plant matrix's cell walls while simultaneously promoting the release of bioactive substances (Medina *et al*., 2017). Plant cell walls burst due to cavitation bubbles created by the acoustic cavitation process by sonication. As a result, the solvent can easily penetrate the extractable substance (Syahir *et al*., 2020).

Phytochemical Analysis

The qualitative phytochemical class analysis of *Zingiber neesanum* extracts were performed by using standard methods (Harborne, 1973; Sofowora *et al*., 1984; Trease *et al*., 1989).

Evaluation Antimicrobial Property

Antimicrobial property was studied by agar well diffusion technique against the clinical isolates *Klebsiella pneumonia, E. coli, S. aureus and Bacillus cereus,* which are identified by morphological culture characteristics, biochemical assays, and molecular evaluation (16srDNA sequence) obtained from Doctor John's Biotech Centre for Research and Development, Kottarakkara. The studies were performed on Mueller Hinton Agar. Samples were dissolved in DMSO (1 mg/mL), 10 µL and 20 µL of the which was used for the study. DMSO was used as negative control and antibiotic discs of Ampicillin (10 µg) and Tetracycline (30 µg) were as positive control. Each bacterial strain designated for evaluation was uniformly spread on MH agar plates employing a sterile swab that dipped in bacterial suspension. Thereafter, wells measuring 6 mm in diameter were created in the agar medium using sterile well borer and the samples were applied to the well. The samples were permitted to diffuse at room temperature for 2 hr in the laminar air flow chamber. The plates were then incubated at a temperature of 37°C for 48 hr. Upon completion of the incubation period, the diameters of the zones of inhibition were measured in millimetres using an electronic vernier calliper. The experiments were conducted in triplicate and data were expressed as mean±standard deviation (Jinu *et al*., 2011).

Anti-Inflammatory Studies by SRBC Membrane Stabilization Assay

Preparation of Sheep Red Blood Cells (SRBC) Suspension

Fresh whole sheep blood was collected from the local butcher shop and mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% NaCl). SRBCs were collected by centrifugation at 3000 rpm for 10 min. The cells were then washed three times with isosaline (0.85% PBS, pH 7.2) and were reconstituted to 10% v/v suspension with respect to packed cell volume in iso-saline.

Hypotonicity induced haemolysis

The alcoholic extract was dissolved in sterile distilled water (1 mg/mL). 5 mL of the hypotonic solution containing various concentrations of the extracts (25, 50, 100 and 200 µg/mL) in distilled water were taken in centrifuge tubes. Similarly, 5 mL isotonic solution containing graded concentrations of the extracts (25 - 200 µg/mL) were also put into duplicate pairs per dose. Control (5 mL) tubes were prepared of the vehicle (distilled water) and similar concentrations of diclofenac sodium respectively as reference standard. 0.1 mL of the erythrocyte suspension was added to each of the tubes and mixed gently. The mixtures were the incubated for 1 hr at room temperature (37°C), and afterwards, centrifuged for 5 min at 3000 rpm. The haemoglobin content of the supernatant was estimated by measuring the absorbance at 540 nm using UV-visible spectrophotometer (Systronics). The percentage haemolysis was measured by assuming that the cells in distilled water has 100% haemolysis. The percent inhibition of haemolysis by the extract was calculated using the following equation:

$$\text{Percentage inhibition of haemolysis} = [1-(A_2-A_1)/(A_3-A_1)] \times 100$$

Where A_1 - absorbance of test sample in isotonic solution, A_2 - absorbance of test sample in hypotonic solution and A_3- absorbance of control sample in hypotonic solution.

Heat induced haemolysis

For this study, the extract and standard drug used were dissolved in phosphate buffered saline. A set of 4 centrifuge tubes containing, 5 mL different concentrations of the extracts or drug (25, 50, 100 and 200 µg/mL) were arranged in triplicate sets in separate tubes. 5 mL of PBS, was used as the vehicle control. 0.1 mL SRBC suspension was added to the tubes and were mixed gently. The tubes were then incubated at 54°C for 20 min in a temperature-controlled water bath. While the other group of tubes were incubated at -10°C in a refrigerator for 20 min. After the incubation period, the tubes were centrifuged at 4000 rpm for 5 min and the haemoglobin content of the supernatant was measured at 540 nm by using a UV-visible spectrophotometer (Systronics). The percentage inhibition of haemolysis by the extract and the standard drug were calculated by the equation:

Percentage inhibition of Haemolysis=1-$[(A_2-A_1)/(A_3-A_1)]$ ×100

Where A_1 = absorbance of test sample unheated, A_2 = absorbance of test sample heated and A_3 = absorbance of control sample heated.

RESULTS

Preliminary Phytochemical Studies

Preliminary qualitative analysis revealed the presence of alkaloids, saponins, reducing sugars, steroids, carbohydrates and terpenoids in the rhizome of *Zingiber neesanum* (Table 1). These phytochemical compounds may contribute to its medicinal potential.

Antimicrobial Potential of *Z. neesanum* Extract

The antimicrobial properties of the rhizome extracts were evaluated using by the agar well diffusion method. Among the tested organisms, the maximum activity by zone of inhibitions observed against *S. aureus* (10.4±1.26) by the ethanolic extract (20 µg), while *E. coli* (7.9±0.86) and *Bacillus cereus* (7.8±1.61) showed moderate activity. *Klebsiella pneumoniae* was found to be resistant against both extracts tested (Figure 1). Hexane extract showed activity against Gram positive bacteria (*Bacillus cereus*, *S. aureus*), in with inhibition zone of inhibition 8.7±0.92 and 7.9±1.24 mm respectively, while it was found to be resistant against Gram negative bacteria (Table 2).

Anti-inflammatory Studies

Anti-inflammatory studies were performed by the heat-induced membrane stabilization and the hypotonicity-induced SRBC membrane stabilization assay. The extracts showed concentration depended activity in both the tested models.

Hypotonicity Induced Haemolysis

The plant extract was found to protect the sheep erythrocyte membrane against lysis induced by hypotonicity (Table 3 and Figure 2). The percentage inhibitions of lysis shown by the extract doses were comparatively higher than that obtained for diclofenac sodium. The plant extracts ZNS (*Zingiber neesanum*) exhibited membrane stabilization effect by inhibiting hypotonicity induced lysis of erythrocyte membrane. The structure and function of erythrocyte membrane is comparable to the lysosomal membrane, and its stabilization by the extracts suggests that it can also stabilize lysosomal membranes. This stabilization is crucial in reducing the inflammatory response by preventing the release

Table 1: Qualitative phytochemical analysis of *Z. neesanum*.

Sl. No.	Phytochemicals	Test	Hexane Extract	Ethanol Extract
1	Alkaloids	Dragendroff's test	Absent	Present
2	Tannin	Braymer's test	Absent	Present
3	Flavonoids	Shibata's reaction test	Absent	Present
4	Carbohydrates	Molisch's test	Absent	Present
5	Reducing Sugar	Fehling's test	Absent	Present
6	Saponin	Foam Test	Absent	Present
7	Cardiac glycoside	Keller-Killani test	Absent	Absent
8	Anthraquinone	Borntrager's test	Absent	Absent
9	Steroids	Liebermann-Burchard Test	Present	Present
10	Terpenoids	Salkowski's Test	Present	Present
11	Amino acid	Ninhydrin test	Absent	Absent
12	Wax and Mucilage	Alcohol test	Absent	Absent

Table 2: Antibacterial sensitivity of ZNS (*Zingiber neesanum*) extracts (Diameter of zone of inhibition in mm±SD, *n*=3).

Sl. No.	Organism	Zone of Inhibition in mm±SD						
		ZNS Ethanolic		ZNS Hexane extract		DMSO	Ampicillin 10 mcg	Tetracycline 30 mcg
		10 µg	20 µg	10 µg	20 µg			
1	*Staphylococcus aureus*	8.8±0.86	10.4±1.26	6.1±1.02	7.9±1.24	0	33.1±0.26	17.7±0.48
2	*Bacillus cereus*	5.7±0.48	7.8±1.61	0	8.7±0.92	0	0	17.6±0.82
3	*Klebsiella pneumoniae*	0	0	0	0	0	9.8±0.72	15.1±1.04
4	*E. coli*	6.7±1.02	7.9±0.86	0	0	0	0	16.9±0.22

Table 3: Anti-inflammatory properties of plant extracts in hypotonicity induced haemolysis model.

Concentration	% Stabilization	
	ZNS extract	Diclofenac sodium
0	0.00	0.00
25	18.84±0.92	10.41±1.01
50	31.15±1.61	15.39±2.09
100	52.88±2.07	27.47±0.97
200	66.14±1.97	64.64±1.62

Table 4: Anti-inflammatory properties of plant extracts in heat induced haemolysis model.

Concentration (µg/mL)	% Stabilization	
	Diclofenac	ZNS
0	0	0
25	9.62±1.62	14.17±1.21
50	17.4±1.04	22.41±1.98
100	29.64±1.16	32.28±1.09
200	59.06±1.02	48.28±1.08

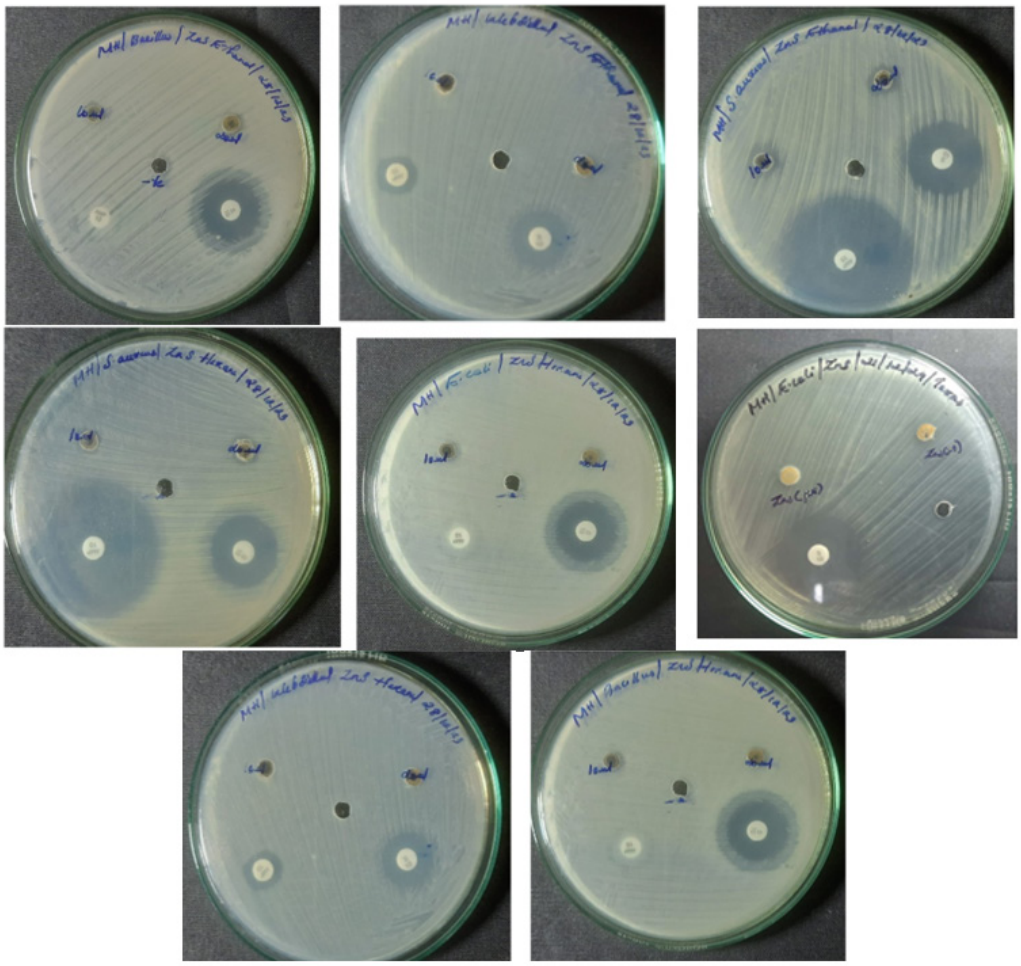

Figure 1: Antimicrobial properties of ZNS extracts.

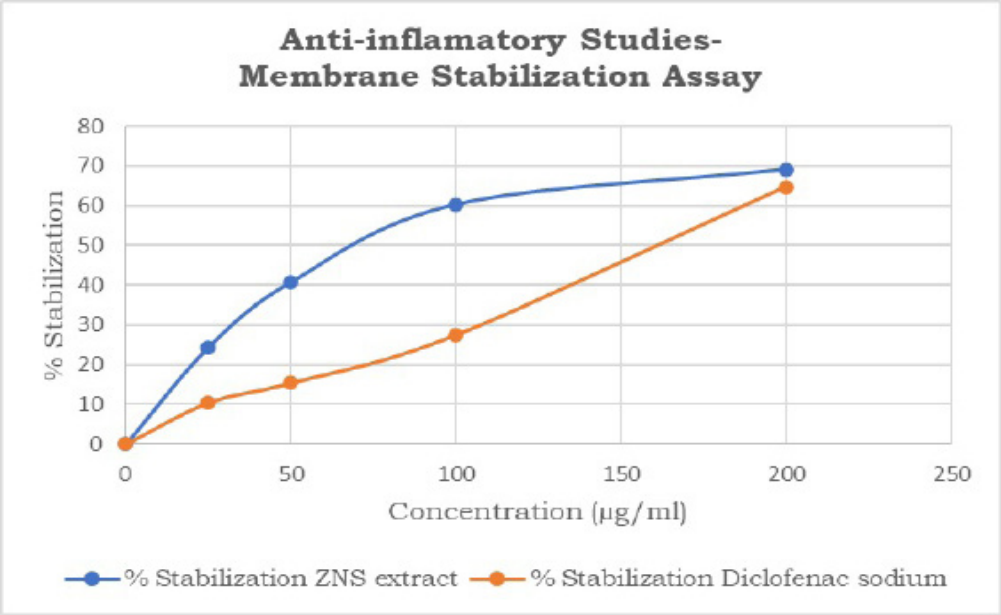

Figure 2: Anti-inflammatory properties of plant extract in hypotonicity-induced haemolysis model.

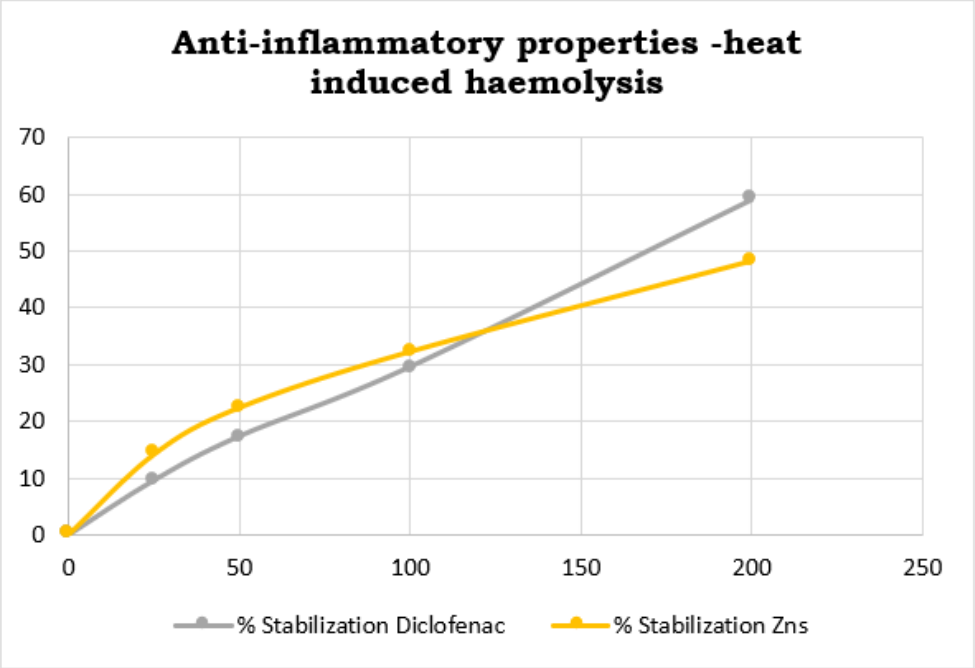

Figure 3: Anti-inflammatory properties of plant extract in heat induced haemolysis model.

of lysosomal contents from activated neutrophils, including bactericidal enzymes and proteases.

Heat-induced haemolysis of the HRBC membrane

The extract was also found to possess anti-inflammatory activity by protecting the human RBC membrane against heat-induced lysis (Table 4 and Figure 3). Both the extract and the standard drug diclofenac sodium were showed concentration depended activity by inhibiting the haemolysis of SRBC induced by heat.

DISCUSSION

Aswati *et al.*, identified the major bioactive phytochemical constituents in *Z. neesanum* rhizome by GC-MS analysis included 2-Methyl-7-nonadecene (13.99%; antimicrobial), Actinomycin C2 (8.57%; antineoplastic) and Deoxyspergualin (12.55%; immunosuppressive) (Aswati *et al.*, 2019). The findings of Judin *et al.*, also supportive to these observations. They reported that the methanolic extract of rhizome shows the

presence of alkaloid, flavonoids, terpenoids, sterol and phenolics (Judin., 2016). The antimicrobial activities of plant species are extensively researched and reported. The possible mechanism of action of these extracts is the interaction of phytochemicals with the microbial biomolecules and affecting its physiological activities. The results of the study by Gonelimali *et al*., indicated that the plant extracts significantly affected the cell membrane of Gram-positive and Gram-negative bacteria, as demonstrated by the decline in pH_{int} as well as cell membrane hyperpolarization (Gonelimali *et al*., 2018). Zingiber spp. could serve as a promising and innovative natural alternative to synthetic food preservatives. This approach aligns with the growing consumer concern about the potential health risks linked to conventional antimicrobial agents in food. Hypertonicity, the state of a solution with a higher solute concentration than a cell's internal environment, can induce haemolysis (Anosike *et al*., 2008). Membrane stabilization by the extracts can prevent the leakage of serum protein and fluids into the tissue (Yesmin *et al*., 2020).

The phytochemical composition of the methanolic extract such as flavonoids and other phenolics might be stabilize the membrane of RBC by precluding the discharge or inhibition of lytic enzymes and other active inflammatory mediators. The genus Zingiber members represent a promising and innovative source of natural bioactive agents, mainly gingerols, shogaols and zingerone (Sharifi-Rad *et al*., 2017).

CONCLUSION

Medicinal plants represent a good source of lead molecules, which can provide in effective drug molecules. The rhizome extracts of *Zingiber neesanum*, shows anti-inflammatory and antibacterial properties due to the presence of rich content of alkaloids, saponins, reducing sugars, steroids, and terpenoids. The presence of such bioactive phytochemical with anti-inflammatory and antibacterial properties of *Zingiber neesanum* indicate the therapeutic potential of the herb.

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ABBREVIATIONS

Zns/ZNS: *Zingiber neesanum*; **SRBC:** Sheep Red Blood Cells; **DMSO:** Dimethyl Sulfoxide; **SD:** Standard Deviation; **UAE:** Ultrasonic Assisted Extraction; **PBS:** Phosphate Buffered Saline; **GC-MS:** Gas Chromatography-Mass Spectrometry; **HRBC:** Human Red Blood Cells.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUMMARY

The plants belong to Zingiberaceae family is abundant in bioactive substances, especially alkaloids, terpenoids and phenolics and flavonoids, which support its medicinal potential. The rhizome extracts of *Zingiber neesanum* show promising antibacterial action against a range of pathogenic bacteria and also show anti-inflammatory activity, underscoring its promise as a medicine. It could be used pharmacologically for development of novel drug molecules. The isolation of these bioactive compounds, along with in-depth studies to analyze their biological activities and clinical trials for developing novel drug formulations, will be conducted and reported in the future.

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