



REVIEW ARTICLE

# Bioevaluation, Pharmacokinetics and Molecular docking study of Phenylpropanoid rich rhizome essential oil of understudied *Zingiber neesatum* from Konkan region of India

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## Abstract

*Zingiber neesatum* a species from the Zingiberaceae family exhibits convincing medicinal applications and is available vastly in the Konkan region. Its anti-inflammatory potential is an unexplored part. This work presents a Bioevaluation, Pharmacokinetics and Molecular docking study emphasizing the anti-inflammatory potential of rhizome oil of this species. An ultrasound-assisted solvent extraction method was employed to isolate oil and characterized by GC/FID and GC/HRMS techniques. The antimicrobial efficacies were checked by the Broth and Agar dilution protocols while the DPPH and ABTS assays were employed to test antioxidant potency. The anti-inflammatory potentials were tested by 3 methods-Heat-induced hemolysis, Inhibition of albumin denaturation and Proteinase inhibitory action. ADMET study was performed by the Swiss-ADME server while the docking was performed with AutoDock 4.2 software with a major component, [E]-3,4-Dimethoxy cinnamic acid methyl ester ([E]-3,4-DCME) using trypsin receptor. A pale yellow-colored essential oil was dominated by Phenylpropanoids (62.09%). Excellent antimicrobial potentials were observed versus *Staphylococcus aureus* and *Candida albicans* while excellent antioxidant activities were observed in both assays. But best anti-inflammatory action was documented in the albumin denaturation method. The pharmacokinetic properties of [E]-3,4-DCME, like high GI absorption, zero Lipinski violation with good bioavailability score etc., were promising. The docking results revealed that [E]-3,4-DCME has substantial binding affinity due to 'H' bonding interactions, and non-bonded Van der Waals and  $\pi$ -alkyl type interactions with the active site residues of a receptor. So, this study concludes that the rhizome oil of this underexplored species could be utilized in developing novel phytopharmaceuticals after further study.

## Keywords

[E]-3,4-Dimethoxy cinnamic acid methyl ester ([E]-3,4-DCME), Molecular docking, Pharmacokinetics, Ultra-sound assisted rhizome essential oil (USAREO), *Zingiber neesatum*

## Introduction

Antimicrobial resistance, a very serious threat to human beings is leading to about 7 lac deaths every year and estimates to be around 10 million by 2050 (1). The present Covid-19 global pandemic also demands to search for novel antimicrobial, antioxidant and anti-inflammatory agents effective against newly emerging bacteria and viruses that are prone to existing drugs.

Plants bear many useful bioactive molecules. WHO has specified a list

of 21000 medicinal plants which are utilized for medical purposes across the world. In India alone, 2500 plants have been investigated and around 150 species are utilized by biopharmaceutical houses as medicines. Being the largest medicinal plant producer country, India is “the botanical garden of the world” (2).

The family Zingiberaceae among the kingdom Plantae is an extensively studied useful family which constitutes rhizomatous aromatic and medicinal plants containing essential oils. The *Curcuma*, *Zingiber*, *Amomum* and *Alpinia* are important genera under the Zingiberaceae family. The genus *Zingiber* is comprised of 85 species grown mostly in Asia, Africa and South America (3). The species of *Zingiber* are known as food preservatives, spices, and also exhibit important medicinal properties. From this genus, *Z. officinale*, *Z. montanum*, *Z. mioga*, *Z. zerumbet*, *Z. ottensii* along with *Z. corralinum* are important species reported for medicinal value (4). *Z. nimmonii* is reported for its excellent antimicrobial activities (5) in south India. In the recent past, zingibers like *Z. anamalayanum* from the Western Ghats had been stated to have antimicrobial and antioxidant activities (6).

The species *Zingiber neesatum* is endemic to the Western Ghat region, one of the biodiversity hotspots. It is reported from Maharashtra, Karnataka, Kerala, Tamilnadu as well as Myanmar (7). But, except for a few reports, considerable research on this species has to be carried out from India (8-10).

As the chemical constituents of essential oils are influenced greatly by terrestrial and ecological factors (11) therefore, an attempt has been made to investigate this species from the Konkan region, a portion of Western Ghat, declared as a world heritage center by UNESCO (12).

The previous literature had intended the composition, antimicrobial and antioxidant activities of this species. However, the anti-inflammatory efficacy of this species has not yet been explored. Moreover, no combined reports on chemical constituents, bio evaluation, pharmacokinetics, and docking study of this species are available to date. This is the foremost study that emphasizes these aspects.

## Materials and Methods

**Plant material:** The 150 g fresh rhizomes of *Z. neesatum* species were gathered from the forest area situated near the village Phalani, located on Lonere-Shriwardhan road, (SH-99), (Lat. N 18° 6' 44.9784" Long. E73° 15' 57.0276") in Sept. 2019. The plant material was collected in the flowering season. The species was validated by Dr. Priyanka Ingle, from the Botanical Survey of India, Pune.

### Isolation of essential oil

#### Solvent Extraction:

The solvent extraction proceeded using 99.99% ethanol as a solvent. The rhizomes were soaked in a beaker comprising a solvent and were enclosed with Al foil. For half an hr, the same was exposed to ultrasonic irradiation (5.5-liter capacity, 230 V, 50 Hz and max. temp. 70 °C). Then, after the centrifuge process, the solvent was removed by a rotary

evaporator. After dehydrating the essential oil with Anhydrous Sod. Sulfate was kept in the freezer and the % yield was calculated after three replications (13).

#### Essential oil analysis:

##### Gas Chromatography-High Resolution Mass spectrometry (GC/HRMS) analysis:

GC/HRMS analysis was performed by using Agilent technologies 7890 Gas chromatograph equipped with JEOL The Accu ToF GCV JMS-T100 GCV MS detector. Helium was the carrier gas used in GC with a flow rate of 1.0 mL/min. with HP5 column also called EB5column (30 m length x 0.25 mm diameter x 0.25 µm thickness) was fortified in GC during analysis. The samples to be analyzed- were diluted in a1: 100 v/v ratios and acetone was used as a solvent. The samples were inserted through an auto-injector (1:10 for a min) with a constant temperature of 250 °C. Initially, the temperature of GC was programmed at 60 °C for 2 min then programmed to 250 °C and increased to 280 °C at 30 °C/min for 10 min. The GC/MS was scanned between 45-650 amu. The mass spectra were recorded at 70 ev (EI) and the compositions of oil were recognized based on R. I., Library MS search (NIST), and by comparing MS with reported literature (14).

##### Gas Chromatography Analysis:

Shimadzu QP-2010 Ultra Gas Chromatograph with HP5MS column (length 30 m, film thickness: 0.25 µm with max. temp. 250 °C) was used for gas chromatographic analysis. Helium was the carrier gas used in the analysis at a flow rate of 1 mL/min. The oven temperature initially was 50 °C which was further programmed to 250 °C. The flame ionization detector was used as a detector in the analysis. The injector and detector temperatures were 260 °C and 280 °C respectively. The percentage composition was calculated by the Area normalization method and was compared with standards.

**In-vitro Antibacterial Study:** The Broth dilution method was used to know the MIC of extracted rhizome essential oils with diluted DMSO, as it does not hamper the MIC of oil (15-17), following standard protocols. The serial dilutions in the primary (1000, 500 and 250 µg/ml) and secondary (200, 100, 50, 25, 12.5, 6.25 µg/mL) screening were prepared. The highest dilution showing at least 99 % inhibition zone was taken as MIC. Two gram-positive bacteria – *S. aureus* (MTCC 96), *S. pyogenes* (MTCC 442), and two gram-negative bacteria- *E. coli* (MTCC 443) and *P. aeruginosa* (MTCC 1688) were tested versus rhizome essential oil. Ampicillin and Ciprofloxacin were used as reference drugs. The results were displayed after a triplicate analysis.

**In- vitro Antifungal Study:** Agar dilution protocol had been employed to assess rhizome essential oil versus some selected fungal strains – *C. albicans* (MTCC227), *A. niger* (MTCC 282) & *A. clavatus* (MTCC 1323) (15).

The stock solutions of isolated rhizome oil were prepared in diluted DMSO, then incorporated in a specified amount of sterile molten dextrose agar for screening antifungal activities. To prepare inoculums, the stock of 100 mL of nutrient broth in 250 mL sterilized and clean conical flasks were heated at 27 °C for 24 hrs. before the experiment. The plates kept in aseptic conditions were allowed to diffuse through a potato-agar dextrose medium. Further, the plates

were heated at 25 °C for 2 days. The dilution which showed 99% inhibition was considered MIC concerning Griseofulvin and Nystatin as reference drugs. The results displayed are the output of the triplicate analysis.

**In-vitro Antioxidant activity:** Two assays viz. DPPH and ABTS were employed to check the anti-oxidant efficacy of isolated rhizome oils following standard protocols.

**DPPH assay:** The scavenging activity was measured using the stable radical DPPH by 96 well plate method. The methanolic DPPH (0.1 mM) solution was made ready and 1.0 mL of this solution was transferred to 3.0 mL of USAREO with varying concentrations (25, 50, 75, and 100) µg/mL. The absorbance was recorded at 517 nm after 30 min (18). The IC<sub>50</sub> values were decided as follows:

$$\% \text{ Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

All the tests were carried out in triplicate and the results are expressed as mean ± SD.

**ABTS assay:** This was done using stable ABTS<sup>•+</sup> radical cation following standard protocols (19). Test sample solutions were mixed with ABTS<sup>•+</sup> solution and the absorbance were read after 1min using UV-Visible spectrophotometer at 734 nm. A Phosphate buffer solution was used as a blank. The % scavenging was decided by the same formula used for the DPPH assay.

#### Calculation of 50 % inhibition concentration (IC<sub>50</sub>)

This was obtained by scavenging 50% of ABTS<sup>•+</sup> with gallic acid as a standard. The results were expressed as mean ± SD, after triplicate analysis.

#### In-vitro Anti-inflammatory Activities:

The anti-inflammatory efficacy of USAREO was evaluated against human RBCs. The three different methods viz. Heat-induced hemolysis, Inhibition of albumin denaturation as well as Proteinase inhibitory action, were employed with five different concentrations of test samples - 25, 50, 75, 100, 125 and 150 µg/ml by following standard protocols with Diclofenac sodium as standard.

Preparation of (10% v/v) suspension of Human RBCs:

The blood sample was transferred to a heparinized centrifuge tube and centrifuged at 3000 rpm for 15 min. The supernatants were removed carefully and the packed red blood cells (RBCs) were washed with fresh normal saline. The washing and centrifugation were repeated five times till clear supernatant. Finally, Human (10% v/v) suspension was prepared and used for further analysis.

#### Assay of Heat-induced hemolysis

The 2 mL reaction mixture (1 mL USAREO +1 mL 10% RBCs suspension) was heated at 56 °C, for 30 min. Then, the tubes were cooled and the mixture was centrifuged for 5 min. and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates (20). The tube containing only saline was taken as a blank.

The % inhibition was decided as follows:

$$\% \text{ Inhibition} = 100 \times (A_2/A_1 - 1)$$

Where: A<sub>1</sub> = Abs. of buffered saline solution.

A<sub>2</sub> = Abs. of the test sample in a hypotonic solution

#### Proteinase inhibitory action

The 0.5 mL reaction mixture (0.06 mg trypsin + 0.25 mL 20 mM Tris-HCl buffer (pH 7.4) + 0.25 mL test sample of different concentrations) was heated at 37 °C for 25 min, by adding 0.25 mL of 0.8% (w/v) casein. The reaction was terminated by adding 0.5 ml perchloric acid (70%). The cloudy suspension was centrifuged and the absorbance was read at 210 nm against the buffer as blank (20, 21). The experiment was executed in triplicates. The % inhibition was decided as follows:

$$\% \text{ Inhibition} = 100 \times (1 - A_2/A_1)$$

Where, A<sub>1</sub> = abs. of the control, and A<sub>2</sub> = abs. of the sample.

#### Inhibition of albumin denaturation

The reaction mixture (test samples +1% aqueous solution of bovine albumin fraction) was taken. The pH of the reaction mixture was adjusted using a small amount of 1N HCl. The samples were incubated at 37 °C for 20 min. and then heated at 57 °C for 20 min. After cooling, the turbidity was measured spectrophotometrically at 660 nm. The testing was repeated in triplicate (20, 22, 23). The % inhibition was decided as follows:

$$\% \text{ Inhibition} = (\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}} - 1) \times 100$$

#### Pharmacokinetics study

An *in-silico* chemical absorption, distribution, metabolism, excretion and toxicity (ADMET) study of [E]-3,4-Dimethoxy cinnamic acid methyl ester ([E]-3,4-DCME) was executed using the Swiss-ADME server to predict its possible use in the drug/s development (24).

#### Molecular Docking study:

Molecular docking was performed to accomplish the anti-inflammatory potential of [E]-3,4-DCME with trypsin receptor using AutoDock 4.2 software. Earlier, it is stated that trypsin possesses anti-inflammatory activity and exhibits a synergistic effect (25). Hence, for molecular docking, trypsin was employed as a receptor to know the inhibitory potential of [E]-3,4-DCME. The crystal structure of trypsin (source code: 1TRN.pdb) was retrieved from the protein database ([www.rcsb.org](http://www.rcsb.org)). The three-dimensional coordinates of [E]-3,4-DCME were retrieved from the PubChem database (PubChem ID: 766745). The blind and local docking approach was used to identify the putative binding mode of the drug compound (<http://autodock.scripps.edu>), similar to earlier studies (26). For the blind docking, the grid box of 126×126×126 with a grid spacing of 0.400 Å was used to cover the entire trypsin molecule. The Lamarckian genetic algorithm (LGA) was utilized with the default parameters. Here, five independent docking runs were performed, each of the 100 runs, a total of 500 output conformations were generated. These output conformations were further clustered using an all-atom RMSD with a cut-off of 4 Å. The resulting output conformations were then compared based on the cluster size, Van der Waals energy, binding energy, etc. (<http://autodock.scripps.edu>). Next, local docking was done by considering the active sites of trypsin observed in the blind docking. Here, a grid box of 60×60×60 grid points with a grid spacing of 0.375 Å was built around the binding pocket of trypsin. The least energy conformation was fur-

ther analyzed using the Discovery Studio visualizer (27).

**Statistical Analysis:** All the results are results of a triplicate analysis and expressed as means  $\pm$  SD errors. One-way ANOVA was employed to get mean differences.

## Results

**Chemical composition:** The average essential oil yield by an ultra-sound assisted solvent extraction was 0.6% (0.9 mL from 150 g rhizome) which is higher than the previous report (8). The chemical compositions of USAREO were detected using GC/GC-FID and GC/HRMS analysis.

**Constituents in Solvent extract:** The solvent extraction resulted in pale yellow-colored essential oil. It constituted 7 components, comprising 99.94% essential oil. The key constituents of oil were [E]-3,4-DCME (62.09%), 2H-1,3-Benzimidazole-2-thione,1,3-dihydroxy,5,6-dimethoxy (25.49%) and 1H-1,3-Benzimidazole,5-methoxy,2-phenyl (8.58%).

The USAREO constituted 62.09 % Phenylpropanoids, 34.07% Benzimidazole Derivatives and 3.78 % other compounds as depicted in Table 1.

### **In-vitro antibacterial activities:**

These were tested by the Broth dilution method. Results are summarized in Table 2. The USAREO showed outstanding activities versus *S. aureus* and *E. coli* with ampicillin as a reference drug.

**In-vitro antifungal activities:** Table 3 summarizes the *in-vitro* potency of rhizome oils against selected fungal strains. Out of the 3 strains used, the essential oils displayed excellent antifungal activity versus *C. albicans* (MTCC-227) concerning Griseofulvin as standard.

**In-vitro antioxidant activities:** The antioxidant efficacy of USAREO were evaluated by 2 assays as depicted in Table 4.

**Table 2.** MICs of USAREO against bacterial pathogens

Test Pathogens	MICs of USAREO ( $\mu$ g/ml)	MICs ( $\mu$ g/ml) of Ampicillin (Standard)	MICs ( $\mu$ g/ml) of Ciprofloxacin (Standard)
<i>E. coli</i> (MTCC-443)	100	100	25
<i>P. aeruginosa</i> (MTCC-1688)	125	100	25
<i>S. aureus</i> (MTCC-96)	62.5	250	50
<i>S. pyogenus</i> (MTCC-442)	125	100	50

Based on table 4, it seems that USAREO has exhibited excellent antioxidant activities in both assays concerning the reference. The IC<sub>50</sub> values of USAREO are very closer to the standard used.

**Table 3.** MICs of USAREO against fungal pathogens

Test pathogens	MICs of USAREO ( $\mu$ g/ml)	MICs ( $\mu$ g/ml) of Griseofulvin (Standard)	MICs ( $\mu$ g/ml) of Nystatin (Standard)
<i>C. albicans</i> (MTCC-227)	250	500	100
<i>A. niger</i> (MTCC-282)	500	100	100
<i>A. clavatus</i> (MTCC-1323)	500	100	100

**In-vitro anti-inflammatory activities:** An anti-inflammatory potency of USAREO were checked by three methods as depicted in Table 5. From table 5, it is clear that the oil sample has exhibited an amazing anti-inflammatory activity. In albumin denaturation, the anti-inflammatory activity value is superior to the reference drug.

### **Pharmacokinetics (ADMET) study of [E]-3,4-DCME:**

The physicochemical, and pharmacokinetics properties along with bioavailability radar and boiled egg graph of [E]-3,4-DCME, are tabulated in table 6 and Fig.1 respectively, retrieved from the Swiss ADME server.

**Table 1.** Chemical composition of *Z. neesatum* rhizome oil

Sr.No.	Retention Time (min)	Components	RI <sup>1</sup>	RI <sup>2</sup>	USASE (%)
1.	17.76	Silacyclobutane ,1-chloro,1-methyl	534	541	0.43
2.	18.35	2,7:3,6 -Dimethanonaphthalene, decahydro	1022	1051	1.38
3.	21.42	2H-1,3-Benzimidazole-2-thione,1,3-dihydroxy,5,6-dimethoxy	2025	2020	25.49
4.	22.42	Phthalic acid,4,5-dimethyl, dimethyl ester	1667	1650	1.32
5.	22.68	N, N-Diacetyl-2,4-dinitroaniline	2220	2200	0.65
6.	22.97	1H-1,3-Benzimidazole,5-methoxy,2-phenyl	2124	2110	8.58
7.	46.07.	[E]-3,4-dimethoxy cinnamic acid methyl ester	1825	1825	62.09
		Total Chemical Components (% composition)	NA	NA	07 (99.94%)
		Phenylpropanoids (7)	NA	NA	62.09 %
		Benzimidazole derivatives (3,6)	NA	NA	34.07 %
		Others (1,2,4,5)	NA	NA	3.78%

RI<sup>1</sup>- Retention index values detected on HP5 column.

RI<sup>2</sup>- Retention index values from literature (Adams 2007).

NA-Not Applicable

USASE-Ultra-Sound Assisted Solvent extraction

**Table 4.** IC<sub>50</sub> values of USAREO in antioxidant assays

Sr. No	Test samples	Mean IC <sub>50</sub> values (µg/ml)	
		DPPH assay	ABTS assay
1.	USAREO	57.59 ± 2.07	56.04 ± 0.55
2.	Standard (Gallic acid)	39.68 ± 0.89	53.14 ± 0.44

From Table 6, it is clear that the [E]-3,4-DCME shows the topological surface area (TPSA) > 30 Å<sup>2</sup> suggesting very good brain penetration and high lipophilicity value, Log Po/w (iLogP) as 2.77. Further, the [E]-3,4-DCME shows good pharmacokinetics such as high GI absorption, no P-glycoprotein (p-gp) indicating better intestinal absorption. The brain-blood-barrier (BBB) permeant is too yes, justify-

**Table 5.** Anti-inflammatory activities of USAREO

Sr. No.	Test samples	Methods used		
		Albumin denaturation (µg/ml)	Membrane stabilization (µg/ml)	Proteinase inhibition (µg/ml)
1.	USAREO	90.18 ± 4.43	83.11 ± 1.73	81.47 ± 0.30
2.	Standard (Diclofenac sodium)	85.84 ± 1.95	89.06 ± 1.20	91.28 ± 2.29

ing its suitability. Also, the interaction with the Cytochrome P family indicates its effectiveness and non-toxic nature. Moreover, [E]-3,4-DCME shows drug-likeness properties like zero Lipinski violation, Ghose, Veber, and Egan parameters are too yes with a very good bioavailability score of 0.55. Overall, [E]-3,4-DCME shows good ADMET properties; hence, it is an ideal candidate as a drug.

Fig. 1(A) indicates the molecule is inside a pink area showing its drug-likeness with bioavailability contour while as shown in Fig. 1(B) the presence of [E]-3,4-DCME in yolk with a red point confirms its high brain penetration probability with no P-gp (28).

Hence, we performed the docking study with the anti-inflammatory trypsin receptor.

### Molecular Docking study:

To study the binding mode and inhibitory mechanism of [E]-3,4-DCME with anti-inflammatory trypsin receptor, docking was employed through AutoDock4.2 (29). The least binding energy conformation of [E]-3,4-DCME with receptor was found to be -6.24 kcal/mol (Fig. 2). The binding mode of interaction of [E]-3,4-DCME is similar to the novel Quinolinyli-thiazolo triazole derivative with trypsin (30). The analysis of trypsin with [E]-3,4-DCME docked complex (Fig. 2) reveals that Gln192 (distance 3.0 Å, angle 113.4) and Gly193 (distance 1.80 Å, angle 159) forms conventional 'H' bonding interactions with drug. Whereas, His57, Asp189, Ser190, Trp215, Gly219, Lys224, Gly226 and Val227 form the carbon-hydrogen bonding interactions with the drug as depicted in Fig. 2B. In addition, Cys220 shows the π-alkyl type interactions with drugs (Fig. 2B).

Fig. 2(A) shows the binding mode of the drug with a receptor, protein is shown here with ribbon and drug with stick model. The Carbon, hydrogen, and oxygen atoms are

**Table 6.** Pharmacokinetic properties of [E]-3,4-DCME

Properties	Values
<b>Physicochemical Properties</b>	
Molecular Formula	C <sub>12</sub> H <sub>14</sub> O <sub>4</sub>
Molecular weight	222.24g/mol
No. of heavy atoms	16
No. of 'H' bond donor	0
No. of 'H' bond acceptors	4
TPSA	44.76 Å <sup>2</sup>
<b>Lipophilicity</b>	
Log Po/w (iLogP)	2.77
<b>Pharmacokinetics</b>	
Water Solubility	1.22e-01 mg/ml; 5.47e-04 mol/l
Class	Soluble
P-gp substrate	No
GI absorption	High
BBB permeability	Yes
Log Kp (skin permeation)	-5.76 cm/s
CYP1A2 inhibitor	Yes
CYP2C19 inhibitor	Yes
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
<b>Druglikeness</b>	
Lipinski	Yes, zero violation
Ghose, Veber, Egan	Yes
Bioavailability score	0.55

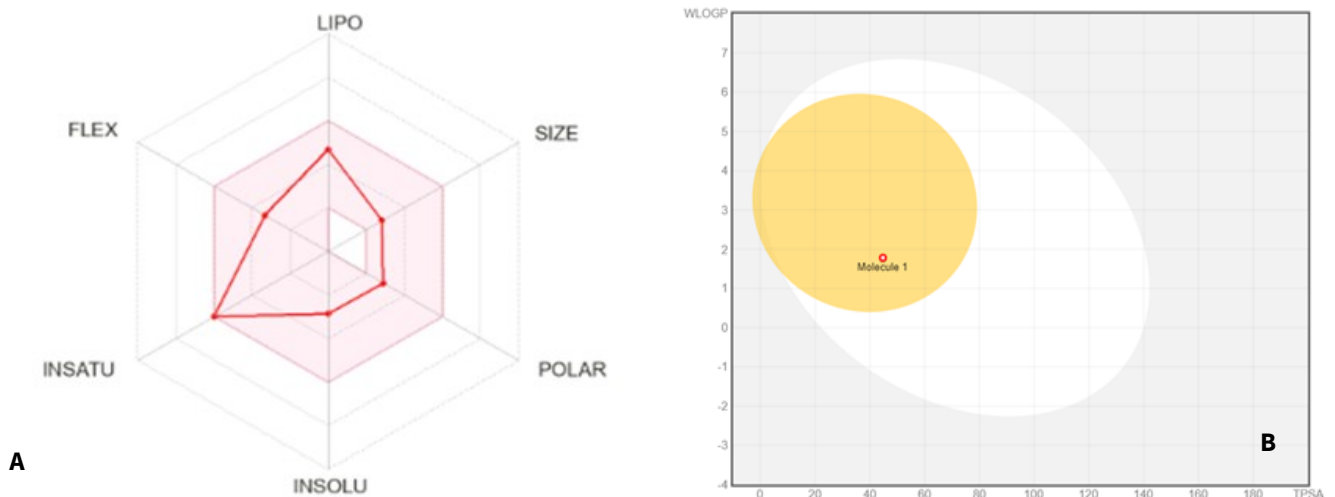
shown in cyan, grey and red color respectively. 2(B) shows, the 2D interactions of the drug with active site residues of trypsin. Gln192 and Gly193 form a conventional hydrogen bond with the drug while Cys220 and His57 form π-alkyl types interactions. The residues are involved in the carbon-hydrogen bonding interactions. The Discovery studio visualizer was employed to generate the 2D interaction network image (27).

The docking results reveal that the [E]-3,4-DCME has substantial binding affinity due to 'H' bonding interactions, and non-bonded Van der Waals and π-alkyl type interactions with the active site residues of a receptor (Fig. 2B).

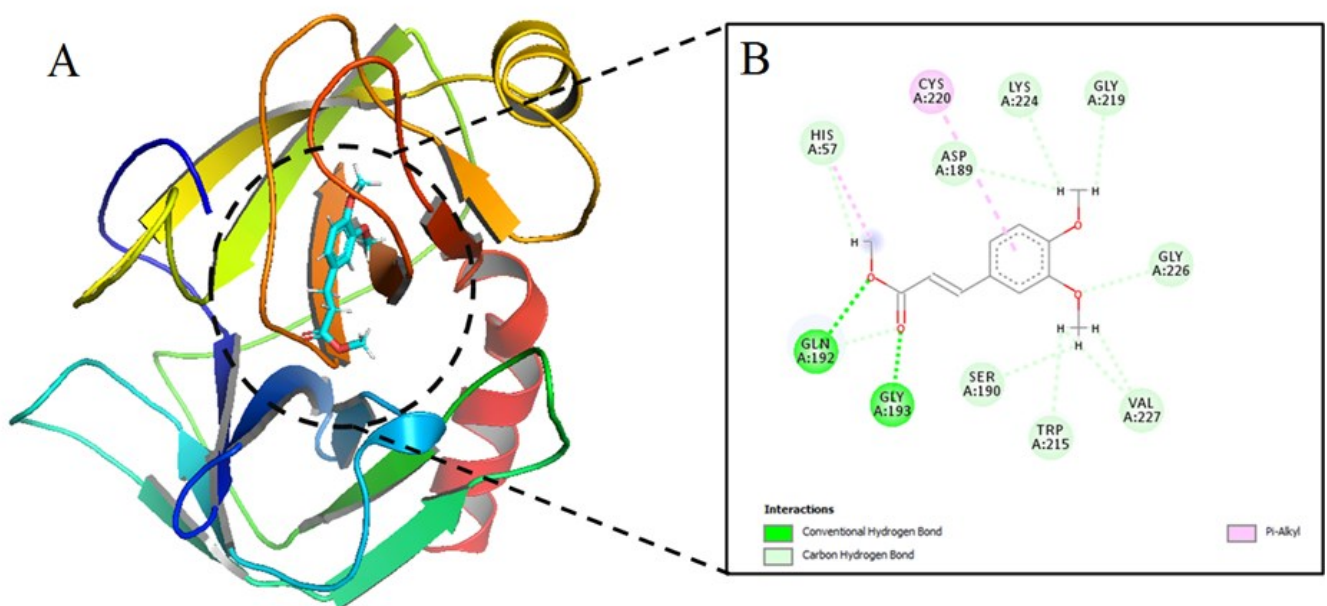
## Discussion

### Constituents in Solvent extract:

It was reported that the total of 61 components with 97.4% oil. (E)-1-(3',4'-dimethoxy phenyl) butadiene was the major component and Phenylbutanoids (54.4%) were dominant in their findings by hydrodistillation (8).



**Fig. 1.** Bioavailability radar (A) and Boiled-egg graph of [E]-3,4-DCME (B). (Molecule 1 represents [E]-3,4-DCME).



**Fig. 2.** Binding mode of [E]-3,4-DCME with trypsin receptor.

It was reported that the Deoxyspergualin (12.55%), 2-Methyl -7-nonadecene (13.99%) and Actinomycin C2 (8.57%) as the important components extracted using 5 solvents viz. isopropanol, ethyl acetate, benzene, methanol and hexane (9).

So, the present results are varying in chemical compositions with reported work in qualitative and quantitative aspects obtained by newer ultra-sound assisted solvent extraction method, owing to different terrestrial and ecological factors.

3,4,5-trimethoxy cinnamic acid ester derivatives (3,4,5-TMCA ester) have been reported to possess excellent antimicrobial, antiviral, and anti-inflammatory activities etc (31). So, methoxy cinnamic acid ester derivatives are important natural products having a wide range of bioactivities. The existence of [E]-3,4-DCME, an important component responsible for the excellent antimicrobial, antioxidant and anti-inflammatory activities as a major component of USAREO encouraged us to study its bioactivities with unexplored anti-inflammatory efficacies (32, 33).

#### ***In-vitro* antibacterial activities:**

Methyl ferulate, an ester derivative of cinnamic acid having a similar structure to [E]-3,4-DCME exhibits excellent antibacterial activities, especially against *S. aureus* and *E. coli* (34). Present outcomes too follow these results, so exceptional antibacterial activities of USAREO can be attributed to [E]-3,4-DCME.

#### ***In-vitro* antifungal activities:**

The excellent antifungal agents like - 2H - 1,3-Benzimidazole-2-thione, 1, 3-dihydroxy, 5, 6-dimethoxy, 1H-1, 3-Benzimidazole, 5-methoxy, 2-phenyl and [E]-3,4-DCME are present in higher amount in USAREO (34, 35). So, the antifungal potency of USAREO can be due to these major components.

#### ***In-vitro* antioxidant activities:**

The excellent antioxidant activities of USAREO can be attributed to the action of major components viz.[E]-3,4-DCME owing to the structural similarity with methyl ferulate (36).

The anti-oxidant activities of USAREO are comparable with a report (9) in which oil exhibited free radical scavenging activity by DPPH assay as  $94.1 \pm 5.4 \mu\text{g/mL}$ . The free radical scavenging activity of USAREO using the ABTS assay was superior to the FRAP assay ( $156.3 \pm 10.4 \mu\text{g/mL}$ ).

#### **In-vitro anti-inflammatory activities:**

It was reported that the Phenylbutanoids, excellent anti-inflammatory agents, as a major part of their work, but haven't tested these activities *in-vivo* or *in-vitro*. These activities are evaluated firstly in this report following phenylpropanoids as majors (8).

Ethyl 3,4,5-trimethoxy cinnamate, the structural analog of [E]-3,4-DCME is an excellent anti-inflammatory agent (37). So, these exceptionally well anti-inflammatory activities of USAREO can be devoted to important anti-inflammatory agents i.e. [E]-3,4-DCME.

#### **Pharmacokinetics (ADMET) study of [E]-3,4-DCME:**

The ADMET study of [E]-3,4-DCME reveals its usefulness to formulate the drug/s. All the parameters explained under 3.6, highlight the non-toxic nature of [E]-3,4-DCME with a good bioavailability score. Bioavailability radar and Boiled-egg graph too support the same.

#### **Molecular Docking study:**

The binding mode of interaction of [E]-3,4-DCME is similar to the novel Quinoliny-thiazolo triazole derivative, with trypsin reported recently. This point strengthens the anti-inflammatory potential of [E]-3,4-DCME and opens the window for its use as an anti-inflammatory agent, apparently after detailed *in-vivo* investigations.

### **Conclusion**

This study reveals that the chemical constituents of *Z. neesatum* rhizome oil are influenced greatly by terrestrial and ecological factors. The present results do vary significantly from the reported literature in qualitative and quantitative aspects. The dominance of Phenylbutanoids (54.43%) was reported earlier (8). However, the phenylpropanoids dominated the USAREO (62.09%) and the percent composition was too high. The USAREO has exhibited excellent antibacterial, antifungal, antioxidant and anti-inflammatory activities. The excellent pharmacokinetic properties of [E]-3,4-DCME, like high GI absorption, zero Lipinski violation with good bioavailability score, etc. support its candidacy as a drug. Further, the exceptional anti-inflammatory activities of rhizome oils too are attributed to a major component, [E]-3,4-DCME, supported by molecular docking results and reported for its distinct taxonomic identity, for the first time. Overall, the rhizome oil of this underexplored species can be utilized in developing novel phytopharmaceuticals and medicines after further study.

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### **Authors contributions**

PL designed the study. PN carried out experimental work and wrote the manuscript. PL, HM, and PN finalized the manuscript.

### **Compliance with ethical standards**

**Conflict of interest :** Authors state no conflict of interest

**Ethical issues :** None

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