In vitro assessment of cytotoxicity, antioxidant and anti-inflammatory activities of *Zingiber zerumbet* rhizome essential oil

Nayak Ananya, Gadnayak Ayushman, Jena Sudipta, Ray Asit and Sahoo Ambika*
Centre for Biotechnology, School of Pharmaceutical Sciences, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar-751003, Odisha, INDIA
*ambikas672@gmail.com

Abstract

The present research deals with studying the phytoconstituents, antioxidant potential, cytotoxicity and anti-inflammatory activities of the Zingiber zerumbet rhizome essential oil (ZZEO). A total of 29 components were identified using GC-MS accounting for 94.68% of the total EO composition. The ZZEO was characterized by sesquiterpenes, comprising of 83.11% of the total composition with zerumbone (68.18%) and α-Humulene (11.72%) as the major compounds. The DPPH and FRAP assays of the rhizome essential oil showed IC_{50} values of 83.90±0.05 mg/ml and 1.98±0.04 mg/ml respectively. The in silico PASS prediction revealed that compounds more than 1% area, such as α-Pinene (0.853>0.005), camphor (0.701>0.015), β-caryophyllene (0.741> 0.011), spathulenol (0.769>0.009) and zerumbone (0.764>0.002) exhibited Pa>0.7 value, confirming ZZEO's anti-inflammatory potential.

The in vitro cytotoxic effect of the ZZEO was evaluated against RAW 264.7 murine-macrophage cell line by using MTT assay, which resulted in very little cytotoxicity (IC_{50}=176.71 µg/ml). Furthermore, at a concentration of 100µg/mL, the rhizome essential oil demonstrated significant anti-inflammatory potential in LPS-activated RAW 264.7 macrophage cells by reducing nitric oxide (NO) production. The study results revealed that the ZZEO is a rich source of bioactive constituent, zerumbone, with potential anti-inflammatory activity, leading to further investigation for the development of anti-inflammatory drugs.

Keywords: Anti-inflammatory activity, Antioxidant activity, Cytotoxicity, Essential oil, GC-MS, Zerumbone.

Introduction

The genus *Zingiber* contains over 141 species and is a major source of essential oil used in the cosmetic, perfume and pharmaceutical sectors[24]. According to phytochemical investigations, the rhizomes of these species contain secondary metabolites that play important roles in human disease therapy. Secondary metabolites are key components of a wide range of industrial products including beverages, perfumes, drugs and nutraceuticals. Terpenes are the predominating classes of secondary metabolites among these natural chemicals with considerable bioactive potential for utilization in medicines. *Zingiber zerumbet*, popularly known as shampoo ginger, is an important medicinal plant of the family *Zingiberaceae*.

The rhizome essential oil of *Z. zerumbet* is frequently utilized in the pharmaceutical industry. The rhizomes of *Z. zerumbet* contain a high concentration of diterpenes and sesquiterpenes which have a wide spectrum of bioactivities. The presence of therapeutically significant sesquiterpenes and monoterpenes in *Z. zerumbet* rhizome has been reported[8]. The *Z. zerumbet* rhizome essential oil possesses a variety of pharmacological activities, including antinociceptive, antimicrobial, antitumor[2], antiproliferative[19], chemopreventive[22], chemotherapeutic[22], anti hypersensitivity, antioxidant, antisecretory[12,21] and anti-inflammatory[28] properties.

One of the main constituents of the terpenoids-rich *Z. zerumbet* rhizome essential oil is zerumbone, a pharmacologically active compound[5,19,14]. Zerumbone has been in high demand due to its pharmacological and therapeutic properties. Monocyclic sesquiterpenoid zerumbone inhibits carcinogen-induced NF-κβ and TNF-β regulated gene expression[9]. It also inhibits IL-6 expression and promotes cell-cycle arrest and apoptosis in cancer cells[1]. According to several researches, zerumbone has little to no cytotoxic impact on normal human endothelial cells and fibroblasts while inducing apoptosis in cancer cell lines. As a result, zerumbone can be regulated at several molecular sites for cancer prevention and therapy[17,20].

Several authors have described the health advantages of *Z. zerumbet.* Its chemical components can decrease cyclooxygenase enzyme activity and synthesis of inflammatory mediators[18,29]. Yob et al[26] demonstrated that the plant *Z. zerumbet* possessed promising anti-inflammatory, antinociceptive, antiproliferative, antitumor, antiplatelet aggregation and larvicidal activities through *in vitro* studies.

Despite all the information available on the EO of *Z. zerumbet*, the investigation dealing with the comparative study has been inadequate. This study aims to compare the antioxidant, cytotoxicity and anti-inflammatory activity of the *Z. zerumbet* rhizome EO through their *in vitro* activity assays like DPPH, reducing power, MTT and NO inhibition tests to evaluate their significance for traditional use along with PASS prediction to know the biological activity of the individual components to strengthen the *in vitro* findings.

Material and Methods

Extraction of rhizome essential oil: The *Z. zerumbet* rhizome samples were collected from the Barunei region of Khordha district, Odisha in 2022. All the collected rhizomes were authenticated by Dr. P. C. Panda, Plant taxonomist and the voucher specimen was deposited to the herbarium of CBT, SOA. The rhizomes were cleaned, chopped and subjected to hydrodistillation for isolation of the essential oil. The pale yellow-colored essential oil was recovered from the distillate and filtered with anhydrous sodium sulfate. The essential oil was stored in sealed vials at 4^0C until studied.

GC-MS Analysis: To analyze the phytochemical composition of the oil, the Gas-chromatographic mass-spectrometer (GC-MS) technique was employed. The GC-MS analysis was done on Clarus 580 GC attached to an SQ8S mass spectrometric detector. The GC was equipped with an Elite-5 MS column, whose film thickness was 30 m×0.25 mm, 0.25 µm. The instrument was operated using the following conditions: oven temperature initially set at 60^0C, then increased to 220^0C (with a ramp of 3^0C/min), final hold for 7 minutes, carrier gas He, injection volume 0.1 µL neat oil. The mass scan was performed between 50 to 600 amu with electron ionization mode at 70 eV voltages. The constituent identification was done by comparing the mass spectra to the NIST08 library followed by matching their experimental RI values with those reported in the literature[3]. To calculate the experimental RI, n-alkanes (C8–C20) series was subjected to GC-MS in similar chromatographic conditions as the sample.

***In silico* PASS Prediction:** The computational-based biological activity was investigated by employing the PASS online tool (http://www.way2drug.com/passonline/). The simplified molecular-input line-entry system (SMILES) notations of all the constituents were used in the PASS prediction. PASS is a sophisticated method that can estimate the biological activity of desired or unknown candidates based on their chemical structures[10]. The PASS prediction delivers values for probable activity (Pa) and probable inactivity (Pi), based on currently available training set data in the PASS library.

DPPH Antioxidant Assay: The essential oil's DPPH free radical scavenging potential was assessed using 1,2-diphenyl-2-picryl-hydrazil (DPPH). 0.004% w/v DPPH radical solution in methanol was prepared. After 1 hr, the absorbance was measured at 517nm on a spectrophotometer. The lower is the absorbance of the DPPH solution, the higher is the DPPH radical scavenging activity. The radical scavenging expressed as a percentage of inhibition was calculated using the following equation:

$$\%\ Inhibition = \frac{A - B}{A} \times 100$$

where A is the absorbance of control i.e. DPPH solution without sample and B is the absorbance of the sample with DPPH solution. IC_{50} value indicates the concentration where the sample shows 50% inhibiting activity.

Reducing Power Assay: The ferric-reducing power assay (FRAP) is a method used to measure antioxidant capacity. This assay deals with the reduction of the ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The reduction of ferric ions is monitored by measuring the change in absorbance at a wavelength of 700nm and can be determined by seeing the decrease in the absorbance. For this study, 1ml of aliquots of various concentrations (100 µg/ml to 1 mg/ml) of standards (Ascorbic acid and BHT) and the *Z. zerumbet* rhizome essential oil (1-15 mg/ml) were taken. The aliquots of samples and the standards were mixed with 0.2M phosphate buffer (pH 6.6) and 1% w/v potassium ferricyanide solution and heated in a water bath at 50^oC for 20min.

To get the supernatant, trichloroacetic acid was added and then mixed with distilled water and 0.1% anhydrous ferric chloride ($FeCl_3$). The absorbance was taken at 700nm after 30min incubation. The activity was calculated by the following equation:

$$\%\ Inhibition = \frac{A - B}{A} \times 100$$

where A is the absorbance of control i.e. ferric solution without sample and B is the absorbance of the sample with ferric solution. IC_{50} value indicates the concentration where the sample shows 50% inhibiting activity.

MTT Assay: MTT assay was used to measure the toxicity of *Z. zerumbet* essential oil on RAW 264.7 (murine macrophage) cell lines. RAW 264.7 cells were cultured at a concentration of 5×10^5 cells/mL in a 96-well culture plate and were incubated. After incubation, the cells were treated with 20 µL of essential oil at concentrations ranging from 6.25µg/mL to 100µg/mL for 1 hour. Following a 24-hour incubation period, MTT reagent (5mg/mL, Himedia) was added to each well and incubated for 1 hour. Afterwards, the supernatant was discarded and 100µL of DMSO was added and mixed to each well to dissolve the formazan crystals that had formed. The absorbance at 570 nm was taken to quantify formazan crystals using an ELISA plate reader[4,16]. The cell viability percentage was calculated by:

$$\%\ Cell\ viability = \frac{Abs\ of\ treated\ cells}{Abs\ of\ Untreated\ cells} \times 100$$

The IC_{50} value for the *Z. zerumbet* essential oil was calculated by plotting a graph of essential oil concentrations against cell viability percentage.

Nitric Oxide Inhibition Assay: RAW 264.7 cells were cultured in 96-well plates with a mass of 2×10^4 cells/well and incubated for 24 hours to allow adhesion. The cells were then treated with lipopolysaccharide (LPS) at a concentration of 1µg/mL for 2 hrs. Following the pre-

treatment, the cells were treated with *Z. zerumbet* rhizome essential oil at doses ranging from 6.25µg/mL to 100µg/mL and incubated for 24 hours at 37°C. The supernatant from each well was collected after the incubation period of 24 hours and it was gently rinsed with cold PBS using centrifugation at 1,000 rpm for 5 minutes. Then 100µL of supernatant was mixed with 100µL of Griess reagent and the mixture was incubated for 10 minutes at 37^0C[15]. At 580 nm, the absorbance was measured. The nitrite content in the culture medium was measured by comparing the absorbance to a sodium nitrite standard curve.

Results and Discussion

Essential oil Composition: The average yield of the hydrodistilled rhizome essential oil of *Z. zerumbet* was 0.63 ± 0.06 %. This is in agreement with a previous report[7]. The phytoconstituents present in the *Z. zerumbet* essential oil were presented in table 1 and listed according to their elution order in the Elite-5 MS column. The GC-MS analysis of the essential oil revealed a total number of 29 compounds which accounted for 94.68±1.22% of the total oil.

Table 1
Volatile compounds identified in the *Zingiber zerumbet* rhizome essential oil

S.N.	Compounds	RT	RI[a]	RI[b]	Area%
1	Tricyclene	5.379	920	926	0.08±0.01
2	α-Pinene	5.656	930	939	1±0.03
3	α-Fenchene	6.144	947	952	2.64±0.09
4	Sabinene	6.875	973	975	0.03±0.01
5	Camphene	7.159	983	954	0.15±0.01
6	β-Pinene	7.741	1003	979	0.09±0.01
7	α-Phellandrene	7.842	1006	1002	0.5±0.01
8	ρ-Cymene	8.343	1020	1024	0.09±0.01
9	Limonene	8.519	1024	1031	0.41±0.01
10	β-Ocimene	8.655	1028	1024	0.29±0.01
11	Eucalyptol	9.535	1052	1038	0.02±0.01
12	γ-Terpinene	10.564	1080	1059	0.03±0.01
13	Terpinolene	10.719	1084	1088	0.01±0.01
14	Linalool	11.146	1096	1096	3.51±0.06
15	Camphor	13.062	1142	1159	2.47±0.04
16	Isoborneol	13.38	1150	1174	0.02±0.01
17	Borneol	14.023	1165	1186	0.02±0.01
18	Terpinen-4-ol	14.443	1175	1184	0.05±0.01
19	α-Terpineol	14.734	1182	1188	0.02±0.01
20	Eugenol	23.257	1382	1356	0.04±0.01
21	β-Caryophyllene	24.516	1412	1426	1±0.03
22	α-Humulene	26.249	1454	1446	11.72±0.3
23	δ-Cadinene	29.207	1527	1516	0.24±0.02
24	Elemol	29.748	1541	1528	0.06±0.01
25	Germacrene	30.324	1556	1519	0.11±0.01
26	E-Nerolidol	30.655	1564	1543	0.01±0.01
27	Spathulenol	31.021	1574	1583	1.25±0.02
28	β-Eudesmol	33.748	1645	1619	0.52±0.02
29	Zerumbone	37.83	1757	1733	68.18±0.42
	Total identified				94.68±1.22
	Monoterpene hydrocarbons (1-10, 12,13)				5.32±0.22
	Monoterpene alcohols (14, 16,17,19)				3.58±0.09
	Monoterpene ketones (15)				2.47±0.04
	Menthane monoterpenoids (18)				0.05±0.01
	Sesquiterpene hydrocarbons (21-25)				13.14±0.64
	Sesquiterpene alcohol (26-28)				1.79±0.05
	Cyclic sesquiterpene (29)				68.18±0.42
	Ethers (11)				0.02±0.01
	Phenols (20)				0.04±0.01

Note: Data are represented as mean ± SD. RT= Retention Time. [a] Retention Index (RI) calculated against homologous n-alkane series (C_8-C_{20}) on the Elite-5 MS column. [b] RI from literature.

Among all, cyclic sesquiterpene (68.18±0.42%) was the predominant and most abundant class of compounds among others, followed by sesquiterpene hydrocarbons (13.14±0.64%), monoterpene hydrocarbons (5.32±0.22%), monoterpene alcohols (3.58±0.09%) and monoterpene ketones (2.47±0.04%). Zerumbone (68.18±0.42%) was found to be the major constituent followed by α-humulene (11.72±0.3%) and linalool (3.51±0.06%). α-pinene (1±0.03%), α-fenchene (2.64±0.09%), camphor (2.47±0.04%), β-caryophyllene (1±0.03%) and spathulenol (1.25±0.02%) were among the other chemicals that were found to have compositions more than 1%. A total of twenty-one constituents had compositions that were less than 1 %.

Dash et al[7] reported zerumbone to be the major constituent of ZZEO. According to Srivastava et al[23] and Yu et al[27], the essential oil of *Z. zerumbet* contains about 86% sesquiterpenoids, with zerumbone being the most abundant. They also discovered caryophyllene in the *Z. zerumbet* essential oil. Chane-Ming et al[6] also showed that the EO of *Z. zerumbet* was rich in zerumbone, α-pinene and camphene.

Possible biological activity analysis (PASS): The possible biological activity of the phytoconstituents analyzed through GC-MS was assessed by PASS prediction (Table 2). Briefly, 12 out of the 29 phytocompounds in *Z. zerumbet* essential oil showed anti-inflammatory and antioxidant activity with a Pa value of more than 0.7. According to PASS prediction, those compounds that exhibit Pa>0.7 can be considered as most potential in case of having biological activity and those that exhibit Pa within 0.7 to 0.5, can be considered with moderate activity. Among the 12 compounds exhibiting Pa>0.7, 5 phytocompounds, such as α-pinene (0.853>0.005), camphor (0.701>0.015), β-caryophyllene (0.741> 0.011), spathulenol (0.769>0.009) and zerumbone (0.764>0.002) are with an area percentage more than 1% in the essential oil. Scientifically, PASS prediction of individual compounds confirmed that the *Z. zerumbet* essential oil could be used as a natural anti-inflammatory and antioxidant regime after some pharmacological validation.

Antioxidant Activities: DPPH free-radical scavenging and the reducing power test were used to assess the antioxidant properties of *Zingiber zerumbet* essential oil. Ascorbic acid and BHT were taken as the standard (positive control). In the DPPH assay, the 50% inhibitory concentration (IC_{50}) values of sample concentration were used to calculate the sample's ability to decrease free radicals. The antioxidant activity was assessed based on the IC_{50} value (Table 3). Ascorbic acid and BHT, the positive controls, showed IC50 values of 0.01±1.48 mg/ml and 0.02±1.21 mg/ml respectively.

The IC50 value of *Z. zerumbet*essential oil was determined to be 83.90±0.05 mg/ml indicating weaker DPPH radical scavenging potential than the positive control. In the reducing power assay, the effective concentration (EC50) of the sample solution required to reduce Fe^{3+} to Fe^{2+} was found to be 1.98±0.04 mg/ml, 0.01±1.33 mg/ml and 0.02±1.62 mg/ml for *Z. zerumbet* essential oil, ascorbic acid and BHT respectively. Therefore, the rhizome essential oil exhibited a weaker EC50 value than positive control in reducing power assay.

Table 2
***In silico* PASS prediction bioactivities of phyto-constituents analyzed from *Z. zerumbet* rhizome essential oil**

S.N.	Compounds	Canonical smiles	PASS Prediction (Pa > Pi)	
			Activity	**Pa > Pi**
1	Tricyclene	CC1=CCC2CC1C2(C)C	2-Methylacyl-CoA dehydrogenase inhibitor	0.037>0.021
			2-Oxoaldehyde dehydrogenase (NADP+) inhibitor	0.256>0.054
			3'-Nucleotidase inhibitor	0.113>0.112
			4-Alpha-glucanotransferase inhibitor	0.083>0.075
2	α-Pinene	CC1(C2CCC1C(=C)C2)C	Anticarcinogenic	0.265>0.075
			Antiinflammatory	0.853>0.005
			Antiinflammatory, intestinal	0.232> 0.142
			Antiinflammatory, ophthalmic	0.283> 0.106
			Oxidoreductase inhibitor	0.475> 0.083
3	α-Fenchene	CC(C)C12CCC(=C)C1C2	Oxidizing agent	0.368> 0.027
			Oxidoreductase inhibitor	0.431> 0.097
			Nitric oxide scavenger	0.316> 0.005
			Nitric oxide antagonist	0.317> 0.012
			Oxygen scavenger	0.350> 0.130
4	Sabinene	CC1(C2CCC(C2)C1=C)C	Antiinflammatory	0.611> 0.029
			Oxidizing agent	0.399> 0.022
			Oxidoreductase inhibitor	0.461> 0.088
			Nitrite reductase (NO-forming) inhibitor	0.386> 0.043
			Nitric oxide antagonist	0.317> 0.012
			Nitric oxide scavenger	0.300> 0.007

			Anticarcinogenic	0.214> 0.106
5	Camphene	CC1(C2CCC(=C)C1C2)C	Oxygen scavenger	0.466> 0.063
			Nitric oxide scavenger	0.307> 0.006
			Antiinflammatory	0.396> 0.098
			Antiinflammatory, ophthalmic	0.329> 0.044
			Anticarcinogenic	0.259> 0.078
6	β-Pinene	CC1=CCC(C=C1)C(C)C	Oxidoreductase inhibitor	0.755> 0.010
			Antiinflammatory	0.644> 0.024
			2-Oxoaldehyde dehydrogenase (NADP+) inhibitor	0.623> 0.005
			Oxygen scavenger	0.619> 0.019
			Nitric oxide scavenger	0.346> 0.004
			Antiinflammatory, intestinal	0.364> 0.035
7	α-Phellandrene	CC1=CC=C(C=C1)C(C)C	Antiinflammatory	0.610> 0.029
			Oxygen scavenger	0.358> 0.123
			Oxidizing agent	0.261> 0.057
			Antioxidant	0.157> 0.094
8	ρ-Cymene	CC1=CCC(CC1)C(=C)C	Antiinflammatory	0.599> 0.032
			Antioxidant	0.570> 0.005
			Nitrite reductase (NO-forming) inhibitor	0.548> 0.014
			Oxygen scavenger	0.494> 0.053
			Anticarcinogenic	0.444> 0.025
			Nitric oxide antagonist	0.402> 0.007
			Nitric oxide scavenger	0.397> 0.003
9	Limonene	CC(=CCC=C(C)C=C)C	Nitrite reductase (NO-forming) inhibitor	0.437> 0.031
			Nitric oxide scavenger	0.330> 0.004
			Oxygen scavenger	0.410> 0.090
			Oxidizing agent	0.344> 0.033
			Antiinflammatory, ophthalmic	0.327> 0.046
			Nitric oxide antagonist	0.227> 0.028
10	β-Ocimene	CC1(C2CCC(O1)(CC2)C)C	Oxidoreductase inhibitor	0.582> 0.043
			Oxygen scavenger	0.537> 0.039
			Antiinflammatory	0.470> 0.066
			Nitric oxide scavenger	0.329> 0.004
			Antiinflammatory, intestinal	0.311> 0.063
11	Eucalyptol	CC1=CCC(=CC1)C(C)C	Oxygen scavenger	0.571> 0.030
			Oxidizing agent	0.489> 0.009
			Antiinflammatory	0.528> 0.049
12	γ-Terpinene	CC1=CCC(=C(C)C)CC1	2-Methylacyl-CoA dehydrogenase inhibitor	0.037>0.021
			2-Oxoaldehyde dehydrogenase (NADP+) inhibitor	0.256>0.054
			3'-Nucleotidase inhibitor	0.113>0.112
			4-Alpha-glucanotransferase inhibitor	0.083>0.075
13	Terpinolene	CC(=CCCC(C)(C=C)O)C	Antiinflammatory	0.791>0.007
			Nitric oxide scavenger	0.381>0.003
			Oxygen scavenger	0.380>0.107
			Antioxidant	0.251>0.036
14	Linalool	CC1(C2CCC1(C(=O)C2)C)C	Nitric oxide scavenger	0.348>0.004
			Oxygen scavenger	0.416>0.086
15	Camphor	CC1(C2CCC1(C(C2)O)C)C	Oxidoreductase inhibitor	0.701>0.015
			Antiinflammatory	0.538>0.046
			Nitric oxide scavenger	0.431>0.002
16	Isoborneol	CC1(C2CCC1(C(C2)O)C)C	Oxidoreductase inhibitor	0.700>0.015
			Antiinflammatory	0.538>0.046
			Nitric oxide scavenger	0.431>0.002
			Oxygen scavenger	0.348>0.131

17	Borneol	CC1=CCC(CC1)(C(C)C)O	Oxidoreductase inhibitor	0.636>0.026
			Antiinflammatory	0.609>0.030
			Oxygen scavenger	0.422>0.083
			Nitric oxide scavenger	0.322>0.005
18	Terpinen-4-ol	CC1=CCC(CC1)C(C)(C)O	Antiinflammatory	0.651>0.023
			Oxidoreductase inhibitor	0.478>0.082
			Oxygen scavenger	0.416>0.086
			Nitric oxide scavenger	0.305>0.006
19	α-Terpineol	COC1=C(C=CC(=C1)CC=C)O	Free radical scavenger	0.563>0.007
			Oxidoreductase inhibitor	0.545>0.057
			Antioxidant	0.463>0.008
			Antiinflammatory	0.491>0.060
20	Eugenol	CC1=CCCC(=C)C2CC(C2CC1)(C)C	Antiinflammatory	0.745>0.011
			Nitric oxide antagonist	0.485>0.005
			Non-steroidal antiinflammatory agent	0.435>0.018
			Nitric oxide scavenger	0.264>0.015
21	β-Caryophyllene	CC1=CCC(C=CCC(=CCC1)C)(C)C	Antiinflammatory	0.741> 0.011
			Oxidizing agent	0.448> 0.014
			TNF expression inhibitor	0.427> 0.048
			Nitric oxide antagonist	0.367> 0.008
			Oxygen scavenger	0.415> 0.087
22	α-Humulene	CC1=CC2C(CCC(=C2CC1)C)C(C)C	Antiinflammatory	0.492>0.059
			Oxidoreductase inhibitor	0.492>0.077
			Nitric oxide scavenger	0.296>0.008
23	δ-Cadinene	CC(=C)C1CC(CCC1(C)C=C)C(C)(C)O	Antiinflammatory	0.793>0.007
			Nitric oxide antagonist	0.410>0.007
			Nitric oxide scavenger	0.257>0.018
			Antioxidant	0.168>0.081
24	Elemol	CC1=CCCC(=CCC(CC1)C(=C)C)C	Antiinflammatory	0.602>0.031
			Nitric oxide antagonist	0.472>0.005
			Nitric oxide scavenger	0.287>0.010
			Oxidoreductase inhibitor	0.344>0.134
25	Germacrene	CC(=CCCC(=CCCC(C)(C=C)O)C)C	Antiinflammatory	0.800>0.007
			Nitric oxide scavenger	0.481>0.002
			Antioxidant	0.431>0.010
26	E-Nerolidol	CC1(C2C1C3C(CCC3(C)O)C(=C)CC2)C	Prostaglandin-E2 9-reductase inhibitor	0.651>0.020
			Oxidoreductase inhibitor	0.533>0.062
			Antiinflammatory	0.521>0.051
			Nitric oxide scavenger	0.265>0.015
27	Spathulenol	CC12CCCC(=C)C1CC(CC2)C(C)(C)O	Antiinflammatory	0.769>0.009
			Oxidoreductase inhibitor	0.509>0.071
			Transcription factor NF kappa B inhibitor	0.398>0.007
			Nitric oxide scavenger	0.293>0.009
28	β-Eudesmol	CC1=CCC(C=CC(=O)C(=CCC1)C)(C)C	Antiinflammatory	0.831>0.005
			TNF expression inhibitor	0.536>0.022
			Glyoxylate oxidase inhibitor	0.521>0.015
			Oxidoreductase inhibitor	0.484>0.080
29	Zerumbone	CC1=CCC2CC1C2(C)C	Prostaglandin E1 antagonist	0.764>0.002
			Macrophage colony stimulating factor agonist	0.509>0.061
			Antiinflammatory	0.490>0.060
			(R)-6-hydroxynicotine oxidase inhibitor	0.468>0.058
			Glyoxylate oxidase inhibitor	0.415>0.029
			Prostaglandin-A1 DELTA-isomerase inhibitor	0.401>0.048

Tian et al[25] demonstrated that the fresh rhizome essential oil of *Z. zerumbet* exhibited weak free-radical-scavenging activity as well as antibacterial and cytotoxic activities against various human tumor cell lines in a time and concentration-dependent manner. Similarly, Hemn et al[12] discovered that zerumbone, the main component of *Z. zerumbet* essential oil, is a potent antioxidant that suppresses free radical formation *in vivo*, implying that the monoterpenes or sesquiterpenes found in the essential oil have antioxidative activity. Though the DPPH and FRAP assays are used to measure the free radical-reducing abilities, they are not suitable for studying the efficiency of lipid peroxidation inhibition. Different approaches for assessing antioxidant capability *in vitro* and *in vivo* may produce inconsistent results.

Cytotoxicity Study: Cytotoxicity assay of *Z. zerumbet* oil was done using the MTT colorimetric assay. The anti-inflammatory potential of essential oils has been widely examined to mitigate the resistance development to multiple drugs and side effects. The current study aimed to evaluate the cytotoxic effect of the essential oil of *Z. zerumbet* against RAW 264.7 cell line using MTT assay. Cells treated with different concentrations of essential oil (ranging from 6.25 to 100µg/mL) for 24 hr did not exhibit a significant reduction in viability compared to untreated control RAW 264.7 cells. The observations in statistical data of cell cytotoxicity study by MTT assay suggest that *Z. zerumbet* oil showed IC_{50} at 176.71 µg/ml against Raw 264.7 cells (Figure 1). *Z. zerumbet* showed non-toxicity on Raw 264.7 cells till the 50 µg/mL concentration was greater than 70% cell viability and decided 50 µg/mL maximum concentrations as an optimum concentration for further studies.

Recent studies have revealed the significant cytotoxic activity of zerumbone against leukemia, lung cancer, colon cancer, skin cancer, ovarian cancer, liver cancer and breast cancer. Zerumbone, a key compound in *Z. zerumbet*, has emerged as a highly active anti-cancer natural product[22]. Zerumbone exhibited selective cytotoxic activity to human tumor cell lines, demonstrating significantly lower cytotoxicity towards human normal cell lines compared to tumor cells[24]. Zerumbone, the principal compound in *Z. zerumbet*, was mainly responsible for cytotoxic activity. Huang et al[13] have shown zerumbone, the major compound of *Z. zerumbet* as an effective antitumor compound in both *in vitro* and *in vivo* models.

Table 3
Antioxidant activities of *Zingiber zerumbet* essential oil using DPPH and FRAP assays

Samples/Standards	IC50 of DPPH radical scavenging activity	EC50 of FRAP radical scavenging activity
Essential oil	83.90±0.05 mg/ml	1.98±0.04 mg/ml
Ascorbic acid	0.01±1.48 mg/ml	0.01±1.33 mg/ml
BHT	0.02±1.21 mg/ml	0.02±1.62 mg/ml

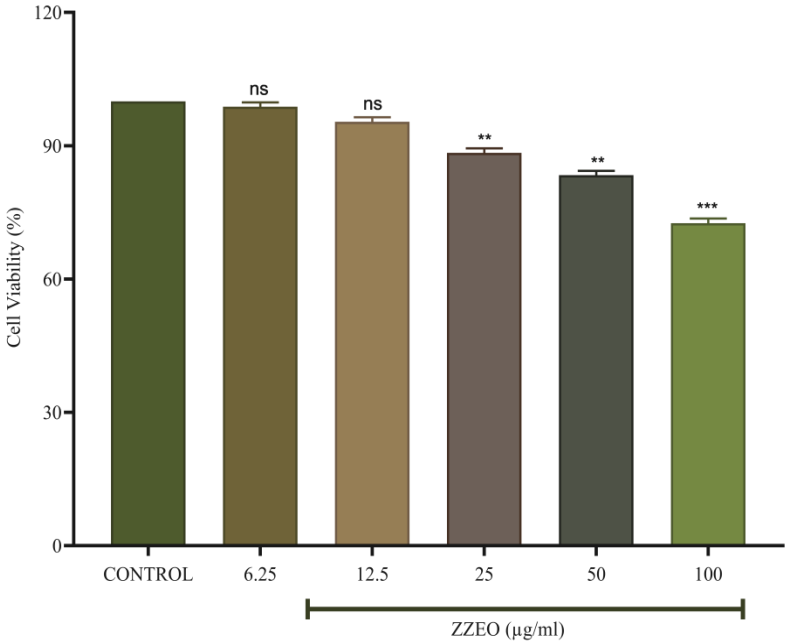

Figure 1: Effects of *Zingiber zerumbet* essential oil on cell viability of the RAW 264.7 cells after 24hr using MTT assay. Data are presented as means ± SD of different experiments. *p<0.05, **p<0.01 and ***p<0.001 represent significant statistical differences relative to the control group performed using one-way ANOVA with Dunnett's Multiple Comparison post-test (ns- non-significant).

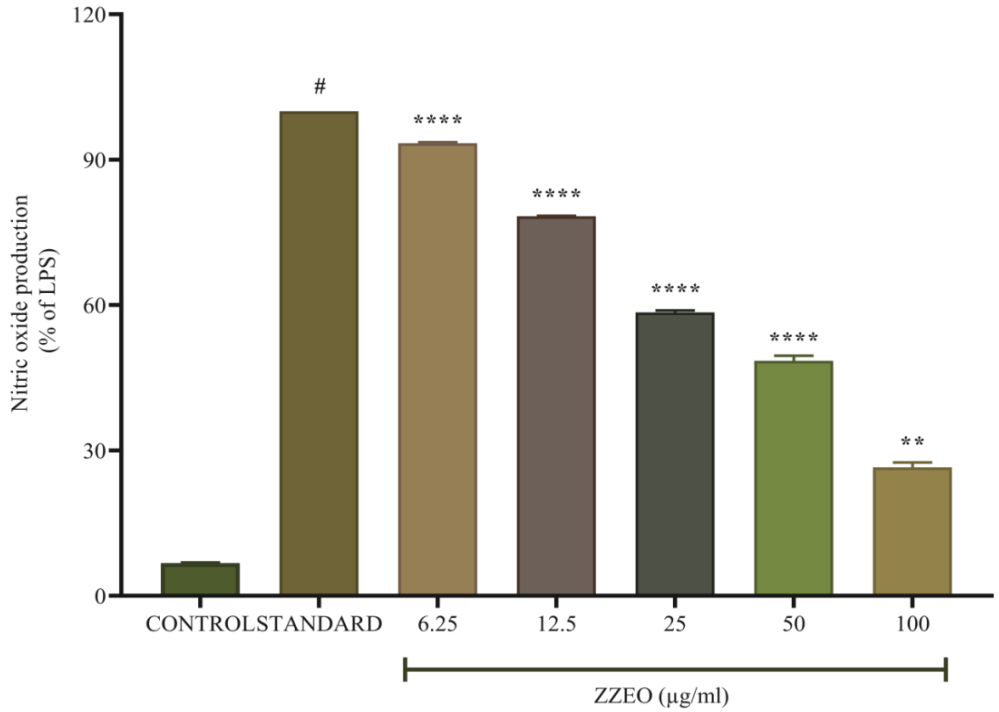

Figure 2: Effects of *Zingiber zerumbet* essential oil on nitric oxide production on LPS induced RAW 264.7 cells. Data are presented as means ± SD of different experiments. **p<0.01 and ****p<0.0001 represent significant statistical differences relative to the control group performed using one-way ANOVA as compared with LPS treated group (ns- non-significant).

Nitric Oxide Inhibition Assay: The effect of *Z. zerumbet* rhizome essential oil on nitric oxide (NO) production was examined on LPS-stimulated RAW 264.7 murine macrophage cells. Lipopolysaccharide (LPS) was used to induce inflammation and stimulate the release of Nitric Oxide from macrophage cells. Nitric oxide is considered a mediator for pathological reactions, particularly in acute inflammatory responses[11]. The incubation of macrophages with the essential oil resulted in the inhibition of LPS-induced nitrite accumulation in a dose-dependent manner (Figure 2).

The nitrite concentration was assessed following the treatment with LPS (1 µg/mL) alone (Standard) and in the addition of *Z. zerumbet* rhizome essential oil at varying concentrations (6.25, 12.5, 25, 50 and 100 µg/mL). Adding 100 µg/mL of rhizome essential oil, the LPS-induced NO production in RAW 264.7 cells was notably reduced to nearly 30% compared to LPS alone treated cells. Murakami et al[17] have shown that the major compound zerumbone of *Z. zerumbet* inhibits NO and PGE_2 production in LPS-stimulated RAW 264.7 cells.

Conclusion

The antioxidant effect of rhizome essential oil was shown to be weak in this investigation. The results showed that *Zingiber zerumbet* rhizome essential oil has less cytotoxicity and a high anti-inflammatory effect by reducing NO generation in the RAW 264.7 murine macrophage cell line. The current *in vitro* investigations show that *Zingiber zerumbet* essential oil can be utilized to treat a variety of inflammatory disorders. However, adequate confirmation through animal models is required before putting the essential oil through clinical trials.

Acknowledgement

The authors are indebted to the President, SOA and Dean, SPS for providing infrastructure and support for the research.

References

1. Abdelwahab S.I., Abdul A.B., Zain Z.N.M. and Hadi A.H.A., Zerumbone inhibits interleukin-6 and induces apoptosis and cell cycle arrest in ovarian and cervical cancer cells, *International Immunopharmacology*, **12(4)**, 594-602 **(2012)**

2. Abdul A.B., Abdelwahab S.I., Al-Zubairi A.S., Elhassan M.M. and Murali S.M., Anticancer and antimicrobial activities of zerumbone from the rhizomes of Zingiber zerumbut, *IJP-International Journal of Pharmacology*, **4(4)**, 301-304 **(2008)**

3. Adams R.P., Identification of Essential Oil Component by Gas Chromatography/Mass Spectrometry, 4th ed., Allured Publishing Corporation, Carol Stream, IL, USA, 1–811 **(2007)**

4. Alley M.C., Scudiero D.A., Monks A., Hursey M.L., Czerwinski M.J., Fine D.L., Abbott B.J., Mayo J.G., Shoemaker R.H. and Boyd M.R., Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay, *Cancer Research*, **48(3)**, 589-601 **(1988)**

5. Baby S., Dan M., Thaha A.R., Johnson A.J., Kurup R., Balakrishnapillai P. and Lim C.K., High content of zerumbone in volatile oils of *Zingiber zerumbet* from southern India and Malaysia, *Flavour and Fragrance Journal*, **24(6)**, 301-308 **(2009)**

6. Chane-Ming J., Vera R. and Chalchat J.C., Chemical composition of the essential oil from rhizomes, leaves and flowers of *Zingiber zerumbet* Smith from Reunion Island, *Journal of Essential Oil Research*, **15(3)**, 202-205 **(2003)**

7. Dash B., Ray A., Sahoo A., Kar B., Chatterjee T., Halder T., Chandra Panda P., Patnaik J., Ghosh B. and Nayak S., A combined approach using ISSR and volatile compound analysis for assessment of genetic and phytochemical diversity in *Zingiber zerumbet* (L.) from Eastern India, *Journal of Essential Oil Bearing Plants*, **22(1)**, 31-49 **(2019)**

8. Dash B., Sahoo A., Ray A., Jena S. and Nayak S., Identification of chemical constituents of *Zingiber zerumbet* rhizome extract using GC/MS, *Journal of Biologically Active Products from Nature*, **10(5)**, 411-417 **(2020)**

9. Giang P.M., Son P.T., Jin H.Z., Lee J.H. and Lee J.J., Comparative study on inhibitory activity of zerumbone and zerumbone 2, 3-epoxide on NF-κB activation and NO production, *Scientia Pharmaceutica*, **77(3)**, 589-596 **(2009)**

10. Goel R.K., Singh D., Lagunin A. and Poroikov V., PASS-assisted exploration of new therapeutic potential of natural products, *Med. Chem. Res.*, **20**, 1509-1514 **(2011)**

11. Guzik T., Korbut R. and Adamek-Guzik T., Nitric oxide and superoxide in inflammation, *Journal of Physiology and Pharmacology*, **54(4)**, 469-487 **(2003)**

12. Hemn H.O., Noordin M.M., Rahman H.S., Hazilawati H., Zuki A. and Chartrand M.S., Antihypercholesterolemic and antioxidant efficacies of zerumbone on the formation, development and establishment of atherosclerosis in cholesterol-fed rabbits, *Drug Design, Development and Therapy*, **9**, 4173-4208 **(2015)**

13. Huang G.C., Chien T.Y., Chen L.G. and Wang C.C., Antitumor effects of zerumbone from *Zingiber zerumbet* in P-388D1 cells in vitro and in vivo, *Planta Medica*, **71(03)**, 219-224 **(2005)**

14. Madegowda B.H., Rameshwaran P., Nagaraju N.P. and Murthy P.S., *In vitro* Mycological activity of essential oil from *Zingiber zerumbet* rhizomes, *Journal of Essential Oil Research*, **28(1)**, 81-88 **(2016)**

15. Mohanty S., Ray A., Sahoo C., Sahoo A., Jena S., Panda P.C. and Nayak S., Volatile profiling coupled with multivariate analysis, antiproliferative and anti-inflammatory activities of rhizome essential oil of four Hedychium species from India, *Journal of Ethnopharmacology*, **317**, 116835 **(2023)**

16. Mosmann T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *Journal of Immunological Methods*, **65(1-2)**, 55-63 **(1983)**

17. Murakami A., Takahashi D., Kinoshita T., Koshimizu K., Kim H.W., Yoshihiro A., Nakamura Y., Jiwajinda S., Terao J. and Ohigashi H., Zerumbone, a Southeast Asian ginger sesquiterpene, markedly suppresses free radical generation, proinflammatory protein production and cancer cell proliferation accompanied by apoptosis: the α, β-unsaturated carbonyl group is a prerequisite, *Carcinogenesis*, **23(5)**, 795-802 **(2002)**

18. Nayak A., Gadnayak A., Dash K.T., Jena S., Ray A., Nayak S. and Sahoo A., Exploring molecular docking with MM-GBSA and molecular dynamics simulation to predict potent inhibitors of cyclooxygenase (COX-2) enzyme from terpenoid-based active principles of Zingiber species, *Journal of Biomolecular Structure and Dynamics*, **41(20)**, 10840-10850 **(2023)**

19. Padalia R.C., Verma R.S., Chauhan A., Singh V.R., Goswami P., Singh S., Verma S.K., Luqman S., Chanotiya C.S. and Darokar M.P., *Zingiber zerumbet* (L.) Roscoe ex Sm. from northern India: Potential source of zerumbone rich essential oil for antiproliferative and antibacterial applications, *Industrial Crops and Products*, **112**, 749-754 **(2018)**

20. Prasannan R., Kalesh K.A., Shanmugam M.K., Nachiyappan A., Ramachandran L., Nguyen A.H., Kumar A.P., Lakshmanan M., Ahn K.S. and Sethi G., Key cell signaling pathways modulated by zerumbone: role in the prevention and treatment of cancer, *Biochemical Pharmacology*, **84(10)**, 1268-1276 **(2012)**

21. Sidahmed H.M.A., Hashim N.M., Abdulla M.A., Ali H.M., Mohan S., Abdelwahab S.I., Taha M.M.E., Fai L.M. and Vadivelu J., Antisecretory, gastroprotective, antioxidant and anti-Helicobcter pylori activity of zerumbone from *Zingiber zerumbet* (L.) Smith, *PloS One*, **10(3)**, e0121060 **(2015)**

22. Singh Y.P., Girisa S., Banik K., Ghosh S., Swathi P., Deka M., Padmavathi G., Kotoky J., Sethi G., Fan L. and Mao X., Potential application of zerumbone in the prevention and therapy of chronic human diseases, *Journal of Functional Foods*, **53**, 248-258 **(2019)**

23. Srivastava A.K., Srivastava S.K. and Shah N.C., Essential oil composition of *Zingiber zerumbet* (L.) Sm. from India, *Journal of Essential Oil Research*, **12(5)**, 595-597 **(2000)**

24. Thorat Abhijit S. and Attarde Sanjay B., Status of solid waste generation in municipal corporation cities of Maharashtra in India: a review, *Res. J. Chem. Environ.*, **27(3)**, 129-145 **(2023)**

25. Tian M., Wu X., Hong Y., Wang H., Deng G. and Zhou Y., Comparison of chemical composition and bioactivities of essential oils from fresh and dry rhizomes of *Zingiber zerumbet* (L.) Smith, *BioMed Research International*, **2020(1)**, 9641284-9641292 **(2020)**

26. Yob N.J., Jofrry S.M., Affandi M.M.R., Teh L.K., Salleh M.Z. and Zakaria Z.A., *Zingiber zerumbet* (L.) Smith: a review of its ethnomedicinal, chemical and pharmacological uses, *Evidence-Based Complementary and Alternative Medicine*, **2011(1)**, 543216-543227 **(2011)**

27. Yu F. et al, Isolation and functional characterization of a β-eudesmol synthase, a new sesquiterpene synthase from *Zingiber zerumbet* Smith, *FEBS Letters*, **582(5)**, 565-572 **(2008)**

28. Zakaria Z.A., Mohamad A.S., Ahmad M.S., Mokhtar A.F., Israf D.A., Lajis N.H. and Sulaiman M.R., Preliminary analysis of the anti-inflammatory activity of essential oils of Zingiber zerumbet, *Biological Research for Nursing*, **13(4)**, 425-432 **(2011)**

29. Zakaria Z.A., Mohamad A.S., Chear C.T., Wong Y.Y., Israf Z. and Sulaiman M.R., Antiinflammatory and antinociceptive activities of *Zingiber zerumbet* methanol extract in experimental model systems, *Medical Principles and Practice*, **19(4)**, 287-294 **(2010)**.

(Received 01st April 2024, accepted 03rd June 2024)