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RESEARCH ARTICLE

Zingiber zerumbet rhizome extract inhibits cell migration and induces apoptosis in MCF-7 breast cancer cells

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Abstract

Zingiber zerumbet (L.) Roscoe ex Sm. (Zingiberaceae) is used in an extensive range of conventional medicine practices. This study demonstrates the chemical constituents of the rhizome extract of *Z. zerumbet* and evaluates its antiradical, antiproliferative and apoptosis-inducing potential against breast cancer cells. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified zerumbone (94.88 ± 0.02) to be the main component of the methanol extract along with humulene epoxide (3.05 ± 0.06) and caryophyllene oxide (2.07 ± 0.04). The extract exhibited notable antiradical activity with IC_{50} value of 261.66 ± 0.20 and 159.45 ± 0.15 µg/mL for DPPH and ABTS assays respectively. While the reducing power assay indicated effective activity in the range, 200–500 µg/mL. Moreover, cell viability assay exhibited MCF-7 cells to be the most sensitive among the cell lines examined. DNA fragmentation analysis and Annexin V-Fluorescein Isothiocyanate/propidium iodide (Annexin V-FITC/PI) dual staining confirmed the induction of apoptosis in MCF-7 cells. The changes in nuclear morphology were further validated using 4′,6-diamidino-2-phenylindole (DAPI) staining. Additionally, the cell migration assay presented the anti-metastatic potential of the extract. Findings of this study demonstrated that *Z. zerumbet* exerts cytotoxic and apoptosis induction effects against MCF-7 cells, indicating its potential significance for future preclinical studies in breast cancer.

Keywords: anti-metastatic; apoptosis; cytotoxicity; phytochemistry; *Zingiber zerumbet*; zerumbone

Introduction

Cancer is a foremost cause of death worldwide and the overall mortality rate is still far too high. According to World Health organisation (WHO), nearly 10 million deaths (nearly 1 in 6 deaths) were recorded in 2020 (1). Additionally, based on statistics, from official database of WHO, breast cancer is responsible for the highest mortality among females, accounting for approximately 244361 deaths (about 21 %) in the lower middle-income countries in 2022. In India, it is the most prevalent malignancy and a leading cause of cancer related mortality among women, with approximately 190000 new cases and 100000 deaths reported in 2022. The traditional treatments include radiotherapy, surgery, hormonal therapy and chemotherapy. However, the efficacy of these treatments is quite unsatisfactory as they still cause several severe side effects and at best, only extend lifespan for a few years.

Nowadays herbal remedies are broadly used to treat several disorders because as plants contain numerous phytochemicals like alkaloids, terpenoids and polyphenols, with proven pharmacological activities, including anticancer effects. Various phytochemicals are being utilised as a prospective anticancer drug (2). Many traditional medicinal systems such as Ayurveda, Traditional Chinese Medicine and Native American Herbal Medicine have long used plant extracts for the treatment of various ailments, including cancer, owing to their various bioactive compounds that act synergistically and selectively target cancer related pathways while sparing normal cells (3). With advancements in technology, researchers can identify specific plant extracts tailored to individual patients or cancer types, leading to a more personalised approach to cancer treatment. Apart from this, many plant components possess antioxidant potential that help strengthen the natural defences of the body against cancer. Moreover, their immunomodulatory effects can improve immune responses and also improve the overall effectiveness of treatment. Hence, nowadays there is an attention rising on this herbal therapy worldwide.

Zingiber zerumbet (L.) Roscoe ex Sm., (common name: shampoo ginger), a rhizomatous perennial herb (up to 2 m tall) with oblong-lanceolate leaves and white flowers, is an important member of the family Zingiberaceae having various biological activities. *Zingiber zerumbet* naturally propagates in the moist and shaded parts of the hill slants. This plant is inherent to India as well as the Malaysian Peninsula (4). Every single part viz. leaf, inflorescence and rhizome of this herb is traditionally used for treatments of several diseases like joint pain, asthma, worms, leprosy, cough and other skin disorders (4). Among the reported compounds present in *Z. zerumbet*, zerumbone (a monocyclic

sesquiterpene) is known to inhibit nuclear factor kappa-light-chain -enhancer of activated B cells (NF-kB) activation. It also suppresses tumour necrosis factor-beta (TNF-β) controlled gene expression triggered by carcinogens. Moreover, it inhibits the expression of interleukin-6 and stimulates apoptosis in malignant cells (5). Therefore, rhizome extract of *Z. zerumbet* has been widely studied to analyse its phytoconstituents through various chromatographic techniques (6) and to check its efficiency in an extensive range of biological potential (7).

One of the most prospective uses of aromatic compounds is to be used as antioxidant agents (8–10). Oxidative stress is crucial in the progression of cancer as it promotes DNA damage, genomic instability and the triggering of oncogenic signalling pathways. Reactive oxygen species (ROS) are generated during normal cellular metabolism and in response to environmental stress. These ROS can induce mutations in key genes such as TP53 and activate oncogenes like *RAS* and *MYC*, ultimately leading to malignant transformation. (11). During tumour progression, moderate levels of ROS further enhance cancer cell proliferation, survival and angiogenesis by activating redox-sensitive signalling cascades such as PI3K/Akt, MAPK and NF-κB (12). Additionally, ROS facilitate metastasis through the upregulation of matrix metalloproteinases and remodelling of the tumour microenvironment. Cancer cells often adapt to oxidative stress by upregulating antioxidant defences, contributing to resistance against therapies that rely on ROS-mediated cytotoxicity (13). Therefore, finding of naturally occurring antioxidants having potential to protect human beings damage caused by oxidative stress has increased. The phytochemicals scavenge ROS and enhance the activity of endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), thereby reducing oxidative damage to cellular components (14). Studies have demonstrated that *Z. zerumbet* extract can decrease lipid peroxidation and restore redox balance in various disease models, including cancer and inflammation (15). Zerumbone, the major sesquiterpene of *Z. zerumbet*, also modulates redox-sensitive signalling pathways such as NF-κB and Nrf2, contributing to its cytoprotective and anti-inflammatory effects (16). These antioxidant actions highlight the potential of *Z. zerumbet* as a natural therapeutic agent in oxidative stress-related disorders. Several researchers have also reported the anti-oxidant activity (5, 6) and cytotoxic activity (5, 17) of *Z. zerumbet.* Furthermore, many investigations have revealed that zerumbone isolated from *Z. zerumbet* extract exhibits cytotoxic activity against breast cancer cell lines (18–22). These studies also demonstrated the molecular mechanisms involved by which zerumbone prevents tumour growth and development. Likewise, various reports are also available describing the cytotoxic activity of *Z. zerumbet* extract towards breast cancer (17, 23). Standard chemotherapeutic drugs like doxorubicin and cisplatin are effective in targeting rapidly dividing cancer cells, they often induce significant systemic toxicity and drug resistance (24). In contrast, *Z. zerumbet* extract exhibits selective cytotoxicity towards cancer cells, inducing apoptosis through mitochondrial pathways (14). Although *Z. zerumbet* and its major compound zerumbone have shown promising anticancer properties, existing reports mainly focus on the cytotoxic effects of the extract, with limited systematic evidence linking its phytochemical profile with antioxidant activity and apoptosis mediated antiproliferative effects in breast cancer cells. To the best of our knowledge these reports have only given the idea about cytotoxic effect of the extract. However, the molecular mechanism behind the activity is hardly known. Therefore, this research was intended to analyse the chemical constituents of *Z. zerumbet* extract and to elucidate its antiradical activity along with its cytotoxicity on different cancer (HeLa, MCF-7, HepG2, HT-29, A549, HCT 116, BT-474 and SKBR3) and normal (HEK 293) cell lines. Additionally, in this research the connection of the cytotoxic activity of *Z. zerumbet* rhizome methanolic extract (ZZRME) with probable apoptosis, DNA degradation, changes in nuclear morphology and cell migration, was also studied in breast cancer cells.

Materials and Methods

Plant material and extract preparation

Zingiber zerumbet (Fig. 1) rhizomes were collected from Daspalla, (latitude 20° 19' 52.17" N, longitude 84° 51' 11.57" E), Nayagarh, Odisha at an altitude of 121 m above the sea level in the month of

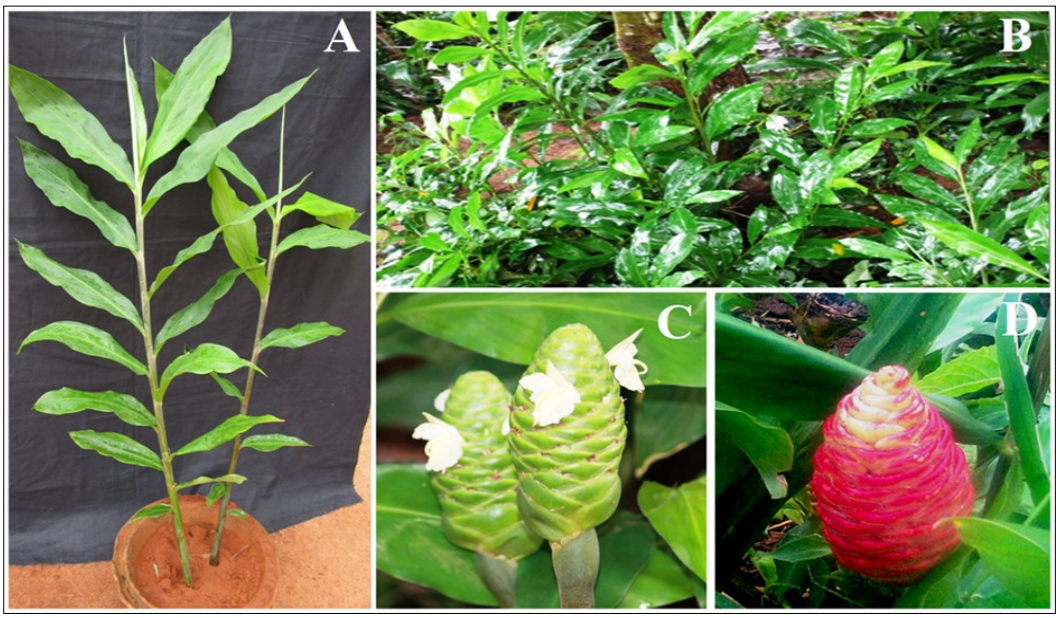

Fig 1. Different parts of *Zingiber zerumbet*, (A): complete plant; (B): *Zingiber zerumbet* at its natural habitat; (C): Inflorescence with flower; (D): inflorescence (mature stage).

November 2022. The collected specimen was taxonomically identified and authenticated by Prof. Pratap Chandra Panda, Centre for Biotechnology, Siksha 'O' Anusandhan (Deemed to be University) Bhubaneswar. The specimen (voucher specimen no.: 2022/CBT, 20th November 2022) was submitted at the herbarium of the same organisation. The collected rhizomes were dried in shade and crushed into powder. Rhizome powder (50 g) was weighed and taken for methanol (300 mL, Merck, Germany) extraction using a Soxhlet's apparatus for 12 hr. The prepared extract (ZZRME) was filtered using a filter paper (Whatman®, Grade-1) and vaporised to dryness using rotary vacuum evaporator (Buchi, Labortechnik AG, Switzerland) at a temperature of 50 °C and kept at 4 °C for future use. Extract yield was calculated as follows.

Extract yield (%) = [Weight of extract (g) /Net rhizome powder taken (g)] X 100

Gas chromatography-mass spectroscopy (GC-MS) analysis

The rhizome extract (ZZRME) was analysed for its chemical composition using a gas chromatograph (THERMO TRACE 1300) equipped with a mass detector (THERMO TSQ 8000). Prepared extract (0.1 µL) was injected in split less mode. Helium (He) gas (at a constant flow rate of 1 mL per min.) was used as a carrier gas. The TG 5MS silica capillary column, measuring 30m X 0.25 mm and 0.25 µm, was employed. An electron ionisation energy method was employed for detection. Similarly, 3 temperature parameters are maintained: 230 °C for the ion source, 250 °C for the injector and 280 °C for the transfer line. The electron ionisation (EI) source was configured to operate at 70 eV. The mass spectrometer was scanned with a mass range of 50–600 amu. Components were resolute by reading the mass spectrum of GC-MS referring the National Institute of Standards and Technology (NIST) library. The Retention Index (RI) was calculated using straight-chain n-alkanes ranging from C8 to C20 (Sigma Aldrich, USA) under the same operating conditions. To further confirm the identification of compounds, the RI values obtained from the experiment were compared with those documented in previous literature (25, 26). Additional confirmation of zerumbone was carried out by co-injecting authentic reference standard of zerumbone (Sigma Aldrich, USA) in Clarus 580 gas chromatograph (Perkin Elmer Inc, USA) equipped with a flame ionisation detector (FID). The experiment was conducted in triplicate by running the same sample in GC-MS.

Antiradical activity

The antiradical activity of an extract should be determined through multiple investigational test procedure as each technique delivers different data regarding the antiradical potential of the tested extracts (27). For each assay, the ZZRME was dissolved in methanol and filtered to ensure that the test solution was homogeneous and clear. Required numbers of working concentrations were freshly prepared from the stock solution via serial dilution. To ensure reproducibility, all experiments were conducted in triplicate. Results are shown as mean±SD. Positive controls, such as recognised antioxidant standards like ascorbic acid (Hi-Media Pvt. Ltd., Mumbai, India) and butylated hydroxytoluene (BHT, Hi-Media Pvt. Ltd., Mumbai, India) was used concurrently at different concentrations (methanolic solution) ranging from 1, 2, 5, 10 and 20 µg/mL to validate the performance and sensitivity of experiment. Methanol alone was used as the negative control to adjust background interference. All biological assays included adequate blank and reagent-only controls were used.

Radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The electrons contribution capability of *Z. zerumbet* extracts was measured using methanolic solution of DPPH [Hi-Media Pvt. Ltd. (Mumbai, India; Assay: >85 %)]. The influence of the extracts on DPPH was estimated by following the procedure by previous researchers (28) with necessary modifications. Briefly, 1 mL of extract at different concentrations (100–500 µg/mL), prepared in methanol, was mixed with 1 mL of a methanolic DPPH solution. The reaction mixture was then incubated for 30 min at RT in dark condition. The sample absorbance was noted at 517 nm and the inhibition percentage of DPPH radical was calculated using the below mentioned formula.

$$\% \text{ inhibition} = [(A_c - A_S)/A_c)] \text{ X } 100.$$

Where, A_c: absorbance of the control and A_S: absorbance of the sample taken.

The exact concentration of the extract at which it could inhibit 50 % of the DPPH radicals was declared as IC_{50} value.

Radical scavenging activity by 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The ABTS radical scavenging capacity of ZZRME were measured following the protocol (29) with slight alteration. In this assay, a reaction mixture containing 2.6 mM ammonium persulfate, 7.4 mM ABTS and 1mL of ZZRME at various concentrations (100–500 µg/mL) was prepared and incubated in dark at room temperature. Absorbance of the sample was recorded at 734 nm taking methanol as blank. The inhibition percentage of ABTS radicals was acquired by using the formula used in DPPH assay. The concentration of the tested extracts that inhibited 50 % of the ABTS radicals was considered as IC_{50} value. Ascorbic acid and BHT were taken as positive standards.

Reducing power assay (RPA)

Reducing power assay method is functional on the theory that compounds having reduction potential, react with Fe^{3+} (ferric form) to form Fe^{2+} (ferrous form) further interact with ferric chloride forming ferric-ferrous complex with absorption maxima (λ_{max}) at 700 nm. The reducing power property of ZZRME was assessed referring the protocol of (30). A 1 mL of ZZRME sample (dilution range: 100–500 µg/mL) was added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 1 % potassium ferricyanide solution (2.5 mL), incubation of the reaction mixture was followed for 20 min using a water bath at 50 °C. To this, 10 % trichloroacetic acid (2.5 mL) was added and followed by centrifugation at 3000 rpm for 10 min. The supernatant (2.5 mL) was then added to distilled water (2.5 mL) and 0.1 % ferric chloride (0.5 mL) solution. The sample absorbance was noted at 700 nm through UV-visible spectrophotometer in contrast to methanol as blank.

Cytotoxic activity

Culture of cell lines

Human adenocarcinoma cells (HeLa), human breast adenocarcinoma cells (MCF-7, SKBR3), hepatocellular carcinoma (HepG2), colorectal adenocarcinoma (HCT 116, HT-29) human lung adenocarcinoma (A549) ductal carcinoma (BT-474), human embryonic kidney (non-cancerous) cells (HEK 293) were obtained from Imgenex India Pvt. Ltd., Bhubaneswar. Cells were grown in T25 culture flask (Eppendorff, Hamburg, Germany) using the respective media viz. HeLa, MCF-7, HepG2, HT-29, A549 and BT-474 cells were

cultured using Dulbecco's Minimum Essential Medium (DMEM) (Gibco, Germany) similarly, HCT 116 cells were maintained using Roswell Park Memorial Institute (RPMI 1640) medium and for SKBR3 culture, McCoy's media was used. All culture media, except McCoy's medium, were supplemented with 10 % fetal bovine serum (FBS; Gibco, Germany). McCoy's medium was supplemented with 15 % FBS. In addition, all media contained 1 % of 1x antibiotic-antimycotic solution (Gibco, Germany), 2 mM L-glutamine (Sigma-aldrich, Munich, Germany) and the required amount of $NaHCO_3$ (Sigma-aldrich, Munich, Germany)] The cell lines were maintained at 37°C in a humidified atmosphere with 5 % CO_2. Prior to seeding (after achieving 80 % confluency) using a 0.25 % trypsin-EDTA solution, cells were separated for 2 to 3 min at 37 °C. The addition of full growth medium with 10 % FBS was done to neutralise the trypsin activity. Before seeding for the tests, centrifugation and resuspension in new medium were performed.

Cell viability/cytotoxicity assay

Water soluble tetrazolium-1 (WST-1) assay was carried out to check the effects of ZZRME on the viability of HeLa, MCF-7, HepG2, HT-29, A549, HCT 116, BT-474 SKBR3 and HEK 293 cells. Here, HEK 293 was taken as normal cell. Briefly, cancer cells were plated in flat bottom 96 well plates (Thermo Scientific, Germany). Each well contained 10×10^3cells. The plates were incubated with 5 % CO_2 at 37 °C for 24 hr. After incubation (at 80 % confluency), cells were treated with ZZRME dissolved in 0.1 % dimethyl sulfoxide (DMSO, Sigma-Aldrich, Munich, Germany) at different concentrations ranging from 50–600 µg/mL (*i.e.,* 50, 100, 200, 300, 400, 500 and 600 µg/mL). Dimethyl sulfoxide (0.1 %) was taken as negative control. Then the plates were again incubated in 37 °C at 5 % CO_2 incubator for 72 hr. Next to incubation, cell proliferation reagent WST-1 (Roche diagnostic India Pvt Ltd, Mumbai, India) was added in all wells at a conc. of 10 µL per 100 µL of cell supernatant followed by incubation of 2 hr and the absorbance was recorded using a micro plate reader at wavelengths of 450 and 620 nm. Final reading was evaluated by deducting the background absorbance from original reading (absorbance obtained at 450 nm absorbance obtained at 620 nm). The IC_{50} values were calculated for all tested samples. All experiments were carried out in triplicate. Percentage of alive cells was calculated taking the following formula:

Percentage of alive cells (%) = (absorbance of sample/absorbance of control) x 100

DNA fragmentation analysis

To confirm the cell death because of apoptosis, DNA fragmentation assay was carried out. Briefly, MCF-7 cells were treated with *Z. zerumbet* extract for 24 hr. Cell alone of respective cells were used as negative control. Cells were then harvested, washed with Phosphate-buffered saline (PBS), 100 µL of 100 mM lysis buffer [NaCl (5M), Tris (pH-8.5), EDTA (0.5M), proteinase K (10 µg/mL) and SDS (10 %)] was mixed to the cell pellet, then incubated on ice for 30 min. The supernatant was collected in a micro centrifuge tube and to it equal quantity of phenol: chloroform: isoamyl alcohol mixture (25:24:1) was added followed by mixing of sodium acetate (50 mM) and centrifugation (at 4000 rpm for 10 min). The supernatant was collected and the preceding step was carried out again. Later collecting the supernatant, same vol. of isopropanol was added to the tubes followed by centrifugation. The pellet was washed twice with 70 % ethanol. The pellets were dehydrated and resuspended in Tris-Ethylenediaminetetraacetic acid (EDTA) buffer ($T_{10}E_1$) buffer containing 10 mM Tris-HCl, 1mM EDTA, (pH 8.0) followed by electrophoresis. Extracted DNA (12 µL, dissolved in $T_{10}E_1$ buffer) was loaded on to a 1 % agarose gel, in Tris-acetate-EDTA (1X TAE) buffer and containing 0.5 µg/mL ethidium bromide along with a 50 bp DNA ladder (Thermo Scientific), Electrophoresis was carried out at 60 volts for 2 hr and the gel was visualised using a molecular Imager (Gel Doc XR, BioRad, USA).

Detection of nuclear morphological changes by 4′,6-diamidino-2-phenylindole (DAPI) staining

The changes in nuclear morphology were examined using DAPI staining. For this study, MCF-7 cells were seeded at a density of approximately $1x10^5$ cells per culture dish (35 mm) and cultured upon 80 % confluence. Cells were treated with ZZRME at the IC_{50} concentrations for 48 hr, while untreated cells served as the negative control. Culture media supplemented with the appropriate concentration of ZZRME were refreshed every 24 hr, to ensure a consistent nutrient supply, maintain pH balance and minimize the build-up of metabolic waste. After incubation, cells were harvested following trypsinization steps, washed twice with PBS (1X, ice cold) and then fixed with paraformaldehyde (3.7 %) for 15 min at RT. Then the cells were washed with 1X PBS and stained with 5 µg/mL of DAPI followed by 15 min of incubation at RT in a dark room. After incubation, cells were again rinsed twice using sterile PBS (1X) and observed under a fluorescence microscope (Nikon, Japan) with 40X magnification. All experiments were conducted in triplicate and at least 3 randomly selected microscopic fields were examined per replicate to ensure consistency of observations.

Detection of apoptosis by Annexin V-FITC assay and flow cytometry

The apoptosis induction potential of ZZRME in MCF-7 cells was measured by flow cytometer using Annexin V-FITC conjugated apoptosis detection kit (Abeomics, San Diego, USA). Flow cytometric analysis was performed with ZZRME treated cells (extract dissolved in DMSO at IC_{50} conc. along with control cells. The cells were then incubated for 24 hr at 37 °C in CO_2 incubator with 5 % CO_2 humidified atmosphere. Cells were harvested (following trypsinization steps) washed with PBS and stained with 5 µL of Annexin V-FITC conjugated (0.5 µg/10^6 cells) to spot the apoptotic cell percentage. Propidium iodide (0.1 µg/10^6 cells) was too added to the cell samples to assess the dead cell percentage and incubated for 20 min in dark at RT. Stained cells were acquired and analysed. For each sample, a minimum of 10000 events (cells) were collected. To properly calibrate instrument parameters and gating boundaries, all flow cytometry experiments included fluorescence-minus-one (FMO), unstained and single-stained compensation controls. Acquisition of data was carried out using FACSCalibur™ flow cytometer (BD Biosciences) in filter-1 (FL1) channel to distinguish Annexin V-FITC stained cells and filter-2 (FL2) channel to detect PI-stained cells. Cell Quest software was used for this data analysis.

Cell migration assay

The wound healing assay was performed to check the cell migration inhibition capability of ZZRME on MCF-7 cells. MCF7 cells were maintained in a six well plate up to 80 % confluency was attained. Confluent cells were cultured in media containing 0.5 % to 1 % FBS after scratch creation to reduce proliferation during the scratch assay. This condition was selected in order to inhibit proliferation while preserving cell viability and facilitating the evaluation of

migratory behaviour. In the cell monolayer, a linear wound was created using a sterile 10 µL micro tip and washed with PBS. Using an inverted microscope, the scratched area was photographed. Then the cells were treated and incubated (for 0, 12, 24 and 36 hr) with ZZRME, while untreated cells served as negative controls. Culture media supplemented with the appropriate concentration of ZZRME were refreshed after 24 hr, to ensure a consistent nutrient supply, maintain pH balance and minimize the build-up of metabolic waste. The images were taken at 0, 12, 24 and 36hr and the evaluation of cell migration was studied as the migration rate percentage using the following formula.

% of Gap closure = (Distance covered after particular time point/ area of the original wound) X 100

All experiments were conducted in triplicate wells per treatment conditions and 3 independent fields per well analysed to account variability.

Statistical analysis

Results are expressed as the mean ± SD for 3 independent experiments. Graphical representations were carried out using GraphPad Prism software (version 7). To assess the statistical variations in the concentration of extract components (%), a one-way ANOVA was conducted, followed by the Tukey HSD test at a 95 % confidence level, utilizing the statistical software Minitab 17.

Results

Zingiber zerumbet, with its bioactive compound zerumbone is recognized for its bioactivity particularly for cytotoxic potential and henceforth, we tried to study and characterize the chemical compounds present in ZZRME by chromatographic approaches.

Gas chromatography-mass spectroscopy (GC-MS) analysis

The chemical profile of ZZRME was assessed by GC-MS analysis, which resulted the identification of total 3 constituents (Table 1; Fig. 2) that were identified by matching the retention indices value and mass spectra of the obtained peaks with the published RI and the NIST library. This study exhibited zerumbone (94.88 ± 0.02) to be the predominant compound in the prepared extract. Besides zerumbone, humulene epoxide (3.05 ± 0.06) and Caryophyllene oxide (2.07 ± 0.04) were found to be the additional constituents. All the compounds obtained in the tested extract were oxygenated sesquiterpenes. The obtained total ion chromatogram of ZZRME and standard zerumbone was depicted in the figure (Fig. 2). The spectra extracted at retention time (RT) of 17.18 min presented abundance ion fragments at m/z of 107.1, 135.1, 163.1 and 218.2, which was like that of zerumbone (Fig. 2).

Antiradical activity

In this study, The IC_{50} value DPPH scavenging activity of ZZRME was found to be 261.66 ± 0.20 µg/mL this result was compared with the positive controls (ascorbic acid and BHT) as well (Table 2). Figure (Fig. 3A) portrays the DPPH scavenging potential of different concentrations of ZZRME which expresses that the scavenging potential of ZZRME increased with increasing concentration. Therefore, the radical scavenging is proportionate to the amount of hydrogen donors in the ZZRME.

The antiradical ability of ZZRME was also determined by means of ABTS radical scavenging assay. The present study exhibited IC_{50} valueof 159.45 ± 0.15µg/mL. The ABTS scavenging activity of various concentrations of ZZRME was depicted in Figure (Fig. 3B). In this study, ZZRME showed better scavenging activity in ABTS assay as compared to DPPH assay showing lower IC_{50} value though both are based on the same principle. The result showed that the reducing power of ZZRME increased with its increasing concentration and the extract concentration ranging from 200–500 µg/mL was found to be the effective inhibitory concentration for reducing Fe^{3+} ions (Fig. 3C).

Cell viability/cytotoxicity assay

The cytotoxic effect of ZZRME was assessed by performing WST-1 assay. It demonstrated that, ZZRME has inhibitory effect on proliferation of all selected doses on HeLa, MCF-7, HepG2, HT-29, A549, HCT 116, BT-474 SKBR3 in comparison with the controlled cells (deprived of ZZRME treatment) after 72 hr deprived of affecting the viability of normal human embryonic kidney (HEK 293) cells. The graph (Fig. 4) explains that the cell viability percentage declined in a concentration dependent manner. According to the result, MCF-7 cells were found to be the most sensitive cell line towards ZZRME among the tested cell lines, with IC_{50} values of 162.7±1.32 µg/mL after 72 hr of incubation. The result also showed stronger cytotoxic effect of ZZRME against HeLa cells

Table 1. Chemical profile of *Zingiber zerumbet* rhizome methanolic extract obtained through GC-MS analysis

Sl.No	Compound	RI_{exp}	RI_{lit}	MW (g/mol)	Molecular formula	Compound type	Concentration % (mean ± SD)
1	Caryophyllene oxide	1583	1582	220.35	C15H24O	Oxygenated sesquiterpene	2.07 ± 0.04^{c}
2	Humulene epoxide	1605	1608	220.35	C15H24O	Oxygenated sesquiterpene	3.05 ± 0.06^{b}
3	Zerumbone	1732	1732	218.33	C15H22O	Oxygenated sesquiterpene	94.88 ± 0.02^{a}

Data are represented as mean ± SD (n = 3). Means followed by different superscript letters in the same column are significantly different according to Tukey's test ($p < 0.05$). Compounds are listed in order of their elution on the TG 5MS column; RI_{exp}: Retention indices calculated against homologous n-alkane series (C8 – C20) on the same column; RI_{lit}: RI from literature (Adams 2007); MW: Molecular weight.

Table 2. Antiradical activities of *Zingiber zerumbet* rhizome methanolic extract using DPPH, ABTS assays

Sl. No	Samples/Standards	IC_{50} (µg/mL, mean ± SD)	
		DPPH (IC_{50})	ABTS (IC_{50})
1	ZZRME	261.66 ± 0.20^{a}	159.45 ± 0.15^{a}
2	ascorbic acid	4.71 ± 0.05^{c}	1.71 ± 0.20^{c}
3	BHT	16.98 ± 0.02^{b}	14.48 ± 0.20^{b}

IC_{50} value: Concentration at which the antiradical activity was 50 %; BHT: Butylated hydroxytoluene. Values are means ± standard deviation (n=3). Means followed by different superscript letters in the same column are significantly different according to Tukey's test ($p < 0.05$).

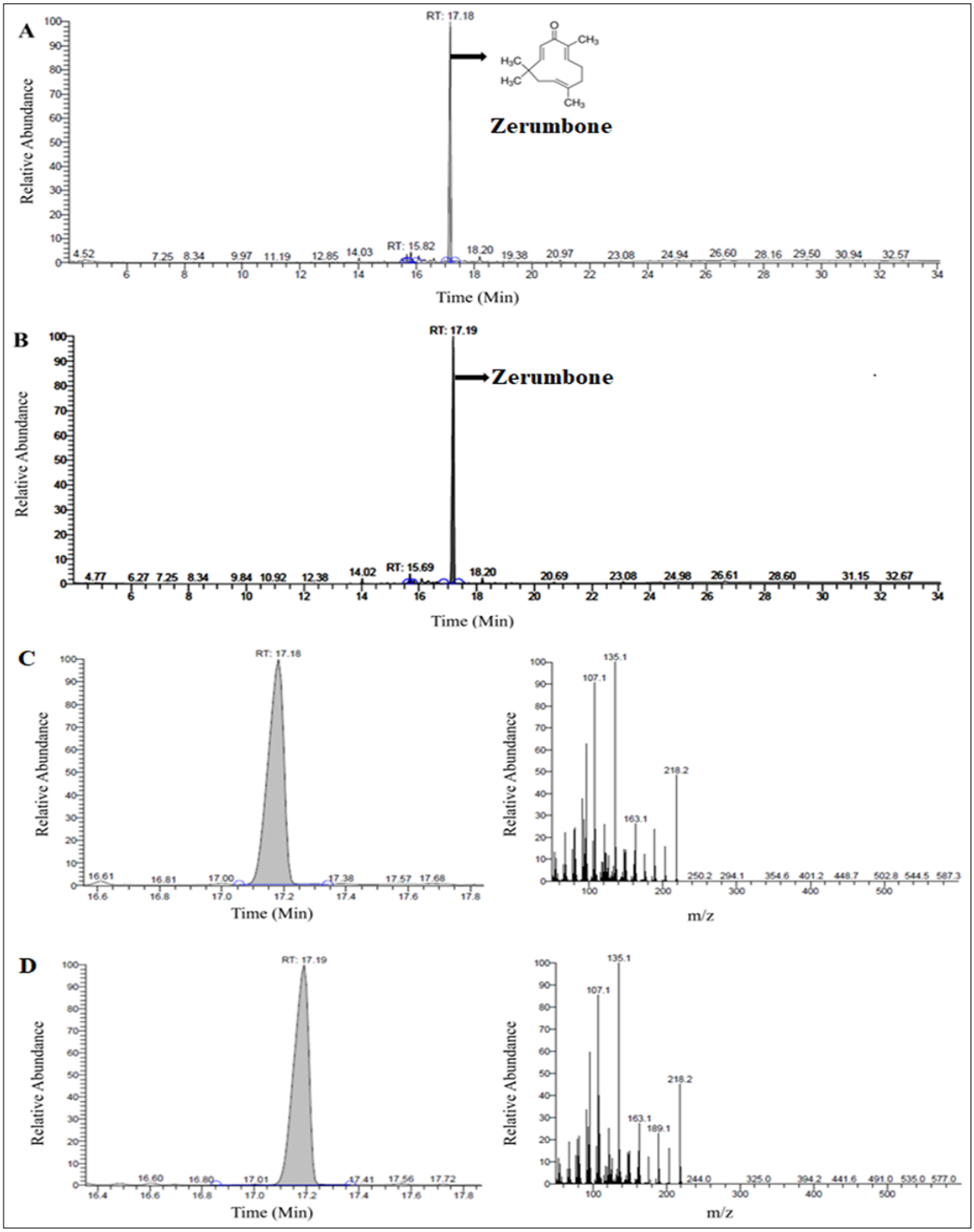

Fig. 2. GC-MS analysis showing: (A)- Chromatogram of *Zingiber zerumbet* rhizome methanolic extract; (B)- Chromatogram of zerumbone standard; (C)- MS fragments of ZZRME extract; (D)- MS fragments of zerumbone standard. GC-MS: Gas chromatography-mass spectrometry; TIC:Total ion chromatogram.

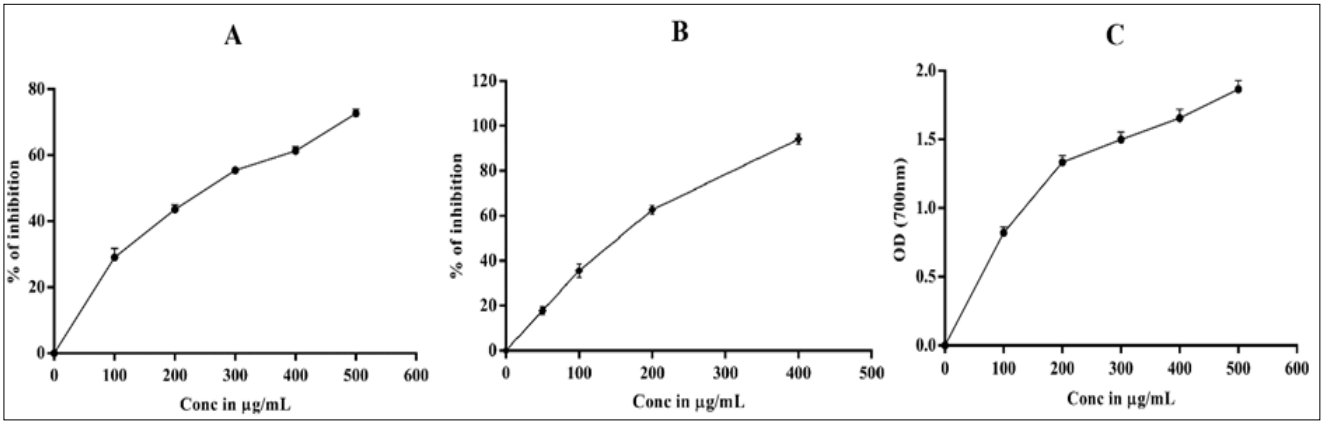

Fig. 3. Antiradical activity of *Zingiber zerumbet* rhizome methanolic extract: (A)- DPPH assay; (B)- ABTS assay; (C)- Reducing power assay. DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid).

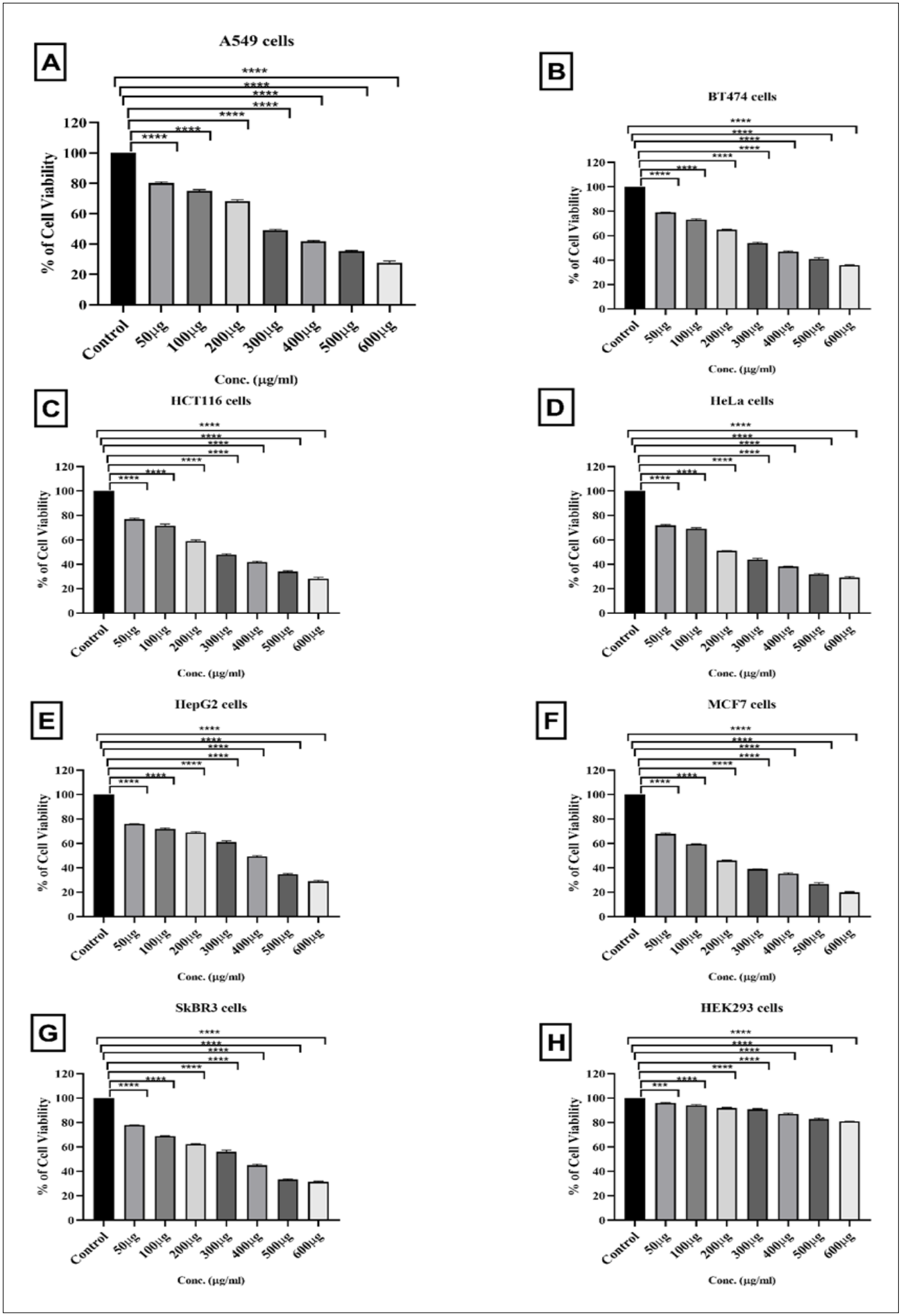

Fig. 4. Dose-response curve presenting percentage cell viability of different cancer and normal (HEK293) cells at different concentrations of *Zingiber zerumbet* rhizome methanolic extract at 72 hr. The figure shows the percentage of cell viability of different cancer cell lines and normal HEK293 cells following treatment with increasing concentrations of ZZRME for 72 hr. Cell viability was assessed to evaluate the cytotoxic effect of ZZRME in a dose-dependent manner. HEK293: human embryonic kidney cells.

with IC_{50} values below 200 µg/mL. The cytotoxic activity of ZZRME on tested cancerous cell lines from strongest to weakest was found to be in the order: MCF-7 (IC_{50}:162.7±1.32 µg/mL) >HeLa (IC_{50}:189.5±1.12 µg/mL) > BT474 (207.4±0.72 µg/mL) > SKBR3 (IC_{50}:211.7±2.41 µg/mL) > HCT 116 (IC_{50}:217.9±2.56 µg/mL) > A549 (IC_{50}:228.4±2.13 µg/mL) > HT-29 (IC_{50}:277.8±1.49 µg/mL) > HepG2 (IC_{50}:330.2±0.72 µg/mL). Instead, it exhibited more viable cells with range of 96.32-81.11 % after treatment with 50–600 µg/mL of ZZRME on normal HEK 293 cells (Fig. 4). Which established that ZZRME is nontoxic towards normal cells. In addition, less proliferation was noticed after 72 hr of the extract treatment, which indicated that ZZRME acts in a time dependent manner. Based on the results obtained from WST-1 assay, MCF-7 cell was selected with the dose of 162.7 µg/mL (IC_{50} concentration) for further cytotoxicity studies.

DNA fragmentation analysis

These cytotoxicity data are further supported by analysing the effect of ZZRME on DNA fragmentation in ZZRME treated MCF-7 cells. This analysis exhibited a smearing pattern representing internucleosomal DNA degradation in ZZRME treated MCF-7 cells at IC_{50} conc., which is an attribute of apoptosis, whereas a compact band of DNA was seen in untreated control cells (Fig. 5).

Fig. 5. DNA fragmentation assay of MCF-7 cells treated with *Zingiber zerumbet* rhizome methanolic extract. DNA fragmentation patterns of MCF-7 human breast cancer cells analyzed by agarose gel electrophoresis. M: 50bp DNA ladder; 1: untreated control MCF-7 Cells; 2: MCF-7 Cells treated with ZZRME at the IC_{50} concentration. The presence of fragmented DNA indicates apoptosis induction.

Detection of nuclear morphological changes by fluorescence microscopic analysis

The nuclear morphology changes were studied using DAPI staining in MCF-7 cell treated with ZZRME. Microscopic observation exhibited that the untreated cells reserved their normal MCF-7 shape which contains the intact nuclear structure whereas the ZZRME treated cells (IC_{50} conc.) showed nuclear fragmentation and chromatin condensation with occurrence of apoptotic bodies, as highlighted by red colour circles (Fig. 6). Nuclear morphological changes indicative of apoptosis such as chromatin condensation and nuclear fragmentation-were consistently observed in treated cells under fluorescence microscopy. However, no quantitative statistical analysis was performed at this stage and the findings are based on qualitative visual assessment.

Detection of apoptosis by Annexin V-FITC assay and flow cytometry

The apoptosis induction ability of ZZRME against MCF-7 cells was analysed by measuring the percentage of apoptotic cells by Annexin V-FITC/PI dual-staining experiment. Flow cytometry of stained cells could separate the cells *viz.*, viable, early apoptotic, late apoptotic and necrotic cells. According to the results obtained, an increase in the percentage of late apoptotic cells was noted after treatment with the IC_{50} dose of ZZRME for 24 hr in MCF-7 cells. The percentage of cells increased to 93.60 % with ZZRME treatment as compared to the control cells (0.49 %). The histogram of apoptotic and necrotic MCF-7 cells obtained through flow cytometry is shown in Figure (Fig. 7). This Annexin V-FITC/PI assay also proposed that the principal reason of cell death after ZZRME treatment is apoptosis.

Cell migration assay

To analyse the anti-metastatic activity of ZZRME on MCF-7 cells, Cell migration activity was carried out using wound healing assay (Fig. 8). The result showed that treatment with ZZRME significantly reduced the wound closure of MCF-7 cells after 12, 24 and 36 hr of incubation. ZZRME treatment (IC_{50} dose) presented the percentage wound closure of 39.2, 42.24 and 45.51 % at 12, 24 and 36h of incubation respectively. Whereas the percentage wound closure in control cells (cell alone) was noted to be 45.23, 67.46 and 75.38 % at 12, 24 and 36 hr of incubation correspondingly.

Discussion

Zingiber zerumbet is reported to exhibit significant cytotoxic activities against various cancer cells (7). Earlier studies have identified zerumbone (13.00 & 13.35 %) as major constituent in methanol extract of *Z. zerumbet* rhizome (31), which were collected from Solo and Yogyakarta, Indonesia respectively. Likewise, another report also showed zerumbone to be the major constituent of aqueous extract where rhizomes were sampled from rural areas of Thiruvananthapuram, Kerala (32). In agreement with these reports, our GC-MS analysis confirmed zerumbone as the foremost constituent, however, the present study revealed a notably high content of zerumbone i.e., 94.88 ± 0.02 %. Many researches have revealed that the biosynthesis of secondary metabolites is influenced by ecological factors (33). Moreover, medicinal plants have more medicinal ability in their natural habitat (34). Hence the obtained high amount of zerumbone might be due to the natural habitat and the geography of the collection site.

Plants belong to Zingiberaceae family have been reported to acquire significant hydroxyl radical scavenging potential (35). In the present study, the difference in the result of DPPH and ABTS assay is due to dissimilar reaction stoichiometry exists between DPPH and ABTS radical, along with the antioxidants present in ZZRME. Moreover, resonance stabilizes the DPPH radical attributable to the occurrence of the double bond and steric factors in the side chain (36). Reducing power assay was also employed in this research to examine the effective extract concentration of ZZRME. Earlier report has also revealed that antiradical / antioxidant activities of some plant extracts are directly proportional to the reducing power (37). In

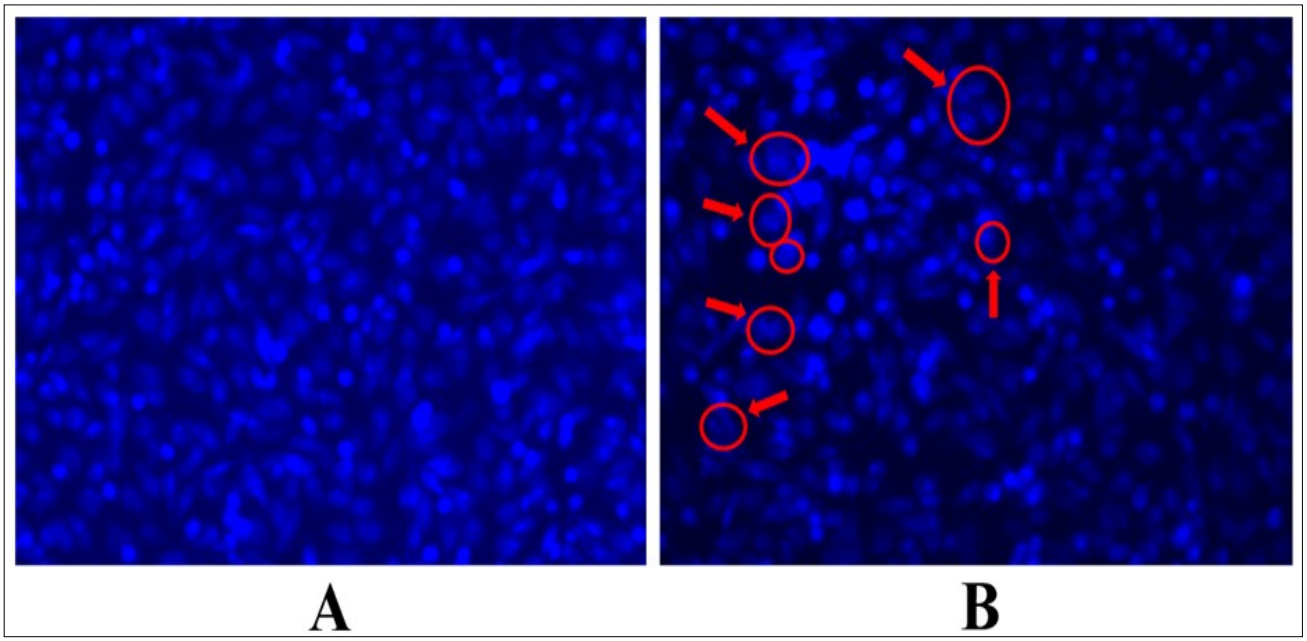

Fig. 6. DAPI staining: A-Untreated MCF-7 cells; B- MCF-7 cells treated with IC_{50} concentration of *Zingiber zerumbet* rhizome methanolic extract at 24hr. Images were captured at 40× magnification. DAPI: 4′,6-diamidino-2-phenylindole. DAPI: 4′,6-diamidino-2-phenylindole.

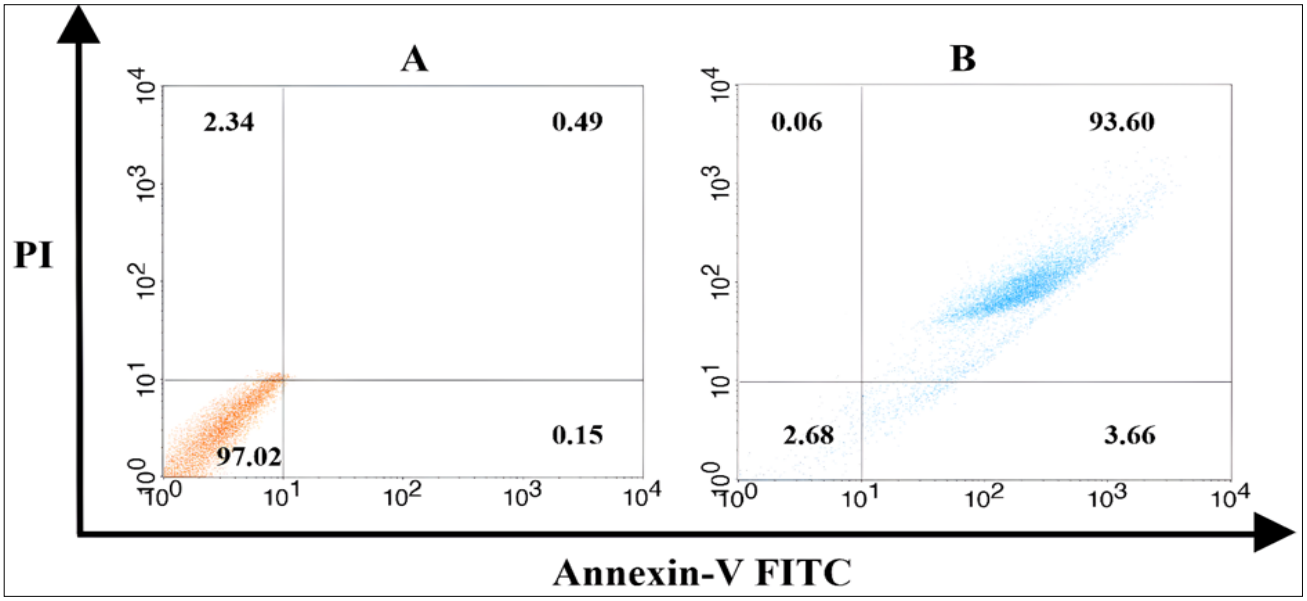

Fig. 7. Flow cytometric analysis of apoptosis in MCF-7 cells using Annexin V–FITC/PI staining. Representative flow cytometry plots show the distribution of viable, early apoptotic, late apoptotic and necrotic MCF-7 human breast cancer cells. A- Untreated control cells; B- cells treated with the IC_{50} concentration of ZZRME. Annexin V-FITC/PI double staining was used to distinguish apoptotic and necrotic cell populations.

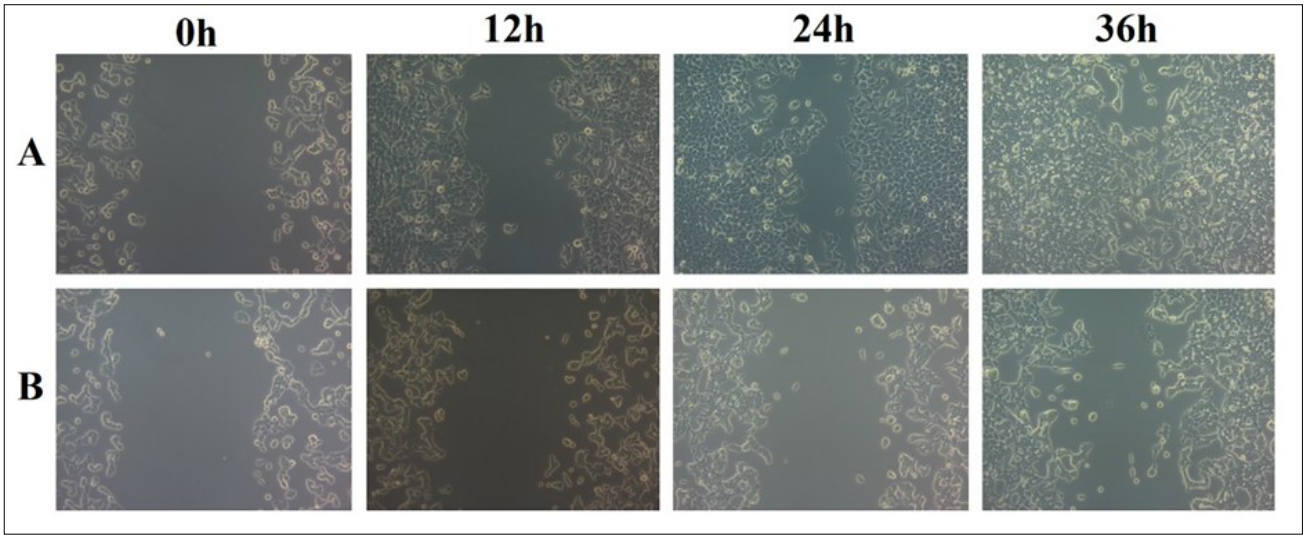

Fig. 8. MCF-7 cells: A- Control; B- treated with IC_{50} concentration of *Zingiber zerumbet* rhizome methanolic extract, showing wound closure ability of ZZRME.

this study, the reducing power of ZZRME increased with its increasing concentration. According to the previous report, zerumbone is a strong antioxidant which suppresses the generation of free radicals (38). It also significantly reduces the malondialdehyde value and increases the superoxide dismutase value. Moreover caryophyllene oxide, another vital constituent of ZZRME was reported to contain notable radical scavenging potential (39). Additionally, in their study caryophyllene oxide was reported to be the major constituent of *Piper miniatum* essential oil showing prominent radical scavenging activity. Hence, the obtained antiradical action of ZZRME might be attributed due to the presence of high amount zerumbone along with a notable amount of caryophyllene oxide present in the extract. A primary limitation of this antiradical study is that the experiments were conducted *in vitro*, which cannot entirely replicate the complexity of antiradical potential within the system. The *in vivo* systems, like animal models, offer a more accurate depiction of the human body. However, in the future, the results from *in vitro* assays will be valuable for conducting *in vivo* validation of antiradical potential of ZZRME.

Gchromatography-mass spectrometry analysis in the present study confirmed the presence of zerumbone, known for its significant cytotoxic activity against various cancer cell lines. In addition, caryophyllene oxide previously reported to exert cytotoxic effects in PC-3 cells (40) was also detected. The combined presence of high levels of zerumbone and caryophyllene oxide may therefore account for the observed cytotoxic activity of the extract. The studied cancer cell lines varied cytotoxic responses most likely reflect variations in their molecular makeup. The hormone receptor-positive breast cancer lines MCF-7 and BT474 showed increased sensitivity to ZZRME, presumably as a result of their inherent apoptotic signalling pathways. In contrast, A549 and HepG2 cells, which are recognized for having high levels of detoxifying enzyme expression and MDR activity, demonstrated decreased sensitivity. In the present study, ZZRME demonstrated an IC_{50} beyond 48 hr in the tested cancer cell lines, highlighting the time-dependent nature of its bioactivity. This delayed cytotoxic effect may stem from the prolonged time required for certain phytochemicals to penetrate cell membranes or overcome inherent cellular defence mechanisms. This phenomenon is particularly significant when targeting cancer stem cells, which are characterized by their resilience and quiescent behaviour. Thus, the observed delay in cytotoxicity could be attributed to these factors. Although ZZRME exhibited dose-dependent cytotoxic effects, the IC_{50} values obtained were in the µg/mL range, indicating moderate cytotoxic activity rather than high potency, which is typical for crude plant extracts. Furthermore, variations in proliferative index, receptor expression and p*53* status could all be factors in the observed disparities in responses. In such type of study, the capability of the selected drug to discriminate among malignant and non-cancerous cells is a vital example in the discovery of operative agent in the field of chemotherapy as the available drugs nowadays reportedly target cancerous as well as normal cells.

This data is furthermore supported by DNA fragmentation assay. A characteristic feature of apoptosis is the disintegration of DNA by a precise nuclease i.e., caspase-activated DNase (CAD). Caspase-activated DNase activation by the caspase cascade leads to a perticular cleavage of DNA at internucleosomal linker sites, making nucleosomes (41). Earlier, DNA fragmentation analysis is accomplished by several researchers to conform induction of apoptosis (33). Therefore, in the present investigation, the smearing pattern of DNA in extract treated cells confirmed that ZZRME induced DNA fragmentation in MCF-7 cells. The precise size range of the DNA pieces was unknown, even though the existence of DNA fragmentation was verified by gel electrophoresis. This might have given more information about the stage of apoptosis. More sensitive methods, including capillary electrophoresis or TUNEL assays, will be used in future research to more precisely measure and describe DNA fragmentation. Furthermore, DAPI labelling revealed significant nuclear morphological changes in ZZRME-treated cells, such as chromatin condensation, nuclear fragmentation and apoptotic body formation, supporting the conclusion that ZZRME largely cause apoptotic cell death.

Recent research has shown that zerumbone, a major bioactive compound from *Z. zerumbet*, has anticancer properties *via* intrinsic mitochondrial apoptotic pathway, which involves upregulation of pro-apoptotic proteins (Bax), downregulation of anti -apoptotic proteins (Bcl-2), cytochrome c release and activation of caspases-9 and caspase-3 in various cancer models. These mechanistic insights are likely applicable to our findings, implying that ZZRME may use comparable molecular mechanisms to induce death in MCF-7 cells (42, 43). Together, the nuclear morphological alterations seen with DAPI labelling and the DNA fragmentation pattern support the concept that ZZRME causes apoptosis in breast cancer cells. Further molecular research is needed to determine the precise apoptotic mechanisms implicated. Future research will also examine ZZRME's mode of action in other cancer cell lines with a variety of genetic traits, even if the mechanistic investigations in this study were concentrated on MCF-7 cells because of their great sensitivity to the drug. This will assist in determining if the observed pro-apoptotic and antiproliferative effects are generally preserved or specific to a particular cell type.

It is widely recognized that Annexin V has strong affinity towards the phosphatidylserine which translocate from cytoplasm towards the plasma membrane in apoptotic phases and delivers stimulus for production of anti-inflammatory mediators throughout the early apoptotic stages which is a biomarker of apoptosis (44). Although the present study demonstrates that ZZRME significantly inhibits migration of MCF-7 cells, the underlying molecular mechanisms remain to be fully elucidated. Based on prior reports, it is possible that ZZRME modulates the expression or activity of MMPs, which play a key role in tumour cell invasion. However, this study did not directly assess MMP levels and thus, further investigation is warranted to confirm this proposed mechanism. Therefore, increased percentage in late apoptotic phase of MCF-7 in this research confirmed that, ZZRME induced apoptosis in MCF-7 cells.

One of the trademark steps that have been related with cancer development and metastasis in various cancer cells is the expression of MMP protein (39, 45–47). The MMPs are a different group of zinc-containing calcium-dependent endopeptidases, that control the proteolytic cleavage of extracellular matrix proteins (48). The result proposes that ZZRME restricted the invasion and migration of MCF-7 cells as compared to control hence, the invasion and metastasis of MCF-7 (breast cancer cell) might be supressed due to the inhibition of these MMP proteins because of the ZZRME treatment *in vitro*. *Zingiber zerumbet* rhizome methanolic extract therapy might be reduced MMP expression, which suggests a possible strategy for preventing cancer cell

invasion and metastasis. The MMPs, especially MMP-2 and MMP-9, break down basement membranes and extracellular matrix, which promotes the spread of tumour cells. *Zingiber zerumbet* rhizome methanolic extract may lessen breast cancer cells' capacity to spread by inhibiting MMP activity. The observed reduction in cell migration following ZZRME treatment may be associated with pathways such as MMP regulation or NF-κB signaling, as reported for other phytochemicals; however, these mechanisms were not directly investigated in the present study and therefore remain speculative. Furthermore, mechanistic investigations revealed that the extract effectively induces apoptosis, as confirmed by morphological changes, DNA fragmentation and Annexin V-based assays. The inhibition of cell migration further indicates its potential to suppress metastatic progression. To confirm these effects and evaluate ZZRME's therapeutic potential in preventing metastasis, more *in vivo* research is necessary. Recent studies have demonstrated that zerumbone, a main phytochemical in *Z. zerumbet*, can affect the expression and activity of MMP-2 and MMP-9 by interacting with upstream regulators such as the *NF-κB* and MAPK signalling pathways (46). These data lend support to the concept that ZZRME's anti-metastatic potential is mediated in part by inhibiting MMP production or function, compromising the cellular machinery responsible for ECM remodelling and invasion. However, future research using gelatin zymography, qPCR, or Western blotting for MMPs and their regulators would give more conclusive evidence to support this approach. One of the key limitations of the present study is the *in vitro* nature of the experiments. While the observed anti-migratory and pro-apoptotic effects of *Z. zerumbet* extract in MCF-7 cells are promising, these results require validation in *in vivo* models to assess the extract's therapeutic potential, bioavailability and toxicity in physiological context. Further studies involving animal models and clinical validation are essential to establish translational relevance.

Conclusion

This research provides experimental evidence supporting the anticancer potential of ZZRME and its key bioactive constituent, zerumbone against breast cancer cells. Comprehensive phytochemical profiling confirmed the presence of bioactive compounds which interrelated well with the noticeable antiradical activity observed *in vitro*. The extract exhibited selective cytotoxicity against breast cancer cells. Additionally, mechanistic investigations revealed that the extract effectively induces apoptosis, as confirmed by morphological changes, DNA fragmentation and Annexin V-FITC/PI based assays. The inhibition of cell migration further indicates its potential to suppress metastatic progression. Together, these findings demonstrate that the extract of *Z. zerumbet* has therapeutic importance in breast cancer treatment; besides it can be concluded that *Z. zerumbet* extract has considerable potential of being a new basis for the development of natural therapeutic agents. It contains excessive potential but its use in most of the applications discussed above should also be taken into consideration. This plant may treasure new assistances as natural sources in the pharmaceutical and food industries. The present report will be helpful for further comprehensive study; however, additional *in vitro* investigations and studies using animal models are essential to authenticate these bioactivities of *Z. zerumbet in vivo*.

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Authors' contributions

BD, AR and SN conceived the idea. PCP identified the plant specimen. BD, SJ and AS performed the research. SP, SM, BD, HTB and KGA conducted the experiments. BD and HTB analyzed the results and performed the statistical analysis. BD drafted the manuscript. JA, PCP and SN reviewed and revised the manuscript. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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