

## CHEMICAL CONSTITUENTS OF ESSENTIAL OIL OF THE ENDEMIC ZINGIBER SPECIES (*Zingiberaceae*) IN VIETNAM, AND ITS ACTIVITY

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### ABSTRACT

The purpose of this study is to determine the chemical composition and antimicrobial, antioxidant and cytotoxic activities of essential oil from the rhizome of *Zingiber atroporphyreus* Škorničk. & Q.B. Nguyễn species of Vietnam, an endemic Zingiber species. The essential oil and its bioactivities are unknown. The essential oil (EO) was isolated by hydrodistillation and analysis by gas chromatography-mass spectrometry/flame ionization detection (GC/MS-FID) method. By GC/MS-FID, 33 compounds representing 99.10% of the essential oil were identified. It was found to be a volatile monoterpene-rich natural source,  $\beta$ -pinene makes up 72.0%, follow by  $\alpha$ -pinene with 9.0%. The antimicrobial activity of essential oil exhibited antibacterial activity against fungi, with  $IC_{50}$  values ranging from 2.5 to 14.8 mg/mL depending upon the microbial species. In the DPPH radical scavenging assay, EO possessed the potential antioxidative  $EC_{50}$  values of 6.3 mg/mL. The EO exhibited significant cytotoxic activity against all tested cancer cell lines and can be used as a potential natural source of anticancer agents. To the best of our knowledge, the results of the present study were investigated here for the first time.

**Keywords:** *Zingiber atroporphyreus*, essential oil, antimicrobial, antioxidant and cytotoxic activities, hydrodistillation.

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## INTRODUCTION

*Zingiber* Mill. is a genus of the Zingiberaceae family found widely in Southeast Asia (Mabberley 2008). It comprises 146 species (Kew Royal Botanic Gardens, 2023), of which 38 species are native to Vietnam (Leong-Skornikova et al., 2015; Ly Ngoc Sam, 2016; Le Thi Huong et al., 2019). The plants of this genus are mainly used for spice, food and traditional medicine for a long time, and the rhizomes are used for extracting essential oils. Many types of chemical compounds of *Zingiber* species have been discovered, such as  $\alpha$ -pinene,  $\beta$ -pinene, camphene, geranial, geranyl acetate, linalool, 1,8-cineole, neral,  $\beta$ -caryophyllene, zingiberene, ar-curcumen,  $\beta$ -bisabolene,  $\beta$ -sesquiphellandrene,  $\delta$ -cadinene and  $\beta$ -eudesmol, gingerol, zerumbone and curcumin as well as other derivatives (Sasidharan & Menon, 2010; Mesomo et al., 2013; Ghosh et al., 2011; Le Minh Chau et al., 2014; Le Thi Huong et al., 2019, 2020, 2021; Nguyen Duc Hung et al., 2019; Doan Quoc Tuan et al., 2022; Nguyen Phuong Hanh et al., 2023). The essential oils from *Zingiber* plants have been discovered to possess numerous biological activities, such as anti-inflammatory, anticancer, antimicrobial, larvicidal, antioxidant and hypoglycemic activities (Singh et al., 2008; Wongwarut et al., 2017; Bellik, 2014; El-Baroty et al., 2010; Nguyen Phuong Hanh et al., 2022a, 2022b). Although numerous chemical constituents of *Zingiber* species have been reported, chemical constituents and the bioactivity of essential oils from newly discovered species of this genus was rarely reported.

*Zingiber atroporphyreus* Škorničk. & Q.B. Nguyễn is one of nine endemic species of Vietnam, discovered in 2015, but until now, apart from the taxonomic report, there has not been any research on the phytochemical of this species. Herein, the chemical composition and biological activity of essential oils from the rhizome of *Z. atroporphyreus* were determined for the first time.

## MATERIALS AND METHODS

### Plant material

The rhizome of *Z. atroporphyreus* species were collected from Xuan Son National Parks, Phu Tho province, Vietnam in 2023. The identification of the samples was done by Dr. N.Q. Binh, Vietnam National Museum of Nature, Vietnam Academy of Science and Technology (VAST). The voucher specimen (XS2023) was deposited at the Institute of Ecology and Biological Resources, VAST.

### Isolation of essential oils

Fresh rhizomes (1,300 g) of *Z. atroporphyreus* were cleaned, chopped into small pieces (about 2–5 mm). After that, the material was used for isolation of the essential oils by hydrodistillation for 4.0 hours at normal pressure using a Clevenger type apparatus. The isolated essential oil was preserved in a dark sealed tube under refrigeration (at 4 °C) prior to GC/MS-FID analysis. The oil yields (%) were calculated based on the dry weight of samples basis.

### Gas chromatography-Mass spectrometry, flame ionization detector (GC/MS-FID) analysis

The analysis of the essential oils was done by GC/MS which was carried out using an Agilent GC7890A system with a Mass Selective Detector (MSD) (Agilent 5975C). An HP-5MS fused silica capillary column (60 m  $\times$  0.25 mm i.d.  $\times$  0.25  $\mu$ m film thickness) was used. The injector and detector temperature was set at 250 and 280 °C respectively. Helium was the carrier gas with a flow rate of 1.0 mL/min. The inlet temperature was 240 °C and the oven temperature program was as follows: 60 °C to 240 °C at 4 °C/min. The split ratio was 100:1 and the injection volume were 1  $\mu$ L of essential oils. The MSD conditions were as follows: ionization voltage 70 eV, emission current 40 mA, acquisitions scan mass range 35–450 amu under full scan. FID analysis was carried out using the same chromatographic conditions. The FID temperature was 270 °C. The retention indices (RI) were experimentally determined using the

*n*-alkanes (C8-C20) analyzed under the same GC conditions. MassFinder 4.0 software connected to the HPCH1607, W09N08 libraries, and the NIST Chemistry WebBook was used to match mass spectra and retention indices. To confirm these results, a further comparison was made with data from authentic compounds reported in the original literature.

#### Antimicrobial assay

The antimicrobial activity of essential oil of *Z. atropurpureus* against bacterial (*Staphylococcus aureus* - ATCC 13709, *Bacillus subtilis* - ATCC 6633, *Lactobacillus fermentum* - VTCC N4, *Escherichia coli* - ATCC 25922, *Salmonella enterica* - VTCC and, *Pseudomonas aeruginosa* - ATCC 15442, and one yeast *Candida albicans* - ATCC 10231) strains was already assessed using microdilution broth method (Coset al., 2006; Hadacek & Greger, 2000). The ATCC strains were obtained from the American Type Culture Collection and the VTCC strains were obtained from the Vietnam Type Culture Collection, Institute of Microbiology and Biotechnology, Vietnam National University, Ha Noi. Antimicrobial and antifungal activity

of the samples was determined by Median inhibitory concentration (IC<sub>50</sub>) and minimum inhibitory concentration (MIC) assay against the above microorganism strains. The bacterial culture medium double-strength Mueller-Hinton broth or double-strength tryptic soy broth, while fungi was cultured in double-strength Sabouraud dextrose broth. Test samples were dissolved by DMSO to provide a range of concentrations (16.0, 4.0, 1.0, and 0.25 mg/mL). Microorganisms remained at -80 °C. Before assay, they were activated by culture medium to reach bacterial concentration to  $5 \times 10^5$  and fungi to  $1 \times 10^3$  CFU/mL. The microorganism solution was in 96-well plates which were further incubated at 37 °C within 18–24 hours. The experiment was repeated three times. Positive controls were wells with a bacterial/fungi suspension in growth medium, and culture medium without bacteria/fungi as a negative control. Ampicillin, Cefotaxime and Nystatin were used as a reference compound for Gram (+), Gram (-) and fungal strains, respectively. The results were described as the absorption at 590 nm and calculated by Rawdata software following the below equations:

$$\text{Inhibitory percentage (\%)} = \left( \text{OD}_{\text{contr}(+)} - \text{OD}_{\text{test}} \right) / \left( \text{OD}_{\text{contr}(+)} - \text{OD}_{\text{contr}(-)} \right) \times 100\%$$

$$\text{IC}_{50} = \text{High}_{\text{Conc}} - \frac{(\text{High}_{\text{Inh \%}} - 50) \times (\text{High}_{\text{Conc}} - \text{Low}_{\text{Conc}})}{\text{High}_{\text{Inh \%}} - \text{Low}_{\text{Inh \%}}}$$

Where: The IC<sub>50</sub> value was the concentration of the compound exhibiting inhibitory fifty percentage of bacterial/fungi growth under the assay conditions.

#### DPPH-antioxidant assay

Free radical scavenging activity of essential oil was carried out by 1,1-diphenyl-2-picryl hydrazyl (DPPH). Briefly, a 0.1 mM solution of DPPH in methanol was prepared. This solution (190 µL) was added to 10 µL of different concentrations of essential oil in DMSO (16.0, 4.0, 1.0, and 0.25 mg/mL). The mixture was carried out by a 96-well plate at room temperature for 30 min. Then, absorbance was measured at 517 nm by using

the Biotek tool. The percent DPPH scavenging effect was calculated by using the following equation: DPPH scavenging effect (%) or inhibitory percentage:

$$\text{SC(\%)} = \left[ (\text{H}_0 - \text{H}_1) / \text{H}_0 \right] \times 100\%$$

Where: H<sub>0</sub> was the absorbance of the control reaction, and H<sub>1</sub> was the absorbance in the presence of a test or standard sample. Each experience was triplicated, while quercetin was used as a reference compound. The EC<sub>50</sub> value, also known as the concentration of tested samples that induced half maximal response has been calculated from linear regression of the serial SC values versus the

concentrations by using Table Curve 2Dv4. Correction.

### Cytotoxicity assay

The Hep-2 (hepatocellular carcinoma), MCF-7 (human breast adenocarcinoma) and A-549 (human lung adenocarcinoma epithelial) cell lines were acquired from ATCC (Manassas, VA, USA) and maintained at 37 °C in 5% carbon dioxide (CO<sub>2</sub>) in suitable media (MEME, DMEM) containing 7–10% fetal bovine serum (FBS), penicillin (100 UI/mL), streptomycin (100 mg/mL), and l-glutamine (2 mM). The cytotoxic effect of essential oil was carried out by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

The cells were diluted in 96-well microplates to a density of  $3 \times 10^4$  cells per well in 200 µL mixture. The samples (10 µg/mL) and positive control (ie, ellipticine at concentrations from 0.4 to 1.6 µg/mL) were added to the cells (190 µg/mL) and incubated at 37 °C for 72 hours with 5% CO<sub>2</sub>. A total of 10 µL of MTT (Merck KGaA) was added to the wells, and incubation was continued at 37 °C for 4 hours. Absorbance was recorded at 540 nm using on a Biotek spectrometer (BioTeK Instruments, Inc Highland Park Winooski, USA). The experiment was performed in triplicate. The rate of growth inhibition, IC<sub>50</sub> according to the following equations (1) and (2) and Raw data computer software (Belgium):

$$\text{Inhibition rate (\%)} = \left( \frac{\text{OD}_{\text{con (+)}} - \text{OD}_{\text{sampl}}}{\text{OD}_{\text{con (+)}} - \text{OD}_{\text{con (-)}}} \right) \times 100\% \quad (1)$$

$$\text{IC}_{50} = \text{High}_{\text{Conc}} - \frac{(\text{High}_{\text{Inh\%}} - 50) \times (\text{High}_{\text{Conc}} - \text{Low}_{\text{Conc}})}{\text{High}_{\text{Inh\%}} - \text{Low}_{\text{Inh\%}}} \quad (2)$$

Where: OD<sub>sampl</sub> and OD<sub>con</sub> are the optical densities of the samples and the control, respectively. HighConc/LowConc: Concentration of test agent at high concentration/low concentration; HighInh%/LowInh%: % inhibition at high concentration/% inhibition at low concentration.

## RESULTS AND DISCUSSION

### Chemical composition of the essential oils of *Zingiber atroporphyreus*

The essential oils were obtained by hydrodistillation from the rhizome of *Z. atroporphyreus* a light yellow liquids with a lower density than water. The average yield of essential oils calculated on a dry weight base was 0.27% (v/w).

In the investigation oil, thirty-three components were identified in essential oil from the rhizome of *Z. atroporphyreus*, representing 99.1% of the total oil (Table 1). The data in Table 1 further showed that the main classes of compounds identified was monoterpenes (96.8%), in which the highest concentration of monoterpene hydrocarbons (90.0%) was observed in the oil from the

rhizome of *Z. atroporphyreus* and the most abundant constituents were β-pinene (72.0%) and α-pinene (9.0%), followed by sabinene (2.8%), limonene (1.7%), myrcene (1.4%),... while some compounds oxygen-monoterpenes were detected in a sizeable amount such as 1,8-cineole (0.5%), endo-fenchol (0.44%), trans-sabinol (0.47%), pinocarvone (0.44%), borneol (1.4%), α-terpineol (1.0%) and myrtenol (1.31%), myrtenal (0.47%),... In addition, the number of sesquiterpenes compounds (sesquiterpene hydrocarbons and oxygenated sesquiterpenes) were also present only in small amounts, lesser than 1.0%, including cis-β-elemene (0.8%), α-santalene (0.2), β-caryophyllene (0.42%), β-selinene (0.1%), g-amorphene (0.3%), g-bicyclohomofarnesal (0.3%).

However, compared with essential oil constituents of rhizome *Zingiber pellitum*, which are included in the same group with *Z. atroporphyreus* - terminally flowering inflorescences, has a different chemical composition of the essential oils. In the present study, the content of α-pinene (9.0%) and β-pinene (72.0%) have a much higher

percentage than  $\alpha$ -pinene (3.0%) and  $\beta$ -pinene (3.6%) of *Z. pellitum*. On the contrary, from *Z. pellitum* species was high amounts of 9-*epi*-(*E*)-caryophyllene (7.5%), humulene epoxide II (7.4%),  $\alpha$ -humulene (6.4%) and caryophyllene oxide (5.7%) while these compounds are absent in study species (Nguyen Phuong Hanh et al., 2023).

The present study is the first to provide helpful insight into the chemical profiles of essential oil from the *Z. atroporphyreus*

rhizome. Compared with other *Zingiber* species, *Z. atroporphyreus* essential oil also possesses a comparable quantity of high-value bioactive compounds whose potential activities require extensive exploitation in the future, especially  $\beta$ -pinene (72.0%) has been shown to have powerful biological activity (Uribe et al., 1985). These properties include antimicrobial, antioxidant, anti-inflammatory and gastroprotective (Le Thi Huong et al., 2000; Uribe et al., 1985; Wisit et al., 2022; Yongkyu et al., 2016).

Table 1. Composition of essential oils from the rhizome of *Zingiber atroporphyreus*

Sr. No	RT (min)	RI (Cal.)	RI (Lit.)	Compounds	Content (%)
1	9.56	930	927	$\alpha$ -thujene	0.20
2	9.83	939	932	$\alpha$ -pinene	9.00
3	10.27	953	948	$\alpha$ -fenchene	0.10
4	10.31	954	952	Camphene	1.10
5	11.04	978	978	Sabinene	2.80
6	11.26	986	980	$\beta$ -pinene	72.0
7	11.44	992	990	Myrcene	1.40
8	12.22	1015	1016	$\delta$ -3-carene	0.20
9	12.41	1021	1024	$\alpha$ -terpinene	0.20
10	12.68	1029	1030	o-cymene	0.10
11	12.82	1033	1032	Limonene	1.70
12	12.87	1035	1033	$\beta$ -phellandrene	0.20
13	12.95	1037	1036	1,8-cineole	0.50
14	13.35	1049	1044	( <i>E</i> )- $\beta$ -ocimene	0.20
15	13.83	1062	1062	$\gamma$ -terpinene	0.40
16	14.88	1093	1091	Terpinolene	0.40
17	15.86	1121	1113	endo-fenchol	0.40
18	16.24	1102	1098	6-camphenone	0.10
19	16.81	1148	1148	<i>trans</i> -sabinol	0.50
20	17.62	1172	1171	Pinocarvone	0.40
21	17.75	1175	1175	Borneol	1.40
22	18.11	1185	1187	Terpinen-4-ol	1.00
23	18.56	1198	1198	$\alpha$ -terpineol	1.30
24	18.79	1205	1201	Myrtenol	0.40
25	18.84	1206	1204	Myrtenal	0.50
26	21.89	1295	1298	2-undecanone	0.20
27	25.44	1402	1405	<i>cis</i> - $\beta$ -elemene	0.80
28	26.32	1430	1436	$\alpha$ -santalene	0.20
29	26.49	1435	1437	$\beta$ -caryophyllene	0.42
30	28.61	1503	1502	$\beta$ -selinene	0.10
31	28.78	1508	1510	$\gamma$ -amorphene	0.20

Sr. No	RT (min)	RI (Cal.)	RI (Lit.)	Compounds	Content (%)
32	28.86	1511	1514	$\alpha$ -selinene	0.30
33	37.71	1825	1822	$\gamma$ -bicyclohomofarnesal	0.30
Total					99.1
Monoterpene hydrocarbons (Sr. No. 1–12, 14–16)					90.0
Oxygen-containing monoterpenes (Sr. No. 13, 17–26)					6.8
Sesquiterpene hydrocarbons (Sr. No. 27–32)					2.0
Oxygenated sesquiterpenes (Sr. No. 33)					0.3

Notes: Sr. No: Serial number; RT: Retention times on HP-5MS column; RI (Cal.) = Experimental retention indices; RI (Lit.) = Literature retention indices on HP-5MS column as seen in NIST (National Institute of Science and Technology (2018).

### Antimicrobial activity of *Zingiber atroporphyreus* essential oil

The antimicrobial activity of essential oil from the rhizome of *Z. atroporphyreus* was evaluated against 6 bacterial (three Gram-positive: *Staphylococcus aureus*, *Bacillus*

*subtilis*, *Lactobacillus fermentum* and three Gram-negative: *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*) and one yeast strain (*Candida albicans*) by the microdilution broth susceptibility assay by determining the MIC and IC<sub>50</sub>. The results tested are summarized in Table 2.

Table 2. IC<sub>50</sub> and MIC values of the essential oils from the rhizome of *Zingiber atroporphyreus*

Microorganisms	Values (mg/mL)	
	IC <sub>50</sub>	MIC
Gram-positive		
<i>Staphylococcus aureus</i>	14.8	> 16.0
<i>Bacillus subtilis</i>	5.7	16.0
<i>Lactobacillus fermentum</i>	> 16.0	> 16.0
Gram-negative		
<i>Salmonella enterica</i>	> 16.0	> 16.0
<i>Escherichia coli</i>	2.5	16.0
<i>Pseudomonas aeruginosa</i>	> 16.0	> 16.0
Yeast		
<i>Candida albicans</i>	6.95	16.0

The range of tested concentrations from 16.0, 4.0, 1.0, and 0.25 mg/mL of essential oil showed that the oil exhibited against both the Gram-positive, Gram-negative trains and yeast. For bacteria *S. aureus* and *B. subtilis*, the median inhibitory concentration (IC<sub>50</sub>) displayed values of 14.8 and 5.7 mg/mL, respectively, and 2.5 mg/mL for bacterium *E. coli*, along with 6.0 mg/mL for *C. albicans*. At the same concentration (MIC) of 16.0 mg/mL, the essential oil was effective against *B. subtilis*, *E. coli* and *C. albicans*. In contrast, both the IC<sub>50</sub> and MIC values of the oil on tested bacteria

*L. fermentum*, *S. enterica* and *P. aeruginosa* were much higher than 16 mg/mL. Thus, *Z. atroporphyreus* rhizome essential oil showed strong antibacterial activity against *E. coli*, *B. subtilis*, *S. aureus* and *C. albicans* yeast, and other bacteria such as *L. fermentum*, *S. enterica* and *P. aeruginosa* are less sensitive.

The present study was the first to display the antimicrobial activity of *Z. atroporphyreus* rhizome essential oil. These results add to knowledge about the antimicrobial activity of *Zingiber* species, previously reported for



*Zingiber officinale* (Singh et al., 2008), *Zingiber nudicarpum*, *Zingiber magang*, *Zingiber tamii*, *Zingiber castaneum* and *Zingiber nitens* (Le Thi Huong et al., 2020, 2021, 2022),... In addition to antimicrobial activity, *Zingiber* oils also exhibit a wide range of other interesting biological activities such as mosquito larvicidal (Le Thi Huong et al., 2020) (against *Culex quinquefasciatus*, *Aedes albopictus* and *Aedes aegypti*), anti-inflammatory (Wisit et al., 2022), antioxidant, and cytotoxic (Yongkyu et al., 2016; Thubthimthed et al., 2005).

#### DPPH-antioxidant assay of *Zingiber atroporphyreus* essential oil

Essential oil isolated from the rhizome of *Z. atroporphyreus* were shown to be

substantially antioxidative in the DPPH-radical scavenging assessment. The results from those assays, at concentrations of essential oil ranging from 1.0 to 4.0 mg/mL, the scavenging activity obtained evolved from 4.0% to 38.0%, respectively. For quercetin with concentrations ranging from 0.008 to 0.032 mg/mL, the scavenging activity evolved from 45.5% to 100%. The essential oils exhibited potential antioxidant activity ( $EC_{50} = 8.4$  mg/mL) but weaker than quercetin ( $EC_{50} = 0.01$  mg/mL). As shown in Figure 1, at the concentration of 16 mg/mL, the oil completely controlled DPPH with SC = 100%. This is the first information of testing the antioxidant properties of essential oil of *Z. atroporphyreus* native to Vietnam.

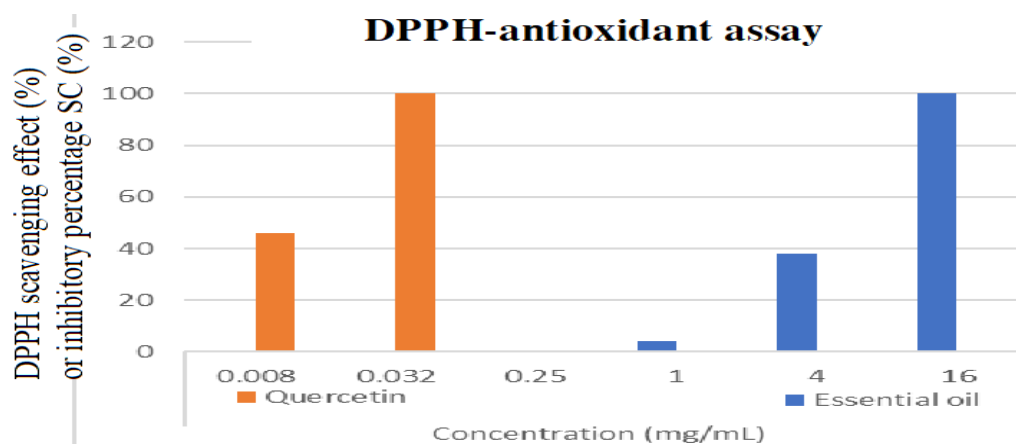


Figure 1. Percent radical scavenging activity of *Zingiber atroporphyreus* rhizome essential oil

#### Cytotoxic activity of *Zingiber atroporphyreus* essential oil

The essential oil isolated from the rhizome of *Z. atroporphyreus* was evaluated for *in vitro* anticancer activity. The cytotoxic effects of essential oil against human breast cancer (MCF7), lung cancer (A549) and liver cancer (HepG2) cell lines were assessed using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Table 3). The obtained results indicated that essential oil was cytotoxic against the A549 cell line with an  $IC_{50}$  value of 119  $\mu$ g/mL. Further, the

cytotoxic potential of essential oil was evaluated against different human cancer cell lines and a positive control assay was carried out with ellipticine. The essential oil displayed activity against all cell lines tested with  $IC_{50}$  values ranging from 119 to 129  $\mu$ g/mL, in which A549 was the most sensitive cell line to the essential oil, with an  $IC_{50}$  of 119  $\mu$ g/mL. This result was comparable to other *Zingiber* species. For instance, essential oil of *Zingiber ottensii* species exhibited inhibitory activity against cancer cell lines (ie, A549, HeLa and MCF-7) with  $IC_{50}$  values ranging from 9.77 to

60.49 µg/mL (Pawaret et al., 2022). For *Zingibermontanum* oil could not inhibit the A549 cell line with IC<sub>50</sub> > 100 µg/mL (Pawaret et al., 2022).

In the present study, GC-MS/FID analysis has shown that the chemical composition of essential oils was different as discussed above. The essential oil contained potential bioactive compounds such as sabinene, terpinen-4-ol, and α-terpineol which were other species that have a minimal quantity or not detected, which has a wide range of biological activity (i.e. antimicrobial, antioxidant (Shamini et al., 2019), anti-inflammatory activity (Hart et al., 2000), anticancer (Shailaja et al., 2019). The cytotoxic properties of *Z. atroporphyreus* essential oil against several human cancer cell lines (MCF7, 549, HepG2) are the first findings, promising anticancer agents for the future.

## CONCLUSION

Determine the chemical composition and antimicrobial, antioxidant and cytotoxic activities of essential oil from the rhizome of *Z. atroporphyreus* Škorničk. & Q.B. Nguyễn species of Vietnam, a new endemic Zingiber species. The essential oil and its bioactivities are unknown.

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