SUPPLEMENTARY MATERIAL

Chemical Profile, Antiproliferative and Antioxidant Activities of Rhizome Oil of *Zingiber anamalayanum* from Western Ghats in India

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Volatile oil from fresh rhizomes of *Zingiber anamalayanum* was isolated by hydrodistillation and characterised by GC-FID and GC-MS. Twenty one out of 24 constituents comprising 99.47% of the oil were identified. Major components in *Z. anamalayanum* rhizome oil were δ -2-carene (52.83%), camphene (9.83%), endo-fenchol (9.42%), iso-dihydrocarveol (6.44%) and cis-p-mentha-2,8-dien-1-ol (5.19%). Monoterpene hydrocarbons in the rhizome oil were 65.81%, followed by oxygenated monoterpenes (23.78%) and sesquiterpene hydrocarbons (9.87%). Physical parameters of rhizome oil were n_D^{20} 1.4031, $[\alpha]_D^{20}$ -16.097° ($c = 1$, CHCl₃) and d^{20} 0.9202. *Z. anamalayanum* rhizome oil showed significant anti-Dalton's Lymphoma Ascitic activity.

Keywords: *Zingiber anamalayanum*, essential oil, gas chromatography, δ -2-carene, camphene, cytotoxic activity.

1. Experimental

1.1. Plant sample

Rhizomes of *Z. anamalayanum* were collected from Minnampara, Nelliampathy in Kerala in October 2013. A voucher specimen (135850 CALI) was deposited at the Calicut University Herbarium, University of Calicut, Kerala.

1.2. Extraction of essential oil

Z. anamalayanum fresh rhizomes (464 g) were subjected to hydrodistillation in a Clevenger-type apparatus for 6 h, and the colourless, pleasant smelling volatile oil (1.4 mL, 0.30%, v/w) was collected, dried over anhydrous sodium sulfate and stored at 4°C.

1.3. Physical parameters

Physical parameters of *Z. anamalayanum* rhizome oil viz., refractive index (J257 Digital Refractometer, Rudolf Research Analytical, USA), specific rotation (Autopol IV Polarimeter, Rudolf Research Analytical, USA) and specific gravity were measured.

1.4. GC-FID analysis

Z. anamalayanum rhizome oil (100 µl) was diluted to 3 ml in acetone and GC-FID analysis was carried out by repeatedly injecting 1 µl each of this diluted oil onto a Shimadzu GC-2010 Plus Gas Chromatograph with AOC-20i autoinjector and FID (Shimadzu, Japan), fitted with a Rxi-5 Sil MS capillary column (5% phenyl 95% dimethyl polysiloxane, non-polar, 30 m x 0.25 mm i.d., 0.25 µm film thickness, Restek, USA). GC operation conditions: injection mode split; split ratio 50; injector temperature 270°C; oven temperature programme 60-250°C (3°C/min); hold time 2 min at 250°C; carrier gas N₂ 3 ml/min and detector temperature 270°C. *Z. anamalayanum* rhizome oil injections were repeated three times under similar experimental conditions. Relative percentages of individual components were calculated from the peak area-percent report of volatiles from GC-FID data.

1.5. GC-MS analysis, LRI determination, identification of constituents

Z. anamalayanum fresh rhizome oil (100 µl) was diluted to 3 ml in acetone and the diluted oil was subjected to GC-MS analysis by splitless injection (1 µl) onto a CP3800 GC-Saturn 2200 ion trap mass spectrometer fitted with a VF-5 (5% phenyl 95% dimethyl polysiloxane, non-polar, 30 m × 0.25 mm i.d., 0.25 µm film thickness, Varian Inc., USA) capillary column. GC-MS operation conditions: injector temperature 220°C, transfer line 240°C, detector temperature 250°C, oven temperature programme 60-246°C (3°C/min), carrier gas He 1.4 ml/min, mass spectra: electron impact (EI⁺) mode 70 eV and ion source temperature 240°C. Linear Retention Indices (LRI) of rhizome oil constituents were determined on the VF-5 column using standard C₅-C₃₀ straight chain hydrocarbons (Aldrich Chemical Company, USA). Individual compounds in *Z. anamalayanum* rhizome oil were identified by Wiley and

NIST database matching, comparison of LRI's and comparison of mass spectra with Adam's database (Table 1) (Adams 2007).

1.6. In vitro antioxidant assays

1.6.1. Superoxide radical scavenging activity

Superoxide scavenging activity of *Z. anamalayanum* rhizome oil was performed by light induced superoxide generation as described in McCord and Fridovich (1965). Phosphate buffer (0.069 M, pH 7.8, 2600 µl), 100 µl of nitro blue tetrazolium (1.5 mM) and 200 µl of potassium cyanide (0.0015% in 10 mM EDTA) each were taken in test tubes. To these solution(s), different concentrations of *Z. anamalayanum* rhizome oil or standard quercetin (1, 5, 10, 25, 50 µg/ml) in 50 µl of methanol were added separately and mixed well. In control tubes instead of rhizome oil, methanol (50 µl) was added along with the reaction mixtures. Then 50 µl of riboflavin (0.12 mM) was added to each mixture of test and control tubes to a final volume of 3 ml. These reaction mixtures were uniformly illuminated with an incandescent lamp for 15 min and optical densities were measured at 560 nm before and after illumination. The % inhibition of superoxide generation was evaluated by comparing the absorbance value of the control and experimental mixtures (Table S1).

1.6.2. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of *Z. anamalayanum* rhizome oil was tested as described in Kunchandy and Rao (1990). Briefly, reaction mixture containing potassium dihydrogen phosphate-KOH buffer solution (0.1M, pH 7.4, 450 µl), 100 µl hydrogen peroxide (0.1 mM), 100 µl EDTA (0.1 mM), 100 µl ferric chloride (0.1 mM), 100 µl ascorbic acid (0.1 mM) and 100 µl deoxyribose (2.8 mM) were taken in test tubes. To these solutions different concentrations of *Z. anamalayanum* rhizome oil or standard quercetin (1, 5, 10, 25, 50 µg/ml) in 50 µl of methanol were added separately. In control tubes instead of rhizome oil, methanol (50 µl) was added along with the reaction mixtures. All the tubes were mixed well and incubated at 37°C for 1 h. To these reaction mixtures (800 µl each), 200 µl of sodium lauryl sulphate (8%), 1.5 ml of acetic acid (20%) and 1.5 ml of thiobarbituric acid (0.8%) were added, and incubated at 100°C for 1 h. These reaction mixtures were cooled, centrifuged, optical densities were recorded at 530 nm and % inhibition(s) were calculated by comparing the absorbance values of the control and experimental tubes (Table S1).

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1.6.3. DPPH radical scavenging activity

Z. anamalayanum rhizome oil or standard compound quercetin (1, 5, 10, 25, 50 µg/ml) was added to 0.2 mM of DPPH (in methanol, 2 ml each) separately, along with the control group without rhizome oil. The tubes were mixed well and incubated at 37°C for 30 min. Absorbances of these mixtures were measured at 517 nm and percentage inhibitions were calculated as % inhibition = (absorbance of control - absorbance with the addition of test sample) x 100/(absorbance of control) (Molyneux 2004) (Table S1).

1.7. Dalton's lymphoma ascites (DLA) cells

DLA cells originally obtained from Amala Cancer Research Centre, Thrissur, India were maintained as transplantable tumors in the peritoneal cavity of mice at JNTBGRI Animal House.

1.8. In vitro cytotoxic activity

Cytotoxicity of *Z. anamalayanum* rhizome oil was tested using Trypan blue exclusion method (Strober 1997). Dalton's lymphoma ascites (DLA) cells were aspirated with phosphate buffered saline (pH 7.4) from the peritoneal cavity of DLA bearing mice. The cell concentration was adjusted to 1×10^6 cells/ml in PBS using a Neubauer counting chamber. Cytotoxicity was assessed by incubating 1×10^6 DLA cells/ml in PBS containing various concentrations of *Z. anamalayanum* rhizome oil (5, 10, 25, 50, 100 µg/ml in 0.1% DMSO) and 0.1% of DMSO separately, at 37°C for 3 h in a water bath incubator. After incubation, the treated cells were added with trypan blue and cytotoxicity was determined under a light microscope. The cells appeared in blue colour was counted as dead while unstained cells were counted as live (Table S2).

Table S1. Antioxidant activity of *Zingiber anamalayanum* rhizome oil.

Concentration ($\mu\text{g/ml}$)	% inhibition					
	Superoxide radical scavenging activity		Hydroxyl radical scavenging activity		DPPH radical scavenging activity	
	Rhizome oil	Quercetin	Rhizome oil	Quercetin	Rhizome oil	Quercetin
1	1.60 ± 0.25	17.04 ± 1.05	15.04 ± 1.00	13.01 ± 1.20	6.04 ± 0.55	7.90 ± 0.22
5	2.59 ± 0.49	21.00 ± 1.30	21.63 ± 1.50	17.04 ± 2.30	10.33 ± 1.13	15.04 ± 1.02
10	7.85 ± 0.48	31.03 ± 2.20	29.34 ± 1.55	24.02 ± 0.70	13.22 ± 0.84	28.03 ± 2.12
25	10.62 ± 0.51	47.04 ± 0.66	33.73 ± 0.24	33.64 ± 1.40	15.34 ± 0.56	42.02 ± 4.14
50	13.76 ± 0.77	70.80 ± 0.14	45.53 ± 1.21	48.03 ± 0.87	18.43 ± 0.30	70.13 ± 1.23

(Values are mean $\pm$ SD, n = 3)Table S2. Cytotoxic activity of *Zingiber anamalayanum* rhizome oil.

Concentration ($\mu\text{g/ml}$)	Rhizome oil (% cell death)
DMSO (0.1%)	0.00
5	9 ± 1.41
10	51 ± 1.41
25	64 ± 5.66
50	89 ± 9.19
100	100 ± 0.00

(Values are mean $\pm$ SD, n = 6)

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