SUPPLEMENTARY MATERIAL

Title: Polyphenolic characteristics, antimicrobial and antioxidant activity of *Zingiber neesanum* (Graham) Ramamoorthy rhizomes and identification of volatile metabolites by GC-MS analysis

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Abstract

Rhizomes of *Zingiber neesanum* (Graham) Ramamoorthy, endemic to Western Ghats and subject to few studies was analysed for bioactivity and phytochemical composition. High total tannin (TT) content in isopropanol extract [55.261±6.623 mg TAE.100g^{-1} DW] contributed to antimicrobial activity against gram negative *E. fecalis* (21.7±0.6 cm) while high total flavonoid (TF) content in ethyl acetate extract [681.94±33.87 mg CE.100g^{-1} DW] accounted for the antifungal activity against *M. rouxii* (9.7±0.6). Isopropanol extracts also showed high anti-oxidant activity as determined by DPPH and FRAP assays. Major bioactive phytochemical constituents in *Z. neesanum* rhizome identified by GC-MS analysis included 2-Methyl-7-nonadecene (13.99%; antimicrobial), Actinomycin C2 (8.57%; antineoplastic) and Deoxyspergualin (12.55%; immunosuppresive).

Keywords: *Zingiber neesanum*; GC-MS, Antioxidant; Anti-microbial; Polyphenolics; Phytochemical

General Experimental Procedures

Plant material

Z. neesanum was collected from their natural habitat from Thirunelly hills, Western Ghats, Kerala (Latitude: 11.9117° N, Longitude: 75.9958°E), India. The rhizomes were planted in pot containing mixture of sand, soil, and cow dung in 1:1:1 and watered every 2 to 3 days with tap water. Plants were grown and maintained under natural light conditions (~12:12h) and temperature (28±5^{o}C).

Polyphenolic Extraction and Profiling

Rhizomes were sliced, air dried at room temperature (37˚C) for three weeks, after which it was ground to a uniform powder of 40 mesh size. Powdered rhizome samples (100 g) were sequentially extracted with hexane, benzene, isopropanol, ethylacetate, and methanol using a soxhlet extractor. Extracts were filtered through Whatman filter paper no. 42 (125 mm) to remove un-extractable matter. Extracts were concentrated to dryness using rotary evaporator under reduced pressure. The dried samples were stored in labeled sterile bottles and kept at -20˚C till further analysis.

Polyphenolic estimations

The five solvent fractions were subjected to estimation of polyphenolics viz., total phenolics (TP), total flavonoids (TF) and total tannins (TT). TP content was estimated by Folin–Ciocalteu colorimetric method with slight modifications using Gallic acid (GA) as standard (Eberhardt et al., 2000). Briefly, aliquot of 0.125 ml of each solvent extract was diluted with sterile water and made up to 3.5 ml, followed by addition of equal amount of Folin–Ciocalteu reagent. After incubation at room temperature for 6 min, 1.25 ml of 7% sodium carbonate was added. Samples were incubated at 30^{o}C for 1.5 hours and absorbance was measured at 760 nm using UV spectrophotometer (UV/Vis, UV 3000, Lab India, India). Quantitative measurements were performed, based on a standard calibration curve prepared using GA as standard at varying concentrations (20 - 120 mg.L^{-1}). Total phenolic content was expressed as gallic acid equivalents (GAE) in mg.100 g^{-1} dry weight (DW) and determined in triplicates.

TF was measured by aluminum chloride colorimetric assay (Zhishen et al., 1999). Briefly, 0.1 mL aliquot of solvent extract were mixed with sterile water and made up to 2.5 ml,

followed by addition of 75 µL of 5% $NaNO_2$. After incubating for 5 min at 30°C, 150 µl of 10% $AlCl_3$ was added and the mixture was incubated at room temperature for 6 minutes. Reaction was stopped by adding 0.5 mL of 1M NaOH and absorbance measured against reagent blank at 510 nm. TF content was quantified in extracts using catechin (20 - 120 $mg.L^{-1}$) as standard and was expressed as mg catechin equivalents (CE).100 g^{-1} DW.

TT content was estimated by Prussian blue method (Graham, 1992) with slight modification. For the assay, 0.1 ml aliquot of solvent extracts were mixed with sterile water and made up to 7 ml. Subsequently 1 ml of potassium ferricyanide (0.008 M) followed by 1 ml of ferric chloride (0.02 M in 0.1 M HCl) was added to the assay mixture. The solution was mixed well and absorbance measured at 700 nm using UV spectrophotometer (UV/Vis, UV 3000, Lab India, India). TT content in extracts was quantified from standard curve prepared using tannic acid as standard (20 - 120 mg. L^{-1}). Results were expressed as mg tannic acid equivalent (TAE).100 g^{-1} DW and determined in triplicates.

Antimicrobial assays

For determination of antimicrobial activity of phytochemicals, the five solvent extracts were screened for their antibacterial and antifungal activities against Gram positive bacteria (*S. aureus*, *E. fecalis*); Gram negative bacteria (*E. coli*, *P. aeruginosa*, *K. pnemoniae*) and fungi (*M. rouxii*, *A. flavus*, *C. albicans*) by agar well diffusion method (Perez et al., 1990). Nutrient agar plates were prepared and seeded with indicator bacterial and fungi strains. A well of 6 mm diameter was made using a sterile cork borer and 50 $\mu g.ml^{-1}$ of respective solvent extracts placed in it. Ampicillin was used as positive control. Zones of growth inhibition around the well were measured after 18 hours of incubation at 37°C for bacteria and 48 hours for fungi at 28°C. Sensitivity of the tested microorganisms to the extracts was determined by measuring the diameter of inhibitory clear zones on the agar surface around the well. Values ≤ 6 mm were considered as not active against tested microorganisms. All experiments were done in triplicate.

Antioxidant assays

Free radical scavenging activity of *Z. neesanum* extracts was determined by 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) method with slight modification (Gulcin, 2006). Increasing concentration of the solvent extracts (20 - 120 $\mu g.ml^{-1}$) were diluted with methanol and made up to 1 ml, followed by addition of 1 ml of methanolic solution of DPPH (100 µM). Resultant mixture was incubated in dark for 30 min and reduction of DPPH radical was determined by

measuring decrease in absorbance at 517 nm. Ascorbic acid was used as positive standard control. IC_{50} values were estimated using each of the extracts to determine the amount required to scavenge 50% of DPPH free radicals.

FRAP assay was done according to the method of Yen and Chen, (Yen and Chen, 1995) with slight modifications. To varying concentration of solvent extracts (20- 120 $\mu g.ml^{-1}$) diluted using deionized water to 1.0 ml, 2.5 ml of phosphate buffer (0.1 M: pH- 6.6) and 2.5 ml potassium ferricyanide (1%) were added. The mixture was incubated at 50˚C for 20 min and the reaction was stopped by addition of 2.5 ml of trichloroacetic acid (10 % w/v). The mixture was centrifuged at 3000 rpm for 10 min and the upper layer of 2.5 ml was mixed with equal volume of deionized water and finally 0.5 ml of freshly prepared ferric chloride solution (0.1% w/v) was added. Amount of Fe (II) was monitored by recording absorbance after 10 minutes at 700 nm with higher absorbance indicative of higher reducing power. Ascorbic acid at varying concentrations (20- 100 $\mu g.\ ml^{-1}$) was used as standard. Anti-oxidant activity was determined as: Scavenging effect (SE) (%) (for DPPH assay) and/or Increase in reducing power (%) (FRAP assay)= $(A_{test}- A_{blank})\ A_{blank}^{-1}$ x 100, where A_{test}: is absorbance of test solution and A_{blank}: is absorbance of blank.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Methanolic extracts were shaken for 90 min at 25°C, centrifuged and filtered using a Whatman filter paper (No. 2). Filtered solvent extract was analysed qualitatively byGC-MS on Hewlett-Packard HP 6890 GC (injector temperature, 220 °C; 1 μL splitless injection) coupled to a HP MS-5973 mass-selective detector in a DB5MS column (30 m×0.32 mm×0.25 μm) with 1.4 ml min−1 helium as carrier gas. The oven temperature was programmed from initial temperature of 60 °C with an increase of 3 °C/min to 240 °C. Identification was based on matching mass spectra in Wiley and National Institute of Standards and Technology (NIST) Mass Spectral Library.

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Figure Legend:

Figure S1: DPPH radical scavenging and FRAP activity assay (IC_{50}) of solvent extracts of *Z. neesanum* rhizomes. Ascorbic acid used as positive control and radical scavenging effect is expressed as ascorbic acid equivalent (AAE) (µg AAE per ml extract). Results are expressed as mean ± SD (n = 3).

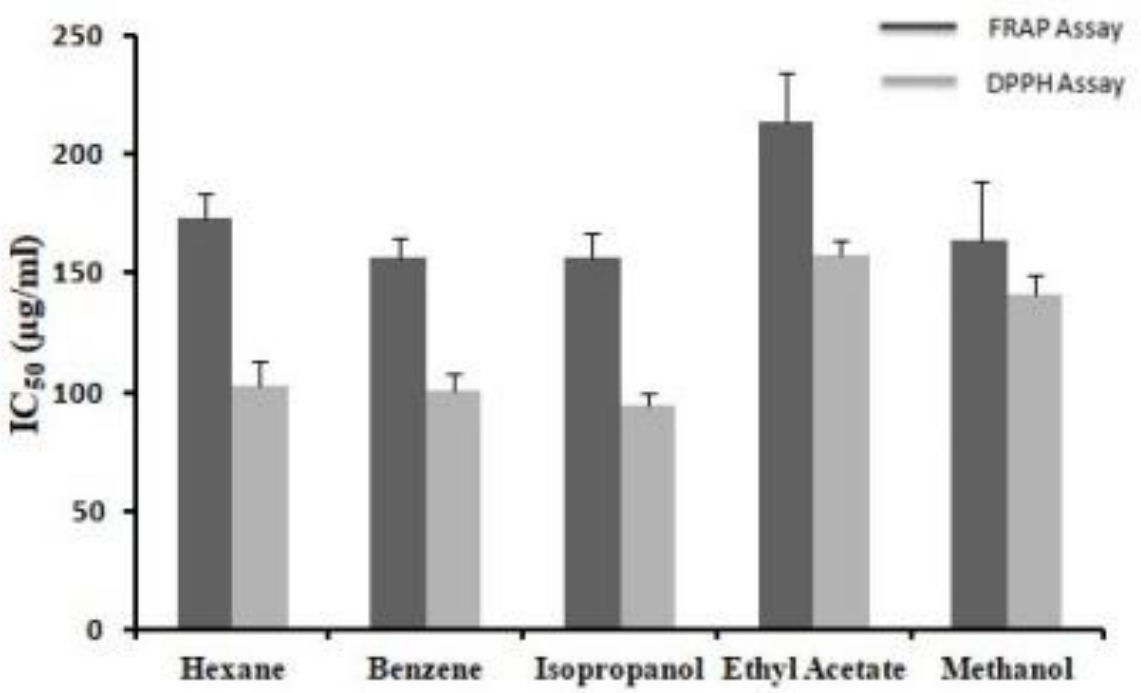

Table S1: Estimation of Polyphenolics in solvent extracts of *Z. neesanum* rhizomes. TP, TF and TT values are means ± SD (n = 5). Total phenol (TP) content expressed as gallic acid equivalents (GAE); the total flavonoid (TF) content expressed as catechin equivalents (CE); and total tannin (TT) content expressed as tannic acid equivalent (TAE).

Polyphenolic	**Hexane**	**Benzene**	**Isopropanol**	**Ethylacetate**	**Methanol**
TP [mg GAE. 100 g^{-1} DW]	41.67±7.67	103.66±27.10	68.09±12.69	92.99±17.97	71.14±20.81
TF [mg CE. 100 g^{-1} DW]	136.11±15.77	44.44±7.89	48.61±11.47	681.94±33.87	43.75±19.09
TT [mg TAE. 100 g^{-1} DW]	2.884±0.531	1.785±0.201	55.261±6.623	2.134±0.345	1.590±0.139

Table S2: Screening of antibacterial activity of solvent extracts of *Z. neesanum* rhizomes by agar well diffusion method. Ampicillin (20 µg.ml^{-1}) was used as positive control. Values are means of triplicate diameter measuring zone of inhibition in mm ± standard deviation. - indicates no growth inhibition (the zone of inhibition $\leq$6 mm).

Test microbes	**Zone of inhibition (mm)***					
	Hexane	**Benzene**	**Isopropanol**	**Ethylacetate**	**Methanol**	**Ampicillin**
S. aureus	-	-	16.3±0.6	8.3±0.0.5	7.0±0.6	8.7±0.5
E. coli	-	-	8.3±0.5	7.7±0.6	7.3±0.6	-
P. aeruginosa	-	-	8.7±0.5	7.3±0.5	7.7±0.0.6	-
K. pnemoniae	-	-	8.0±0.1	-	-	-
E. fecalis	-	-	9.3±0.5	7.7±0.6	7.3±0.5	15.3±0.6
Mucor rouxii	-	-	8.3±0.6	9.7±0.6	-	-
A. flavus	-	-	8.7±0.06	7.7±0.06	-	-

Candida spp.	-	-	7.7±0.6	-	-	-