



# Evaluation of total phenolics, antioxidant and antiproliferative activities of rhizome extracts from select Zingiberaceae species in South India

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## Abstract

Zingiberaceae family members are well known for their ethnobotanical diversity and medicinal importance. This study aimed to evaluate total phenolic content, antioxidant and antiproliferative capacity of five different organic solvent extracts prepared from the rhizomes of *Curcuma mutabilis* (CM), *Curcuma haritha* (CH), *Curcuma neilgherrensis* (CN) and *Zingiber anamalayanum* (ZA), four hitherto unexplored Zingiberaceae species. Folin-Ciocalteu method and DPPH radical scavenging assay were used to determine respectively the total phenolic content and antioxidant capacity. The antiproliferative activity of the extracts were tested against four human cancer cell lines – K562, REH, Nalm6 and MCF7 to ascertain the IC<sub>50</sub> values. Based on total phenolic content, extracts were classified into high-H (> 150 mg GAE/g), medium-M (50-150 mg GAE/g) and low-L (< 50 mg GAE/g) categories. Likewise, percentages of DPPH scavenging activity of extracts were also grouped into high-H (> 50%), medium-M (25 – 50%) and low-L (< 25%) categories. Ten of the twenty extracts exhibited strong cytotoxicity with an IC<sub>50</sub> value less than 30 µg/mL. To our knowledge, this is the first report on quantitative assessment of total phenolics, antioxidant and antiproliferative potential of organic solvent extracts of rhizomes from the above mentioned plants.

**Keywords** : Zingiberaceae, organic solvent extracts, total phenolic content, antioxidant potential, antiproliferative activity.

## Introduction

Plant derived drugs - naturally derived or chemically altered natural products - have had the greatest impact in the area of cancer chemotherapy [1]. Plants continue to be important bioresources for development of anticancer drugs [2,3,4]. Barring the great majority, only a small fraction, as low as 15% of the higher plants, have been estimated to be investigated for bioactive compounds [1]. Zingiberaceae family consists of a large number of medicinal plants and is well known for its use in ethnomedicine. The genus *Curcuma*, comprising of more than eighty species, have been used extensively as medicine, nutraceuticals, agents for flavorings, food color, dyes and cosmetics [5,6,7]. Among them, *C. longa*, the most studied species, is well known to possess tremendous anticancer activity attributable to the presence of curcumin [8,9,10]. Even though a number of compounds with strong anticancer activities have been reported from different genera of Zingiberaceae family [11,12,13], a large number of them are yet to be analyzed for realizing their therapeutic potential.

*Curcuma mutabilis* and *Curcuma haritha* are reported to be endemic to Kerala, while *Curcuma neilgherrensis* and *Zingiber*

*anamalayanum* found in Western Ghats are endemic to south India [14,15]. Major oil constituents from *C. haritha* rhizome have been reported to possess a unique profile in comparison to all other *Curcuma spp.* [16,17]. Leaves of *C. neilgherrensis* have been used in folklore medicine against diabetes mellitus [18] and phytochemical screening of this plant has revealed the presence of various secondary metabolites lending credence to its medicinal usages [19]. Alcoholic extracts of its rhizome have also been shown to possess antifungal activity against *Candida albicans* and *Aspergillus niger* [20]. Rhizome oil of *Z. anamalayanum* has been reported to possess significant antiproliferative activity against Dalton's lymphoma ascites cells [21]. The present study was carried out on select Zingiberaceae species from Kerala, namely, *C. mutabilis* (CM), *C. haritha* (CH), *C. neilgherrensis* (CN) and *Z. anamalayanum* (ZA). Here we report, for the first time, on total phenolic content, antioxidant and antiproliferative activities of organic solvent extracts prepared from rhizomes of these plants. Four human cancer cell lines, K562, REH, Nalm6 and MCF7 were employed in this study.

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## Materials and Methods

### Collection and identification of plant samples

Three members of genus *Curcuma* - *C. mutabilis* (CM), *C. haritha* (CH) and *C. neilgherrensis* (CN) and one from genus *Zingiber* - *Z. anamalayanum* (ZA) - were collected from different locations within Kerala State in the months of September and October 2013-2014. The plants were authenticated by Dr. M. Sabu, Department of Botany (University of Calicut) and a voucher specimen was deposited in the University Herbarium.

### Extract preparation

Rhizomes of all plants were cleaned and dried in shade and ground separately into a fine powder for preparation of the organic extracts. 5.0 g of the rhizome powder was placed in 25 mL each of the different organic solvents - petroleum ether (Pe), chloroform (Ch), ethyl acetate (Ea), acetone (Ac) and methanol (Me) [HPLC grade, SRL Pvt. Ltd., Mumbai, India]. After 24 h shaking on a gyrosaker at room temperature, the extracts were filtered through Whatman No.1 filter paper and the filtrate was kept for complete evaporation of the solvents to obtain the dry extract. Known amounts from each of the different extracts were dissolved in dimethylsulphoxide (SRL Pvt. Ltd., Mumbai, India) to prepare the stock solutions (20mg/mL). Extract stocks were sterilized by filtration through a 0.20µm filter (Genetix Biotech Asia Pvt.Ltd., New Delhi, India) and stored at -20°C.

### Determination of total phenolic content

The total phenolic contents of all the extracts were determined using Folin-Ciocalteu (FC) reagent as previously described [22], using gallic acid as standard. Briefly, 200µL of Folin-Ciocalteu reagent (SRL Pvt. Ltd., Mumbai, India) was mixed with 2.0 mL of extract and kept for 10 minutes at room temperature, followed by addition of 300µL of 15% Na<sub>2</sub>CO<sub>3</sub>. The mixture was allowed to stand for 2 h at 25°C. Absorbance was read at 765 nm and the total phenolic content of different extracts were expressed as gallic acid equivalents (mg GAE/g dry extract).

### Determination of antioxidant activity using DPPH method

Antioxidant activities of all extracts were determined mainly by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. DPPH scavenging activity of each extract was evaluated as described earlier [23]. Briefly, 1.0 mL of 100 mM methanolic solution of DPPH (HiMedia Laboratories, Mumbai, India) was mixed with an equal volume of the extract (1 mg/mL). The mixture was incubated at 25°C for 30 min in the dark. Ascorbic acid was used

as standard. Absorbance was read at 517 nm using a spectrophotometer (Lambda 25, Perkin Elmer, UK) and the activity was calculated using the formula:

$$\% \text{ free radical scavenging activity} = [(Abs_{\text{control}} - Abs_{\text{sample}}) / Abs_{\text{control}}] \times 100$$

### Determination of antiproliferative activity

#### Cell culture

Human chronic myelogenous leukemia cell line - K562, acute lymphocytic leukemia cell lines - REH and Nalm6, were maintained and propagated in RPMI 1640 medium while the human breast cancer cell line - MCF7 was cultured in Dulbecco's Modified Eagle's Medium (HiMedia Laboratories, Mumbai, India). Both media were supplemented with 10% (v/v) fetal bovine serum (Gibco, Thermo Fisher scientific), streptomycin (100 µg/mL) and penicillin (100 U/mL). Cells were cultured in T-25 tissue culture flasks (Nunc, Thermo Scientific, USA) and maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

#### Cytotoxicity assay

A total of 20 organic extracts were screened for antiproliferative activity by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on all four cancer cell lines used for the study. K562, REH and Nalm6 (2 x 10<sup>4</sup> cells/well), and MCF7 cells (6000/well) were seeded into 96-well microtitre plates. Following overnight incubation, cells were treated with varying concentrations (5 – 150 µg/mL) of the different extracts and incubated for a further period of 24, 48 and 72 h. Corresponding amounts of DMSO were used as vehicle control. Cell viability was determined by MTT assay as described by Mosmann et al. [24]. Briefly, MTT (HiMedia Laboratories, Mumbai, India) was added into each well of the 96-well microtitre plate at a final concentration of 500 µg/mL followed by incubation at 37°C in the dark for 3 – 4 h. The supernatants were removed and the insoluble formazan crystals were solubilized in DMSO. Absorbance was measured at 570 nm using microplate reader (Multiscan EX, Thermo scientific, USA) and IC<sub>50</sub> values of all the extracts were determined from the percentage cell viability as given below:

$$\text{Percentage cell viability} = [(Abs_{\text{sample}} - Abs_{\text{blank}}) / (Abs_{\text{control}} - Abs_{\text{blank}})] \times 100$$

#### Statistical analysis

All experiments were performed in triplicate. Results were analyzed for significance by one-way ANOVA using SPSS software version 15.0. The data were recorded as mean ± standard deviation. The means were compared by LSD multiple comparisons at *P* < 0.01.



## Results and Discussion

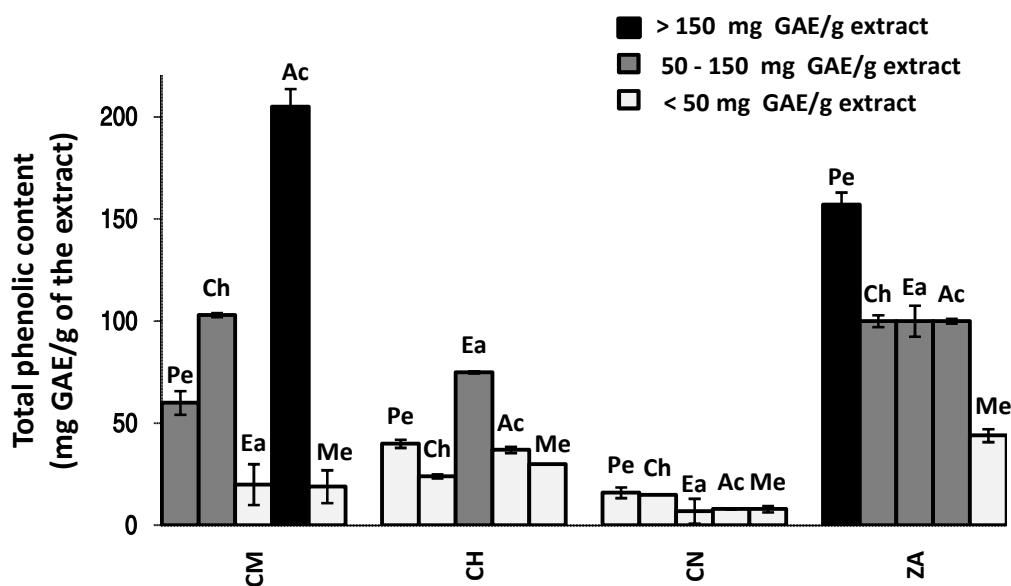
### Extraction yields, total phenolics and antioxidant capacity of rhizome extracts

Percentage yield (*w/w*) of each of the five organic solvent extracts prepared from rhizomes of four select Zingiberaceae species including the plant collection sites have been given in Table 1. The highest yields were obtained in the methanol extracts.

**Table.1** – Collection site and percentage yield of select species of Zingiberaceae

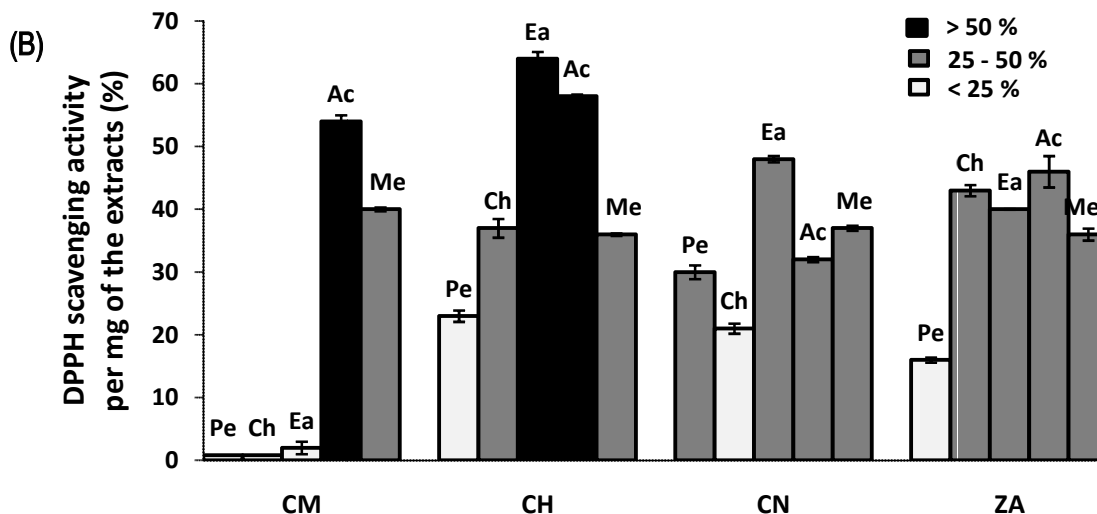
Species name & collection site	Extracts (Rhizome)	Percentage of yield ( <i>w/w</i> )
<i>C. mutabilis</i> Skornick, M. Sabu & Prasanth K. (CM) (Nilambur, Palakkad)	Pe	2.2
	Ch	4.2
	Ea	3.6
	Ac	3.6
	Me	6.8
<i>C. haritha</i> Mangaly & M. Sabu (CH) (Azhinjilam, Calicut)	Pe	0.8
	Ch	2
	Ea	1.3
	Ac	1.8
	Me	2.2
<i>C. neilgherrensis</i> Wight (CN) (Nelliyampathy, Palakkad)	Pe	2.3
	Ch	3
	Ea	3.8
	Ac	6.4
	Me	10
<i>Z. anamlayanum</i> Sujjanapal & Sasidh (ZA) (Nelliyampathy, Palakkad)	Pe	0.2
	Ch	0.7
	Ea	0.5
	Ac	0.4
	Me	1.2

(A)



Total phenolic content of the extracts are given in Fig. 1(A). Based on total phenolic content, extracts were classified into high-H (> 150 mg GAE/g), medium-M (50-150 mg GAE/g) and low-L (< 50 mg GAE/g) categories. CM-Ac and ZA-Pe extracts were found to possess the highest phenolic content followed by that in CM-Pe, CM-Ch, CH-Ea, ZA-Ch, ZA-Ea and ZA-Ac extracts in the medium category. The remaining 12 extracts - CM-Ea, CM-Me, CH-Pe, CH-Ch, CH-Ac, CH-Me, CN-Pe, CN-Ch, CN-Ac, CN-Ea, CN-Me and ZA-Me - possessed low phenolic content. Phenolic compounds are commonly found in both edible and nonedible plants, and they have been reported to have multiple biological effects, including antioxidant activity [25]. The antioxidant capacity of extracts in terms of free radical scavenging activity by DPPH per milligram of extract has been given in Figure. 1(B). As done earlier with respect to total phenolic content, extracts were grouped into high-H (> 50%), medium-M (25 - 50%) and low-L (< 25%) categories depending on the percentage of free radical scavenging activity. Accordingly, CM-Ac, CH-Ea and CH-Ac extracts were found to possess high free radical scavenging activity, while eleven extracts - CM-Me, CH-Ch, CH-Me, CN-Pe, CN-Ea, CN-Ac, CN-Me, ZA-Ch, ZA-Ea, ZA-Ac and ZA-Me exhibited medium activity and the remaining six extracts showed low activity. Antioxidant capacity of plant constituents, attributable mainly to phenolic compounds, is known to protect the human body from free radicals and ROS effects and retard the progress of many chronic diseases like cancer[26,27]. A correlation between high phenolic content and high antioxidant activity was evident in the case of only five extracts - namely, CM-Ac, CH-Ea, ZA-Ch, ZA-Ea and ZA-Ac.





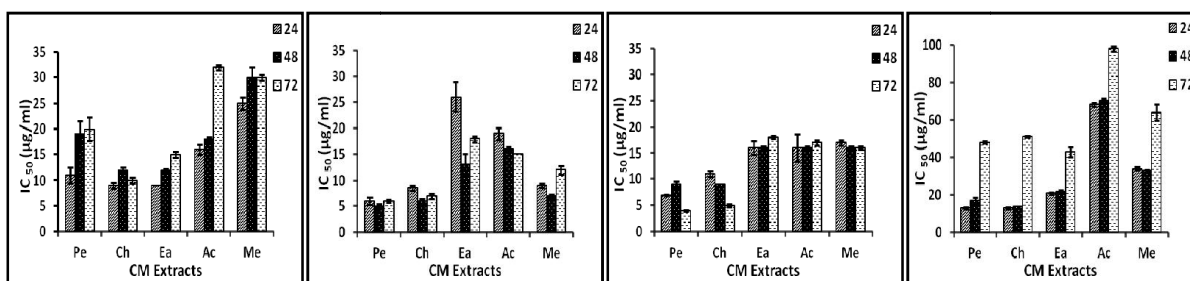
**Figure 1.** (A) Total phenolic content and (B) DPPH radical scavenging activity of different organic solvent extracts from rhizomes of *Curcuma mutabilis* (CM), *Curcuma haritha* (CH), *Curcuma neilgherrensis* (CN) and *Zingiber anamalayanum* (ZA). Data represents mean  $\pm$  S.D. (n=3).

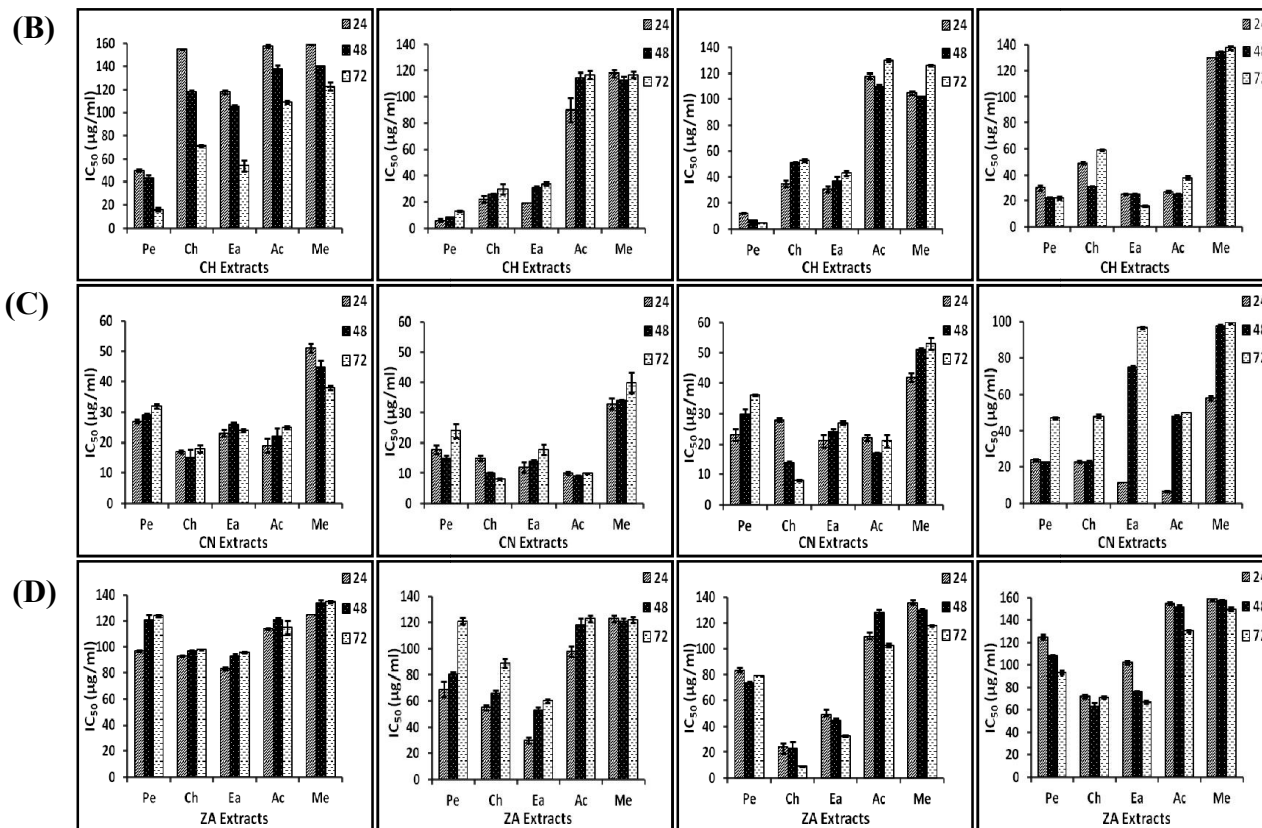
### Antiproliferative activity of rhizome extracts on Human cancer cell lines

Antiproliferative activities of the various extracts were tested against four human cancer cell lines. The results of the MTT assay, following exposure of cells to the extracts for 24, 48 and 72 h are shown in Figure. 2. The extent of cytotoxicity of the different extracts towards different cell lines showed variations. Of the 20 extracts, 10 exhibited potent antiproliferative activity with IC<sub>50</sub> values less than 30  $\mu$ g/mL. As per the guidelines of American National Cancer Institute, the IC<sub>50</sub> limit for selecting a crude extract as a promising candidate for drug development should be lower than 30  $\mu$ g/mL [28]. Notably, the highest antiproliferative activities were exhibited by all extracts of CM, CN (except methanolic) and

CH-Pe with the lowest IC<sub>50</sub> values ranging from 6 to 28  $\mu$ g/mL. CN-Me, CH-Ch, CH-Ea, ZA-Pe, ZA-Ch and ZA-Ea extracts exhibited a relatively medium level of antiproliferative activity with IC<sub>50</sub> values ranging from 31 to 98  $\mu$ g/mL followed by the low cytotoxicities (>100 $\mu$ g/mL) of CH-Ac, CH-Me, ZA-Ac and ZA-Me extracts. Most of the extracts exhibited maximum activity at 24 h of exposure. Longer periods of exposure were found to be ineffective in the case of CM and CN extracts as evidenced by an increase in IC<sub>50</sub> value. Upon extended exposure, the activities of CH and ZA extracts, either decreased or remained stable in terms of IC<sub>50</sub>. Taken together, the results showed that 10 of the 20 extracts tested exhibited promising drug potential exploitable to provide future bioactive compounds for development of new leads for cancer therapy.

### (A)





**Figure 2.** IC<sub>50</sub> values obtained following 24, 48 and 72 h exposure of human cancer cell lines – K562, REH, Nalm6 and MCF7 to different organic solvent extracts from rhizomes of (A) *Curcuma mutabilis* (CM), (B) *Curcuma haritha* (CH), (C) *Curcuma neilgherrensis* (CN) and (D) *Zingiber anamalayanum* (ZA). Data represents mean ± S.D. (n=3).

### Conclusion

In conclusion, the results of this study indicate that the rhizomes of plants taken up for the study possess significant antiproliferative activity. Of all rhizome extracts, CM-Ac showed high phenolic content and good free radical scavenging activity while CM-Pe extract showed high antiproliferative activity against all cell lines. Further studies are underway to identify and characterize the bioactive compounds with antiproliferative activity and to evaluate the molecular pathways affected by the bioactive constituents of the extracts.

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### Conflict of Interest

The authors declare no conflict of interest.

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