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## Phytochemical estimation and *in vitro* antioxidant activity of rhizome of *in vitro* regenerated *Zingiber zerumbet* (L.) Sm.

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### Abstract

*Zingiber zerumbet* (L.) Sm. is an important medicinal plant belongs to the family Zingiberaceae and it is commonly known as Bitter ginger, Pine cone ginger, Pine cone lily. The present study aimed to estimate the phenols and flavonoids in the crude methanolic extract of rhizome and to assess the *in vitro* antioxidant activity by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical assay. The phenols like gallic acid, hydroxyl benzoic acid, coumaric acid, vallinic acid and cinnamic acid and flavonoids like rutin, quercetin, kaempferol and luteolin were estimated with various quantities (mg/g). *In vitro* antioxidant activity of the extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radicals DPPH and ABTS. IC<sub>50</sub> values are 179µg/mL and 77µg/mL for DPPH and ABTS respectively. The findings of the phytochemical and antioxidant properties of the rhizome of *in vitro* regenerated plant are indeed highly valuable to promote the use *Z. zerumbet* as natural sources of phytochemicals and potential antioxidant.

**Keywords:** *Zingiber zerumbet*, phytochemicals, rhizome extract, antioxidant activity, DPPH, ABTS

### Introduction

*Zingiber zerumbet* (L.) Sm. is a monocot herbaceous and rhizomatous perennial plant belongs to the family Zingiberaceae and it is commonly called as bitter ginger and pinecone lily due to its conical shape inflorescence. It is also called as "shampoo ginger" because of the mucilaginous substance present in the inflorescence and is used as shampoo and natural hair conditioner [1]. Traditionally, the *Z. zerumbet* is used in the treatment of swelling, sores and loss of appetite, the juice of the boiled rhizomes is also used as a medicine for worm infestation in children and decoction prepared from the rhizome is used to treat jaundice [2]. In India, the rhizome powder is mixed with ripe *Morinda citrifolia* for the treatment of severe pain, the cooked and softened rhizome is used to treat toothache, cough, asthma, worms, leprosy and other skin diseases and the ground and strained rhizome is mixed with water and drunk to treat stomachache. Various reports have been published regarding the phytochemical content of rhizome of *Z. zerumbet* and other species of Zingiber.

The rhizome of *Z. zerumbet* has been demonstrated to possess multipotential bioactivities such as anti-inflammatory activity [3], antipyretic activity [4], anti-allergic activity [5], immunomodulatory activity [6], antiplatelet aggregation activity [7], antiproliferative activity [8], anti hyperglycemic activity [9], antimicrobial activity [10] and antioxidant activity [11]. The antioxidant activity of the plant extract is mainly attributed to their phenolic constituents such as flavonoids, phenolic acids and polyphenolic compounds which neutralize free radicals by different mechanism including metal chelation and electron donation as reducing agent. Free radicals called Reactive Oxygen Species (ROS) are normal product of human metabolism [12]. Phenols and flavonoids also been reported to possess diverse biological activities, for instance, antiulcer [13].

The present study is aimed to estimate the phenols and flavonoids present in crude methanol extract of rhizome of *in vitro* regenerated *Z. zerumbet*. Based on the phytochemical estimation, it is understood that the molecules may have potential for free radical scavenging activity. Hence, the *in vitro* antioxidant activity study was planned and executed against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radicals.

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## Materials and Methods

**Plant material:** *In vitro* regenerated plants of *Zingiber zerumbet* (L.) Sm. was obtained on the MS medium supplemented with 8.88µM/L of BAP, 1.10µM/L of NAA and 10.86µM/L of AdS. The *in vitro* regenerated plant lets were hardened and establish in field and maintained in the garden of Department of Botany, Pachaiyappa's College, Chennai, Tamilnadu. The rhizomes were collected from 12 months old plant for this study; 500 grams of fresh rhizome were collected separately and were washed thoroughly with tap water to remove the adhered soil particles on the surface of rhizome. These rhizomes were cut in to small pieces, dried in oven (50°C) for about 48 hours and were then coarsely powdered.

### Preparation of Extract

The coarsely powdered sample was extracted in 1:10 ratio at room temperature with 99% methanol. The extract was filtered with Whatman No.1 filter paper and was concentrated by distillation and desiccated. Ultimately 10%w/w of semi solid residues was recovered and the extract was subjected for the estimation of the phenol, flavonoid and *in vitro* antioxidant studies.

### Phytochemical analysis

Extraction and purification of phenolic acids and flavonoids such as gallic acid, hydroxy benzoic acid, coumaric acid, vallinic acid, cinnamic acid, rutin, quercetin, kaempferol and luteolin were done by following the method described by Irakli *et al.* [14] with little modification.

### Quantitation of phenolic acids and flavonoids

A liquid chromatograph from Shimadzu with an LC-10 AT VP pump, an SCL-10A VP, control system, an SIL-10AD VP auto sampler, an SPD 10AV VP spectrophotometric detector, a DGU-14A degasser and a computer system Class VP (version 5.0) were used. The analyses were carried out on a Luna C<sub>18</sub> 250 x 4.6 mm, 5 µm. The mobile phase was composed of different proportions of (A) Acetonitrile (B) methanol and (C) acidified water. The initial mobile phase composition was 5% B and 90% C, followed by a linear gradient to 10% B and 85% C in 5 min; 5-30 min, from 85 to 80% C and B constant; 30-38 min, from 10 to 30% A and 80 to 70% C; 38-50 min, from 30 to 60% A and 70 to 40% C. The post-running time was 5 min. The flow rate was 1 mL/min, the column temperature was set at 25C, and the sample injection volume was 20 L. The acquisitions were performed in the range 190 - 450 nm and the chromatograms were integrated at 260 nm (for 4-hydroxybenzoic acid and vanillic acid), 280 nm (for gallic acid and cinnamic acid), 320 nm (for p-coumaric acid), and 360 nm (for luteolin, kaempferol and quercetin). A stock solution of 1 mg/mL was prepared by dissolving each PA and FL standard in methanol. Working standard solutions were made by gradual dilution with the mixture of acidified water/ACN/MeOH (9:0.5:0.5 v/v/v) to the required concentration, which was based on the sensitivity of detection and the linearity range identified. Identification of PAs and FLs was performed by comparing retention times and absorption spectra of the unknown peaks with reference standards.

### *In vitro* antioxidant activity

#### Scavenging of DPPH radical

The DPPH scavenging activity was carried out by using the little modified method described by Blois [15]. DPPH (Hi

media) of 7.9 mg was dissolved in 100 mL methanol and it was protected from light by covering the test tubes with aluminum foils. DPPH is always prepared freshly and to be used for studies. 1mL of 100 µM DPPH solution was mixed with 3 mL of methanol and an absorbance was taken immediately at 517nm for control reading. 1mL of 100 µM DPPH solution was mixed with an equal volume of each of the various concentrations of test sample (50, 100, 150, 200, 250 and 300 µg/mL) and the mixture was shaken vigorously, covering with aluminum foil and incubate them for 20 minutes at room temperature and an absorbance was taken at 517 nm (UV-visible spectrophotometer, Systronics). The inhibition % were calculated as follows

$$\text{Inhibition (\%)} = \frac{\text{Control Absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

The 50% inhibition (IC<sub>50</sub>) was calculated from the graph plotting inhibition percentage against extract concentrations. Methanol was used as a blank and Butylated hydroxytoluene (BHT) and L-ascorbic acid were used as standard.

#### Scavenging of ABTS radical

ABTS radical cation scavenging activity was measured by using the little modified method described by Prabhakar *et al.*, [16]. In the improved version of ABTS<sup>•+</sup>, a free radical is generated by persulphate oxidation of ABTS<sup>2-</sup>. The ABTS radical cation was produced by reacting ABTS 7 mM solution with 2.45 mM ammonium persulphate. The solution was prepared by mixing 7 mM of ABTS salt with 2.45 mM of ammonium persulfate in 25 mL of distilled water. The solution was incubated at room temperature in the dark for 16h before use. Fresh ABTS solution was prepared for each analysis. Various concentrations of test sample of methanolic extract as mentioned in the DPPH method (1mL) were added to 0.6 mL of ABTS solution and the final volume was mixed up with methanol to make 2 mL. The absorbance was read at 745 nm and the percentage of inhibition was calculated. The standards and the formula for calculating the percentage of inhibition were same as in DPPH radical assay.

#### Statistical Analysis

All the tests were carried out in triplicates and the data were analyzed statistically using the SPSS 16.0 software (SPSS Inc., Chicago, USA) and the mean values are expressed as mean ± SE. The significance of differences among means was carried out at P<0.05 probability level using Duncan's Multiple Range Test (DMRT).

## Results and Discussion

### Estimation of phytochemicals

Methanolic extract of rhizome of *in vitro* regenerated *Zingiber zerumbet*, were subjected to an estimation of phenols and flavonoids the findings are summarized. The rhizome extract consist of some phenols such as gallic acid, hydroxyl benzoic acid, coumaric acid, vallinic acid and cinnamic acid with different quantities expressed as mg/g of crude extract (Table-1 and 2).

Analysis of phenol in rhizome, contain gallic acid (4.00 ± 0.01mg/g), hydroxyl benzoic acid (6.10 ± 0.10), coumaric acid (1.90 ± 0.02), vallinic acid (2.43 ± 0.08) and cinnamic acid (1.43 ± 0.02) (Table-1). Phenolics in food products of plant origin are the secondary plant metabolites that are known to protect plants from UV light, infections or act as attractants for pollinators. Phenolic compounds are secondary

products which possess an aromatic ring bearing a hydroxyl substituent and most are of plant origin [17]. Phenolic compounds are widely found in the secondary product of medicinal plants as well as in many edible plants [18]. Plant phenolics play an important role on the mechanism against diseases and pathogens and also in many physiological events in the plants such as growth vigour, differentiation of flowers and roots, determination of gene activity and characterisation of some developmental stages [19]. In the present study, the presence of five different phenolic compounds such as gallic acid, hydroxy benzoic acid, coumaric acid, vallinic acid and cinnamic acid was confirmed in methanolic extract of rhizome of *in vitro* regenerated *Z. zerumbet* by quantitative analysis. Among these phenolic compound hydroxy benzoic acid showed maximum quantity ( $6.10 \pm 0.10$  mg/g) followed by gallic acid in  $4.00 \pm 0.01$  mg/g and minimum amount of coumaric acid vallinic acid and cinnamic acid were recorded and no significant variation among them in quantity. The similar study was carried out in rhizome of *ex vitro* grown *Zingiber zerumbet* and reported some phenols with different quantities that gallic acid (4.47), hydroxyl benzoic acid (1.81), coumaric acid (1.61), vallinic acid (11.27) and cinnamic acid (1.00) [20]. Gallic acid has remarkable effects on lung cancer cell lines by inducing apoptosis and activating caspases. In murine models, gallic acid seemed to reduce the rate of tumor growth [21]. Cinnamic acid also displays an antitumor activity, namely against colon adenocarcinoma by antiproliferative methods like enzyme induction and modulation of the cAMP signaling pathway [22].

**Table 1:** Quantitative estimation of phenols in methanolic extract of rhizome

Sl. No	Phenols	Composition (mg/g)
1	Gallic acid	$4.00 \pm 0.01$
2	Hydroxy benzoic acid	$6.10 \pm 0.10$
3	Coumaric acid	$1.90 \pm 0.02$
4	Vallinic acid	$2.43 \pm 0.08$
5	Cinnamic acid	$1.43 \pm 0.02$
F- Value		885.412
P- Value		0.00

Values are expressed as Mean  $\pm$  SEM, n=3.

The methanolic extract of rhizome of *in vitro* regenerated plant also contains some flavonoids such as rutin, quercetin, kaempferol and luteolin with various amounts (mg/g) (Table-2). The estimation of flavonoids in the rhizomes was rutin ( $4.22 \pm 0.15$ mg/g), quercetin ( $1.18 \pm 0.19$ ), kaempferol ( $3.53 \pm 0.04$ ) and luteolin ( $3.75 \pm 0.11$ ). Flavonoids are an important group of polyphenols which are widely distributed among the plant kingdom. Over four thousand flavonoids are known to exist and some of them are pigments in higher plants. Quercetin, kaempferol and quercitrin are common flavonoids present in nearly 70% of plants. Other group of flavonoids include flavones, dihydroflavons, flavans, flavonols, anthocyanidins, proanthocyanidins, calchones and catechin and leucoanthocyanidins [23]. Flavonoids, which are generally found in the plant kingdom, may serve specific functions in flower pigmentation, UV-protection, plant defense against pathogens and legume nodulations [24]. In the present study, the presence of four different flavonoids such as rutin, quercetin, kaempferol and luteolin were confirmed in extract of rhizome *in vitro* regenerated *Z. zerumbet* by quantitative estimation. All these four flavonoids are rich in extract of rhizome in different quantities. Rutin ( $4.22 \pm 0.15$ ) and letolin ( $3.75 \pm 0.11$ ) are the maximum and have no

significant variation in quantity, quercetin and kampferol was in minimum amount. This result were coincides with report of Gandhi and Saravanan [20] in the rhizome of *ex vitro* grown *Z. zerumbet* that flavonoids with various quantities like 3.14mg/g of rutin, 2.65 of quercetin, 1.48 of kaempferol and 2.06 of luteolin. Rhizome of *Z. zerumbet*, which is a widely used herb taken before meals especially in Fiji, is reported to be the richest source of kaempferol (240 mg/100 g) when compared to other species of Zingiberaceae [34]. Kaempferol has been studied for potential anticancer properties, in terms of human cell lines and it was proved to be effective against hepatocarcinoma [26]. Jang *et al.*, [27] reported the isolation of aromatic compound and kaempferol derivatives from *Z. zerumbet*. The properties of quercetin, rutin, caffeic acid, vanillic acid and gallic acid of different wine against pathogenic microorganisms was investigated by Vaquero *et al.*, [28]. Flavonoids constitute a wide range of substances that plays an important role in protecting the biological systems against the harmful effects of oxidative processes on macromolecules, such as carbohydrates, proteins, lipids and DNA [29].

**Table 2:** Quantitative estimation of flavonoids in methanolic extract of rhizome

Sl. No	Flavonoids	Composition (mg/g)
1	Rutin	$14.22 \pm 0.15$
2	Quercetin	$1.18 \pm 0.19$
3	Kaempferol	$3.53 \pm 0.04$
4	Luteolin	$3.75 \pm 0.11$
F- Value		175.1
P- Value		0.00

Values are expressed as Mean  $\pm$  SEM, n=3

#### *In vitro* antioxidant activity

The various concentrations of methanolic extract of rhizome of *in vitro* regenerated *Z. zerumbet* were tested for DPPH and ABTS radical scavenging potential. All the six different concentrations of the samples showed the DPPH and ABTS radical scavenging potential with different percentages of inhibition (Table-3) and their IC<sub>50</sub> values were also recorded (Table-4).

The DPPH reaction was very stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH assay is one of the most widely used methods for screening of antioxidant activity, since it can accommodate many samples in a short period and detect active ingredients at low concentration [30]. The six different concentrations (50-300  $\mu$ g/mL) of rhizome extract were showed various percentage of scavenging potential ranging from  $39.80 \pm 0.70\%$  to  $68.28 \pm 0.06\%$  on DPPH radical scavenging (Table-3). The IC<sub>50</sub> values was recorded at 179  $\mu$ g/mL which is about 6-fold higher than the standards L-Ascorbic acid and BHT (27  $\mu$ g/mL) (Table-4). The maximum scavenging ( $68.28 \pm 0.06\%$ ) was recorded at 300  $\mu$ g/mL of rhizome of *in vitro* regenerated *Z. zerumbet*. The DPPH radical scavenging potential of the methanolic extract of rhizome was due to the presence of phenolic compounds. The degree of discoloration indicates scavenging potential of the antioxidant extract which is due to the hydrogen donating or radical scavenging ability [31]. Phenolic compounds are important plant antioxidants which exhibit considerable scavenging activity against free radicals. Thus the antioxidant capacity of a sample can be attributed mainly to its phenolic compound [32]. The effects of antioxidants on DPPH radical scavenging may be due to their hydrogen donating ability [33].

Ghasemzadeh *et al.*,<sup>[13]</sup> reported about 58.22% of inhibition in *Z. officinale* and Jagtap<sup>[34]</sup> reported 56.33% of DPPH radical scavenging activity in methanolic extract of *Zingiber cernuum*. The antioxidant activity is expressed by IC<sub>50</sub> value, which is defined as the effective concentration of substrate that causes 50% loss of the DPPH activity<sup>[35]</sup>. In the present study, the DPPH assay exhibited the IC<sub>50</sub> values at 179µg/mL of rhizome extract. This result support with the report in rhizome extract of *ex vitro* grown *Z. zerumbet* the IC<sub>50</sub>-181 µg/mL and maximum scavenging percentage (65.17) recorded at concentration of 300µg/mL<sup>[20]</sup>. These natural products have shown a higher scavenging ability, indicating that they are potent free-radical inhibitors. Similarly, the results also exhibited the strong radical-scavenging activity against DPPH free radicals, implying that *in vitro* regenerated *Z. zerumbet* has quite potential as natural antioxidant resources. In this study the DPPH radical scavenging activities of rhizome extract (179µg/mL) were compared with L-ascorbic acid (27) and BHT (27) the standard drugs and it is found to be 6-fold higher than that of standards.

**Table 3:** Effect of methanolic extract of rhizome on DPPH and ABTS antioxidant assay

Sl. No	Concentration (µg/mL)	Free Radical Scavenging Activity (Inhibition %)	
		DPPH	ABTS
1	50	39.80±0.70	48.10±0.25
2	100	42.23±0.06	65.06±0.19
3	150	45.88±0.07	77.15±0.56
4	200	55.80±0.04	84.41±0.59
5	250	60.02±0.17	88.79±0.16
6	300	68.28±0.06	91.17±0.40
F-Value		138.3	168.1
P-Value		0.00	0.00

Values are expressed as Mean ± SEM, n=3

**Table 4:** IC<sub>50</sub> value of standards and sample on DPPH and ABTS radical scavenging activity

Sl. No	Sample	IC <sub>50</sub> value (µg/mL)	
		DPPH Radical	ABTS Radical
1	Rhizome extract	179	77
2	L- Ascorbic acid	27	33
3	BHT	27	36

The ABTS radical scavenging potential also assessed with different concentrations as mentioned in DPPH radical assay. All the six different concentrations of the samples showed ABTS radical scavenging potential with different percentages of inhibition (Table-3). The percentage of ABTS radical scavenging potential ranged from 48.10 ± 0.25% to 91.17 ± 0.40%. Leong and Shui<sup>[36]</sup> reported that the ABTS assay is an excellent tool for decisive the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and of chain breaking antioxidants (scavenger of lipid peroxy radicals). The ABTS radical reactions involve electron transfer and the process take place faster rate when compared to DPPH radicals. The decolorization of the ABTS<sup>+</sup> radical also reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical species<sup>[37]</sup>. In the ABTS radical cation scavenging activity, the rhizome of *Z. zerumbet* showed concentration dependent scavenging activity. The present investigation has shown that the methanolic extract of rhizome of *in vitro* regenerated plant exhibited significant ABTS radical scavenging activity. The maximum (91.17 ± 0.40%) of scavenging was observed at

300 µg/mL of concentration. In this study, the ABTS assay exhibited the IC<sub>50</sub> values at 77µg/mL of rhizome extract. This result support with the report in rhizome extract of *ex vitro* grown *Z. zerumbet* the IC<sub>50</sub>-90 µg/mL and maximum scavenging percentage (87.63) recorded at the concentration of 300µg/mL<sup>[20]</sup>. Antioxidant activity was classified as the initial biopotential assessment, since antioxidants have been strongly associated with the defence mechanisms of living cells against oxidative damage<sup>[38]</sup>. Several classes of plant-derived compounds such as flavonoids, phenolics and alkaloids, have also been reported to exhibit antioxidant properties<sup>[39]</sup>. The ABTS radical scavenging activities of rhizome extract (77 µg/mL) were compared with L-ascorbic acid (33 µg/mL) and BHT (33 µg/mL) the standard drugs and it is found to be 2-fold higher than that of standards. The present study clearly indicate that, there is no significant differences in quantity of phytochemicals and potential of *in vitro* antioxidant activity among the methanolic extract of rhizome of *in vitro* regenerated and *ex vitro* grown *Zingiber zerumbet*.

## Conclusion

The results of present study, it may be concluded that the potential antioxidants activity of rhizome of *in vitro* regenerated *Z. zerumbet* could be because of the presence of significant quantities of different phenols and flavonoid compounds present in the rhizome. The findings of the antioxidant properties are indeed highly valuable to promote the use as natural sources of potential antioxidants. The phytochemical data of this study will be helpful for the standardization and quality control of invaluable indigenous drug and can be scientifically validate the use of medicines obtained from the tissue culture derived plants.

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