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Phytochemical Estimation and *In vitro* Antioxidant Activity of Rhizome of *Zingiber zerumbet* (L.) Sm.

Gandhi, K and Saravanan, S*

Post Graduate and Research Department of Botany
Pachaiyappa's College, Chennai-600 030, Tamil Nadu, India.

*Corresponding authors email: sstc1965@yahoo.co.in

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 lilly. 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_{50} values are 181µg/mL and 90µg/mL for DPPH and ABTS respectively. The findings of the phytochemical and antioxidant properties of this plant are indeed highly valuable to promote the use *Z. zerumbet* as natural sources of potential antioxidant.**

Keywords: ***Zingiber zerumbet,*** **Phytochemicals, antioxidant avtivity, DPPH, ABTS.**

1. 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 ginger, pinecone lilly 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 drank to treat stomachache. The young shoots and inflorescence are used as condiments and as food supplements to reduce experimental ulcerative colitis [3]. Various reports have been published regarding the phytochemical content of rhizome of *Z. zerumbet*. Attempts to isolate and identify bioactive compounds from the rhizome have started since 1944 [4].

The rhizome of *Z. zerumbet* has been demonstrated to possess multipotential bioactivities such as antinociceptive activity [3], anti-inflammatory activity [5], antipyretic activity [6], anti-allergic activity [7], immunomodulatory activity [8], antiplatelet aggregation activity [9], antiproliferative activity [10], anti hyperglycemic activity [11], antiamoebic and antigiardial activity [12], antimicrobial activity [13], antimycobacterial activity [14], anthelminthic activity [15], larvicidal activity [16] and antioxidant activity [17]. 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 [18]. Phenols and flavonoids also been reported to

possess diverse biological activities, for instance, antiulcer, [19], cytotoxic and antitumor [20], antiplasmodic [21] and anti depressant activities [22].

The aim of the present study was to analyze the phenols and flavonoids present in crude methanol extract of rhizome of wild *Z. zerumbet.* Based on the phytochemical analysis, 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.

2. MATERIALS AND METHODS

Plant material

The healthy plants of *Z. zerumbet* (L.) Sm. was collected from herbal nursery maintained by Forest department of Tamil Nadu at Pollachi, Coimbatore. The plants were authenticated by taxonomist in the Department of Botany, Pachaiyappa's College, Chennai, Tamilnadu. The rhizomes were collected from 12 months old plant; 500 grams of fresh rhizome were collected separately and were washed with tap water to remove the adhered soil particles on the surface of rhizome. These samples were cut in to small pieces, dried in oven (50ºC) for about 48 hours and were then coarsely powdered.

Preparation of Extract

The coarsely powdered sample was extracted in 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 employed 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* ([23] 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_{18} 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.

Scavenging activity DPPH radical

The DPPH scavenging activity was estimated by using the little modified method described by Blois [24]. 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

$$\text{Inhibition (\%)} = \frac{\text{Control Absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \text{X100}$$

The 50% inhibition (IC_{50}) 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 activity of ABTS radical

ABTS radical cation scavenging activity was measured by using the little modified method described by Prabhakar *et al.*, [25]. In the improved version of $ABTS^{-}$, a free radical is generated by persulphate oxidation of $ABTS^{2-}$. 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 formula for calculating the percentage of inhibition and the standards were also same as DPPH.

Statistical Analysis

All the tetsts 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).

3. RESULTS AND DISCUSSION

Estimation of phytochemicals

Methanolic extract of rhizome, were subjected to an analysis 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 various quantities expressed as mg/g of crude extract (Table 1a and 1b).

Analysis of phenol in rhizome, contain gallic acid (4.47 ± 0.19), hydroxyl benzoic acid (1.81 ± 0.09), coumaric acid (1.61 ± 0.11), vallinic acid (11.27 ± 0.17) and cinnamic acid (1.00 ± 0.01) (Table 1a). 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 [26]. Plant phenolics are a chemically heterogenous group, where some are soluble only in organic solvents and some are water-soluble carboxylic acids and glycosides. Phenolic compounds are widely found in the secondary product of medicinal plants as well as in many edible plants. Another group of phenolics are insoluble polymers [27]. 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 [28]. 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 rhizome extract of *Z. zerumbet* by quantitative analysis. Among these phenolic compound vallinic acid showed maximum quantity (11.27 ± 0. 17 mg/g), followed by gallic acid in 4.47 ± 0. 19 mg/g and minimum amount of coumaric acid vallinic acid and cinnamic acid were recorded and no significant variation among them in quantity. 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 gowth [29]. Cinnamic acid also displays an antitumor activity, namely against colon adenocarcinoma by antiproliferative methods like enzyme induction and modulation of the cAMP signaling pathway [30].

The extract of rhizome also contained flavonoids such as rutin, quercetin, kaempferol and luteolin with varied amount (mg/g) (Table. 1b). The estimation of flavonoids in the rhizome, were 3.14 ± 0.08 of rutin, 2.65 ± 0.03 of quercetin, 1.48 ± 0.15 of kaempferol and 2.06 ± 0.09 of luteolin. 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 [31]. 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 [32]. In the present study, the presence of four different flavonoids such as rutin, quercetin, kaempferol and luteolin were confirmed in rhizome extract of *Z. zerumbet* by quantitative analysis. All these four flavonoids are rich in rhizome in different quantities. Rutin and letolin are the maximum and have no significant variation in quantity, quercetin and kampferol was in minimum quantity. Park *et al.,* [33] revealed that these compounds posses significant anti-inflammatory activity is due to the direct inhibition of initial processes in inflammation. 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 (*Z. officinale*) [34]. Kaempferol has been studied for potential anticancer properties, in terms of human cell lines and it was proved to be effective against hepatocarcinoma. Kaempferol has been proved to reduce tumor cell viability even under hypoxic conditions [35]. Jang *et al.,* [36] reported the isolation of aromatic compound and kaempferol derivatives from *Z. zerumbet*. Vaquero *et al.,* [37] investigated the properties of quercetin, rutin, caffeic acid, vanillic acid and gallic acid of different wine against pathogenic microorganisms. Flavonoids constitute a wide range of substances that plays an important role in protecting biological systems against the harmful effects of oxidative processes on macromolecules, such as carbohydrates, proteins, lipids and DNA [38].

***In vitro* antioxidant activity**

The various concentrations of methanol extract of rhizome of *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. 2a) and their IC_{50} was recorded at (Table. 2b)

The DPPH reaction was very stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. This assay is one of the most widely used methods for screening antioxidant activity, since it can accommodate many samples in a short period and detect active ingredients at low concentration [39]. The six different concentrations (50-300 µg/mL) of rhizome extract were showed various percentage of scavenging potential ranging from 40.88 ± 0.19% to 65.17 ± 0.17% on DPPH radical scavenging (Table. 2a). The IC_{50} was recorded at 181 µg/mL which is about 6-fold higher than the standards L- Ascorbic acid and BHT (27 µg/mL) (Table. 2b). The maximum scavenging (65.17 ± 0.17%) was obtained at 300 µg/mL of rhizome extract. 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 [40]. 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 [41]. The effects of antioxidants on DPPH radical scavenging may be due to their hydrogen donating ability [42]. Ethanol extract of dried rhizome of *Zingiber officinale* exhibited 90.1% of DPPH radical scavenging activity with the IC_{50} concentration of 0.64µg/mL [43]. Ghasemzadeh *et al.,* [19] reported about 58.22% of inhibition in *Z. officinale* and Jagtap [44] reported 56.33% of DPPH radical scavenging activity in methanolic extract of *Zingiber cernuum*. The antioxidant activity is expressed by IC_{50} value, which is defined as the effective concentration of substrate that causes 50% loss of the DPPH activity [45]. In the present study, the DPPH assay exhibited the IC_{50} values at 181µg/mL of rhizome of *Z. zerumbet.* This result coincides with the IC_{50} value of *Z. zerumbet* which was 299µg/mL in *Curcuma xanthorhiza* [46]. 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 *Z. zerumbet* has quite potential as natural antioxidant resources.

The methanol extract of *Z. zerumbet* were tested for ABTS radical scavenging potential with different concentration 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. 2a). The percentage of ABTS radical scavenging potential ranged from 35.05 ± 0.52% to 87.64 ± 0.52%. The extract concentrations of 50, 100 and 150 µg/mL showed 35.05 ± 0.52, 55.46 ± 0.39 and 69.12 ± 1.03 % of inhibition respectively. The 78.24 ± 0.68 % of radical scavenging observed at 200 µg/mL concentration, 83.97 ± 0.52% for 250µg/mL and 300µg/mL concentration showed 87.64 ± 0.52% of inhibition. Leong and Shui [47] 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 peroxyl

radicals). The ABTS radical reactions involve electron transfer and the process take place faster rate when compared to DPPH radicals. The decolorization of the $ABTS^{+}$ radical also reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical species [48]. 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 exhibited significant ABTS radical scavenging activity. The maximum (87.64 ± 0.52%) of scavenging was obtained at 300 µg/mL of rhizome. In this study, the ABTS assay exhibited the IC_{50} values at 90µg/mL of rhizome extract. This result was in agreement with the report of Bhavesh et al., [49] who reported the 50% inhibition of ABTS radical scavenging potential at 78.72µg/mL in *Z. zerumbet.* 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 [50]. Several classes of plant-derived compounds such as flavonoids, phenolics and alkaloids, have also been reported to exhibit antioxidant properties [51]. The ABTS radical scavenging activities of rhizome extract (90) were compared with L-ascorbic acid (33) and BHT (36) the standard drugs and it is found to be 3-fold higher than that of standards.

4. CONCLUSION

From the above results, it may be concluded that the potential antioxidants activity of *Z. zerumbet* could be because of the presence of significant quanties of various phenol and flavonoid compounds present in it. The findings of the antioxidant properties of *Z. zerumbet* are indeed highly valuable to promote the use as natural sources of potential antioxidants. This should lead to a better understanding of the antioxidant activity and the active principles and furthermore, may allow for rational recommendations regarding their uses in folk medicine systems. The phytochemical data of *Z. zerumbet* will be helpful for the standardization and quality control of invaluable indigenous drug and can be scientifically validate the use of *Z.zerumbet* in traditional folk medicine.

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APPENDIX - A

TABLE 1a: Quantitative estimation of phenols in rhizome of *Zingiber zerumbet*

Sl.No	Phenols	Composition (mg/g)
1	Gallic acid	4.47 ± 0. 19[b]
2	Hydroxy benzoic acid	1.81 ± 0. 08[a]
3	Coumaric acid	1.60 ± 0. 10[a]
4	Vallinic acid	11.27 ± 0. 17[c]
5	Cinnamic acid	1.69 ±0.16[a]
F- Value		760.501
P- Value		0.00

Values are expressed as Mean ± SEM, n=3

TABLE 1b: Quantitative estimation of flavonoids in rhizome *Zingiber zerumbet*

Sl.No	Flavonoids	Composition (mg/g)
1	Rutin	3.14 ± 0.08[c]
2	Quercetin	2.65 ± 0.03[b]
3	Kaempferol	1.48 ± 0. 15[a]
4	Luteolin	3.33 ±0.017[c]
F- Value		85.495
P- Value		0.00

Values are expressed as Mean ± SEM, n=3

TABLE 2a: Effect of methanolic extract of rhizome *Zingiber zerumbet* on DPPH and ABTS antioxidant assay

Sl No	Concentration (µg/mL)	Free Radical Scavenging Activity (Inhibition %)	
		DPPH	ABTS
1	50	40.88±0.19[a]	35.05±0.52[a]
2	100	43.79±0.16[b]	55.46±0.39[b]
3	150	44.24±0.09[b]	69.12±1.03[c]
4	200	55.36±0.07[c]	78.24±0.68[d]
5	250	57.81±0.17[d]	83.97±0.52[e]
6	300	65.17±0.17[e]	87.64±0.52[f]
F-Value		4.065E3	991.753
P-Value		0.00	0.00

Values are expressed as Mean ± SEM, n=3

TABLE 2b IC_{50} value of standard and rhizome extract of *Zingiber zerumbet* on DPPH and ABTS radical scavenging activity

Sl. No	Sample	IC_{50} value (µg/mL)	
		DPPH Radical	ABTS Radical
1	Rhizome extract	181	90
2	L- Ascorbic acid	27	33
3	BHT	27	36