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Antioxidant Activities and Cytotoxicity of *Zingiber zerumbet* (L.) Smith Rhizome

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ABSTRACT

Zingiber zerumbet (L.) Smith, popularly known as shampoo ginger, is one of the most commonly used ingredients in Indo-Malaysian traditional medicines. The antioxidant and cytotoxic activities of the ethanolic extract of *Z. zerumbet* rhizome (ZZ) was investigated. 2,2-di(4-tert-octylphenyl)-1-picryl-hydrazyl (DPPH) and hydroxyl radical scavenging assays showed significant radical scavenging activities of ZZ. The extract was rich in polyphenol and flavonoids. Cytotoxicity was assessed *in vitro* by trypan blue exclusion test. Human peripheral blood lymphocyte cells were incubated in different concentrations of ZZ (0, 15, 30, 60, 120, 300 and 600 µg/ml) for 3 h at 37 °C. The rhizome extract was found to be cytotoxic at concentrations higher for human consumption. In addition, HPLC analysis revealed ZZ as a rich source of kaempferol. Based on the results of the present investigation the rhizome may be used safely as a therapeutic antioxidant.

Keywords: *Zingiber zerumbet*; Ethanolic Extract; Antioxidant Activities; Cytotoxic; Kaempferol

1. Introduction

In the past few years phytomedicines have witnessed a great deal of scientific attention mainly concentrated on their role in preventing diseases by oxidative stress including cancer [1] aging [2], atherosclerosis [3], cardiac arrest [4], immune disruption [5] and brain dysfunction [6]. *Zingiber zerumbet* (L.) Smith, an important member of the family Zingiberaceae has been traditionally used as a folk medicine across the globe especially in the southern part of Asia including India [7]. *Z. zerumbet* is known to have certain medicinal properties like anti-inflammatory [8], anti tumor [9], anti-allergic [10], anti pyretic [11], anti platelet aggregation activities [12]. This plant is reported to contain sesquiterpenoids, flavonoids, aromatic compounds, vanillin, kaempferol derivatives and other polyphenolic compounds [13-15]. Polyphenolic compounds are reported to have multiple biological effects including antioxidant activity [16]. These are aromatic benzene rings with substituted hydroxyl groups and are secondary metabolites, ubiquitous to the plant kingdom [17]. Antioxidant capacity of *Z. zerumbet* demands more study.

Plants that are rich in phenolic contents are often reported to possess cytotoxicity [18, 19]. Nevertheless, information on cytotoxic effect of *Z. zerumbet* rhizome is scarce.

In the present work, antioxidant activities of ethanolic extract *Z. zerumbet* rhizome (ZZ) have been studied. ZZ has been evaluated for its cytotoxicity in human peripheral blood lymphocyte cells. The study focuses on characterization and quantification of one of the major active constituent kaempferol using high performance liquid chromatography (HPLC). In addition, total polyphenol content and flavonoid content of ZZ was determined.

2. Materials and Methods**2.1 Test Chemicals**

Kaempferol standard (CAS 520-18-3), 2,2-di(4-tert-octylphenyl)-1-picryl-hydrazyl free radical (DPPH, CAS 84077-81-6), phosphoric acid (H₃PO₄) were purchased from Sigma-Aldrich Fine Chemicals, St. Louis, USA. Ethanol and Aluminium chloride anhydrous (CAS 7446-70-0) were obtained from Merck Specialities (pvt.) Ltd., India.

Acetonitrile (HPLC grade) was obtained from Qualigen Fine Chemicals, USA. Other reagents like 2 deoxy-D-ribose sugar (CAS 533-67-5), di-sodium salt of ethylene diamine tetra acetic acid (EDTA) disodium salt, thiobarbituric acid (TBAR), trichloroacetic acid (TCA), tris buffer, phosphate-buffered saline (PBS) (Ca²⁺, Mg²⁺ free), RPMI-1640 media, Histopaque, quercetin were purchased from Hi Media, India. Gallic acid, ascorbic acid, Folin–Ciocalteu reagent were purchased from SRL, India. All other chemicals like di-potassium-hydrogen phosphate (K₂HPO₄), hydrogen peroxide (H₂O₂), potassium di-hydrogen phosphate (KH₂PO₄), Ferric chloride (FeCl₃), sodium hydroxide (NaOH), sodium carbonate (Na₂CO₃) were purchased locally and were of analytical grade.

2.2 Preparation of ZZ

Plant material of *Z. zerumbet* were collected locally, authenticated by plant taxonomist and voucher specimen was submitted to the herbarium of the department of Botany, University of Calcutta, Kolkata. A total of 100 gm of the dried rhizome was soaked in 1L of ethanol at room temperature (35.5 °C) and kept in dark for 7 days. The extract was filtered and evaporated to dryness. Finally 3.45 gm of semi-solid mass of ZZ was recovered and stored at -20 °C.

2.3 Determination of Total Polyphenol Content (TPC)

The total polyphenol content of the extract was determined according to the method of McDonald *et al.* [20] and Roy *et al.* [21] with modification. Briefly, 0.5 ml of extract (50 µg/ml) was mixed with Folin–Ciocalteu reagent (5 ml, 1:10 dilution with distilled water) and further neutralized by aqueous Na₂CO₃ (4 ml, 1 M) solution. The reaction mixture was then allowed to stand for 15 min at room temperature. The absorbance of the reaction mixture was measured at 765 nm using a UV–visible spectrophotometer [Beckman Coulter, USA]. The calibration curve was prepared using solutions of gallic acid (standard) in ethanol with final concentrations in reaction mix ranging from 0–35 µg/ml. The total polyphenol content was expressed in terms of milligram of gallic acid equivalent per gram of extract (mg GAE/g). Three replicates were performed. Results are represented as mean ± standard deviation.

2.4 Determination of Total Flavonoids

The method of Ordonez *et al.* [22] and Taie *et al.* [23] was followed for the estimation of total flavonoid content with minor modifications. Briefly, 0.5 ml of 2% AlCl₃ in ethanol solution was added to 0.5 ml of the extract (50 µg/ml). The reaction mixture was then allowed to stand for 1h at room temperature. The absorbance was measured at 420 nm using a UV–visible spectrophotometer [Beckman Coulter, USA] against the sample blank. Total flavonoid content was calculated as quercetin equivalent (mg/g) obtained from calibration curve (0-33 µg/ml). The total flavonoid content was expressed in terms of milligram of quercetin equivalent per gram of extract (mg QUE/g). Three replicates were performed. Results are represented as mean ± standard deviation.

2.5 HPLC Analysis of Phenolic Compounds

HPLC analysis was carried out using a High Performance Liquid Chromatography apparatus equipped with a Agilent DAAD detector (Agilent, USA) and an Agilent Eclipse plus C18 column (100 mm×4.6 mm, 3.5 µm) (Agilent, USA). The mobile phases

were (A) 0.1% phosphoric acid and (B) acetonitrile. The gradient was linear from 94 to 92.5% A for 4 min; stand on 92.5% for 3 min, from 92.5 to 10% A for 8 min, from 10 to 6% A for 5 min and 6% A for 5 min followed by washing with B and reequilibration of the column for 5 min. The flow rate was 0.5 ml/min and injection volume was 20 µl. UV–vis absorption spectra were recorded on-line from 190 to 600 nm during the HPLC analysis. Standard kaempferol (Sigma-Aldrich, Co. USA), were used to calibrate the standard curve. The DAAD detection was conducted at 254 nm for the quantification. The compound was identified and quantified by comparing both retention times and UV–vis spectra with those of pure standard. The results were expressed as microgram of kaempferol per one gram of ZZ.

2.6 Detection of Hydroxyl Radicals by Deoxyribose Assay

The assay was performed as described by Halliwell *et al.* [24] and Stoilova *et al.* [25]. All solutions were freshly prepared. One ml of the reaction mixture contained 100 µl of 28 mM 2-deoxy-ribose (dissolved in KH₂PO₄–K₂HPO₄ buffer, pH 7.4), 500 µl solution of various concentrations of the extract (at final concentration of 15, 30, 60 and 120 µg/ml in the reaction mix), 200 µl of 200 M FeCl₃ and 1.04 mM EDTA (1:1 v/v), 100 µl H₂O₂ (1.0 mM) and 100 µl ascorbic acid (1.0 mM). After an incubation period of 1 h at 37°C the extent of deoxyribose degradation was measured by the TBA reaction. 1.0 ml of TBA (1% in 50 mM NaOH) and 1.0 ml of 2.8% TCA were added to the reaction mixture and the tubes were heated at 100 °C for 20 min. After cooling, the absorbance was read at 532 nm against a blank (containing only buffer and deoxy-ribose). The percentage inhibition (I %) was calculated by the formula: $I\% = 100 - (\text{Abs.}_{\text{sample}} / \text{Abs.}_{\text{control}}) \times 100$

The IC₅₀ value represents the concentration of the compounds that caused 50% inhibition of radical formation. Quercetin was used as a positive control. The data obtained at each point were the average of three measurements.

2.7 Scavenging effect on 2, 2-di (4-tert-octylphenyl)-1-picryl-hydrazyl free radical

The DPPH radical scavenging capacity was determined using the method described by Liu *et al.* [26] and Lu *et al.* [27]. 10 µl ethanolic extracts (at final concentration of 15, 30, 60 and 120 µg/ml in the reaction mix) were mixed with 3 ml of 6×10⁻⁵ M DPPH in ethanol. After 30 min of incubation in the dark at room temperature, the absorbance at 517 nm was measured against blank. The inhibition percentage of DPPH radical was calculated according to the formula [28]:

$$\text{DPPH radical scavenging capacity \%} = [(A_{\text{DPPH}} - A_{\text{EXTR}}) / A_{\text{DPPH}}] \times 100$$

Where, A_{DPPH} is the absorbance of the control solution (containing only DPPH), and A_{EXTR} is the absorbance in the presence of antioxidant. The IC₅₀ value represents the concentration of the compound that caused 50% inhibition of radical formation. Ascorbic acid was used as a positive control. The data obtained at each point were the average of three measurements.

2.8 Isolation of Human Peripheral Blood Lymphocytes

Human peripheral blood was obtained by venipuncture from healthy 23-year-old male donors (non-smokers and not under any medication) into vacutainers (heparin). Lymphocytes were isolated from fresh blood according to the method of Bøyum [29]. Fresh blood (1 ml) was diluted with equal volume of PBS and was

layered over 3 ml of Histopaque and centrifuged at $800\times g$ for 40 min. The buffy coat was aspirated into 3–5 ml of PBS and was centrifuged at $250\times g$ for another 10 min. The supernatant was discarded and the pellet was suspended in RPMI-1640 media. All experiments were conducted in accordance with the Institutional guidelines and approved by ethical committee, University of Calcutta.

2.9 Cytotoxicity Assay

Cytotoxic effects of ZZ at various concentrations (15, 30, 60, 120, 300, 600 $\mu\text{g/ml}$) were studied by the trypan blue dye exclusion test [30]. Lymphocytes (2×10^5 cells/ml) were incubated at 37°C with different concentrations of ZZ in RPMI-1640 media. After 3 h the media was washed by centrifugation, and fresh media was added. EC_{50} value was determined that represents the effective concentration of the extract that causes 50% death of the cells.

2.10 Statistical Analysis

All experiments were done in triplicate, and data were expressed as Mean \pm SD of 3 independent experiments. The data were analysed using the Statistical Programme–SigmaStat3.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) test, at $P \leq 0.05$, was done. 50% inhibitory concentrations ($\text{IC}_{50}/\text{EC}_{50}$)

were calculated by plotting the data in the graph as concentration versus percentage inhibition/scavenging.

3. Results

3.1 Total Polyphenol and Flavonoid Content

The total polyphenol content of ZZ was 33.64 ± 3.22 mg GAE/g, as determined by Folin-Ciocalteu method, determined with reference to standard curve and is reported as gallic acid equivalents with reference to standard curve ($y=0.088x+0.132$, $R^2=0.99$). Total flavonoid content of ZZ was 26.79 ± 0.68 mg quercetin equivalent/g of the extract (QUE/g) determined with reference to standard curve ($y=0.037x+0.0017$, $R^2=0.99$) (Table 1).

3.2 HPLC analysis of Phenolic Compounds

HPLC analysis at 254 nm along with gradient elution program revealed number of peaks in the chromatogram of the extract (Figure 1). A comparison between retention time, spectra of the sample peak and the reference standard confirmed kaempferol as one of the major components in ZZ. Using the standard curve of the reference standard ($y=92.68x-310.80$; $R^2=0.993$; 25–100 $\mu\text{g/ml}$) the amount of kaempferol was 11.63 ± 0.09 mg/g of extract (Figure 1, Table 1).

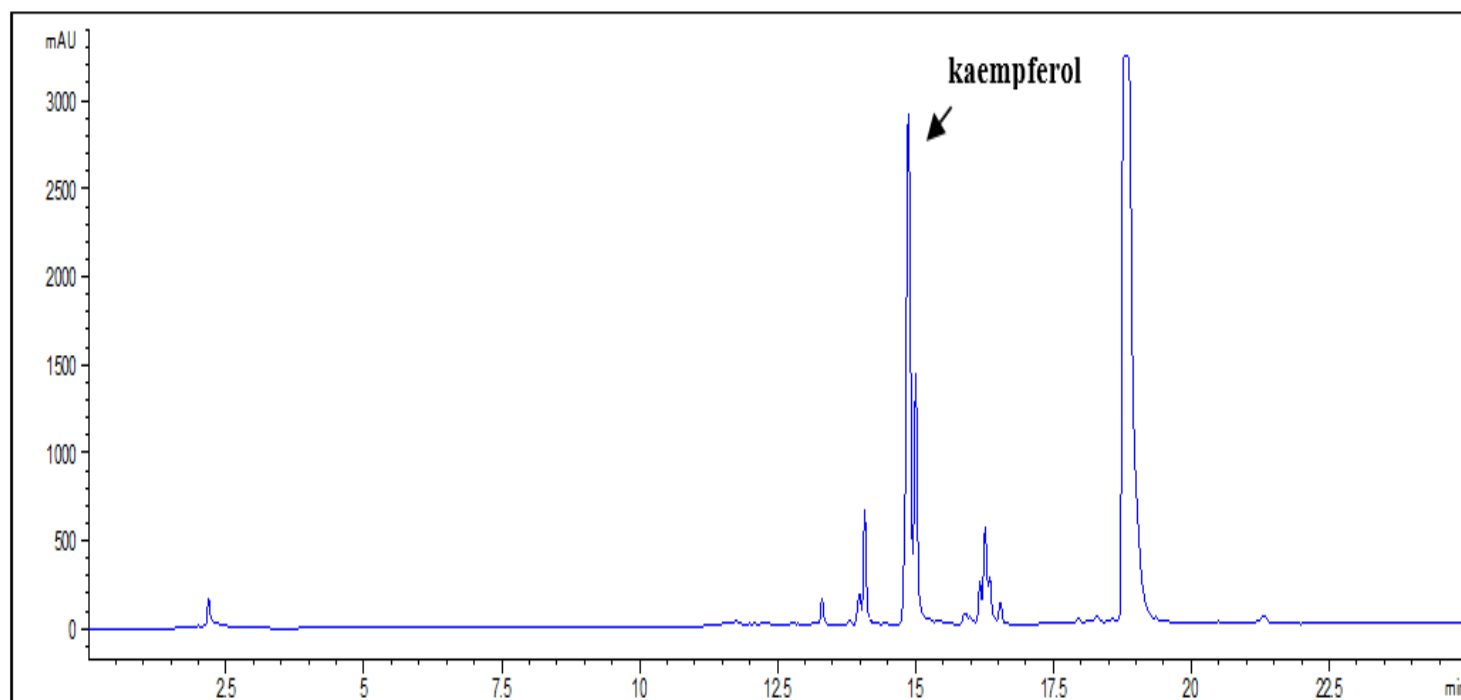


Fig 1: High performance liquid chromatography analysis of *Zingiber zerumbet* rhizome extract at 254 nm

3.3 Detection of Hydroxyl Radicals by Deoxyribose Assay

Hydroxyl radicals are formed in free solution after incubation of Fe-EDTA with H_2O_2 and ascorbic acid at pH 7.4. These hydroxyl radicals were detected by their ability to degrade 2-deoxy-D-ribose sugar in fragments that on heating with TBA at acidic pH forms pink coloured complex [25]. In comparison with the control ZZ significantly inhibited ($p\leq 0.05$) the degradation of the sugar by the

hydroxyl radicals (69.86–83.01%) at different concentrations of ZZ (15, 30, 60 and 120 $\mu\text{g/ml}$; Figure 2, Table 1). The IC_{50} value for inhibition of degradation of sugar was 13.24 $\mu\text{g/ml}$. Quercetin was used as a positive control. The IC_{50} value of quercetin was 2.46 $\mu\text{g/ml}$.

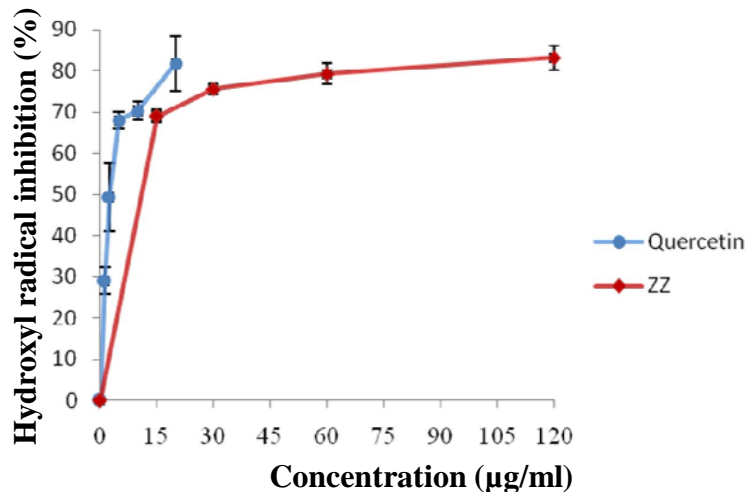


Fig 2: Inhibition of the radical degradation of 2-deoxy-D-ribose sugar

3.4 Scavenging effect on 2, 2-di(4-tert-octylphenyl)-1-picryl-hydrazyl free radical

Acceptance of an electron from an antioxidant molecule resulted in bleaching of purple colour of DPPH free radicals. The ability of scavenging DPPH by antioxidant molecules is assessed spectrophotometrically by the quantitative estimation of the

discoloration of the same. ZZ at various concentrations (0, 30, 60 and 120 µg/ml) showed a weak effect in inhibiting DPPH. The value was significant ($p \leq 0.05$) at the concentrations tested. IC_{50} was 417.14 µg/ml for ZZ and 2.71 µg/ml for ascorbic acid (Figure 3, Table 1).

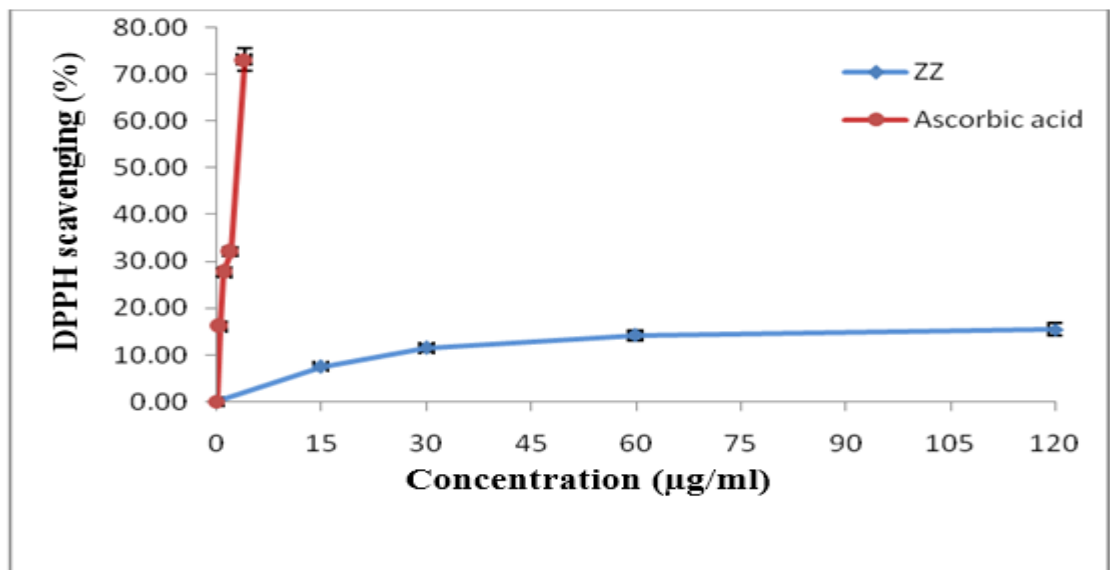


Fig 3: DPPH free radical-scavenging activity

Table 1: Extraction yield, phenolic content and antioxidant activities of ZZ

Extraction yield (%)	Total polyphenol (mg GAE/g of ZZ) [mean±SD]*	Total flavonoid (mg QUE/g of ZZ) [mean±SD]*	Kaempferol (mg/g of ZZ) [mean±SD]*	IC_{50} (µg/ml) Hydroxyl scavenging activity	IC_{50} (µg/ml) DPPH scavenging activity
3.45	33.64±3.22	26.79±0.68	11.63 ±0.09	13.24	417.14

*mean of three replicates; SD- standard deviation

3.5 Cytotoxicity assay

The cytotoxicity of ZZ is shown in Figure 4. The % viable cells ranged between 12.33 - 99.33%. The numbers of viable cells

decreased significantly at concentrations 300 $\mu\text{g/ml}$ and above. The EC_{50} value of ZZ was 385.07 $\mu\text{g/ml}$.

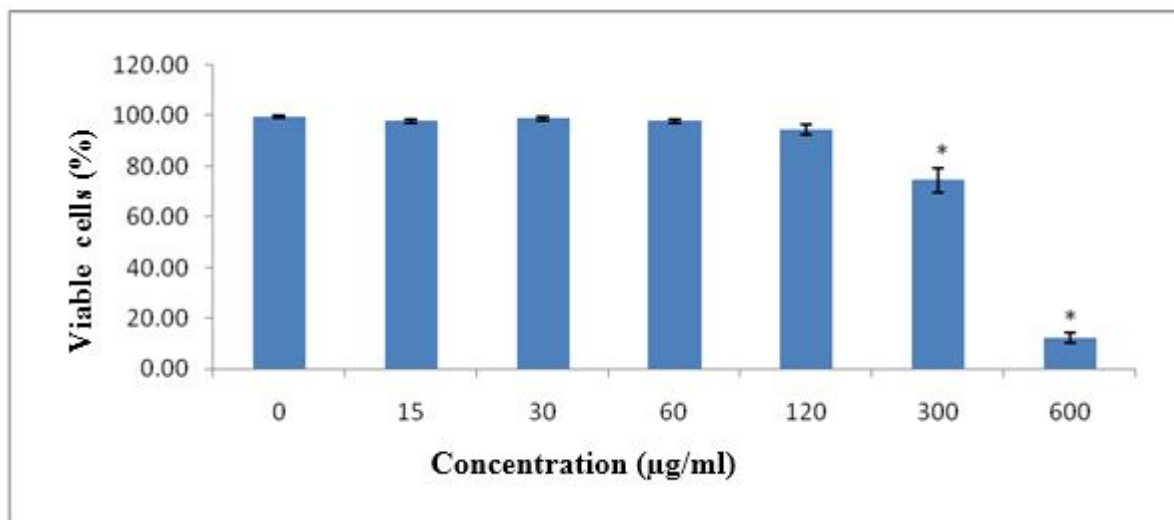


Fig 4: Viable lymphocyte cells at different concentration of ZZ by trypan blue assay
*Significantly different from control ($p \leq 0.05$)

4. Discussion

The rich world of herbs with thousands of species is being extensively explored traditionally as herbal medicines since ages. The rhizome of *Z. zerumbet* finds use in traditional medicines. In the present study, the ZZ was studied for its antioxidant and cytotoxic properties.

Antioxidants are micro constituents present in the diet that can scavenge reactive oxygen species (ROS) by terminating the oxidizing chain reaction [31, 32]. These ROS is considered to play a very vital role in the pathogenesis of several degenerative diseases from cardiovascular diseases to the carcinogenesis [33, 34]. The model of DPPH scavenging antioxidant assay have gained wide acceptance for its capacity to rapidly screen samples of interest [35]. Several members of the family Zingiberaceae were reported to possess significant DPPH scavenging activities; however a few were also known to show low scavenging capacity [36]. Although not much data on antioxidant activity of *Z. zerumbet* is available, solvent extracts (ethanol, methanol and isopropanol) of *Z. zerumbet* spent have shown fair DPPH scavenging activity [37]. In contrast our study revealed comparatively lower DPPH scavenging activity of ZZ. Such discrepancy may be related to the difference in the extraction method, cultivars, maturity and other environmental factors that have been known to directly influence the antioxidant capacity of fruits and vegetables [38, 39].

The hydroxyl radical is an extremely reactive free radical found in biological systems and has been implicated as highly damaging species in free radical pathology capable of damaging every molecule found in living cells [35, 40]. Plants of Zingiberaceae have been reported to possess considerable hydroxyl radical scavenging activity [41]. The results of the present study showed that ZZ is a powerful OH scavenger in competition with 2-deoxy ribose sugar. The hydroxyl radical scavenging capacity of ZZ is much higher than its DPPH scavenging activity, as evident by respective IC_{50} values. Steric accessibility is the major determinant for DPPH assay, since small molecules have better access to the radical sites than the larger ones [42]. In addition, DPPH is stable, long-lived nitrogen radical unlike radicals present in living organisms and has

no similarity to the highly reactive and transient radicals [43-44]. Thus, antioxidants that react quickly with these radicals may react slowly or may be inert to the DPPH radical [44].

In our study HPLC analysis confirmed the presence of kaempferol along with the appreciable amount of polyphenol and flavonoid compounds. These phenolic compounds are known to impart its biological activities *in vitro* and *in vivo* including anti-inflammatory [45], antimicrobial [46], antimutagenic [47] and antitumor effects [48]. In agreement with the findings of Lako *et al.* [39], presence of high amount of kaempferol in ZZ was confirmed by HPLC analysis. Kaempferol is reported to possess strong hydroxyl radical scavenging activity [49] which might have contributed to similar activity of ZZ in our study.

Although antioxidants of plant origin are rapidly gaining popularity, their safety is of great concern. A few data are available on the cytotoxic effects of the plants, belonging to the family Zingiberaceae [50]. Crude ethanol extracts of the rhizome of *Z. zerumbet* showed cytotoxicity against brine shrimp [51]. Tushar *et al.* [52] reported cytotoxicity of *Z. zerumbet* in Raji cells at concentrations 320 $\mu\text{g/ml}$ and above. Our study on lymphocyte cells are in agreement to that of Tushar *et al.* [52].

The demonstrated DPPH and hydroxyl radical scavenging activities of ZZ substantiated its potential as a strong natural antioxidant. Several authors reported direct correlation between phenolic compounds and antioxidant activities of different plant extracts [27, 53-54]. The abundance of phenolic compounds as estimated in ZZ including kaempferol as a major flavonoid, might have contributed to its antioxidant properties. ZZ with cytotoxicity at only higher doses can be used as a therapeutic antioxidant in near future.

5. Conclusion

Z. zerumbet possesses antioxidant properties and the extract is non cytotoxic at low concentrations. Further toxicological studies are warranted for its use as phytomedicine.

The authors wish to state that there is no conflict of interest in carrying this work.

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