

Phytochemical Analysis and *in vitro* Antioxidant Activity of *Zingiber officinale*

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ABSTRACT

Introduction: Antioxidant activity of the methanol extract of *Zingiber officinale* was determined by Reducing power assay, Superoxide anion scavenging activity assay, Hydroxyl radical scavenging activity assay, Nitric oxide scavenging activity assay, DPPH free radical scavenging assay, and hydrogen peroxide method. **Methods:** Preliminary phytochemical screening revealed that the extract of *Z. officinale* possesses flavonoids, volatile oil and phenolic materials. In the present investigation, quantitative estimation of flavonoids and phenols was carried out by colorimetric methods, using aluminum chloride method and Folin Ciocalteu reagent respectively. **Result:** The extract showed significant activities in all antioxidant assays compared to the standard antioxidant in a dose dependent manner and remarkable activities to scavenge reactive oxygen species (ROS) may be attributed to the high amount of hydrophilic phenolics. The IC₅₀ values of all parameters were determined while ascorbic acid was used as standard. **Conclusion:** The results obtained in the present study indicate that *Z. officinale* extract is a potential source of natural antioxidant.

Keywords: Antioxidant, Ascorbic acid, DPPH, *Zingiber officinale*

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INTRODUCTION

Interest in the search for new natural antioxidants has grown over the past years because reactive oxygen species (ROS) production and oxidative stress have been shown to be linked to diseases such as cancer, cardiovascular disease, osteoporosis, and degenerative diseases. Natural antioxidant substances are believed to play a potential role in interfering with the oxidation process by reacting with free radicals, chelating catalytic metals and scavenging oxygen in biological systems^[1]. Natural antioxidants are widely used because they are regarded as safer and causing fewer adverse reactions but the synthetic antioxidants which have been restricted by legislative rules because they are suspected to have some toxic effects and as possible carcinogens^[2-4]. Plants constitute an important source of active natural products which differ widely in terms of structure and biological properties. They have played a remarkable role in the traditional medicine of various countries. In recent years, the prevention of cancer and cardiovascular

diseases has been associated with the ingestion of fresh fruits, vegetables or teas rich in natural antioxidants^[5]. The protective effects of plant products are due to the presence of carotenoids^[7], flavonoids^[8], anthocyanins and other phenolic compounds^[9]. Phenolic compounds are commonly found in both edible and non-edible plants. They are important in the plant for normal growth development and defense against infection and injury. The presence of phenolic compounds in injured plants may have an important effect on the oxidative stability and microbial safety. Although phenolic compounds do not have any known nutritional function, they may be important to human health because of their antioxidant potency^[10-12].

Z. officinale is known as zanjabeel in unani system of medicine and is used not only as a dietary ingredient but also for its medicinal value. *Z. officinale* is known for its effect on the gastrointestinal system, and in traditional system of medicine. When used as a culinary herb, *Z. officinale* can be useful as a carminative, diuretic and antiemetic^[13]. The dried rhizomes have been used as a

primary ingredient for stomachics which are used to treat nausea, indigestion and flatulence^[14]. In cases of abdominal pain, the ground rhizome is steeped in hot water and drunk^[15]. Several pieces of the rhizome of *Z. officinale* have been used in a decoction along with the aerial parts of *Ocimum americanum* and *Xylopia aethiopica* in order to treat colic, constipation or irregularly^[16]. Often *Z. officinale* rhizomes has been chewed raw and the juices swallowed to alleviate general abdominal pain^[17].

Z. officinale has also been used as a popular respiratory aide. Decoctions of *Z. officinale* rhizome have been mixed with milk in order to suppress coughing^[18]. In cases of persistent cough or bronchitis, the rhizome has been chewed raw^[16].

Free radical scavenging activity of *Z. officinale* by DPPH method has been reported^[19].

In the present investigation phytochemical screening, quantitative estimation of total phenolic and total flavonoid has been carried out followed by antioxidant activity correlation between antioxidant activity and total phenolic and total flavonoid content were also investigated in order to establish if there is a relationship between these groups of phytochemical and antioxidant activity. Antioxidant activity *Z. officinale* was evaluated by Reducing power method, Superoxide anion scavenging method, Hydroxyl radical scavenging method, DPPH radical scavenging method, Nitric oxide scavenging method, Hydrogen Peroxide scavenging method for the first time.

MATERIALS AND METHODS

Chemicals and reagents

Folin Ciocalteu reagent, 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH), Nitro blue tetrazolium (NBT), phenazine methosulfate, nicotinamide adenine dinucleotide, ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyano ferrate [$K_3Fe(CN)_6$], and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. Catechin and rutin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade available commercially.

Preparation of extract

The rhizomes of *Z. officinale* were coarsely powdered and extracted with mixture of methanol by a soxhlet apparatus at 60°C. The solvent was completely removed by rotary evaporator (Rotavapor® R-210, BUCHI

Corporation) and obtained greenish gummy exudates. This crude extract was used for phytochemical analysis and evaluation of antioxidant activity.

Phytochemical screening

The freshly prepared extract of *Z. officinale* was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using standard procedures^[20, 21].

Determination of total phenolic content

Total phenols were determined by Folin Ciocalteu reagent^[22]. A dilute extract of plant extract (0.5 ml of 10mg/ml) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm (Schimadzu UV-Vis 1601). The standard curve was prepared using 25, 50, 100, 150, 200, 250, 300 µg/ml solutions of gallic acid in methanol.

Determination of total flavonoid content

Aluminum chloride colorimetric method was used for flavonoids determination^[22]. Plant extract (0.5 ml of 10mg/ml) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm (Schimadzu UV-Vis 1601). The calibration curve was prepared by preparing rutin solutions at concentrations 10 to 100 µg/ml in methanol.

In vitro Antioxidant Activity

Reducing power assay

The reducing power of *Z. officinale* was determined according to the method described^[23]. Different concentrations of *Z. officinale* extract (10 µg/ml – 50 µg/ml) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%) and the absorbance was measured at 700 nm (Schimadzu UV-Vis 1601). Increased absorbance of the reaction mixture

indicated increased reducing power. Ascorbic acid was used as a reference standard. Phosphate buffer (pH 6.6) was used as blank solution.

Superoxide anion scavenging activity assay

The scavenging activity of the *Z. officinale* towards superoxide anion radicals was measured^[24]. Superoxide anions were generated in a non-enzymatic phenazine methosulfate nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of phosphate buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 μ M) solution, 0.75 ml of NADH (936 μ M) solution and 0.3 ml of different concentrations (10 μ g/ml – 50 μ g/ml) of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 μ M) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm (Schimadzu UV-Vis 1601) was measured in spectrophotometer. The super oxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance of the extract or standard.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction^[25]. Reaction mixture contained 60 μ l of 1.0 mM FeCl_2 , 90 μ l of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 μ l of 0.17 M H_2O_2 , and 1.5 ml of extract at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm (Schimadzu UV-Vis 1601) was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated.

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance of the extract or standard.

Nitric oxide scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method^[26]. Sodium nitroprusside in

aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of *Z. officinale* extract at various concentrations (10 μ g/ml – 50 μ g/ml) and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm. The nitric oxide radicals scavenging activity was calculated.

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance of the extract or standard.

Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH)

The free radical scavenging capacity of the extracts was determined using DPPH^[27]. DPPH solution (0.004% w/v) was prepared in 95% methanol. Methanolic extract of *Zingiber officinale*, was mixed with 95% methanol to prepare the stock solution (1 mg/ml). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes then *Z. officinale* extract was added followed by serial dilutions (10 μ g/ml to 50 μ g/ml) to every test tube so that the final volume was 3 ml and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (Schimadzu UV-Vis 1601). Ascorbic acid was used as a reference standard and dissolved in double distilled water to make the stock solution with the same concentration (1 mg/ml) followed by serial dilutions (10 μ g/ml to 50 μ g/ml). Control sample was prepared containing the same volume without any extract and reference ascorbic acid.

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance of the extract or standard.

Scavenging of Hydrogen Peroxide

The ability of *Z. officinale* to scavenge hydrogen peroxide was determined according to the method^[28]. A solution

of hydrogen peroxide (2 mM) was prepared in phosphate buffer (pH 7.4) and concentration was determined spectrophotometrically at 230 nm (Schimadzu UV-Vis 1601). *Z. officinale* extract (5 µg/ml – 25 µg/ml) in distilled water was added to a hydrogen peroxide solution (0.6 ml, 2 mM) and the absorbance of hydrogen peroxide at 230 nm was determined after 20 min against a blank solution in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of *Z. officinale* and standard compounds was calculated by using the above equation.

RESULTS AND DISCUSSION

Phytochemical screening

Preliminary phytochemical screening of the extract of *Z. officinale* revealed the presence of various bioactive components of which flavonoids, phenolics, sterols, alkaloid, proteins and amino acids were the most prominent.

Total phenolic content

Phenolics are the most wide spread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers^[29]. Therefore, in the present study, total phenolic content present in extract was estimated using modified Folin-cioalteau method. In *Z. officinale* extract, the phenolic content was found to be $1.45 \pm 0.01\%$ w/w.

Total flavonoid content

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process^[30, 31]. Therefore, in the present study, total flavonoid content present in extract was estimated using Aluminum chloride colorimetric method. In *Z. officinale* extract, the flavonoid content was found to be $0.84 \pm 0.03\%$ w/w.

In vitro Antioxidant Activity

Reducing power assay

The reducing power of *Z. officinale* extract was very potent and the power of the extract was increased with quality

of sample. The plant extract could reduce the most Fe³⁺ ions, which had a lesser reductive activity than the standard of ascorbic acid. Increased absorbance of the reaction indicated increased reducing power. The reductive capability of the plant extract compared to ascorbic acid is shown in fig. 1.

Superoxide anion scavenging activity assay

It is well known that superoxide anions damage biomolecules directly or indirectly by forming H₂O₂, OH, peroxy nitrite or singlet oxygen during aging and pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation^[32]. The superoxide anion radical scavenging activity of *Z. officinale* extract assayed by the PMS-NADH system is shown in fig. 2. The superoxide scavenging activity of *Z. officinale* extract was increased markedly with the increase in concentrations. Thus, higher inhibitory effects of the rhizomes extracts on superoxide anion formation noted herein possibly renders them as a promising antioxidants. The half inhibition concentration (IC₅₀) of *Z. officinale* extract was 45.94 µg/ml while IC₅₀ value for ascorbic acid was 16.15 µg/ml. These results

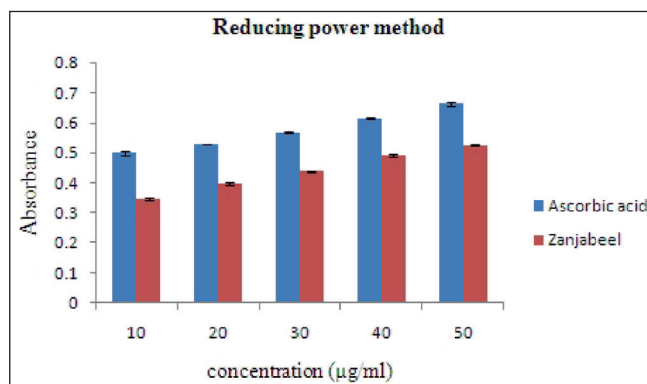


Figure 1. Reducing power assay.

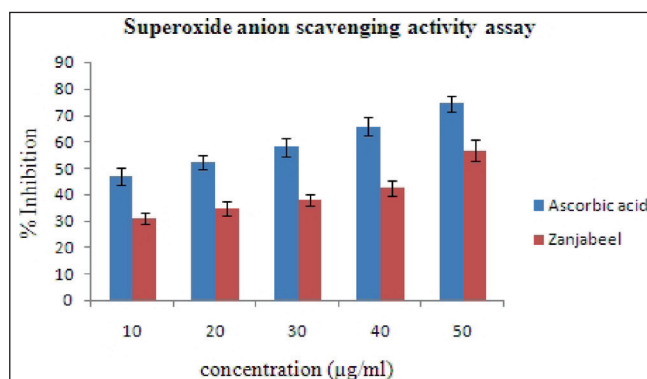


Figure 2. Superoxide anion scavenging activity assay.

suggested that *Z. officinale* extract has a potent superoxide radical scavenging effects.

Hydroxyl radical scavenging activity assay

Activity of the rhizomes extract on hydroxyl radical has been shown in fig. 3. Hydroxyl radical is highly reactive oxygen centered radical formed from the reaction of various hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecule. *Z. officinale* extract exhibited concentration dependent scavenging activity against hydroxyl radical generated in a Fenton reaction system. The IC₅₀ value was found to be 22.36 µg/ml while IC₅₀ value for ascorbic acid was 1.27 µg/ml.

Nitric oxide scavenging activity assay

Z. officinale extract showed moderately good nitric oxide scavenging activity between 10 and 50 µg/ml in fig.4. The percentages of inhibitions were increased with increasing concentration of the extracts. IC₅₀ value for scavenging of nitric oxide for *Z. officinale* extract was 44.65 µg/ml, while IC₅₀ value for ascorbic acid was 3.90 µg/ml. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological condition.

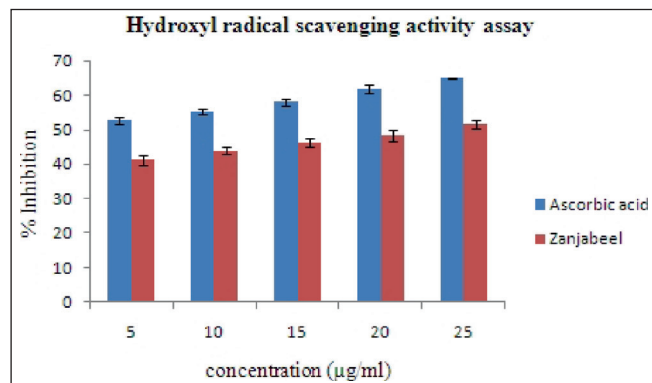


Figure 3. Hydroxyl radical scavenging activity assay.

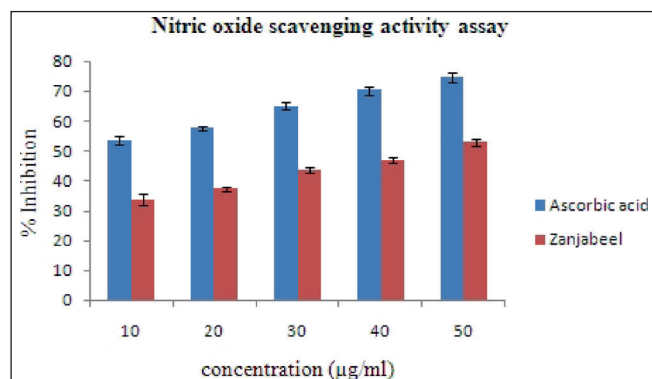


Figure 4. Nitric oxide scavenging activity assay.

Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical

Fig. 5 shows the DPPH radical scavenging activity of the *Z. officinale* extract, compared with ascorbic acid, as standard. IC₅₀ values of extract and standard were 24.97 µg/ml and 7.79 µg/ml, respectively. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability^[33]. Though the DPPH radical scavenging abilities of the extracts were less than those of ascorbic acid, the study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

Scavenging of Hydrogen Peroxide

As shown in fig. 6, *Z. officinale* extract also demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner with an IC₅₀ of 15.24 µg/ml, while IC₅₀ value for ascorbic acid was 2.60 µg/ml. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H₂O₂ can probably react with Fe²⁺, and possibly Cu²⁺ ions to

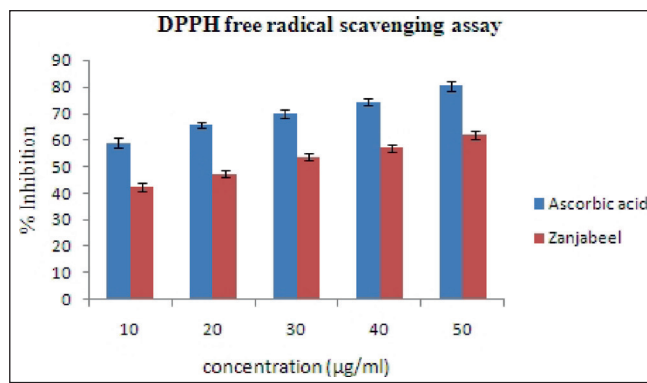


Figure 5. Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical.

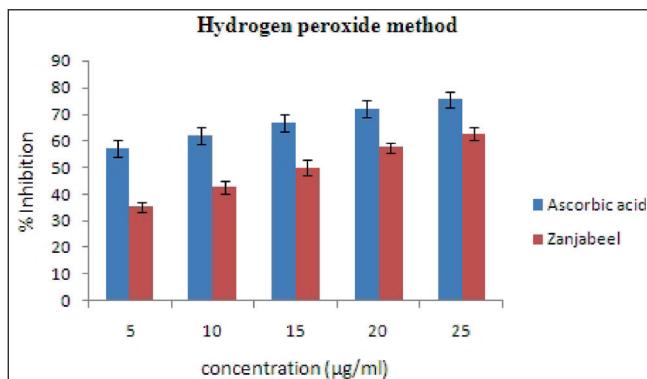


Figure 6. Scavenging of Hydrogen Peroxide.

form hydroxyl radical and this may be the origin of many of its toxic effects^[34]. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The decomposition of H₂O₂ by *Z. officinale* extract may at least partly result from its antioxidant and free radical scavenging activity.

Phytochemical screening of the *Z. officinale* revealed the presence of phenolic and flavonoid glycosides. Flavonoids have been shown to have antioxidant, antibacterial anti-inflammatory, antiallergic activity etc.^[35]. The presence of phenolic and flavonoids in the drug extract is likely to be responsible for the antioxidant activity. These compounds are reported to be antioxidant or free radical scavengers^[36]. The gingerols, shogols, which are a homologous series of phenols^[37]. The essential oil, which is a mixture of monoterpenic and sesquiterpenic compounds, might be contributed to its antioxidant potential of the drugs.

CONCLUSION

The results obtained in the present study indicate that *Z. officinale* extract exhibits free radical scavenging, reducing power. The overall antioxidant activity of *Z. officinale* extract might be due to its flavonoid, polyphenolic and other phytochemicals constituents^[37]. The findings of the present study suggested that *Z. officinale* could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases. Further research work is required to isolate phytoconstituents responsible for antioxidant activity.

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