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ANTIOXIDANT, CYTOTOXIC AND PHYTOCHEMICAL ACTIVITY OF SOLVENT EXTRACTS OF *ZINGIBER ZERUMBET*

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ABSTRACT

Zingiber zerumbet belongs to family Zingiberaceae. Rhizomes of the plant are used in medicine and ornamental purposes. In the present study leaf extracts of the plant were explored for their phytochemical, cytotoxic and antioxidant properties. Extracts of leaf of the species showed the presence of medicinally active metabolites like alkaloids, tannins and cardiac glycosides. Methanol extract showed antioxidant activity equivalent to the standard in DPPH radical scavenging assay. All the extracts exhibited mild cytotoxic activity against brine shrimp larvae.

KEYWORDS: *Zingiber zerumbet*, brine shrimp assay, TLC, Methanol extract, DPPH, antioxidant.

INTRODUCTION

Zingiber zerumbet belonging to family Zingiberaceae is a perennial, aromatic and tuberose plant known as bitter ginger.[1] *Z. zerumbet* is traditionally found throughout Asia, and is used in foods, beverages and for ornamental purposes.[2] The genus *Zingiber* contains approximately 85 species majority of which founds mention in Ayurvedic and Siddha system of medicines.[3] A number of bioactive compounds gingerol, shogaol and other gingerones with anti-inflammatory properties have been isolated from the rhizome of *Zingiber gingerale*. Rhizome of *Z. zerumbet* is used in folk medicines as maceration and infusion of fresh rhizome in tinctures and poultices.[4] The cone-shaped flowers of *Zingiber zerumbet* are long-lasting and are employed in craft arrangements for ornamental purposes. The rhizome is used as a tonic and as a stimulant. The rhizome serves as a seasoning in foods, while the floral buds are consumed as vegetables. The rhizome of

ginger has been extensively used with remarkable therapeutic effects for the treatment of inflammation, diarrhea, stomach cramps, bacterial infections, fever, flatulence, allergies and poisoning.[5] Powdered rhizome is used to treat ear infections, toothache and, in the form of tea, to treat stomach disease.[6] In the present study leaves of the plant has been explored for their medicinal potential.

MATERIALS AND METHODS

Plant collection and preparation of Solvent extract

Fresh leaves of *Zingiber zerumbet* were collected from the medicinal germplasm garden of Regional plant resource center (RPRC), Bhubaneswar. Leaves were weighed and washed with running tap water to remove dust and impurities. After drying, weight of leaves was again taken for the determination of moisture content. Moisture content of the leaves was calculated by using the following formula:

$$\text{Moisture content (\%)} = \frac{Fw - Dw}{Fw} \times 100$$

Where, Fw = Fresh weight of leaf sample

Dw = Dry weight of powdered leaf sample

Further, Leaves were dried in shade till complete drying followed by grinding in grinder(Lexus make) to make fine powder for the preparation of solvent extracts.

Solvent extraction

Solvent extraction was done by using Soxhlet extraction method. 14.91gm of leaf powder of *Zingiber zerumbet* was taken in a thimble made up of cellulose and was subjected for serial extraction with different solvent Hexane, Chloroform, Ethyl acetate and Methanol on the basis of their increasing polarity. 400ml of solvent was taken in a round bottom flask and refluxed continuously for 2-3 days so as to get the maximum number of molecules in a particular solvent. After extraction the extract was concentrated by using Buchhi(R-200) Rotavapour under vacuum at 45-50°C depending upon the boiling point of the solvent. Yield of the solvent extracts was also recorded. Concentrated extracts were transferred to screw cap vials and extract yield was calculated by using the formula.

$$\textbf{Percentage yield of extract} = \frac{\textbf{Extract weight} \times 100}{\textbf{Powdered weight}}$$

Phytochemical analysis

Phytochemical analysis was conducted using the standard protocols.[7] Brief account of tests conducted were as follows.

1. Test for Alkaloid: Alkaloids tests were done by using 3 different reagents.

- **Dragendroff' s test** –To 1ml of extract 2ml of 1%HCL was added and boiled for few minutes after boiling 2 -3 drops of Dragendroff ‘s reagent was added and sample was observed for reddish brown precipitate.
- **Wagner's test-**To 100µl of extract 2ml of diluted HCl was added followed by 1ml of Wagner's Reagents drop wise. Formation of reddishbrown precipitate indicates the presence of alkaloids.
- **Mayer's test** - To 100µl of methanolic extract 2ml of diluted HCl and 1ml of Mayer's Reagent was added. Formation of yellow cream colour represents the presence of alkaloids.

2. Test for flavonoids: To1 ml of methanolic extract 1ml of 10% NaOH was added from the side of the tube, drops of conc. HCL was added. Yellow colour turns to colourless which indicates presence of flavonoids.

3. Test for Anthraquinone: To 1ml of extract 2ml of 5% KOH was added and was observed for pink colouration.

4. Test for Saponin: To 1ml of extract 2ml of NaHCO3 was added which on shaking forms lather if saponin is present.

5. Test for Terpenoids: To 1ml of extract,400 microlitre of chloroform and 4-5 drops of conc.H_2SO_4 was added from the wall of the test tube. Reddish brown precipitate indicates the presence of terpenoids.

6. Test for cardiac glycosides: To 5ml of extract 2ml of glacial acetic acid, few drop of $FeCl_3$ and conc.H_2SO_4 was added from the wall of the test tube. Presence of cardiac glycosides is determined by Reddish brown ring.

7. Test for Tannin: It can be observed by 2 methods.

- 1ml of extract was boiled and few drops of $FeCl_3$ were added to it. The sample was observed for blue, black and green colour.

- To 1ml of extract 500 microliter of lead acetate was added which gives yellow colour.

8. Test for Phlobotanin: To 1ml of extract 1% HCl was added and boiled, formation of precipitation occurred on positive test.

Determination of total phenolic content

The total phenolic content (TPC) of the leaf extract was determined using the method of Yaldiz *et al.*[8] First standard was calculated by using Gallic acid and then leaf extract of different solvents i.e. hexane, chloroform, ethyl acetate, methanol were used.

Concentrations of 1mm, 0.5mm, 0.25mm, 0.125mm, 0.062mm, 0.031mm of gallic acid and plant extracts were prepared in methanol. To 0.5 ml of test sample (gallic acid or leaf extract), 1.5ml (1: 10 v/v diluted with distilled water) FolinCiocalteau´s reagent was added and allowed to stand for 5 min at 22˚C. After 5 min, 2.0ml of 7.5% of sodium carbonate was added. These mixtures were incubated for 90 min in the dark after slight shaking. After incubation development of blue colour was observed. Absorbance of different samples was measured at 725nm using spectrophotometer. The phenolic content was calculated as gallic acid equivalents GAE / g on the basis of standard curve of gallic acid. All the experiments were carried out three times.

Antioxidant activity

Qualitative analysis (TLC based antioxidant assay)

TLC is one of the most widely used and potent techniques to resolve mixture of plant compounds. It is also called DPPH (2, 2- diphenyl – 1 – picrylhydrazyl) assay. The TLC sheets supplied by merck, Germany (TLC silica gel 60 F 254) was used as stationary phase. The developed TLC was sprayed with 0.2% DPPH in methanol as indicator as per the standard protocol.[9] The presence of antioxidant compounds detected by yellow spots against purple background on the TLC sheet.

$$\underset{\text{(violet colour)}}{\mathbf{DPPH}} + \mathbf{AH} \rightarrow \underset{\text{(yellow colour)}}{\mathbf{DPPH - H}} + \mathbf{A^-}$$

Three types of solvents were prepared for TLC chromatography techniques.

BEA -Benzene: Ethanol: Ammonium hydroxide (90: 10: 1)[Non polar / Basic]

EMW -Ethyl acetate: Methanol: water (40: 5.4: 4) [Polar / neutral]

CEF - Chloroform: Ethyl acetate: Formic acid (5: 4: 1) [Acidic]

Qualitative screenings of the constituents in each of the plant extracts of *Zingiber zerumbet* for antioxidant activity was done by TLC based antioxidant assay. The silica coated TLC sheet was activated at 100degree for 2 minutes. The sample were loaded on the TLC sheet with the help of micro tips by leaving 1cm from the bottom of the sheet. Chromatogram was run upto 8cms in the three different solvents as mentioned above. After drying the DPPH solution was sprayed on the TLC sheet. The yellow bands against the purple background representing the antioxidant bands were counted and Rf values of all the antioxidant bands were calculated using the following formulae.

Retardation factor = Distance travelled by the extract

Total distance travelled by the solvent

Quantitative anti-oxidant Analysis

Quantitative analysis was done by two popular methods as follows.

DPPH free radical scavenging assay

For DPPH free radical scavenging assay 1mM DPPH (2, 2-diphenyl-1-picrylhydrazyl) solution was prepared by adding 4mg of DPPH dissolved in 10ml methanol. DPPH assay was done by serial dilution method starting from concentration of plant extracts (7.8µg/ml, 15.62µg/ml, 31.25µg/ml, 62.5µg/ml, 125µg/ml, 250µg/ml, 500µg/ml, 1000µg/ml) was prepared in methanol. 1ml of each sample was taken in the test tubes and 500µl of DPPH solution was added. For control, each test tube contained 1ml methanol and 500µl DPPH**.** Samples were incubated for 30 minutes at room temperature in dark. All the samples were taken in triplicate and complete set of experiment was repeated three times. Optical density (OD) was measured at 517nm in spectrophotometer. The percentage of free radical scavenging activity was calculated from the following formula.

Percentage free radical scavenging [DPPH] = [(Ac – As) ÷ Ac] × 100

Where, Ac = Absorbance of control and As = Absorbance of sample.

FRAP ASSAY (Estimation of total antioxidant activity)

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay as per the standard protocol.[10] Spectroscopic method is based upon the ability of antioxidants to reduce Fe+3 to Fe+2 in the presence of TPTZ, forming an intense blue Fe+2-TPTZ complex with an absorption maximum at 593 nm. The decrease in absorbance is proportional to the antioxidant present. The FRAP reagent (300 mM Acetate buffer pH 3.6: 40 mM Dilute HCl: 10 mM TPTZ: 20 mM FeCl3.6H2O in the ratio of 10: 1: 1) was prepared and then

incubated at 37^{o}C in a water bath for 10 minutes. Absorbance of FRAP reagent was taken at 0th minute (t0) which was the control of the experiment. Ascorbic acid was taken as standard. A total of 100 µL of sample/standard and 300µl of distilled H2O was then added to the FRAP reagent and incubated at 37°C for 4 minutes. A reagent blank was prepared as described above but 100µl of distilled H2O was added instead of test sample. Duplicate test tubes were taken and absorbance was measured at 593nm. Ascorbic acid was taken as standard and 1.0mM to 0.1mM concentration of standard was prepared for the FRAP assay and based on the observations a standard curve was plotted. A number of dilutions of each sample extract were tested allowing dose response curves to be produced.

Cytotoxic activity

Brine shrimp (Artemia salina) mortality assay

Cytotoxic activity study was carried out by brine shrimp lethality assay using standard protocol.[11] Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. Brine shrimp (*Artemia salina*) eggs were hatched in artificial sea water, which was prepared using black salt 2 gm/ 200 ml distilled water. The eggs were incubated for 24 hours at temperature of about 28° C to get the desired growth of the larvae for biological evaluation. For each dose level 3 replicates were used. To each test tube of control, positive control and extracts, 20 numbers of brine shrimp and volume was made up to 10ml by adding salt water. Cytotoxic assay was carried out at three doses 500, 1000 and 2000µg/ml. Motility assessment of larvae was conducted at each hour up to four hours.

Motility readings were graded as below.

4+ = high motile

3+ = motile

2+ = sluggish

1+ = slow

Nil = no activity

After 24 hrs, the number of survived larvae in the control and experimental tubes were counted. From this data, the percentage (%) of inhibition of the brine shrimp was calculated for each concentration using the following formula.

$$\% \text{ Inhibition} = \frac{\text{No of larvae (control)} - \text{No. Of larvae(experiment)}}{\text{No. of larvae in control}} \times 100$$

RESULTS AND DISCUSSION

Moisture content of leaves of *Zingiber zerumbet* was 71.53 %. As can be seen from the Table1, highest yield was obtained in methanol extract followed by hexane, chloroform and ethyl acetate in descending order. Overall it can be concluded that polar molecules outnumber the nonpolar ones.

Table 1: Yield of different solvent extracts.

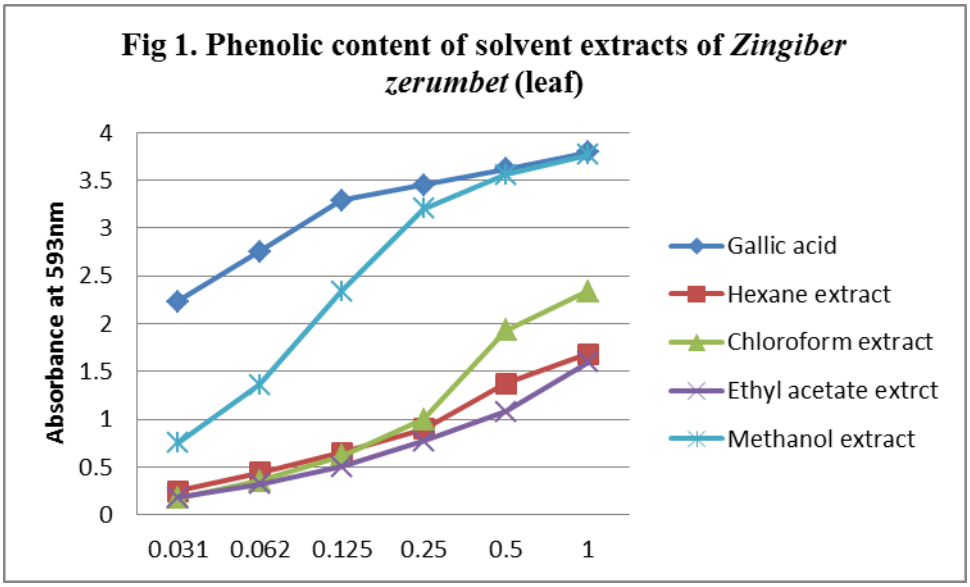
Solvent extract	Percentage
Hexane	3.57%
Chloroform	2.75%
Ethyl acetate	9.54%
Methanol	15.74%

Presence of alkaloid was predominant as can be seen in Table 2. it was found positive in all three tests. Tannin was present in all the extract. Cardiac glycoside was present in all the extracts except chloroform extract. Thus, just like the rhizome of the plant leaf extracts also possessed medicinally important molecules like alkaloids, tannins and cardiac glycosides which are invariably used as anti inflammatory and antioxidant purpose.[12]

Table 2: Phytochemical analysis of leaf extracts of *Zingiber zerumbet.*

Secondary metabolite	Hexane extract	Chloroform extract	Ethyl acetate extract	Methanol extract
Alkaloids	+	+	+	+
Mayer's test	+	+	+	+
Wagner's test	+	+	+	+
Dragendroffs test	+	+	+	+
Flavonoids	-	-	-	-
Anthraquinone	-	-	-	-
Saponin	-	-	-	-
Tannin	+	+	+	+
Terpenoids	-	-	-	-
Phlobotanin	-	-	-	-
Cardiac glycoside	+	-	+	+

As can be seen from Fig 1 at higher dose, absorbance of methanol extract was similar to the standard gallic acid suggesting an amount of Phenolic content equivalent to gallic acid. Whereas other extract possessed less content when compared with the standard molecule.



Antioxidant activity of solvent extracts of *Zingiber zerumbet* was explored using qualitative as well as quantitative assays.

Qualitative Antioxidant Activity

Antioxidant activity of solvent extracts of *Zingiber zerumbet* was conducted using TLC based DPPH antioxidant assay. Best separation was obtained in BEA solvent where hexane, chloroform and ethyl acetate extract showed antioxidant bands respectively. Whereas a complete yellow streak was obtained in methanol extract which suggested presence of antioxidant molecules in close proximity. Thus all the extracts of *Zingiber zerumbet* had antioxidant potential.

Table 3: TLC Based DPPH ASSAY of solvent extracts of *Zingiber zerumbet.*

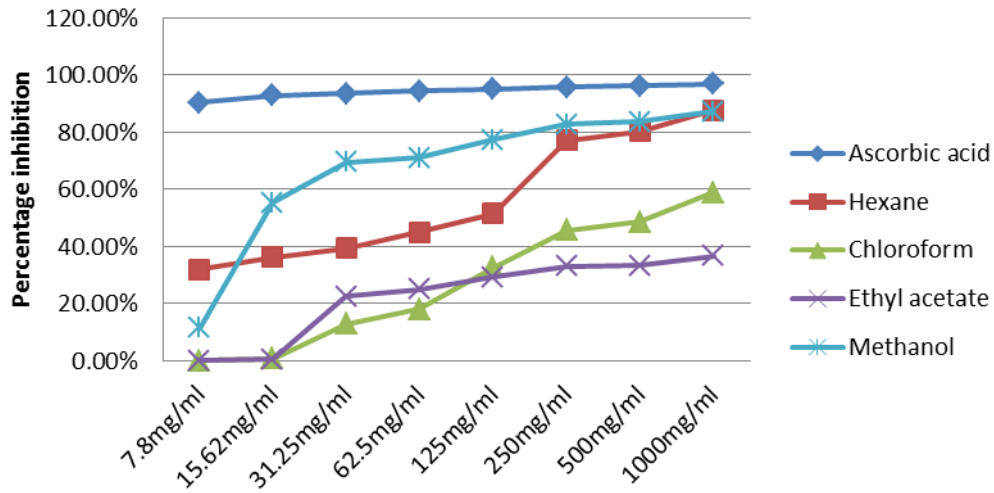
SAMPLE	SOLVENT	No. of bands	Rf values
HEXANE	BEA	10	0.28, 0.35, 0.38, 0.42, 0.53, 0.56, 0.65, 0.7, 0.79, 0.8
	CEF	Nil	Nil
	EMW	1	0.65
CHLOROFORM	BEA	7	0.27, 0.34, 0.38, 0.41, 0.49, 0.75, 0.8
	CEF	1	0.75
	EMW	Nil	Nil
ETHYL ACETATE	BEA	2	0.75, 0.77
	CEF	Nil	Nil
	EMW	Nil	Nil
METHANOL	BEA	Complete yellow streak	Infinite number of antioxidant molecules in close proximity.
	CEF	Nil	Nil
	EMW	Nil	Nil

Quantitative Antioxidant assay

DPPH free radical scavenging assay

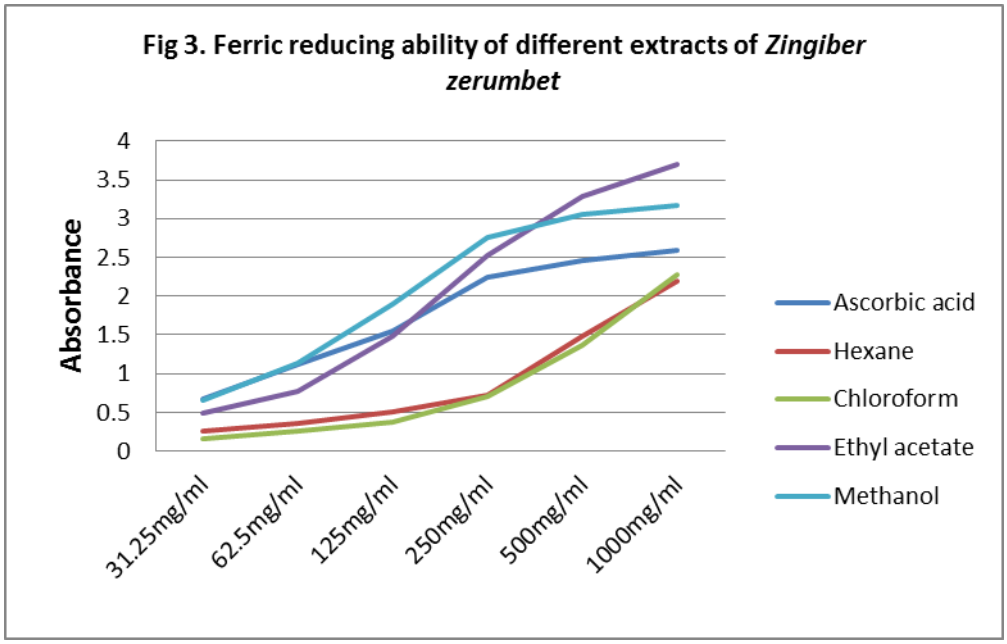
The antioxidant activity of different extracts of *Zingiber zerumbet* was analyzed with DPPH, a stable free radical. As DPPH picks up one electron in the presence of free radical scavenger, the absorption decrease and the resulting discoloration related to the number of electrons gained. As can be observed from the (Fig 2), all solvents showed less amount of antioxidant activity as compared to the standard ascorbic acid. However, hexane and methanol extract showed more than 80% inhibition at higher dose of 1mg/ml.

Fig 2. DPPH radical scavenging assay of solvent extracts of *Zingiber zerumbet*



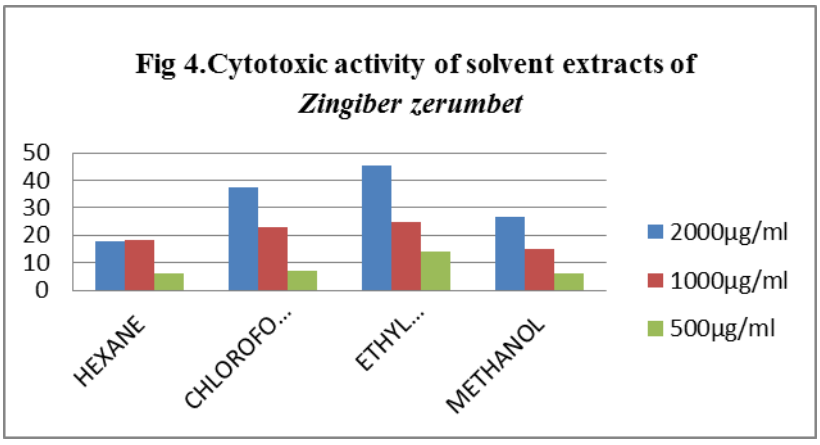
Ferric reducing antioxidant power assay

The antioxidant can donate an electron to free radicals, which leads to neutralization of the radical. Reducing power was measured by direct electron donation in the reduction of Fe3+ to Fe2+ in the presence of TPTZ. The product was visualized by forming an intense blue colour complex and then measured at 593nm. As can be seen from the Fig 3, Ethyl acetate extract showed better antioxidant value at higher dose in comparison to the standard molecule.



Cytotoxic activity using brine shrimp assay

All the extracts were tested in three doses (2000, 1000, 500 microgram/ml). Cytotoxic activity was found highest in ethyl acetate extract (45.16%) at the dose 2000microgram/ml followed by chloroform extract which showed 37.2% activity at the dose 2000microgram/ml. Remaining extracts showed mild activities against brine shrimp assay.



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