



# IN-VITRO ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF *ALPINIA CALCARATA* IN ANDAMAN ISLANDS

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## Abstract

The ethanolic and aqueous extracts of rhizomes of *Alpinia calcarata* Roscoe, from Zingiberaceae family, are known to possess anti-bacterial, anti-fungal, antihelmintic, antinociceptive, anti-inflammatory, antioxidant, aphrodisiac, gastro protective, and anti-diabetic properties. Its decoction is widely used to treat cough, stomach ache, respiratory ailments, bronchitis, asthma, rheumatism and diabetes in traditional system of medicine. *In vitro* antioxidant and free radical scavenging activity of cold ethanolic extract (CEE), cold methanolic extract (CME) and cold water extracts (CWE) of *Alpinia calcarata* from Andaman Islands were analysed. Highest IC<sub>50</sub> value was found in ethanolic extract (36 µg/ml) suggesting high efficiency in quenching 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical followed by methanolic (142.5 µg/ml) and aqueous extract (176 µg/ml).

**Key words :** *Alpinia calcarata*, antioxidants, methanolic, ethanolic extracts.

## Introduction

The *Alpinia calcarata* (fig. 1), a rhizomatous perennial herb of Zingiberaceae family is native to Asia, Australia and the Pacific Islands. The herb lacks true stem, but have pseudo stems usually up to 3-8 meters long which are composed of the overlapping leaf sheaths, each flower is about 4cm long. The inflorescence takes the form of a spike, a panicle, or a raceme and may be hooded in bracts and bracteoles. The fruit appears as a rounded, dry or fleshy capsule. They grow from thick rhizomes. The mature rhizomes are branched and dense with a light to dark brown colour. The phytochemical screening revealed the presence of polyphenols, tannins, flavonoids, steroid glycosides and alkaloids in the extracts and essential oil of this plant. Also, the Essential oils and extracts from this plant were found to possess wide range of pharmacological and biological activities.

Phytochemicals are known for their presumed role in the prevention of various chronic diseases including cancers and cardiovascular diseases. The ethanolic and aqueous extracts of the rhizomes are known to possess anti-bacterial, anti-fungal, anti-helmintic, antinociceptive, anti-inflammatory, antioxidant, aphrodisiac, gastro protective, and anti-diabetic properties thus its decoction is widely used to treat cough, stomach ache, respiratory

ailments, bronchitis, asthma, rheumatism and diabetics. The herb is commonly employed in the indigenous medicinal formulations for the treatment of indigestion, throat inflammation, voice improvement and alleviation of impurities present in the blood. Arawwawala *et al.* (2012) reported the anti-inflammatory and antioxidative properties of *A. calcarata* in hot ethanol extract and hot water extract. The cytotoxic properties of *A. calcarata* rhizome alcoholic extract have been investigated against Ehrlich Ascites Carcinoma (EAC) tumour bearing Swiss Albino mice. Pesticidal effect of *A. calcarata* essential oil has also been found against *Periplaneta americana*. Roots, rhizomes and leaves of *A. calcarata* have been reported to possess many phytochemicals such as protocatechinic acid, 1, 8-cineole, quercetin, β-pinene, 4-O-methyl-syringic acid, methyl cinnamate, vanillic acid and a number of diterpenes. Kong *et al.* (2002) isolated several diterpenes from the rhizomes of *A. calcarata* such as calcaratarins A-E, sesquiterpenes such as shyobunone and coumarins such as herniarin and reported that the two bis-labdanic diterpenoids exhibited cytotoxic activity against human KB cells in vitro. Apart from this, some benzenoids such as protocatechuic acid, alkaloids and different flavonoids were isolated from leaves of *A. calcarata* available in India. Plant genetics and cultivar,

soil composition and growing conditions, maturity state and post-harvest conditions affect the quantity and quality of the polyphenols present in plant food.

### Phytochemical properties

A number of benzenoids such as vanillic acid, protocatechuic acid, syringic acid, alkaloids and flavonoids have been isolated from the leaves of *A. calcarata* in India. According to Arambewela *et al.* (2004), at least eighteen volatile compounds were identified from the essential oils of rhizomes, roots and leaves of *A. calcarata* and among the isolated compounds, 1, 8 - cineol was found to be the major constituent in rhizomes and leaf oil while fenchyl acetate was present in the roots. Qualitative phytochemical analysis of the hot ethanol extract and hot water extract revealed the presence of tannins, polyphenols, steroid glycosides, flavonoids and alkaloids in *A. calcarata* rhizomes.

Raj *et al.* (2011), Arambewela *et al.* (2005) have confirmed its antihyperglycemic activity through hot ethanolic extract and hot water extract in normal glycaemic diabetic rats with improvement in oral glucose tolerance, reduction in plasma triglyceride and total cholesterol level. However, in streptozotocin (STZ)-induced and alloxan-induced diabetic rats, neither the hot ethanolic extract nor the hot water extract could reduce the blood glucose levels. Hot ethanolic extract has been effective in decreasing glucose absorption in the small intestine and increasing glycogen accumulation in tissues. According to Raj *et al.* (2011) ethanolic extract exhibited better results in improving glucose tolerance than the aqueous extracts. Similar hypoglycaemic activity has been claimed by Akhtar *et al.* (2002) through methanolic and aqueous extracts of rhizome of *Alpinia galanga* in normal rabbits.

Anti-inflammatory activity, probably due to the inhibition of histamine and prostaglandin synthesis, of *A. calcarata* was shown through hot water extract and hot ethanolic extract by Arawwawala *et al.* (2012), Hema and Nair (2009) concluded that flavonoids and other more polar constituents from *A. Calcarata* have considerable chemical similarity with those of *A. katsumadai*. Similarly, Ahmed *et al.* (2015) found that leaf extract of *Alpinia nigra* (Gaertn.) Burt showed the presence of medicinally active secondary metabolites such as alkaloids, glycosides, cardiac glycosides, flavonoids, steroids, tannins, anthraquinone glycosides, and saponins. The leaf extract (2 mg/disc) showed mild antibacterial activity compared to tetracycline (50 mg/disc). Whereas, free radical scavenging activity was reported by Ramya *et al.* (2015), concluding that majority of the secondary metabolites was

extracted through ethanol.

*In-vivo* cytotoxic activity was reported by Perveen *et al.* (2012) which can be considered as a probable new source of antitumor agents available through ethanolic extract of *A. calcarata* against Ehrlich Ascites Carcinoma (EAC) tumour bearing Swiss Albino mice, leading to elevation of the depleted haematological cell counts.

Information regarding phenolic content and different antioxidant activities of *Alpinia calcarata* was barely reported and especially for the population growing in tropical climate like Andaman Islands where plant is subjected to more oxidative stress vis-a-vis to climatic pattern. Therefore, *A. calcarata* from Andaman Islands were chosen for the analysis.

### Objectives

1. To study the phytochemical composition of the *Alpinia calcarata* grown in the Andaman Islands.
2. To reveal the relationship between phenolic content and different antioxidant activities.

### Materials and Methods

After harvest, the *Alpinia calcarata* rhizome was washed in tap water to remove mud, clay and sand particles adhered to it followed by drying in cabinet dryer at 50° C for 2-3 days to reduce the moisture content to 6-7% (wet basis). After drying turmeric slices were grinded into powder form by using high speed blender. One gram sample was mixed with 50 ml of each of solvent (80% methanol, 80% ethanol and distilled water) and kept for 1-2 day followed by centrifugation at 8000 rpm for 10 min followed by filtering through Whatman No. 1 filter paper (fig. 2). The extract was concentrated by rotary evaporator and kept at (-4°C) for analysis.

### Total Phenolics Content (TPC)

The Folin-Ciocalteu colorimetric method was used to measure the total phenolic content (Bao *et al.*, 2005). Briefly, 200 µl of the extractions were oxidized with 1 ml of 0.5 N Folin-Ciocalteu reagent and then the reaction was neutralized with 1 ml of the saturated sodium carbonate (75 g/L). The absorbance of the resulting blue color was measured at 760 nm with a UV-2600 spectrophotometer (Simadzu, Japan) after incubation for 2 h at room temperature. Quantification was done on the basis of the standard curve of gallic acid. Results were expressed as milligram of gallic acid equivalent (mg GAE) per gram of flour weight.



**Fig. 1 :** *Alpinia calcarata*.

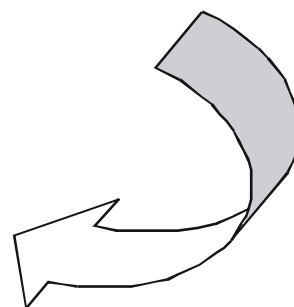
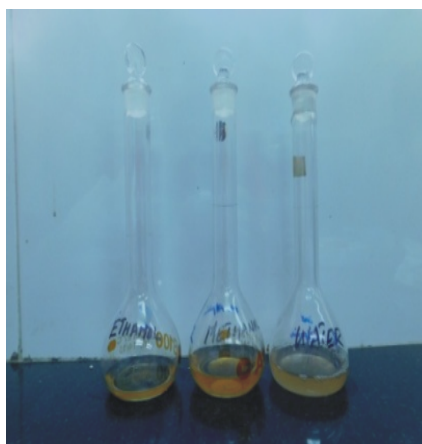
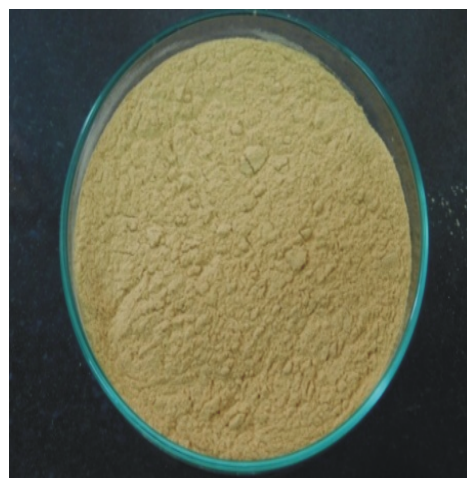
**Flavonoids content**

Total flavonoids content was determined by a colorimetric method (Bao *et al.*, 2005). 0.5 ml extracts were added to 15 ml polypropylene conical tubes containing 2 ml ddH<sub>2</sub>O and mixed with 0.15 ml 5% NaNO<sub>2</sub>. After reacting for 5 min, 0.15 ml 10% AlCl<sub>3</sub>.6H<sub>2</sub>O solution was added. After another 5 min, 1 ml 1 M NaOH was added. The reaction solution was well mixed, kept for 15 min and the absorbance was determined at 415 nm. Qualification was done using the Rutin as standard and the results was expressed as milligrams of rutin equivalent (mg RE) per gram of flour weight.

**Estimation of free radical scavenging activity**

**DPPH radical scavenging activity**

Total antioxidant activity was obtained by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Rattanachitthawat *et al.*, 2010) with some modification. The working solution of DPPH was freshly prepared by diluting 3.9 mg of DPPH with 95% ethanol to get



**Fig. 2 :** Preparation of extracts for phytochemical analysis.

with an absorbance of  $0.856 \pm 0.05$  at 517 nm. The extract (2.5 mg/ml) was mixed with 1.5 ml of working DPPH and the absorbance of the mixture immediately measured spectrophotometrically after 10 min. Total antioxidant activity of the extract was expressed as mg BHA/g sample equivalent, obtained from the calibration curve.

$$\% \text{ inhibition of DPPH radical} = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$$

Where,  $A_{\text{control}}$  is the absorbance of the control (without extract) and  $A_{\text{sample}}$  is the absorbance in the presence of the extract/standard. The IC<sub>50</sub> (concentration for 50% inhibition) values of all the methods were calculated by using linear regression analysis. All the experiments were repeated thrice. Higher the IC<sub>50</sub> value, lower is the scavenging activity.

#### ABTS radical scavenging activity

The total antioxidant capacity was determined by a colorimetric method (Re *et al.*, 1999) with a little modification. First, ABTS<sup>+</sup> solution prepared and pH was adjusted to about  $0.784 \pm 0.01$  with 80% ethanol. Then, 3.9 ml ABTS<sup>+</sup> cation solution was added to 0.1 ml (2.5 mg/ml) of extracts and mixed thoroughly. The mixture was incubated for 6 min at room temperature and absorbance was taken at 734 nm. Results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC,  $\mu\text{M}$  Trolox equivalents per 100 g dry weight).

$$\% \text{ inhibition of ABTS radical} = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$$

#### Ferric Reducing-Antioxidant Power (FRAP) Assay

Benzie and Strain (1996) method, based on the reduction of the complex of ferric iron and 2,3,5-triphenyl-1,3,4-triazole-2-azoniacyclopenta-1,4-diene chloride (TPTZ) to the ferrous form at low pH was used. Briefly, 0.9 ml of prepared FRAP reagent is mixed with 0.1 ml of diluted sample and the absorbance at 595 nm was recorded after a 15 min incubation at 37°C and the results were expressed in mM of Fe<sup>2+</sup> equivalents per g dry weight.

#### Nitric oxide scavenging activity

Nitric oxide scavenging activity was measured using the procedure described by Marcocci *et al.* (1994). SNP (10 mM) in phosphate buffer saline (PBS) was mixed with different concentrations of the extract and incubated at 25°C for 150 min. The samples were added to Griess reagent (1% sulphanilamide, 2% H<sub>3</sub>PO<sub>4</sub> and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl-ethylene-di-amine was read at 546 nm and

referred to the absorbance of standard solutions of ascorbic acid treated in the same way with Griess reagent as a positive control. The percentage of inhibition was measured by the following formula:

$$\% \text{ inhibition of NO radical} = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$$

#### Metal Chelating Activity (MCA)

The chelating of ferrous ion was measured using the method of Dinis *et al.* (1994). The crude extracts were reacted with 0.05 mL of 2.0 mM FeCl<sub>2</sub>. The mixture was then added with 0.2 mL of 5.0 mM ferrozine. After which, the reaction was shaken and incubated at room temperature for 10 min and the absorbance of the red color was measured at 562 nm. EDTA was used as a positive control. The percentage of metal chelating activity was calculated by the following equation:

$$\% \text{ Metal chelating activity} = (A_c - A_s) / A_c \times 100$$

Where, 'Ac' is the absorbance of the control and 'As' is the absorbance of the extract/standard.

#### Hydrogen Peroxide Scavenging (H<sub>2</sub>O<sub>2</sub>) Assay

Scavenging activity of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was estimated using the method of Ruch *et al.*, 1984 with little modification. Extract at various concentration was mixed with 0.6 ml of 4 Mm H<sub>2</sub>O<sub>2</sub> prepared in phosphate buffer saline (pH 7.4) and was incubated for 10 min. The absorbance of solution was taken at 230 nm against a blank solution containing extract in PBS without H<sub>2</sub>O<sub>2</sub>. Ascorbic acid was used as positive control. The amount of nitric oxide radical inhibition is calculated following this equation:

$$\% \text{ inhibition of H}_2\text{O}_2 \text{ radical} = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$$

#### Reducing Power (RP) Assay

This method involves in the mechanism of single electron transfer system using ferric to ferrous reducing activity as determined spectrophotometrically from the formation of Perl's Prussian blue colour complex. This method is based on the principle of increase in the absorbance of the reaction mixtures described by Oyaizu (1986). 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of K<sub>3</sub>Fe(CN)<sub>6</sub> (1% w/v) are added to 1.0 mL of sample dissolved in distilled water. The resulting mixture is incubated at 50°C for 20 min, followed by the addition of 2.5 mL of Trichloro acetic acid (10% w/v). The mixture is centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of FeCl<sub>3</sub> (0.1%, w/v). The absorbance is then measured at 700 nm against blank

sample. The higher the absorbance of the reaction mixture the greater is the reducing power.

## Results and Discussion

### Phenolic and Flavonoid Content

Total phenolic and flavonoid content of three extract is shown in table 1. Methanolic extract had highest phenolic (21.08 mg/g GAE) followed by ethanolic (17.52) and aqueous extract (8.91 mg/g). Similar results were reported by Wong et al. (2008) in other species of *Alpinia*. They reported that the phenolic content of *Alpinia galanga* had 2.14 mg GAE/g which was 10 times less than our result. The reason may be due to different growth period, geographic location, storage type, genetic diversity etc. Similarly, the flavonoid content varied between 60.7 mg rutin/g (methanolic extract) and 14.3 mg rutin/g (aqueous extract). *Alpinia* species is rich in flavonoids such as kaempferol, quercetin and proanthocyanidins (Williams and Harborne, 1977), which contribute toward the TPC values reported in this work. Methanol, besides having higher extraction efficiency, is more efficient in cell wall degradation as compared with other two solvents (Lapornik *et al.*, 2005). The higher values for methanolic extract may be due to the extraction power of solvent (80% methanol) to the flavonoid compounds to the dilution media. The levels of total phenolics determined in this way are not absolute measurements of the amounts of phenolic compounds, but are in fact based on their chemical reducing capacity relative to gallic acid. Antioxidant index of vegetables have been found to be a combined measure of quality and quantity of antioxidants in vegetables (Elliot, 1999). The phenolic compounds are secondary metabolites which are derived from pentose phosphate, shikimate, and phenylpropanoid pathways in plants. Aside with being responsible for the color (such as yellow, orange, red, and blue pigments), taste and flavor (such as vanillin and eugenol) of foods one of the major polyphenol characteristics is they are the dominant antioxidants that exhibit scavenging efficiency on free radicals, and reactive oxygen species are numerous and widely distributed in the plant kingdom (Prior and Cao, 2000; Zia-Ul-Haq *et al.*, 2011).

### In-vitro Free Radical-Scavenging Activity

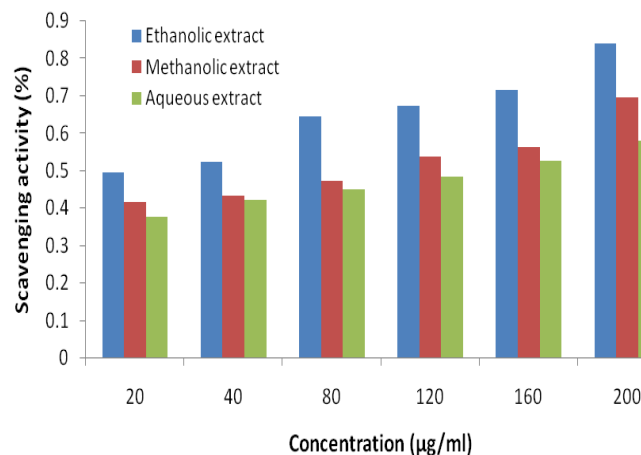
The scavenging activity is attributed to the presence of phenolic hydroxyls, particularly in the 32 OH and 42 OH of the three-carbon chain. Antioxidants are the compounds responsible for the reduction of oxidative degradation by scavenging free radicals, per-oxide radicals, metal chelators and metal reducing agents.

### DPPH Radical Scavenging Activity

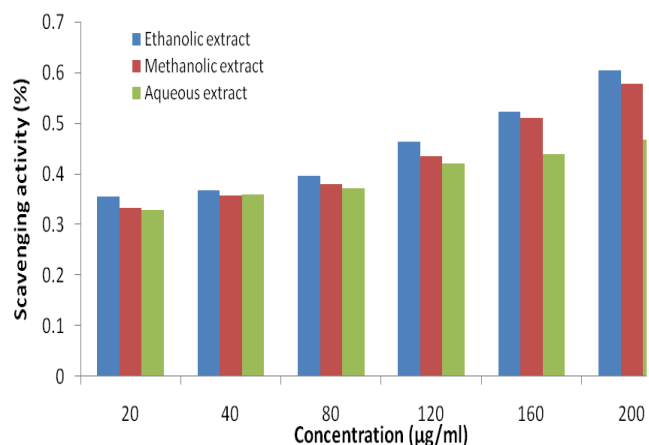
The free radical-scavenging activity of the extracts of tested extracts measured through DPPH- method is presented in table 1 with BHT as reference compound. The method is based on the reduction of methanolic DPPH- solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extracts were able to reduce the stable radical DPPH- to the yellow-coloured diphenyl-picryl-hydrazine. Results from present study indicated highest DPPH activity in ethanolic extract (5.82 mg BHT/g) followed by methanolic extract (547.5 mg/g) and aqueous extract (532.5 mg/g). There was steady increase in the inhibition of the radicals with concomitant increase in concentration of the extract establishing dose dependence of the extract in scavenging DPPH radicals. DPPH radical scavenging activity of three extracts were increased with increasing concentration (20-200 µg/ml), being highest in ethanolic extract (83.9%) and lowest in aqueous extract (58.01%) at 200 µg/ml as shown in fig 3. The higher scavenging activity was due to high level of phenolic content in methanolic extract. Phenolic compounds are high level antioxidants (Hall and Cuppett, 1997) because they possess the ability to adsorb and neutralize free radicals, quench active oxygen species and decompose superoxide and hydroxyl radicals (Duh *et al.*, 1999). IC<sub>50</sub> (concentration required for 50% reduction of scavenging activity) is shown in table 2. Smaller IC<sub>50</sub> value correlated with higher DPPH scavenging activity. Highest IC<sub>50</sub> value was found in ethanolic extract (36 µg/ml) suggesting high efficiency in quenching DPPH radical followed by methanolic (142.5 µg/ml) and aqueous extract (176 µg/ml).

### ABTS Radical Scavenging Activity

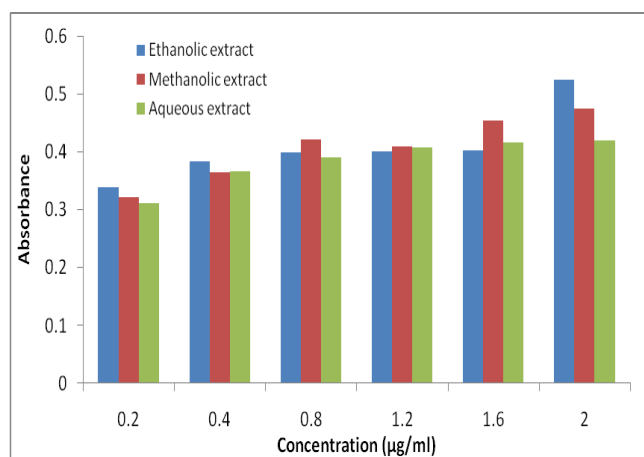
The ABTS assay is based on the inhibition by antioxidants of the absorbance of the ABTS radical cation (ABTS<sup>+</sup>) (Sanchez-Moreno, 2002). ABTS activity was found to be highest in methanolic extract (7.1 mg trolox/g) and lowest in aqueous extract (5.6 mg/g) as shown in table 1. A concentration-dependent activity was observed in this assay (fig. 4). The highest activity in methanolic extract corresponds to high phenolic content compared to other two extract. With the increase in concentration (20-200 µg/ml), the scavenging activity of powder to ABTS radical scavenging activity increased from 35.5 to 60.5 % (ethanolic extract) to 33.2 to 57.8% (methanolic extract) and 32.8 to 46.9% (aqueous extract) (fig. 4). So, at higher concentration, the extracts had high scavenging activity. As compared to DPPH activity, the scavenging effect is very low. The relative low scavenging



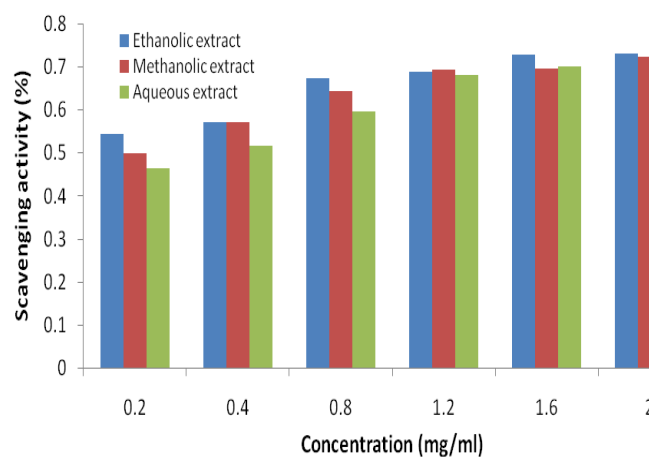
**Fig. 3 :** DPPH radical scavenging activities of *Alpinia calcarata* rhizome powder.



**Fig. 4 :** ABTS radical scavenging activities of *Alpinia calcarata* rhizome powder.



**Fig. 5 :** FRAP activities of *Alpinia calcarata* rhizome powder.



**Fig. 6 :** Nitric oxide radical scavenging activity of *Alpinia calcarata* rhizome powder.

potential for aqueous extract may be due to its low extract potential to remove the phytochemicals from its complex tissue to the solvent medium. Different radical scavenging capacity of the methanol extract against the tested radicals (DPPH and ABTS) may be due to the different mechanisms involved in the radical-antioxidant reactions. These assays differ from each other in terms of substrates, probes, kinetic factors and quantification methods. Radical systems in antioxidant evaluations may influence the difference in the results obtained in an experiment (Yu *et al.*, 2002). Some compounds, though possessed ABTS<sup>+</sup> scavenging activity, did not exhibit DPPH scavenging activity (Wang *et al.*, 1998). Thus, comparison of assays is difficult and ranking of antioxidant activity is strongly dependent on the test system and on the substrate to be protected by the antioxidants (Frankel and Meyer, 2000). Due to low power of aqueous extract, its IC<sub>50</sub> value is two times higher than corresponding ethanolic extract (table 2).

### Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe<sup>3+</sup>-TPTZ] complex and producing a coloured ferrous tripyridyltriazine [Fe<sup>2+</sup>-TPTZ]. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom. FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction (Guo *et al.*, 2003). FRAP assay was used by several authors for the assessment of antioxidant activity of various food product samples (Halvorsen *et al.*, 2006; Pellegrini *et al.*, 2003). Halvorsen *et al.* (2006) suggested most of the secondary metabolites are redox-active compounds that will be picked up by the FRAP assay. In current study, FRAP activity was measured to be 217.22, 190 and 150.94 mg Fe<sup>2+</sup>/g in ethanolic, methanolic and aqueous extract. With the

**Table 1 :** Phytochemicals in *Alpinia calcarata* rhizome powder.

Extract	Phenol (mg GAE/g)	Flavonoids (mg rutin/g)	DPPH (mg BHT/g)	ABTS (mg trolox/g)	FRAP (mg Fe <sup>2+</sup> /g)	NO (mg Vit-C /g)	MCA (mg EDTA/g)	H <sub>2</sub> O <sub>2</sub> (mg Vit-C/ g)	RP (mg BHT/g)
Ethanolic	17.52	60.7	5.82	6.96	2.17	11.65	11.90	14.05	11.02
Methanolic	21.08	47.3	5.25	7.1	1.90	11.40	12.35	15.76	15.01
Aqueous	8.91	14.3	5.52	5.6	1.59	11.05	11.70	11.97	6.1

**Table 2 :** IC 50 values ( $\mu\text{g/ml}$ ) *Alpinia calcarata* rhizome with different antioxidant methods.

	DPPH	ABTS	NO	MCA	H <sub>2</sub> O <sub>2</sub>
Ethanolic extract	36	141.75	83	2083	534
Methanolic extract	142.5	146.25	167	1389	715
Aqueous extract	176	237.1	191	2005	912

increase in concentration of sample (0.2-2 mg/ml), the absorbance value increased, being maximum in ethanolic extract (0.52) and minimum in aqueous extract (0.46) as shown in fig. 5. According to Oktay *et al.* (2003) positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species. Recently, Li *et al.* (2003) reported that the presence of phytochemicals in several plants significantly correlated with high FRAP values. Since, the extract have the ability to scavenge free radicals, thereby preventing lipid oxidation via a chain breaking reaction; they could serve as potential nutraceuticals when ingested along with nutrients. In this study, the ethanolic extract possesses the better hydrogen donation capacity which suppresses the free radicals.

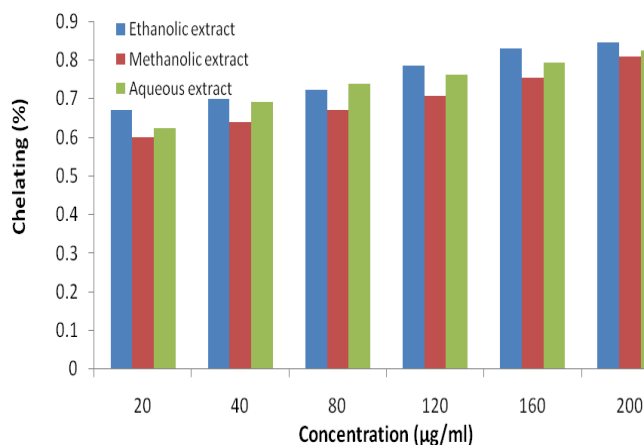
#### Nitric oxide Scavenging Activity

The toxicity and damage caused by NO and O<sub>2</sub> multiplies as they react to produce reactive peroxynitrite, which leads to serious toxic reactions with biomolecules, such as protein, lipids, and nucleic acids. High concentration of nitric oxide (NO) has deleterious effects, and therefore, it is necessary that the production of NO be tightly regulated. Here, *Alpinia* rhizome powder extract effectively reduced the generation of NO<sup>•</sup> radical where the activity was highest in ethanolic extract (11.65 mg Vit-C/g) and lowest in aqueous extract (11.05 mg Vit-C/g). With the increase in concentration (0.2-2 mg/mL), the scavenging activity increased from 46.5 to 73%, 49.2 to 72% and 46.8 to 71% for ethanolic, methanolic and aqueous extract (fig. 6). Increasing the sample concentration range from 0.2 to 2 mg/mL, the scavenging effect also increased in the dose dependent manner. Hence the ethanolic extract of *A. calcarata* has better nitric oxide radical scavenging activity in competing with

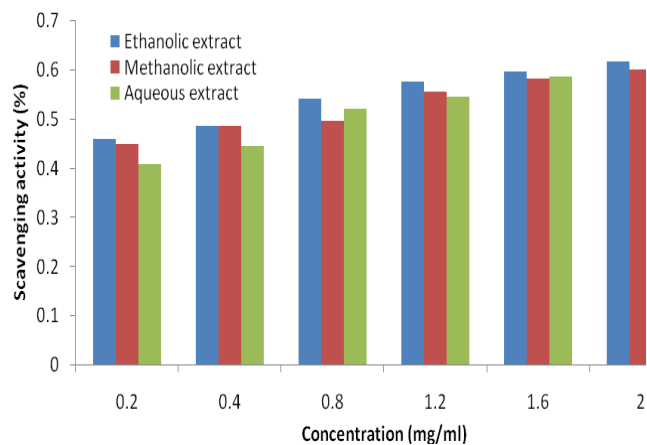
oxygen to react with nitric oxide and thus the inhibition of generation of anions were accordance with the earlier reports of Balakrishnan and Kokilavani (2011). Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity suggesting the possibility of utilizing tropical rice as a viable source of antioxidant for nutraceuticals and functional foods to curb against NO radical mediated disorder in the body. IC50 was found to be 83, 167 and 191  $\mu\text{g/mL}$  for ethanolic, methanolic and aqueous extract suggesting higher efficiency of ethanolic extract for 50% inhibition to NO radicals.

#### Metal Chelating Activity (MCA)

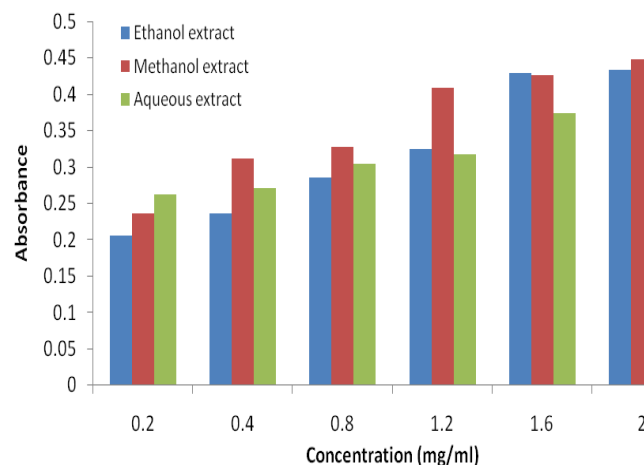
Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanisms, since it reduces the concentration of the catalysing transition metal in LPO (Duh *et al.*, 1999). The complex formation can be disrupted by the presence of other complexing agents which cause a decrease in the red colour intensity of complexes. Substances or samples that can reduce its colour intensity can be considered as antioxidant through the mechanism of inhibition of heavy metal. It was reported that chelating agents that form  $\sigma$ -bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Kumaran and Karunakaran, 2007). Here, it is interesting to note that the MCA of all extracts were at par with each other (11.7-12.35 mg EDTA/g) which showed the effectiveness of rhizome to defend for oxidative damage to cell even with aqueous extract. From fig. 7, it is seen that with the



**Fig. 7 :** Metal chelating scavenging activities of *Curcuma longa* L. powder.



**Fig. 8 :** Hydrogen peroxide radical scavenging activities of *Alpinia calcarat* rhizome.



**Fig. 9 :** Reducing power of *Alpinia calcarat* rhizome powder.

increase in the concentration of (20-200 µg/mL) in all three extracts, the chelating activity increased in dose dependent manner where it was highest in ethanolic extract (84.6%) and lowest in methanolic extract (80.7%) at 200 µg/mL. Similar results were also reported by Wong *et al.* (2008). This suggests that ligands in *A. calcarata* rhizome compete well with ferrozine. Ligands in both parts of the plant effectively sequester ferrous ions by intercepting all coordination sites of metal ions, thus suppressing the formation of hydroxyl radical via Fenton reaction. The results of the present study suggest that *A. calcarata* rhizome exhibits good chelating activity on ferrous ions, probably due to their content of flavonoids (Hendrich *et al.*, 1999).

#### Hydrogen Peroxide Radical Scavenging Activity

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it can give rise to hydroxyl radical in the cells. Thus the removal of H<sub>2</sub>O<sub>2</sub> is

very important for antioxidant defence in cell or food systems (Halliwell, 1991). Thus, the removing of H<sub>2</sub>O<sub>2</sub> is very important for antioxidant defence in cell or food systems. H<sub>2</sub>O<sub>2</sub> can cross membranes and may oxidize a number of compound, probably react with Fe<sup>2+</sup> and possibly Cu<sup>2+</sup> to form hydroxyl radical and this may be the origin of many of its toxic effects. In the current study, the H<sub>2</sub>O<sub>2</sub> activity of all three extract varied between 11.97 mg vit-C/g (aqueous extract) and 15.76 mg vit-C/g (methanolic extract). With the increase in concentration (0.2-2 mg/mL), the scavenging activity increased from 45.9 to 61.6%, 44.9 to 60.1% and 40.9 to 60.6% for ethanolic, methanolic and aqueous extract (fig. 8).

This shows that *A. calcarata* rhizome extract presents a good ability to inhibit the formation of OH radical. Removal of OH radical is one of the most important effective defences of a living body against disease. Therefore any compound with antioxidant activity might contribute towards the total and partial alleviation of this damage. The IC<sub>50</sub> values for ethanolic, methanolic and aqueous extracts were found to be 534, 715 and 912 µg/mL respectively (table 2).

#### Reducing power

The reducing properties are generally associated with the presence of reductones (Pin-Der & Duh, 1998), which have been shown to exert antioxidant action by breaking the free radical chain, by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing capacity of extracts suggested that reductone-associated and hydroxide groups of compounds can act as electron donors and can react with free radicals to convert them to more stable products,



and thereby terminate radical chain reactions. The reducing power of the rhizome increased and correlated well with the increasing concentration. A high absorbance value of the sample indicates its strong antioxidant activity. With the increase in concentration from 0.2 to 2 mg/ml (fig. 9), the absorbance increased from 20 to 43% (ethanolic extract), 23% to 44.8% (methanolic extract) and 26.3 to 39.3% (aqueous extract). From this study, it is revealed that the extracts of *A. calcarata* was weak in donating electrons to reactive radicals and reducing them into more stable and unreactive species.

### Conclusion

In living systems free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases and extensive lysis. Many synthetic drugs protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicine. The determination of total phenolics and flavonoid content along with the total antioxidant capacity of three extract of *A. calcarata* rhizome showed that this plant can be one of the potential sources of safer natural antioxidants. On the basis of the results of this present study, the decreasing order of the antioxidant activity of the extracts assayed through all the three extracts was found to be in the order of ethanol > ethanol > water extract. This showed ethanol was the powerful solvent to extract phytochemical in the study in dose dependent manner. Since reactive oxygen species are thought to be associated with food deterioration and the pathogenesis of chronic infections, and inflammatory diseases, the observed inhibitory potential may partially explain the helpful effects of *A. calcarata* in treating different disease conditions. It is however, worthwhile to further investigate the *in vivo* potentials, different antioxidant mechanisms which may appear to be a most promising candidate. The extracts can be used for minimizing or preventing lipid oxidation in food or pharmaceutical products, delaying the formation of toxic oxidation products, maintaining nutritional quality, and prolonging the shelf life of food or pharmaceutical materials.

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