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Evaluation of Antioxidant Activities of *Alpinia galanga* (L.) Willd

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Present research was designed to evaluate the free radical scavenging capacities and antioxidant activities of rhizome extracts of *Alpinia galanga* prepared in different solvent systems (60% aqueous methanol, 60% aqueous ethanol and distilled water) using different *in vitro* chemical assays. Antioxidant components such as total phenolic content (TPC), total flavonoid content (TFC) and ascorbic acid contents of the ginger species were screened. Antioxidant assays employed included sulphur free radical reactivity assay, ferric ion reducing power assay, DPPH free radical scavenging capacity assay, hydroxyl radical scavenging assay, nitric oxide scavenging activity assay and hydrogen peroxide scavenging assay. The obtained data reveal that the plant extracts contained significant amount of the observed antioxidant components and also exhibited significant free radical scavenging capacities. Methanol (60%) extract exhibited highest antioxidant activity than other solvents. The polyphenolic constituents of the plant extracts appear to be largely responsible for the radical scavenging capacity. The plant extracts act as promising source of antioxidants, and may be useful for development of nutraceuticals and pharmaceutical drugs.

Keywords: Antioxidant, phytochemicals, free radicals, *Alpinia galanga,* reactive oxygen species (ROS).

Free radicals and ROS are highly reactive atoms or group of atoms produced constantly in our body during normal metabolic functions or introduced from the environment such as exposure to solar radiation, air pollution, ionizing radiations and smoking. Overproduction of free radicals may results into oxidative stress, which contributes to more than hundred disorders in human including hypertension, atherosclerosis, rheumatoid arthritis, diabetes mellitus, stroke, myocardial infarction, reperfusion injury, gastritis, Parkinson's disease, haemorrhagic shock, coronary heart diseases, neuro-degeneration, cataract, carcinogenesis, inflammatory disorders, AIDS as well as age-related brain disorders[1,2]. Antioxidants present in plants play vital role in neutralizing free radicals and oxygen-derived species by interfering with oxidation process, chelating catalytic metals and also act as oxygen scavengers[3]. ROS, superoxide anion, hydroxyl radical and hydrogen peroxide that are generated as by-products of biological reactions or from exogenous factors react with nearly every molecule found in living cells including DNA, if excess ROS are not eliminated by antioxidant

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system. Since many antioxidants such as citric acid, propyl gallate, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) are reported to have many side effects including toxicity and cause lipid alteration as well as carcinogenesis[4,5], attention is being focussed on the development of new, safe and non-toxic, economic and powerful natural antioxidants.

Plants are important source of phytochemicals with potential therapeutic effects. Presence of a diverse group of phytochemicals such as phenolic acids, flavonoids, steroids, glycosides, alkaloids, tannins, lignins, stilbenes, anthocyanins etc. contribute to the biological activities of plants including anti-oxidant activity[6,7]. Phenolic compounds or polyphenols are a group of heterogeneous plant secondary metabolites with an aromatic ring and are ubiquitously distributed in the plant kingdom. Flavonoids are also widely distributed in almost all plant families and they easily scavenge aqueous free radicals because of their amphipathic characteristics[8]. This group of compounds have potential to inhibit ROS, damage by haem protein/peroxide mixtures and lipoxygenase and cyclooxygenase enzymes *in vitro*[9].

Alpinia galanga (L.) Willd. (commonly called greater galanga) is a perennial aromatic rhizomatous herb of Zingiberaceae family which is widely cultivated in South East Asia. The plant is widely used as spices for flavouring food as well as herbal remedy in traditional system of medicine such as Ayurveda, Unani, Chinese and Thai folk medicine[10]. The rhizome has a wide range of application as herbal medicine in the treatment of rheumatoid arthritis, ulcer, whooping colds in children, asthma, bronchitis, intermittent fever, stomachache and colic, vomiting, indigestion[11,12]. It is also used as remedy for halitosis, dyspepsia, diabetes and sexual impotency. Phytochemical constituents of the plants have been reported to possess anti-inflammatory, analgesic, antifungal, antibiotic, antibacterial, antiulcer, and anticancer properties[13,14,12,15]. These multiple pharmacological and medicinal properties of *Alpinia galanga* have triggered our interest to evaluate its antioxidant potential. In the present study, antioxidant activities were assessed using sulphur free radical reactivity assay, ferric ion reducing power assay, DPPH free radical scavenging capacity assay, hydroxyl free radicals scavenging assay, nitric oxide scavenging activity assay and hydrogen peroxide scavenging activity assay. Antioxidant components such as total phenolic content, total flavonoid content and ascorbic acid contents were also estimated.

MATERIALS AND METHODS

Reagents and chemicals

Ascorbic acid, curcumin, Folin-Ciocalteu, TCA, DPPH, glutathione and quercetin were obtained from Sigma-Aldrich, USA. $FeSO_4$, Sodium carbonate, gallic acid, potassium hexacyanoferrate, sodium salicylate, metaphosphoric acid, naphthylethylenediamine dihydrochloride, Na_2-EDTA, trichloroacetic acid, and nitroblue tetrazolium (NBT) were procured from HiMedia, India. Other chemicals and reagents were of analytical grade obtained from Merck, India.

Preparation of extract

Fresh rhizomes of mature plants of *Alpinia galanga* (L.) Willd. were collected and washed thoroughly in tap water. Outer scales of rhizomes were removed by a sharp scalpel. One gram dry weight equivalent of fresh rhizomes relative to the moisture content was ground using mortar and pestle, and the paste was treated with 10 ml of 60% aqueous methanol, distilled water, or 60% aqueous ethanol and homogenized separately for each extract. The homogenate was collected and centrifuged at 3000 X g for 10 min to get a clear supernatant. Finally, the clear supernatant was decanted and filtered through Whatman No. 1 filter paper and stored at 4°C.

Determination of antioxidant components

Antioxidant phytochemicals such as total phenolics, total flavonoids and ascorbic acid content were estimated as per the procedures of our earlier studies[16,17,18] . Total phenolic content was expressed as gallic acid equivalents (GAE) in $mg.100g^{-1}$ The concentration range of gallic acid used for standard curve was 2-16$\mu g.ml^{-1}$, and the equation of standard gallic acid curve was $y = 0.0757x + 0.0443$ ($r^2 = 0.9926$). For the estimation of total flavonoid content, quercetin at the concentration range of 5 to 100 $\mu g.ml^{-1}$ was used as a standard compound and the equation of the calibration curve was $y = 0.0074x + 0.0019$ ($r^2 = 0.9974$). For the measurement of ascorbic acid

content, the concentration range of ascorbic acid used for the construction of calibration curve was 0.32-3.52 µg.ml^{-1} and equation of the calibration curve was found to be y = - 0.2535 x + 0.9755 (r^2=0.9879).

Determination of antioxidant potentials

Potential of the plant extract to scavenge free radicals were determined through sulphur / thiyl free radical reactivity assay[19], ferric ion reducing power assay, DPPH free radical scavenging capacity assay, hydroxyl radicals scavenging assay, nitric oxide scavenging activity assay and hydrogen peroxide scavenging assays following the methods published elsewhere[20,21,22].

RESULTS AND DISCUSSIONS

Determination of antioxidant components

Total phenolic content

Phenolic compounds are secondary metabolites possessing high antioxidant properties that are derived from the pentose phosphate, shikimate and phenylpropanoid pathways in plants[23]. Their antioxidant activity is mainly due to their redox properties which allow them to act as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers. Total phenolic content (TPC) of the plant extracts was expressed as gallic acid equivalent (GAE) in 100g of sample. 60% methanol extract showed highest TPC than that of 60% ethanol and aqueous extracts (Table 1). TPC of 60% methanol, 60% ethanol and aqueous extracts of the plant were found to be 184.52 ± 0.41, 152.96 ± 0.57 and 116.38 ± 0.38 mg GAE.100g^{-1} respectively.

Total flavonoid content

Flavonoids are secondary metabolites with scavenging properties against most oxidizing molecules, including singlet oxygen, and various other free radicals implicated in several diseases[24]. They possesses therapeutic potential, including cardioprotective, anti-inflammatory, antimicrobial, and antitumor[25]. Potential health benefits of flavonoids are due to their antioxidant activities which can be attributed to the phenolic hydroxyl groups attached to the flavonoid structure[26]. Hydroxyl group present in the molecular structure of flavonoids could donate an electron (H^+) to radicals such as hydroxyl ($HO^•$), superoxide ($O_2^•$) and peroxyl ($ROO^•$) and thus neutralizing them[27]. Total flavonoid content (TFC) of the plant extracts were expressed as quercetin equivalent (QE) in 100g of sample. 60% methanol extract showed highest TFC than that of 60% ethanol and aqueous extracts. TFC of 60% methanol, 60% ethanol and aqueous extracts of the plantwere found to be 278.15 ± 0.42, 234.54 ± 0.63 and 203.86 ± 0.47 mg QE.100g^{-1} respectively (Table 1).

Ascorbic acid content

Ascorbic acid acts as a powerful antioxidant in fighting free-radical mediated diseases. It can regenerate other antioxidants within the body, including alpha-tocopherol or vitamin E[28,29]. Unlike to most other animals, human are not able to synthesize ascorbic acid. So, it must be obtained from diet and, must be obtained regularly as it cannot be stored in the body. Ascorbic acid is an excellent source of electrons and directly react with or neutralize hydroxyl, alkoxyl and lipid peroxyl (ROO^a%) radicals as well as radical form of other antioxidants such as glutathione radical and vitamin E radical, thereby regenerating these antioxidants[30]. Due to soluble in water, it can combat free radical damages both inside and outside the cells. Ascorbic acid content as determined from the calibration curve was found to be 35.28 ± 0.36, 48.74 ± 0.41 and 54.19 ± 0.57 mg.100^{-1}g for 60% methanol, 60% ethanol and aqueous extracts respectively (Table 1). The antioxidant activity of rhizome might be attributed to its phenolic compounds, flavonoids and ascorbic acid contents.

Sulphur / thiyl free radical reactivity assay

Thiyl free radicals (GS') are formed in gamma irradiated aqueous solution of glutathione. Oxidation of curcumin due to GS' results in the depletion of chrome orange-yellow compound curcumin. Antioxidant potentials of rhizome extract in terms of thiyl free radical scavenging capacity was determined by their ability to protect curcumin from sulfur free radicals. A substantial depletion of curcumin was observed when the reaction mixture was supplemented with different doses of plant extracts (200, 500 and 700 µL) (Fig. 1). Inhibition of curcumin depletion was found to increase with increasing extract dose which indicates that the plant possess bioactive molecules having antioxidant properties thereby protecting the curcumin molecules present in the

reaction mixture by scavenging thiyl free radicals. The crude rhizome extract at the concentration of 6 mg.ml^{-1} inhibited curcumin depletion in the reaction mixture at 60 Gy radiation exposure to

Table 1. Antioxidant components of the rhizome extracts of in different solvent systems

Solvent type for plant extract	Total phenolic content (mg GAE.100g^{-1})	Total flavonoid content (mg QE.100g^{-1})	Ascorbic acid content (mg.100g^{-1})
60% Methanol	184.52 ± 0.41	278.15 ± 0.42	35.28 ± 0.36
60% Ethanol	152.96 ± 0.57	234.54 ± 0.63	48.74 ± 0.41
Aqueous	116.38 ± 0.38	203.86 ± 0.47	54.19 ± 0.57

Values are expressed as mean ± S.D. (n=3). Values are statistically significant at p d" 0.05 as determined by Duncan's test. ANOVA does not apply between columns.

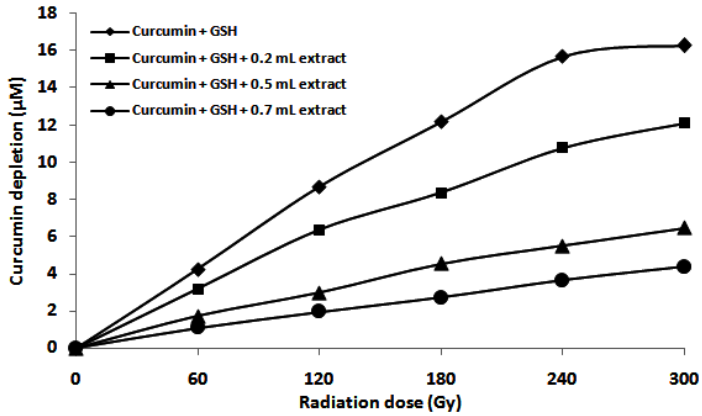

Fig. 1. Graphical representation of protection of curcumin by 60% methanol extract of *Alpinia galanga*

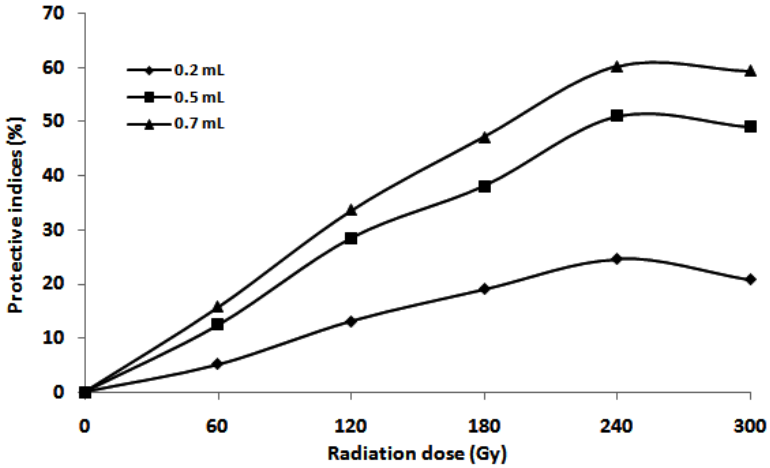

Fig. 2. Protective indices of 60% methanol extracts of plant rhizomes at different extract concentrations against Sulphur / thiyl free radical mediated curcumin depletion

the extent of 3.18 µM. Under the same treatment of radiation exposure, these values were changed to 1.72 µM. at the extract concentration of 16 mg. ml^{-1},which is further shifted to 1.08 µM at the extract concentration of 24 mg.$ml.^{-1}$ Similar trend in the protection of curcumin depletion were observed in other cases of radiation exposure. Protective Index was found to be low at the lower dose of 60 Gy, and increased until the dose reached 240 Gy but declined with further increase in radiation dose (Fig. 2). Increase in PI at lower radiation dose can be due to formation of lesser population of thiyl free radicals that were able to compete with curcumin, whereas after a threshold dose, thiyl free radical generation increases, resulting in greater damage, causing depletion in curcumin.

Fe^{3+} reducing power assay →

For the measurement of the reducing ability, the reduction of $Fe^{3+} \rightarrow Fe^{2+}$ was analysed with the plant extracts. Antioxidants are able to donate electrons to reactive radicals, reducing them into more stable and unreactive species. The production of Perl's Prussian blue colouration due to the formation of Fe^{2+} can be monitored at absorbance of 700 nm by spectrophotometer. The reducing capacity of a compound may serve as a

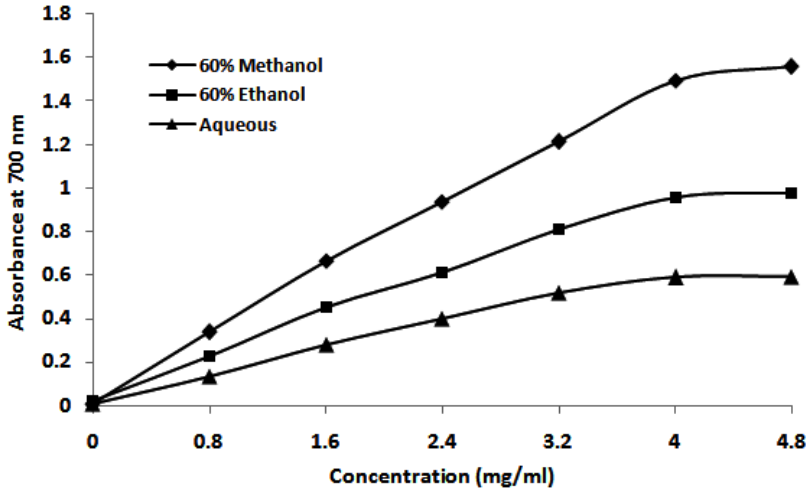

Fig. 3. Ferric ion reducing curve of *Alpinia galanga* in different solvent systems

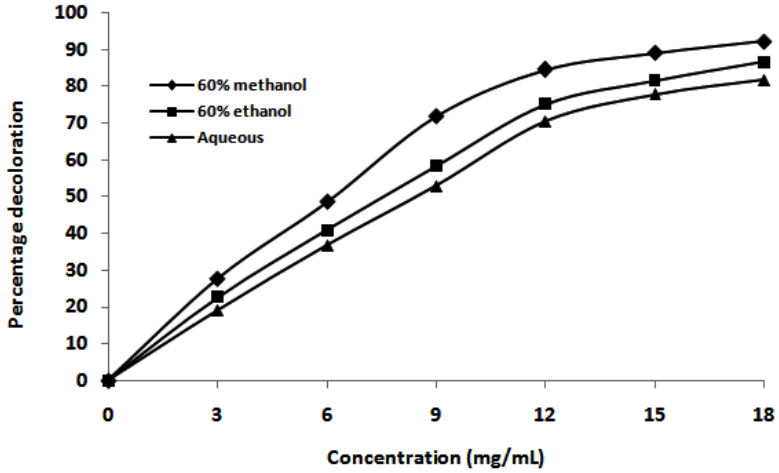

Fig. 4. DPPH free radical scavenging activity in different solvent systems

significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging. Basically, reducing power is associated with the presence of reductones that break the free radical chain by donating a hydrogen atom[31]. Antioxidants present in the plant extracts can donate electrons to free radicals, which lead to the neutralization of the radicals. Fig. 3 shows the reductive ability of the crude extracts of the plant rhizomes. The reducing power of plant extracts was increased with increased dosage.

DPPH free radical scavenging capacity assay

DPPH assay has been widely used to evaluate the free radical scavenging capacity of the plant extracts or their compounds as it is simple, rapid and highly sensitive. Due to the presence of an odd electron, this nitrogen-centred radical gives a maximum absorption at about 515 nm and accepts an electron from an antioxidant compound

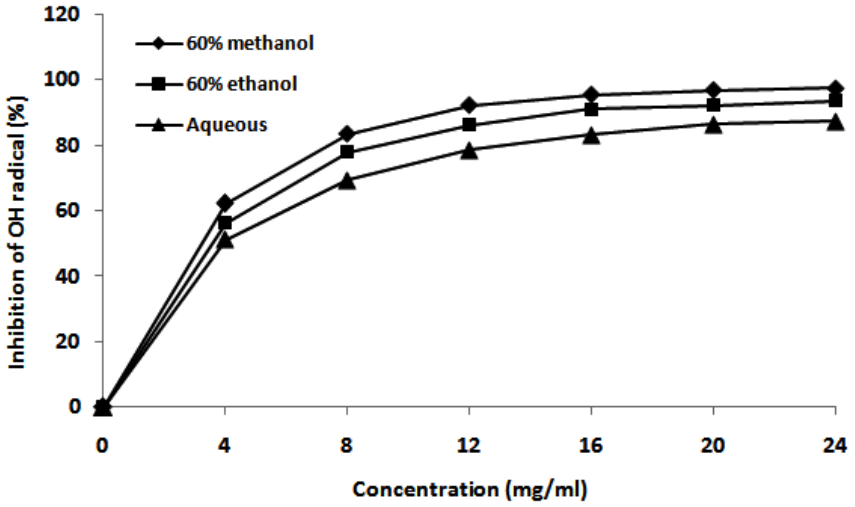

Fig. 5. Hydroxyl radical scavenging activity in different solvent systems

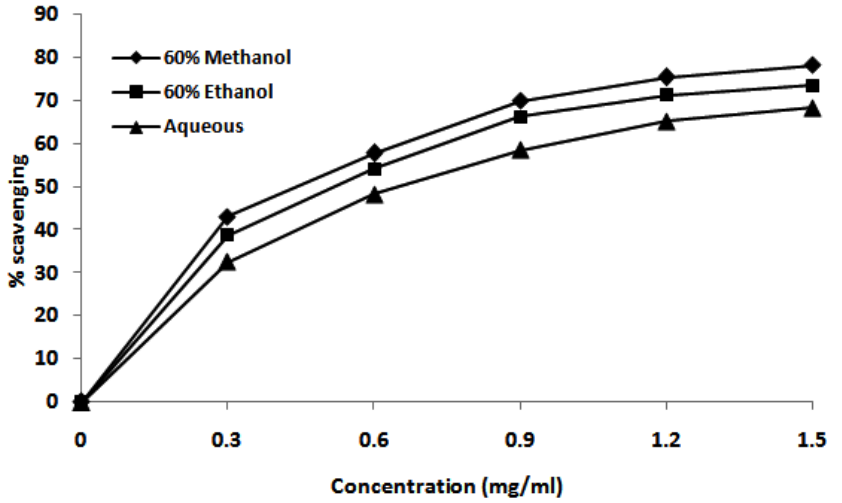

Fig. 6. Nitric oxide scavenging curve in different solvent systems

which acts as a hydrogen donor. The scavenging activity of the plant extracts was monitored based on the amount of DPPH radicals remaining in the test sample using a spectrophotometer. Ascorbic acid was used as standard compound because it impairs the formation of free radicals in the process of intracellular substance formation throughout the body. The purple coloured solution of DPPH free radical was decolorized to its yellow coloured hydrazine, when supplemented with different concentrations of plant extracts to the 100 ìM solution of DPPH. The degree of decoloration increased with increasing concentration of the plant extracts. Rhizome extracts in different solvent systems (aqueous, 60% ethanol and 60% methanol) showed good scavenging capability against DPPH free radical. At extract concentration of 18 mg/ml, plant extracts inhibited 81.75%, 86.61% and 92.4% for aqueous, 60% ethanol and 60% methanol extracts respectively (Fig. 4). The highest scavenging activity was observed in the plant extracts made in 60% methanol with an IC_{50} of (7.22 ± 0.57 mg/ml). This is followed by the 60% ethanol extract (8.54 ± 0.38 mg/ml) and water fraction (9.33 ±0.46 mg/ml) (Table 2). Ascorbic acid, used as standard compound for this experiment has IC_{50} 5.07µg.ml^{-1}.

Hydroxyl radical scavenging assay

Hydroxyl radical is known to be the most powerful oxidizing radical of all the reduce forms of dioxygen and responsible for the oxidative damage of most biomolecules in living cells, such as sugars, proteins, DNA, polyunsaturated fatty acid in membranes and most of the biological molecules it contacts and are capable of abstracting hydrogen atoms from membrane lipids through peroxidation reaction of lipids[32,33]. It generates various products from the DNA bases which mainly include C-8 hydroxylation of guanine to form 8-oxo-7, 8 dehydro- 2 deoxyguanosine,

Table 2. Free radical scavenging capacities through different antioxidant assays using different solvent systems expressed as IC_{50}

Type of solvent	DPPH assay IC_{50} (mg/ml)	Hydroxyl IC_{50} (mg/ml)	Nitric IC_{50} (mg/ml)	Hydrogen peroxide IC_{50} (mg/ml)
60% Methanol	7.22 ± 0.57	4.37 ± 0.43	0.61 ± 0.46	1.87 ± 0.39
60% Ethanol	8.54 ± 0.38	5.54 ± 0.61	0.69 ± 0.61	2.29 ± 0.56
Aqueous	9.33 ± 0.46	7.06 ± 0.54	0.82 ± 0.42	2.74 ± 0.42

Values are expressed as mean ± S.D (n=3). Values are statistically significant at $p \leq 0.05$ as determined by Duncan's test. ANOVA does not apply between columns

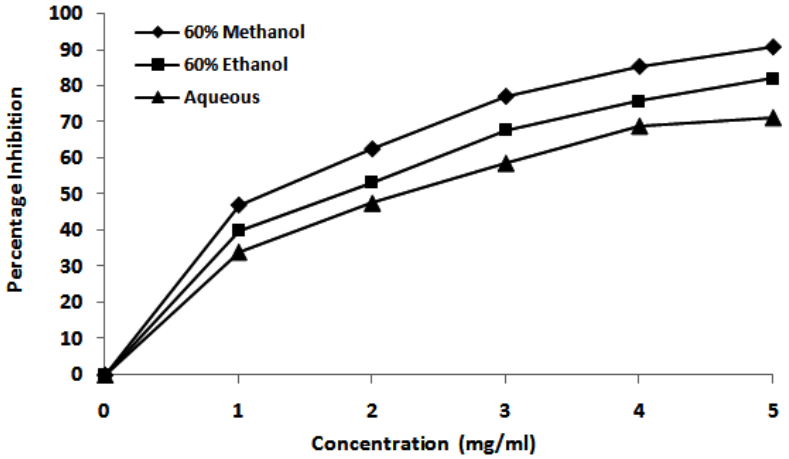

Fig. 7. H_2O_2 scavenging activity in different solvent systems

a ring opened product[34]. Thus, removal of OH radical is very important for the protection of living systems. In the present study, the plant extracts exhibited a concentration dependent scavenging activity against hydroxyl radicals generated. 60% methanolic extracts of plant rhizomes showed relatively maximum degree of scavenging activity towards hydroxyl free radicals than 60% ethanol and aqueous extracts. The degree of inhibition increased with increasing concentration of the extracts. At extract concentration of 24 µg/ml, it exhibited 87.57%, 93.58% and 97.62% inhibition for aqueous, 60% ethanol and 60% methanol extracts respectively (Fig. 5). IC_{50} of this assay were found to be 4.37 ± 0.43, 5.54 ± 0.61 and 7.06 ± 0.54 mg/ml for extracts dissolved in 60% methanol, 60% ethanol and distilled water respectively (Table 2). IC_{50} of curcumin for this experiment was found to be 26.36 µg.ml.$^{-1}$

Nitric oxide scavenging activity assay

NO radical plays a multiple role in diverse biological systems including an effector molecule, neuronal messenger, vasodilatation and antimicrobial as well as antitumor activities[35]. Inhibitors of nitric oxide are known to be beneficial on some aspect of inflammation and tissue damages associated with inflammatory diseases. Nitric oxide radical scavenging assay is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions which can be measured by using Griess reagent[36]. Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25°C for 150 min resulted in the generation of NO. The plant extracts effectively reduced the generation of NO as indicated by the significant decreased in the absorbance of the reaction mixtures with increasing amount of plant extracts. Fig. 6 illustrates a significant decrease in the NO radical due to the scavenging activity of plant extracts. 60% methanolic extract showed maximum percentage of scavenging activity, followed by 60% ethanolic and aqueous extracts. At extract concentration of 1.5 µg/ml, it showed 68.45%, 73.51% and 78.24% inhibition for aqueous, 60% ethanol and 60% methanol extracts respectively. Lowest IC_{50} values for both samples were observed in case of 60% methanolic extracts as compare to 60% ethanolic and aqueous extracts (Table 2). IC_{50} were found to be 0.61 ± 0.46, 0.69 ± 0.61 and 0.82 ± 0.42 mg/ml for plant extracts dissolved in 60% methanol, 60% ethanol and distilled water respectively. Ascorbic acid has IC_{50} of 7.90 µg ml^{-1} for this experiment.

Hydrogen peroxide scavenging assay

Hydrogen peroxide can be produced either in biological systems or through *in vivo* by several oxidizing enzymes such as superoxide dismutase. It is a weak oxidizing agent and can cross membranes which may results into oxidation of a number of compounds. For instance, it deactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase[37]. It is toxic and induces cell death *in vitro*. It is used in the respiratory burst of activated phagocytes[38]. In our study, the ability of plant extracts to scavenge hydrogen peroxide is shown in Fig. 7. Here, plant extracts of different solvents showed hydrogen peroxide decomposition activity in a concentration dependent manner. At extract concentration of 5 mg /mL, it exhibited 71.22%, 82.19% and 90.88% inhibition for plant extracts dissolved in distilled water, 60% ethanol and 60% methanol respectively (Fig.7). Maximum scavenging activity was observed in case of 60% methanolic extracts as indicated by its lowest IC_{50} value, followed by 60% ethanol and aqueous extracts (Table 2). IC_{50} were found to be 1.87 ± 0.39, 2.29 ± 0.56 and 2.74 ± 0.42 mg.mL^{-1} respectively for extracts dissolved in 60% methanol, 60% ethanol and distilled water respectively.

CONCLUSION

The results obtained in the present study indicate that rhizome extract of *Alpinia galanga* used both as non-conventional food plant and also as herbal medicine exhibit significant free radical scavenging and reducing power activity. The rhizome extract prepared in 60% methanol exhibit highest antioxidant activity as compared to the other rhizome extracts which might be attributed to its phytochemical constituents including phenolic and flavonoid compounds. The findings of the present study demonstrate that *Alpinia galanga* rhizome can be a noteworthy resource of natural antioxidant as can be vouched from the efficient free radical scavenging activities with possible applications in pharmacology and medicine for countering the oxidation of cells in the human body caused by free

radicals. Supplementation of this non-conventional rhizomatous food plant in the dietary intakes would significantly help in enhancing cellular free radical defensive processes thereby protecting cells from undergoing subsequent damages and possible neoplastic transformations.

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