Evaluation of anti-oxidant and cytotoxic properties of tropical ginger, *Zingiber montanum* (J. König) A. Dietr.

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Abstract

Many members belonging to the family Zingiberaceae are well known for their uses in traditional medicine for curing various ailments since times immemorial. The rhizomes of some medicinal Zingiberaceae are widely used in the dietary intakes. Curcumin present in turmeric and gingerol in ginger have been known to possess anti-oxidant properties. The northeast India, which lies within the Indo-Burmese mega-biodiversity 'hotspot' region, is a genetic treasure house of biological resources with good representation of Zingiberaceous species. The present studies were conducted to assess the free radical scavenging antioxidant properties of rhizome extract of Zingiber montanum (J. König) A. Dietr [=Z. cassumunar Roxb.] using various chemical assay systems like diphenyl picrylhydrazyl (DPPH), superoxide (O_2^-) and hydroxyl (OH^-) radical scavenging methods. Increased percent of DPPH decoloration from 50-500 µg/ml indicated concentration dependent scavenging activity of DPPH radicals by the crude extract of this species. Even at a low concentration of 1 µg/ml, the rhizome extract showed strong (~75%) OH^- scavenging activity. Similarly, the crude extract showed a concentration dependent inhibition of O_2^- radical production where a concentration of 50 µg/ml almost showed 100% inhibition. Cytotoxicity was assessed by MTT assay using NIH 3T3 fibroblast cell line. Only 28% cytotoxicity was observed up to a concentration of 100 µg/ml. The results strongly support the therapeutic use of crude rhizome extract of Z. montanum for its dietary intake and use as traditional medicine, thereby suggesting its potential as promising radioprotective agent.

Introduction

Manipur, which lies within the Indo-Burmese mega-biodiversity '*hotspot*' region in the northeast India, is a genetic treasure house of rich biological resources. This active '*centre of speciation*' represents a zone of gene diversity

for a variety of wild as well as domesticated plants, and a secondary centre for several economically important medicinal and aromatic plants. These gene pools are invaluable resources and their sustainable utilization can, through biotechnological interventions, bring about economic growth of the region. Bioprospects of these biological wealth scattered in the potentially useful plants as '*bio-active molecules*' need to be fully explored. These are the molecules, which would help in designing and manufacturing various plant-based drug formulations.

A wide variety of phytochemicals including polyphenolics, carotenoids, terpenoids, coumarins, saponins, phytosterols, curcuminoids, etc., have been identified in several plants. The most publicized phytochemicals with antioxidant profiles have been vitamins C, E and beta-carotene. Flavonoids are widely distributed in plants and other plant products, and are powerful inhibitors of lipid peroxidation, reactive oxygen species (ROS) scavengers, inhibitors of damage by haem protein/ peroxide mixtures, metal ion binding agents and inhibitors of lipoxygenase and cyclooxygenase enzymes *in vitro*. The degree of hydroxylation and relative position of –OH groups are of prime importance in determining antioxidant activity. In whole animals, flavonoids have been reported to exert anti-inflammatory and anti-cancer effects (Read, 1995).

Frankel *et al*. (1995) reported that plant phenols in red wine exerted cardioprotective effect. Keli *et al*. (1996) suggested an inverse relationship in the incidence of coronary heart disease and stroke in elderly men with dietary intakes of flavonoids from tea, fruits and vegetables in human populations. Phenolic substances have been found to possess anti-carcinogenic and anti-mutagenic activities, the majority of these naturally occurring phenolics retain antioxidative and anti-inflammatory properties which appear to contribute to their chemopreventive or chemo-protective activity (Surh, 1999). The human body is constantly under attack from free radicals, which are highly reactive chemical entities and are fundamental to any biochemical processes representing aerobic life. They are continuously produced by the body's normal use of oxygen, such as respiration and cell-mediated immune functions, and are generated through a variety of environmental agents. Free radicals can react readily with various biomolecules, such as DNA, proteins and lipids, to cause cellular lesions, which, in turn, lead to various human diseases (Halliwell and Gutteridge, 1999). *In vitro* generated sulfur free radicals have been suitably used in experiments against a reference molecule such as curcumin, beta-carotene or retinol (Devi *et al*., 1992; D'Aquino *et al*., 1994) for rapid evaluation of antioxidant potentials.

The rhizomes of tropical ginger, *Zingiber montanum* (J. König) A. Dietr. (syn. *Z. cassumunar* Roxb.), abundantly found in Manipur are used in diarrhoea, colic, and used as stimulant, carminative, for flavouring food

preparations and substituting for true ginger as antidote for snakebites, and in asthma, ascites, anemia, bruises, bronchitis, dropsy and fever, and for treatment of intestinal disorders, swellings, rheumatism, numb feet and painful parts. Antioxidant molecules already reported from this plant are alflabene, cassumunene, cassumunaquinones I, II, cassumunins A, B, C and cassumunarins A, B, C (Dinter *et al*., 1980; Masuda and Jitoe, 1994; Jitoe *et al*., 1992). Our investigations on sulfur free radical reactivity using curcumin as a reference indicator, and its inhibition by various crude extracts of fourteen medicinal Zingiberaceous species *in vitro* showed that *Z. montanum* [as *Z. cassumunar* in the work] exhibited maximum antioxidant property (Chirangini *et al*., 2004). In this paper, attempts have been made to screen antioxidant potentials using DPPH, hydroxyl and superoxide radical scavenging assays and cytotoxicity using NIH 3T3 mouse fibroblast cell lines.

Materials and Methods

The tropical ginger, *Zingiber montanum*, has rootstocks that are perennial. Rhizomes are bright yellow inside with strong camphoraceous scent. Leaves are oblong-lanceolate and hairy underneath. The spike-like inflorescence is oblong with ovate, reddish bracts. From the bracts, pale yellow colored flowers come out in acropetal succession. The corolla tube is as long as the bract with whitish segments, upper portion being broader and more concave. The most beautiful part of the flower, the lip or the labellum, is yellowish white in coloration with a deeply bifid midlobe. The basal auricles are large, oblong, and obtuse. The male part of the flower, the stamens are yellowish white, but shorter than the lip. The ovary is 3-celled and the ovules are many arranged in the inner angle of the cells. Plants collected from wild natural wetland habitats of Manipur grown in the Experimental Field, Department of Life Sciences, Manipur University, since July 2000, were used in these experiments. Morpho-taxonomic characters were properly recorded. Healthy, uninfected and unbruised fresh rhizomes were used for all the experiments. Herbarium voucher have been collected and deposited at Herbarium of Manipal University, Imphal (MU/LSD/Herb.32): India, Manipur, Imphal, Thoubal & Bishenpur Districts, 22.VI.2000, *Chirangini et al. 32.*

Preparation of the Zingiber montanum *extract*

Fresh rhizomes were washed and cleaned thoroughly in running tap water. The roots were removed along with the outer scales. After drying in between the folds of the filter paper, rhizomes were weighed and crushed with the

help of mortar and pestle. Then, it was homogenized in absolute methanol (1gm/ml). The crude extracts obtained were centrifuged twice and filtered, using Whatman No. 1 filter paper, till a clear supernatant was obtained. The supernatant was vacuum evaporated till dryness. The residue obtained was kept at 4°C for future use.

I. Antioxidative capacity - The antioxidative capacity of Z. montanum extract was examined by comparing it to the activity of known antioxidants, such as ascorbic acid, by the following chemical assays - scavenging of DPPH radical and oxygen radicals such as superoxide, and hydroxyl radicals.

DPPH assay

The DPPH assay was carried out as described by Cuendet et al. (1997) with slight modification. The reaction mixture consisted of 250 µM DPPH in 100% methanol with 50-500 µg/mL of the crude extract of Z. montanum or 0.01-0.1 mM of vitamin C. After a 30-min incubation period in the dark at room temperature, the absorbance was read against a blank at 517 nm. Percentage inhibition was determined by comparison with a methanol treated control group. The percentage of DPPH decoloration was calculated as follows:

$$\% \text{ DPPH decoloration} = [1 - \text{O.D. sample}/ \text{O.D. control}] \times 100$$

The degree of decoloration indicates the free radical scavenging efficiency of the substances. Values are presented as mean ± standard deviation of three determinations.

Hydroxyl radical scavenging activity assay

Hydroxyl radical scavenging activity assay was carried out by measuring the competition between deoxyribose and the extracts for hydroxyl radicals generated from the Fe(II)/ascorbate/EDTA/H_2O_2 system. The attack of the hydroxyl radical to deoxyribose leads to thiobarbituric acid reactive substances (TBARS) formation (Kunchandy and Rao, 1990). Various concentrations of the extracts were added to the reaction mixture containing 2.8 mM deoxyribose, 25 µM $FeCl_3$, 100 µM EDTA, 100 µM ascorbic acid, 2.8 mM H_2O_2, and 5 mM phosphate buffer (pH 7.4), making up a final volume of 1.0 mL. The reaction mixture was incubated at 37 °C for 1 h. The formed TBARS were measured by the method of Ohkawa et al. (1979). One milliliter of thiobarbituric acid (TBA, 1% w/v in 50 mM NaOH) and 1 mL of trichloroacetic acid (TCA, 2.8% w/v) were added to test tubes and incubated at 100°C for 30 min. After the mixtures cooled, absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Reactions were carried out in triplicate.

Inhibition (*I*) of deoxyribose degradation in percent was calculated in the following way:

$$I = (A_0 - A_1)/A_0 \times 100$$

where A_0 is the absorbance of the control reaction (containing all reagents except the test compound) and A_1 is the absorbance of the test compound.

Inhibition of superoxide radical

Superoxide radical generated by the hypoxanthine/xanthine oxidase system was determined spectrophotometrically by monitoring the product of nitroblue tetrazolium (NBT). Various concentrations of the extracts were added to the reaction mixture containing 100ml of 30 mM EDTA (pH 7.4), 10ml of 30 mM hypoxanthine in 50 mM NaOH, 200ml of 1.42 mM NBT and the final volume of 3 ml was made up by 50 mM PO4 Buffer (pH 7.4). After adding 100ml of 0.5 U/ml xanthine oxidase, the reaction mixture was incubated for 30 min at 25°C. The absorbance was read at 560 nm and compared with control samples in which the enzyme, xanthine oxidase, was not included.

The percent inhibition of superoxide radicals was calculated from the optical density of the treated and control samples.

$$\text{Inhibitory effect (\%)} = [(A_{560\ \text{control}} - A_{560\ \text{sample}})/A_{560\ \text{control}}] \times 100$$

II. Cytotoxicity studies - *In vitro* cytotoxic effect of crude extracts of *Z. montanum* was studied on normal mouse embryo fibroblast cell (NIH/3T3). The methanol extract was dissolved in dimethylsulphoxide (DMSO). The cell line NIH/3T3 was provided by National Centre for Cell Science, Pune, India.

Cell culture conditions

Stock cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum supplemented with 0.04M $NaHCO_3$, 0.006% penicillin and 0.025% streptomycin at 37°C in an atmosphere of 5% CO_2 and 95% humidity. The medium was changed every three days. Monolayer cells were plated out at $2x10^4$ cells/well in 96-well microtitre plate. The cell growth was found to be exponential during 2-3 days in the medium.

MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] easily

enters cells. The esterases present in viable cells can cleave MTT to form purple-colored formazan crystals which are then solubilized. Color produced is directly proportional to cell viability.

The cytotoxic effect of the crude extracts, expressed as cell viability, was assessed by MTT staining experiment (Mosmann, 1983). Briefly, confluent cultures of NIH/3T3 cells were treated with medium containing the methanol extract of Z. montanum at concentrations from 2-400 µg/mL. The extract was first dissolved in absolute DMSO and then in DMEM. The final concentration of DMSO in the test medium and controls was <1%. Cells were exposed for 48 hr to test medium with or without the extracts. The medium was removed and 100 µl of MTT solution (1 mg/ml in PBS) was added to each well of 96 multiwell plates and the plates were incubated for additional 3 hr at 37°C. Finally, 100 µl of 10 % (w/v) sodium dodecyl sulfate in 0.01N HCl was added to each well and the absorbance was measured at 550 nm using the ELISA reader (Biotek System). Each concentration of plant extract was tested in hexaplicate and repeated twice in separate experiments.

Percentage viability was calculated from the following relation:

$$\% \text{ Viability} = [1 - OD_{sample}/OD_{control}] \times 100$$

Results and Discussion

DPPH Assay: As shown in Fig. 1, Vitamin C and methanol extract of *Z. montanum* were able to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine. The strongest effect was observed in 0.1mM Vitamin C with 91% DPPH decoloration. Up to 86% DPPH decoloration was observed in case of 500 µg/mL *Z. montanum* extract that is having similar effect with 0.08mM Vitamin C (84%).

Hydroxyl radical ($OH^{\cdot}$) scavenging: When the methanol extract of Z. montanum was incubated with the reaction mixture used in the deoxyribose degradation assay, they removed hydroxyl radicals from the sugar and prevented its degradation. As shown in Fig. 2, Z. montanum extract, even at a concentration of 1 µg/mL there is 59% scavenging of the $OH^{\cdot}$ radical showing very potent activity. The following results show that there is no dose dependent reponse for $OH^{\cdot}$ radical scavenging capacity.

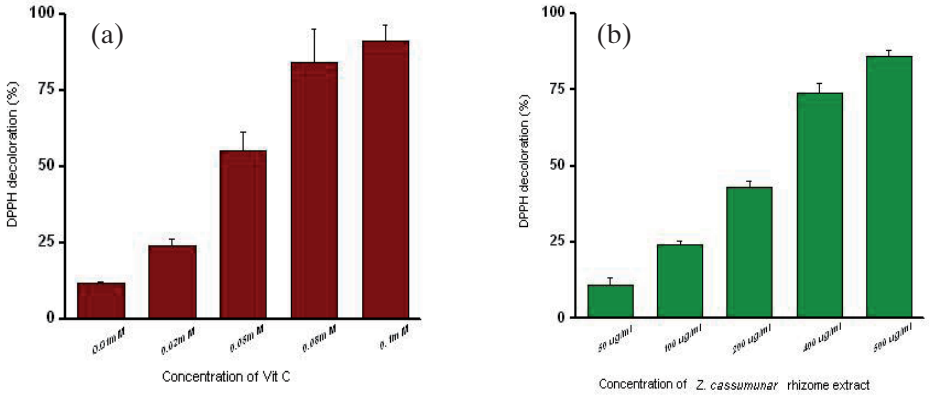

Figure 1. DPPH radical scavenging activity of (a) Vitamin C and (b) *Z. montanum* rhizome.

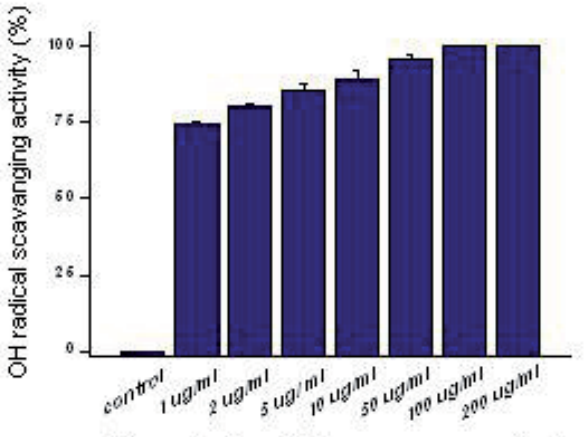

Figure 2. Hydroxyl radical (OH^-) scavenging activity of *Z. montanum* rhizome extract.

Inhibition of superoxide radical: Methanol extract of *Z. montanum* were found to scavenge the superoxide radicals generated from the hypoxanthine/ xanthine oxidase method. There is a dose dependent response of the compound as well as the extract (fig. 3). The results do point towards an increased trend in the response with small increases in the concentration of the extract.

Cytotoxicity testing by MTT assay: Methanol extracts of *Z. montanum* tested for cytotoxicity against normal mouse fibroblast cell line using standard MTT assay showed very low toxicity up to 100ug/ml (fig.4). Only 24% of the cells survive the toxic effect of *Z. montanum* extract at a concentration of 200 µg/mL.

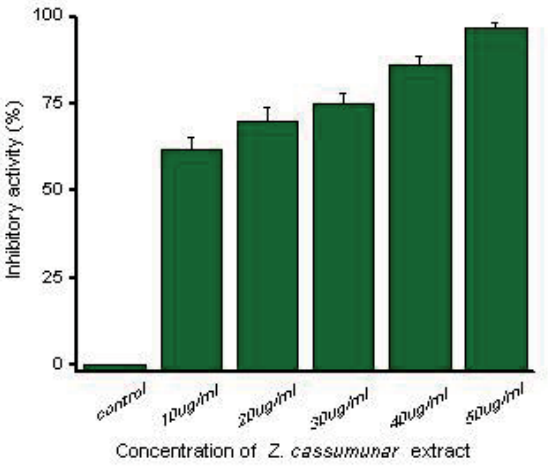

Figure 3. Inhibition of superoxide radical production by *Z. montanum* rhizome.

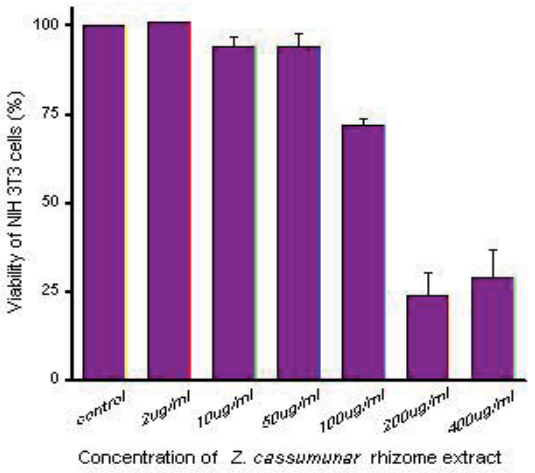

Figure 4. Cytotoxicity of methanolic extracts of *Z. montanum* rhizome.

Sulfur free radicals ($GS^{\cdot}$) formed in gamma irradiated aqueous glutathione (GSH) solution could easily oxidize the chrome orange-yellow compound curcumin - its depletion increasing with increasing dose of radiation. Supplementation of the crude rhizome extract reduced the depletion of curcumin significantly. The inhibition of curcumin depletion in the rhizome extract-added reaction solution varied with the species showing different protective indices (PIs). A relative comparison of PIs at 75 Gy exposure showed *Zingiber cassumunar* [currently accepted name *Z. montanum*] > *Kaempferia galanga* > *Hedychium flavascens* > *Zingiber officinale* >

Hedychium coccineum > *Curcuma caesia* > *Curcuma amada* > *Alpinia allughas* > *Curcuma leucorhiza* > *Hedychium coronarium* > *Alpinia galanga* (Chirangini *et al*., 2004).

Free radicals generated either by endogenous metabolism or external environmental agents are harmful to cellular constituents, such as proteins, lipids, DNA and carbohydrates, and result in possible alteration of cell function (Davies *et al*., 1987; Dezwart *et al*., 1999; Gebicki & Gebicki, 1999). ROS are known to be carcinogens and act at several stages in malignant transformation (Cerutti, 1994), including permanent DNA sequence changes in the form of point mutations, deletions, gene amplifications, and rearrangements which may result in the activation of proto-oncogenes or the inactivation of tumor-suppressor genes (Hsie *et al*., 1986; Moraes *et al*., 1990). The role of these ROS in oxidative damage to the membranes and mechanism of apoptotic death of thymocytes have been well elaborated (Bhosle *et al*., 2002; Mishra & Hota, 2003; Pandey & Mishra, 2003).

The body's antioxidant defense system is composed of various antioxidants present in the plasma or biological fluids in a reduced form. While scavenging/neutralizing the free radicals, they are either oxidized or exhausted. An external anti-oxidant can prevent oxidative damage by inhibiting the generation of reactive species, scavenging free radicals, or raising the endogenous level of antioxidant defense. To maintain antioxidant level in the body, external supplementation is necessary for healthy living (Halliwell & Gutteridge, 1989). Supplementation of natural antioxidants through a balanced diet could be more effective, and also more economical than supplementation of an individual antioxidant, such as Vitamin C or E, in protecting the body against oxidative damage under various conditions (Wang *et al*., 1996). It has been known that several medicinal plants contain '*active principles*' possessing antioxidant properties. In Manipur, a number non-conventional and under-used plant-based food, particularly belonging to the family Zingiberaceae, possessing rich antioxidant properties are consumed by the people which perhaps may be the basis for low incidence of cancers (Chirangini *et al*., 2004).

Although some work has been done on the radioprotective effect of curcumin extracted from *Curcuma longa* (Inano & Onado, 2002) and ginger rhizome (Jagetia *et al*., 2003), detailed studies have not been carried out as yet on the potential antioxidant properties of *Z. montanum*. The present studies made using the DPPH, hydroxyl and superoxide radical scavenging assays, therefore, have reaffirmed the antioxidant potentials of *Z. montanum* in a much more elaborate manner, and are hence quite relevant. Although the crude extracts of these various plants have numerous medicinal potentials, clinical applications can be made only after extensive research on the bioactivity, mechanism of action, pharmaco-therapeutics

and toxicity studies of the different compounds present in these plants. Recent years have seen an increased enthusiasm in treating various diseases with natural products. Many phytonutrients or phytochemicals having very high antioxidant profile need to be investigated for their application as anti-tumour or radioprotective agents to inhibit acute and chronic effects and even mortality after irradiation. It is expected that some novel compounds may turn out to have very rich radioprotective property which could be comparatively better than that of amifostine, the only agent that reduces radiation induced toxicity during clinical trials.

It can be concluded that Z. montanum rhizome extract possesses significant radical scavenging and anti-oxidant properties. Besides being an efficient scavenger, cytotoxicity of Z. montanum rhizome extract above 100 µg/ml indicate its significant potential as an anti-tumor agent. The results shown above do strongly support the therapeutic applicability of Z. montanum extract for its dietary intake and use in traditional system of medicine. Based upon these significant anti-oxidant properties, there is an urgent need for investigation of the rhizome extract of Z. montanum for its radioprotective activity using suitable in vivo mammalian test systems.

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