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Research Article

ANTIOXIDANT ACTIVITY OF ENZYMATIC EXTRACTS OF *CURCUMA ZEDOARIA* (CHRISTM.)

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ABSTRACT

Curcuma zedoaria (Christm.) belongs to family Zingiberaceae shows active antioxidant enzymes like catalase, guaiacol peroxidase, glutathione peroxidase and superoxide dismutase which have various biological activities. The enzymatic leaf extracts of this plant have been analyzed for their free radical-scavenging activity in different *in vitro* systems, e.g. DPPH free radical scavenging activity, hydroxyl free radical-scavenging activity and total antioxidant activity. The free radical scavenging activities were compared with standard antioxidant like ascorbic acid. The DPPH radical scavenging activity was found to be 39.7± 0.2 with 200 µg/ml of the enzymatic extract. Total antioxidant activity was measured and it was found to be 121± 0.7(mg AE /g) and 91.3 ± 0.5 (mg TE/g) with 100 µl of the enzymatic extract of leaf samples. The maximum antioxidant activities were found in catalase, glutathione peroxidase and superoxide dismutase enzyme where as the guaiacol peroxidase shows poor antioxidant activity as compared to ascorbic acid. The hydroxyl radical scavenging activity was found to be 48.23±0.05 with 50 µl of the enzymatic extract. Therefore, it is suggested that *C. zedoaria* could be a potential source of natural antioxidant that could have great importance in therapeutic agent in preventing or slowing down the progressive ageing and age associated oxidative stress related degenerative diseases.

Keywords: *Curcuma zedoaria,* DPPH, Hydroxyl radical scavenging assay, Total antioxidant assay, Catalase, Peroxidase, Superoxide dismutase.

INTRODUCTION

Curcuma zedoaria (Christm.) of Family- Zingiberaceae is commonly known as 'yellow zedoary' which is used in Indian system of medicine since time immemorial. The plant is found in eastern Himalayas, Odisha, Chittangang, Bengal and Kerala also often cultivated throughout India. The plant is aromatic, pungent, bitter in taste, useful in flatulent colic, indigestion, used as an ingredient in bitter tincture of zedoary as well as anti-periodic pills. It is also used in some traditional eastern medicines, acts as an agent for blood purifier and used as antivenom against the bite of Indian cobra. This has been used to treat coronary heart disease, liver cancer, anemia, chronic pelvic inflammation and helps prevent leukopenia due to cancer therapies. In Ayurvedic medicine, plant pacifies vitiated pitta, kapha, indigestion, flatulence, dyspepsia, skin diseases, cough, bronchitis, urinary retention, allergy, leucoderma and as general tonic. A paste of rhizome is useful externally for cuts, wounds, itching and in sprains. The rhizomes are source of Shoti Starch, used as a food for babies and convalescents, recovering from chronic stomatitis. It is cooling and demulcent. *C. zedoaria* (Christm.) has been reported to have analgesic effect[1], anti-allergic[2], anti-inflammatory[3], anti-metastasic[4], antioxidant[5] and hepatoprotective properties[6]. The free radical mediated damage may play in many disorders like Coronary heart diseases, diabetes and cancer etc.

Free radicals are atoms or molecules that have one or more unpaired electrons in their atomic structures and are therefore highly reactive. Oxygen is the most ubiquitous of all biologically important chemicals species and is a major source of reactive oxygen species (ROS). The increase conditions in the formation of reactive oxygen species (ROS) such as H_2O_2 (hydrogen peroxide), O_2 – (superoxide) and OH (hydroxyl) radicals, through enhanced leakage of electrons to molecular oxygen[7]. ROS acts as a second messenger which is involved in the stress signal transduction pathway[8], but excessive ROS production can cause oxidative stress, which damages the plants by oxidizing photosynthetic pigments, membrane lipids, proteins and nucleic acids[9]. The generated ROS are detoxified by the antioxidants present in the body. Antioxidants have been reported to prevent oxidative damage caused by free radical and can be used in cardiovascular and anti-inflammatory diseases[10]. To keep the levels of active oxygen species under control, plants have non-enzymatic and enzymatic antioxidant systems to protect cells from oxidative damage[11]. Oxidative stress results from an imbalance between the oxidant productions. Oxidative stress is associated with many of the risk factors implicated in the path physiology of atherosclerosis, including diabetes, hypercholerimia, renal failure, aging, hypertension and smoking. As the demand of natural food additives is increasing day by day, so the herbal medicinal plant species can be a better option for the replacement of synthetic antioxidant agents. The antioxidant system, the first line of defense against free radicals includes a number of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), gluaicol peroxidase (GP), and non-enzymatic antioxidants such as glutathione (GSH), protein-SH, ascorbic acid and dietary antioxidants etc. So in this study, we evaluated the level of antioxidant enzymes activity and experimented the effect of enzyme extracts of *Curcuma zedoaria* (Christm.) instead of crude extracts on different antioxidant assays.

MATERIALS AND METHODS

Plant Material collection

The leaves of *C. zedoaria* (Christm.) of family Zingiberaceae were collected from the wild areas of Koraput and maintained in the medicinal garden. The fresh leaves were collected in cold and dark condition and washed thoroughly under running tap water to remove the dust particles.

Extraction of enzyme

The antioxidant enzyme was extracted from the frozen leaves of *C. zedoaria* following the method of Abedi and Pakniyat[12] with little modification. The sample was made to a fine powder with liquid nitrogen and addition of chilled extraction buffer [1mM PMSF, 0.05M Tris, 6mM cystine chloride, 0.5M sucrose and 6 mM ascorbic acid] and (pH-8) was maintained. Then the homogenate was centrifuged at 10,000 rpm for 15 minutes at 4°C and the supernatant was used for enzyme activity determinations.

Enzymatic antioxidants in *Curcuma zedoaria* (Christm.)

Assay of catalase

Catalase activity was determined by measuring the inhibition rate of hydrogen peroxide (H_2O_2) at 240 nm according to the method described by Sinha[13].

Assay of Guaiacol peroxidase

The peroxidase activity was determined by measuring the increase of absorbance at 420 nm as a result of oxidation of guaiacol according to the method described by Addy and Goodman[14].

Assay of Superoxide _dismutase

Superoxide dismutase activity was determined by measuring the inhibition rate of nitroblue tetrazolium (NBT) at 560 nm Das *et al*[15].

Assay of Glutathione Peroxidase

Glutathione peroxidase activity was assayed using the method described by Rotruck *et al*[16]. Oxidation of NADPH was recorded spectrophotometrically at 340nm and the enzyme activity was calculated as n moles NADPH oxidized/min/mg of enzyme, using extinction coefficient of 6.22 × 103M−1 cm−1.

ANTIOXIDANT ABILITY ASSAYS

DPPH radical scavenging activity

DPPH solution (0.1mM) was prepared in methanol by dissolving 1.9mg of DPPH in methanol and the remaining volume was made 100ml with methanol. The solution was kept in darkness for 30 minutes to complete the reaction. The free radical scavenging activity of the enzymatic plant extracts was determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH).This antioxidant activity was measured by following the method described by Ilhami *et al*.[17] Briefly, 1ml of 0.1mM methanolic DPPH solution was added to 3ml of enzymatic extracts, at different concentration (60,100,120,180,200μg/ml).The mixture was vigorously shaken and left to stand for 30 minutes under subdued light. The absorbance was measured at 517 nm in a UV spectrophotometer. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid, which is a good antioxidant, was taken as a standard in this study. The DPPH radical scavenging activity was calculated by using the following equation:-

$$\text{DPPH Scavenging activity (\%)} = (1\text{-As/Ac}) \times 100$$

Where, As is the absorbance of the sample and Ac is the absorbance of the control, respectively. All extracts were analyzed in triplicates.

Hydroxyl radical scavenging activity

Deoxy ribose assay was used to detect hydroxyl radical scavenging activity and iron binding ability of them sample. Hydroxyl radicals are generated in a reaction mixture containing ascorbate, H_2O_2 and iron–III-EDTA at pH 7.4 and measured by their ability to degrade the sugar deoxyribose. The extent of inhibition is dependent on the concentration of the scavenger and its rate constant for reaction with hydroxyl radicals. A mixture of 0.5ml of 3mM deoxyribose solution, 0.1mM ferric chloride solution, 0.1mM solution of EDTA, 0.1mM solution of ascorbic acid and 2 mM solution of phosphate buffer was taken in a series of test tube and final volume was made up to 3ml. The reaction mixture was incubated at 37ºC for 1 hr. To this 0.5ml of Trichloro Acetic Acid (TCA) and Thio Barbituric Acid (TBA) was added and incubated for 20 minutes at 37º. Then, 10-50 μg/ml of various concentration of the test samples were added to the above mixture and absorbance was measured at 532nm. 0.1mM solution of ascorbic acid was prepared and used as reference standard. The hydroxyl radical scavenging assay was determined by the method of Kunchandy and Rao[18] with slight modification. All extracts were analyzed in triplicates.

Inhibition of degradation of deoxyribose was calculated by using the formula:-

$$I\,\% = A_c - A_s / A_c * 100$$

Where, As is the absorbance of the sample and Ac is the absorbance of the control.

Determination of total antioxidant capacity by phosphomolybdenum assay

The total antioxidant capacity of the plant extracts was measured by the method described by Prieto *et* al[19]. 0.3 ml of the sample solution was mixed with 3ml of the reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) against a blank containing 100 μl of methanol mixed with 900 μl of reagent solution. The absorbance of the test sample was measured at 695 nm. The antioxidant activity was expressed as vitamin C equivalent (mg AE/g extract) and trolox (mg TE/g extract). Extracts were analyzed in triplicates.

Statistical Analysis

For all the experiments, three samples were analyzed and all the assays were carried out in triplicate. The results are expressed as mean ±standard deviation.

RESULT AND DISCUSSION

The body posses defense mechanisms against free radical-induced oxidative stress, which involve preventative mechanisms, repair mechanisms, physical defenses and antioxidant defenses. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) etc., while non-enzymatic antioxidants are ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids and etc. All these act by one or more of the mechanisms like reducing activity, free radical-scavenging, potential complexion of pro-oxidant metals and quenching of singlet oxygen. Reactive oxygen species (ROS) get special attention due to many factors such as drought, cold, heat, herbicides and heavy metals, because they harm the cell by raising the oxidative level through loss of cellular structure and function[20] . In the present study, the DPPH radical scavenging activity, OH radical scavenging activity and total antioxidant capacity of the enzymatic extracts *C. zedoaria* (Christm.) was investigated.

DPPH free radical scavenging activity

Table-1 shows the result of the DPPH free radical scavenging activity of the enzymatic extracts in *C. zedoaria* (Christm.). The highest DPPH radical scavenging activity recorded was 39.7± 0.2 at the concentration of 200μg/ml. Reports of antioxidant activities of rhizome crude extracts in *C. zedoaria*[21, 22, 23]. In our experiment there is a non-enzymatic antioxidant i.e. ascorbic acid which was taken as standard. No report was found regarding enzyme levels in relation to antioxidant activity of curcuma. Few research studies have been undertaken on the antioxidant activity of enzymes in medicinal plants using DPPH scavenging assay[24, 25].

Table 1: DPPH free radical scavenging activity of ascorbic acid and *Curcuma zedoaria* (Christm.) enzymatic leaf extracts.

Concentration of the sample in (μg/ml)	Antioxidant activity of Ascorbic acid % (Mean ± SE)	Antioxidant activity % (Mean ± SE)
60	62 .6±0.6	31.2± 0.3
100	68.3 ±0.8	36.1± 0.8
120	72 ±0.2	36.6± 0.1
180	79.2 ±0.1	39.5± 0.6
200	80.3 ±0.1	39.7± 0.2

Values are represented in Mean ± SD of three replicates.

Hydroxyl free radical scavenging activity

Hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism among the ROS[26], which could be formed during superoxide anion and hydrogen peroxide, in metal ions, such as copper or iron, and cause the ageing of human body and some diseases[27].The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells[28].In the present study, the enzymatic extracts the leaf samples of *C. zedoaria* (Christm.) were evaluated for their hydroxyl radical scavenging activity(Table-2). The hydroxyl radical scavenging activity was found to be 48.23±0.05 of the enzymatic extracts as compared to ascorbic acid (standard), which was found to be 60.33 ± 0.40 at the concentration of 50 μl. Similar studies have been undertaken in other medicinal plants like *Adhatoda vasica, Sesbania grandiflora*[24], *Curcuma aromatia*[20] etc.

Table 2: Hydroxyl radicals scavenging activity of *Curcuma zedoaria* (Christm.) enzymatic leaf extracts.

Concentration of the sample (µg/ml)	Standard (Ascorbic acid) (Mean ±SD)	Hydroxyl radicals scavenging activity (Mean ±SD)
10	21.67 ± 0.81	19.42±0.04
20	29.50 ± 0.83	21.48±0.04
30	37.58 ± 0.20	28.45±0.04
40	42.50 ± 0.83	36.52±0.04
50	60.33 ± 0.40	48.23±0.05

Values are represented in Mean ±SD of three replicates.

Total antioxidant capacity

Fig.1 shows the total antioxidant capacity, which was reported as ascorbic acid and trolox equivalents. It was found to be 121± 0.7(mg AE /g) and 91.3 ± 0.5 **(**mg TE/g) with 0.3 ml of the enzymatic extract of leaf samples of *C. zedoaria* (Christm.) by phosphomolybdate method. The method is based on the reduction of Mo (VI) – Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. There is a little information about total antioxidant activity of *Curcuma* species except *Curcuma alismatifolia* extract showed strong total antioxidant capacity[29].

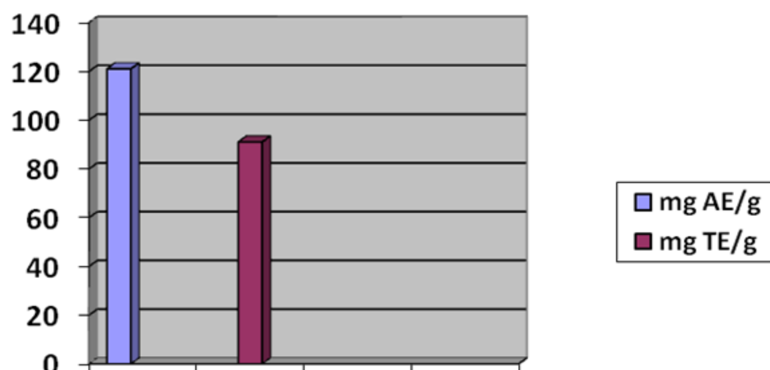

Fig. 1: Total antioxidant capacity by phosphomolybdenum assay of *Curcuma zedoaria* (Christm.)

AE- Ascorbic acid equivalents; TE- Trolox equivalent

Levels of antioxidant enzymes

Fig. 2, shows the level of the active antioxidant enzymes of the leaf samples of the plant *C. zedoaria* (Christm.) and was found to be 58.4± 1.5 U/mg catalase, 82.6± 2.6 U/mg superoxide dismutase, 7.21± 1.8 U/mg guaiacol peroxidase and 39.8± 0.3 U/mg glutathione peroxidase enzymes respectively. In comparison to catalase, superoxide dismutase and glutathione peroxidase, the concentration of guaiacol peroxidase enzyme was low. Enzymatic activities of the enzyme extracts of rhizome in *C. zedoaria* has also been reported[30], which was found to be 0.63 U/mg of protein, 16.60 of protein, and 19.59 U/mg of peroxidase, superoxide-dismutase, and catalase respectively. Similarly, the enzymatic activities of rhizome in *C. longa* has also been reported[31], which was found to be 61.25±2.54 U/mg of catalase,110.90± 1.55 U/mg of superoxide-dismutase, 42.94±4.17 U/mg of glutathione peroxidase and 8.21± 0.09 U/mg guaiacol peroxidase respectively.

Our result on superoxide dismutase activity agrees with the SOD activities in roots and rhizomes of *Smilax zeylanica*[32]. The observation recorded higher amount of enzyme activity in leaves in comparison to rhizome in *C. zedoaria* (Christm.) as recorded by Loc et al,2008 [30].

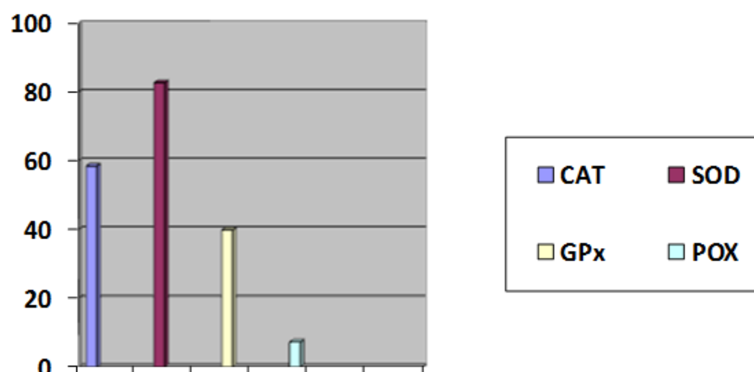

Fig. 2: Levels of antioxidant enzymes in *Curcuma zedoaria* (Christm.).

CAT- Catalase; SOD-Superoxide dismutase; GPx-Glutathione peroxidase; POX-Guaiacol peroxidase.

In the study, the activity of peroxidase, SOD,catalase, glutathione peroxidase indicates the medicinal plant *C. zedoaria* (Christm.) as a highly potential antioxidant. The moderate activity of DPPH and high hydroxyl radical scavenging activity and total antioxidant capacity suggests a good correlation between SOD and OH radical and catalase with DPPH and a non-commitment of guaiacol peroxidase and glutathione peroxidase. However, the data obtained on enzymatic antioxidant activity in comparison to enzyme levels in medicinal plants needs an extensive investigation.

CONCLUSION

The results concluded that the enzymatic antioxidant activity studied in DPPH, OH and total antioxidant capacity assay of different enzymes of *C. zedoaria* (Christm.) are equally potential in

degradation of free radicals in comparison with ascorbic acid, a non-enzymatic antioxidant. Therefore, it is suggested that *C. zedoaria* (Christm.) could be a potential source of natural antioxidant. Further research is recommended for exploitation of enzyme contents of medicinal plants using widely used tests like DPPH scavenging assay, OH radical assay and total antioxidant assay instead of crude extracts.

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