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Research article

COMPARATIVE ANTIOXIDANT ACTIVITY OF NON-ENZYMATIC AND ENZYMATIC EXTRACTS OF *CURCUMA ZEDOARIA, CURCUMA ANGUSTIFOLIA* AND *CURCUMA CAESIA.*

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ABSTRACT: Objective – To compare the efficacy for antioxidant activity of both the crude (non-enzymatic) and enzymatic extracts of three important medicinal plants *Curcuma zedoaria, Curcuma caesia* and *Curcuma angustifolia* respectively. **Methods -** Both the enzymatic and crude extracts of the rhizome and leaves of these plants have been analyzed for their free radical-scavenging activity in different *in vitro* systems, e.g. DPPH radical scavenging activity, hydroxyl radical-scavenging activity and different antioxidant enzymatic assay. **Results –** *C. zedoaria* exhibited the highest DPPH scavenging activity with 62.31±0.5 followed by *C. caesia* which was found to be 55.32±0.2 and *C. angustifolia* with 54.21±0.6 at 200µg/ml of the crude extracts respectively. In case of enzymatic extracts, *C. angustifolia* shows the highest DPPH scavenging activity with 40.6± 0.5 at 220µg/ml, followed by *C. zedoaria* with 39.6± 0.2 and *C. caesia* with 31.2± 0.8 at 200µg/ml respectively. The hydroxyl radical scavenging activity of *C. zedoaria* was found to be 46.03± 0.21, *C. angustifolia* with 47.24± 0.20 and *C. caesia* with 40.26± 0.01 of the crude extracts respectively as compared to ascorbic acid (standard), which was found to be 52.33 ± 0.40 at the concentration of 50 µg/ml. The maximum antioxidant activity were found in catalase, superoxide dismutase and glutathione peroxidase enzyme respectively where as the guaiacol peroxidase shows poor antioxidant activity in all the above plants. **Conclusions –** These findings indicate that the non-enzymatic extracts prove to be a better scavenger of free radical in comparison to enzymatic extracts in all the three *Curcuma* species. Further, the levels of enzymatic activity (catalase, peroxidase and superoxide-dismutase) have a good correlation with the antioxidant activity of these three important medicinal plant species. The potentiality of three curcuma species are credited with higher non-enzymatic extract (crude) as well as antioxidant enzymes which needs further research on medicinal , cosmetic and other pharmaceutical applications.
Key words: - *Curcuma zedoaria, Curcuma caesia*, *Curcuma angustifolia,* DPPH antioxidant activity, hydroxyl scavenging activity, antioxidant enzymes.

INTRODUCTION

The genus *Curcuma,* a member of the Zingiberaceae family, comprises of 80 species, some of which have been used in traditional systems of medicine (Ayurveda, Siddha, Unani) for a long time. Among them, *Curcuma zedoaria,* commonly known as 'yellow zedoary', is found in eastern Himalayas, Odisha, Chittangang, Bengal and Kerala also often cultivated throughout India. It possesses anti-inflammatory and antimicrobial properties and is used in traditional medicine. It has been reported to have analgesic effect [1], anti-allergic [2], anti-inflammatory [3], antimetastasic [4], antioxidant [5] and hepatoprotective properties [6].

The rhizome is used in dysentery, diarrhea and cough and also as anti venom for the cobra bite.[7] *Curcuma caesia* is commonly known as black turmeric which is a perennial herb found throughout the Himalayan region, North-east and central India. The paste of rhizome is used traditionally for the treatment of leucoderma, asthma, tumor, piles etc. Essential oil of *C. caesia* has been known for its antifungal activity[8].

Curcuma angustifolia is commonly known as East Indian arrowroot. It is nutritive and used as agreeable, non-irritating diet in certain chronic diseases, convalescence in fever, in irritation of alimentary canal, pulmonary organs and also used in consumption, excessive thirst, jaundice and kidney disorder [9]. Rhizomes are used in inflammation, bone fracture, intestinal diseases by some tribal of Madhya Pradesh and Chhattisgarh states of India [10]. These non- conventional species of *Curcuma* produce starchy rhizomes which are used as remedies for infections, inflammations, gastric and skin disorders but have not been evaluated scientifically for pharmacological activity. The rhizomes of these species are aromatic. Medicinal uses of rhizome arise from the bioactive components. Bioactive components are responsible for antioxidative and anti-inflammatory properties, wound-healing, hypoglycemia, anticoagulant, antimicrobial activities[11,12]. All most all species of *Curcuma* contains antioxidant activity, the pharmacological effects and prospectus for future clinical use had been tried so far [13-14].

Curcuma plants (rhizomes and leaves) have a camphoraceous aroma and contain many functional compounds such as phenolics, flavonoids and different antioxidant enzymes. Since free radicals are the cause for several major disorders, evaluation of antioxidant compounds activity in plants could result in the discovery of natural antioxidants with pharmacological and food value. The importance of phenolic compounds in plants as natural antioxidants and their use as substitutes to synthetic antioxidants in food additives is well known [15-16]. So in this study, we evaluated the level of antioxidant enzymes and experimented the effect of both the enzyme and crude extracts of the three important non-conventional species of *Curcuma* on different antioxidant assays, in order to explore their pharmacological potential.

MATERIALS AND METHODS

Plant material collection

The rhizomes of the three species were collected from the local tribes of Koraput, Odisha. The fresh rhizomes were washed thoroughly under running tap water to remove the dust particles and make them free from contaminants.

Extraction of crude extract

The rhizomes were cut into small pieces and shade dried for 4-5days. The dried rhizomes were coarsely powdered.Then the ground rhizome materials (50g) were extracted successively with methanol (500ml) using a Soxhlet apparatus for 8-12 h at the temperature of 55-60^{o}C and the solvent was evaporated to dryness under reduced pressure at 90^{0}C by Rotary vacuum evaporator. The residues were weighed and stored at 4 ºC until use.

Extraction of enzyme

The enzyme was extracted from the frozen rhizome samples of *Curcuma* [17]. 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 5,000 rpm for 10 minutes at 4°C and the supernatant was used for enzyme activity determinations.

Enzymatic antioxidants

Assay of catalase

Catalase activity was determined by measuring the inhibition rate of hydrogen peroxide (H_2O_2) at 240 nm [18].

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 [19].

Assay of Superoxide dismutase

Superoxide dismutase activity was determined by measuring the inhibition rate of nitrobluetetrazolium (NBT) at 560 nm [20].

Assay of Glutathione Peroxidase

Glutathione peroxidase activity was determined by measuring the oxidation of NADPH at 340nm [21].

Antioxidant ability assays

DPPH radical scavenging activity

The free radical scavenging activity of both the crude and enzymatic plant extracts was determined by 1, 1- diphenyl-2-picrylhydrazyl (DPPH) [22]. Briefly, 1ml of crude extracts was added to 3ml of 0.1mM methanolic DPPH solution at different concentration (60, 100, 120,180, 200µg/ml). In case of enzymatic extracts, 3ml of extracts was added to1ml of 0.1mM methanolic DPPH solution at different concentration (100, 125,150,180, 200, 220µg/ml). Then, both 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-

DPPH Scavenging activity (%) = (1-As/Ac) ×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, H2O2 and iron–III-EDTA at pH 7.4 and measured by their ability to degrade the sugar deoxyribose [23]. 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 (crude extracts) were added to the above mixture and the absorbance was measured at 532nm. 0.1mM solution of ascorbic acid was prepared and used as reference standard. All extracts were analyzed in triplicates.

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

I% = Ac- As/Ac*100.

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

RESULTS

Both the enzymatic and crude extracts of the rhizome and leave samples of the three important plants of *Curcuma* species (*C. zedoaria, C.caesia* and *C.anguistifolia)* plants have been analyzed for their free radical-scavenging activity in different *in vitro* systems, e.g. DPPH radical scavenging activity, hydroxyl radical-scavenging activity and different antioxidant enzymatic assay.

DPPH free radical scavenging activity

Table-1 shows the result of the DPPH free radical scavenging activity of the crude extracts of the rhizome samples in the three important species of *Curcuma.* Among them, *C. zedoaria* shows the highest DPPH scavenging activity with 62.31±0.5 followed by *C. caesia* which was found to be 55.32±0.2 and *C. angustifolia* with 54.21±0.6 at 200µg/ml of the crude extracts respectively. In case of enzymatic extracts (Table-2), *C. angustifolia* shows the highest DPPH scavenging activity with 40.6± 0.5 at 220µg/ml, followed by *C. zedoaria* with 39.6± 0.2 and *C. caesia* with 31.2± 0.8 at 200µg/ml respectively. In our experiment there is a non-enzymatic antioxidant i.e. ascorbic acid which was taken as standard [24]. 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.

Hydroxyl free radical scavenging activity

In the present study, the enzymatic extracts the rhizome samples of these plants were evaluated for their hydroxyl radical scavenging activity (Table-3). The hydroxyl radical scavenging activity of *C. zedoaria* was found to be 46.03± 0.21, *C. angustifolia* with 47.24± 0.20 and *C. caesia* with 40.26± 0.01 of the crude extracts respectively as compared to ascorbic acid (standard), which was found to be 52.33 ± 0.40 at the concentration of 50 µg/ml. All the assays were carried out in triplicate. The results are expressed as mean ±standard deviation.

Table-1: DPPH free radical scavenging activity of ascorbic acid and three *Curcuma* species (crude extracts).

Conc. of extracts (µg/ml)	Antioxidant activity (%)			
	Ascorbic acid	*C. zedoaria*	*C. caesia*	*C. angustifolia*
60	63 .61±0.6	50.11±0.2	38.24±0.4	42.20±0.3
100	69.36 ±0.8	52.42±0.4	45.64±0.8	49.13±0.1
120	72.32 ±0.2	57.06±0.4	49.44±0.3	50.17±0.1
180	78.21 ±0.1	61.03±0.6	54.50±0.3	52.35±0.4
200	79.30 ±0.1	62.31±0.5	55.32±0.2	54.21±0.6

Table-2: DPPH free radical scavenging activity of ascorbic acid and three *Curcuma* species (enzymatic extracts).

Conc. of extracts (µg/ml)	Antioxidant activity (%)			
	Ascorbic acid	*C. zedoaria*	*C. caesia*	*C. angustifolia*
100	69.36 ±0.8	30.1± 0.8	21.2± 0.7	
125	73.1 ±0.2	35.6± 0.1	24.1± 0.4	30.2± 0.6
150	75.3±0.7	37.2± 0.3	27.6± 0.4	32.8± 0.4
180	79.2 ±0.1	38.1± 0.6	30.5± 0.2	35.2± 0.7
200	80.3 ±0.1	39.6± 0.2	31.2± 0.8	38.3± 0.5
220	82.1±0.5	---	---	40.6± 0.5

Table-3: Hydroxyl free radical scavenging activity of ascorbic acid and three *Curcuma* species (crude extracts).

Conc. of extracts (µg/ml)	Antioxidant activity (%)			
	Ascorbic acid	*C. zedoaria*	*C. caesia*	*C. angustifolia*
10	36.67 ± 0.81	29.08± 0.21	30.39± 0.27	34.23± 0.42
20	39.50 ± 0.83	39.23± 0.31	33.48± 0.24	37.28± 0.27
30	43.58 ± 0.20	40.34± 0.23	37.43± 0.23	41.23± 0.11
40	46.50 ± 0.83	43.02± 0.22	39.31± 0.21	45.41± 0.22
50	52.33 ± 0.40	46.03± 0.21	40.26± 0.01	47.24± 0.20

Fig-1: *Curcuma zedoaria, Curcuma caesia* and C*urcuma angustifolia* plants maintained in the medicinal garden.

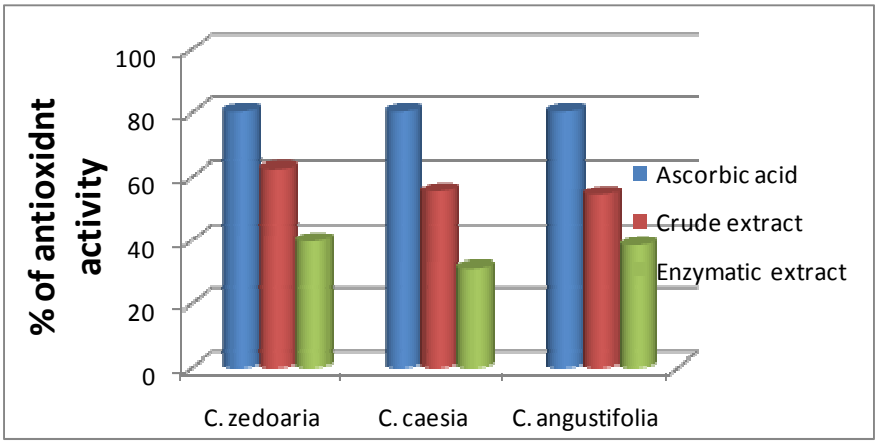

Fig-2: Comparison between the crude and enzymatic extracts of the three *Curcuma* species with ascorbic acid in DPPH assay.

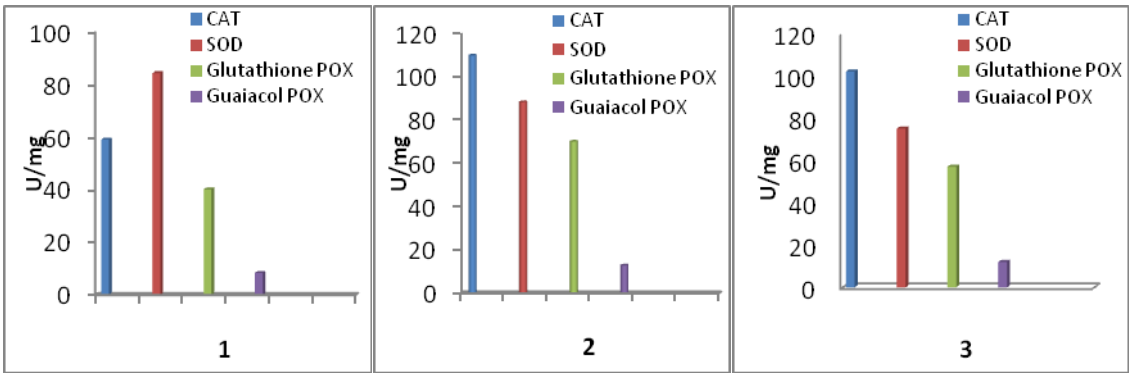

Fig-3: Levels of antioxidant enzymes in (i). *Curcuma zedoaria* (ii). *Curcuma caesia and* (iii). *Curcuma angustifolia* respectively.

Levels of antioxidant enzymes.

The level of the active antioxidant enzymes of the leaf samples of these plants of *Curcuma* species was evaluated (Fig-III). The active antioxidant enzymes of *C. zedoaria* was found to be 59.2± 1.5 U/mg catalase, 84.5± 2.6 U/mg superoxide dismutase, 8.4± 1.8 U/mg guaiacol peroxidase and 40.2± 0.3 U/mg glutathione peroxidase enzymes respectively. The level of the active antioxidant enzymes of *C.angustifolia* was found to be 102±2.5 U/mg catalase, 75.2±2.6 U/mg superoxide dismutase, 12.02±1.7 U/mg guaicol peroxidase and 57.1± 0.2 U/mg glutathione peroxidase enzymes respectively. Similarly, the level of the active antioxidant enzymes of *C.caesia* was found to be 110.01±1.5 U/mg catalase, 88.4±2.6 U/mg superoxide dismutase, 12.9±1.8U/mg guaicol peroxidase and 70.2±0.3 U/mg glutathione peroxidase respectively. In comparison to catalase, superoxide dismutase and glutathione peroxidase, the concentration of guaiacol peroxidase enzyme was low in case of three plants.

DISCUSSION

Free radicals are the cause for several major disorders. So, evaluation of antioxidant activity in plants could result in the discovery of natural antioxidants with pharmacological and food value. The importance of phenol compounds in plants as natural antioxidants and their use as substitutes to synthetic antioxidants in food additives is well known [14, 15]. Therefore, these observations could help in developing new drugs for the therapeutic use in human-beings. However, only limited work has been done on *Curcuma* species [25] .Therefore, the present work was aimed to analyze the antioxidant potential of these non-conventional species of *Curcuma*.

DPPH assay is often used to evaluate the ability of antioxidants to scavenge the free radicals from the supplied samples, whereby the free radicals cause biological damage through oxidative stress and such processes leads to many disorders like neurodegenerative disorders, cancer and AIDS. [26] Therefore, DPPH assay is an effective method to measure their scavenging power. The principle of the DPPH is based on the color changes from purple (DPPH solution) to yellow. [21] The color changes can be measured quantitatively at the absorbance 517nm.In our study, *C. zedoaria* was found to be more significant than the other two species of *Curcuma* in case of crude extracts. But, in case of enzymatic extracts, *C. caesia* was found to be less significant than the two other species. Few research studies have been undertaken on the antioxidant activity of enzymes in medicinal plants using DPPH scavenging assay [27, 28]. A comparative account between crude and enzymatic extracts of the three *Curcuma* species in DPPH assay is presented in Fig-2.

Hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism among the ROS [29] 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 [30].

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 [31]. In the present study, the crude extracts the rhizome of the three plants of *Curcuma* species were evaluated for their hydroxyl radical scavenging activity. In this case, *C.angustifolia* was found to be more significant than the other two species. Recently, a study on hydroxyl scavenging activity has been studied. Our results vary slightly due to effect of solvents [32].

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 [1]. In our study, the level of guaiacol peroxidase enzyme was low in comparison to catalase and superoxide dismutase, glutathione peroxidase. Our findings are in accordance with the enzyme extracts of rhizome in *C. longa* and *C. aromatica* [33].
In the study, the activity of peroxidase, SOD,catalase, glutathione peroxidase indicates these medicinal plants are highly potential antioxidant. However, irrespective of concentrations, crude extracts proves to be a superior antioxidant. The natural enzyme concentrations reveal high catalase activity followed by SOD, glutathione peroxidase and guaiacol peroxidase respectively. In our experiment, there is a correlation between catalase activities and scavenging of hydrogen radical and superoxide dismutase and glutathione peroxidase activity are indicators OH radical scavengers. However, guaiacol peroxidase plays a minor role in enzymatic antioxidant test. Although, crude extracts are invariably known for its phenol content [34], sometimes there are no correlation between phenol content of crude extract and antioxidant activity [35]. Hence, using of enzymatic extracts in DPPH free radical test provides an additional support to the conventional non enzymatic tests along with natural antioxidant enzyme activity as a defense system in plants. The results concluded that the antioxidant activity study of enzymatic extracts and the crude extracts employing DPPH and OH radical scavenging assay reveals higher activity of crude extracts (non enzymatic) in comparison to enzymatic extracts in the rhizomes of the *Curcuma* species. High amount of catalase activity and moderate amount of SOD and Glutathione peroxidase may be responsible for H+, OH- and hydrogen scavenging in both the enzymatic and non- enzymatic test in addition to secondary metabolites present in the plant. Therefore, it is suggested that these three species of *Curcuma* could be the potential source of natural antioxidant. Further research is recommended for exploitation of both crude and enzyme contents of medicinal plants using widely used tests like DPPH scavenging assay, OH radical assay etc.

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