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In vitro prevention of oxidative damage by *Curcuma amada* in goat liver slices exposed to oxidative stress

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ABSTRACT

Liver, the largest organ in vertebrate body, has a pivotal role in regulation of physiological processes. It is involved in several vital functions such as metabolism, secretion and storage. Furthermore, detoxification of a variety of drugs and xenobiotics occurs in liver. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages in liver. Even common dietary antioxidants can provide such protection from liver damage caused by oxidative mechanisms of toxic chemicals. A phytotherapeutic approach to modern drug development can provide many invaluable drugs from traditional medicinal plants. Search for pure phytochemicals as drugs is time consuming and expensive. In the present study one such medicinal plant *Curcuma amada* has been tested for their antioxidant activity in precision cut goat liver slices exposed to oxidative stress by hydrogen peroxide treatment. The activity of enzymic antioxidants and the levels of non enzymic antioxidants which decreased initially by H_2O_2 treatment was found to be increased on treatment with methanolic extract of both the leaves and rhizomes of *Curcuma amada*.

Key words: *Curcuma amada*, liver, antioxidants, phytochemicals, hydrogen peroxide, oxidative damage

INTRODUCTION

In the human and animal body, Reactive Oxygen Species (ROS) can be neutralized by antioxidant defence systems including antioxidant enzymes and antioxidant compounds [1]. Oxidative stress occurs when the production of reactive oxygen species overrides the antioxidant capacity in the target cell, resulting in the damage of macromolecules such as lipids, nucleic acids and proteins, causing alterations in the target cell function and leading to cell death [2]. Oxidative stress significantly impacts multiple cellular pathways that can lead to the initiation and progression of varied disorders throughout the body [3].

Antioxidant supplements or foods rich in medicinal plants are used to help the human body in reducing oxidative damage by free radicals and active oxygen. Currently, research interest has been focussed on the role of antioxidants as well as antioxidant enzymes, in the treatment and prevention of many diseases [4]. Antioxidants may guard against ROS toxicities by the prevention of ROS construction, by disruption of ROS attack, by scavenging reactive metabolites and converting them to less reactive molecules or by enhancing the resistance of sensitive biological target to ROS attack [5].

Precision-cut tissue slices represent an organ mini-model that closely resembles the organ from which it is prepared, with all cell types present in their original tissue-matrix configuration [6]. Organ slices, an *in vitro* model representing the multicellular, structural and functional features of *in vivo* tissue, is a promising model for elucidating mechanisms of drug-induced organ injury and for characterizing species susceptibilities. The liver is the major organ used in organ slice studies [7]. In this model, cells are refrained in an environment with normal cell–cell and cell–matrix contacts, and remain to express high levels of metabolic enzymes [8]. This tissue shows a high rate of free radical generation with high metabolic and detoxifying capacity [9]. The advantage of precision-cut liver slices is based on the juxtaposition of cellular assays and tissue morphology [10]. Hence in the present study precision cut goat liver slices subjected to oxidative stress by H_2O_2 was used for evaluating the antioxidant potential of the methanolic extract of leaves and rhizomes of the medicinal plant *Curcuma amada*.

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MATERIALS AND METHODS

Plant material

Curcuma amada rhizomes were procured from Arya Vaidya Pharmacy, Centre for Indian medicinal plant heritage, Kanjikode, Kerala and were grown as pot culture in our university herbal garden. Both leaves and rhizomes were collected fresh for the study. Previous studies conducted by us showed that the methanolic extract of the leaves and rhizomes were rich in antioxidants [11].

Leaves **Rhizomes**

Figure 1: *Curcuma amada* leaves and rhizomes

Extract preparation

The leaves and rhizomes collected fresh were rinsed with tap water blotted dry using a filter paper and used for extract preparation. The components present in the leaves and rhizomes were extracted using methanol. The methanolic extract prepared after evaporation of methanol was dissolved in

DMSO (Dimethyl sulfoxide) [20mg plant extract in 50µl DMSO]. For each antioxidant assay 0.25mg of rhizome extract and 0.15mg of leaf extract dissolved in 5µl DMSO were used.

Preparation of goat liver slices

The goat liver was collected fresh from a slaughter house, plunged into cold sterile PBS and maintained at 4ºC till use. Thin slices of 1mm thickness were treated with the oxidising agent H_2O_2 (200µM) and/or rhizome and leaf extract and incubated at 37ºC with mild shaking for one hour. Appropriate controls were also set up. The homogenate was used for the assay.

Treatment groups

1.Liver slices
2.Liver slices + H_2O_2
3.Liver slices+ methanolic extract of *C.amada* leaves (CAL)
4.Liver slices+ CAL+ H_2O_2
5.Liver slices+ methanolic extract of *C.amada* rhizomes (CAR)
6.Liver slices+ CAR+ H_2O_2

Determination of the activity of enzymic antioxidants

Assay of Superoxide dismutase (SOD)

The incubation medium contained a final volume of 3ml, 50mM potassium phosphate buffer, 45µM Methionine, 5.3µM Riboflavin, 84µM NBT and 20mM potassium cyanide and enzyme source. The tubes were placed in an aluminium foil-lined box maintained at 25ºC and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of the amount of SOD giving 50% inhibition of the reduction of NBT [12].

Assay of Catalase

3ml of H_2O_2 in phosphate buffer (0.067M, pH 7.0) was taken in a quartz cuvette and the baseline was adjusted at 240nm. 20µl of homogenate was added rapidly and mixed thoroughly. The time interval required for decrease in absorbance by 0.05 units was recorded at 240nm. The concentration of H_2O_2 was calculated using the extinction coefficient 0.036 per µM/cm. One unit is the amount of enzyme activity required to decrease the absorbance at 240nm by 0.05 units [13].

Assay of Peroxidase

3ml of pyrogallol solution (0.05M in 0.1M phosphate buffer) and 0.2ml of the homogenate were pipetted out into cuvette. Adjusted the spectrum to read zero at 430nm. 0.5ml of H_2O_2 was added in the test cuvette. The change in absorbance was recorded every 30 seconds upto 3 minutes. One unit of peroxidase activity is defined as the change in absorbance/ min at 430nm [14].

Assay of Glutathione reductase

The assay system contained 1ml of potassium phosphate buffer (0.12M, pH7.2), 0.1ml EDTA (15mM), 0.1ml Sodium azide (10mM), 0.1ml of oxidized glutathione (6.3mM) and 0.1ml of liver homogenate. The final volume was made up to 2ml using water. Then 0.1ml 0f 6.3mM NADPH was added. The absorbance at 340nm was recorded at an interval of 15 seconds for 2-3 minutes. The enzyme activity was expressed as mM NADPH oxidized/min/g liver [15].

Assay of Glutathione S-transferase

The substrates for GST (1mM GSH and 1mM CDNB, 0.1ml each) were taken in a test cuvette along with 0.1M phosphate buffer (pH 6.5) to make a volume of 2.9ml. The reaction was started by the addition of 0.1ml of the enzyme source to this mixture. The readings were recorded against distilled water blank for a minimum of 3 minutes. The complete assay mixture without the enzyme source served as the control. The enzyme activity was determined by recording the changes in absorbance at 340nm. One unit of GST activity is defined as the nM of CDNB conjugated/minute [16].

Determination of the level of non-enzymic antioxidants

Estimation of Ascorbic acid

Aliquots (0.2 to 1.0ml) of the working ascorbate solution (1mg/ml 4%TCA) were made upto 2.0ml with 4% TCA. 0.5ml DNPH reagent (2% DNPH in 9N H_2SO_4) was added to each tube, followed by two drops of 10% thiourea solution and incubated at 37ºC for 3 hours. The osazone crystals formed were dissolved by the addition of 85% H_2SO_4 (2.5ml) in cold. To the blank alone, DNPH reagent and thiourea were added after the addition of H_2SO_4. After incubation for 30min at room temperature the absorbance was read at 540nm [17].

Estimation of Tocopherol

The liver homogenate (1.5ml), 1.5ml standard (10ng/l alcohol) and water (1.5ml) were pipetted out into three centrifuge tubes namely test, standard and blank respectively. To all the tubes, xylene (1.5ml) was added, stoppered, mixed well and centrifuged. The xylene layer (1.0ml) was taken and transferred to another set of stoppered tubes, 1.0ml of 2,2' dipyridyl (1.2g/l n-propanol) was added to each and mixed. The reaction mixture (1.5ml) was taken in a spectrophotometric cuvette and the extinction of test and standard were read against the blank at 460nm. 0.33ml Ferric chloride solution (1.2g/l ethanol) was added and after exactly 15 minutes, the absorbance of the red colour was read against blank at 520nm. The amount of tocopherol in the sample was calculated using the formula,

Tocopherol (µg) = [(Reading of standard at 520nm- Reading at 450nm)/ (Reading at 520nm)]X 0.29 x 0.15

The results are expressed as µg tocopherol/g sample [18].

Estimation of Vitamin-A

Liver homogenate (1.0ml) was mixed with 1.0ml of saponification mixture (2N KOH in 90% alcohol) and refluxed for 20 minutes at 60ºC in the dark. All the steps subsequent to saponification were carried out in the dark. Vitamin A was extracted twice with 10ml of petroleum ether (40-60ºC). The extracts were pooled, washed thoroughly with water and the layers were separated using a separating funnel. When the petroleum ether fraction was clear, a pinch of sodium sulphate (anhydrous) was added to remove the excess moisture. The volume of the extract was noted and a fraction (1.0ml) was evaporated to dryness at 60ºC. The residue was dissolved in 1.0ml of chloroform. Aliquots of the standard (vitamin A palmitate) were pipetted out into a series of clean, dry test tubes in the concentration ranging from 0 to 7.5µg. The volume in all the tubes was made up to 1.0 ml with chloroform. TCA reagent (2.0ml) was added rapidly, mixed well and the absorbance of blue colour was read immediately at 620nm in a spectrophotometer. The vitamin A level was expressed as µg/g tissue [19].

Estimation of Reduced glutathione

0.1ml of sample was made upto 1ml with 0.2M Sodium phosphate buffer (pH 8). 2.0ml of freshly prepared DTNB solution (0.6M in phosphate buffer) was added and the intensity of the yellow color formed was read at 412nm in a spectrometer after 10 minutes. A standard curve of GSH was prepared between the concentration range of 2 to 10nM. The values were expressed as nM GSH/g liver [20].

Statistical analysis

The parameters of the experiment are expressed as Mean ±S.D. Statistical evaluation of the data was done using one way ANOVA with the level of significance at $P<0.001$ in sigma stat package version 3.1.

RESULTS AND DISCUSSION

The activities of all the enzymic antioxidants analyzed decreased significantly on exposure to H_2O_2. The activities of enzymic antioxidants in the liver slices upon exposure to the leaf and rhizome extracts significantly

Table1. Effect of *C.amada* leaf and rhizome extracts on the activities of enzymic antioxidants in goat liver slices exposed to H_2O_2 *in vitro*

Parameters	Liver slices	Liver slices+ H_2O_2	Liver slices+ CAL	Liver slices+ CAL+ H_2O_2	Liver slices+ CAR	Liver slices+ CAR+ H_2O_2
SOD[1]	11.49±1.2	8.45±0.56[a]	13.91±0.34[a,d]	11.7±0.19[b,c]	16.22±0.46[a]	13.52±0.09[a,b,c]
CAT[2]	360±28.28	138.9±4.08[a]	329.7±17.84	296.2±18.23[a,b]	324.55±21.86	319.39±17.85[b]
POD[3]	32.28±0.2	23.18±0.72[a]	47.57±0.11[a]	47.64±2.38[a,b]	49.02±0.44[a]	46.15±2.38[a,b]
GR[4]	7.25±0.16	4.29±0.86[a]	9.98±0.12[a,d]	9.56±0.08[a,b]	8.96±0.32[a,d]	8.52±0.05[a,b]
GST[5]	0.44±0.03	0.26±0.002 [a]	0.47±0.03	0.38±0.007 [b,c]	0.43±0.03	0.41±0.08 [b]

Values are expressed as Mean± SD of triplictes, values are expressed in Units/g liver tissue
1- 1 Unit is defined as the amount of enzyme that gives 50% inhibition of the extent of NBT reduction in 1 min
2- 1 Unit is defined as the amount of enzyme required to decrease the absorbance by 0.05 units at 240nm
3- 1 Unit is defined as the Change in absorbance at 430nm per minute.
4-1 Unit is defined as the milli moles of NADPH oxidized / min.
5- 1 Unit is defined as the nano moles of CDNB conjugated / min.
a- Statistically significant (P<0.05) compared to untreated control
b- Statistically significant (P<0.05) compared to H_2O_2 alone treated group
c- Statistically significant (P<0.05) compared to the respective extract treated group
d- Statistically significant (P<0.05) compared to the rhizome extract treated group

Table2. Effect of *C.amada* leaf and rhizome extracts on the levels of non-enzymic antioxidants in goat liver slices exposed to H_2O_2 *in vitro*

Parameters	Liver slices	Liver slices+ H_2O_2	Liver slices+ CAL	Liver slices+ CAL+ H_2O_2	Liver slices+ CAR	Liver slices+ CAR+ H_2O_2
Vitamin-C (mg/g liver)	0.54±0.02	0.23±0.002[a]	0.86±0.009[a]	0.75±0.011[a,b]	0.71±0.02[a,d]	0.64±0.012[a,b]
Vitamin-E (µg/g liver)	3.05±0.13	1.07±0.19[a]	3.19±0.33	2.64±0.2[b,c]	3.15±0.13	2.78±0.13[b]
Vitamin-A (µg/g liver)	72.01±0.33	54.53±0.44[a]	78.4±0.66[a,d]	71.62±0.44[b,c]	81.71±0.76[a]	76.16±0.98[a,b,c]
Reduced glutathione (mM/g liver)	5.92±0.05	3.95±0.05[a]	5.75±0.04[a]	5.52±0.02[a,b,c]	6.35±0.04[a,d]	5.65±0.03[a,b,c]

a-Statistically significant (P<0.05) compared to untreated control
b-Statistically significant (P<0.05) compared to H_2O_2 alone treated group
c-Statistically significant (P<0.05) compared to the respective extract treated group
d-Statistically significant (P<0.05) compared to the rhizome extract treated group

increased compared to untreated control. The presence of the extracts along with the oxidant, recorded an increase in the activities of enzymic antioxidants when compared to the oxidant treated group. The same trend was recorded in the level of non-enzymic antioxidants also in which the exposure of liver slices to leaf and rhizome extract significantly increased the non-enzymic antioxidants levels which were initially diminished by the oxidant treatment.

Our results correlated with the study which reported that the 43kD protein fraction from the leaves of *Cajanus indicus* significantly improved SOD, catalase and glutathione S-transferase activities in chloroform administrated mice liver *in vivo* [21]. The above findings also correlated with another study which showed increased level of enzymic antioxidants in liver of arthritis rats treated with *Withania somnifera* root powder extract [22]. *Trianthema decanda* root extract administration to Wistar rats also improved the antioxidant status by increasing the activities of liver CAT, SOD, GPx and GR in rats subjected to CCl_4 treatment [23]. The level of GST was found to be increased in the heart of myocardial injured rats after administration with *Ficus hispida* extract [24].

Increase in the level of vitamin C in the liver of Wistar rats was observed after administration of ethanolic extract of *Piper longum* in alloxan induced diabetic rats [25]. The results obtained correlated with the report that the administration of saponin containing compounds from *Aesculuc hippocastanum, Medicago sativa* and *Spinacia oleracea* reversed the plasma carotene and retinol level to normal in rats exposed to X-radiation [26]. *Operculina turpethum* extract administration also increased the level of enzymic antioxidants (CAT, SOD and GPx) and non-enzymic antioxidants vitamin E and C in the liver of Sprague–Dawley rats [27]. The level of glutathione was found to increase in rat liver after treatment with *Helicteres isora* extract in rats subjected to oxidative stress by CCl_4 [28].

The administration of *Solanum nigrum* leaf extracts to the Swiss albino mice also showed an increase in the activities of enzymic and levels of non-enzymic antioxidants in the liver of mice subjected to oxidative stress [29].

Administration of methanolic extract of *Nyctanthes artortris-tis* leaves also improved the enzymic antioxidant status of goat liver slices exposed to oxidative stress [30] and *Alternanthera sessilis* leaf extract treatment also increased the antioxidant status of rat liver which was decreased by oxidant treatment [31].

CONCLUSION

Thus, the results of the study using precision-cut goat liver slices revealed that the methanolic extract of leaves and rhizomes of *Curcuma amada* possess antioxidant principles that can fight against free radical mediated disorders.

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