WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 4, Issue 2, 927-939. Research Article ISSN 2277– 7105

# EVALUATION ON ANTIOXIDANT ACTIVITY OF CURRY LEAF EXTRACTS IN CISPLATIN INDUCED WISTAR RATS

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Article Received on
15 Nov 2014,

Revised on 16 Dec 2014,
Accepted on 07 Jan 2015

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## ABSTRACT

Reactive oxygen species (ROS) are harmful molecules which are continuously produced and constantly attack or damage the molecules within cells. The process of oxygen species damage to the cells is referred to as oxidative stress. ROS cause cancer, aging and heart diseases. Glutathione, Lipid peroxides, superoxide dismutase, glutathione reductase and catalase play a very important role in removing free radicals. Cancers related to lungs, gastrointestinal tract, ovarian, bladder and testis are treated by cisplatin by damaging the tumors via apoptosis and also by the activation of various signal pathways. Curry leaves possess antioxidative, antimicrobial and cholesterol reducing activities are reported already in different studies. Our observation in animal model also shows curry leaves has significant antioxidant activity and therefore inclusion in our daily food is expected to protect the body from oxidative stress.

**KEYWORDS:** Free Radicals, Curry Leaves, Antioxidant Enzymes, Cisplatin, Wistar Rats.

## INTRODUCTION

Free radicals are harmful molecules derived from oxygen, that are produced and constantly attack and damage the molecules within cells. The process by which oxygen species damage to the cells is referred to as oxidative stress.[1] Cells utilise antioxidants to remove free radicals.[2-4] In case, any imbalance between the production of reactive oxygen species and

antioxidant enzymes defence mechanisms leads to an oxidative stress, that causes aging processes and even in some pathological conditions. ROS can cause liver damage, carcinoma, aging and cardiac diseases.[5-7] Cisplatin is used for treating cancers related to bones, blood vessels etc.[8,9] The discovery of cisplatin have shown interest in the platinum(II)-compounds and other metal-containing compounds as potential anticancer drugs. Cisplatin reacts with cellular components such as DNA, proteins, RNA, membrane phospholipids and microfilaments. The lysis of Escherichia coli cells containing bacteriophage λ was observed when treated with the cisplatin and also shared by other DNA-damaging agents. DNA is an important cellular target. Cytotoxicity caused by the cisplatin depends on the cell type and concentration used. In addition, it damages the tumors through apoptosis and also by the activation of various signal pathways. Cisplatin causes multiorgan toxicity by inducing redox imbalance in cells. Cisplatin is used in the treatment related to gastrointestinal, lung, ovaries, and testis cancers.

## MATERIALS AND METHODS

### Chemicals

Cisplatin was purchased from the Sigma chemicals, St. Louis Co., MO and USA. This cisplatin compound was dissolved in 0.9% normal saline solution to obtain the final concentration of the 3 mg/kg body weight of the animal.

### Preparation of aqueous curry leaf extract

The method of preparation of *Murraya Koenigii* leaf extract was followed as according to zhang etal. ( 2011) with modification.The dried curry leaf dusts were soaked overnight in double distilled water (7.5 g per 100 ml), filtered through the clean cotton cloth.The obtained filtrate was centrifuged by using a REMI cold – centrifuge at 5000 rpm for 10 min.The supernatent, thus obtained was filtered again through the clean cloth, and that supernatent was collected in a sterile polypropylene tubes and later frozen at - 20˚C.The tubes were then lyophilized and the aqueous extract, was stored at - 20˚C until further use. The exact amount of this material was always freshly dissolved in the distilled water to give a appropriate concentration of this solution was fed to the rats. In case if any solution was left it should be discarded.The obtained yield of curry leaves aqueous extract was 15.59 ± 0.36% ( w/w).

### Preparation of Wistar Rats

Wistar rats were maintained in animal house under controlled conditions ( 12h Light / dark cycle with relative humidity of 40 – 60%, temperature 25 ± 2˚C). The animals of all groups were maintained on a diet containg 18% protein.The 18% protein diet was used as it is considered as an adequate ( normal) dietary protein level, which was used earlier ( Chatterjee etal., 1984).The Carbohydrates content of the diet was at 71%. The other ingredients of the diets and supply of vitamins were the same as reported earlier ( Chatterjee etal., 1984).The treatment of rats was carried out as per the schedule mentioned below:

**Group I:** Control rats ( C) treated with a normal saline every alternate day for a period of 21 days.

**Group II:** Curry leaf aqueous extract was treated to rats; dose, 100 mg/kg body weight, administered orally every alternate day for 21 days.

**Group III:** Cisplatin treated rats; was subcutaneously injected, dose of 3 mg / kg in 0.9 % normal saline body weight, given every alternate day for a period of 21 days.

**Group IV**: *Murraya koenigii* aqueous extract treated rats; dosage given 100 mg/kg body weight, administered orally , given every alternate day for a period of 21 days and Cisplatin was administered subcutaneously to the same group of rats at a dose of 3 mg / kg in 0.9 % normal saline body weight, given every alternate day for a period of 21 days one hour after administration of the aqueous extract.

| Animal group | Treatment | Route of administration | No of Animals used | Parameters |
|---|---|---|---|---|
| Group –I | Control | ------ | 6 | Body weight determination. **Blood analysis** Glutathione. Lipid peroxidation Superoxide dismutase Glutathione reductase Serum catalase |
| Group-II | Curry leaves extract 100mg/kg | Orally | 6 | |
| Group-III | Cisplatin 3 mg/kg | Intraperitoneally | 6 | |
| Group-IV | Cisplatin+ Curry leaves extract 3mg/kg + 100mg/kg | Intraperitoneally + Orally | 6 | |

**Estimation of Reduced Glutathione (GSH)**

Total glutathione (GSH) content in blood was measured by the method of Tietze (1969)[10] using dithionitro benzene and expressed as mmol/ml. 0.2 ml of blood was made upto 1.0 ml by addition of 5% TCA and the protein in the sample was precipitated by centrifugation. For the assay 0.2 ml of the protein free supernatant was used. 2.0 ml of DTNB was mixed with

0.2 ml of the supernatant and the final volume was made up to 3.0 ml with phosphate buffer and against the blank, the optical density was measured at 412 nm in a spectrophotometer within 60 seconds. The blank consist of 2.0 ml of DTNB and 0.2 ml of TCA, finally it was made upto 3.0 ml with phosphate buffer. The standard glutathione was prepared in separate tubes at a concentration range of 5 to 20 µg were treated with 2.0 ml of DTNB and then the volume was made up to 3.0 ml with phosphate buffer. The blank and the standard were also measured at 412 nm. The amount of reduced Glutathione in the plasma was expressed in mg/dl.

**Lipid peroxidation**

The lipid peroxidation levels in serum was measured as thio barbituric acid reactive substances (TBARS) following the method described by Satoh (1978).[11] Take a labeled micro-centrifuge tube in that 0.5 ml of serum sample was mixed with 0.5 ml sterile distilled water. The blank sample was also prepared by adding 2 ml of the reagent to 1 ml distilled water.

For each sample 0.2 ml of Sodium dodecyl sulphate, 1.5 ml of TBA and 1.5 ml of glacial acetic acid was added, and for 10 minutes the micro tubes were incubated in a boiling water bath. Then the tubes were kept for 15 min in room temperature and tubes are centrifuged at 1,000×g for 10 min at 4°C. Finally, the obtained supernatants were transferred in to new labeled tubes, and absorbances of all the samples were measured at 535 nm against a blank sample. Finally by adding the malondialdehyde index ($1.056\times10^5$ $M^{-1}$ $cm^{-1}$) the Antioxidative activity was calculated.

**Antioxidant enzymes**

**Estimation of Superoxide dismutase (SOD) (Superoxide: Oxidoreductase) (E.C. 1.15.1.1).**

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity in erythrocytes was performed according to Nandi and Chatterjee (1988). Chloroform 0.15 ml and absolute ethanol of 0.25 ml was added to 1 ml of sample. After completion of shaking for 15 minutes, the obtained suspension was centrifuged from that the obtained supernatant was the enzyme extract. The reaction mixture for auto – oxidation consists of Tris – HCl buffer 2ml, pyrogallol 0.5 ml and 1.5 ml of distilled water. At first, the rate of auto-oxidation of pyrogallol was measured at an interval of one minute for 3 minutes. In the assay mixture the enzymes consist of Tris – HCl buffer 0.1 m and pyrogallol 0.5 ml of the enzyme preparation and water gives a final volume of 4

ml. The unit of activity is asserting as the amount of enzyme that forbid the rate of auto-oxidation of pyrogallol by 50% under standard conditions and was expressed as u/g protein.

**Estimation of Glutathione reductase (GR) (E.C. 1.6.4.2)**

Glutathione reductase activity in erythrocytes was described by Goldberg, D. M. and Spooner R. J. (1983).[12] 0.5 ml of whole blood was centrifuged for 5 min at 2000 rpm. Carefully remove the plasma and buffy coat, without removing too many erythrocytes. The erythrocytes should be washed three times thoroughly in 0.9% NaCl and centrifuge for 5 min at 2000 rpm after each wash. Cell lysis is done by resuspending them in the cold redistilled water and back up to 0.5 ml. Leave it for 10 min at + 2 - + 8℃. C. Lysate is centrifuged for 5 min at 2000 rpm for removing stroma. For assay, take 100 µl of lysate with 1.9 ml of 0.9% NaCl solution to that add diluted lysate 40 µl, substrate 1 ml and NADPH 200 µl. Mix it well. After 5 minutes read the absorbance at 340nm.Glutathione reductase activity in the serum was determined by following the oxidation of NADPH to NADP during the reduction of oxidized glutathione (Goldberg and Spooner, 1983) and expressed as mmol of NADPH oxidized/ min/ml.

**Estimation of Serum catalase (Hydrogen –Peroxide: Oxidoreductase) (EC1.11.1.6)**

The activity of serum catalase activity was described by Goth (1975).[13] For the assay, optimal conditions were as follows: 0.2 ml of serum was incubated in 1.0 ml of substrate at $37^0$ C for 60 s.

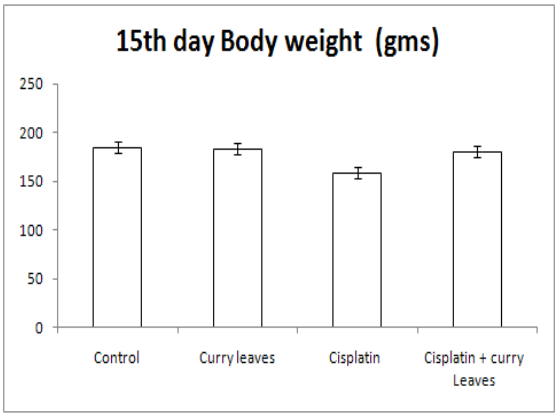
The activity of serum catalase is linear up to100 kU/l. In case catalase activity exceeded 100 kU/l the serum was diluted with the phosphate buffer (2- to lo-fold) and the assay was repeated. 1 unit catalase decomposes 1 pmol of hydrogen peroxide/l min under these conditions.

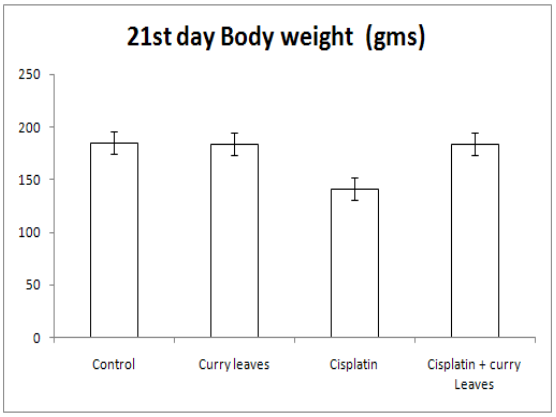
**RESULTS**

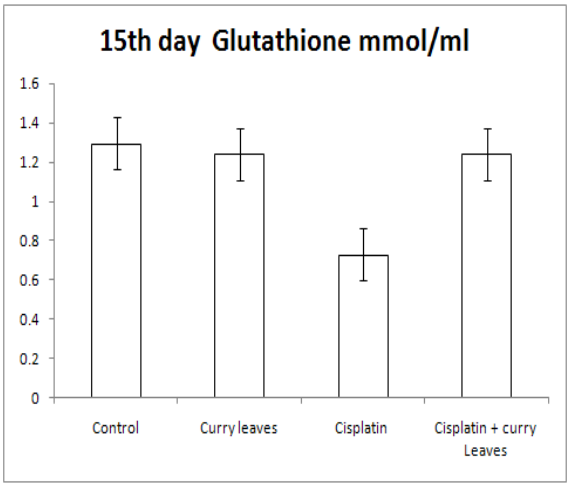
| Parameter | Control | Curry leaves | Cisplatin | | Cisplatin + curry Leaves | |
|---|---|---|---|---|---|---|
| | | | Day 15th | Day 21st | Day 15th | Day 21st |
| Body weight g ms | 185 ± 3.725 | 183.42 ± 2.401$^{ns}$ | 158.80 ± 0.66$^{***}$ | 141.14 ± 3.879$^{***}$ | 180.36 ± 0.364$^{***}$ | 183.45± 2.754$^{***}$ |
| Glutathione mmol/ml | 1.295 ± 0.048 | 1.24 ±0.014$^{ns}$ | 0.728 ± 0.013$^{***}$ | 0.753 ± 0.042$^{***}$ | 1.238 ± 0.005$^{***}$ | 1.185 ± 0.057$^{***}$ |
| LPO n. moles/ ml | 3.590 ± 0.597 | 2.963 ± 0.39$^{ns}$ | 4.646 ± 0.15$^{***}$ | 4.924 ± 071$^{***}$ | 3.5003 ± 0.13$^{***}$ | 3.573 ± 0.681$^{***}$ |
| SOD Units/ gm | 0.498 ± 0.014 | 0.480 ±0.0141$^{ns}$ | 0.368 ± 0.030$^{***}$ | 0.426 ± 0.008 | 0.473 ± 0.012$^{***}$ | 0.488 ± 0.024$^{***}$ |

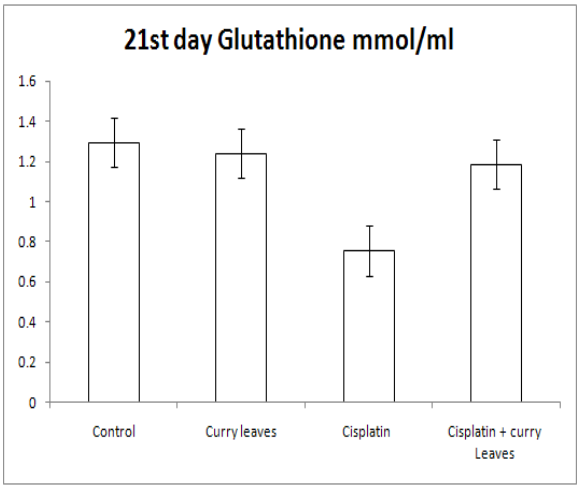
| | | | | | | |
|---|---|---|---|---|---|---|
| protein | | | | | | |
| Glutathione Reductase mmol of NADPH oxidized/ min/ml Or Units /ml serum | 1.165 ± 0.05 | 1.081 ±0.069$^{ns}$ | 1.655 ± 0.09$^{***}$ | 1.77 ± 0.04$^{***}$ | 1.030 ± 0.031$^{***}$ | 1.032 ± 0.013$^{***}$ |
| Catalase Units / L serum | 2.527 ± 0.098 | 2.498 ±0.043$^{ns}$ | 3.864 ± 0.05$^{***}$ | 4.018 ± 012$^{***}$ | 2.256 ± 0.01$^{***}$ | 2.335 ± 0.018$^{***}$ |

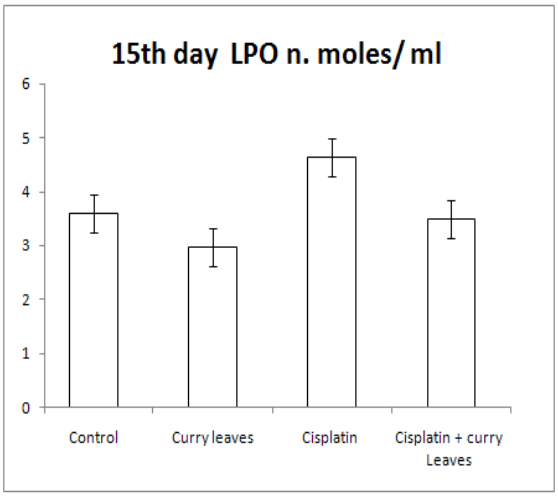
Values are mean ± standard error of mean of six male Wistar Rats. *** p<0.0001

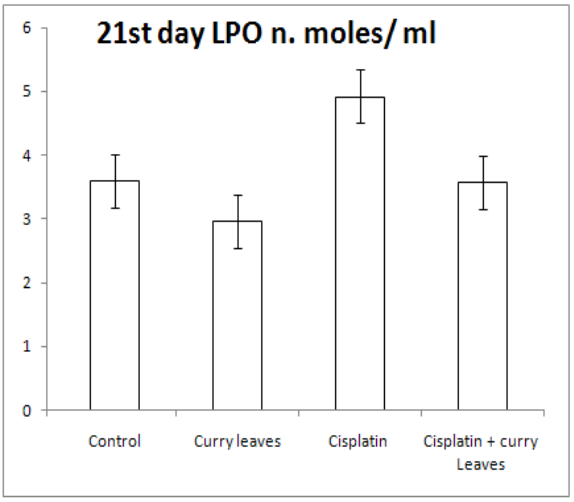


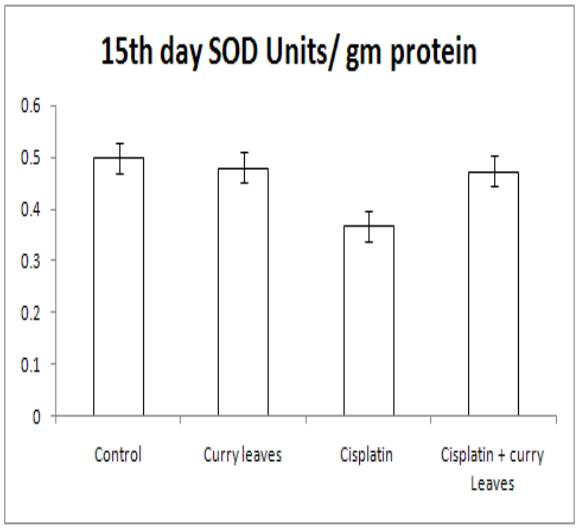



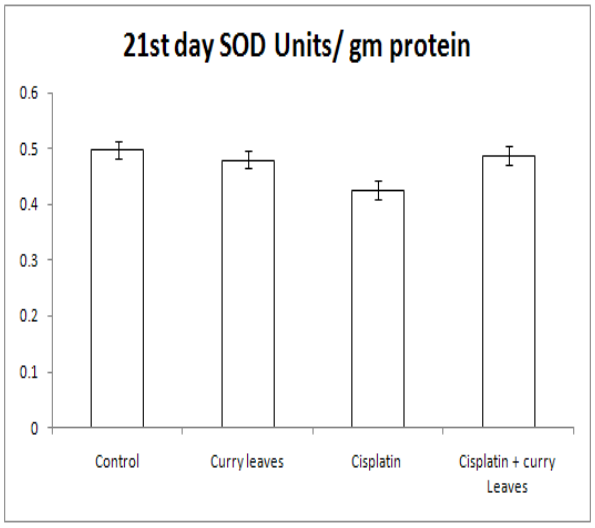



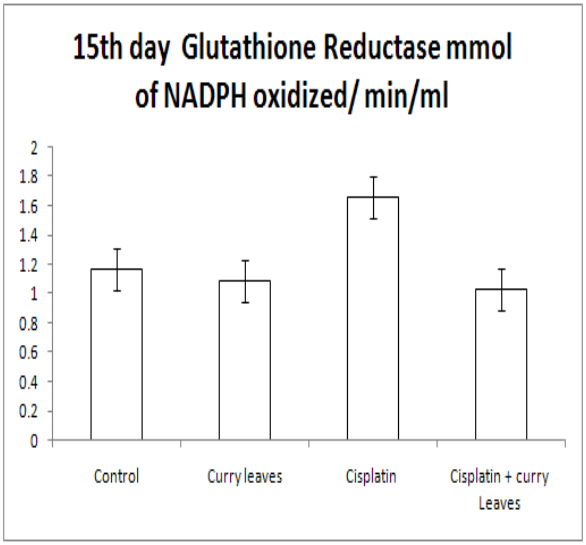



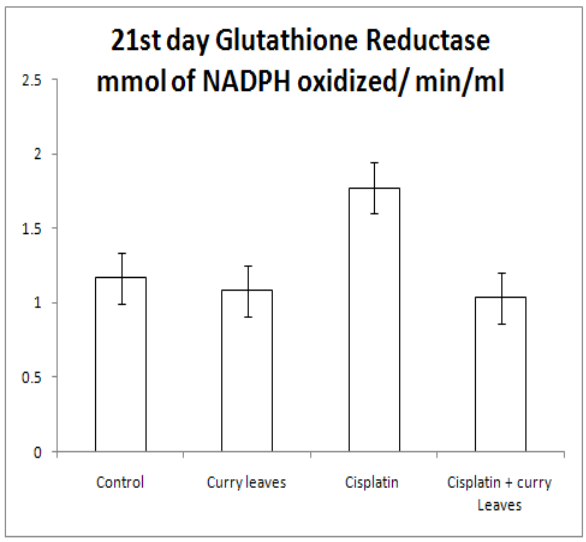

15th day LPO n. moles/ ml
6
5
4
3
2
1
0
Control
Curry leaves
Cisplatin
Cisplatin + curry Leaves

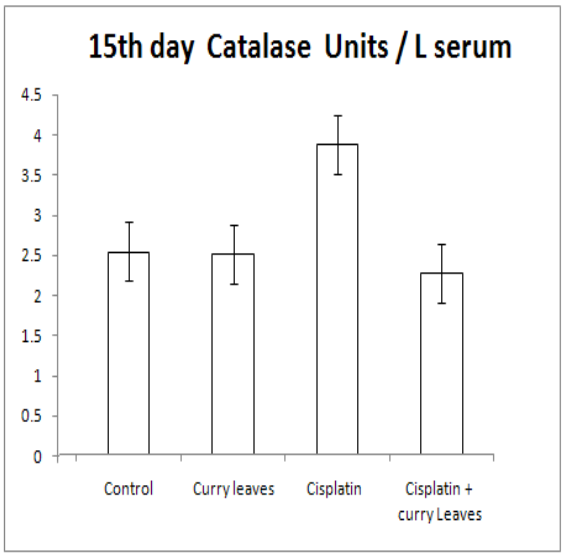

21st day LPO n. moles/ ml
6
5
4
3
2
1
0
Control
Curry leaves
Cisplatin
Cisplatin + curry Leaves

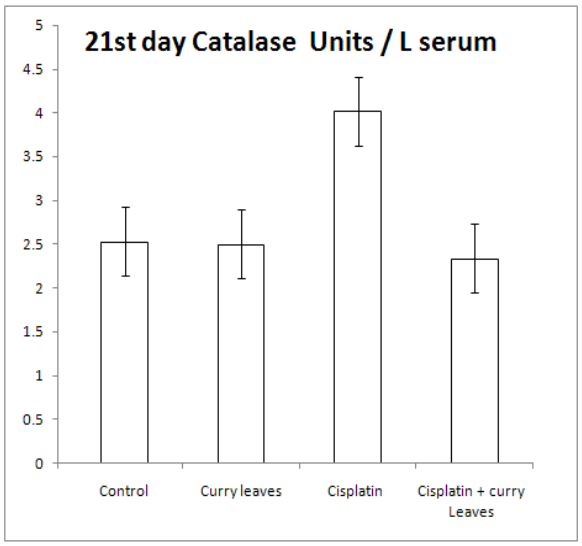

15th day SOD Units/ gm protein
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Curry leaves
Cisplatin
Cisplatin + curry Leaves


21st day SOD Units/ gm protein
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Curry leaves
Cisplatin
Cisplatin + curry Leaves


15th day Glutathione Reductase mmol of NADPH oxidized/ min/ml
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Curry leaves
Cisplatin
Cisplatin + curry Leaves


21st day Glutathione Reductase mmol of NADPH oxidized/ min/ml
2.5
2
1.5
1
0.5
0
Control
Curry leaves
Cisplatin
Cisplatin + curry Leaves




## DISCUSSION

### Body weight

The net loss of body protein is likely a major factor responsible for decreased body weight caused either by deficiencies of some amino acids or in proteins synthesis including antioxidant enzymes. Essential amino acids such as arginine and histidine, or non protein amino acids such as citrulline, taurine or small peptides such as GSH and carnosine or nitrogenous metabolites such as creatine and uric acid directly scavenge oxygen free radicals. Any form of free radicals has the capacity to cross blood brain barrier and destroy the cells which gives signals to the cells to destroy the protein present especially in skeletal muscle.

As per our study compared with control, in the animals treated with cisplatin, body weight decreases drastically both on 15th and 21st day (≈ 30 gms). This may be because of free radicals induced by cisplatin influences nerve signal in order to degrade the skeletal muscle protein.

Treatment With either with curry leaves alone or curry leaves along with cisplatin there is not much significant reduce in the body weight indicates the component present in curry leaves protects the degradation of muscle protein.

### Glutathione

Glutathione is a cysteine-containing peptide found in most forms of living organisms in increased concentration. The thiol group in glutathione moiety is a reducing agent that can be reversibly oxidized. Its central role include in maintaining the cell's redox equilibrium and cellular antioxidants. GSH helps the body to eliminate toxins and pollutants. GSH has a

crucial role in regulating a normal balance between oxidatitive and antioxidatitive condition which gives an impact on many vital functions at cellular level such as the DNA synthesis and its repair, the protein secretion, synthesis and its activation, maintaining the reduced form in thiol group of protein, increasing immunity, maintain homeostasis of nitric oxide, the neurotransmitter receptors activity and antioxidant enzymes regulation. The lower level of GSH is related to different physiological and biochemical disturbances.

As per our study compared with control, the Glutathione level in the serum of the animal treated with cisplatin decreases drastically both on 15th and 21st day (≈ 0.5 mmol/ml). This may be because of free radicals induced by cisplatin influences the maximum utilization of glutathione present in the blood in order to provide antioxidant effect.

With either by curry leaves alone or curry leaves along cisplatin there is not much significant reduce in the glutathione levels indicates the component present in curry leaves acts as an antioxidant rather than glutathione.

**Lipid peroxide**

Polyunsaturated fatty acids (PUFAs) act as excellent substrates for lipid peroxidation due to the presence of active bis-allylic methylene groups. The activated methylene units which contain carbon-hydrogen bonds have lower bond dissociation energies, making these hydrogen atoms more easily abstracted in radical reactions.[14] The PUFA has more susceptibility towards peroxidation which increases the number of unsaturated sites in the lengthy fatty chain.[15]

As per our study compared with control, the generation of Lipid peroxide level in the serum of the animal treated with cisplatin increases drastically both on 15th and 21st day (≈1.5 nmol/ml). This may be because of free radicals induced by cisplatin binds with the free lipids/ fats and increases the levels of lipid peroxide present in the blood.

With either by curry leaves alone or curry leaves along cisplatin there is a significant reduction in the Lipid peroxide levels indicates the component present in curry leaves acts as a antioxidant and prevent the synthesis and release of lipid peroxides.

**Glutathione reductase**

Glutathione Reductase (GR) is present in all tissues and blood cells for glutathione metabolism. It contains flavin that helps to protect erythrocyte from hemolysis. This enzyme

converts oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH and regulates intracellular GSH/GSSG ratio of about 500 in red blood cells.[16] GR is also required for reducing protein thiols to their native state. This enzyme is highly conserved in all groups of species. In bacterial genome, yeasts and animals only one GR gene is found but in plant genomes two GR genes are enclosed. Under optimal conditions, GSH and GR involves in the detoxifying Hydrogen peroxide. Alteration GH level always correlated with oxidative stress in cell induced by many factors including toxic chemicals, pollutants, immune response like inflammation and different diseases centric towards red blood cell defects.

As per our study compared with control, the generation of glutathione reductase level in the serum of the animal treated with cisplatin increases drastically both on 15$^{th}$ and 21$^{st}$ day (≈ 0.6 Units/ml). This may be because of free radicals induced by cisplatin helps to generate glutathione reductase by the cells to prevent the damage.

With either by curry leaves alone or curry leaves along cisplatin there is a significant reduction in the glutathione reductase levels indicates the component present in curry leaves acts as antioxidant and prevent the synthesis and release of glutathione reductase.

**Superoxide Dismutase**

Superoxide dismutase (SOD) is used as the first line defense to defend upon superoxide radicals that are generated because of oxidative stress. SOD discovered by McCord and Fridovich, plays a vital role to defend when biological cells are exposed to oxygen. SOD belongs to the enzymes group those catalyses the O2– dismutation and produces $H_2O_2$. This mechanism of SOD is considered as an antioxidant system that prevents cellular damage from free superoxide anion toxicity. There are many types of SOD which are known by the metal ion it utilizes for its mechanism. Three major isoforms are found in mammals with different tissue distributions.

Cu/Zn - SOD (SOD1) distributed widely in the liver particularly in cytoplasm, lysosomes, and nucleus of mammalian cells.[17, 18] SOD1 is a homodimer contains 153 amino acids with one Cu and Zn ion in each 16KDa subunit. In the active of the SOD 1 enzyme the histidine residue that contains imidazolate ligands hold the copper ion. The stability of the enzyme depends upon the interaction with zinc ion (Zn 2 +)[19]

As per our study compared with control, the utilization of superoxide dismutase level in the serum of the animal treated with cisplatin decreases drastically both on 15$^{th}$ and 21$^{st}$ day (≈ 0.1 Units/g). This may be because of free radicals induced by cisplatin are counter acted by superoxide dismutase by the cells to prevent the damage.

With either by curry leaves alone or curry leaves along cisplatin the superoxide dismutase levels are normal indicates the component present in curry leaves acts as antioxidant and maintains the level of synthesis and release of Superoxide dismutase.

**Catalase**

Catalse (CAT) is the major marker for antioxidant enzymes.[20] It is the first to be purified and crystallized which has been known for is relation towards aging, cancer and diabetes.[21] It is found in almost every cell and in particularly use oxygen for detoxification process to produce Hydrogen peroxide. Catalase reacts and catalyses the $H_2O_2$ into water and oxygen.[22, 23] Catalase can also use to detoxify some toxic substances through peroxidase.[24] Many evidences support that the changes of catalase activity and its regulation are very vital in response to stress resulting protection towards cellular damage from the toxicity of hydrogen peroxide.[25]

As per our study compared with control, the generation of catalase level in the serum of the animal treated with cisplatin increases drastically both on 15$^{th}$ and 21$^{st}$ day (≈ 1.5 Units/l). This may be because of free radicals induced by cisplatin helps to generate catalse by the cells to prevent the damage.

With either by curry leaves alone or curry leaves along cisplatin there is a significant reduction in the catalase levels indicates the component present in curry leaves acts as antioxidant and prevent the synthesis and release of catalase.

**CONCLUSION**

Our study shows curry leaves has better antioxidant activity and therefore inclusion in our daily food is expected to protect the body from oxidative stress. Any formulation containing curry leaves extracts may have beneficial effect to overcome oxidative stress during viral infection such as Influenza etc. However inclusion of curry leaves for garnishing using oil may be undesirable or even may not have potentiality to overcome oxidative stress. It is

advisable to include curry leaves as a dry powder form in the food preparation. Use of garnished curry leaves has any positive antioxidant effect need to be studied.

**ACKNOWLEDGEMENTS**

I would like to thank Dr.Shridhar Reddy, Department of Life Sciences, REVA University, Bangalore and Dr. V.Palanivel, Associate Professor, Dept.of.Pharmacology, Padmavathi College of Pharmacy & Research Institute, Periyanahalli (P.O), Dharmapuri Tamil Nadu for continuous support and encouragement throughout this research work.

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