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Antimutagenic /Antigenotoxic Activity of Bioactive Compounds of *Curcuma caesia rox b* against Cyclophosphamide induced Hepatotoxicity and Nephrotoxicity in Mice

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Abstract: ***Objective: The present study has focused on the antimutagenic effects of bioactive compounds of Curcuma caesia rox b (Black turmeric) against mutagenic effects of cyclophosphamide induced hepatotoxicity and nephrotoxicity in mice. Bioactive compounds in plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. These are produced within the plants associated with plant growth and development and are regarded as products of biochemical "side tracks" in the plant cells which also play an important role in curing various kinds of diseases like cancer, diabetes etc. Method: The present study evaluated the bioactivity of proteins in aqueous extracts of rhizomes of the species. This species are used in traditional Indian medicine for their antimicrobial, anticancer, anti-inflammatory properties. Total phenol content was estimated by the Folin-Ciocalteau method. The highest total phenol content was exhibited by aquoeous extracts. This species showed a significant antioxidant activity in aquoeous extract. Result: C. caesia proteins showed highest antioxidant potential. The C2-C3 double bond of flavonoids increases the radical scavenging activity. Plant derived compounds are regarded as a substantial source for novel lead structures to develop medicines and biocides natural products. This species has to be analysed further for identifying its various medicinal properties.***

Keywords: Curcuma caesia, Biochemical assays, Protien estimation, Antioxidant activity

1. Introduction

Antimutagen is described as an agents that reduces the apparent yield of spontaneous or induced mutation. Antimutagens have been classified into two major classes one is desmutagens: that inactivate the chemical interactions before the mutagen attacks the genes and the other is Bio-antimutagens: that stop the mutation process once after the genes are damaged by mutagens [1]. Cyclophosphamide, an anticancer agent used as a mutagen with anti-metabolites activity, it exerts its activity by prohibiting DNA chain elongation. Antimutagenesis are considered as one of the most feasible ways for prohibiting the negative effects of genotoxicants including carcinogens.

Medicinal plants are the blessings for any country which contribute a lot for traditional health management as well as providing lead compounds for modern drug discovery and india has long history of using medicinal plants for medicinal purpose as mention in ayurveda. Curcuma also called Black turmeric (common name) like many other plants, has been used widely in traditional medicine since ancient time and till now as anti-fungal activity Banerjee and Nigam [2], smooth muscle relaxant and anti-asthmatic activity Arulmozhi et al. [3], bronchodilating activity Paliwal et al. [4], antioxidant activity Mangla et al. [5], anxiolytic and CNS depressant activity, locomotor depressant, anti-convulsant Karmakar et al. [6], anthelmintic activity Gill et al. [7], anti-bacterial activity Rajamma et al. [8], anti-ulcer activity Das et al. [9]. C. caesia is a large genus belonging to the family Zingiberaceae. It comprises about 70 species of rhizomatous herbs distributed mostly in Southeast Asia as wild and cultivated plants [10] The phytochemical studies of *C. caesia* revealed the presence of multiple phytoconstituents like essential oils with camphor, ar-turmerone, (Z) ocemene, ar-curcumene,1,8-cineole, elemene, borneol, bornyl acetate, curcumene, etc [11]. The varities of molecules or bioactive compounds contained in plants have been proved to combat complicated diseases, based on this natural product scientists have always focused on the isolation of bioactive compounds. In addition, it was reported that the giant pharmaceutical companies were also capitalizing these scopes for incorporating new drugs in the market [12], [13], [14], [15], [16]. The rhizomes of this have a high economical importance because of its putative medicinal properties. Arulmozhi DK *et al* in 2006 have reported that rhizomes are used in the treatment of smooth muscle relaxant activity and Sasikumar B in 2005 has also found the importance of the species in treating various diseases like haemorrhoids, leprosy, asthma, cancer, epilepsy, fever, wound, vomiting, menstrual disorder, anthelmentic, aphrodisiac, inflammation, gonorrhoeal discharges, etc [17],[18]. Interaction of the free radicals such as hydrogen peroxides, superoxide anions, and organo peroxides, etc produced by drugs, ultraviolet radiations, ionising radiations, pollution with polyunsaturated fatty acids, nucleotides and disulphide bonds has been implicated as the major factor to cause the oxidation of the biological compounds i.e oxidative damage and this leads to mutations and many degenarative diseases like emphysema, cardiovascular, inflammatory diseases, cataracts, etc [19], [20], [21]. Cellular system of this plant has developed many endogenous antioxidants such as superoxide dismutase (SOD), catalase, glutathione, glutathione peroxidases and reductase, and nonenzymatic antioxidants like vitamin E (tocopherols and tocotrienols), vitamin C, etc to neutralise the free radicals[22],[23]. This has triggered to search for effective antioxidant agents from various sources including

plants. Many researchers have investigated that the increase levels of antioxidants present in plants are believed to decrease the oxidative damage and its harmful effects [24]. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) are preferable but can cause serious ill effects in human health as per recent reports by Lobo *et al* in 2010. C. Ruan in 1989 have reported the importance of bioactive compounds such as flavonoids, phenolics, carotenoids, coumarins, anthraquinones, tannins, terpenoids, saponins that play a prominent role in inhibiting human carcinogenesis and repair the cell mutations [25].

2. Materials and Methods

Plant material collection and extraction: The Rhizomes of *Curcuma caesia Rox B* were collected during December 2013 from the region of Nambol, Brishnupur District, Manipur, India. Fresh Rhizomes were thoroughly washed, sliced and shade dried in a hot air oven for 6 hours. Dried rhizomes were then ground to fine powder by electronic mill. Then 100g of it was successfully extracted with various solvents starting from least polar to more polar, i.e. from petroleum ether to ethyl acetate, ethanol, methanol and then finally to water through soxhlet at a temperature of 50–60 °C for a period of 12-24 h. The crude extracts of each solvent were dried in water bath and kept for further uses.

Phenolic compound and their biological activities: The phenolic compounds are the products of pentose phosphate, shikimate and phenylpropanoid pathways in plants and are characterized due to the presence of aromatic ring bearing one or more hydroxyl groups [26]. It has been found that the phenolics compounds may contribute directly to the antioxidant action due to the presence of hydroxyl functional groups around the nuclear structure.

Antioxidant activity: Mechanisms of antioxidant action can include suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation; scavenging ROS; and upregulation or protection of antioxidant defenses. Phenolic compound such as flavanoids known to inhibit the enzymes involved in ROS generation.

Lipid peroxidation is a common consequence of oxidative stress. Flavanoids (bioactive compound) present in this species protect lipids against oxidative damage by various mechanisms. Free metal ions enhance ROS formation by the reduction of hydrogen peroxide with generation of the highly reactive hydroxyl radical. Due to their lower redox potentials flavonoids (Fl-OH) are thermodynamically able to reduce highly oxidizing free radicals (redox potentials in the range 2.13-1.0 V).

Biochemical assays: SGOT and SGPT are the enzymes found mainly in liver cells, mucles, skeletal muscles and in kidneys. Injury to these tissue results in the release of these enzymes in the blood. The elevated levels of SGOT and SGPT were assayed according to the method provided by Reitman and Frenkel (1957) method using commercial kit [27].

DPPH radical scavenging assay: The antioxidant activity of extracts was determined on the basis of the scavenging activity of the stable DPPH free radical by the method described by Braca *et al*., 2001 [28].

Assay of lipid peroxidation: Quantification of lipid peroxidation is essential to assess oxidative stress in pathophysiological process. Lipid peroxidation forms Malondialdehyde (MDA) and 4-hydroxynonenal(4-HNE), as natural bi-products. Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage. Lipid peroxidation was assayed according to the method of Halliwell and Gutteridge in1989 [29].

Protien estimation: Protein estimation was performed following Lowry's et al, 1951. 0.025g of tissue was homogenized in 1ml of 1x PBS (phosphate buffer saline). 0.5ml of the homogenate was diluted to 6ml PBS. From this 0.5ml of the diluted sample was mixed with 0.7ml of lowry's solution and vortex it and incubated for 20 min followed by the addition of 0.1ml of Folin ciocalteau reagent. Vortexed it and incubated for 30 min and read the absorbance at 750nm.

Determination of tissue reduced Glutathione (GSH): Meister and Anderson in 1983 reported that glutathione plays a very important fundamental role in cellular defence system against reactive free radicals and other oxidant species. According to Anderstam *et al*., in 1992, depletion of GSH results in enhanced lipid peroxidation and Comporti in 1987 have reported that excessive lipid peroxidation can cause increase GSH consumption [30].

Reduced glutathione in the tissue was determined according to the method of Moron et al., 1979. For the estimation 0.1g of the sample were homogenized with 5%TCA and centrifuged at 10,000 rpm for 10 min at 4°c. Then supernatant was used for the estimation of GSH. To 0.1 ml of supernatant, 1.0 ml of phosphate buffer then 2.0 ml of freshly prepared DTNB (Ellman's reagent) solution was added and the intensity of the yellow colour formed was read at 412nm in a spectrophotometer after 10 mins. The GSH content will be calculated with thw help of the standard curve obtained different concentrations of GSH and DNTB mixtures are expressed as nmoles of GSH/ g tissue.

Assay of Glutathione reductase: Glutathione reductase was assayed by the procedure adopted by David and Richard(1983) [31]. To 0.1 ml of sample, 1ml of potassium buffer (0.12m pH 7.2), 0.1ml of EDTA, 0.1 ml of sodium azide and 0.1 ml of oxidized glutathione were added and the volume was made up to 2ml with water. The mixture was kept at room temperature for three mins and 0.1 ml of NADPH was added. The absorbance at 340nm was recorded at intervals of 15 seconds for 2 to 3 mins. One unit of GR is expressed as FM of NADPH oxidized/ minute/gram. The GR activity was calculated by the formula,

$$U/ml = \Delta A340nm/min * 3/(6.22)(0.1)$$

Where 6.22-millimeter extinction coefficient of β-NADPH; 0.1-volume of enzyme used for assay; 3-volume of reaction mixture.

3. Results

Free radicals or reactive oxygen species are introduced in to the living system as a product of normal metabolic function or from the environment. Plants have evolved a well regulated mechanism for scavenging ROS, generally through the production of various antioxidative enzymes such as Superoxide dismutase, Peroxidase, Glutathione peroxidase, Ascorbate oxidase, glucose 6- Phosphate-Dehydrogenase and Glutathione reductase. And the present study concerns about the Glutathione reductase, Glutathione reduced, lipid peroxidase and protein content. These enzymes are usually considered to be the most predominant ROS-scavenging in plant systems [32].

Table 1: SGOT and SGPT analysis of aqueous extract of Curcuma Caesia

	SGOT(Means±S.D)	SGPT(Means±S.D)
Negative control	15±3.81	21.66±1.81
Positive control	147.22±4.22	48±2.24
!00mgAECC+CP	66.11±3.73	36.66±1.42
250mgAECC+CP	45±3.52	8.33±1.76
500MGAECC+CP	17.77±3.11	6±1.53

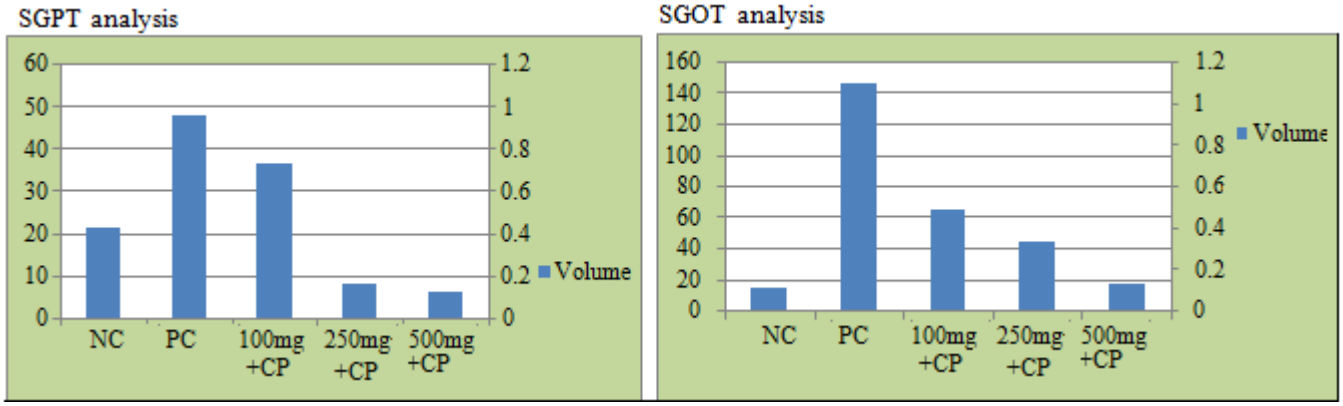

Figure 1: SGPT and SGOT analysis

Intraperitoneal injection of Cp in mice resulted in marked impairment of liver and kidney functions as reflected by increase in the levels of serum SGOT and SGPT as compared with normal control rats (table1) (fig1). On the other hand, oral administration of aqueous extract of rhizome of Curcuma caesia efficiently alleviated the altered serum marker enzymes that is the leaking of such enzymes is blocked by the administration of extracts.

Table 2: Lipid peroxidation assay of liver and Kidney:values are represented as %inhibitory effect

	LPO(Liver) Mean±S.D	LPO(Kidney) Means±S.D
Negative control	22.40±0.019	41.48±0.013
Positive Control	33.66±0.017	23.88±0.182
100mgAECC+CP	30.98±0.018	35.20±0.021
250mgAECC+CP	27.64±0.024	31.76±0.016
500mgAECC+CP	24.90±0.023	28.00±0.019

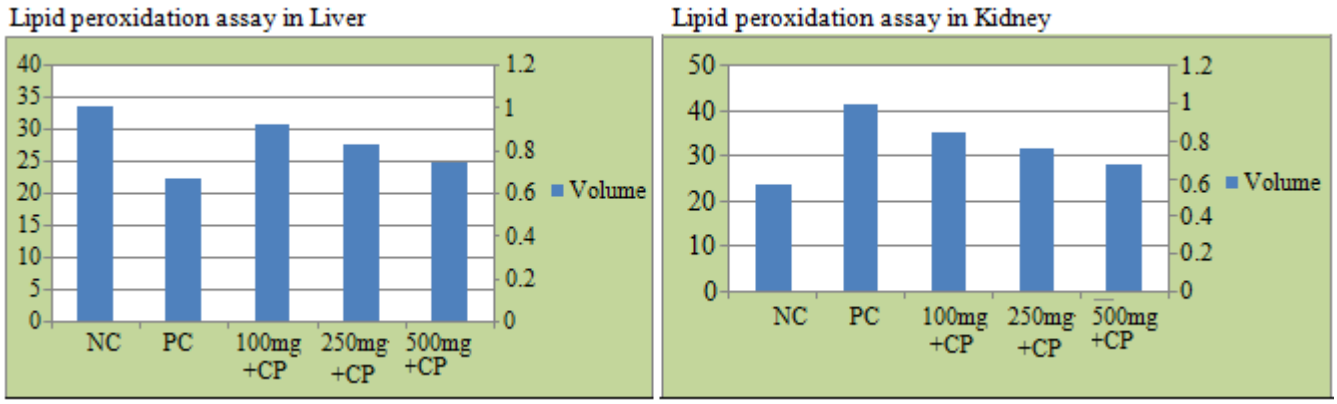

Figure 2: Lipid peroxidation in tissues

Cyclophosphamide is an inactive cytostatic and in the liver cells, it metabolized into active metabolites. During bioactivation ROS are also formed, which decreases the antioxidative capacity [33]. The two active metabolite of cyclophosphamide is phosphoramide mustard and acrolein. Lipid peroxidation is one of the main reasons of cyclophosphamide induced toxicity, due to the production of acrolein. Kim *et al* in 2012 have reported that Malondialdehyde is the end product of lipid peroxidation, which is a reactive aldehyde and marker of oxidative stress[34]. This formation of MDA was indicated by the presence of pink colour and it was found to be increase in positive control mice and there was a marked decrease in the formation of pink colour gradually in the extract treated mice, which indicates that the aqueous of rhizome of Curcuma caesia is effective in treatment of lipid peroxidation in table2 and fig2.

Table 3: Protein content in liver and kidney:

	Liver(Means±S.D) (µmole/mg)	Kidney(Mean±S.D) (µmole/mg)
Negative control	4.69±.08	9.31±.04
Positive control	1.38±0.11	3.46±0.08
100mg+CP	5.31±0.05	5.08±0.05
250mg+CP	9.08±0.02	7.85±0.06
500mg+CP	16.54±0.03	11.23±0.11

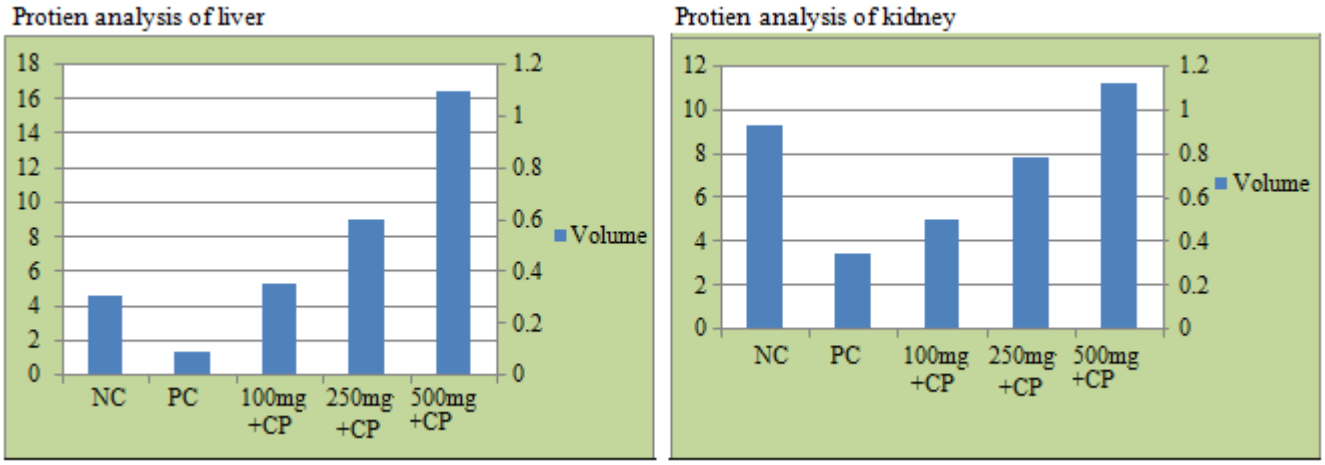

Figure 3: Protein content assay of liver and kidney

Protiens are macromolecules that act as alternate energy sources are in short supply. They are the building block of any organism. Protien content of liver and kidney were analysed and the results obtained are represented in table3. The protein contents was found to be decreased in positive control group as compared to normal groups. But also it was observe that there was significant increase in the level of proteins in the extract and CP treated groups in both the tissues showing inhibitory effects of the extracts used against cyclophosphamide as shown in fig3.

Table 4: Analysis of GSH in liver and Kidney:

	Liver (Mean± S.D) nmole/mg	Kidney (Means±S.D) nmole/mg
Negative control	1.3±0.09	1.5±0.01
Positive control	0.35±0.03	0.4±0.001
100mg+CP	0.3±0.002	1±0.011
250mg+CP	0.5±0.005	1.15±0.006
500mg+CP.,	0.95±0.009	1.3±0.005

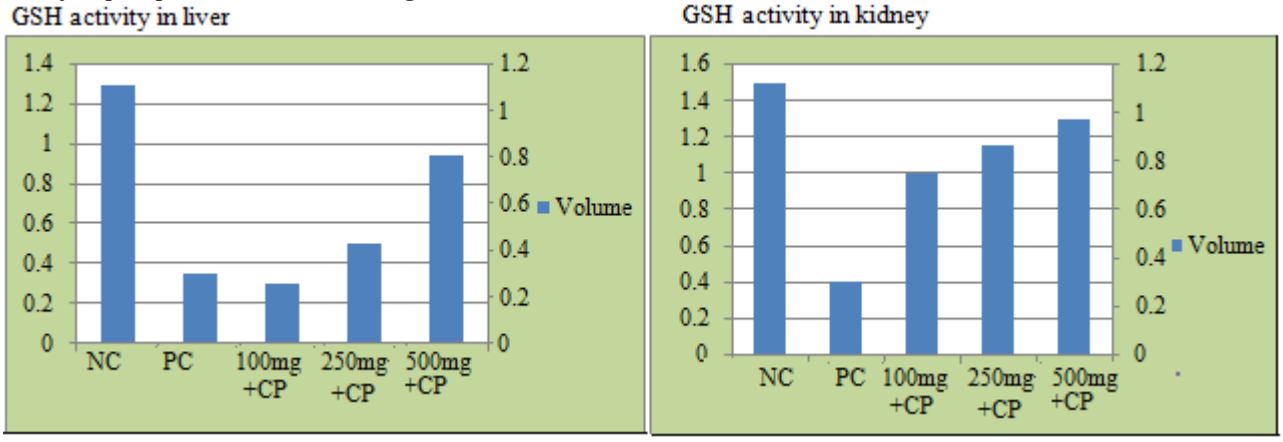

Figure 4: Analysis of reduced glutathione in both liver and kidney

Reduced glutathione an important antioxidant that is found to detoxify toxic substances by conjugation,reported by Peklak *et al* in 2005. The acrolein produced by cyclophosphamide reduced the level of GSH was reported by Abraham *et al*., 2008, which is the immediate results of lipids damage in tissues and protien damages [35]. The level of reduced glutathione was estimated and the results were represented in table4. Following CP injection, GSH was significantly declined in CP- injected mice as compared to normal ones was shown in fig4. Administrations of extract significantly ameliorated the altered GSH content.

Table 5: Assay of GR activity in liver and kidney

	Liver(NADPH oxidized per min per gram)	Kidney(NADPH oxidized per min per gram)
Negative control	9.49±0.146	10.52±2.182
Positive control	9.06±0.050	6.84±0.072
100mg+CP	7.82±0.243	7.21±0.094
250mg+CP	8.84±0.139	8.49±0.121
500mg+CP	9.48±0.117	9.08±0.089

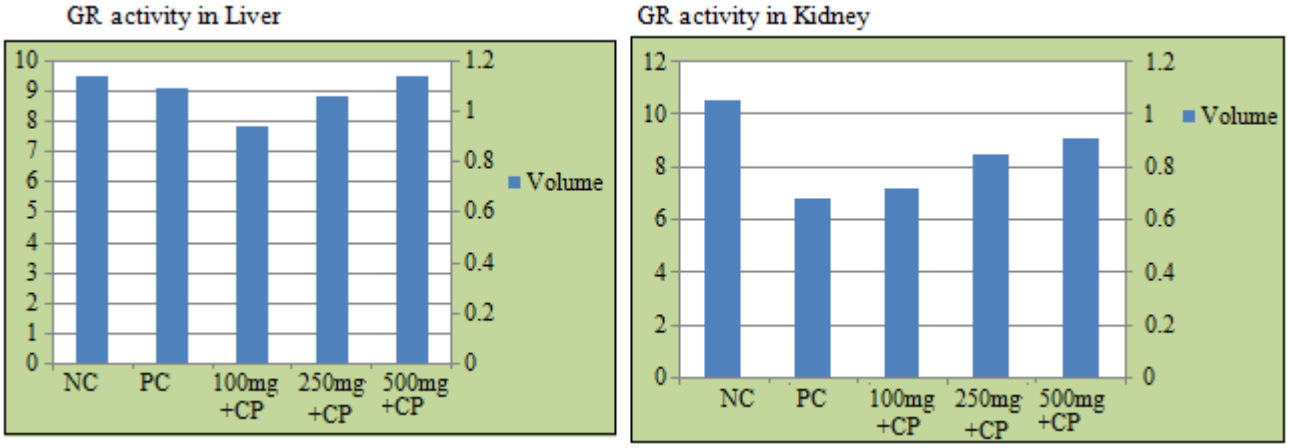

Figure 5: Analysis for GR activity in both Liver and Kidney

Glutathione reductase catalyzes the conversion of oxidized glutathione to reduced glutathione employing NADPH as substrate. The amount of NADPH utilized is a direct measure of enzyme activity discussed David and Richard in 1983. The activity of GR was assessed and the results obtained are shown in the above table5 and fig5. From the result it was observe that the ability to reduced glutathione oxidase to reduced glutathione decreases in positive control as compare to normal control. But the conversion increases in case of cyclophosphamide and extract treated mice in a dose dependent manner. Gossett, *et al* in 1996 proved that GR is a ubiquitous enzyme and may be a rate limiting enzyme for defense against active oxygen toxicity[36].

4. Discussion

The present study deals with the biochemical indices monitored in the liver and kidney are useful "markers" for assessment of tissue damage. The measurement of activities of various enzymes in the tissues and body fluids plays a significant role in disease investigation and diagnosis as discussed by Malomo in 2000. Tissue enzymes can also indicate tissue cellular damage caused by chemical compounds long before structural damage that can be picked by conventional histological techniques (Akanji,1986). Cyclophosphamide induced hepatotoxicity and nephrotoxicity involves cellular injury leading to the production of free radicals. Free radical alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids in the presence of oxygen to produce lipid peroxides as a consequence of which, the cytosolic enzymes SGOT and SGPT leach out leading to their raised levels in the blood, depression in proteins synthesis, and many other cytosolic enzymes [37].

Lipid peroxidation is the degradation of lipids that occurs as a result of oxidative damage and is an useful marker for oxidative stress. Polyunsaturated lipids are susceptible to an oxidative attack, typically by reactive oxygen species, resulting in a well –defined chain reaction with the production of end products such as malondialdehyde (MDA). Lipid peroxidation may contribute to the pathology of many diseases including atherosclerosis, diabetes and Alzheimer's. The present study shows that CP treated mice alleviates the level of peroxides in both the tissues i.e Liver and kidney. But the administration of extract in CP treated mice showed a marked concentration dependent decrease in the level of peroxides. Glutathione also plays an important role in the kidney and takes part in a transport system involved in the reabsorption of aminoacids (Ellman,..1959).Glutathione content are of significant importance because it helps in the conversion of oxidized glutathione (G-S-S-G) to reduced (GSH) by glutathione reductase. Glutathione reductase minimize or remove cellular reactive radical cascades and decrease cytotoxic oxidative damage in cells. Moreover, bioactive compounds inducing antioxidative enzymes or decreasing free radicals levels could decreases mutation production and cancer inhibition because they might reduce intracellular oxidative stress and DNA damage as discussed by Yen and chen in 1998. It is understood that defense against oxidative stress is primarily dependent upon combine action between exogenous and endogenous antioxidants. Since Curcuma Caesia has been known for its medicinal properties like higher antioxidant activity, high phenolic, flavanoid, curcumin content as referred in the papers (Mangla,2010;Krishnaraj *et al*,2010; Karmakar,2011; Sarangthem and Haokip,2010),it comes to the conclusion that it can protect the tissue damages caused by free radicals produced from Cyclophosphamide.

5. Conclusion

From this study it is clear that the bioative compounds of the medicinal plant Curcuma caesia play a vital role in many kinds of diseases such as hepatoxicity and nephrotoxicity in Animal models. Cyclophosphamide causes nephrotoxicity by alkylation of renal cells and hepatic cells by Cys sulfhydryl group of acrolein. This acrolien is one of the active metabolite of cyclophosphamide. Renal cell and hepatic cells alkylation leads to variable reduction in glomerular filtration rate as well as tubular dysfunction resulting from acute renal failure and many metabolite functions done by hepatic cells. Cyclophosphamide also generates the free radicals that cause renal and damage. Various pharmacological interventions may help in reducing oxidative stress and tissues damage. From these studies, we can end that formation of free radicals can be prevented by an antioxidant. Herbal drugs are radialy available, economic and without any side effects as medicinal herbs as the potential source of therapeutics aids have attained a significant role in the health system all over the world for

both humans and animals not only in the diseases condition but also as potential material for maintaining proper health.

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