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Response Pattern of Antioxidants to Lipid Peroxide Concentration in Carbon Tetrachloride-Induced Hepato-Toxicity Is Tightly Logistic in Rabbits

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Research Article

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ABSTRACT

Logistic response of antioxidants to lipid peroxide concentration in carbon tetrachloride toxicity in rabbit liver was evaluated. Carbon tetrachloride (CCl_4), ethanol extracts of C*hromolaena odorata* (ETECO), sylimarin (a known hepatoprotective agent) and water, were used to induce variations in the oxidant/antioxidant balance in the test and control animals. This was used as a model to study the delicate balance between the activities and/or the intracellular concentrations of these antioxidants and lipid peroxide. Concentrations of lipid peroxidation product (malondialdehyde) were estimated to access the degree of oxidation of the polyunsaturated fatty acids in the liver tissue. Glutathione (GSH) concentration was estimated to capture the non-enzymatic antioxidant concentration, while glutathione-s-transferase (GST), superoxide dismutase (SOD), and catalase (CAT) activities were assayed in the liver to assess the enzymatic antioxidant activities. Results obtained from this study showed that the concentrations of lipid peroxidation product (malondialdehyde) varied in a logistic fashion with the non-enzymatic antioxidant (glutathione) and the enzymatic antioxidants (glutathione-s-transferase, superoxide dismutase, and catalase). The concentration of the peroxidation product and the concentration/activity of the antioxidants were inversely related, maintaining a highly logistic relationship (R^2 = 0.99). The non-enzymatic antioxidant (GSH) concentration and the enzymatic antioxidant (GST, SOD, and CAT) activities were found to be directly related in a sigmoidal manner (R^2 = 0.98). These observations indicated that oxidant/antioxidant concentrations and activities in a rabbit liver tissue is tightly related and mathematically associated.

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1. INTRODUCTION

Fitting a curve or an equation to observed data is an important step towards a deeper analysis of a scientific or technological problem. A 'good fit' establishes the existence of a functional relationship among the variables. Understanding the response of tissue enzymatic and non-enzymatic antioxidant to oxidative damage resulting from chemical intoxication requires a deeper analysis of the generated data. Carbontetrachloride (CCl_4) has been one of the most intensively studied hepatotoxicants to date (Adinarayana et al., 2011) and provides a relevant model for other halogenated hydrocarbons that are used widely (Clawson, 1989; Dahm and Jones, 1996; Weber et al., 2003). It consistently produces liver injury in many species (Chavda et al., 2010). Carbontetrachloride is well known to be converted by cytochrome P-450-mixed function oxygenases in smooth endoplasmic reticulum of liver into toxic metabolite, mainly trichloromethyl radical ($CCl_3^{\bullet}$). This free radical in the presence of oxygen may cause peroxidation of lipids on target cell resulting in extensive damage.

Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, like trichloromethyl radical ($CCl_3^{\bullet}$) from carbontetrachloride, which start chain reactions that damage cells. Antioxidants on the other hand terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or polyphenols. Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1997). Defence against free radical-induced oxidative stress involve among others, the antioxidant mechanism. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants.

During hepatic injury, superoxide radicals generate at the site of damage and modulate superoxide dismutase (SOD) and catalase (CAT) activity, resulting in the loss of activity and accumulation of superoxide radical, which damages liver. Decreased CAT activity is linked up to exhaustion of the enzyme as a result of oxidative stress caused by CCl_4. A great number of reactions proceeding in cells are coupled with transfer of redox equivalents. So, maintenance of a particular redox state in cytoplasm is an important condition for the normal life of the cell. Redox activity, resistance to auto oxidation, high concentration and ability to maintain a reduced state, make glutathione (GSH) the most important intracellular redox buffer.

The glutathione *S*-transferases (GST) show high activity with lipid peroxides (Sharma et al., 2004). These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism (Hayes et al., 2005).

Under normal conditions, there is a balance between both the activities and the intracellular concentrations of these antioxidants. This delicate balance is essential for the survival of

organisms and their health (Reed, 1993). The relationship that exists between these antioxidants and lipid peroxides and the delicate balance between the activities and the intracellular concentrations of these antioxidants are not well documented. We wish to show that response of antioxidants to oxidative damage in carbontetrachloride intoxicated rabbit liver is logistic.

2. MATERIAL AND METHODS

2.1 Collection and Preparation of Plant Samples

Fresh aerial parts of *Chromolaena odorata* were collected from Egbu and Ihiagwa in Owerri, Imo State and authenticated by a plant taxonomist, at the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria. Voucher specimen has been retained at the authors' laboratory.

The leaves were shed, dried at 30^0C and then reduced to a coarse powder in a mill (Kenwood BL357). A 500g portion was extracted with 2 litre ethanol by shaking for 48hrs. Soluble extract was recovered by distillation under reduced pressure at 49^oC in a Buchi rotavapour (Switzerland). The extract was then dried to solid form in vacuum desiccator (CNS Simax), and stored in a freezer (4.0 ^{0}C) until needed.

2.2 Animals

Thirty white New Zealand male rabbits acquired from an animal breeder in Owerri, Imo State, Nigeria were maintained under standard environmental condition (28-30 ^{O}C, 60-70 % relative humidity, 12-h dark / light cycle) in stainless steel cages with free access to standard laboratory animal diet (Vital finisher) and drinking water.

2.3 Induction of Hepatic Injury

Seven days after acclimatization, animals (rabbits) were separated into five groups of six animals each. Group I served as normal control (NC) which received food and water only throughout the treatment period.Group II served as intoxicated controls (CCl_4 group) which received food and water *ad libitum* and carbon tetrachloride (0.2ml/kgbw in liquid paraffin 1:1) on day 7. Groups III served as intoxicated tests (ETECO test) that received food and water *ad libitum*, received ethanol extracts of *C. odorata* (400mg/kg body weight of animal) in two divided equal daily doses and carbon tetrachloride (0.2ml/kgbw in liquid paraffin 1:1) on day 7. Group IV received food and water *ad libitum* and received ethanol extracts of *C. odorata* (400mg/kg body weight of animal) in two divided equal daily doses but did not receive carbon tetrachloride. Group V received food and water *ad libitum* and received Sylimarin (50mg/kg body weight of animal) daily and CCl_4 on day 7. At the end of the seven-day pre-treatment and subsequent intoxication with carbon tetrachloride, animals were allowed for 48hrs. Animals were anaesthetized and sacrificed by cervical dislocation.

2.4 Preparation of Liver Homogenate

The liver was removed rapidly, and cut into separate portions. Hepatic tissues was homogenized in KCl [10 mM] phosphate buffer (1.15%) with ethylene-di amine tetra acetic acid (EDTA; pH 7.4) and centrifuged at 12,000×*g* for 60 min. The supernatant (post

mitochondrial fraction) was used to assay for the enzymes (glutathione-*s*-transferase, superoxide dismutase and catalase), glutathione and thiobarbituric acid reactive substances.

2.5 Estimatioin of Lipid Peroxides

Lipid peroxidation in the supernatant fractions was determined spectrophotometrically by assessing the concentration of thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et al. (1979) as described by Liu et al. (1990). The results were expressed in malondiadehyde (MDA) formed relative to an extinction coefficient of 1.56×10^6 mol/cm.

2.6 Determination of Glutathione Concentration

Reduced glutathione (GSH) was estimated by its reaction with dithio-bis-2-nitrobenzoic acid (DTNB) that gives a yellow coloured complex with absorption maximum at 412 nm (Raja et al., 2007).

2.7 Assay of Glutathione-S-Transferase (GST) Activity

Glutathione-*s*-transferase activity was estimated by the method of Habig et al. (1974). The reaction mixture consists of 2.75mL of sodium phosphate buffer (0.1 M; pH 7.4), 0.1mL reduced glutathione (l mM), 0.1mL supernatant in a total volume of 3.0 mL. The changes in the absorbance was recorded at 340 nm and enzymes activity was calculated as nanomoles of 1-chloro-2,4-dinitro benzene (CDNB) conjugate formed/min/mg protein using a molar extinction coefficient of $9.6 \times 10^3 M^{-1}\ cm^{-1}$.

2.8 Assay of Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD) activity was assayed as formerly described by Fridovich (1989). The ability of the superoxide dismutase to inhibit the autoxidation of adrenalin was the basis of the SOD assay.

2.9 Assay of Catalase (CAT) Activity

Catalase activity was assayed by the method of Aebi (1974). A 0.1ml portion of supernatant was added to cuvette containing 1.9mL of 50mM phosphate buffer (pH 7.0). Reaction was started by addition of 1.0mL of freshly prepared 30mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240nm using the equation for a first-order reaction.

2.10 Data Analysis

Results of groups were calculated as means ± SD, subjected to one-way analysis of variance (ANOVA) and significant difference between means were determined at alpha = 0.05 using Excel+Analysi-it (Leeds UK). Mathematical relationships between parameters were estimated by iterative minimization of least squares using Levenberg-marquardt algorithm (Table curve 2D systat USA) Marquardt (1964).

3. RESULTS

3.1 Effect of Ethanol Extracts of *Chromolaena odorata* on Liver Superoxide Dismutase Activity in Carbon Tetrachloride Induced Hepatotoxicity

Result obtained (Figure 1A) showed that Liver Superoxide dismutase activity in carbon tetrachloride intoxicated animals was significantly decreased when compared to normal control and ethanol extracts of *C. odorata* control. Ethanol extract of *C. odorata* significantly raised the activity of Liver Superoxide dismutase in the intoxicated group, but could not restore SOD to their normal control activity.

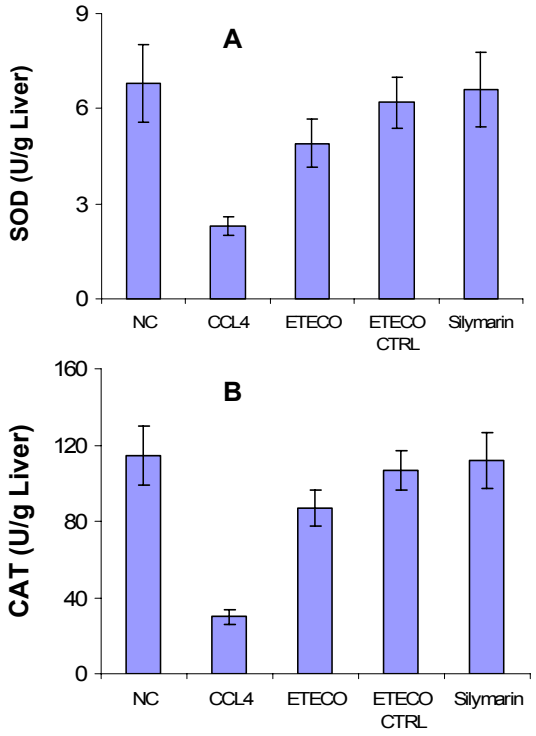

Fig. 1. Effect of ethanol extracts of *Chromolaena odorata* on (A) superoxide dismutase (SOD) and (B) catalase activity (CAT) in carbon tetrachloride induced liver intoxication in rabbits.

3.2 Effect of Ethanol Extracts of *Chromolaena odorata* on Liver Catalase Activity in Carbon Tetrachloride-Induced Hepatotoxicity

Our result (Figure 1B) shows that Liver catalase activity in carbon tetrachloride intoxicated animals was significantly decreased when compared to normal control and ethanol extracts of *C. odorata* control. Ethanol extract of *C. odorata* significantly raised the activity of Liver catalase in the intoxicated group; it however, could not restored catalase activity to a level not significantly different from the normal control level.

3.3 Effect of Ethanol Extract of *C. odorata* on Hepatic Glutathione

Carbon tetrachloride intoxication in the animals produced a decrease in glutathione concentration (Figure 2A). This decrease in glutathione concentration was reversed by treatment with ETECO which significantly increased the glutathione concentration.

3.4 Effect of Ethanol Extract of *C. odorata* on Hepatic Lipid Peroxidation

Intoxication of rabbits with carbon tetrachloride produced an elevated MDA concentration which was significantly higher than the normal control and the ethanol extracts of *C. odorata* treated control (Figure 2A). Treatment with ethanol extracts of *C. odorata* significantly lowered MDA concentration in the intoxicated animals.

3.5 Effect of *C. odorata* Ethanol Extract on Hepatic Glutathione-S-Transferase Activity

Our result (Table 1) showed that liver glutathione-S-transferase activity in carbon tetrachloride intoxicated animals was significantly decreased when compared to normal control (NC) and ethanol extracts of *C. odorata* control. Ethanol extract of *C. odorata* significantly raised the activity of liver catalase in the intoxicated group; it however, could not restored glutathione-S-transferase activity to a level not significantly different from the normal control activity.

Table 1. Effect of ethanol extract of *C. odorata* (400mg/kgbw) on some oxidative stress parameters in carbon tetrachloride intoxicated rabbits

Parameters	Normal Control	Carbon tetrachloride (CCl_4)	ETECO Test	ETECO Control	Silymarin (50mg/kgbw)
MDA nMol/gLiver	8.0 ± 0.38	25 ± 1.2	15 ± 0.7	10 ± 0.48	9.5 ± 0.9
GSH µMol/gLiver	650 ± 18	300± 12	400 ± 15	590± 32	625 ± 21
GST (µmol/min/mg Protein $\times 10^{-4}$)	1535 ± 120	635 ± 43	1026 ± 90	1420 ± 86	1420 ± 110
SOD U/gLiver	6.8 ± 1.20	2.3 ± 0.30	4.9 ± 0.76	6.2 ± 0.80	6.6 ± 1.18
CAT U/gLiver	114.30±15.4	30.00 ± 3.90	87.0± 9.70	106.80 ±10.60	112.0 14.65

3.6 Response of Lipid Peroxidation to Variation in Glutathione Concentration in Carbon Tetrachloride Induced Hepatotoxicity

Mathematical modeling of relationship existing between GSH and MDA (Figure 2B) shows that Lipid peroxidation responds to variation in Glutathione concentration by a mathematical relationship as shown in Table 2.

Table 2. Mathematical model of relationship existing between some oxidative stress parameters in rabbit liver exposed to carbon tetrachloride

Oxidative Stress Parameter		Equations / Empirical values *Logistic dose response (LDR abcd)……(1)* *Sigmoid model (abcd)……..(2)* $y = a + \frac{b}{1 + \left(\frac{x}{c}\right)^d}$ ……..(1) $y = a + \frac{b}{1 + Exp\left(-\frac{(x-c)}{d}\right)}$ ……..(2)				Procedure Levenberg Marquardt r^2 Coef Det	Robust Minimization Lorentzian DF Adj r^2	Error Fit Std Err
y	*x*	*a*	*b*	*c*	*d*	r^2	r^2	
GSH	MDA	0.28385073	0.45612445	11.394014	4.1086707	0.991	0.964	0.026
GST	MDA	622.170210	8699.16990	4.1471393	3.2874775	0.990	0.987	0.038
GSH	GST	0.25519249	0.39894404	875.93975	119.69472	0.983	0.932	0.036
CAT	SOD	-419.04569	582.726598	-2.365309	3.8503139	0.999	0.999	0.076

3.7 Response of Glutathione-S-Transferase (GST) Activity to Lipid Peroxidation in Carbon Tetrachloride Induced Hepatotoxicity

Mathematical modeling of relationship existing between GST and MDA (Figure 2C) glutathione-s-transferase responds to induction of lipid peroxidation in Carbon tetrachloride induced hepatotoxicity is governed by a logistic dose response with mathematical relationship as shown in Table 2. As peroxidation increases, activity of GST was decreased.

3.8 Response of Glutathione-S-Transferase (GST) Activity to Depletion of Glutathione in Carbon Tetrachloride Induced Hepatotoxicity

Mathematical modeling of relationship existing between GST and GSH (Figure 2D) shows that Glutathione-S-transferase's activity responds to variation in glutathione concentration in carbon tetrachloride induced hepatotoxicity is governed by a sigmoid abcd equation (Table 2) Depletion of glutathione concentration resulted in a reduction in GST enzyme activity, while increase in glutathione concentration resulted to increases in GST enzyme activity.

3.9 Response of Superoxide Dismutase (SOD) and Catalase (CAT) Activity in Carbon Tetrachloride Induced Hepatotoxicity

Mathematical modelling of relationship existing between superoxide dismutase and catalase activity (Figure 2E) showed that their responds to induction of lipid peroxidation in Carbon tetrachloride induced hepatotoxicity is governed by a sigmoid abcd equation (Table 2). As peroxidation increases, activities of superoxide dismutase and catalase were decreased. Table 3 showed that superoxide dismutase and catalase had a high negative Pearson correlation coefficient (r = -0.99846 and -0.99589 respectively).

Table 3. Pearson correlation coefficient *(*r) between the antioxidant parameters and malondialdehyde

Pearson correlation coefficient (r)	MDA	GSH	GST	SOD	CAT
MDA	1				
GSH	-0.95327	1			
GST	-0.98757	0.98582	1		
SOD	-0.99846	0.959608	0.987102	1	
CAT	-0.99589	0.92844	0.970487	0.995112	1

4. DISCUSSION

During hepatic injury, superoxide radicals generate at the site of damage and modulate SOD and CAT, resulting in the loss of activity and accumulation of superoxide radical, which damages liver. Decreased CAT activity is linked up to exhaustion of the enzyme as a result of oxidative stress caused by CCl_4. The restoration of SOD and CAT activities in CCl_4-treated animals using ethanol extract of *C. odorata* (Figure 1A and 1B) is an indication of its antioxidant property.

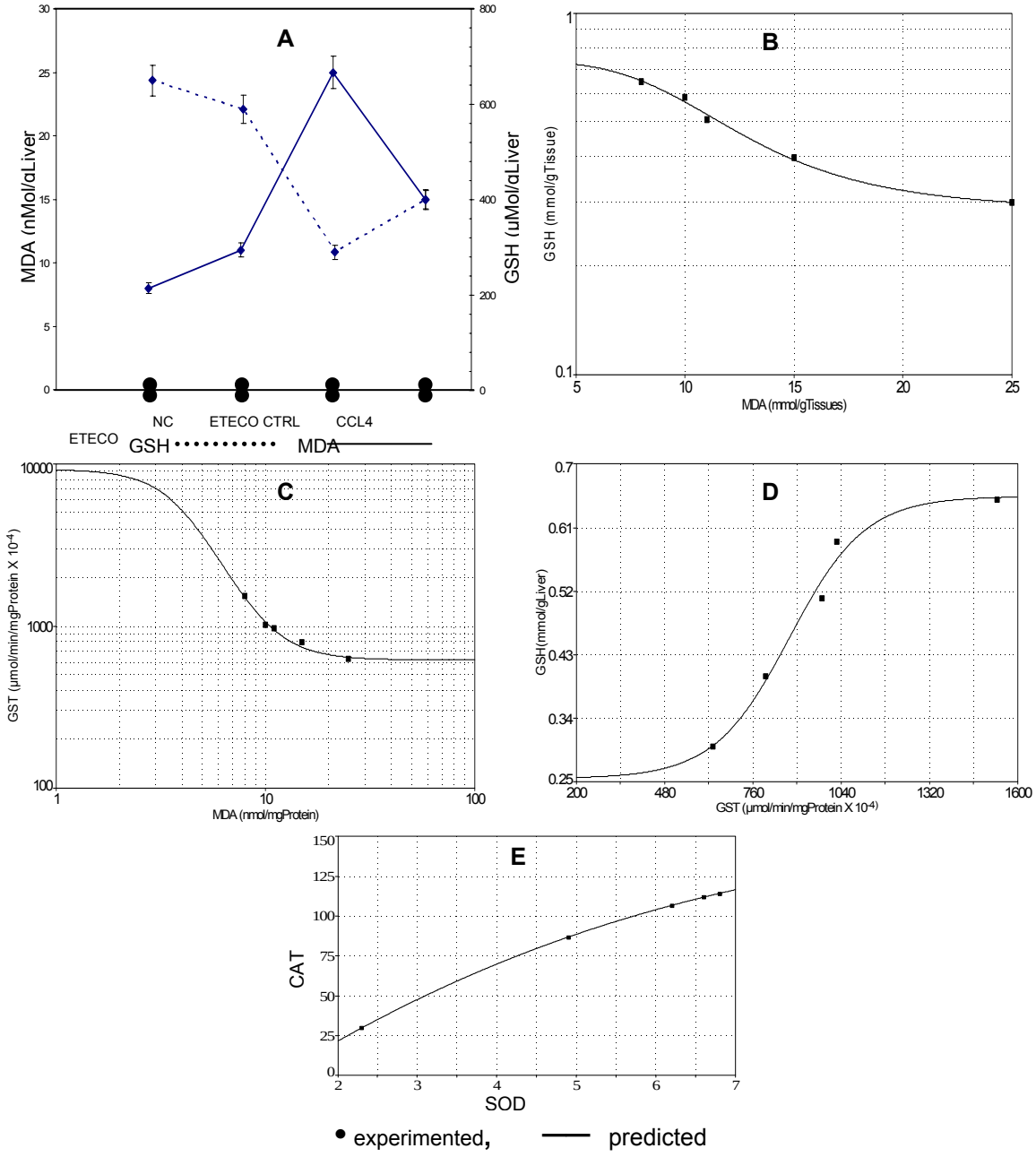

Fig. 2. Relationship existing between GSH, GST, MDA, CAT and SOD. Response of lipid peroxidation to glutathione concentration (A, B), glutathione-s-transferase responds to lipid peroxidation in Carbon tetrachloride induced hepatotoxicity (C), glutathione-s-transferase responds to glutathione concentration (D) and catalase (CAT) activity in response to superoxide dismutase (SOD) activity.

Reduced glutathione (GSH) constitutes the first line of defense against free radicals. Due to its central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants (Meister and Anderson, 1983). Glutathione concentration in tissues affected malondialdehyde concentration (Figure 2A). Malondialdehyde concentration is directly proportional to/and indicative of degree of lipid peroxidation and related inversely to glutathione concentration in a dose dependent fashion that mimicked logistic dose response model abcd (R^2 = 0.991). Higher glutathione concentration indicated higher antioxidant status. The above observations meant that peroxidation in carbon tetrachloride intoxicated rabbits increased strictly in a mathematical fashion that is related to antioxidant status.

Cellular responses to chemical perturbations are known to follow mathematical models. The inverse association seen in glutathione and malondialdehyde concentrations are because glutathione works to protect the cell against oxidative attack and peroxidation, so if glutathione protection is overwhelmed, peroxidation increases. Reduction in liver glutathione seen in association with increased lipid peroxidation in CCl_4-treated rabbits indicated an antioxidant diminution resulting from increase in oxidative stress which may have resulted to damage to the hepatic cells. Increase in hepatic glutathione concentration in ETECO treated rabbits could either be due to an effect on the *de novo* synthesis of GSH, its regeneration or both. Ko et al. (1995) had suggested that as a consequence, hepatic glutathione concentration could be sufficiently maintained to counteract the increased formation of free radicals as in the case of carbon tetrachloride toxicity.

There was a strong relationship between the MDA concentration, GSH concentration (r =-0.95327) and GST activity (r = -0.98757). GST plays a physiological role in initiating the detoxification of potential alkylating agents Raja et al. (2007). Chemicals like chloroform and CCl_4 alter the hepatic GST activity (Aniya and Anders, 1985). GST activity was significantly ($p < 0.05$) reduced in CCl_4-treated rabbits and upward reversal was observed with treatment using ethanol extract of *C. odorata*. This may be attributed to substrate (glutathione) activation or a direct action of extract on the hepatic GST activation, the mechanism of which is not clearly understood.

Glutathione-s-transferase responds to induction of lipid peroxidation in carbontetrachloride-induced hepatotoxicity is governed by a logistic dose response model (R^2 = 0.990) (Figure 2C). The mathematical association between GST and MDA seem to be an indirect one. Glutathione depletion related directly to GST activity. This direct relationship affected GST /MDA association. Glutathione-s-transferase activity responded to induction of lipid peroxidation in a logistic dose response manner which is inversely related. Mathematical modeling of relationship existing between GST and GSH (Figure 2D) showed that glutathione-s-transferase's activity responded to variation in glutathione concentration in Carbon tetrachloride induced hepatotoxicity by a sigmoid abcd equation: Depletion of glutathione concentration resulted in a reduction in GST enzyme activity, while increase in glutathione concentration resulted to increases in GST enzyme activity. Glutathione-s-transferases may act as binding proteins, which are primarily involved in the neutralization of harmful exogenous or endogenous compounds by enzymatic conjugation with the scavenger peptide glutathione (GSH) and/or by direct binding of non-substrate ligands (Beckett et al., 1993), such as bilirubin (Carmagnol, 1981). The other major function is to protect against oxidative damage to lipids and nucleic acids and participate in the metabolism of some steroids and leukotrienes. Increase in glutathione-s-transferases activity seen in pre-treatment with ethanol extract of *C. odorata* or sylimarin indicated that activation of glutathione-s-transferases in the presence of the extract could be substrate activation since pre-treatment brought about increase in glutathione concentration. Measurement of

glutathione-s-transferases activity had been considered useful for the evaluation of prophylactic treatment in trials of antioxidant strategies Chiang et al. (2007). Consideraions/conclusions of this nature needed a strong mathematical basis as provided in this study. We confirm that association of glutathione-s-transferases activity with oxidant product/antioxidant concentration is strong enough to make it useful.

5. CONCLUSION

We have shown that variation in antioxidants (superoxide dismutatse, catalase, glutathione, glutathione-s-transferases) activity/concentration to lipid peroxide concentration in carbon tetrachloride-induced hepatotoxicty in rabbit liver follow a logistic pattern. It is expected that in future, predictions on the oxidant stress level could be made simply from antioxidant activity/concentrations and vice-versa.

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