Comparative Study of Tissue Oxidative Stress in *Hydrocotyl bonarienssis* Treated Rats Fed with Dietary Galactose

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ABSTRACT
Studies on toxicity of galactose and its management have been largely concentrated on the lens. The present study compare the oxidative stress that occurred in various tissues of rats placed on dietary galactose and simultaneously administered with different doses of *Hydrocotyl bonariensis* leave extract. Male rats weanling rats placed on dietary galactose and administered (orally) with different doses of the extract were used in the study. After four (4) weeks of treatment a significant increased level of lens and liver peroxidation with reduced activity of catalase, superoxide dismutase and reduced glutathione (GSH) level was observed in rats placed on dietary galactose, whereas the kidney was not significantly affected (though superoxide dismutase activity of the kidney increases). Administration of 500mgKg[-1] of the extract lowered the level of peroxidation of these tissues and significantly increased the catalase activity and GSH level, while superoxide dismutase activity only increased in the lens and the kidney. Though treatment with 1000mgKg[-1] of the extract reduced the level of peroxidation of these tissues, it does not increase the antioxidant status. Our findings suggests that, the lens and liver is the most at risk of oxidative stress generated with dietary galactose but not the kidney and that *Hydrocotyl bonariensis* leave possess active components that may be beneficial in reducing the effect of these oxidative stress when administered at lower dose.

Key Words: Tissues, Oxidants, peroxidation, Antioxidants, Galactose, *Hydrocotyl bonariensis*.

INTRODUCTION

Research has long implicated free radical oxidative stress resulting from hyperglycemia as one source of long term complications of excess blood sugar (Brownlee et al 1988; Hunt and Wolf 1991). Galactose is an essential component of cerebrosides and mucopolysaccharides, and interference of galactose utilization by a tissue is thought to create a shortage in these vital macromolecules (Cordes 1960). Galactose, flooding into the retinal tissue in excess, initiates non-enzymatic glycation and enters into the polyol pathway to generate free radicals and induce oxidative damage, as well as activating protein kinase C and other diabetes- like abnormalities (Kern and Engerman 1996; Kowluru et al 2000).

The polyol pathway has been implicated as the primary cause of cataractogenesis in diabetics. The formation of hydroxyl radical was detected in sugar cataracts induced by galactose in rats using ESR spin trapping method with a spin trappin agent DMPO (Kubo et al 1999). Polyol accumulation of lenses in the same group simultaneously peaked. It is suggested that hydroxyl radical was produced in proportion to polyol accumulation in the early cataract stage. Aldose reductase is the key enzyme for the polyol pathway. This enzyme has been found to play a pivotal role in the development of "sugar" cataracts (Pallavi 2003). Aldose recuctase reduces the unused glucose to sorbitol which is then oxidized to fructose. In uncontrolled diabetics, polyol pathway is highly favors which ultimately lead to production of sorbitol. An increase in the concentration of glucose contributes to an enhanced activity of the two enzymes used in the polyol pathway, aldose reductase and sorbitol dehydrogenase. With the increased activity of these two enzymes, there is a consequent reduction in the NADPH: NADP$^+$ ratio and the NADH: NAD$^+$ ratio (Giugliano et al 1996). The decrease concentration of these cofactors may cause an inhibition in enzymes which are NADPH-dependent and lead to shortage of NADPH available for many pathways it is involved in. Consequently, there may be decreased synthesis of reduced glutathione (GSH), nitric oxide, mynositol and taurine. These may cause an increase in free radical production which may lead to ischaemia. Sorbitol may glycate nitrogen on proteins and the products of these glycations are referred to as advanced glycation products (AGEs) (Brownlee 2000). Galactose is also a substrate for aldose reductase, producing an alcohol galactitol and sorbitol, which accumulates in the lens. The result is the swelling of fibres, disruption of the normal cytoskeletal architecture and lens opacification (Thomas et al 2004). Galactitol is not however a substrate for sugar alcohol dehydrogenase and thus accumulates rapidly, producing the same osmotic effects and the same consequence as sorbitol. Galactokinase is the first enzyme in the metabolism of galactose; the conversion of galactose to galactose-1-phosphate. Galactokinase deficiency results in galactosemia, galactosuria and cataracts. Cataracts

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form in galactokinase deficient individuals because ingested galactose cannot be metabolized and is therefore available for reduction to galactitol through the action of aldose reductase.

The study of the enzymatic mechanism involved in the reduction of galactose to galactitol has revealed that the lens is a particularly favourable site for the accumulation and production of galactitol. Galactose concentration must be fairly high before the enzyme aldose reductase can convert significant amounts of the sugar to the alcohol form. In other tissues, even though the organ may be exposed to high levels of galactose, the phosphorylation mechanism is sufficiently active to keep the sugar at low levels (Kinoshita et al 1962)

Severe oxidative stress has been reported to cause cell damage and death. In mammalian cells, oxidative stress appears to lead to increase in the levels of free Ca^{2+} and iron within the cells. It has been suggested that excessive rises in intracellular free Ca^{2+} may cause DNA fragmentation by activating endonucleases (Okezie 1998). The importance of oxidative stress injury depends on the molecular target, the severity of the stress and the mechanism by which the oxidative stress is imposed. The toxic effects of free radicals are neutralized by antioxidant system, both enzymatic and non enzymatic (Suryanarayana et al 2003; Spector 1991; Jacques and Chylack 1991). Human defense against free radical induced damage include the enzymes catalase and glutathione peroxidase (both of which remove hydrogen peroxide) as well as superoxide dismutase which catayzes the dismutation of superoxides to the less toxic hydrogen peroxide which is then completely detoxified by catalase and/ or glutathione peroxidase.

No drug is available that has been proven to prevent or reverse the progression of senile cataract. New medical advances such as surgery and lens replacement have a high degree of efficacy, but are not available or too expensive for most of the world's population. The use of traditional medicines, mainly derived from plant sources, has been a major part in the management of many chronic ailments (Swaston et al 1999; Grover et al 2002). Moreover, there is a renewed interest in recent times to identify as many plant sources as possible for their therapeutic values (Chang 2000; Grover 2002). A number of observational studies suggest that intake of foods containing micronutrients having antioxidant potential may be protective against cataract (Jacques and Chylack 1991). In a study by Ansari and Srivastava (1990), antioxidant supplementation was found to inhibit the development of cataract in experimentally induced diabetes rats. Curcumin, a widely used spice in India has also been shown to have significant antioxidant activity both *in vitro* and *in vivo* and show some anticataractogenic properties (Anil Kumar et al 2005).

Hydrocotyl bonariensis Lam., Apiacea (Large leave pennyworth) is an herbaceous plant with prostate, creeping or floating stems, roots forming at nodes. There are approximately 402 species in this genus. They are mostly found in Africa and America. Its common name in West Africa, Nigeria is Karo. Its medicinal use as emetics, diuretics and laxatives has been reported (Evans 1992). The use of the leave of the plant in treating various symptoms of ophthalmic diseases is quite gaining popularity among some local people in Western Nigeria and this is without scientific proof since no data has been sighted in the literature to support this use. An on going investigation in our laboratory indicates the protective effect of the leave of the plant in galactose model of experimental cataract and its ability to reduce cataract progression following its administration after cataract onset. The study also indicated a reduction in oxidative stress and significant increases in antioxidant status with the administration of the extract.

Although, the toxicity of galactose on the lens has been studied for over 70 years, and galactose induced cataract is well accepted as an animal model for an investigation of the cataracts in human (Suryanarayana et al 2003; Ai et al 2000), very few study have been reportedly carried out on the investigation into the interactions of excess dietary galactose and oxidative stress in extra-ocular tissue. In the current study, we have investigated whether generation of oxidative stress in rats placed on dietary galactose can adversely affect other tissue apart from the lens and the efficacy of administration of *Hydrocotyl bonariensis* leave extract in combating this stress in various tissues.

MATERIAL AND METHOD

Plant authentication and extraction
Mature fresh leaves of *Hydrocotyl bonariensis* were collected from a local garden and were authenticated at the herbarium of the Botany Department, University of Lagos, Nigeria. The herbarium voucher number is 13478. The leaves were then oven dried at $40^{o}C$ for 24 hours and further blended using a local kitchen blender. The blended leaves were soaked in water at room temperature for 48 hours. The extract was then sieved into a clean container and further concentrated using a rotary evaporator at $40^{o}C$. The concentrated product was then lyophilized.

Experimental subjects

Thirty (30) male rats [21days old] having an average body weight of 30g bred in the animal house, Department of Biochemistry, Olabisi Onabanjo University, Ago-Iwoye, Nigeria, were used in the study. The rats were randomized into five (5) groups of 6 rats each labeled A-E. Each group was fed a different diet and treated as follows; Group A: Rats were fed a normal stock diet based on the AIN-93 (Reeves, 1997) formula; Group B: Rats were fed 30% galactose in the above diet; Group C: Rats in this group received the group B diet and were administered with $500mgKg^{-1}$ extract; Group D: Rats in this group received the group B diet and administered with $1000mgKg^{-1}$ extract; Group E: rats in this group were kept on the AIN-93 stock diet and administered with $1000mgKg^{-1}$ extract. Animals were housed in individual cage in a temperature and humidity controlled room having a 12hr light and dark cycle. All of the animals had free access to their respective feed and clean drinking water. Their weights were monitored weekly through out the research period. The experiment was carried out for four (4) weeks after which food were withdrawn from the animals overnight, blood was collected from five (5) rats in each group by cardiac puncture into lithium heparinised tube after diethyl ether anesthesia. The rats were then sacrificed by cervical dislocation. Eyes were removed from each rat and lenses were dissected by posterior approach. The kidney and liver were also removed from all the rats sacrificed, cleaned of blood, weighed and then homogenized.

Care of animal

The care of the animals was in accordance with the U. S. Public Health Service Guidelines (NRC, 1999) and approved by the Olabisi Onabanjo University, College Of Health Sciences, Animal Ethics Committee.

Preparation of crude homogenate

[a] **Lens:** A pair of lenses from rat in each group were pooled together and homogenized in 10%, 100mM ice-cold potassium phosphate buffer, pH 6.2. The homogenate was centrifuged at 15,000xg for 30minutes at 4^oC (Reddy et al 2004). All the biochemical parameters were analyzed with the soluble fraction of the lens homogenate except the lens MDA which was estimated in the total homogenate.

[b] **Liver and Kidney:** The liver and the kidney of each animal were homogenized separately in 10% ice cold phosphate buffer, pH6.2. It was then centrifuged at 12,000xg for 5 minutes at 4^oC. The supernatant was carefully removed and used for all biochemical analysis except for the MDA that was determined in the total homogenate.

Biochemical Analysis

Malonaldialdehyde (MDA) was estimated as Thiobarbituric acid reacting substances (TBARS) as described by Buyan et al, (1981). Reduced glutathione (GSH) was assayed for by the method of Beutler et al (1963); Catalase activity was assayed for by the method of Sinha (1972). The method of Del Maestro et al (1983) was used to determine superoxide dismutase activity.

Validation of biochemical analysis

For validation of the aforementioned assay, all the parameters were repeatedly analyzed in the tissues of healthy rats. Reproducible assay characterized by SEM and obtained in repeated determinations (n=5) were noted.

Statistics

Statistical analysis was carried out with ANOVA and Duncan Multiple range test, with a $p<0.05$ considered significant.

RESULTS

Effect of treatment on tissue peroxidation

Shown in table 1 is the level of peroxidation of the liver, kidney and the lens after four (4) weeks treatment period. The result indicated that there was a significant increase in the liver and lens MDA of rats placed on dietary galactose but the value in the kidney was not significantly different from that of the normal control group. With administration of the extract at all the tested doses, the observed MDA value in the liver and the lens was significantly lowered than the untreated group (test control) but not different from the normal

control value. Treatment with the extract was not seen in the study to also cause a significant variation in the level of kidney MDA value.

Table 1. Peroxidation level of various tissues

Group/ Treatment	TBARS [nmol MDA/g tissue]		
	Liver	**Kidney**	**Lens**
A (Normal control)	16.11±1.48	4.99±0.48	1.47 ± 0.15
B (Galactose Diet; Test Control)	19.47±1.04^x	4.12±0.56	2.24 ± 0.13^x
C (Galactose Diet + 500mg/ kg Extract)	15.85±1.59	4.37±0.31	1.77 ± 0.22
D (Galactose Diet + 1000mg/ Kg Extract)	14.44±0.89	4.74±0.55	1.65 ± 0.28
E (Normal Control + 1000mg/ Kg extract)	16.83±2.01	4.94±0.38	1.42 ± 0.28

Note:
- Results presented are mean ± SEM (n=5).
- Mean values were compared using one-way ANOVA. Level of significance was evaluated using Duncan's multiple range test (DMRT).
- ^{X} denotes that value is different from others in the same column ($p<0.05$) but not different from value with a similar superscript.

Effect of treatment on antioxidant status
With dietary galactose, there was a significant decrease in the liver and lens catalase activity whereas the activity in the kidney was not significantly affected (Table 2). Administration of the extract at 500 mgKg^{-1} raised the catalase activity of both the liver and the lens to pretreatment level. Though the observed liver catalase activity at 1000 mgKg^{-1}was noticed not to be significantly different from the normal control value, the activity of the enzyme in the lens was significantly lowered than the normal control value and was also lowered than that of the group placed on galactose diet not treated with the extract (test control, group B). Treatment was also not observed to significantly alter the activity of the enzyme in the kidney.

Table 2. Catalase activity of various tissues after treatment

Group/ Treatment	Catalase activity (μg/mg protein) x10^3		
	Liver	**Kidney**	**Lens**
A (Normal control)	10.37± 0.03	1.12± 0.12	3.18± 0.35
B (Galactose Diet; Test Control)	8.39± 0.02^x	1.04 ±0.13	2.85 ± 0.09^x
C (Galactose Diet + 500mg/ kg Extract)	10.38± 0.04	1.34 ±0.07	3.19 ± 0.07
D (Galactose Diet + 1000mg/ Kg Extract)	10.35 ±0.29	0.96 ±0.12	2.25 ± 0.07$^{\#}$
E (Normal Control + 1000mg/ Kg extract)	10.42± 0.04	1.03 ±0.03	2.95 ± 0.47$^{¢x}$

Note:
- Results presented are mean ± SEM (n=5).
- Mean values were compared using one-way ANOVA. Level of significance was evaluated using Duncan's multiple range test (DMRT).
- The asterisk (*) and $^{\#}$ denotes that value is significantly different ($p<0.05$) from others in the same column but not different from value with a similar superscript, while $^{¢}$ denotes that value is not different from A and C.

Shown in table 3 is the result of treatment on the tissue's reduced glutathione concentration. The result indicates that with dietary galactose, there occurred a significant reduction in the GSH level of the liver and of the lens whereas that of the kidney was not significantly affected. Simultaneous administration of the extract at 500mgKg^{-1} however prevent this reduction as the observed GSH level in both the liver and lens of group C rat was not significantly different from that of the normal control (group A). When the extract was administered at 1000mgKg^{-1} however, the liver GSH level was not significantly different from that of the

untreated group, but lowered than that of the normal control. Neither dietary galactose nor the administration of the extract was noticed to have significantly altered kidney GSH level.

Table 3. Reduced glutathione (GSH) content of various tissues

Group/ Treatment	GSH Level [μg/g tissue] x 103		
	Liver	Kidney	Lens
A (Normal control)	14.80±0.05	8.98±0.18	7.10 ± 0.13
B (Galactose Diet; Test Control)	12.75±0.02*	8.01±0.10	6.31± 0.12*
C (Galactose Diet + 500mg/ kg Extract)	14.08±0.82	8.88±0.10	7.18 ± 0.03
D (Galactose Diet + 1000mg/ Kg Extract)	12.87±0.03*	8.68±0.21	6.67 ± 0.23*¢
E (Normal Control + 1000mg/ Kg extract)	14.10±0.02	8.99±0.06	6.99± 0.17

Note:
- Results presented are mean ± SEM (n=5).
- Mean values were compared using one-way ANOVA. Level of significance was evaluated using Duncan's multiple range test (DMRT) at $p \leq 0.05$.
- The asterisk (*) denotes that value is significantly different ($p < 0.05$) from others in the same column but not different from value with a similar superscript while ¢ indicates that values are not different from A, C and E.

Presented in Table 4 is the observed superoxide dismutase activity of various tissues after the treatment period. In rats placed on dietary galactose, a significant reduction in the lens and kidney superoxide dismutase activities was observed, whereas, the activity of the enzyme in the liver was not significantly affected. Administration of the extract at 500mgKg^{-1} however, significantly raises the activities of the enzyme above the pretreatment values in both the lens and the kidney. At 1000mgKg^{-1} administration, the superoxide dismutase activities of all the tissues studied were not different from the control activity. The enzyme activity in the lens at this dose was also not different from that of the untreated group (test control). Neither dietary galactose nor treatment with the extract was noticed to significantly alter the liver superoxide dismutase activity.

Table 4. Superoxide dismutase activity after treatment period

Group/ Treatment	SOD Activity [ng/mg protein] x 10^3		
	Liver	Kidney	Lens
A (Normal control)	6.62±1.37	1.60±0.18	1.39±0.12
B (Galactose Diet; Test Control)	6.37±0.28	0.82±0.14*	0.64±0.31*
C (Galactose Diet + 500mg/ kg Extract)	9.58±0.35*	2.10±0.39#	1.61±0.31
D (Galactose Diet + 1000mg/ Kg Extract)	6.10±0.18	1.21±0.52	1.13±0.23*¢
E (Normal Control + 1000mg/ Kg extract)	6.16±1.39	1.44±0.68	1.42±0.22

Note:
- Results presented are mean ± SEM (n=5).
- Mean values were compared using one-way ANOVA. Level of significance was evaluated using Duncan's multiple range test (DMRT) at $p \leq 0.05$.
- The asterisk (*) and # denotes that value is significantly different ($p < 0.05$) from others in the same column but not different from value with a similar superscript while ¢ indicates that value is not different from A, C and E.

DISCUSSION

Oxidative stress is caused by an imbalance between the production of reactive oxygen and biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. A previous study by Struther et al (2001) reported that in the galactose fed model of experimental cataract, the liver is exposed to the highest concentration of the oxidative stressor and thus may bear the brunt of the damage. Another study in rats exposed to cadmium (50mg Cd/l) and ethanol (5gEtOH/ Kg body weight/24h,

intragastrically) for 12 weeks in drinking water reported an increased in MDA concentration in the liver, whereas, the parameter was unchanged in the kidney (Cotter et al 1992). Our findings in this study indicate that when rats are placed on dietary galactose, the liver along with the lens faces the greater risk of oxidative damage.

An interesting finding from this study indicates that with cataract development, a reduction in liver catalase activity occurred whereas the enzyme activity in the kidney is not altered. This report is similar to the report of Struther et al (2001) but differ from the observation reported with cadmium and ethanol administration, where a decrease in catalase activity of both the liver and kidney was reported. Low levels of antioxidants, or inhibition of the antioxidant enzymes has been reported to cause oxidative stress and may damage or kill cells (Ho et al 2004). GSH is an extremely important cell protectant. It is known to directly quench reactive hydroxyl free radicals, other oxygen- centered free radicals, and radical centers on DNA and other biomolecules. In healthy cells and tissues, more than 90% of the total glutathione is in the reduced (GSH) state and less than 10% exists in the disulfide form (GSSG). It is known as substrate in both conjugation and reduction reactions catalyzed by glutathione-S- transferase enzymes (Kidd 1997). We report based on the data generated in this study that the reduced activity of catalase and reduced GSH content may be implicated in damage to the liver during cataract development resulting from dietary galactose and that this may be prevented with *Hydrocotyl bonariensis* extract. The results obtained in the study also suggest that with administration of *Hydrocotyl bonariensis*, both glutathione and catalase offers effective protection against oxidative stress generated. Our investigation also revealed that the level of GSH is highest in the liver compared to the kidney and the lens.

Superoxide dismutase catalyzes the detoxification of superoxide to the less toxic hydrogen peroxide. Superoxide dismutase without glutathione peroxidase or catalase to remove the hydrogen peroxide is of little value (Okezie 1998). Though kidney SOD activity was seen in this study to reduce with galactose diet and increase with the administration of the extract, this may not indicate that the kidney is oxidatively stressed during galactose induced catractogenesis since an increased MDA value was not observed in the kidney. Again, we hypothesized that were the kidney to be oxidatively challenged, its antioxidant system may not be able to provide enough protection since we did not observed a corresponding increase in the kidney catalase activity and GSH content was not observed in the kidney in this study. Previous authors have reported different results on the variation of antioxidants status during the development of various types of cataracts. When the activity of SOD was compared in the brain, kidney, liver and eye of normal and hereditary model of cataracts in rat, Komada et al (2000) reported that though the activity in the lens and brain was decreased, the activity in the kidney and liver was not affected. Another study by Suryanarayana et al (2005) reported that SOD activity of the lens was not significantly altered with cataract induced by streptozocin administration and that administration of preventive therapy (curcumin and turmeric) does not significantly altered lens SOD activity. Strother et al (2001) again reported a significant increase in the kidney SOD activity in diabetic rat while an increase that was not significant was observed in the kidney enzyme activity of galactose fed rat. The same study further reported that the SOD activity of the liver was not significantly altered in both diabetic and galactose fed rat. The present result provides evidence that in galactose fed rat, there is a decrease in the lens and kidney SOD but the liver SOD is not affected. Result obtained here may also corroborate the fact that the antioxidant potential of *Hydrocotyl bonariensis* and invariably, its anticataractogenic activities is best exerted at lowered dosage.

CONCLUSION

Our findings have provided information that during cataractogenesis, the liver is also at a greater risk of oxidative damage. The kidney however may not directly suffer this fate. We also report here that both catalase enzyme and reduced glutathione are potent antioxidant in combating this damage and that *Hydrocotyl bonariensis* possess significant antioxidant property that may offer protection from galactose induced oxidative damage in both the lens and the liver.

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