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Use of *Gliricidia sepium* aqueous leaf extract as an antisickling agent: Oxidative stress biomarkers in wistar rats exposed to the extract

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Abstract
Gliricidia sepium aqueous leaf extract is being used in the management of sickle cell disease without regard for harmful effects; hence this study was aimed at assessing the effect of ingestion of *Gliricidia sepium* leaf extract on antioxidant parameters and lipid peroxidation in Wistar rats. The acute oral toxicity of the leaf extract (LD_{50}) and sub-chronic toxicity studies were determined according to Organization for Economic Cooperation and Development (OECD) Limit test 423 and OECD 407 guidelines respectively. Markers of lipid peroxidation and antioxidant factors were assayed using standard techniques at the end of the procedures. In the acute toxicity test, no death or sign of toxicity was observed for up to 14 days, vitamin C level and SOD activities were significantly higher ($p<0.05$) in experimental rats than the control but there was no significant difference between the test group and control for other parameters. In the sub-chronic toxicity study, there was no significant difference ($p>0.05$) between the control and the experimental rats for all the parameters except for SOD which was significantly higher ($p<0.05$) in the experimental rats than the control. In conclusion, *Gliricidia sepium* aqueous leaf extract is relatively safe for human consumption and may not induce oxidative stress imbalance.

Keywords: *Gliricidia sepium*, lipid peroxidation, oxidative stress, Wistar rats, antioxidant factors

Introduction
Herbal products' contribution to modern medicine is well known, and life in most parts of Africa begins and ends with herbal medicine [1]. The World Health Organization [2] reported that 80% of the emerging world's population relies on traditional medicine for therapy.

Gliricidia sepium, a leguminous tree that belongs to the family Fabaceae is one of such herbal formulation used for various ethno-medicinal purposes [3]. It is known by the common name "mother of cocoa" and the Yoruba name in the South-Western Nigeria is Agunmaniye. It originated in Central America and is used in many tropical and sub-tropical countries. This plant was introduced in Philippines and Sri Lanka in 1600s and 1800s, respectively to provide shade to tea plants [4]. Extracts of leaf, flower and bark of *Gliricidia sepium* have been used in the treatment of patients with pathogenic bacterial infections, skin diseases [5, 6], insecticidal, nematicidal and antibacterial properties [7], it has also been reported to possess antioxidants [8], and as antisickling agent[9].

Sickle cell anaemia is an inherited chronic disease caused by abnormal haemoglobin called sickle haemoglobin (HbS) and in which the red blood cells (RBC) become crescent-shaped instead of disc-shaped [10]. Sickle cell disease (SCD) is a hereditary blood disorder caused by a single amino acid substitution (Glu→Val) at the sixth position of the beta-globin chains of haemoglobin. This single amino acid substitution causes a significant reduction in the solubility of the deoxy form of sickle haemoglobin (deoxy-HbS), causing polymer formation inside the red blood cells [11]. Through a complex interplay of adhesive events among blood cells, these altered erythrocytes, can obstruct the vasculature producing episodes of pain, haemolytic anaemia, organ injury, and early mortality [11].

Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are created as a consequence of ATP (adenosine triphosphate) production by the mitochondria. These bye-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from cellular redox process. These species play a dual role as both toxic and beneficial compounds. The delicate balance between their two antagonistic effects is clearly an important aspect of life. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, they generate oxidative stress which plays a major part in the development of chronic and degenerative ailments such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases [12]. However, the human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ, or externally supplied through foods and/or supplements. Endogenous and exogenous antioxidants act as "free radical

scavengers" by preventing and repairing damages caused by ROS and RNS, and therefore can enhance the immune defense and lower the risk of cancer and degenerative diseases [12].

Many plants with medicinal properties have been consumed and are still being consumed without any regard for their possible harmful effects. *Gliricidia sepium* has been widely used in the treatment of many diseases in humans [9]. However, limited data exist with respect to its potential toxic effects. Hence, this study is aimed at investigating the effect of intake of leaf extract of *Gliricidia sepium* on antioxidant parameters and lipid peroxidation in Wistar albino rats.

Methods

Plant collection and identification

Fresh leaf of *Gliricidia sepium* was collected from Osogbo, Osun State, Nigeria. The plant was identified and authenticated at the Herbarium unit of Botany Department by Mr. G.A Ademoriyo, Obafemi Awolowo University, Ile Ife, by comparing with established Herbarium specimen with voucher number IFE/17460 and deposited at the Herbarium.

Preparation and extraction

Fresh leaves of *Gliricidia sepium* were collected and air dried at room temperature over a period of 6 weeks. The dried leaves were ground manually using mortar and pestle. One gram (1 g) of the grinded plant material was soaked in 5 mLs of 80% methanol for 72 hours on a mixer to ensure maximum extraction by maceration technique at room temperature. This was followed by periodic stirring [13]. Resulting crude extract was filtered using Whatman number 1 filter paper and the filtrate was concentrated in an oven at 48℃ to obtain 63.4g crude extract.

Experimental animals

A total of 68 Wistar rat of both sexes, weighing between 150g to 170g were obtained from the animal house of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria.

They were allowed to acclimatize for a period of 2 weeks before the commencement of the study. They were maintained in clean metabolic cage-sand, placed in a well-ventilated room conditioned with a temperature of 26ºC to 28ºC, photoperiods of 12 hours light and 12 hours darkness; humidity of 40% to 60%.The animals were fed with rat pellet obtained from Vital feeds Nigeria Limited and were also allowed access to drinking water *ad libitum*.

Cleaning of the animal cages was carried out on regular basis. All the experimental protocols were in compliance with our Institutional Animal Ethics Committee guidelines as well as internationally accepted practices for use and care of laboratory animals as contained in US guidelines [14], and also in accordance with the recommendations of the International Association for the Study of Pain (IASP) [15].

Experimental Design

Acute oral toxicity study

Acute oral toxicity study, Limit Test, was performed in accordance with the procedures outlined by the Organization for Economic Co-operation and Development Guidelines 423 [16]. Eighteen Wistar rats of both sexes were used for this study. The rats were randomly divided into 3 groups of 6 animals each (3 males and 3 females) with the first group as the control.

The extract was administered to the rats in groups 2 and 3 in single oral doses of 2000mg/kg and 5000mg/kg body weight respectively, dissolved in 1ml distilled water, by intra gastric gavage using oral cannula, one animal per day starting with group 2. The control group (Group 1) received an equal volume of distilled water as vehicle.

Observations of toxic symptoms were made and recorded within the first 4 hours and subsequently for 24 hours after administration of the extract. Behavioural parameters and mortality were also monitored closely for 14 days. Lethal dose in 50% of the total population (LD_{50}) was interpolated using OECD method [17].

Sub-chronic toxicity study

Sub-chronic toxicity study was carried out in accordance with OECD 407 guidelines [18]. Fifty rats of both sexes, were divided into 5 groups of ten 10 rats each. Group 1 served as the control and received normal saline as vehicle. Graded doses of the extract were administered orally to the rats in groups 2, 3, 4 and 5. The doses given to the groups were 250 mg/kg, 500 mg/kg, 750 mg/kg and 1000 mg/kg body weight respectively daily for 28 days. All the rats had free access to food and water throughout the duration of the experiment and were observed daily for general symptoms of toxicity and mortality.

Sample collection

At the end of the 28 days period, the animals were fasted overnight, and anaesthetized using chloroform anaesthesia. Blood samples were collected from the animals through cardiac puncture using 10ml syringe, into lithium heparin containers. The rats were later sacrificed through lumbar dislocation.

The blood collected was then centrifuged at 4000 RPM for 10 minutes. The plasma of each sample was separated and kept frozen at -20ºC until required for analysis

Estimation of antioxidant and lipid peroxidation parameters

The biochemical analyses were performed at the Chemical Pathology Laboratory of Usmanu Danfodiyo University, Sokoto. The separated plasmas were used for the assays of Vitamin A [19], Vitamin C [20], Vitamin E [22], Superoxide dismutase [22], Catalase [23], Glutathione peroxidase [24] and Malondialdehyde [25].

Statistical analysis

The data obtained from these studies were analysed using statistical package for social sciences (SPSS for Windows Inc., Chicago, IL, USA, version 20.0). Data were expressed as mean $\pm$standard deviation (S.D). Student t-test at 95% confidence interval was used to evaluate the significance between the mean values of the measured parameters in respect of tests and control groups. A mean difference was considered statistically significant when $P \leq 0.05$.

Results

Acute toxicity study

Table 1 shows the result of acute oral toxicity (LD_{50} determination) in Wistar rats. The result showed that no behavioural changes or death was recorded in both the control and the treated groups after 24 hours and up to 14 days. This indicates that the LD_{50} of *Gliricidia sepium* is greater than 5000 mg/kg.

Figure 1 shows Oxidative stress biomarkers in Wister rats in acute oral toxicity study.

Figure 2 shows Oxidative stress biomarkers in Wister rats in acute oral toxicity study.

Sub-acute toxicity

Figure 3 shows Oxidative stress biomarkers in Wister rats in sub-chronic toxicity study.

Figure 4 shows Oxidative stress biomarkers in Wister rats in sub-chronic toxicity study.

Table 1: Acute oral toxicity (LD_{50}) study of *Gliricidia sepium* aqueous leaf extract in Wistar rat

Groups	Dosage/kg body weight	Observation period	Behavioural changes	Mortality
Control	**Distilled water**	**Up to 48 hours**	**None**	**None**
Group 2				
Day 1:1st rat	2000mg/kg	Up to 72 hours	None	None
Day 2:2nd rat	2000mg/kg	Up to 72 hours	None	None
Day 3:3rd rat	2000mg/kg	Up to 72 hours	None	None
Group 3				
Day 1:1st rat	5000mg/kg	Up to 72 hours	None	None
Day 2:2nd rat	5000mg/kg	Up to 72 hours	None	None
Day 3:3rd rat	5000mg/kg	Up to 72 hours	None	None

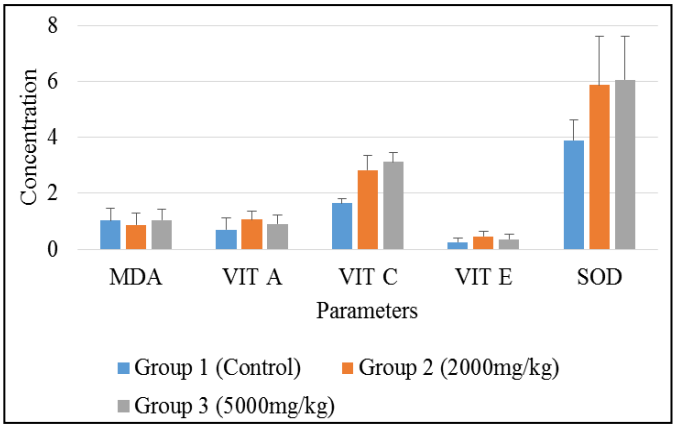

Fig 1: Oxidative stress biomarkers in Wistar rats in acute oral toxicity study

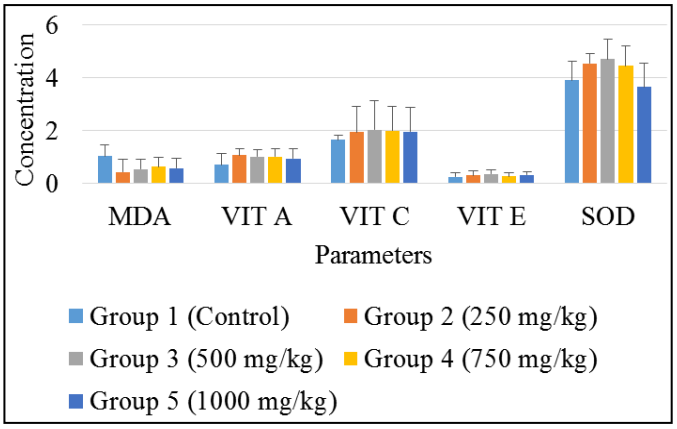

Fig 3: Oxidative stress biomarkers in Wistar rats in sub-chronic toxicity study

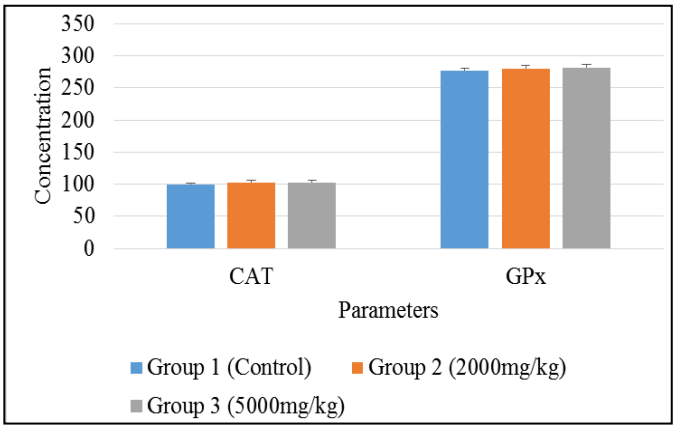

Fig 2: Oxidative stress biomarkers in Wistar rats in acute oral toxicity study

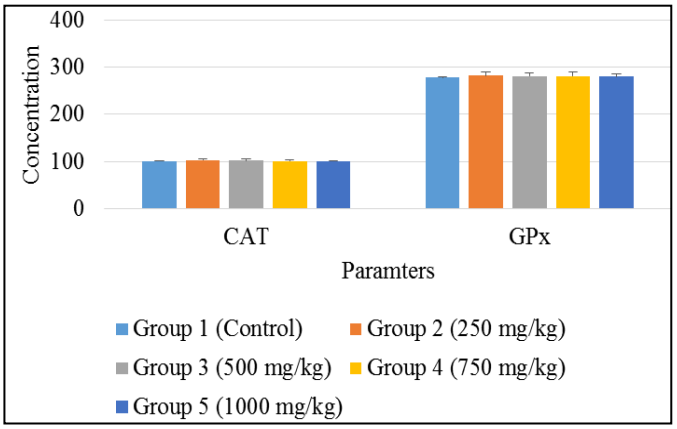

Fig 4: Oxidative stress biomarkers in Wistar rats in sub-chronic toxicity study

Discussion

In the present study, single dose of oral administration of *Gliricidia sepium* to Wistar rats at 5000 mg/kg body weight had no effect on mortality and clinical signs such as changes in the skin and fur, eyes and mucus membrane (nasal), respiratory rate, autonomic effects (salivation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (ptosis, drowsiness, gait, tremors and convulsion). Generally, there was no test substance related mortality observed at 5000 mg/kg [26]. Therefore, no acute toxicity was found in rats treated with *Gliricidia sepium* and the approximate medium acute toxicity lethal value (LD_{50}) were determined to be higher than 5000 mg/kg and as such could be generally regarded as safe for use. This finding is in agreement with Clarke and Clarke [27], who reported that any compound or drug with oral LD_{50} estimates greater than 1000 mg/kg body weight could be considered to be of low toxicity and safe. However, Zbinden and Roversi [28] suggested that variables such as animal species, strain, age, gender, diet, bedding, ambient temperature, caging conditions, and time of the day can all affect the LD_{50} values obtained and as such are considerable uncertainties in extrapolating the LD_{50} obtained for species to other species.

Ingestion of *Gliricidia sepium* leaf extract did not induce lipid peroxidation, as MDA concentrations in acute oral toxicity study did not show significant difference ($p<0.05$) between the control and experimental rats. Although there was no significant difference ($p>0.05$) in the MDA values between the control and experimental rats in the sub-chronic toxicity study, the post-hoc Duncan test revealed that the MDA values in all the experimental rats were significantly lower than the control rat.

Malondialdehyde is a metabolic product of peroxidative reactions (auto-oxidative) of lipids exposed to oxygen; it serves as a reliable marker of lipid peroxidation [25]. Lipid peroxidation products (MDA, and conjugated diene compounds) are cytotoxic and mutagenic [26]. MDA is a reactive aldehyde compound and is one of the many reactive electrophile species that cause toxic stress in the cells and form covalent protein adducts that are referred as advanced lipoxidation end-products (ALE), in analogy to advanced glycation end-products (AGE) [27].

In both acute oral and sub-chronic toxicity studies, Vitamins A, C and E concentrations in the experimental rats were higher than the control rats although the increases were not significant with the exception of Vitamin C which was significantly higher ($p<0.05$) in the experimental rats than the control group. Vitamins A, C and E are antioxidant vitamins. Antioxidants are substances that delay or inhibit oxidative damage to a target molecule [28]. Oxidation is a chemical reaction that can produce free radicals, leading to chain reactions that may damage the cells. Vitamins A, C or thiols and vitamin E terminate these chain reactions. The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention [29].

Increase in the concentration of vitamins in treated rats is an indication that *G. sepium* possess antioxidant property In both acute and sub-chronic toxicity studies, the activities of antioxidants enzymes (SOD, CAT and GPx) were also higher in treated rats than the control group but the increases were not significant ($p>0.05$), with the exception of SOD which was significantly higher in both acute and sub-chronic toxicity studies in treated rats than the control group. These enzymes are involved in the neutralization of ROS/RNS [29]

Ingestion of *Gliricidia sepium* leaf extract did not cause adverse effect on the activities of these enzymes. In this study, ingestion of *Gliricidia sepium* leaf extract neither induces lipid peroxidation nor affects antioxidant enzymes or vitamins. Oxidative stress is a harmful condition that occurs when there is an excess of ROS and/or a decrease in antioxidant levels, this may cause tissue damage by physical, chemical, psychological factors that lead to tissue injury in human and causes different diseases [30]. In conclusion, since LD_{50} of *Gliricidia sepium* leaf extract greater than 5000mg/kg, it is relatively safe for human consumption. Oxidative stress markers analysed also revealed that the leaf extract may not induce oxidative stress disorder but may be useful in the management of oxidative stress diseases.

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Competing interests

Authors have declared that no competing interests exist.

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