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DYE EXTRACT FROM THE HEARTWOOD OF *PTEROCARPUS ERINACEUS* (AFRICAN ROSEWOOD) AMELIORATES CYCLOPHOSPHAMIDE - INDUCED TOXICITY IN RAT'S BRAIN AND LIVER

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ABSTRACT

The use of dyes in beverage food and drinks has increased in recent times, due to their dual role as nutritional and therapeutic supplement. This study sought to assess the ability of dye extracts from the heartwood of *Pterocarpus erinaceus* (African Rosewood) to ameliorate cyclophosphamide- induced toxicity in rat's brain and liver and assess the antioxidant properties of the dye extract *in vitro*. The dye was prepared from the heartwood part of *P. erinaceus* and yielded red colourant. Wistar strain albino rats were placed on diet containing 0.5 and 1.0% red dye prepared from *P. erinaceus* for 14 days. However, intraperitoneal administration of cyclophosphamide (75 mg/kg of body weight) 24 hours before the termination of the experiment, caused a significant ($P < 0.05$) increase in brain malondialdehyde (MDA) content and serum activities of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase in rats fed diet without the dye supplement; while, there was significant decrease ($P < 0.05$) in brain MDA content and serum enzyme activities in rats fed diet with the dye supplement. The high protective effect of the dye could be attributed to its high antioxidant properties as typified by its high reducing power, free radical scavenging and Fe^{2+} chelating ability. Dietary supplementation of *P. erinaceus* as food colorants could ameliorate toxicities induced by cyclophosphamide.

Keywords: *Pterocarpus erinaceus*; dye; food colourant; cyclophosphamide; antioxidant; brain; liver

INTRODUCTION

An interest in natural antioxidants, especially of vegetal origin, has greatly increased in recent years (Adefegha and Oboh, 2011; 2012; 2013; Adefegha *et al.*, 2014). Natural antioxidants can protect the human body from free radicals that may lead to the aging process and cause some chronic diseases including cancer, cardiovascular diseases and cataract as well as retard lipid oxidative rancidity in foods (Kinsella *et al.*, 1993; Lai *et al.*, 2001; Adefegha and Oboh, 2011). Natural antioxidants include tocopherols and phenolic compounds which may act to confer an effective defense system against free radical attack (Shahidi, 1997). Many researches

supported the theory that free radicals cause oxidative damage and contribute to the development of several degenerative diseases and the aging process (Cutler, 1991; Ames *et al.*, 1993; Gey, 1993, Yu *et al.*, 2002; Oboh and Rocha, 2007; Adefegha and Oboh, 2011; 2012; 2013; Adefegha *et al.*, 2014). Phenolic compounds are able to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation (Shahidi, 1997). Although the interest in phenolic compounds is related primarily to their antioxidant activity, they also show important biological activity *in vivo* and may be beneficial in combating diseases related to excessive oxygen radical formation exceeding

the antioxidant defense capacity of the human body (Morello *et al.*, 2004; Adefegha *et al.*, 2014).

Pterocarpus is a large genus, occurs throughout the tropics and it belongs to a huge family; Leguminosae and subfamily; papilionoidea (Dalziel *et al.*, 1955; Keay, 1989). It is sometimes called African Rosewood or African teak (Yoruba – Osun dudu). The tree about 12 – 15m high and up to 2m in girth and slightly buttressed in large trees, is the most widespread *Pterocarpus* in savanna areas. The fruit which is the distinctive characteristic feature of *Pterocarpus* species is a winged more or less circular, one-seeded fruits (Hutchinson *et al.*, 1958). Its sharply prickly fruits distinguish it from all other indigenous species (Keay, 1989). *Pterocarpus erinaceus* is the chief source of native red wood or barwood outside the forest region (Dalziel *et al.*, 1955; Hutchinson *et al.*, 1958; Keay, 1989). The rose red dye of this plant can be extracted hot with organic hydroxyl solvent for several hours. When mixed with palm oil, it is used for smearing the body as a cosmetic. Medicinally, it is used for treatment of diarrhoea and dysentery. Lotion from the bark is used for ringworm of the scalp and infusions of the leaves are used for fevers (Dalziel *et al.*, 1955).

Among the common therapeutic modalities of cancer, chemotherapy plays an important role. Most of the synthetic chemotherapeutic agents available today are immunosuppressant, cytotoxic and exert several side effects (Diwanay *et al.*, 2004). Cyclophosphamide is a cytotoxic alkylating drug with a high therapeutic index and broad spectrum of activity against a variety of cancers (Dollery, 1999). It is inactive in *in-vitro* but is activated to intracellular alkylating metabolites, 4-hydroxy cyclophosphamide and phosphoramidate mustard by hepatic cytochrome P450 monooxygenase system (Colvin, 1997). Major toxic side effects of cyclophosphamide are hematopoietic depression, gastrointestinal toxicity and hemorrhagic cystitis (Salvin *et al.*, 1975). Cytotoxicity towards normal host tissue is the primary dose-limiting factor in cyclophosphamide therapy that reduces quality of life and restricts treatment protocol. Hence there is a continued interest and need for the identification and development of non-toxic and effective chemopreventive compound that

can reduce the side effects of cyclophosphamide. Protection against chemically induced toxicity and cancer using synthetic or natural compounds, constitute a promising means of disease control and prevention (Ma and Kineer, 2002). The use of plants or their active principle in the prevention or treatment of chronic disease is based on the experience of traditional system of medicine from ethnic societies, but their use in modern medicine suffer from lack of scientific evidences. Now-a-days, many medicinal plants have attracted the interest of scientists in this field and plant extracts used in traditional therapy are being reviewed for their chemopreventive activities. Earlier experimental studies in our laboratory have demonstrated that hot short pepper (Oboh and Ogunraku, 2010) and polar with non-polar extract of Annatto seeds (Oboh *et al.*, 2011) possessed chemoprotective activities induced by cyclophosphamide. Therefore this study sought to evaluate the intervention of *Pterocarpus erinaceus* (African Rosewood) dye antioxidants and its potential interactions in drug-induced toxicities.

MATERIALS AND METHODS

Sample collection

Heartwood of *Pterocarpus erinaceus* were purchased from Akure main market in Akure, Ondo State, Nigeria, and authentication of the plant was carried out in the Department of Biology, Federal University of Technology, Akure, Nigeria. The water used was glass-distilled, while the chemicals were of analytical grade.

Sample preparation

The red dye from *P. erinaceus* was prepared using the method described by Furniss *et al.* (1978) and modified by Adetuyi *et al.* (2005). The heart wood was sun-dried, pulverized and boiled in water (1:5) for 30 mins. The water soluble extract was subsequently evaporated to dryness.

Phytochemical Screening

The methods described by Trease and Evans (1978) were used for phytochemical screening of red dye extract of *P. erinaceus*, for the presence of bioactive compound. The test for tannins was carried out by subjecting 3 g of each plant extract in 6 mL of distilled water, filtered and ferric chloride reagents added to the filtrate. For cardiac glycosides, Killer-Kiliani test was adopted (0.5 g of extract was

added to 2 mL of acetic anhydride plus H₂SO₄). The test for alkaloids was carried out by subjecting 0.5 g of aqueous extract in 5 mL of 1% HCl, boiled, filtered and Mayer's reagent added. The extract was subjected to frothing test for the identification of saponin. The extract was also tested for free glycoside bound anthraquinones. Five grams of extract was added to 10 mL of benzene, filtered and ammonia solution added. The presence of flavonoids was determined using 1% aluminum chloride solution in methanol concentrated HCl, magnesium turnings, and potassium hydroxide solution. These qualitative tests were based on the colour change as indication of positive test.

Determination of total phenol content

The total phenol content of the extract was determined using the method reported by Singleton *et al.* (1999). Appropriate dilution of the extract were oxidized with 2.5 ml 10% Folin-Ciocalteu's reagent (v/v) and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 mins at 45°C and the absorbance was measured at 765 nm in the spectrophotometer. The total phenol content was subsequently calculated as tannic acid equivalent.

1,1-diphenyl-2 picrylhydrazyl (DPPH) free-radical scavenging ability

The free-radical scavenging ability of the extract against DPPH (1,1-diphenyl-2 picrylhydrazyl) free radical was evaluated as described by Gyamfi *et al.* (1999). Briefly, appropriate dilution of the extracts (1 ml) was mixed with 1 ml, 0.4 mM methanolic solution containing DPPH radicals; the mixture was left in the dark for 30 mins and the absorbance was taken at 516 nm. The DPPH free-radical scavenging ability was subsequently calculated.

Determination of reducing property

The reducing property of the extract was determined by assessing the ability of the extracts to reduce FeCl₃ solution as described by Oyaizu (1986). In total, 2.5 ml aliquot was mixed with 2.5 ml 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 mins and then 2.5 ml 10 % trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 mins. 5 ml of the supernatant was mixed with an equal volume of water and 1 ml 0.1% ferric chloride. The absorbance was measured at 700 nm and

the ferric reducing antioxidant property was subsequently calculated.

Fe²⁺ chelating ability

The Fe²⁺ chelating ability of the extract was determined using a modified method of Minotti and Aust (1987) with a slight modification by Puntel *et al.* (2005). Freshly prepared 500 μmol L⁻¹ FeSO₄ (150 μL) was added to a reaction mixture containing 168 μL of 0.1 mol L⁻¹ Tris-HCl (pH 7.4), 218 μL saline and the extract (25 μL). The reaction mixture was incubated for 5 min, before the addition of 13 μL of 0.25% 1, 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe²⁺ chelating ability was subsequently calculated with respect to the reference (which contains all the reagents without the test sample).

Lipid peroxidation assay

Preparation of Tissue homogenates

The rats were decapitated under mild diethyl ether anaesthesia and the cerebral tissue (whole brain) was rapidly dissected and placed on ice and weighed. This tissue was subsequently homogenized in cold saline (1:10 w/v) with about 10-up-and -down strokes at approximately 1200 rpm in a Teflon glass homogenizer. The homogenate was centrifuge for 10 min at 3000g to yield a pellet that was discarded, and a low-speed supernatant (S1) containing mainly water, proteins and lipids (cholesterol, galactolipid, individual phospholipids and gangliosides) that was kept for lipid peroxidation assay (Belle *et al.*, 2004).

Lipid peroxidation and Thiobarbituric acid reactions

The lipid peroxidation assay was carried out using the modified method of Ohkawa *et al.* (1979). Briefly, 100 μl S1 fraction was mixed with a reaction mixture containing 30 μl of 0.1 M pH 7.4 Tris-HCl buffer, extract (0 – 100 μl) and 30 μl of the pro-oxidant (250 μM freshly prepared FeSO₄). The volume was made up to 300 μl by water before incubation at 37°C for 1hr. The colour reaction was developed by adding 300 μl 8.1% sodium dodecyl sulphate (SDS) to the reaction mixture containing S1; this was subsequently followed by the addition of 600 μl of acetic acid/HCl (pH 3.4) mixture and 600 μl 0.8% thiobarbituric acid. This mixture was incubated at 100°C for 1hr. Thiobarbituric acid reactive species (TBARS) produced was measured at 532 nm and the absorbance was compared with that of a standard curve using malondialdehyde (MDA).

Bioassay

The ability of the dietary inclusion of red dye extract from Heartwood of *P. erinaceus* to prevent cyclophosphamide - induced toxicities in rat's brain was evaluated using the method reported by Bhatia *et al.* (2006). Wistar strain albino rats weighing 220-250g were purchased from the Biochemistry Department, University of Ilorin, Nigeria, and acclimatized for 2 weeks, during which period they were maintained *ad libitum* on commercial diet. The rats were acclimatized for four weeks during which period they were maintained *ad libitum* on commercial growers (Guinea feeds). The rats were subsequently divided into four treatment groups. Animals in group 1 and 2 were fed the basal diet [Corn flour (67%), Groundnut cake (14%), Fish meal (5%), Groundnut oil (10%), Vitamin-mineral premix (4%)], while animals in group 3 were fed basal diet containing 0.5% supplementation of the red dye from heart wood of *P. erinaceus* (at the expense of the premix), and those in group 4 were the basal diet containing 1.0% supplementation of the red dye from heart wood of *P. erinaceus* (at the expense of the premix). 0.5 - 1.0% red dye extract inclusion gave the desired colouring of the diet. The experiment lasted 14 days. The rats in groups 2 - 4 were injected intraperitoneally with cyclophosphamide (75 mg/kg of body weight) 24 hours before the termination of the experiment (Bhatia *et al.*, 2006; Oboh and Ogunraku, 2010), while group 1 served as the control. The malondialdehyde (MDA) content of the brain was determined as described earlier, and serum activities of glutamate oxaloacetate transferase (SGOT), glutamate pyruvate transferase (SGPT), alkaline phosphatase (ALP) and total bilirubin were determined using diagnostic kits.

Data Analysis

The results of the three replicates were pooled and expressed as mean \pm standard error (S.E.). Student t-test, one-way analysis of variance (ANOVA) and the least significance difference (LSD) were carried out (Zar, 1984). Significance was accepted at $p \leq 0.05$.

RESULTS

The inclusion of red dye from *P. erinaceus* (0.5 & 1.0%) in rat's diet did not cause any significant ($P > 0.05$) change in the average daily feed intake (12.7 - 13.2g) and weight gain (1.0 - 1.2g), when compared with those rats fed diet

without the dye supplement [feed intake (12.1g); weight gain (0.9g)]. Furthermore, the intraperitoneal administration of a single dose of cyclophosphamide (75mg/kg of body weight) 24 hrs prior to the termination of the experiment caused a significant ($P < 0.05$) increase in the MDA content of the brain (Figure 1) of those rats placed on diet without the red dye from *P. erinaceus* (151.1%) when compared with those placed on diet with red dye (101.4-112.0%) and the control (diet not supplement with dye and were not given cyclophosphamide administration). Also, the intraperitoneal administration of a single dose of cyclophosphamide caused a significant ($P < 0.05$) significant rise in the serum AST, ALT, alkaline phosphatase and total bilirubin content in rats fed diet without red dye supplement from *P. erinaceus* when compared with rats fed diet with red dye supplement and the control group (Table 1). However, there was significant decrease ($P < 0.05$) in the marker enzymes activities and metabolite levels of rats previously placed on dietary inclusion of red dye supplement from *P. erinaceus*.

The total phenol content of red dye from *P. erinaceus* is presented in Table 2. The result revealed that the dye had high total phenol content (32.5 %). The antioxidant indices of red dye from *P. erinaceus* typified by the DPPH radical scavenging ability, reducing power and Fe^{2+} chelating ability are presented in Table 2. The result revealed that there was dose-dependent response in the ability of the red dye extract to scavenge DPPH free-radicals, reduce Fe^{3+} to Fe^{2+} and chelate Fe^{2+} .

Furthermore, the ability of red dye extract to inhibit lipid peroxidation was determined on rat's brain tissue *in vitro* (Figure 2). Incubation of the rat brain in the presence of $FeSO_4$ caused a significant increase ($P < 0.05$) in the MDA content of the pancreas (218.33%). However, the introduction of red dye extract from *P. erinaceus* caused a significant decrease ($P < 0.05$) in the MDA content of Fe^{2+} - stressed brain homogenates (46.37 - 139.23 %) in a dose-dependent manner (1 - 4 μ g/mL).

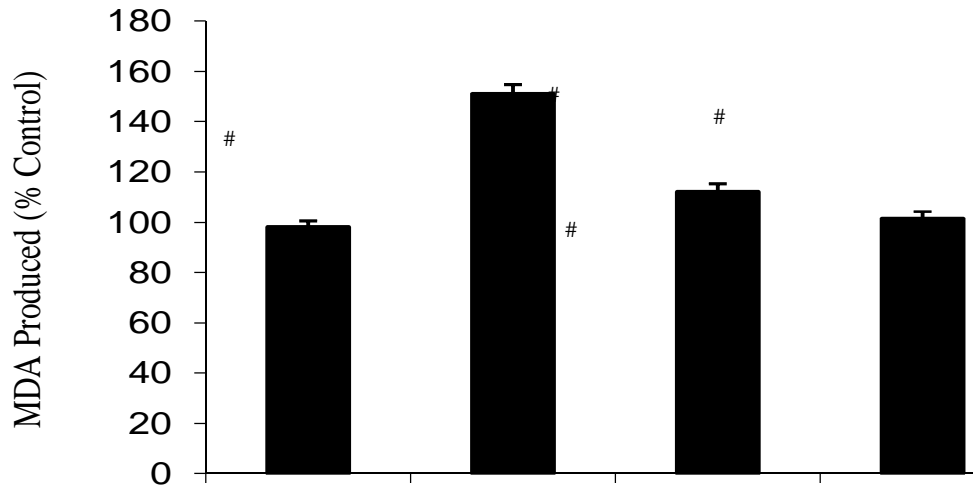


Figure 1: Inhibition of cyclophosphamide- induced lipid peroxidation in rat's brain (in vivo) by supplementation with red dye from *Pterocarpus erinaceus*

#Values are significantly ($P < 0.05$) different from the stressed group

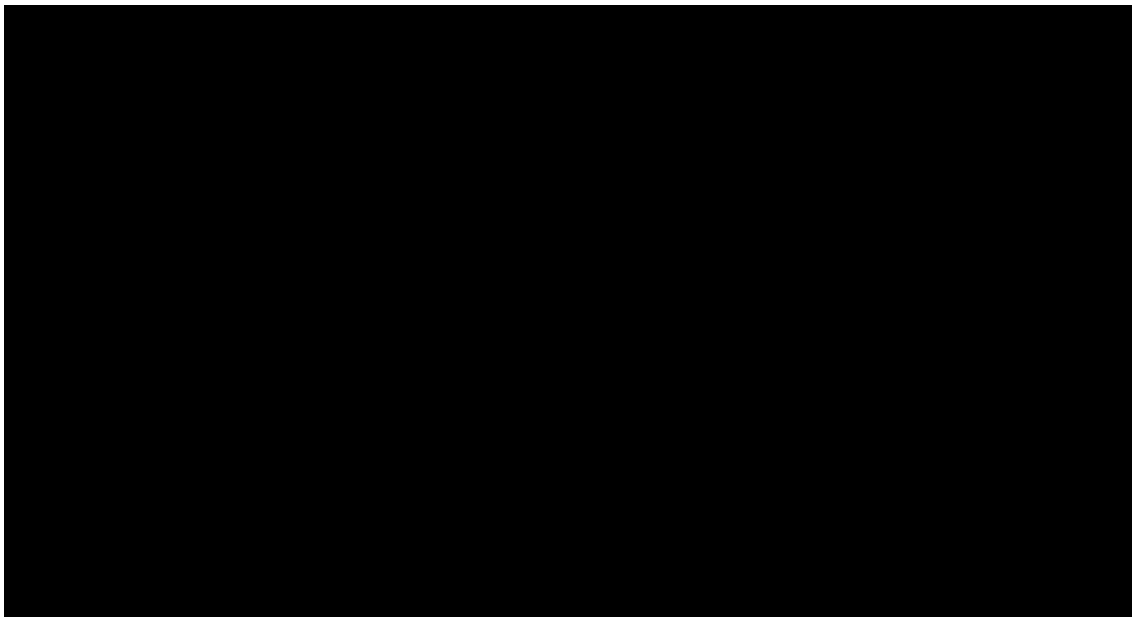


Figure 2: Inhibition of Fe (II) induced Lipid Peroxidation in Rat's Brain by red dye extract from *Pterocarpus erinaceus* -in vitro

Values represent means of triplicate readings

Values showed significant difference ($P < 0.05$) at the concentration tested

Table 1: Inhibition of cyclophosphamide- induced elevated serum ALT, AST, ALP and Total bilirubin by inclusion of red dye from *Pterocarpus erinaceus* in rat's diet

	ALT (UI/L)	AST (UI/L)	ALP (UI/L)	Total bilirubin (mg/dl)
Basal	4.1 ± 0.8*	7.3 ± 1.1*	9.8 ± 0.5*	0.4 ± 0.1*
Stressed	28.3 ± 1.4	21.2 ± 1.0	39.5 ± 1.1	1.5 ± 0.2
0.5% PTE	22.5 ± 1.1*	17.4 ± 1.0	32.7 ± 0.7*	1.3 ± 0.2
1.0% PTE	18.2 ± 0.8*	13.5 ± 0.5*	24.3 ± 0.6*	0.9 ± 0.1*

Values represent means of 5 readings.

*Values are significantly (P<0.05) different from the stressed group

Control – Basal diet only without cyclophosphamide administration

Stressed - Basal diet with cyclophosphamide administration

0.5% PTE – 0.5% Red dye supplemented diet with cyclophosphamide administration

1.0% PTE - 1.0% Red dye supplementation with cyclophosphamide administration

Table 2. Total Phenol, DPPH free radical scavenging ability, reducing power and Fe (II) chelating ability of red dye extract from *Pterocarpus erinaceus*

Parameter	Contents
Total phenol (%)	32.5 ± 1.0
DPPH Free radical scavenging ability (%)	63.7 ± 1.2
Reducing property (OD ₇₀₀)	0.3 ± 0.1
Fe (II) Chelating ability (%)	71.2 ± 1.5

DISCUSSION

Attention is being focused on food and food additives, for their therapeutic uses due to the twin problems of high prices and side effect of imported drug formulations. Traditional use of decoction of *Pterocarpus erinaceus* as food additive and in folklore has been reported (Dalziel, 1955; Keay, 1989). Therefore, the ameliorative effect of dietary inclusion of red dye from *P. erinaceus* on cyclophosphamide-induced toxicity on the brain and liver was assessed.

Cyclophosphamide is used in the treatment of chronic and acute leukemia, multiple myeloma, lymphomas and rheumatic arthritis and also in the preparation for bone marrow transplantation (Mythili *et al.*, 2004). The administration of intermittent massive dosage of cyclophosphamide has been shown to be advantageous in chemotherapy (Senthilkumar *et al.*, 2006). Cyclophosphamide toxicity is mediated by free radical mechanism through the formation of intracellular phosphamide mustard and acrolein (Mythili *et al.*, 2004). Acrolein produces highly reactive oxygen free radicals and disrupts the redox antioxidant status by altering the cell membrane structure and function and enhancing the production of lipid peroxidation in the brain cells ((Mythili *et al.*, 2004). The brain cells are readily susceptible to oxidative stress due to their limited access to the bulk of both exogenous and endogenous antioxidants in the body (Oboh and Rocha, 2007b).

Lipid peroxidation is thought to proceed via radical indicated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids (Halliwell and Gutteridge, 1989). Malondialdehyde (MDA) is one of the end products of lipid peroxidation and extent of lipid peroxidation is measured by estimating MDA levels (Murray and Granner, 2000). The non significant difference in the average daily feed intake and weight gain between rats in all groups may indicate that the inclusion of the red dye in diet, neither affect the appetite of the rats for the feed nor suppress the

growth of the rats. However, the intraperitoneal administration of a single dose of cyclophosphamide (75mg/kg of body weight) 24 hrs prior to the termination of the experiment caused a significant ($P<0.05$) increase in the MDA content of the brain (figure 1) of those rats placed on diet without the red dye from *P. erinaceus* when compared with those placed on diet with red dye and the control (diet not supplement with dye and were not given cyclophosphamide administration. The significant ($P<0.05$) increase in the MDA content in the brain of rats fed diet without red dye supplementation, but administered with cyclophosphamide could have resulted from reactive oxygen species (ROS) production from the drug (Bhatia *et al.*, 2006).

Increased serum activities of aspartate aminotransferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and total bilirubin content is well known diagnostic indicators of liver injury (David and Johnston, 1999). Intraperitoneal administration of a single dose of cyclophosphamide (75mg/kg of body weight) 24 hrs prior to the termination of the experiment also caused a significant ($P<0.05$) significant rise in the serum AST, ALT, alkaline phosphatase and total bilirubin content in rats fed diet without red dye supplement from *P. erinaceus* when compared with rats fed diet with red dye supplement and the control group (Table 1). Moreover, the ameliorative effect of dietary supplementation with red dye from *P. erinaceus* on rat's brain and liver could be attributed to the presence of some biologically active phytochemicals in the dye. Investigation of the phytochemicals present in the *P. erinaceus* dye showed that it contained alkaloid, phlebotannin and anthraquinone.

Polyphenols are the most abundant antioxidants in the diet (Scalbert *et al.*, 2005). They remove free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α -tocopherol radicals, and inhibit oxidases (Amic *et al.*, 2003). It is well established that some polyphenols,

administered as supplements or with food, do improve health status, as indicated by several biomarkers closely associated with some diseased states (Vita, 2005; Keen *et al.*, 2005; Sies *et al.*, 2005). The total phenol content of red dye from *P. erinaceus* is presented in Table 2. The result revealed that the dye had high total phenol content (32.5 %). Prevention of the chain initiation step by scavenging various reactive species such as free radicals is considered to be an important antioxidant mode of action (Dastmalchi *et al.*, 2007). The free radical scavenging ability of the red dye from *P. erinaceus* is also presented in Table 2 and the result revealed that the high free radical scavenging ability (63.7%) of the dye may have contributed significantly to the ability of red dye supplementation in diet to ameliorate cyclophosphamide-induced oxidative stress in the brain and liver. Subsequently, the ability of the dye extract to reduce Fe (III) to Fe (II) was investigated and presented in table 1. Reducing power can be a novel antioxidation defence mechanism; the two mechanisms that are available to affect this reducing power are by electron transfer and hydrogen atom transfer (Dastmalchi *et al.*, 2007). Subsequently, the ability of antioxidants to chelate and deactivate transition metals prevent such metals from participating in the initiation of lipid peroxidation and oxidative stress through metal catalyzed reactions; hence the ability of phenolic extract to chelate transition metals is considered an antioxidant mechanism (Puntel *et al.*, 2005). Iron (Fe), an essential metal needed for normal cellular physiology is present in biological systems bond to several protein moieties such as hemoglobin, ferritin etc. It may also exist in free forms in which it is able to participate in Fenton reaction with OH^{\bullet} and Fe^{3+} as products. Recent reports have revealed that one of the reasons why the brain is prone to oxidative stress may be due to accumulation of Fe in the brain (Herbert *et al.*, 1994). The high Fe (II) chelating ability of the red dye as shown in table 1 may have contributed to the protective effect of the red dye

supplemented diet on cyclophosphamide induced oxidative stress in brain. Furthermore, the ability of red dye extract to inhibit lipid peroxidation was determined on rat's brain tissue *in vitro* (Figure 2). The results revealed that the red dye extract caused a dose-dependent inhibition in MDA production in the brain tissue. This clearly indicates that the red dye from *P. erinaceus* exerted an antioxidant effect. The inhibition of Fe (II) induced lipid in rat brain homogenates (*in vitro*) by the red dye extract (Figure 2) agreed with the fact that dietary supplementation of red dye from *P. erinaceus* could have protected the rat's brain from cyclophosphamide-induced oxidative stress (Figure 1). Therefore, the ameliorative effect of the red dye supplemented diet could be attributed to the high phenolic content and antioxidant activity as typified by high reducing power, free radical scavenging, Fe (II) chelating ability and inhibition of lipid peroxidation in the brain.

CONCLUSION

Red dye from *Pterocarpus erinaceus* showed strong inhibition of cyclophosphamide-induced toxicities in brain and liver. This protective effect of the red dye could be attributed to the high total phenolic content and antioxidant properties as typified by high reducing power, Fe (II) chelating ability, free radical scavenging ability and inhibition of lipid peroxidation in the brain.

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