

INVESTIGATIONS INTO THE ANTIOXIDANT AND ANTI-INFLAMMATORY POTENTIALS OF THE ETHANOLIC EXTRACT OF *CITRUS SINENSIS*, LINN. (SWEET ORANGE) STEM-BARK.

Asaolu, F. T.¹, Areola J. O.¹, Tijani A. S.² and Babalola O. O.¹

¹Department of Biochemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria

²Department of Biochemistry, Faculty of Science, Bowen University, Iwo, Nigeria

Corresponding Author: doctorbablo@yahoo.com +2348037143321

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ABSTRACT

This study was designed to investigate the antioxidant and anti-inflammatory potentials of the ethanolic extract and fractions of *Citrus sinensis* stem-bark, investigate and to evaluate the hepatoprotective potential of the most active fraction (EAF) of the ethanolic extract against acetaminophen-induced acute hepatic injury. The ethanolic extract and the fractions were screened for their secondary metabolites using established methods. The total phenolics and total flavonoid contents of the ethanolic extract and the fractions the antioxidant capacities namely DPPH radical scavenging assay, Ferric reducing power (FRAP) assay, Total antioxidant assay, Nitric oxide inhibitory assay and ABTS (2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay and the *in vitro* anti-inflammatory potential of the extract and fractions were all evaluated by established methods, Hepatoprotective potential of the ethyl acetate fraction was carried out by administering the EAF and standard drug Legalex 70 (Silymarin) (p.o) for 7 consecutive days. The phytochemical screening showed the presence of tannins, flavonoids, terpenoids, cardiac glycosides, saponins and alkaloids in the extract and fractions. The result also showed that the ethyl acetate fraction showed the highest scavenging radical activities in Nitric oxide with an IC₅₀ of 68.85±1.43µg/ml, ABTS with an IC₅₀ of 0.42±0.00mg/ml and FRAP value 1.48±0.02mgAA/g also indicated the ethyl acetate fraction to be the most active. Ethyl acetate fraction gave the highest phenolic content (2.03±0.04 mg GAE/g of fraction) while dichloromethane fraction showed the highest flavonoid content (0.38±0.01mgQUE/g) and DPPH scavenging radical with an IC₅₀ of 0.15±0.03mg/ml. In membrane stability, ethylacetate fraction also showed the highest stability of 88.69±4.17%. Additionally in the *in vivo* study, administration of acetaminophen showed significant changes in the level of the plasma and liver parameters measured when compared to the control group and it was indicative of liver damage. Pre-treatment with ethylacetate fraction (250 and 500mg/kg) and 50mg/kg legalex 70 for 7 days significantly (p<0.05) reduced the level of plasma marker enzyme (ALT, AST and GGT), total bilirubin and total protein. Increased antioxidant level (SOD, CAT, GPx and GSH) in liver tissue was also observed. In conclusion, it is probable that the antioxidant, anti-inflammatory and hepatoprotective ability of this plant extract could be attributed to the presence of flavonoids and tannins in the plant extract/fractions.

INTRODUCTION

Plants are sources of natural antioxidants and some of its constituents had been reported to possess antioxidative properties (Exarchou *et al.*, 2002). The potential of the antioxidant constituents of plant materials for the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists and food manufacturers as consumers move towards the development of functional foods with specific health effects (Lo liger, 1991). Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical-induced tissue injury. Also some plant species have been investigated in the search for novel antioxidants (Chu, 2000; Mantle *et al.*, 2000;

Koleva *et al.*, 2002; Oke and Hamburger, 2002) but generally there is still a demand to find more information concerning the antioxidant potential of plant species.

Medicinal plants play a pivotal role in the health care of ancient and modern cultures. Ayurveda, the Indian system of medicine mainly uses plant-based drugs or formulations to treat various human ailments because they contain the components of therapeutic value (W.H.O., 1993). In addition, plant-based drugs remain an important source of therapeutic agents because of the availability, relatively cheaper cost and non-toxic nature when compared to modern medicine (Agbor and Ngogang, 2005). Many herbs contain antioxidant compounds which protect the cells

against the damaging effects of reactive oxygen species.

The generation of large amount of free radicals, particularly reactive oxygen species and their high activity plays an important role in the progression of a great number of pathological disturbances like inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, cancer, Parkinson's disease, Alzheimer's disease, etc (Mensor *et al.*, 2001; Parejo *et al.*, 2002; Hou *et al.*, 2003; Orhan *et al.*, 2003; Tepe *et al.*, 2005; Ozgen *et al.*, 2006). Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, cause depletion of immune system antioxidants, change in gene expression and inducing abnormal proteins (Tepe *et al.*, 2005).

Free radicals are chemical species that possess an unpaired electron in the outer (valence) shell of the molecule. They are formed when oxygen is metabolized in the body. Thus, free radicals are highly reactive and can react with proteins, lipids, carbohydrates and DNA. Oxidative stress results in the damage of biopolymers including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates. Lipid peroxidation is oxidative deterioration of polyunsaturated lipids and it involves ROS and transition metal ions. It is a molecular mechanism of cell injury leading to yield of a wide range of cytotoxic products, most of which are aldehydes like malondialdehyde (MDA), 4-hydroxynonanal (HNE). Oxidative stress causes serious cell damage leading to a variety of human diseases like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders, etc. (Peterhans, 1997)

Liver disorders continue to be a major cause of morbidity and mortality throughout the world and there is renewed interest in the discovery of novel compounds that can be used to fight these diseases (Chrungoo *et al.*, 1997). Numerous studies have sought to validate the traditional use of antihepatotoxic medicinal plants by investigating the biological activity of extracts of such plants. In Nigeria for instance, numerous medicinal plants and their formulation are for such liver disorders

in ethnomedical practices as well as in the traditional system of medicine (Adeshina and Sofowora, 1982). Of the numerous phytochemicals present in these active extracts, tannins and flavonoids have been suggested to be responsible for antihepatotoxic activity by increasing the antioxidant status. In this regard, antioxidant agents of natural origin have attracted special interest because of the potential they possess in the maintenance of health and protection from some age-related degenerative disorders such as neurodegenerative diseases (Perry *et al.*, 2003), coronary heart diseases and cancer (Velioglu, 1998). This study is therefore designed to investigate the possible antioxidant, anti-inflammatory and hepatoprotective potential of ethanolic extracts of *Citrus sinensis*.

MATERIALS AND METHOD

Plant Materials

The stem-bark of *Citrus sinensis*, L. was collected from a farm in Babajakan Village, Aiyedaade Local Government, Osun State, Nigeria. The plant material was identified and authenticated at the IFE Herbarium, Department of Botany, Obafemi Awolowo University Ile-Ife Osun State with the voucher specimen code 17179.

Experimental Animals

Eighteen albino mice of either sex, bred from the same colony, weighing between 20 – 30 g and twenty five wistar albino rats of either sex, weighing between 100 – 160 g were obtained from the Animal House Faculty of Pharmacy, Obafemi Awolowo University. The animals were housed in a clean polypropylene cage and maintained under standard laboratory conditions. They were fed with standard pellet diet and water *ad libitum*. The animals were acclimatized to laboratory conditions for two weeks prior to experiment.

Extraction of Plant Materials

The stem-bark of plant material was air-dried at room temperature and were mechanically crushed into powdered. Five hundred grams of plant material was suspended in 2 litres of 70% (v/v) of ethanol for 72 hours with occasional agitation. The resulting mixture obtained was then filtered using a piece of white cotton gauze and concentrated in a rotary following concentration to dryness *in vacuo* at 40°C on rotary

evaporator. The crude ethanolic extract (25.0 g) was then suspended in 200 ml of distilled water in a separating funnel, followed by partitioning with dichloromethane (100 ml x 3). The dichloromethane fractions that were obtained were then concentrated to dryness *'in vacuo'* to yield dichloromethane fraction (DCMF). The aqueous layer was collected and partitioned with ethyl acetate (100 ml x 3). The ethyl acetate fractions that were obtained were then concentrated to dryness *'in vacuo'* to yield ethyl acetate fraction (EAF). The aqueous fraction was equally partitioned with n-butanol (100 ml x 3), fractions that were obtained were then concentrated to dryness *in vacuo* to give butanol fraction (BF) and aqueous fraction (AF). The fractions that were obtained were used for analyses.

Acute Toxicity Test

Acute toxicity of *C. sinensis* stem-bark was carried out according to the procedure of Lorkes *et al.*, (1983)

Phytochemical Screening

Chemical tests were carried out on the ethanolic extracts and the fractions for the presence of alkaloids, tannins, flavonoids, terpenes etc using standard procedures as described by Harborne (1973), Sofowora (1993) and Trease and Evans (2002).

IN VITRO ANTIOXIDANT ASSAY

Determination of Total Phenolic Content

The total phenolic of the extract was determined using the Folin-Ciocalteu's reagent reaction method of Singleton and Rossi (1965) as described by Gulcin *et al.* (2003).

Determination of Total Flavonoids Content

The determination of the total flavonoids content of the plant extract and fractions was based on the aluminium chloride colorimetric method according to Zhilen *et al.* (1999) and as described by Miliauskas *et al.* (2004).

Determination of DPPH Radical Scavenging Activity

The hydrogen or radical scavenging properties of the extract and fractions were determined using the stable radical DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) according to the method

of Blois (1958) as described by Brace (2001).

Determination of Nitric Oxide Radical Inhibition Activity

Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitric ions which were measured by Griess reaction (Macocci *et al.*, 1994).

Ferric Reducing Antioxidant Power Assay (FRAP)

The FRAP assay used antioxidants as reductants in a redox-linked colorimetric method with absorbance measured with a spectrophotometer (Benzie and Strain, 1999).

Total Antioxidant Capacity

The total antioxidant capacity of the extract and fractions were determined according to the method of Prieto *et al.* (1999).

Assay for ABTS⁺ Radical Scavenging Activity

The ABTS⁺ radical scavenging activity of sample extract and fractions was determined by the ABTS radical cation decolourisation assay as described by Re *et al.* (1999).

ANTI-INFLAMMATORY

Membrane Stabilizing Activity

The red blood cell membrane stabilizing activity assay was carried out as described by Oyedapo *et al.* (2010) using 2% bovine red blood cells with paracetamol as standard drug.

In vivo Hepatoprotective Activity:

Hepatoprotective activity of *Citrus sinensis* stem-bark was investigated for Acetaminophen induced hepatotoxicity in rats using the method described by (Radhika *et al.*, 2012). Twenty five Wistar albino rats of either sex were divided into five (5) groups of five (5) animals. The normal control group (group I) was given 2% tween 20, Acetaminophen at dose of 2g/kg bwt, p.o was given to group II animals as toxic dose for inducing hepatotoxicity and Legalex 70 (Silymarin) (50mg/kg, p.o) plus 2 g of acetaminophen to group III animals. Two doses of *C. sinensis* stem-bark ethyl acetate fraction i.e., 250mg/kg, p.o plus 2 g of acetaminophen and 500mg/kg, p.o plus 2 g of acetaminophen were administered to group IV and V animals

respectively. The treatment was given for seven days consecutively and after 24 h of last treatment, blood was collected by ocular puncture and analysed for various plasma and liver biochemical parameters.

Collection and Preparation of Blood Sample

Blood was collected by ocular puncture into a heparinized tube. Blood samples collected were centrifuged for 10 minutes at 3000 rpm and the supernatants obtained were stored in the refrigerator for further analyses.

Preparation of Liver Homogenate

The liver sample (1g) from each rat was transferred into a mortar and homogenized with 10 ml of 100 mM phosphate buffer. The resulting homogenate was centrifuged at 3000 rpm for 15 min and the supernatant obtained (10% w/v liver homogenate) was collected and stored at - 4°C for biochemical analyses.

Determination of Biochemical Parameters

Estimation of Alanine Aminotransferase (ALT)

Alanine aminotransferase catalyses the transfer of an amino group from alanine to α -Ketoglutarate with the formation of pyruvate and glutamate, by the method of Reitman and Frankel (1957).

Estimation of Aspartate Aminotransferase (AST)

Aspartate aminotransferase catalyses the transfer of α -amino group from aspartate and to α -ketoglutarate with the formation of oxaloacetate and glutamate., by the method of Reitman and Frankel, 1957.

Determination of Gamma Glutamyl Transferase

Gamma Glutamyl Transferase activity was determined by Szasz 1969

Total Bilirubin

Total bilirubin was determined by the method of Jendrassik and Grof, 1938

Protein Determination

Protein determination was carried out according to the method of Bradford (1976)

Determination of Superoxide Dismutase (SOD) Activity in Liver Homogenate

The levels of SOD activity was determined by the method of Misra and Fridovich (1972).

Determination of Catalase Activity

Catalase activity was determined according to the method of Sinha (1972)

Estimation of Glutathione Peroxidase (GPx)

Glutathione peroxidase (GPx) was measured by the method described by Rotruck *et al.* (1973)

Statistical Analysis

The results are expressed as mean \pm SEM using Graph Pad InStat Graphical-Statistical Package version 5. The difference between groups was analyzed by One-way Analysis of Variance (ANOVA) followed by Turkey Kramer multiple comparisons test with 5% level of significance ($P < 0.05$).

RESULTS

Table 1 shows the results of the phytochemical screening of the stem bark extract and fractions of the *Citrus sinensis*, Table 2 shows the values of the phenolic and the flavonoid content of the extract and fractions, Tables 3, 4, 5 and 6 show the results of the DPPH scavenging activity, the percentages of the Nitric oxide radical inhibition, ABTS radical scavenging activities, and total antioxidant and ferric reducing power (FRAP) of the extract and the fractions respectively. Table 7 shows the results of the membrane-stabilising activity of the crude extract and fraction of the stem bark on the bolvin red blood cells subjected to heat and hypotonic stress while Tables 8 and 9 show the changes in concentrations of the plasma and hepatic metabolite and enzyme activities in rats pretreated with the ethyl acetate fractions before the administration of the hepatotoxic dose of acetaminophen.

Table 1: Phytochemical Constituents of *Citrus sinensis* Stem-bark

Constituents	Alkaloids	Saponins	Cardiac glycoside	Tannins	Flavonoids	Terpenoids
Crude extract	-	+	+	+	+	+
DCMF	-	-	+	+	+	+
EAF	-	-	+	+	+	+
n-BF	+	-	+	+	+	-
AqF	-	+	-	+	+	-

+ = Presents; - = Absents. DCMF: Dichloromethane fraction, EAF: Ethyl acetate fraction, n-BF: Butanol fraction and AqF: Aqueous fraction

Table 2: Phenolic and Flavonoid Content of *C. sinensis* Stem Bark

Constituents	Total phenolic content	Total flavonoid content
	Gallic Acid Equivalent (mg/g)	Quercetin Equivalent (mg/g)
Crude extract	1.39±0.05	0.10±0.01
DCMF	1.43±0.08	0.38±0.01
EAF	2.03±0.04	0.14±0.02
n-BF	1.49±0.04	0.12±0.02
AqF	0.74±0.04	0.04±0.00

Data are expressed as mean ± S.E.M. (n = 3). DCMF: Dichloromethane fraction, EAF: Ethyl acetate fraction, n-BF: Butanol fraction and AqF: Aqueous fraction.

Table 3: DPPH Radical Scavenging Activity of Extract and Fractions of *C. sinensis*

Conc. (mg/ml)	Crude extract (% inhibition)	DCMF (% inhibition)	EAF (% inhibition)	n-BF (% inhibition)	AqF (% inhibition)	Vit. C (µg/ml)	% inhibition
0.5	81.62±2.95	87.26±2.29	84.65±0.25	83.69±0.54	65.42±0.99	10	40.43±0.33
0.25	61.54±3.00	65.58±0.19	59.99±0.33	60.80±0.43	44.04±2.50	8.0	28.98±0.13
0.125	50.17±4.30	51.39±3.08	49.86±1.41	41.67±0.34	26.25±3.02	6.0	17.28±0.07
0.0625	41.11±5.68	40.01±6.79	34.95±2.32	26.60±2.31	18.45±0.33	4.0	11.26±0.13
0.03125	30.07±2.58	29.76±2.28	24.16±0.80	18.63±0.93	10.01±0.20	2.0	7.50±0.13
IC ₅₀ (mg/ml)	0.16±0.04	0.15±0.03	0.19±0.00	0.22±0.00	0.34±0.00	IC ₅₀ µg/ml	12.92±0.09

Data are expressed as Mean ± S.E.M (n= 3). DCMF: Dichloromethane fraction, EAF: Ethyl acetate fraction, n-BF: Butanol fraction and AqF: Aqueous fraction, Vit. C: Vitamin C

Table 4: Nitric Oxide Radical Inhibition Assay of Extract and Fractions of *C. sinensis*

Conc. (µg/ml)	Crude extract (% inhibition)	DCMF (% inhibition)	EAF (%inhibition)	n-BF (% inhibition)	AqF (% inhibition)	Vit.C Conc. µg/ml	% inhibition
100	60.70±0.04	39.46±0.54	63.38±0.46	55.84±0.41	73.54±1.44	125	86.86±3.53
80	53.15±0.94	29.54±2.54	56.30±1.25	47.96±4.22	51.69±2.44	62.5	52.86±3.72
60	41.57±1.01	16.20±0.97	43.24±1.29	41.55±1.20	28.58±0.13	31.25	34.98±1.61
40	22.46±0.14	7.13±0.00	40.42±0.02	32.92±7.35	20.72±0.61	15.625	16.61±1.06
20	11.59±0.95	1.71±0.50	27.33±0.66	31.07±6.56	10.83±0.95	7.8125	8.07±0.83
IC ₅₀ (µg/ml)	78.81±0.73	123.89±3.67	68.85±1.43	83.58±6.61	76.53±0.37		64.15±3.57

Data are expressed as Mean ± S.E.M (n= 3). DCMF: Dichloromethane fraction, EAF: Ethyl acetate fraction, n-BF: Butanol fraction and AqF: Aqueous fraction, Vit. C: Vitamin C

Table 5: Percentage ABTS Radical Scavenging Activity of Extract and Fractions of *C. Sinensis*

Conc. (mg/ml)	Crude extract (% inhibition)	DCMF (% inhibition)	EAF (% inhibition)	n-BF (% inhibition)	AqF (% inhibition)	Trolox conc.	(% inhibition)
1.00	82.91±7.35	70.09±8.17	95.97±1.65	70.56±4.44	59.37±0.75	0.5	58.92±5.22
0.50	32.68±3.45	29.68±0.15	77.01±0.27	50.99±1.36	37.03±5.09	0.4	46.57±3.41
0.25	23.91±1.57	18.67±2.47	29.95±2.29	28.98±6.52	23.99±1.95	0.3	34.79±0.31
0.125	14.47±3.07	15.37±3.97	23.94±0.55	21.83±0.27	17.09±3.75	0.2	21.98±0.10
0.0625	9.67±0.38	12.52±0.52	17.49±1.01	13.59±1.99	14.84±1.95	0.1	11.98±0.49
0.03125	7.42±0.38	9.15±0.89	4.85±2.29	8.15±1.63	8.55±0.75		
IC ₅₀ (mg/ml)	0.62±0.06	0.73±0.08	0.42±0.00	0.61±0.04	0.79±0.01		0.41±0.06

Data are expressed as Mean ± S.E.M (n = 3). DCMF: Dichloromethane fraction, EAF: Ethyl acetate fraction, n-BF: Butanol fraction and AqF: Aqueous fraction.

Table 6: Total Antioxidant Assay and Ferric Reducing Power (FRAP) of Extract and Fractions of *C. sinensis*

Constituents	Total antioxidant assay	FRAP assay
	Ascorbic Acid Equivalent (mg/g)	Ascorbic Acid Equivalent (mg/g)
Crude extract	1.88±0.13	1.24±0.03
DCMF	1.81±0.01	1.25±0.07
EAF	1.60±0.09	1.48±0.02
n-BF	1.83±0.09	1.35±0.03
AqF	1.52±0.43	0.92±0.01

Data are expressed as mean ± S.E.M. (n = 3). DCMF: Dichloromethane fraction, EAF: Ethyl acetate fraction, n-BF: Butanol fraction and AqF: Aqueous fraction.

Table 7: Membrane Stability Activity of Crude Extract and Fractions of *C. sinensis* Stem-Bark on Bovine RBC Subjected to Heat and Hypotonic Stress.

Conc. (µg/ml)	Paracetamol (% stability)	Crude extract (% stability)	DCMF (% stability)	EAF (% stability)	n-BF (% stability)	AqF (% stability)
50	55.56±1.75	60.49±0.59	58.10±16.05	72.57±6.64	42.64±0.53	15.08±3.63
100	57.07±0.00	47.17±3.82	68.17±12.17	54.47±2.26	27.37±0.00	15.27±0.00
150	71.72±2.74	72.28±4.45	77.23±8.00	76.34±1.25	15.38±0.00	20.56±0.00
200	62.12±2.55	44.19±1.62	74.37±5.15	80.46±5.18	34.21±3.68	12.20±1.51
250	70.71±0.25	27.01±1.87	84.49±7.57	84.01±7.16	54.21±1.58	19.09±4.59
300	62.12±2.57	41.00±5.04	74.28±3.12	84.49±4.79	34.74±7.37	17.59±0.79
350	77.78±5.69	47.28±0.74	77.99±4.96	88.69±4.17	17.95±0.00	35.87±13.35

Data are expressed as mean ± S.E.M. (n = 3). DCMF: Dichloromethane fraction, EAF: Ethyl acetate fraction, n-BF: Butanol fraction and AqF: Aqueous fraction.

Table 8: Changes in Plasma Metabolites and Enzyme Activities in Acetaminophen Treated Rats Pretreated with EA Fraction of *C. sinensis* Stem-Bark

Parameters	Group I	Group II	Group III	Group IV	Group V
Total Bilirubin (mg/dl)	0.09±0.01	0.41±0.08 ^a	0.13±0.02 ^b	0.18±0.06 ^b	0.14±0.02 ^b
Total Protein (mg/ml)	2.37±0.05	1.07±0.04 ^a	1.67±0.03 ^b	1.24±0.04	1.42±0.05 ^b
AST (IU/dl)	38.29±7.24	107.81±4.89 ^a	66.32±0.53 ^b	94.03±0.98	84.74±1.58 ^b
ALT(IU/dl)	34.08±1.05	79.75±9.37 ^a	39.30±0.81 ^b	51.7±1.70 ^b	42.7±1.38 ^b
GGT (IU/dl)	0.41±0.06	2.43±0.31 ^a	0.58±0.12 ^b	0.98±0.17 ^b	0.75±0.06 ^b

Values are expressed as mean± S.E.M. n = 5 in each group. Group I: control, Group II: paracetamol treated (toxic control), Group III: Silymarin (50mg + PCM), Group IV: Ethyl acetate fraction (250mg + PCM), Group V: Ethyl acetate fraction (500mg + PCM). ^a p<0.05 compared to the control group, ^b p<0.05 compared to the paracetamol group.

. ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, GGT = Gamma glutamyl transpeptidase.. ALT = Alanine aminotransferase

Table 9: Changes in Hepatic Enzyme in Acetaminophen Treated Rats Pretreated with EAF of *C. sinensis* Stem-Bark

Parameters	Group I	Group II	Group III	Group IV	Group V
ALT(IU/g)	46.40±0.60	13.4±0.80 ^a	37.5±3.90 ^b	23.95±2.45	33.87±3.14 ^b
SOD	5.18±0.14	1.35±0.21 ^a	3.41±0.41 ^b	2.96±0.05	4.19±0.60 ^b
CAT	115.35±11.67	44.54±0.21 ^a	85.08±2.66 ^b	71.65±0.17	103.39±7.28 ^b
GPx	1.62±0.08	0.34±0.05 ^a	1.21±0.18 ^b	1.13±0.01 ^b	0.56±0.00
GSH	1.01±0.00	0.37±0.01 ^a	0.96±0.09 ^b	0.58±0.13	0.99±0.11 ^b
Protein(mg/ml)	1.74±0.10	0.50±0.03 ^a	1.38±0.04 ^b	1.40±0.26 ^b	1.1±0.29

Values are expressed as mean± S.E.M. n = 5 in each group. Group I: control, Group II: paracetamol treated (toxic control), Group III: Silymarin (50mg + PCM), Group IV: Ethyl acetate fraction (250mg + PCM), Group V: Ethyl acetate fraction (500mg + PCM). ^a p<0.05 compared to the control group, ^b p<0.05 compared to the paracetamol group. . ALT = Alanine aminotransferase, SOD = Units/mg protein CAT = μ mole of H₂O₂ consumed/ min/mg protein GPx = μ moles of GSH oxidized/mg protein

DISCUSSION

Phytochemical screening of ethanolic extract and fractions of *C. sinensis* stem-bark using a standard procedure gave positive test for the presence of tannins, flavonoids, cardiac glycosides, terpenoids, saponins and alkaloids. The literature revealed that medicinal plants are backbone of traditional medicine and biological activities of the plants extract/fractions are due to the presence of primary and secondary metabolites (Malairajan *et al.*, 2006). Phenolic chemical constituents such as phenolic acids, flavonoids and tannins played the major role of antioxidant capacity of plants and biological activities may be related to their antioxidant activity (Chung *et al.*, 1998) while tannins contributed a major role as antihemorrhagic, antihyper cholesterol,

hypotensive and cardiac depressant agent (Price *et al.*, 1987). Steroids, terpenoids and saponins were reported to have analgesic, hypocholesterolemic, anti-diabetic properties (Sayyah *et al.*, 2004; Malairajan *et al.*, 2006). Therefore it is probable that the presence of flavonoids and tannins in all the fractions and crude extract is likely to be responsible for the free radical scavenging effects observed. Since flavonoids and tannins are phenolic compounds and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers (Polterait, 1997).

The results of the acute toxicity study indicate that the LD₅₀ of the ethanolic extract of *C. sinensis* stem-bark is more than 5000 mg/kg. This

finding, therefore suggests that the extract at the limit dose tested is essentially non-toxic and safe in oral formulation.

The flavonoid content of the crude ethanolic extract and the fractions of the stem-bark varied from $0.04 \pm 0.00 \text{ mg/g}$ to $0.38 \pm 0.01 \text{ mg/g}$. The dichloromethane fraction with the total flavonoid of $0.38 \pm 0.01 \text{ mg/g}$ had the highest amount among the extract/fractions in this study while ethyl acetate fraction had the highest amount of total phenol in this study. The compounds such as flavonoids, which contain hydroxyls, are responsible for the radical scavenging effect in the plants (Younes, 1981; Das and Pereira, 1990). The high contents of these phytochemicals can be responsible for the radical scavenging activity of the plant.

From the Table 4, the crude extract/fractions showed moderately good nitric oxide scavenging activity. The percentages of inhibitions were increased with increasing concentration of the extract/fractions. Ethyl acetate fraction showed the highest scavenging activity with the IC_{50} value of $68.85 \pm 1.43 \mu\text{g/ml}$ while IC_{50} value for ascorbic acid was $64.15 \pm 3.57 \mu\text{g/ml}$. The extract inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide.

The decolorization of ABTS^+ cation reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to deactivate these radical species (Pellegrini *et al.*, 1999). In the present study, the crude extracts/fractions of the investigated plants showed strong decolorizing effect towards ABTS^+ cation in a dose-dependent manner. It is noteworthy that the ethyl acetate fraction showed the highest ABTS^+ scavenging activity with the IC_{50} of $0.42 \pm 0.00 \text{ mg/ml}$ compared to the IC_{50} value of trolox $0.41 \pm 0.06 \text{ mg/ml}$. ABTS^+ radical generated from oxidation of ABTS^+ by potassium persulphate, is a good tool for determining the antioxidant activity of hydrogen-donating and chain breaking antioxidants (Leong and Shui, 2002). This assay is applicable for both lipophilic and hydrophilic antioxidants. The radical-scavenging activity of the ethyl acetate fraction of *C. sinensis* stem-bark were estimated by comparing the IC_{50} value of

ABTS^+ radicals which is almost equal to that of trolox. These extract/fractions exhibited high radical-scavenging activities when reacted with the ABTS radicals.

The antioxidant capacity of the crude extract/fractions was measured spectrophotometrically through phosphomolybdenum method, which was based on the reduction of Molybdenum (VI) to Molybdenum (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of ethanolic extract/fractions of *C. sinensis* stem-bark was found to decrease in this order: ethanolic extract > butanol fraction > dichloromethane fraction > ethyl acetate fraction > aqueous fraction (Table 6).

In the reducing power assay, the presence of antioxidants in the extract/fractions of *C. sinensis* stem-bark would result in the reduction of Fe^{3+} /ferricyanide complex to its form. The reducing power of the compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al.*, 1995). In this assay, the yellow color of the test solution was changed to blue. The reducing power increased with increasing the phenolic content of the extract/fractions. It was found that the ethyl acetate fraction has the highest reducing powers of all the extract/fractions. This implies that these extract/fractions have significant ability to react with free radicals to convert them into more stable nonreactive species and to terminate radical chain reaction.

The result of the *in vitro* antioxidant in this study has clearly demonstrated that the ethyl acetate fraction of *C. sinensis* stem-bark showed higher level of antioxidant constituent (phenolic compounds) as well exhibit antioxidant activity as evidenced by the relatively high free radical scavenging activity in the nitric oxide, 2, 2'-azinobis (3-ethyl-benzothiazoline 6-sulfonate) (ABTS) and Ferric reducing antioxidant power (FRAP) assay therefore ethyl acetate was considered as the most active fraction and was used for the *in vivo* study.

Stabilization of the RBCs membrane was studied to establish the mechanism of anti-inflammatory

action of *C. sinensis* stem-bark extract/fractions. The extract/fractions were effective in inhibiting the heat and hypotonic induced hemolysis at different concentrations. This provide evidence for membrane stabilization as an additional mechanism of *C. sinensis* stem bark anti-inflammatory effect. In Table 7, the results revealed that dichloromethane fraction, ethylacetate fractions and crude extract contained principles that protected the erythrocytes membranes effectively and compare favourable with standard anti-inflammatory drug (Acetaminophen). *C. sinensis* stem-bark extract/fractions exhibited membrane stabilization effect by inhibiting hypotonicity and heat-induced lysis of erythrocyte membrane. The erythrocyte membrane is analogous to the lysosomal membrane (Chou, 1997) and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extracellular release (Murugasan *et al.*, 1981). Some of the Non-Steroidal Anti-inflammatory Drugs (NSAIDs) are known to posses membrane stabilization properties which may contribute to the potency of their anti-inflammatory effect. It has been reported that flavonoids exert profound stabilizing effects on lysosomes both *in vitro* and *in vivo* in experimental animals (Middleton, 1996) while tannin and saponins have the ability to bind cations and other biomolecules, and are able to stabilize the erythrocyte membrane (Oyedapo, 2001). The high membrane-stabilizing activity of the stem extract/fractions of *C. sinensis* observed in this investigation may be due to its high flavonoid and tannin content.

In the acetaminophen-induced hepatotoxicity, a model of oxidative stress was employed in this study, oxidative liver damage was established by the significant increases in serum levels of AST, ALT, GGT, total bilirubin and total protein while in the liver, there was a concomitant decrease of these hepatic marker enzymes as well as oxidative stress markers such as SOD, GPx, and GSH. Several studies have demonstrated the induction of hepatocellular damage or necrosis by

acetaminophen higher doses in experimental animals and humans (Vermeulen *et al.*, 1992). For screening of hepatoprotective agents, paracetamol-induced hepatotoxicity has been used as a reliable method. Paracetamol is metabolized primarily in the liver and eliminated by conjugation with sulfate and glucuronide, and then excreted by the kidney. Moreover, acetaminophen hepatotoxicity has been attributed to the formation of toxic metabolites, when a part of paracetamol is activated by hepatic cytochrome P₄₅₀ to a highly reactive metabolite Nacetyl- p-benzoquinoneimine (NAPQI) (Mitchel *et al.*, 1976). Toxic metabolites (N-acetyl- p- benzoquineimine) can alkylate and oxidise intracellular GSH, which results in liver GSH depletion subsequently leading to increased lipid peroxidation by abstracting hydrogen from a polyunsaturated fatty acid and ultimately, causing liver damage due to higher doses of paracetamol (Mitchell *et al.*, 1973; Grypioti, 2006). Reactive metabolites can exert initial cell stress through a wide range of mechanisms including depletion of glutathione (GSH) or binding to enzymes, lipids, nucleic acids and other cell structures (Pauli-Magnus *et al.*, 2005).

In the assessment of liver damage by acetaminophen, the determination of enzyme levels such as AST, ALT is largely used. Necrosis or membrane damage releases the enzyme into circulation and hence it can be measured in the serum. High levels of AST indicates liver damage, such as that caused by viral hepatitis as well as cardiac infarction and muscle injury, AST catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore ALT is more specific to the liver, and is thus a better parameter for detecting liver injury. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Drotman and Lawhan, 1978, Oyedapo 2001). Serum bilirubin and total protein levels, on the other hand, are related to the function of hepatic cell.

As observed in this study, the administration of ethyl acetate fraction of *C. sinensis* stem-bark at different doses levels (250 and 500mg/kg b.w) ameliorated the extent of plasma and liver biochemical derailments observed such as the increased levels of the plasma enzymes and

bilirubin and also by Silymarin at a dose of 50 mg/kg. The amelioration of increased plasma enzymes in acetaminophen induced liver damage by the test fraction may be due to the prevention of the leakage of intracellular enzymes by its membrane-stabilizing activity. This is in agreement with the commonly-accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew and Joice, 1987). Effective control of bilirubin and total protein levels points towards an early improvement in the secretory mechanism of the hepatic cells.

The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been distributed by a hepatotoxin. Both Silymarin and the ethyl acetate fraction decreased acetaminophen-induced elevated enzyme levels in tested groups, indicating the protection of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells.

Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in liver injury (Curtis and Mortiz, 1972, Kessler 2003). SOD has been reported as one of the most important enzymes in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical. A higher dose (500 mg/kg) of the ethyl acetate fraction was observed to increase the level of SOD as produced by Silymarin, the standard hepatoprotective drug.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues, and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Chance and Greenstein, 1992). Therefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. Also, a higher dose (500 mg/kg) of ethyl acetate fraction increases the level of CAT as also

produced by silymarin, the standard hepatoprotective drug.

GSH is a non-enzymatic biological antioxidant present in the liver. It removes free radical species such as hydrogen peroxide, superoxide radicals and maintains membrane protein thiols. Also it is a substrate for glutathione peroxidase (GPx) (Prakash *et al.*, 2001). The GSH depletion in hepatic mitochondria is considered the most important mechanism in the acetaminophen induced hepatotoxicity. Reduced GSH level was depleted in acetaminophen treated group may be due to conjugation of GSH with NAPQI to form mercapturic acid (Mitchell *et al.*, 1973). Administration of the ethyl acetate fraction at a lower dose (250mg/kg) increases the level of GPx while a higher dose (500mg/kg) increases the level of GSH as produced by Silymarin, the standard hepatoprotective drug.

In conclusion the result of this study shows that the stem-bark of *C. Sinensis* possessed potent antioxidant properties and anti-inflammatory potential. However, the protective effect of the Ethyl acetate fraction of *C. sinensis* on acetaminophen induced hepatotoxicity in rats appears to be related to enhancement of antioxidant enzyme levels in addition to free radicals scavenging action and membrane stability effect. The phytochemical studies reveal the presence of flavonoids and tannins in all the test fractions and crude extract. Flavonoids are hepatoprotectives (Seevola *et al.*, 1984; Wegner and Fintelmann, 1999). The observed antioxidant, anti-inflammatory and hepatoprotective activities of *C. sinensis* stem-bark may be due to the presence of flavonoids and tannins.

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