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In vivo antioxidant activity of methanolic extract of stem bark of *Spondias mombin* L. on carbon tetrachloride induced oxidative stress in wistar rats

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ABSTRACT

In this study, antioxidant activity of methanol extract of stem bark of Spondias mombin (MESPM) was investigated using carbon tetrachloride (CCl_4)-intoxicated rat liver as the experimental model. The intoxicated rats were administered MESPM for 14 days (daily, orally at the dose of 200 mg/kg and 400 mg/kg body weight) and silymarin at the dose of 50 mg/kg body weight. In vivo antioxidant activities of SPMME have been evaluated by using a range of methods such as measurement of lipid peroxidation by thiobarbituric acid reactive substances (TBARS), 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferric reducing antioxidant of plasma (FRAP) assay. Oral administration of MESPM and reference drug silymarin in intoxicated rats reduced significantly TBARS level in both serum and liver tissues and also increased significantly FRAP values and percentage inhibition of DPPH radical compared to CCl_4 group. At the dose of 400 mg/kg body weight, MESPM restored significantly of these values towards normal as a reference drug silymarin compared to control group in all assays. S. mombin possesses in vivo antioxidant activity and can be employed in protecting tissue from oxidative stress.

Keywords: *Spondias mombin*, antioxidant *in vivo*, CCl_4 (carbon tetrachloride), oxidative stress.

INTRODUCTION

There is increasing interest in the use and measurement of antioxidant capacity in food and pharmaceutical preparations and in clinical studies. The interest is mainly due to the role of reactive oxygen species (ROS) in aging process and pathogenesis of many diseases in which, ROS are mainly involved [1]. In fact, many studies have shown that these ROS, including oxygen free radicals are causative factors in the etiology of degenerative disorders diabetes, atherosclerosis, rheumatic disorders, aging, cancer, cardiovascular, some hepatopathies and other serious organ damage [2-4]. So, at high levels, free radicals and oxidants generate oxidative stress, a deleterious process that can damage important cellular components especially proteins, nucleic acids and polyunsatured fatty acids in cell membranes and plasma lipoproteins. Fortunately, there is controlled naturally by various beneficial compounds known as antioxidants.

Many plants extracts and plant products have been shown to have significant antioxidant activity [5]. Recently, there has been growing scientific interest to find naturally occurring antioxidants because of established carcinogenicity of synthetic ones [6]. Additionally, it has been suggested that natural antioxidants are more effective than the synthesized compound in preventing oxidative stress-related effects due to synergistic interactions among plant components that improve the bioavailability of the antioxidant agents [7].

Spondias mombin is a flowering plant with several reported medicinal properties. It belongs to the family Anacardiaceae and grows mostly in the rain forest and coastal areas of the world to a height of 15- 22m. The trunk

has deep incisions in the bark, which produces a brown resinous substance. The plant has compound leaves which are located at the ends of the branches. Each leaf has an odd number of leaflets, from 9-19. The leaflets are opposite except for the terminal ones. Crushed leaves have faint turpentine-like smell. The trunk and bark are gray and sometimes have distinct bur, blunt, gray spines [8]. In Côte d'Ivoire, it is known by various names ("ngoua" in Attié and Abbey, "trouma" in Baoulé, **"**ningon" in Malinké and "titi" in Bété). All parts of the plant are reported to be medicinally useful. A decoction of the bark is known to relief severe cough with inflammatory symptoms while dry powdered bark is applied as a wound dressing in circumcision. The bark is also used in Côte d'Ivoire to treat sores, bronchitis, nausea, as a poison antidote as well as to facilitate parturition and used to relieve pain and prevent excessive bleeding during menstruation and also considered effective against the hemorrhoid [9, 10]. The leaves are commonly used for the treatment of eye diseases, diarrhea, dysentery and painful colic [11]. Many scientific studies have shown that it is antiepileptic, antipsychotic, sedative [12], anti fertility [13], anti helmintic [14], anti microbial [15], anti malarial [16], molluscicidal [17], haemostatic function [18], increased capillary permeability [19], anti inflammatory [20]. It has also been reported to have blood lipid-lowering activity [21].

In a previous study, we have shown that methanolic extract had antioxidant activity *in vitro* due to the presence of phenolic compounds [22]. In fact, auhors showed that total phenolic, flavonoid et flavonol content of methanolic extract of *S. mombin* was found to be 343.5 ± 6.44 mg gallic acid equivalent (GAE)/g, 11.28 ± 0.45 mg and 31.73 ± 0.54 mg quercetine equivalent (QE)/g of extract. Also, the IC_{50} values for this extract *in vitro* free radical scavenging activity evaluated using DPPH was 5.83 ± 0.88 µg/mL and comparable to ascorbic acid(4,56 ± 0,22 µg/mL), a standard molecule.

Phenolic compounds are prototypic chain breaking antioxidant; their protective effect against lipoperoxidative damage depends on the hydrogen-donating capacity of hydroxyl group in each molecule [23]. So it was of importance to study the antioxidant activity of methanolic extract in *vivo* to confirm their activity in intact biological system.

Thus, the aim of this work was to evaluate the *in vivo* antioxidant activity of methanolic extract of stem bark of *Spondias mombin* on CCl_4-induced oxidative stress in rats in order to give an explanation about utilization of that plant in the folk medicine.

This evaluation was done by using the ferric reducing/antioxidant power (FRAP) assay, inhibition of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical assay and thiobarbituric acid reactive substances (TBARS) assay in serum and liver homogenates.

EXPERIMENTAL SECTION

Chemicals

All reagents used for antioxidant activity assessment were of analytical grade. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,4,6-tris-(2- pyridyl)-s-triazine (TPTZ), 1,1,2,2-tetramethoxypropan (MDA) were obtained from Sigma Aldrich (St Louis, MO, USA), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium dodecyl sufate (SDS) and $FeCl_3,6H_2O$, $CH_3COONa,3H_2O$ and $FeSO_4,7H_2O$ from Prolabo (France) and Merck (Darmstadt, Germany). All solvents used in this investigation were also of analytical grade. Methanol, carbon tetrachloride (CCl_4), acetic acid, pyridine, butanol and hydrochloric acid were obtained from Merck (Darmstadt, Germany).

Collection of plant

Stem bark of *Spondias mombin* was collected from Affery (Region of Adzopé, south of Côte d'Ivoire) in February 2013, and identified by Professor Professor Aké Assi Laurent (Laboratory of Botany, University Felix Houphouët Boigny, Côte d'Ivoire). A voucher specimen of the plant n^o 15778 was enregistered in herbarium of National Floristic Center (University Félix Houphouët Boigny, Côte d'Ivoire). The collected plant material was air dried in darkness at room temperature (20^{o}C). Stem bark were cut up and stored in tight-seal dark containers until needed.

Preparation of extract

One hundred grams (100g) of washed, air dried powdered stem of the plants were extracted with 1.5 L of methanol at room temperature for 48 hours with stirring at interval. The methanolic solution obtained were filtered using a Buckner funnel and Whatman N^o 1 and concentrated to dryness at 40^{o}C using a rotary evaporator under reduced pressure. The dried extracts were stored at 4^{o}C for subsequent analysis. The methanolic extract of stem bark of *Spondias mombin* was named MESPM.

Animals

Albino Wistar rats strain weighing 130-170 g of both sexes were used for acute toxicity and antioxidant activity studies. The animals were bred and maintained in the animal house of the Department of Pharmaceutical and Biological Sciences of University Felix Houphouet Boigny (Abidjan, Côte d'Ivoire). The animal house was maintained under standard hygienic conditions, at a room temperature of 27±1°C and room humidity (60 %±10 %) with a 12 h day and night cycle. The study protocol was approved by the Ethical Guidelines of University (Côte d'Ivoire) Committee on Animal Resources.

Acute toxicity studies

Acute toxicity studies were performed following OECD guidelines [24]. MESPM was orally administered at doses of 300 and 2000 mg/kg to separate groups of animals. The animals were observed continuously for 2 h looking for any symptoms of toxicity and /or death. They remained under observation for a period of 2 weeks and until the end of the study. 1/10th and 1/5th LD_{50} cutoff value of the extract were selected as screening doses for the antioxidant study.

Determination of *in vivo* antioxidant activity

Albino Wistar rats weighing 130-170 g of both sexes were used for the study. The animals were divided into 5 groups consisting of 6 animals each as known below. Oxidative stress was induced in animals by administration of carbon tetrachloride (CCl_4) intraperitoneally (i.p).

Group I served as normal control were untreated. Group II animals served as CCl_4 control, received by intraperitoneal injection (ip) 2 mL/kg body weight (bw) CCl_4 in paraffin (30 %) for three consecutive days. Group III served as a standard, and was administered orally sylimarin in a dose of 50 mg/kg during 14 successive days. Group IV and V were treated with daily doses of 200 and 400 mg/kg, p.o, respectively of MESPM for 14 days. The animals of Group III, IV and V were injected in i.p with CCl_4 (30 % in liquid paraffin) on days 12, 13 and 14.

Twenty four hours after the last treatment i.e. on 15th day, animals were anaesthetized using a light ether anesthesia and blood collected from retro orbital plexus. The blood samples were allowed to clot and the serum was separated by centrifugation at 2500 rpm for 15 min at 37° C and kept at – 4^{O}C. Furthermore, liver was rapidly excised, rinsed in ice-cold saline, and a 10% w/v homogenate was prepared using 0.15M KCl, centrifuged at 3000 g for 20 min at 4°C. Serum and supernatant obtained were used for the estimation of *in vivo* antioxidant activity

Antioxidant activity

Estimation of thiobarbituric acid reactive substances (TBARS) in liver homogenate

Thiobarbituric Acid Reactive Substances (TBARS) assay is the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. The reaction mixture consisted of 0.2 ml of 8.1 % sodium lauryl sulphate, 1.5 ml of 20 % acetic acid solution adjusted to pH 3.50 with sodium hydroxide and 1.5 ml of 0.8 % aqueous solution of thiobarbituric acid was added to 0.2 ml of 10 % (w/v) of liver homogenate. The mixture was brought to 4.0 ml with distilled water and heated at 95 °C for 60 min. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) was added and centrifuged at 3000 rpm for 10 min. The organic layer was discarded and absorbance of the clear upper (n-butanol) layer was measured using spectrophotometer at 532 nm. The calibration curve was obtained using different concentrations of 1, 1, 3, 3-tetramethoxypropane as standard to determine the concentration of TBA-MDA adducts in samples [25].

Estimation of TBARS in serum

To precipitate the serum proteins, 2.5 mL of TCA 20% (m/V) was added into 0.5 mL of the sample, which was then centrifuged at 1500 × *g* for 10 min. Then 2.5 mL of sulfuric acid (0.05 $M.L^{-1}$) and 2 mL TBA (0.2 %) was added to the sediment, shaken, and incubated for 30

min in a boiling water bath. Then, 4 mL n-butanol was added, and the solution was centrifuged, cooled and the supernatant absorption was recorded at 532 nm using a spectrophotometer. Concentration of thiobarbituric acid reactive substances was read from standard calibration curve, which was plotted using 1, 1, 3, 3 –tetramethoxy propane [26].

Determination of Ferric Reducing Antioxidant of Plasma (FRAP)

The FRAP assay, developed by Benzie and Strain [27] as a direct method for measuring the total antioxidant power of biological fluids, was adopted in this study. At low pH, reduction of a ferric 2, 4, 6-tripyridyl-s-triazine [Fe (III) - TPTZ] complex to the ferrous 2, 4, 6-tripyridyl-s-triazine [Fe (II)-TPTZ] complex, which has an intense blue color, can be monitored by measuring the change in absorption at 593 nm.

Briefly, 30 µL of serum was mixed with 90 µL of deionized water and then added to 900 µL of a FRAP reagent. The FRAP reagent was prepared fresh prior to the experiments with 5 mL of 0.3 M acetate buffer (pH 3.6) combined with 0.5 mL of 10 mM TPTZ in 40 mM HCl and 0.5 mL of 20 mM $FeCl_3$. The absorbance at 593 nm was measured at 37°C with a spectrophotometer.

Blank control received water only. Aqueous solutions of $FeSO_4$, 7H2O (7.81-1000 µM) were used for the calibration and the results were expressed as FRAP value (µM Fe (II)) of the samples.

DPPH radical scavenging activity

The free radical scavenging activity of serum samples were measured by the DPPH method proposed by Hasani et al. [28] with a slight modification. Briefly, 50 µl of each sample were added to 950 µl of DPPH in methanol solution (100 µM) in a test tube. After incubation at 37°C for 30 min in darkness, followed by a centrifugation at 3000 g for 5 min, the absorbance of supernatant was determined at 517 nm using spectrophotometer. A methanolic solution of DPPH was used as control and the percentage of DPPH radical scavenging activity was calculated according to the following equation:

Scavenging activity (%) = [(absorbance of the control – absorbance of the sample) /absorbance of the control] × 100.

Statistical analysis

All results are presented as mean ± SEM. Statistical analyses of the data were carried out using ANOVA and Tukey's multiple comparison analysis, and values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of plant extract on lipid peroxidation in serum and liver

TBARS concentrations in serum and liver homogenates of all experimental rats are shown in Figures 1 and 2. The level of TBARS was significantly ($p < 0.001$) increased in CCl_4 control group as compared to untreated control animals (normal control). Treatment with SPMME (200 mg/kg and 400 mg/kg) reduced significantly the TBARS levels when compared with negative control group ($p < 0.05$) and brought them near to normal level. The effect of SPMME at the dose of 400 mg/kg was comparable with that the standard reference silymarin.

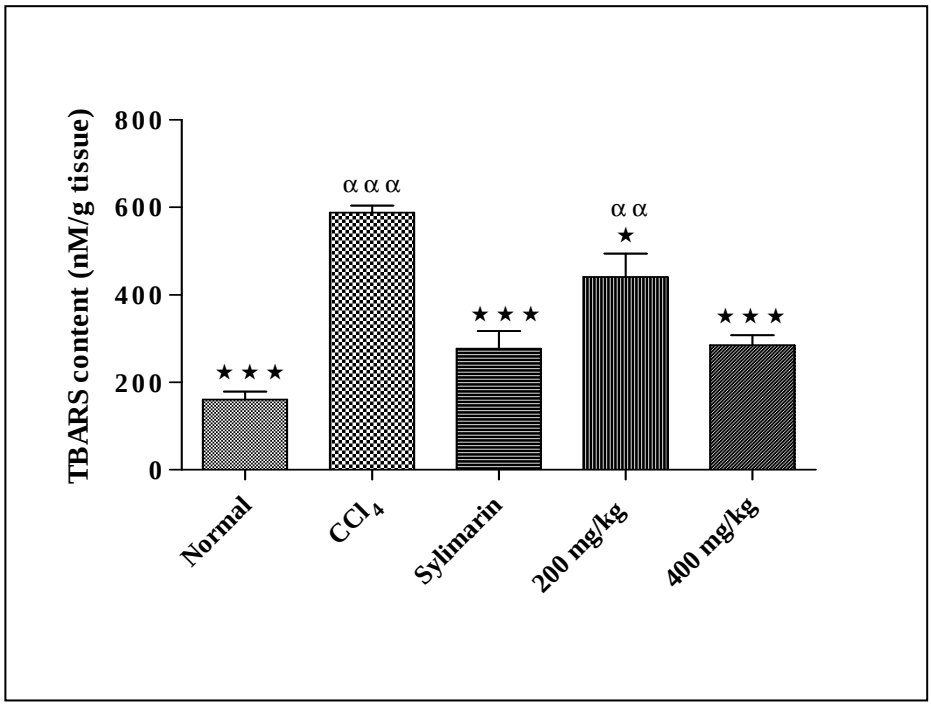

Figure 1: Effect of methanolic extract of stem bark of *S. mombin* and sylimarin on liver tissue TBARS concentration on rats induced oxidative stress with CCl_4

*Values are mean ±SEM; n=6 animals in each group. *p < 0.05, ** p <0.001 indicate significance from CCl_4 group and $^{\alpha\alpha}$ p < 0.001 indicates significance from normal group.*

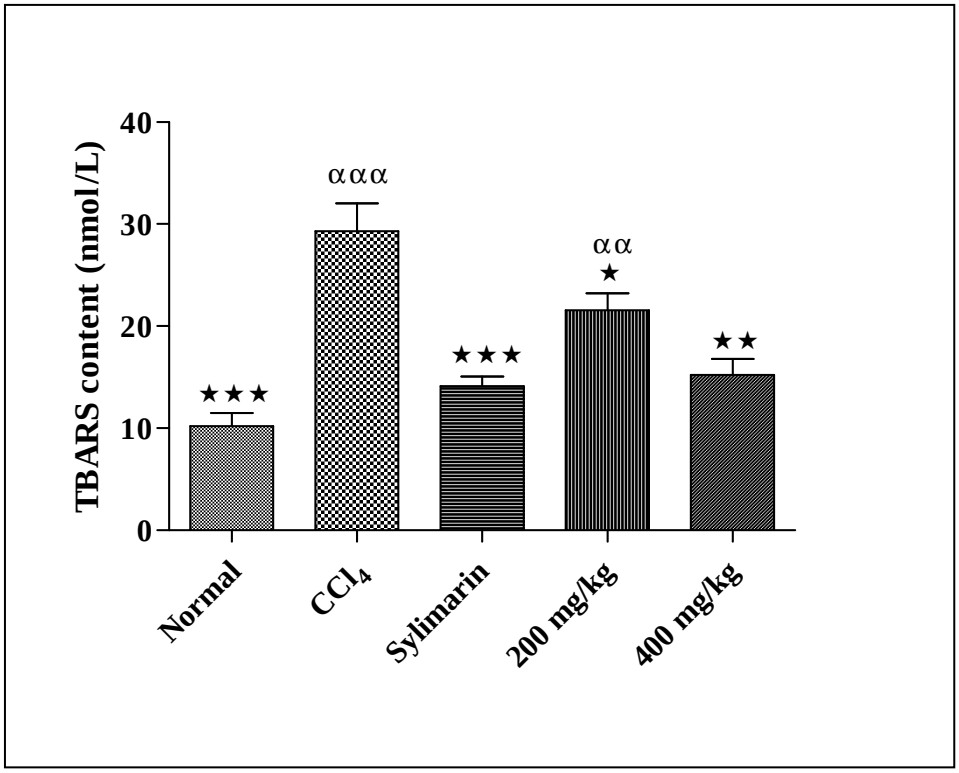

Figure 2: Effect of methanolic extract of stem bark of *S. mombin* and sylimarin on serum TBARS concentration on rats induced oxidative stress with CCl_4

*Values are mean ± SEM; n=6 animals in each group, *p < 0.05, ** p <0.001, *** p <0.0001 indicate significance from CCl_4 group and $^{\alpha\alpha}$ p < 0.001, $^{\alpha\alpha\alpha}$ p < 0.0001 indicate significance from normal group.*

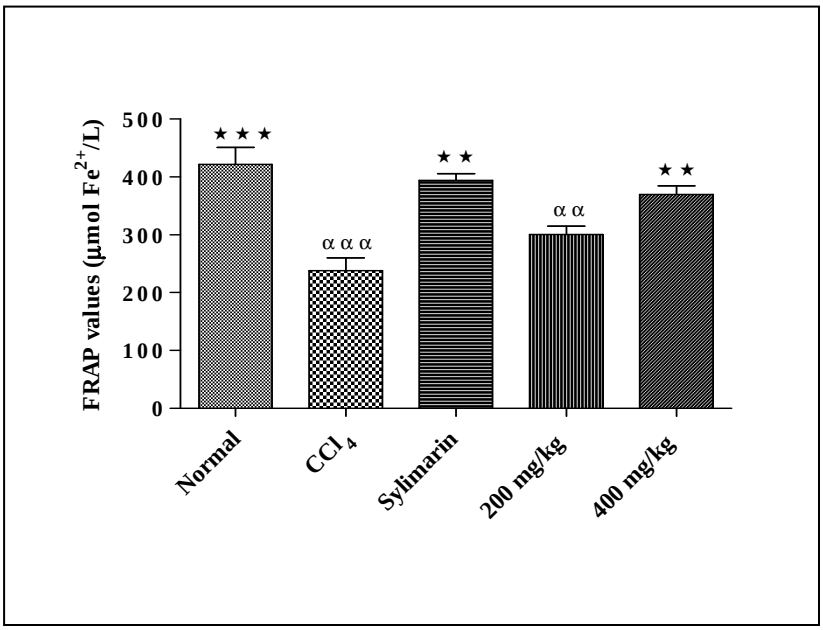

Figure 3: Effect of methanolic extract of stem bark of *S. mombin* and sylimarin on ferric reducing antioxidant power (FRAP) in rats subjected to CCl_4 induced oxidative stress

*Values are mean ± SEM; n=6 animals in each group. ** p <0.001, *** p <0.0001 indicate significance from CCl_4 group, $^{\alpha\alpha}$ p < 0.001, $^{\alpha\alpha\alpha}$ p < 0.0001 indicate significance from normal group.*

Measurement of FRAP value in serum

The effect of MESPM on FRAP value are summarized in Figure 3. The results showed that tetrachloride carbon significantly ($p < 0.001$) decreased the serum FRAP level in CCl_4 induced group as compared to normal control group showing a liver disorders in rats. Prior administration of MESPM at 400 mg/kg and sylimarin (50 mg/kg)

significantly increased the CCl_4-induced elevated level of FRAP value ($p < 0.01$). So, group of the rats treated with MESPM (400 mg/kg) and the drug reference brought CCl_4-induced elevated FRAP value close to the normal ($p > 0.05$).

Effect of plant extract on DPPH radical scavenging

The results of scavenging activity of crude methanol extract of MESPM at a dose of 200 mg/kg and 400 mg/kg on rats intoxicated with carbon tetrachloride were illustrated in Figure 4. It showed that percentages inhibition of DPPH radical in serum of normal group, group treated with SPMME (400 mg/kg) and reference group (silymarin) which are respectively 32.12 ± 2.50 %, 30.20 ± 4.71 % and 29.57 ± 5.08 % were no significantly different ($p > 0.05$). However, inhibition effect on DPPH radical increased significantly in all groups compared to CCl_4 group ($p < 0.05$) except in animals treated with MESPM at the dose of 200 mg/kg ($p > 0.05$).

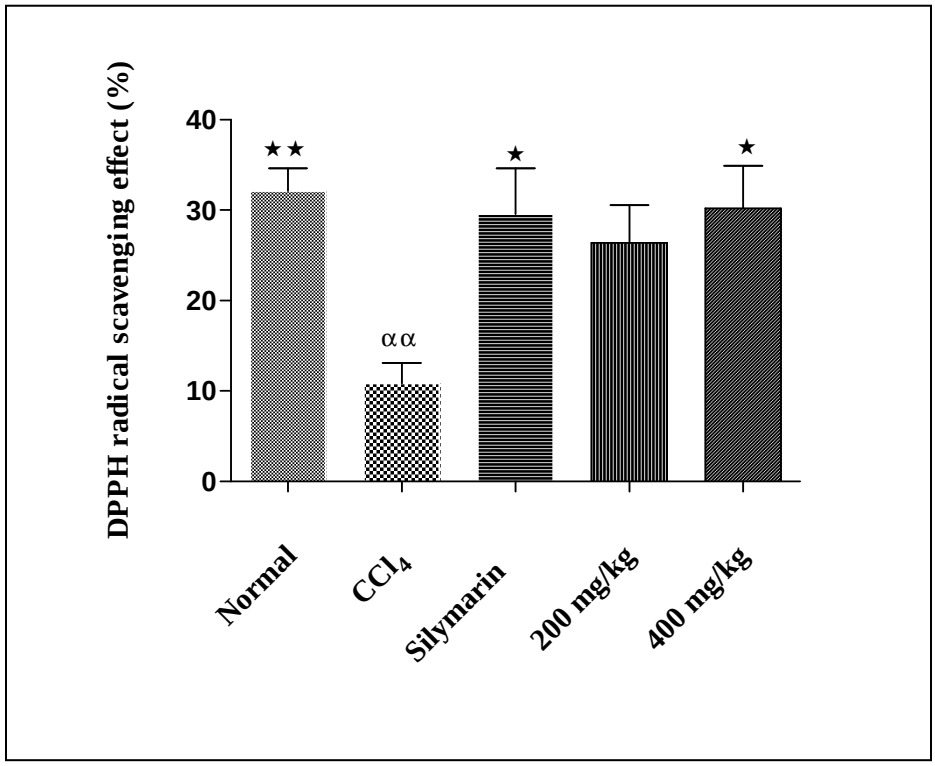

Figure 4: effect of varied doses of methanolic extract of stem bark of *S. mombin* and sylimarin on DPPH radical scavenging on serum of rats administered CCl_4

*Values are mean ±SEM; n=6 animals in each group. *p < 0.05, ** p <0.001 indicate significance from CCl_4 group, $^{\alpha\alpha}$ p < 0.001 indicates significance from normal group.*

DISCUSSION

The powerful oxidants including superoxide anions, hydroxyl radicals and hydrogen peroxide are known as reactive oxygen species (ROS) [29]. At high concentrations, they induce oxidative stress, a deleterious process that can damage all cell structures. They trigger lipid peroxidation, leading to decrease of membrane fluidity, increase of the leakiness, and damage membrane proteins, inactivating receptors, enzymes, and ion channels, *etc*. Peroxidation of polyunsaturated fatty acids also generates free radicals [3, 30]. Therefore, antioxidants are needed in different compartments of the body such as the circulating system, the cell cytoplasm where most cellular activities occur, and across the blood-brain-barrier and central nervous system. The body antioxidant pool can be supplied by diet including plants. Interestingly, many medicinal plants have shown notable antioxidant properties and plant derived products are largely used as antioxidants [31].

Many studies have investigated the role of antioxidant drugs and plant-derived compounds in the prevention of oxidative stress. In the present study, the methanolic extract of stem bark of *S. mombin* was evaluated for *in vivo* antioxidant activity using CCl_4-intoxicated rats. CCl_4 is a widely used toxicant for experimental induction of liver toxicity in laboratory animals [32]. The hepatotoxic effects are largely based on membrane lipid peroxidation and induction of trichloromethyl radical that results in severe cell damage. In fact, trichloromethyl and its peroxy

radicals are capable of binding to proteins or lipids, initiating lipid peroxidation and liver damage, thus playing a significant role in the pathogenesis of diseases [33].

In present study, elevated level of TBARS in CCl_4- treated rats indicates excessive formation of free radicals and activation of lipid peroxidation system resulting in hepatic damage. TBARS produced as products of lipid peroxidation that occurs in hydrophobic core of bio-membranes [34]. Treatment with methanolic extract of *S. mombin* MESPM was able to reduce the level of lipid peroxides in a dose dependent manner as compared with hepatotoxic group indicating anti-lipid peroxidative effect of this plant. Our previous study showed that methanolic extract of *S. mombin* had a high level of total phenolics, flavonoids and flavonols which are known to be excellent antioxidants [22] and numerous studies suggest that dietary intake of plant polyphenol antioxidants may have positive effects in oxidative -stress related pathologies [35]. These antioxidative constituents present in *S. mombin* might be responsible for the, antilipid peroxidation. In other hand, a high positive correlation is found to occur between lipid peroxidation process in liver and serum. As shown by literature data, increased peroxidation of lipids in many tissues and organs leads to their 'leakage' to the blood circulation and shift to other organs and tissues [36]. The current study results seem to suggest that the elevation of serum TBARS level with CCl_4 is mainly due to the rise in the liver.

Other methods such as the ferric reducing antioxidant of plasma (FRAP) and the DPPH radical scavenging activities have been developed to determine plasma antioxidant capacity. All two methods are widely recognized and used in different clinical studies of oxidative stress-related pathological conditions [37-39].

In the FRAP method the ability of the sample to reduce the ferric ion is used as a criterion on antioxidant capacity. Ascorbic acid, α-tochopherol, uric acid, bilirubin and phenolic compounds were found to have ferric-reducing activity, but this method was suggested to be unsuitable for proteins, glutathione and lipoic acid [27, 40]. DPPH is a stable commercial available free radical using as a popular marker for screening of free radical scavenging activity of compounds or biological samples. This method measures hydrogen atom or electron-donating activity and the scavenging activity related to the structure of the active substances [41]. Since the activities of antioxidant may vary in different biological systems, it is necessary to employ several methods to measure total antioxidant capacity based on different principles. Data obtained by DPPH and FRAP assays indicate that administration of MESPM in intoxicated rats effectively inhibits oxidative stress by increasing DPPH radical scavenging percentage and FRAP level. In fact, the plasma contains a network of endogenous antioxidants such as (albumin, bilirubin, reduced glutathione and uric acid) as well as exogenous antioxidants derived from food. These antioxidants may act in a complementary and synergistic manner to provide a better protection against ROS [42]. The increase in plasma antioxidant capacity is probably attributed to the elevated levels of exogenous antioxidants acquired following treatment with *S. mombin* extract.

CONCLUSION

In conclusion, present results demonstrate that methanolic extract of stem bark of *S. mombin* (MESPM) exhibited strong antioxidant activity and free radical scavenging effect *in vivo*. So, MESPM is able to protect various pathological conditions, including oxidative stress. Further work is necessary to isolate active principles from MESPM and elucidate the actual mechanism involved in the antioxidant activity.

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