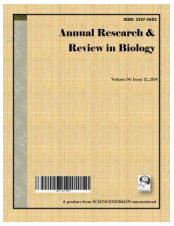

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Anti-hemolytic, Anti-lipid Peroxidation, Antioxidant Properties and Acute Toxicity of *Xanthium strumarium* Leaves Extracts

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Authors' contributions

This work was carried out in collaboration between all authors. Author TG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors LA, SK, NEC and AB managed the analysis of the study. Authors SA, FZ and SB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The present study was undertaken to evaluate the *in vitro* and *in vivo* antioxidant effects of different extracts prepared from the leaves of *Xanthium strumarium.* Polyphenols and flavonoids contents in all extracts were determined by spectrophotometric assays, antioxidant and antiradical capacities of the extracts were assayed using 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) radical scavenging assay, reducing power, β-carotene and anti-hemolytic assay. In addition, the *in vivo* antioxidant activity of three concentrations of leaves crude extract was investigated. Antioxidant activity of the

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crude extract was examined using anti-hemolytic assay and the determination of Glutathione and malondialdehyde (MDA) contents and catalase activity. *In vitro* antioxidant assays showed that crude extract and its fractions have strong effects in scavenging DPPH and reducing power. These activities decreased in the following order: ethyl acetate extract (EAE) > aqueous extract (AqE) > crude extract (CrE) > chloroform extract (ChE). The β-carotene bleaching assay showed that the CrE had the highest antioxidant activity followed by the EAE, AqE and the ChE. However, the anti-hemolytic test demonstrated that the ChE was the most effective in protecting red blood cells, followed by the EAE, AqE and the CrE. Three concentrations of leaves crude extract were tested for the *in vivo* antioxidant assays, and anti-hemolytic Catalase activity and the content of both MDA and Glutathione (GSH) were estimated. Among these tests, *X. strumarium* crude extract exhibited a potent inhibition of lipid peroxidation.
It was concluded that *X. strumarium* extracts contain high phenolic content and have powerful antioxidant capacity *in vitro* and *in vivo*. These extracts were found to be safe with no toxic effects. These findings support the traditional use of this plant as an anti-inflammatory remedy.

Keywords: Xanthium strumarium L.; acute toxicity; In vitro antioxidant activity; polyphenols; antioxidant enzymes.

1. INTRODUCTION

Reactive oxygen species (ROS) are normal components of healthy cells, they are also mediators of the first defensive actions of cells and involved in phagocytosis, apoptosis and detoxification [1]. Overproduction of ROS and oxygen-derived free radicals may cause damage to lipids, proteins and nucleic acids and subsequently contribute to a variety of chronic diseases as cardiovascular diseases, atherosclerosis, cancer and aging [2]. In order to reduce negative effects caused by ROS, both synthetic and natural antioxidants are used. The BHT (butylate hydroxytoluene) a common synthetic antioxidant, used as a food additive is considered to be responsible for liver carcinogenesis [3,4]. Recently there has been increasing interest in discovering natural antioxidants. Plants used in traditional medicine usually present a large spectrum of natural antioxidants which can reduce nocive effects of ROS.

Polyphenols are strong antioxidants. Phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins, are present in the different plant parts (roots, leaves, branches/stems, barks, flowers and fruits). The antioxidant properties of phenolic acids and flavonoids are due to their redox properties, ability to chelate metals, quenching of singlet oxygen [5,6] and stabilizing lipid peroxidation [2].

Xanthium strumarium L (family Compositae) is a cocklebur commonly found as a weed in roadsides and hedges. The genus *Xanthium* includes 25 species, all of the American origin. *Xanthium spinosum* L. and *Xanthium strumarium* L. are medically used in Europe, North America and Brazil; *Xanthium canadens* Mill. is used in North America and Brazil and *Xanthium strumarium* L. in China, India and Malaysia [7]. *Xanthium strumarium* L. is used in traditional Chinese medicine to treat nasal sinusitis, headache, urticaria and arthritis. It has also been mentioned to possess curative effects against chronic bronchitis, chronic rhinitis, allergic rhinitis, lumbago and other ailments [8]. According to the bibliography, fruits of *Xanthium strumarium* L. known as *Xanthii Fructus* were the part of plant the most used in the traditional medicine [9].

The aerial parts of the plant contain a mixture of alkaloids, sesquiterpene lactone; xanthinin, xanthumin, xanthatin, xanthostrumarin, atractyloside, carboxyatractylate (a hypoglycemic agent), phytosterols, hydroquinone and caffeolquinic acids [7].

Xanthium strumarium known as “Chobaita” grew in Algeria, but is practically not used in traditional medicine, it's used is limited to the Algerian east, to treat headache as cataplasm .In the present study, the *in vitro* and *in vivo* antioxidant activities of different fractions, prepared from *Xanthium strumarium* (XS) leaves methanolic extract, were examined.

2. MATERIALS AND METHODS

2.1 Plant Material

Leaves of *Xanthium strumarium* were collected from Beni Aziz (Setif) Algeria, in July-August 2013. The plant was identified by Pr. Oudjhih

Bachir, Elhadj Lakhdar University, Batna, Algeria, with the reference number 164/ISVSA/DA/UHLB1/13.

2.2 Animal Material

Male Nmris mice (weighing between 25-30 g) were purchased from the Pasteur Institute of Algeria. Mice were kept under controlled environmental conditions (24ºC and 12 h light-dark cycle) and fed a normal laboratory diet. The experimental protocol was approved by the Ethics Committee of the University of Sétif 1. All procedures were performed in compliance with laws and institutional guidelines. The animals were housed for a period of at least seven days for acclimatization before the experiments.

2.3 Extraction and Fractionation Procedure

Leaves of *Xanthium strumarium* were air-dried in the dark, then powdered. Extraction was carried out three times with Methanol 98% at room temperature with a ratio of 1:10 W/V. The macerates were evaporated until dryness under reduced pressure on a rotavapor at 40ºC [10].

Fractionation of the extract was effectuated using liquid-liquid extraction [11]. Crude extracts were successively extracted with different solvents of increasing polarity: hexane, chloroform and ethyl acetate. The obtained organic layer of each partition was evaporated under reduced pressure on a rotavapor at 40ºC to dryness.

2.4 Determination of Total Phenols and Flavonoids Contents in Extracts

Folin-ciocalteu method [12] was used to estimate the content of total phenolics in the extracts. 1 ml of the Folin-ciocalteu reagent (diluted to 1/10) was added to 200 µl of extract dissolved in methanol or water. After 4 min, 800 µl of carbonate of sodium were added, and the mixtures were incubated for 1 h and 30 min in the dark. Absorbance was measured at 765 nm. The concentration of total polyphenols was expressed as milligrams equivalent gallic acid per gram extract (mg EGA/g E).

Aluminium Trichloride method described by Boussoualim et al. [13] was used to estimate flavonoids in the different extracts. 1 ml of each extract (prepared in methanol or distilled water) with adequate dilutions, was added to 1 ml of $AlCl_3$ solution (2% in methanol). After 10 minutes of incubation and reaction, the absorbance was registered at 430 nm. The concentration of flavonoids was expressed as milligrams equivalent quercetin per gram extract (mg EQ/g).

2.5 *In vitro* Antioxidant Activities

2.5.1 DPPH radical scavenging assay

In this method, 2,2′-diphenylpicrylhydrazyl (DPPH) was used as this free radical is relatively stable [14]. Antioxidants reduce the DPPH purple into a yellow product. 50 µl of different concentrations of extracts were added to 1250 µl of DPPH (0.004% in methanol). After 30 min of incubation in dark, the absorbance was measured at 517 nm. Results are expressed as inhibition percentage (I %).

$$I\ \% = [(A_C - A_E) / A_C] \times 100$$

A_C: absorbance of the control.
A_E: absorbance in presence of the extract.

Inhibitory concentration of 50 % of DPPH (IC_{50}) of each extract is then calculated from the equation, which determines the inhibition percentage in function of the inhibitory concentration. IC_{50} is expressed in mg/ml and compared with that of BHT.

2.5.2 β-carotene / linoleic acid bleaching assay

In this test, antioxidant capacity of the extracts was determined by measuring the inhibition of the oxidative degradation of β-carotene (discoloration) by the products of linoleic acid degradation [15]. The emulsion of β-carotene/ linoleic acid is prepared by solubilizing 0.5 mg of β-carotene in 1 ml of chloroform, 25 µl of the linoleic acid and 200 mg of Tween 40 were added. Chloroform was evaporated by the rotavapor. 100 ml of distilled water saturated with oxygen were added, the emulsion is agitated vigorously. 350 µl of the extracts or of BHT were added to 2.5 ml of the emulsion. The kinetics of the discoloration of the emulsion in presence of the extracts, or the BHT, was measured at 490 nm at 0, 1, 2, 3, 4, 6, 12, 24 and 48 hours. The antioxidant capacity of the extract was calculated from the equation:

$$AA\ \% = (A_E / A_{Et0}) \times 100$$

A_E : absorbance in the presence of the extract at the time of the lecture.
A_{Et0} : absorbance in the presence of the extract at 0 hour.

2.5.3 Reducing power assay

The reducing power of the extracts was determined using the method described by Bencheikh et al. [16]. Extracts possessing reducing power react with potassium ferricyanid to form the potassium ferricyanide, which react with ferric chloride to obtain ferric ferrous complex absorbing at 700 nm. 2.5 ml of different concentrations of the extracts were added to 2.5 ml of phosphate buffer (0.2 M and pH 6.6) and 2.5 ml of potassium ferricyanide (1 %). After an incubation at 50°C for 20 min, 2.5 ml of trichloroacetic acid (TCA) (10%) were added, the mixture was centrifuged (3000 rpm for 10 minutes). 2.5 ml of distilled water and 0.5 ml of ferric chloride (0.1%) were added to 2.5 ml of the supernatant, and the absorbance was measured at 700 nm. Ascorbic acid was used as a positive control. The increase in the absorbance of the mixture indicates an increase in the reducing ability of the extracts.

2.5.4 Anti-hemolytic activity

The resistance of erythrocyte treated by plant extracts to radical attack by the AAPH [2,2'-azo-bis (2-amidinopropane) HCl], was accessed according to the protocol described by Zerargui et al. [17]. The blood used in this test was obtained by decapitation of mice. Blood collected in tubes containing Etylene Diamine Tetra-Acetic acid (EDTA) was centrifuged for 15 min at 3000 rpm at 4ºC. The recovered red blood cells (RBCs) were washed three times with Phosphate Bufer Saline (PBS) to obtain a hematocrit of 2%.

The radical attack is induced by the addition of AAPH to the erythrocyte suspension and the extracts. The mixtures were incubated at 37ºC. The kinetics of the gradual disappearance of erythrocytes was followed by the measurement of the decrease in absorbance at 630 nm. The resistance of RBCs to the radical attack was expressed by the time required to the lyses of 50% of erythrocytes. Ascorbic acid was used as a standard antioxidant. Positive control contains RBCs and AAPH.

2.6 Acute Toxicity Test

Acute toxicity study was performed according to OECD (Organisation for Economic Co-operation and development) guidelines 425 [18]. The animals were divided into two groups of five animals each. Crude extract was administrated orally, the first dose was 2000 mg/Kg, animals were observed if any sign of toxicity appears for 14 days. If no mortality is observed at this dose, the same procedure will be repeated for the dose of 5000 mg/Kg. Results of this test permit to determine the doses which will be used in the *in vivo* tests.

2.7 *In vivo* Antioxidant Activities

Animals were divided into 5 groups: negative control (received only water), positive control (received ascorbic acid as a standard antioxidant) and the other three groups received leaves crude extract of *X. strumarium* (200 mg/Kg, 400 mg/Kg and 600 mg/Kg). This treatment was carried out orally for 21 days) [19].

After 21 days of treatment, mice were sacrificed. Blood samples were collected in tubes containing an anticoagulant; a volume of blood was used directly for testing the anti hemolytic activity, the rest was centrifuged at 3000 g at 4ºC for 15 min. Livers and kidneys were removed, washed and homogenized in ice-cold KCl (1.15%) with a ratio of 1/10, and then centrifuged at 5000 rpm for 15 min at 4°C, the resulting supernatant was used for the determination of catalase activity, malondialdehyde (MDA) and glutathione contents in organs.

2.7.1 Anti-hemolytic activity

Anti-hemolytic activity was carried according to Trabsa et al. [14] with slight modifications. Blood was diluted in PBS. Then the AAPH was added, and the mixtures were incubated at 37ºC. Kinetics of the gradual disappearance of erythrocytes were followed by the measurement of the decrease in the absorbance at 630 nm. The resistance of erythrocytes to the radical attack was expressed as the time required for the lysis of 50% of erythrocytes.

2.7.2 DPPH radical-scavenging activity of plasma

The appropriate volume of DPPH (0.004% in methanol) was added to plasma, after incubation for 30 min, the mixture was centrifuged and the absorbance of the supernatant was measured at 517 nm.

2.7.3 Reducing power assay

Phosphate buffer (0.2 M and pH 6.6) and potassium ferricyanide (1%) was added to the plasma. Mixtures were incubated at 50°C for 20 min, and then TCA (10%) was added, the mixtures were centrifuged (3000 rpm for 10 min).

Distilled water and ferric chloride (0.1%) were added to the supernatant, and the absorbance was measured at 700 nm.

2.7.4 Catalase activity

Catalase activity was determined according to Bouaiz et al. [15] with slight modifications. The reaction mixture was composed of organ homogenate and H_2O_2 19 mM in a phosphate buffer (50 mM, pH 7.4). The decrease in H_2O_2 was measured spectrophotometrically at 240 nm for 30 seconds at room temperature. The molar extinction coefficient of H_2O_2 is 43.4 $M^{-1}.cm^{-1}$ and catalase activity was expressed as UI/mg protein.

2.7.5 Anti-lipid peroxidation activity

Anti-lipid peroxidation activity was determined using the method described by Zerargui et al. [17]. Since MDA is an end-product of oxidation of the polyunsaturated fatty acids by ROS. The principle of this assay depends on the reaction of MDA with thiobarbituric acid (TBA) and forming a pink colored MDA-TBA complex. Briefly, 500 µl of TCA (20%) and 1 ml of TBA (0.67%) were added to 500 µl of the homogenate. Mixtures were incubated at 100ºC for 15 minutes and cooled. 4 ml of n-butanol were added, followed by a centrifugation at 3000 rpm for 15 minutes. The absorbance of the supernatant was registered at 530 nm. The molar extinction coefficient of MDA is 1.56 $.10^5$ $M^{-1}.cm^{-1}$ and concentration of MDA was expressed as nmol/g of tissue tested.

2.7.6 Determination of glutathione content

Total glutathione was determined according to Bentahar et al. [20]. In this test, 5,5'- Dithio-bis-(2-nitrobenzoic acid) (DTNB) is used. DTNB oxidizes GSH, forming a colored compound called 5-thio-2-nitrobenzoic acid (TNB) with (ε_{412}=13600 $M^{-1}.cm^{-1}$). The reaction mixture was composed of the homogenate (diluted in phosphate buffer; 0.1 M and pH 8.0) and DTNB. After 5 minutes of incubation, the absorbance was measured at 412 nm. Blank was prepared by replacing the homogenate by the buffer. Concentration of GSH was expressed as µmol/g of tissue.

2.7.7 Determination of protein concentration in homogenates

Protein content of the homogenates was estimated according to Gornall et al. (1949) [21] using the Biuret reagent and bovine serum as a standard. The reaction mixture was composed of 1 ml of biuret reagent and 25 µl of the homogenate or the standard. 10 min later, the absorbance was measured at 540 nm.

2.8 Statistical Analysis

The results were expressed as mean ± SD. All comparisons were performed by the analysis of variance (ANOVA) followed by Tukey's test. All calculations were performed using Graph Pad software version 5.0. $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ were considered as indicative of significance, as compared to control groups.

3. RESULTS AND DISCUSSION

3.1 Extraction and Determination of Total Polyphenols and Flavonoids Content

The different groups of compounds which compose the crude extract were separated using solvents with increased polarity as described by Markham [11]. This allows access to lipids (using hexane) in the first step followed by aglycon flavonoids (using chloroform) then mono- and di-glycosylated flavonoids (using ethyl acetate) and finally tri- and tetra- glycosylated flavonoids and other hydrophilic molecules (in the aqueous phase). The solubility of phenolic compounds depends on the solvents used and the degree of polymerization of the phenols and the interaction of polyphenols with other compounds. For this reason, it is recommended to use methanol to extract phenols [22].

Extraction was performed using pure methanol 98%, it allows the extraction of phenols and flavonoids. To increase the extraction yield, maceration was carried out during 7 days and repeated twice. Yields of extraction are shown in Table 1. Yield of extraction was 21% for crud extract (CrE) of leaves. Concerning the fractionation, chloroform extract (ChE) and aqueous extract (AqE) have approximately the same yield of extraction (8.44 and 8.14% respectively) and ethyl acetate extract (EAE) had the lowest yield (1.09%).

The content of total polyphenols in the extracts of leaves of *X. strumarium* was higher in the EAE (166.26 ± 27.98 mg EGA/g E), followed by the CrE (85.77 ± 4.98 mg EGA/g E), the AqE (75.24 ± 13.31 mg EGA/g E) and the ChE (70.58 ± 3.3

mg EGA/g E). As for the content of flavonoids, EAE contains the highest value (29.037 ± 3.14 mg EQ/ g E), followed by the ChE (17.26 ± 2.75 mg EQ/ g E), then the CrE (11.76 ± 1.39 mg EQ/ g E) and finally the AqE (10.6 ± 1.615 mg EQ/ g E).

The yield of extraction of the crude extract of *X. strumarium* was higher than the one found by Scherer and Godoy [23]. On the other hand, the content of phenolics compounds was very higher than that cited by Nair and Sheela [24]. But, according to the same authors, the content of flavonoids was lower. This may be due to the method of estimation used.

3.2 *In vitro* antioxidant Activities

Antioxidant activity should not be concluded based on a single antioxidant test model. In practice, several in vitro test procedures are carried out for evaluating antioxidant activities with the simple of interest [25]. In this way, different tests were applied.

Results of the *in vitro* antioxidant activities and scavenging effects were summarized in Table 2.

3.2.1 DPPH radical scavenging assay

The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. It is a simple and rapid test, thus it is widely used to investigate the scavenging activities of different extracts. After the reaction, color of the mixture changed from purple to yellow [26]. Based on the results, EAE, AqE and CrE exhibit free radical scavenging abilities and can be considered as primary antioxidants.

Results of DPPH scavenging activity are represented in Fig. 1. EAE was characterized by the lower IC_{50} (0.017 ± 0.0004 mg/ml), followed by AqE (0.046 ± 0.0006 mg/ml) then CrE (0.084 ± 0.0004 mg/ml) ($P < 0.001$) and finally ChE (0.234 ± 0.017 mg/ml). IC_{50} of BHT was 0.087 ± 0.001 mg/ml. CrE and BHT have nearly the same IC_{50} (Table 2). The IC_{50} of the crude extract was nearly the same found by Kamboj and Saluja [7].

Table 1. Yields of extraction of *X. strumarium* and total phenolics and flavonoids content in the different extracts

Extracts	Yield %	Total phenolics°	Flavonoïds°°
CrE	21	85.77 ± 4.98	11.76 ± 1.39
ChE	8.44 ± 1.96	70.58 ± 3.3	17.26 ± 2.75
EAE	1.09 ± 0.43	166.26 ± 27.98	29.037 ± 3.14
AqE	8.14 ± 1.28	75.24 ± 13.31	10.6 ± 1.615

°: equivalent of milligrams of gallic acid per gram of extract weight (mg EGA/g E).
°°: equivalent of milligrams of quercetin per gram of extract weight (mg EQ/ g E).
Values were expressed as means ± SD (n = 3).

Table 2. Antioxidant activities; scavenging of DPPH, β-carotene bleaching, reducing power and anti hemolytic activity

Extracts	DPPH[1] (mg/ml)	β-Carotène[2] AA%	Reducing power[3] EC_{50}(mg/ml)	HT_{50}[4] (min)
CrE	0.084± .0003^{ns}	76.56± 4.2***	0.059 ± 0.001***	83.89 ± 4.82***
ChE	0.234 ± 0.017***	22.06±0.47***	0.120 ± .0045***	99.96 ± 3.31***
EAE	0.017 ± 0.0004***	71.66±2.89***	0.017 ± 0.00014^{ns}	98.73 ± 5.99***
AqE	0.046 ± 0.0006***	58.82±2.88***	0.036 ± 0.0016***	74.38 ± 2.33**
BHT	0.087 ± 0.001	98.89±1.37	-	-
Ascorbic acid	-	-	0.0149 ± 0.0004	81.91 ± 4.71***
Positif Control	-	-	-	57. ± 3.4

*[1] Comparison with BHT, ***: P ≤ 0.001. [2] Comparison with BHT, ***: P ≤ 0.001. [3] Comparison with Ascorbic acid, ***: P ≤ 0.001.[4] Comparison with Positive Control (contains RBCs and AAPH), ***: P ≤ 0.05. ns: not significant.*
Values were expressed as means ± SD (n = 3).

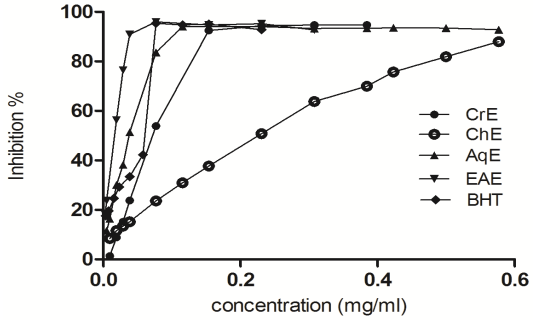

Fig. 1. DPPH scavenging activity of *X. strumarium* extracts and BHT. Values were expressed as the mean of triplicate.

3.2.2 β-carotene bleaching assay

In the β-carotene / acid system, free linoleic acid radical formed upon the abstraction of a hydrogen atom linoleic from one of its diallylic methylene groups attacked the β-carotene molecule, which lost the double bonds and therefore its characteristic orange colour [27].

The antioxidant activity was determined by using a β-carotene /linoleic acid system; results are represented in Fig. 2. In our study, CrE had the highest antioxidant activity (76.66%) followed by EAE (71.66%) then AqE (58.82%) and finally ChE (22.06%) ($P \leq 0.001$). CrE and EAE extracts have nearly the same antioxidant activity, and BHT had the stronger antioxidant activity.

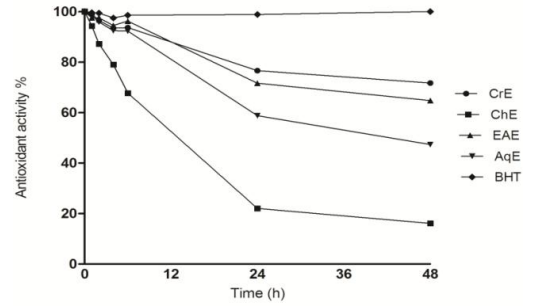

Fig. 2. Kinetic of antioxidant activity of *X. strumarium* extracts and BHT during 48 hours, using β-carotene/linoleic acid bleaching assay. Values are expressed as means of triplicate.

3.2.3 Reducing power assay

The reducing power is associated with antioxidant activity. It may serve as a significant indicator of antioxidant activity by electron donating ability [28]. Reducing power is apparently dependent on the hydrophilic characteristic of the extract.

Results are represented in Fig. 3. EAE showed the lower EC_{50} (0.017 ± 0.00015 mg/ml), followed by AqE (0.036 ± 0.0016 mg/ml) then CrE (0.059 ± 0.001 mg/ml) and finally ChE (0.12 ± 0.0045 mg/ml) (Tab. 2). Ascorbic acid which was used as an antioxidant standard had an EC_{50} of 0.015 ± 0.0004 mg/ml. Reducing power of all extracts was lower than that of ascorbic acid ($P \leq 0.001$). The EC_{50} of the crude extract was very lower than that found by Kamboj and Saluja [7].

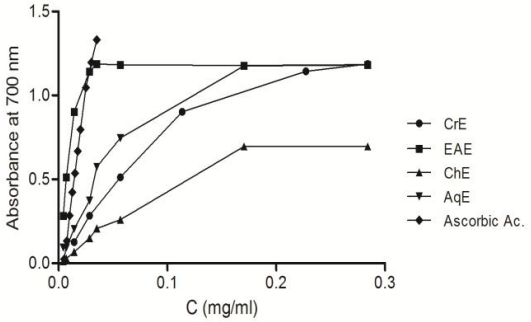

Fig. 3. Reducing power of *X. strumarium* extracts and ascorbic acid. Values were expressed as the mean of triplicate

3.2.4 Anti-hemolytic activity

Erythrocytes are often used as biological model to investigate oxidative damage in biomembranes because of their high vulnerability to peroxidation, since their membranes are rich in polyunsaturated fatty acids, and they have high concentrations of oxygen and hemoglobin which promote the oxidative process [29]. The different extracts tested protected effectively the erythrocytes membranes.

Resistance of erythrocytes treated by the extracts of *X. strumarium* and radical attack by the AAPH, were appraised and results are represented in Fig. 4. The hemolytic activity of AAPH-generated $ROO^{\cdot}$ was followed continuously at 620 nm to verify changes in the turbidity.

Positive control had an HT_{50} of 57.51 min. All extracts were more effective than positive control and ascorbic acid. ChE had the higher HT_{50} (99.96 min), followed by EAE (98.73 min)

then CrE (83.89 min) and finally the AqE (74.38 min). Ascorbic acid which was used as an antioxidant standard had an HT_{50} of 81.91 min (Table 2).

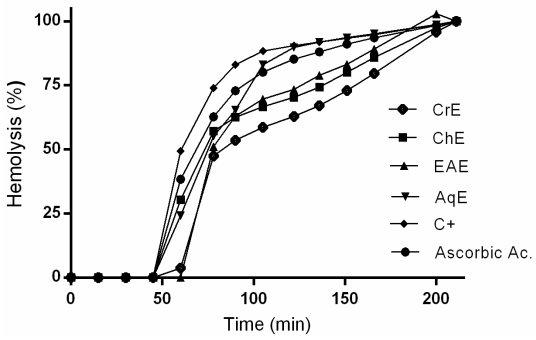

Fig. 4. Time course of the effect of *X. strumarium* extracts and ascorbic acid on AAPH induced-hemolysis in RBCs. C^+ contains RBCs and AAPH. Values are expressed as the mean of triplicates

For the *in vitro* antioxidant assays applied, EAE had the stronger effect in scavenging of DPPH, β-carotene bleaching test and reducing power. Except in the hemolytic assay, ChE had the stronger effect. These differences were due to the chemical composition of each extract. EAE is very rich in both polyphenols and flavonoids. Flavonoids possess powerful antioxidant characteristics [30].

3.3 Acute Toxicity of Crude Extract

Acute toxicity study was performed according to OECD [18] (Organisation for Economic Co-operation and development) guidelines 425. Doses 2000 mg/Kg and 5000 mg/Kg were found to be safe for animals. No mortality or toxic symptoms were observed during the duration of the study. This is in concordance with the literature which mentioned that adult leaves of *X. strumarium* are not toxic [23].

3.4 *In vivo* Antioxidant Activities of Crude Extract

It is important to appraise the total antioxidant capacity in the plasma after treatment with leaves extract since the non-enzymatic antioxidant defenses also play a major role against oxidative stress [31]. The doses of the crude extract of *X. strumarium* increased the DPPH scavenging capacity of the plasma, but in the case of reducing capacities of the plasma, only the higher dose (600 mg/kg) was effective.

Plasma of animals treated with the three concentrations of *X. strumarium* extract has a scavenger effect on DPPH with a significant difference ($P \leq 0.01$) compared with the negative and positive controls. There is no significant difference between control group and positive control ($P > 0.05$).

Concerning the reducing capacities of the concentrations tested, results indicated that there is no significant difference compared with negative and positive controls. There was also no significant difference between the highest dose of extract (600 mg/Kg) and positive control.

The three concentrations of *X. strumarium* extract did not protect cells from hemolysis, since there was no significant difference between the control group and the treated groups ($P > 0.05$). However positive control had a significant effect ($P \leq 0.05$) compared with control group. Results of the *in vivo* antioxidant activities of the plasma and total bloods are summarized in Table 3.

Catalase and GSH are important in the inhibition of free radical formation and usually used with MDA as biomarkers to indicate ROS production [32]. The enhanced activities of antioxidant enzymes may provide an effective defense against the damaging effects of free radicals [33].

Table 3. *In vivo* effects of different concentrations of leaves of *X. strumarium* extract on plasma antioxidant activities; scavenging DPPH, reducing power and anti hemolytic effect

Treatment	DPPH (%)	Reducing power (Abs at 700 nm)	HT_{50} (min)
C group	4.88 ± 0.48	0.18 ± 0.027	104.48 ± 1.056
PC 100 mg/Kg	4.075 ± 0.38^{ns}	0.25 ± 0.045^{ns}	127.017 ± 8.83^{ns}
200 mg/Kg	$11.73 \pm 0.47^{***}$	0.16 ± 0.005^{ns}	102.68 ± 4.15^{ns}
400 mg/Kg	$13.067 \pm 0.43^{***}$	0.21 ± 0.046^{ns}	104.92 ± 3.84^{ns}
600 mg/Kg	$13.24 \pm 0.59^{***}$	$0.24 \pm 0.035^{*}$	96.15 ± 3.013^{ns}

C group: untreated group; PC: Positive Control group (group treated with ascorbic acid. Comparison was with C group (P ≤ 0.05). ns: not significant. Data were expressed as means ± SEM; (n = 6-8).

Table 4. *In vivo* effects of different concentrations of *X. strumarium* leaves extract on liver and kidney catalase activity, MDA and GSH contents

Treatment	Liver			Kidney		
	CAT (µmole/min /mg protein)	MDA (nmole/g tissue)	GSH (µmole/g studied tissue)	CAT (µmole/min /mg protein)	MDA (nmole/g tissue)	GSH (µmol/g tissue)
C group	15.38 ± 2.59	189.67 ± 6.8	16.22 ± 0.90	21.45 ± 1.98	170.33 ± 9.45	12.52 ± 0.76
$PC_{100\ mg/Kg}$	26.35 ± 3.5 ^{ns}	138.35 ± 22.22 ^{ns}	16.48 ± 0.62 ^{ns}	35.67 ± 7.22 ^{ns}	164.9 ± 16.51^{ns}	13.26 ± 0.79 ^{ns}
200 mg/Kg	8.45 ± 1.85^{ns}	205.38 ± 17.97^{ns}	15.40 ± 0.454^{ns}	19.82 ± 3.81^{ns}	135.6 ± 10.6^{ns}	12.21 ± 0.54^{ns}
400 mg/Kg	16.35 ± 3.33^{ns}	150.63 ± 22.86 ^{ns}	15.006 ±0.575^{ns}	20.31 ± 3.372^{ns}	134.97 ± 6.616^{ns}	14.332 ±0.79^{ns}
600 mg/Kg	24.34 ± 5.06 ^{ns}	112.5 ± 17.007 ^{ns}	16.68 ± 0.895^{ns}	27.75 ± 3.29^{ns}	113.28 ± 8.39*	14.91 ± 0.99^{ns}

C group : untreated group ; PC: Positive Control group (group treated with ascorbic acid. Comparison was with C group (P ≤ 0.05). ns: not significant. Data were expressed as means ± SEM; (n = 6-8)

Catalase directly converts H_2O_2 into oxygen and water, playing an important role in H_2O_2 metabolism [2]. By this way, catalase is an important enzyme as an antioxidant enzyme. In this study, catalase activity was determined in liver and kidney.

Lipid peroxidation is the process that involves the chain reaction of free radicals with polyunsaturated fatty acid. These reactions lead to hydroperoxide generation and lipid breakdown into lower molecular weight fragments such as ketones, alcohol, hydrocarbon, acids and epoxides. Therefore, inhibition of lipid peroxidation is of great importance in disease process involving free radicals [34]. As MDA is a naturally occurring product of lipid peroxidation, the level of MDA is measured as a biomarker of lipid peroxidation [35] and must decrease in presence of strong antioxidants.

Glutathione is a cysteine-containing peptide found in most forms of aerobic life. It is a strong reducing agent owing to the presence of -SH group in its cysteine. On the other hand, GSH is used (as a co-substrat) by peroxidase family of enzymes like GPx to scavenge H_2O_2 and form water [36].

Results of catalase activity, MDA and glutathione contents in organs are represented in Table 4.

Results of this study indicate that there is no significant difference in catalase activity in liver and kidney for the three doses tested compared to control group ($P > 0.05$), and there is no significant difference between positive control (ascorbic acid 100 mg/Kg) and the higher dose of the extract (600 mg/Kg) ($P > 0.05$).

According to the results obtained, there was a correlation between the MDA content decrease in both liver's and kidney's homogenates and the increase of doses of *X. strumarium* extract administered.

Results of this study indicate that there is no significant difference in GHS content in liver and kidney for the three doses tested compared to control group ($P \leq 0.05$).

Among these tests, *X. strumarium* crude extract showed a potent inhibition of lipid peroxidation.

5. CONCLUSION

In vitro antioxidant activities of different fractions of the crude *X. strumarium* leaves extracts showed strong scavenging activity toward DPPH, a significant protection of β-carotene from bleaching and a potent reducing power. *In vivo* assays demonstrated that the crude extracts concentrations of 400 mg/Kg and 600 mg/Kg exhibited catalase activity similar to that of ascorbic acid and provoked a decrease in the concentration of MDA, and increase of GSH. These results suggest that these plant fractions had potent antioxidant activities and they could be used where these activities are warranted.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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