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Study II on *Chrozophora oblongifolia* Aerial Parts: Assessment of Antioxidant Activity, Hepatoprotective Activity and Effect on Hypothalamic-gonadal Axis in Adult Male Rats

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ABSTRACT

Objectives: The present study aimed at the investigation of antioxidant activity, hepatoprotective activity in addition to the effect on hypothalamic-gonadal axis in adult male rat of total methanolic extract and different fractions of *Chrozophora oblongifolia* aerial parts. **Methods:** Antioxidant activity was evaluated by DPPH spectrophotometric method, Hepatoprotective activity was evaluated using CCl_4 induced liver damage method by measuring serum marker enzymes, SGOT, SGPT and total bilirubin. The effect on hypothalamic-gonadal axis in adult male rats was estimated by measuring serum level of testosterone, follicle stimulating hormone and leutinizing hormone by immune enzymatic assay kit using ELISA reader. **Results:** The methanol fraction showed highest antioxidant activity followed by total extract and ethyl acetate fraction concentrate, while the methylene chloride and *n*-hexane fractions showed lowest antioxidant activity. The total methanolic extract in addition to ethyl acetate and methanol fractions exhibited significant decrease in SGOT and SGPT enzymes and total bilirubin, indicating their effectiveness in protecting the liver normal functional status. The total methanolic extract of aerial and fruit parts, each showed marked increase in serum level of total testosterone, follicle stimulating hormone and leutinizing hormone, when administered orally for 28 successive days compared to control group. **Conclusion:** Total methanol extract of the aerial parts of *Chrozophora oblongifolia* has significant antioxidant and hepatoprotective activities. Also extract of aerial parts including fruits has increased rat serum level of gonads, suggesting *Chrozophora oblongifolia*, as a valuable biological source of drugs enhances fertility.

Keywords: *Antioxidant; Chrozophora oblongifolia; Fertility; Hepatoprotective activity*

INTRODUCTION

Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) are constantly produced during normal cellular metabolism or by other exogenous means including the metabolism of environmental toxins or carcinogens, by ionizing radiation and by phagocytic cells involved in the inflammatory response. When the cellular concentration of oxidant species is increased to an extent that exceed the endogenous antioxidant defense system, oxidative stress occurs, leading to lipid, protein, and DNA damage. In addition, ROS, particularly H_2O_2, are potent regulators of cell replication and play an important role in signal transduction, thus, oxidative damage is considered the main factor contributing to carcinogenesis and evolution of cancer[1]. Due to their ability to scavenge and reduce the production of free radicals and act as transition metal chelators; natural phenolic compounds can exert a major chemopreventive activity[2]. Liver is involved with almost all the biochemical pathways of growth, fight against disease, nutrient supply, energy

provision and reproduction The major functions of the liver are carbohydrate, protein and fat metabolism, detoxification, secretion of bile and storage of vitamin. Thus, to maintain a healthy liver is a crucial factor for overall health and well being. But it is continuously and variedly exposed to environmental toxins and abused by poor drug habits, alcohol and prescribed and over-the-counter drug, which can eventually lead to various liver ailment like hepatitis, fibrosis, cirrhosis, fatty liver and others[3]. The importance of fertility and procreation as a factor for survival of the human race is a crucial factor. On reviewing available literature, the use of herbs has a long history in for fertility regulation. Nowadays the effect of plant-derived chemicals on the endocrine system and the activity of sexual organs have induced a great interest[4]. And since the fruit part of *Chrozophora oblongifolia* are used by natives living in Saint-Catherin (Sainai, Egypt, were the plant was collected), to increase sexual activity in goat and sheep males, it was encouraging to undertake this present study, as previous phytochemical and biological study of *Chrozophora oblongifolia* aerial parts, has led to the isolation of five compounds *viz*; are lupeol, 1-octacosanol, 4-hydroxybenzoic acid, methyl gallate and amentoflavone, it also showed significant antiviral and wound healing activities[5].

MATERIALS AND METHODS

Plant materials

The aerial parts of *Chrozophora oblongifolia* were collected in March 2013 from Saint Catherin, South Siniai, Egypt. The plant was identified and authenticated by Prof. Dr. Salah El-Naggar Prof. of Botany and Plant Taxonomy, Faculty of Science Assuit University, Assuit, Egypt. A voucher specimen (COE-1) was kept in the Department of Pharmacognosy, Fculty of Pharmacy, Al-Azhar University, Assuit, Egypt.

Instruments

Spectroscopic data were measured using UV-visible spectrophotometer (Schimadzu, UV 240, Kyoto, Japan). ELISA micro plate reader, spectrophotometer (Tecan Group Limited.-Sunrise, Crailsheim**,** Germany) was used for estimation of hypothalamic-gonadal hormones according to assay kit protocol (GenWay Biotech Inc. San Diego, CA, USA).

Chemicals

2, 2-Diphenyl-1-picryl hydrazyl (DPPH) (obtained from Sigma-Aldrich Chemicals Co. Germany). Ascorbic acid and quercetin as an antioxidant standard (were obtained from Sigma-Aldrich Chemicals Co, Germany), and other chemicals used were of high analytical grade and obtained from Merck chemical Co, Germany and El-Nasr chemical Co, Egypt. NaCl (0.9%) obtained from (El-Nasr Pharmaceutical and Chemical Co., Egypt) (ADWIC). Sylimarin as standard hepatoprotective drug obtained from (Chemical Industries Development Co., Egypt). Tween 80 and CCl_4 as hepatotoxin obtained from El-Nasr Pharmaceutical and Chemical Co., Cairo, Egypt (ADWIC). Thiopental sodium injection (500mg) obtained from Egyptian International Pharmaceutical Industry Co Cairo, Egypt (EIPI Co).

Animals for hepatoprotective activity

The healthy adult Wistar albino rats (35 rat) weighing 250–250 g, were housed under standard environmental conditions of temperature and humidity (25±0.50 ◦C) and 12 h light/dark cycle) were utilized for the studies. The animals were fed with standard pellet diet and water. The rats were divided into seven groups (5 rats in each).

Animals for effect of hypothalamic-gonadal axis in adult male rats

The healthy adult male Wistar albino rats (15 rat) weighing 250–300 g, were housed under standard environmental conditions of temperature and humidity (25 ± 0.50°C) and 12 h light/dark cycle) were utilized for the studies. The animals were fed with standard pellet diet and water. The rats were divided into three groups (5 rats in each). The animal handled according to Ethical Guidelines of Animal House, Faculty of Medicine, Assuit University.

Methods

Extraction and fractionation

The air-dried powdered aerial parts (4 Kg) of *Chrozophora oblongifolia* were extracted by maceration in methanol (70%) till complete exhaustion [three times each 8 L, overnight]. The methanolic extracts were concentrated under reduced pressure till constant weight to give a dark brown syrupy residue (430g). A part of the methanolic extract (400 g) was subjected to successive solvent fractionation on VLC with *n*-hexane, methylene chloride (MC), ethyl acetate (EtOAC), methanol (MeOH) and finally with aqueous methanol till complete exhaustion in each case to give *n*-hexane (30 g), MC (5 g), EtOAC (30 g), MeOH (180 g) and aqueous methanol (70 g) sub fractions. Three hundred grams of fruit part of *Chrozophora oblongifolia* were extracted with methanol (70%) till complete exhaustion to yield 70 g of total methanolic extract concentrate.

Antioxidant activity

Antioxidant activity was measured by spectrophotometric method[6]; DPPH 10×10^{-5} M solution was prepared by dissolving 40 mg of DPPH in

1000 ml ethanol. In the assay 0.2 ml of ethanolic solution of different fractions of *Chrozophora oblongifolia* aerial parts of different concentrations (0.0625, 0.125, 0.25, 0.5, 1mg/ml) was mixed with 2 ml of ethanolic solution of DPPH (0.1mM). Similarly; 0.2ml ethanolic solution of ascorbic acid and quercetin of different concentrations (0.625, 0.125, 0.25, 0.5, 1mg/ml) were mixed with 2 ml of DPPH solution. A mixture of 0.2 ml of ethanol and 2 ml of ethanolic solution of DPPH (0.1mM) served as control. After mixing, all the solutions were incubated in dark for 30 min. Absorbance was measured at λ_{max} 517 nm. The experiments were carried out in triplicate manner using ascorbic acid and quercetin as a referance standards and antioxidant activity was calculated by using this formula[7]:

$$\% \text{ antioxidant activity} = \frac{\text{absorbance of control - absorbance of tested sample}}{\text{absorbance of control}}$$

Hepatoprotective activity

The tested samples (total methanolic extract, *n*-hexane, ethyl acetate and methanol fractions as well as silymarin was dissolved in distilled water with the aid of 2% tween-80 to obtain concentration of 400 mg/ ml of each tested sample.

Experimental design[8]

Rats were divided into 7 groups each of 5 animals. **Group 1** control (vehicle): received 1% *v/v* Tween-80 in distilled water (5 ml/kg body weight, p.o.) single daily dose for 7 consecutive days, **group 2** (CCl_4 induced): received CCl_4 in olive oil (1:1 *v/v*, 1.5 ml/kg body weight i.p.) single dose on the 6th day, **group 3** (standard): received standard drug Sylimarin (400 mg/kg body weight p.o.) single daily dose for 7 consecutive days, **group 4** (test 1): received total methanolic extract (400 mg/kg body weight, p.o.) single daily dose for 7 consecutive days, **group 5** (test 2): received *n*-hexane fraction (400 mg/kg body weight, p.o.) single daily dose for 7 consecutive days, **group 6** (test 3): received ethyl acetate fraction (400 mg/kg body weight, p.o.) single daily dose for 7 consecutive days and **group 7** (test 4): received methanol fraction (400 mg/kg body weight, p.o.) single daily dose for 7 consecutive days. Groups from 3 to 7 received CCl_4 in olive oil (1:1 *v/v*, 1.5 ml/kg body weight i.p.) single dose on the 6th day only in addition to their basic treatments.

Sample collection

All rats were sacrificed by cervical decapitation separately after 24 hrs of the last treatment. Blood samples were collected from each group into sterilized dry centrifuge tubes, followed by rotation at 3000 rpm for 10 minutes to obtain clear serum. Liver was excised immediately, washed with normal saline and preserved in 10% buffered neutral formalin solution for histopathological examination.

Estimation of biochemical markers

The clear serum obtained after centrifugation was used for the estimation of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and total bilirubin (TB). All the biochemical parameters were measured spectrophotometrically.

Estimation of AST and ALT

It based on the reaction of ketoglutrate with aspartate in case of AST (=SGOT) to form glutamate and oxaloacetate. In case of ALT (=SGPT) it is based on the reaction of ketoglutrate with alanine to form glutamate and pyruvate. The formed ketone (pyruvate or oxaloacetate) reacts with 2, 4–dinitro phenyl hydrazine hydrochloride in alkaline solution to give colored complex, that determined spectrophotometrically at λ_{max} 505 *nm*[9].

Estimation of total bilirubin

It is based on that, the serum bilirubin reacts with diazotized sulphanilic acid to give a purple azo-bilirubin dye, which measured colorimetrically at 540 *nm*[10-11].

Effect on hypothalamic-gonadal axis in adult male rats

The tested samples (total methanolic extract of aerial parts and total methanolic extract of fruit was dissolved in distilled water with the aid of 2% tween-80 to obtain concentration of 200mg/ml of each tested sample.

Experimental design

The animal groups were treated according to the following procedure[12]. **Group 1** control (vehicle): Received 1% *v/v* Tween-80 in distilled water (5 ml/kg body weight, p.o.) single daily dose for 28 successive days, **group 2** (total methanolic extract of aerial parts): received 200 mg/kg body weight p.o.) single daily dose for 28 successive days and **group 3** (total methanolic extract of fruit): received 200 mg/kg body weight p.o.) single daily dose for 28 successive days.

Sample collection

All rats were anesthetized by administering thiopental sodium (50 mg /kg, i.p)[13] after 24 hrs of the last treatment. Blood samples were collected from each group by cardiac puncture into sterilized dry centrifuge tubes, followed by rotation at 3000 rpm for 10 min to obtain clear serum.

Table 1. Antioxidant activity of total extract and different fractions of *Chrozophora oblongifolia* aerial parts in comparison with standard antioxidants (Ascorbic acid and quercetin)

Fraction	Concentration (mg/ml)				
	1	0.5	0.25	0.125	0.062
	Antioxidant activity				
Ascorbic acid	93.70±0.1%	92.70±0.03%	91.70±0.03%	90.50±0.03%	88.90±0.06%
Quercetin	91.20±0.03%	89.60±0.08%	89.20±0.1%	87.40±0.03%	86.10±0.06%
Total extract	88.00±0.08%	86.10±0.10%	85.70±0.08%	64.80±0.10%	45.00±0.05%
***n*-hexane Fraction**	49.00±0.10%	48.40±0.10%	19.40±0.06%	12.70±0.06%	10.30±0.10%
Methylene chloride Fraction	60.60±0.06%	52.40±0.03%	42.70±0.03%	27.00±0.07%	19.40±0.03%
Ethyl acetate Fraction	70.00±0.03%	68.00±0.10%	64.40±0.06%	62.40±0.08%	60.60±0.5%
Methanol Fraction	90.00±0.03%	88.40±0.03%	84.80±0.1%	76.00±0.06%	64.60±0.08%

Values are expressed as mean ± SE M; n = 3

Estimation of serum level of total testosterone, FSH and LH

Serum concentration of total testosterone, luteinizing and follicle stimulating hormones was measured following an immune-enzymatic method with an ELISA reader, according to the standard protocol given in assay kit.

Statistical Analysis

Experimental results are expressed as mean ± standard error. Results were statistically analyzed using analysis of variance (one-way ANOVA) followed by Tukey's t test for comparison between different groups. SPSS 20 version was used for the statistical analysis.

RESULTS AND DISCUSSION

Antioxidant activity

The obtained results (**Table 1**) indicated that, the methanol fraction showed the highest antioxidant activity followed by total extract and ethyl acetate fraction. The methylene chloride and *n*-hexane fractions showed the lowest antioxidant activity. The highest antioxidant activity of methanol, total extract and ethyl acetate fractions could be attributed to presence of polyphenolic compounds in these fractions. The major polyphenolics responsible for free radical scavenging activity are flavonoids. The activities are closely related to the chemical structure of their aglycone moieties and also the position of hydroxyl groups. The antioxidant activity of the aglycone is more potent than their corresponding glycosides that are in good agreement with the published data[14-16]. The presence of ortho-dihydroxylation of the B-ring of the flavonoid molecule, C2-C3 double bond and 4-oxo group of the ring C in addition to the presence of both 3-and 5-hydroxyl moiety of the rings C and A, play an important role in radical scavenging activity of the flavonoids[14,16,17]

Hepatoprotective activity

Carbon tetrachloride is one of the most commonly used hepatotoxins in the experimental study of liver diseases. The hepatotoxic effects of CCl_4 are largely due to its active metabolite, trichloromethyl radical[18]. These activated radicals bind covalently to the macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides. This lipid peroxidative degradation of biomembranes is one of the principle causes of hepatotoxicity of CCl_4[19]. This is evidenced by an elevation in the serum marker enzymes namely SGOT, SGPT, ALP, total bilirubin and decrease in protein. The diagnosis of organ disease/damage is aided by measurement of a number of non-functional plasma enzymes characteristic of that tissue or organ. The amount of enzyme released depends on the degree of cellular damage, the intracellular concentration of the enzymes and the mass of affected tissue. The concentration of the enzyme released reflects the severity of the damage. SGOT and SGPT are enzymes normally present in the liver, heart, muscles and blood cells. They are basically located within hepatocytes.

Table 2. Effect of the total methanolic extract and fractions of *Chrozophora oblongifolia* aerial parts on AST (SGOT), ALT (SGPT) and total bilirubin on CCl_4 treated rats

Animal group	Concentration mg/kg	SGOT (IU/L)	SGPT (IU/L)	Total bilirubin mg/dL
Control (vehicle)	----	69.60±.1.26	57.70±1.54	0.46±0.01
CCl_4 treated	----	3260±2.12	302.30±1.46	1.03±0.022
Sylimarin	100	165.9±1.42	155.50±1.42	0.60±0.03
Total extract	400	234.3±2.19	211.20±1.67	0.78±0.02
***n*-hexane fraction**	400	301.5±0.94	292.80±1.48	0.85±0.01
Ethyl acetate fraction.	400	201.9±0.81	194.20±0.62	0.72±0.01
Methanol fraction	400	176.9±1.39	160.40±1.79	0.58±0.00

Values are mean ± SE M; number of rats used in each group= 5

Table 3. Effect of total methanolic extracts of aerial parts and fruit part of *Chrozophora oblongifolia* on serum level of androgens in adult male rats

	Testosterone(ng/ml)	FSH (MIU/ml)	LH (MIU/ml)
Control group	0.418±0.023	0.112±0.02	0.706±0.016
Group 1	2.06±0.078	0.408±0.016	1.324±0.020
Group 2	1.79±0.025	0.452±0.026	1.25±0.018

Values are expressed as mean ± SE M; n = 5
Group: group 1 treated with total methanolic extract of aerial parts.
Group: group 2 treated with total methanolic extract of fruit.

So, when liver cells are damaged or dies transaminases are released into blood stream, where they can be measured and considered as an index of liver injury[19].

The obtained results (**Table 2**) showed that; the total methanolic extract in addition to ethyl acetate and methanol fractions of *Chrozophora oblongifolia* aerial parts exhibited significant decrease in SGOT and SGPT enzymes and total bilirubin in comparison with CCl_4 treated group. Decrease in serum enzymes and bilirubin after treatment with the total methanolic extract and some fractions in liver damage induced by CCl_4, indicated the effectiveness of the total extract and fractions in normal functional status of the liver. The obtained activity of total methanolic extract, ethyl acetate and methanol fractions may be attributed to presence of phytoconstituents as tannins and flavonoids in the total extract and these fractions[20]. The lower hepatoprotective activity of *n*-hexane fraction may be attributed to lack of phytoconstituents as flavonoids and tannins.

Effect on hypothalamic-gonadal axis in adult male rats

Hypogonadism is a clinical condition in which low level of serum sex hormones, including testosterone in males, as well as estradiol and progesterone in females, is found in association with specific signs and symptoms. These signs and symptoms may include diminished libido and sense of vitality, erectile dysfunction, dysmenorrhea, reduced muscle mass and bone density, depression and anemia. By restoring these serum sex hormones level to the normal range using such agents as hormone supplement therapy, many of these symptoms can be relieved[21].

Administration of total methanolic extract of aerial parts and total methanolic extract of fruits of *Chrozophora oblongifolia* in dose of 200 mg/kg body weight to male Wistar albino rats for 28 successive days showed marked increase in serum level of total testosterone, FSH and LH in comparison with control group (**Table 3**).

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are called gonadotrophins because they stimulate the gonads, the testes in males. In the testes, LH binds to receptors on Leydig cells, stimulating synthesis and secretion of testosterone. The increase in the concentration of LH significantly stimulates the synthesis and release of high levels of testosterone in blood. This leads to assume that some phytoconstituent present in the methanolic extract of both aerial parts and fruits may possibly mimic the function of LH to stimulate interstitial cells[12].

Sexual desire may be enhanced directly by increasing serum testosterone level or by having testosterone like effect. Luteinizing hormone (LH) and Follicle Stimulating Hormone (FSH) produced by anterior pituitary lobe are necessary for maintaining testosterone levels such that as LH and FSH increases so do the testosterone. Medicinal plants claimed to have aphrodisiac potential apart from being able to increase the concentration of free testosterone should also cause increase in the concentrations of serum LH and FSH. The saponins present in the plant extracts might have assisted in stimulating an increase in the body natural endogenous testosterone levels by raising the levels of LH. Studies have implicated the saponins components of plants in enhancing aphrodisiac properties due to its androgen increasing property[22].

CONCLUSION

The methanol fraction of *Chrozophora oblongifolia* aerial parts showed the highest antioxidant activity followed by total extract then ethyl acetate and *n*-hexane fractions showed the lowest antioxidant activity. The total methanolic extract in addition to ethyl acetate and methanol fractions of aerial parts exhibited significant decrease in serum level of SGOT and SGPT enzymes and total bilirubin in comparison with CCl_4 treated group and indicating that these fractions exhibit great degree of hepatoprotective activity. Also the total methanolic extract of aerial parts and fruit part showed marked increase in serum level of gonads in its stimulating hormones from pituitary gland in adult male rats suggesting *Chrozophora oblongifolia*, as a valuable biological source of drugs enhances fertility.

Conflict of Interest

The authors declare that they don't have any conflict of interest.

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