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RESEARCH ARTICLE

Protective effect of aqueous extract of *Anacyclus pyrethrum* root on atrazine-induced male reproductive disorders in rats.

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Abstract

The current study was designed to evaluate the protective effect of the aqueous extract of *Anacyclus pyrethrum* (AP) root on reproductive system disorders induced by atrazine (ATZ) in male rats. Male Wister albino rats were divided into 5 groups: control, corn oil, AP root aqueous extract (150 mg/kg bw), ATZ (200 mg /kg bw) and AP plus ATZ groups. Obtained results indicated that administration of ATZ to male rats produced significant decreases in the body weight gain, relative testis and epididymis weights and sperm count as well as serum concentrations of dihydroepiandrosterone (DHEA), testosterone (T), follicle stimulating hormone (FSH), luteinizing hormone (LH), high density lipoprotein-cholesterol (HDL-C), and testicular levels of alkaline phosphatase (ALP), acid phosphatase (ACP), gamma glutamyl transferase (γ-GT), total antioxidant capacity (TAC), reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) besides serum and testis total proteins (TP). On contrary, rats administered ATZ showed significant increases in the serum levels of ALP, ACP, γ-GT, low density lipoproteins-cholesterol (LDL-C) and serum and testis levels of total lipids (TL), total cholesterol (TC), total triglycerides (TG) and phospholipids (PL) and testicular contents of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), nitric oxide (NO), protein carbonyl (PC), percent of apoptotic cells and p53. However, treatment of ATZ-exposed male rats with water extract of AP roots ameliorated the reproductive adverse effects induced by ATZ in the tested parameters and returned most of them to almost the control levels. In conclusion, the present findings provided evidence that the aqueous extract of AP root can be used effectively in the protection against male sexual disorders resulted from herbicide ATZ exposure, mainly due to their bioactive constituents that are able to reduce oxidative stress and apoptosis, enhance the antioxidant status and improve the testicular function.

INTRODUCTION

For many years, it has been known that some pesticides can cause endocrine disruption in both human, and many wild and domestic animals. Such endocrine disruption has the potential to comprise proper development in organisms leading to reproductive, behavioral, immune system and neurological problems as well as the development of cancer **(Sanderson *et al*., 2000).** ATZ is an organophosphate herbicide which is widely used against long and broadleaf herbaceous plants in the fields of corn, sorghum, sugarcane and cotton, as well as gardens **(Seiler *et al*., 1992)**. ATZ is a common name for 2-chloro-4-(ethylamino)-4-(isopropylamino)-s-triazine, and has a chemical formula C8H14ClN5 **(Cai *et al*., 2003).** ATZ and its metabolites are widely persistent in the water and are mostly

found in the soil. So that, the prolonged use of ATZ, in addition to its persistence, could represent the risk of its retention in crops and soils. Moreover, this compound may pass from surface to the ground water causing contamination of natural water sources **(Mudiam *et al*., 2011).**

ATZ is an endocrine disruptor that demasculinizes and feminizes the gonads of male vertebrates by means of the reduction in androgen levels and the induction of estrogen synthesis **(Hayes *et al*., 2011).** A number of studies have suggested that ATZ might have adverse effects on the reproductive function **(Kniewald *et al*., 2000; Trentacoste *et al*., 2001 and Cooper *et al*., 2007).** It has been reported that ATZ has the ability to reduce the weights of pituitary, prostate and seminal vesicle in rats. It is also can modify the testicular functions as indicated by its lowering effect on spermatozoa count, viability and motility **(Kniewald *et al*., 2000 and Trentacoste *et al*., 2001)**. Moreover, ATZ can cause infertility in men living in agricultural areas **(Swan, 2006)**.

Recently, medicinal plants have attracted world-wide interest due to their bioactive constituents and their potential role for drug discovery **(Hamilton, 2004).** Many populations rely on medicinal plants because they are easily available at an inexpensive price and their phototherapeutic properties in treating variety of diseases **(Bellakhdar *et al*., 1991).** Pellitory (AP) is one of the most common herbal plants which have medicinal properties, particularly its root part. It has been reported that, AP root has a pungent efficacy which used in relieving toothache, promoting a free flow of saliva and making it popular as a food spice **(Kumar *et al*., 2012)**. AP root extract in traditional medicine has been widely used as an aphrodisiac and as a medicine for rejuvenation and vitality. So that, in male rats the administration of alkylamide-rich extracts of AP improved male sexual behaviors **(Sharma *et al*., 2009).** Experimental studies have also shown that substances found in herbs have androgenic effects **(Sharma *et al*., 2009)**, which play an important role in fertility and in the treatment of sexual dysfunction in both males and females **(Etuk and Muhammad, 2009)**.On the other hand, antioxidant activity of AP roots extract has been reported, and attributed to their bioactive phytochemical constituents such as phenol, flavonoids, alkaloids and tannins **(Sujith *et al*., 2011)**. Antioxidants have been reported to prevent oxidative tissue damage induced by free radicals and reactive oxygen species and thus may prevent a variety of diseases **(Daniel *et al*., 1998)**. Therefore, the present study was the first which was designed to determine whether water extract of AP root can protect against ATZ-induced reproductive disorders in male rats. The study included evaluation of levels of androgens, activity of testicular enzymes, and other testicular biomarkers.

2. MATERIALS AND METHODS

2.1. Chemicals:

The herbicide ATZ was purchased from Misr Company for Agriculture Chemicals, Egypt. AP was purchased from HARAZ Medical Planta, Cairo, Egypt. AP was approved from Botany Department, Faculty of Science, Mansoura University. Corn oil was purchased from local market at Mansoura city. Kits was purchased from Biodiognostic Company for Chemicals, Cairo, Egypt.

2.2. Preparation of AP root aqueous extract:

For the preparation of aqueous extract of AP root, roots were washed and dried at room temperature. Then, 100 g of dried roots were powdered and extracted by refluxing with distilled water for one hour. Thereafter, it was decanted and centrifuged for 30 min at 855 g. The supernatant was further filtered to eliminate any residues and the filtrate was dried in an oven at 40°C to make a powder. The solid residue was stored in desiccator prior use for subsequent experiment. At the time of experiment, the powder was weighed and dissolved in known volume of distilled water to determine the concentration of used dose, then kept in dark bottle **(Selles *et al*., 2012).**

2.3. Experimental animals:

This study was performed on male Wistar albino rats (*Rattus rattus*), weighing 160±10 gm. Rats were obtained from the company for biological product & vaccines, Cairo, Egypt. They were housed in stainless steel cages at a well ventilated animal house. Rats were fed on adequate diet and given water *ad libitum*. Care and use of the animals were conducted under supervision of the Animal Ethics Committee of Mansoura University, Egypt.

2.4. Experimental design:

After one week of adaptation, rats were randomly divided into five groups of six animals each as follows:

1. **Control group:** received no treatment.
2. **Corn oil group:** received (0.2 ml/kg bw) corn oil **(Dehkhargani *et al*., 2012)**.
3. **AP group:** received aqueous extract of AP roots at a dose level of 150 mg/kg bw **(Selles *et al*., 2012)**.
4. **ATZ group:** received ATZ dissolved in corn oil at a dose level of 200 mg/kg bw **(Dehkhargani *et al*., 2012)**.

5. **AP+ATZ group:** received the same used doses of AP and ATZ concomitantly.

All treatments involved corn oil, AP root extract and ATZ were given daily to the rats using the stomach tube for 60 days.

2.5. Blood and tissue sampling:

At the end of the treatment period, the rats were fasted over night, and then sacrificed under ether anesthesia. Blood samples were collected in clean dry centrifuge tubes and the sera were separated by centrifugation at 855 g for 10 minutes and then quickly frozen at -20°c for further biochemical analysis. After blood collection, the abdomen of slaughtered rats were immediately dissected by longitudinal incision and the two testes and epididymis were quickly removed and weighed. The right testis was used for biochemical estimations, while the left one was fixed in 10% neutral formalin for histopathological study. On the other hand, the epididymis was weighed and used to prepare solution for the sperm count.

2.6. Preparation of tissue homogenate and epididymal solution:

A portion of the right testis of each rat was weighed, homogenized in cold distilled water using teflon homogenizer, centrifuged for 10 min at 855 g and the resultant supernatants of all samples were stored at -20°c until analysis of selected biochemical parameters. Another portion from the right testis was weighed, homogenized with phosphate buffer solution (pH 7.4), centrifuged at 10,000 g for 20 minutes and the supernatant was separated for NO analysis **(Montgomery and Dymock, 1961).** The third portion of the testis was kept at -20°c for flow cytometric measurement**.**

Epididymal sperm count was done by squeezing the epididymis in 5 ml of normal saline solution. The final concentration of the epididymal fluid must contain appropriate amount of formalin to kill the sperms. Counting was done using haemocytometer and total number of sperm per gram of epididymis was then calculated according to method of **Adeeko and Dada (1998)**.

2.7. Measurement of biochemical parameters:

Body weight gains (%) in different animal groups were calculated from recorded body weights at the start and at the end of the experiment. Relative weights of both testis and epididymis were calculated as the ratio of organs weight to animal body weight (g/100g bw). Serum T and DHEA levels were estimated using kits supplied by Rock Diagnostics GmbH, D-68298 according to the methods of **Tietz (1992)** and **Longcope (1996)** respectively. Serum concentrations of FSH and LH were measured by radioimmunoassay following the method of **Picard *et al.* (2008).** Testis and serum activities of ALP, ACP and γ-GT were determined by the methods of **Belfield and Goldberg (1971), Kind and King (1954)** and **Szasz (1969)** respectively. The concentrations of TP, TL, TC, TG and PL were estimated following the procedures of **Gornal *et al.* (1949)**, **Zollner and Kirsch (1962)**, **Allain *et al.* (1974)**, **Fassati and prencipe (1982) and Connerty *et al.* (1961)** respectively. Level of serum HDL-C was evaluated by the method of **Lopez-Virella *et al.* (1977)**, while LDL-C level was calculated according to the following equation: LDL-C = TC – (HDL-C + TG/5) as described by **Ahmedi *et al.* (2008)**. Cardiac risk ratio (CRR) was assessed by the equation: CRR = TC/HDL-C, as described by **Malaspina (1981)**, while atherosclerosis index (AI) was calculated according to the equation: AI= LDL-C/HDL-C, as described by **Mertz (1980)**. The concentrations of MDA, H_2O_2 and NO in the testis was measured following the methods of **Ohkawa *et al.* (1979)**, **Fossati *et al.* (1980) and Montgomery and Dymock (1961)** respectively. Testicular contents of TAC and GSH were determined using the procedures of **Koracevic *et al.* (2001)** and **Beutler *et al.* (1963)** respectively. Activities of antioxidant enzymes SOD, GPx and GST in the testis were estimated by the methods of **Nishikimi *et al.* (1972)**, **Paglia and Valentine (1967) and Habig *et al.* (1974)** respectively, using kits supplied by Bio-diagnostic Company, Cairo, Egypt. The concentration of PC in the testis was measured as protein hydrazone derivatives, using 2,4-dinitrophenyle hydrazide **(Smith *et al.*, 1991).** The percentage of both apoptotic cells and p53 in the testis was evaluated by the flow cytometer according to the method describe by **Tribukait *et al.*, (1975).** The flow cytometer used in this work is flow activated cell sorter (FACS) calibur flow cytometer (Becton Dickinson, sunnyvale, CA, USA) equipped with a compact air cooked low power 15 mwatt Argon ion laser beam (488 nm). The instrument is placed in Children Hospital, Mansoura University, Mansoura, Egypt.

2.8. Statistical analysis:

All obtained data were represented as means ±SE. One way analysis of variance (One-way ANOVA) followed by Duncan test was used to evaluate the obtained results. The differences among means of investigated groups were considered statistically significant when *P* values equal or less than 0.05 **(Snedecor and Cochran, 1982)**.Values of significance have been denoted by distinct letters in the tables.

3. RESULTS

3.1. Effects of either corn oil or water extract of AP root on biochemical parameters:

Obtained results of different animal groups have been illustrated in tables (1, 2, 3 and 4). As shown in these tables, administration of corn oil (0.2ml/kg) or water extract of AP root (150 mg/kg) separately to the intact rats did not produce any significant changes in all the tested parameters in comparison with normal rats, indicating their non-toxic effects at the applied doses.

Table 1. Body weight gain%, relative testis and epididymis weights, sperm count, serum DHEA, T, FSH and LH hormones; and serum and testis enzymes ALP, ACP and γGT of different animal groups. Values are means ± SE of six animals for each group.

Parameters		Animal groups				
		control	Corn oil	AP	ATZ	AP+ATZ
Body weight gain%		52.7 ±1.26[a]	53.29±1.28[a] (+1.11)	55.88± 1.42[b] (+6.03)	46.67± 1.12[a] (-11.44)	53.32±1.28[a] (+1.18)
RTW(g/100g bw)		0.74±0.019 [a]	0.73 ±0.017 [a] (+2.82)	0.76 ±0.027 [a] (+7.04)	0.52 ±0.012 [b] (-26.76)	0.71±0.024 [a] (+4.23)
REW (g/100g bw)		0.27±0.007 [a]	0.26 ±0.007 [a] (-3.70)	0.27 ±0.008 [a] (0)	0.23±0.006 [b] (-14.81)	0.25±0.005 [c] (-7.41)
Sperm count (x10^4/g)		5.06±0.13 [a]	5.07±0.15 [a] (+0.20)	5.29±0.16 [a] (+4.55)	2.15±0.08 [b] (-57.51)	4.75±0.12 [a] (-6.13)
Serum	DHEA (ng/ml)	5.95±0.15 [a]	5.54±0.18 [a] (-6.89)	5.94±0.19 [a] (-0.17)	3.57±0.10 [b] (-40)	5.61±0.14 [a] (-5.71)
	T (ng/ml)	5.07±0.14 [a]	4.87±0.18 [a] (-3.94)	5.27±0.20 [a] (+3.94)	1.58±0.06 [b] (-68.84)	4.51±0.12 [c] (-11.05)
	FSH(ng/ml)	1.59±0. 10 [a]	1.65±0. 04 [a] (+3.77)	1.75±0. 06 [a] (+10.06)	1.00±0. 04 [b] (-37.11)	1.95±0. 05 [c] (+22.64)
	LH (ng/ml)	1.16±0. 049 [a]	1.25±0. 031 [a] (+7.76)	1.24±0. 039 [a] (+6.90)	0.93±0. 031 [b] (-19.83)	1.17 ±0. 033 [a] (+0.86)
	ALP (U/L)	102.37±2.48 [a]	103.53±2.48 [a] (+1.13)	98.97±2.37 [a] (-3.32)	230.10±5.68 [b] (+124.77)	106.17±2.58 [a] (+3.71)
	ACP(IU/L)	41.27±1.11 [a]	43.46±1.09 [a] (+5.31)	42.57±0.96 [a] (+3.15)	71.40±1.76 [b] (+73)	44.35±1.06 [a] (+7.46)
	γGT (U/L)	10.16±0.25 [a]	10.03±0. 24 [a] (-1.28)	10.16±0.25 [a] (0)	16.59±0.40 [b] (+63.29)	12.87±0.31 [c] (+26.67)
Testis	ALP (U/g)	31.18±0.75 [a]	30.75±0.74 [a] (-1.38)	32.55±0.61 [a] (+4.39)	18.55±0.57 [b] (-40.51)	28.96±0.72 [c] (-7.12)
	ACP(IU/g)	2.78±0.07 [a]	2.75±0.07 [a] (-1.08)	2.79±0.17 [a] (+0.36)	1.44±0.06 [b] (-48.20)	2.43±0.06 [c] (-12.59)
	γGT (U/g)	8.89±0.28 [a]	8.94±0.25 [a] (+0.56)	9.11±0.27 [a] (+2.47)	3.26±0.11 [b] (-63.32)	7.46±0.26 [c] (-16.09)

AP=*Anacyclus pyrethrum* root extract. **ATZ**=Atrazine. **RTW**=Relative testis weight. **REW**=Relative epididymal weight. Similar letters refer to non significant changes (***P* > 0.05**). Different letters refer to significant changes (***P* ≤ 0.05)**. The values in parentheses represent the percentage of changes from control.

3.2. Effects of ATZ (200 mg/kg) on biochemical parameters:

Table (1) shows that administration of ATZ (200 mg/kg) daily for 60 days to male rats induced significant decreases in body weight gain%, relative weights of testis and epididymis, and epididymal sperm count; and serum concentrations of T, DHEA, FSH and LH hormones. Also, treatment with ATZ inhibited the activities of testicular biomarker enzymes including ALP, ACP and γ-GT. On contrary, serum levels of ALP, ACP and γ-GT showed significant increase in ATZ-treated rats in comparison with normal group. In table (2), male rats treated with ATZ showed significant increases in serum and testis contents of TP, TL, TC, TG and PL; and serum LDL-C, CRR and AI levels, when compared to normal group. In contrast, ATZ caused significant decrease in serum HDL-C of the treated rats. As shown in table (3), administration of ATZ to male rats significantly increased testicular content of the oxidative stress markers; MDA, H_2O_2, NO and PC. Concurrently, marked decreases in the antioxidant parameters including TAC, SOD, GSH, GPx and GST were recorded in the testis of ATZ-treated rats. In addition, administration of ATZ caused significant increase in the percent of apoptotic cells and apoptotic marker (p53).

3.3. Effects of water extract of AP roots on ATZ-induced changes biochemical parameters:

Collectively, tables (1, 2, and 3) displayed that, administration of aqueous extract of AP root to male rats treated with ATZ-induced reproductive disorders led to marked amelioration in all the tested parameters in both serum and testis which reached, in most of them, to almost the control levels.

Table 2. Total proteins and lipid profile in serum and testis of different animal groups. Values are means ± SE of six animals for each group.

Parameters		Animal groups				
		control	**Corn oil**	**AP**	**ATZ**	**AP+ATZ**
Serum	TP(g/dL)	7.59 ± 0.22 [a]	7.28 ± 0.17 [a] (-4.08)	7.55 ± 0.18 [a] (-0.53)	5.43 ± 0.18 [b] (-28.46)	7.53 ± 0.17 [a] (-0.79)
	TL(g/dL)	425.15 ± 10.19 [a]	433.98±10.41 [a] (+2.08)	408.09 ±7.12 [a] (-4.01)	671.56 ±16.08 [b] (+57.96)	438.45±10.50 [a] (+3.13)
	TC(g/dL)	56.83 ± 1.36 [a]	57.05 ± 1.37 [a] (+0.39)	55.68 ± 1.20 [a] (-2.02)	96.73 ± 2.32 [b] (+70.21)	59.16 ± 1.42 [a] (+4.10)
	TG(g/dL)	61.09 ± 1.46 [a]	62.11 ± 1.49 [a] (+1.67)	57.90 ± 1.52 [a] (-5.22)	79.02 ± 1.89 [b] (+29.35)	61.24 ±.47 [a] (+0.25)
	PL(g/dL)	7.82 ± 0.19 [a]	8.08 ± 0.19 [a] (+3.32)	7.45 ± 0.18 [a] (-4.73)	14.21 ± 0.34 [b] (+81.71)	7.50 ± 0.18 [a] (-4.09)
	HDL-C (mg/dL)	43.17 ± 1.04 [a]	43.00 ± 1.03 [a] (-0.39)	44.53 ± 1.07 [a] (+3.15)	32.70 ± 0.78 [b] (-24.25)	43.09 ± 1.03 [a] (-0.19)
	LDL-C (mg/dL)	1.44± 0.06 [a]	1.62 ± 0.04 [a] (+12.50)	0.87 ± 0.03 [a] (-39.58)	48.22 ± 1.15 [b] (+3248)	3.82 ± 0.10 [c] (+165.28)
	CRR	1.32±0.034 [a]	1.33±0.034 [a] (+0.8)	1.25±0.032 [a] (-5.30)	2.96 ±0.076 [b] (+124.24)	1.37±0.035 [a] (+3.79)
	AI	0.033±0.0008 [a]	0.038±0.0009 [a] (+15.15)	0.020±0.0005 [a] (-39.39)	1.475±0.0381 [b] (+4369)	0.089±0.0023 [c] (+169.69)
Testis	TP(mg/g)	35.23 ± 0.88 [a]	34.31±0.84 [a] (-2.61)	36.45 ± 0.91 [a] (+3.46)	21.06 ± 0.53 [b] (-40.22)	34.94 ± 1.09 [a] (-0.82)
	TL(mg/g)	101.11 ± 2.48 [a]	96.29 ± 2.31 [a] (-4.77)	94.64 ± 2.73 [a] (-6.40)	158.94 ± 4.35 [b] (+57.20)	115.51± 2.87 [c] (+14.24)
	TC(mg/g)	27.44 ± 0.79 [a]	27.88 ± 0.79 [a] (+1.60)	25.58 ± 0.62 [a] (-6.78)	37.12 ± 1.05 [b] (+35.28)	28.11 ± 0.67 [a] (+2.44)
	TG(mg/g)	34.10 ± 0.82 [a]	34.31 ± 1.00 [a] (+0.62)	34.09 ± 0.82 [a] (-0.03)	30.44 ± 0.50 [b] (-10.73)	35.38 ± 0.85 [a] (+3.75)
	PL(mg/g)	10.28 ± 0.25 [a]	10.00 ± 0.24 [a] (-2.72)	9.59 ± 0.27 [a] (-6.71)	17.97 ± 0.43 [b] (+74.81)	11.31 ± 0.28 [c] (+10.02)

AP=*Anacyclus pyrethrum* roots extract. **ATZ**=atrazine. Similar letters refer to non significant changes (***P* > 0.05)**. Different letters refer to significant changes (***P* ≤ 0.05)**. The values in parentheses represent the percentage of changes from control.

Table 3. Oxidative stress markers, antioxidants, % of apoptotic cells and p53 in testis of different animal groups. Values are means ± SE of six animals for each group.

Parameter	Animal groups				
	control	Corn oil	AP	ATZ	AP+ATZ
MDA (nmol/g)	18.87±0.45 [a]	19.80±0.47 [a] (+4.93)	18.48±0.50 [a] (-2.07)	23.93±0.57 [b] (+26.82)	19.35±0.46 [a] (+2.54)
H_2O_2(mM/g)	20.79±0.50 [a]	21.63±0.53 [a] (+4.04)	20.32±0.55 [a] (-2.26)	41.91±1.01 [b] (+101.59)	35.69±0.86 [c] (+71.67)
NO(µmol/g)	16.21±0.39 [a]	16.01±0.38 [a] (-1.23)	14.85±0.36 [a] (-8.39)	48.24±1.15 [b] (+197.59)	23.98±0.57 [c] (+47.93)
PC(µmol/g)	0.18±0.004 [a]	0.18±0.004 [a] (0)	0.17±0.004 [a] (-5.56)	0.24±0.006 [b] (+33.33)	0.19±0.005 [a] (+5.56)
TAC (mM/g)	1.21±0.018 [a]	1.23±0.020 [a] (+1.65)	1.27±0.026 [a] (+4.96)	0.44±0.011 [b] (-63.64)	1.17±0.029 [a] (-3.31)
GSH (mg/g)	5.38±0.13 [a]	5.40±0.13 [a] (+0.37)	5.49±0.23 [a] (+2.04)	2.71±0.07 [b] (-49.62)	5.27±0.13 [a] (-2.04)
SOD (U/g)	18.13±0.43 [a]	17.55±0.29 [a] (+3.20)	18.54±0.39 [a] (+2.26)	14.61±0.35 [b] (-19.42)	17.90 ±0.43 [a] (-1.27)
GPx(U/g)	459.63±11.86 [a]	458.80±11.80 [a] (-0.18)	459.31±11.84 [a] (-0.07)	325.96±8.27 [b] (-29.08)	409.91±10.52 [c] (-10.82)
GST (U/g)	0.92±0.022 [a]	0.93 ±0.022 [a] (+1.09)	0.95±0.024 [a] (+3.26)	0.54±0.013 [b] (-41.30)	0.62±0.015 [c] (-32.61)
Apoptosis (%)	18.26 ±0.44 [a]	18.63±0.46 [a] (+2.03)	17.93±0.44 [a] (-1.81)	26.50 ±0.66 [b] (+45.13)	19.73±0.49 [a] (+8.05)
p53 (%)	42.39±1.08 [a]	42.23±1.18 [a] (-0.38)	40.36±0.97 [a] (-4.79)	77.56±1.87 [b] (+82.97)	53.04±1.27 [c] (+25.12)

AP=*Anacyclus pyrethrum* roots extract. **ATZ**=atrazine. Similar letters refer to non significant changes (***P* > 0.05)**. Different letters refer to significant changes (***P* ≤ 0.05)**. The values in parentheses represent the percentage of changes from control.

4. Discussion

It is generally acknowledged that the use of herbicides has large benefits to farmers and crops production as they used effectively to destroy unwanted vegetation. However, the present use of these toxic synthetic chemicals in agriculture can cause negative environmental effects to the public health and society **(Oppenheimer & Donnelly, 1997)**. The organophosphate herbicide ATZ, for example, is a well-known environmental pollutant that was reported to induce disruption in the functioning of the male reproductive tissues **(Kniewald *et al*., 2000 and Liu *et al*., 2014).** In response to this, great concern has been exerted to explore the effective treatment regimens against the herbicides toxicity in both human and animals. In this regard, natural products which extracted from the medicinal plants are attracting the attention of scientists because they are cheaper, safer, eco-friendly and within the reach of the current medical community. There are many reports which demonstrated that the medicinal plants possess bioactive constituents which have a real effect on human's health **(Bencheqroun *et al.*, 2012 and Rani *et al*., 2013).** In particular, phytochemical such as flavonoids are biologically active antioxidants that possess various protective and disease preventive actions **(Karimi *et al*., 2005)**. Experimental studies have also shown that substances, such as flavonoids, found in herbs have androgenic effects **(Sharma *et al*., 2009)**, and this effect can play an important role in fertility and the treatment of gonadal dysfunction in both males and females **(Etuk and Muhammad, 2009)**. Of these medicinal plants, AP has been found to be used effectively for different clinical purposes due to its potent antioxidant activity **(Sujith *et al*., 2011)**, antibacterial effect **(Kumar *et al*., 2012)**, anticonvulsant activity **(Gautam *et al*., 2011),** antidiabetic activity **(Tyagi *et al*., 2011)** and reproductive activity **(Sharma *et al*., 2009).**

4.1. Effect of ATZ on reproductive hormones, sperm count; and weights of body, testis and epididymis

LH and FSH are called gonadotropin hormones because they stimulate the gonads in both sexes. In testes, LH stimulates synthesis and secretion of testosterone by the Leydig cells. While, FSH is critical for sperm production since it supports the function of Sertoli cells, which in turn support many aspects of sperm cell maturation. DHEA is mainly secreted by the adrenal gland, but also secreted by testes and brain. DHEA serves as a precursor to androgen hormones (T). The T hormone plays a role in the spermatogenesis and the physiological functions of Sertoli cells in mammals **(Skinner and Fritz, 1985)**.

In the present study, administration of ATZ (200mg/kg) for 60 days in male rats resulted in marked reduction in the levels of serum DHEA, T, FSH and LH hormones. The obtained decrease in the serum levels of T hormone by ATZ in rats could be attributed to direct toxic effect of this herbicide on the gonads and/or an indirect effect on the pituitary gland. Regarding the direct action of ATZ on testicular function, there are some reports which provide evidence that ATZ could induce damage to Leydig cells and Sertoli cells, and subsequently inhibit the T secretion by Leydig cells. Also, it has been proposed that ATZ inhibits Leydig cell steroidogenesis by inhibiting the expression of genes that are involved in steroidogenesis **(Pogrmic *et al*., 2009)**. In this regard, previous studies and the present one provided strong evidence that ATZ exposure to rats produced testicular oxidative stress thereby it can cause damage to the Leydig cells and subsequently inhibit T production **(Farombi *et al*., 2013 and Abarikwu *et al*., 2009)**. On the other hand, the pituitary gland was considered as a target tissue for ATZ **(Matsumoto *et al*., 1986)**, so that exposure to ATZ could impair pituitary function and hence decrease hormonal production of LH and FSH leading to suppressed testicular function. ATZ-induced changes in reproductive hormones have previously been reported by several studies. In some reports, decreases in serum levels of FSH and LH in ATZ treated rats were documented **(Trentacoste *et al*., 2001 and Pogrmic *et al*., 2009)**. Similarly, serum levels of T, FSH and LH were decreased by 85% after 48 days exposure to high dose of ATZ (300 mg/kg bw/day) in adult male Wistar rats **(Dehkhargani *et al*., 2012)**. It was also demonstrated that ATZ exposure to both postnatal and juvenile male rats caused a significant decrease in both the serum and testicular T levels **(Stoker *et al*., 2000 and Friedmann, 2002)**.

Obtained results also showed body weight loss and decreases in absolute and relative testis and epididymis weights, as well as epididymal sperm count and total protein content in serum and testis following exposure to ATZ. Similarly, published data showed that long-term administration of ATZ at dose levels higher than 200 mg/kg produced a significant body weight loss in the rats **(Stocker *et al*., 2000)**. Also, ATZ usage was found to cause testicular weight loss in rats **(Mokhtari *et al*., 2010)**. However, the decrease in body weight, as well as testis and epididymis weights, might associated with ATZ-caused a reduction in the food consumption and absorption **(Trentacoste *et al*., 2001)**. Also, ATZ is a powerful endocrine disruptor which can cause pituitary weight loss and dysfunction **(Fan *et al*., 2007)**, thereby it can bring about a reduction in the pituitary hormones, including growth hormone and gonadotropin hormones, leading ultimately to decrease in the weights of both whole body and organs. Present findings which indicated a reduction in the sperm count, and total protein content in both serum and testis are in agreement with the previous studies which revealed that ATZ can cause a decrease in sperm count, motility and testicular protein concentration **(Simic *et al*., 1994 and Dehkhargani *et al*., 2011)**. Also, **Yang *et al*. (2014)** recorded an irregular and disordered arrangement of the seminiferous epithelium with decreased spermatozoa number and increased spermatozoa abnormality rate in male rats exposed to ATZ. The decrease in sperm count following exposure to ATZ in rats might be due to several factors including a reduction in the food consumption which leads to decreasing the nutrients that serve as main diet of sperms and a decrease in both testis weight and androgen biosynthesis, as shown in this study and other published data **(Akunna *et al*., 2011)**. Testicular proteins are required for spermatogenesis and sperm maturation **(Kasturi *et al*., 1995)** and a decrease in the testicular content of this vital macromolecule in ATZ-treated rats could contribute to the impairment of sperm maturation with subsequent reduction in sperm count.

4.2. Effect of ATZ on lipid profile

The present study exhibited marked increases in the levels of TL, TC, TG and PL in both serum and testis, and in serum concentration of LDL-C along with decreased serum level of HDL-C in ATZ-treated rats. The results also showed increases in the cardiovascular risk factors CRR and IA. In clinical practice, both CRR and IA are considering risk indicators with greater predictive value than isolated parameters used independently, such as LDL. In this trend, there is shortage in published information regarding the hyperlipidemic effect of ATZ. However, in previous study, exposure of adult male rats to ATZ caused a significant elevation in serum TL, TG, LDL-C and VLDL-C, in addition to decrease in HDL-C **(AL-Attabi and AL-Diwan, 2012)**. In another one, ATZ administration resulted in an increase in the level of serum total lipids, the effect which appeared to be dose dependent **(Santa *et al*., 1986)**. However, the increase in serum level of TC and hence LDL-C and VLDL-C might be attributed to down regulation of the steroidogenesis where cholesterol is the precursor for this vital process resulting in a decrease of the end products (like androgens synthesis in Leydig cells) and elevation of cholesterol **(Pogrmic *et al*., 2009)**. These authors have further demonstrated that the inhibition of steroidogenesis in Leydig cell from ATZ-treated rats was associated with a decline in several factors such as LH receptor, scavenger receptor-B1, 3β-HSD, 17β-HSD and CYP17A1. In particular, scavenger receptor-B1 inhibition results in elevation of LDL-C **(Huszar *et al*., 2000)** whereas over expressions of the receptors have lowered the concentrations of VLDL-C and LDL-C **(Arai *et al*., 1999).** Increased production of LDL-C due to exposure to ATZ in male rats is risky since it trapped in the arterial wall and oxidized via oxidative stress by free radicals under the action of resident cells leading ultimately to atherosclerosis **(Williams and Tabas, 1995)**. On the other hand, HDL-C has been considered an important plasma

antioxidant defense system which can protect against the oxidation and cytotoxicity of LDL-C. Observed decrease in serum level of HDL-C after exposure to ATZ in male rats therefore may allow further accumulation of LDL-C with subsequent increased its deleterious effects.

4.3. Effect of ATZ on oxidative stress markers and antioxidants

It has been reported that, oxidative stress in any tissue results from an imbalance between the production of reactive oxygen species (ROS) and their efficient removal by available antioxidant systems. Many conditions, for example, that are associated with male infertility are inducers of testicular oxidative stress. Exposure to X-irradiation and environmental toxicants including herbicides has been reported to initiate testicular oxidative stress, which leads to an increase in germ cell apoptosis and subsequently hypospermatogenesis **(Turner and Lysiak, 2008)**.

In the present study, exposure of male rats to the herbicide ATZ produced gonadal oxidative stress, as indicated by markedly increased testicular contents of MDA, H_2O_2, NO and PC. In contrast, the levels of all measured antioxidants (TAC, GSH, SOD, GPx and GST) were significantly decreased in the testicular tissue after treatment of rats with ATZ, compared to normal group. Available data in this regard have similarly demonstrated significant production of lipid peroxidation products (MDA) as a result of reduction in the antioxidant enzymes including SOD, GPx and GST in the epididymis and testis of rats treated with ATZ **(Farombi *et al*., 2013)**. Also, comparable significant decrease of the antioxidant parameters accompanied with marked increase in testicular content of MDA were observed in rats administered ATZ (200mg/kg) for 16 day **(Akunna *et al*., 2012)**. In recent study, a decrease in the testicular levels of both TAC and GSH, and an increase in lipid peroxidation were observed in ATZ-treated rats **(Yang *et al*., 2014)**. Adding to this, intoxication with ATZ (300mg/kg) for 15 day resulted in elevated level of H_2O_2 **(Jestadi *et al*, 2014)**. It has been known that H_2O_2 can decompose into toxic hydroxyl radicals, which could contribute to severe oxidative tissue damage leading to enhanced lipid peroxidation. Thus, the present findings of increased testicular H_2O_2 and MDA accompanied with decreased antioxidants could reflect incidence of oxidative tissue damage of cellular membranes of spermatozoa which lead to inhibit their number and to increase their abnormality rate **(Aitken *et al*., 1995 and Akunna *et al*., 2012)**.

Any oxidizing radical is a potential agent of oxidative stress. NO is an important physiological signaling molecule, but when produced in a large excess, it also displays cytotoxicity **(Wei *et al*., 2000)**. It can cause tissue injury, and can interact with superoxide radicals forming peroxy nitrite ($ONOO^-$), another potent oxidizing agent which can oxidize some types of proteins producing free radicals **(Pryor *et al*., 2006 and Szabo *et al*., 2007)**. In particular, NO in the testes plays an important physiological role since it contributes to the regulation of steroidogenesis, vasodilatation; and peristalsis and permeability of seminiferous tubules **(El-Gohary *et al*., 1999)**. Thus, increased production of NO and other reactive oxides is considered to be disruptive to the cellular functions in the testis. In the present study, the elevated level of NO in animal treated with ATZ, therefore, is risky since it can amplify testicular injury and thus impairs its physiological function. Current result comes compatible with previous findings which displayed elevated level of NO in female mice exposed to ATZ **(Park and Bae, 2012)**. Actually, induction of oxidative stress as reflected by increased production of ROS and lipid peroxidation following exposure to ATZ could represent a crucial factor which led to depletion of the antioxidant defense system in the testis and impairment of the reproductive functions. In this concern, the loss of testicular and epididymal weights, the decrease of sperm count, the reduction of serum level of T together with diminished activities of antioxidant parameters in the testis could in large part be attributed to ATZ-induced oxidative stress in the testicular tissue.

4.4. Effect of ATZ on testicular biomarker enzymes

Testes are the most important organs in male reproductive system, which are involved in the functions of spermatogenesis and T secretion. There are many kinds of enzymes which are closely associated with the normal functions of testes, and measurement of their activities is very important to assess the reproductive toxicity **(Hodgen and Sherins, 1973; and Shen and Lee, 1976)**. Of these enzymes, ACP which is mainly localized in the cytoplasm of Sertoli cell is associated with the denaturation of seminiferous epithelium and phagocytosis of Sertoli cells. Also, AKP is a dephosphorylated enzyme which is linked with the division of spermatogenic cells and the transportation of nutrients to spermatogenic cells for proliferation and differentiation. In addition, the enzyme γ-GT is a useful marker of Sertoli cell maturation **(Kumar *et al*., 2000)**. It appears to play an important role in the protection of spermatozoa from oxidative stress, since it plays an important role in the metabolism of extracellular glutathione **(Hinton *et al*., 1998)**. In the current study, the activities of such testicular biomarker enzymes ACP, AKP, and γ-GT were evaluated in both serum and testis following exposure of adult male rats to ATZ. Obtained results showed significant increases in serum activities of mentioned testicular biomarker enzymes concomitant with decreased their testicular activities in animal exposed to ATZ. This finding provided evidence that ATZ harmed testicular tissue and disrupted its function. In this line, there are few published reports which demonstrated increased activity of serum ALP **(Santa *et al*., 1986)** and decreased testicular activity of ACP and ALP following exposure of male rats to ATZ

(Yang *et al*., 2014). However, the increase in the activities of the tested enzymes in the serum and the decrease obtained in the testis of rats exposed to ATZ could be attributed to the increased permeability of plasma membrane or even complete cell damage as a result of ATZ-induced oxidative stress and lipid peroxidation which lead to leakage of these enzymes from the testicular tissue into the blood stream. This suggestion derived support from the present result of increased oxidative stress and published data which exhibited increased oxidative tissue damage as indicated by elevated testicular content of MDA accompanied with appearance of histopathological alterations in the testis of rats treated with ATZ **(Yang *et al*., 2014)**.

4.5. Effect of ATZ on apoptosis in testis

The normal spermatogenesis involves proliferation and maturation of male germ cells via various phases from spermatogonia through spermatocytes and spermatids to spermatozoa **(Griswold, 1998)**. However, a number of germ cells die by apoptosis before reaching maturity, even during physiological conditions, suggesting presence of balance between cell proliferation and cell death in normal spermatogenesis **(Rodriguez *et al*., 1997)**. This indicates that apoptosis is required under normal condition in various types of tissues to regulate the balance between cell death and cell survival. On the other hand, physiological levels of ROS are also required for normal functioning of the testis, like spermatogenesis **(Mathur and D'Cruz, 2011)**. However, it was found that the ROS that are produced during spermatogenesis are involved in the regulation of apoptosis within the testis (**Erkkila *et al*., 1999).** Under stressed condition, pathological levels of ROS are pronounced and can produce oxidative tissue damage and hence apoptosis. The latter (apoptosis) subsequently increased and become pathologic inducing disturbance in the balance between cell death and cell survival, i.e. expression of pro-apoptotic genes is increased and the cell death exceeds the cell proliferation. In the present study, administration of a high dose of ATZ (200 mg/kg) to male rats induced oxidative testicular injury and disrupted its function. This toxic agent, therefore, was found to increase the percentage of apoptotic cells and the apoptotic marker p53 in the testis of treated rats. Increased apoptosis under such stressed condition might led to much loss of essential testicular cells with little regenerative capacity leading ultimately to impaired testicular functions, particularly, the production of spermatozoa and sex hormones, like T. It seems likely that the mechanisms underlying the obtained reduction in both the epididymal sperm count, and serum and testicular testosterone concentrations involved both oxidative stress and accompanying apoptosis. In previous studies, oral administration of the organic herbicide ATZ to male rats was found to cause a state of oxidative stress in the testis and epididymis since it lowered the activity of antioxidant enzymes and increased lipid peroxidation and H_2O_2 synthesis **(Abarikwu *et al*., 2009)**, suggesting that ATZ could induce testicular apoptosis. This finding was further supported by **Farombi *et al*. (2014)** who reported that the toxicity of ATZ in the testicular cells of rats is mediated by mechanisms involving apoptosis, oxidative stress and dysregulation in the expressions of several genes specific for the steroidogenic pathway.

4.6. Effect of aqueous extract of AP roots on ATZ-induced reproductive toxicity

In recent years, there has been a great concern about the use of plant products in affecting fertility of humans. AP, as a well-known medicinal herb, is used to improve sexual function especially in males **(Sharma *et al*., 2013)**. Moreover, it possesses an antioxidant activity which can prevent the oxidative tissue damage induced by free radicals and ROS. Yet, there is no investigation reports the protective effect of aqueous extract of AP roots against ATZ toxicity. Therefore, current study was designed to explore, for the first time, the potential protection afforded by AP extract against the reproductive disorders induced by the toxic herbicide ATZ in male rats.

Obtained results of the present study demonstrated that oral administration of aqueous extract of AP roots daily for 60 days at a dose level of 150 mg/kg caused restoration of reproductive hormones (T, LH and FSH), total proteins and lipid profile levels in serum and testis of ATZ-treated rats. There is no available information on the protective effect of AP on ATZ-induced sexual toxicity. However, recent studies carried out on the efficacy of aqueous extract of AP (150mg/kg) for 28 days on testicular function showed significantly increased reproductive hormones T, FSH and LH in intact adult male rats (**Shahraki *et al*., 2014)**. It has also been demonstrated that AP extract administration to healthy adult male rats produced significant increase in the serum concentrations of mentioned reproductive hormones, and AP extract itself produced testosterone-like effect (androgenic effect) by improving the fertility since it enhanced spermatogenesis and testis weight **(Sharma *et al*., 2013).** These anabolic effects were also validated by the observable increases in the weights of epididymis, seminal vesicles, prostate and the whole animal body in normal rats treated with AP extract **(Sharma *et al*., 2009)**. In agreement with these results, present findings exhibited that aqueous extract of AP root largely ameliorated testis, epididymis and body weights; and sperm count in ATZ-treated rats. Potentially, anabolic effects of AP extracts might be associated with an improvement of testicular content of lipids and proteins, which can positively interfere with the processes of steroidogenesis and spermatogenesis. It has also been reported that treatment of male rats with AP extract significantly increased the seminal fructose content indicating that the aqueous extract of AP enhances the quality of

seminal parameters which eventually assist in better reproductive potency and further suggests that AP extract has androgenic potential **(Sharma *et al*., 2009 and 2013)**. Actually, these published data can confirm that, the AP extracts has a beneficial effect in improving various physiological conditions responsible for exhibiting a better sexual performance which can be useful in the protection of human reproductive system against toxic agents, like ATZ.

It is worthwhile to mention that, the ameliorating impact of AP roots extract on the structure and function of male reproductive system could, primarily, be attributed to its high antioxidant activity due to their phytochemical constituents such as phenols, flavonoids, alkaloids and tannins **(Sujith *et al*., 2011)**. Current study in this concern demonstrated that, treatment of ATZ-treated rats with AP aqueous extract showed significant reductions in the testicular levels of oxidative stress markers including MDA, H_2O_2, NO and PC; with concurrent marked increases in the antioxidant parameters which include TAC, GSH, SOD, GPx and GST in the testis. Therefore, in the present study, obtained decrease in lipid peroxidation and ROS in testis of rats treated with ATZ could be attributed to the antioxidant power of water extract of AP roots along with improved intrinsic antioxidants. Treatment of ATZ-exposed rats with AP extract further potentiated the antioxidant system by restoring the serum level of HDL-C, since this lipid fraction can protect LDL-C and other lipoproteins from oxidative stress induced by free radical species **(Williams and Tabas, 1995)**.

Additionally, it has been reported that ROS which are produced normally during spermatogenesis are involved in the regulation of apoptosis within the testis (**Erkkila *et al*., 1999)** and exposure of rats to ATZ-induced ROS overproduction is pathologic and thus can induce testicular injury. Interestingly, treatment of rats exposed to ATZ with water extract of AP roots containing natural antioxidants lowered ATZ-induced apoptosis in the testis to a level close to the control value. Also, AP extract significantly inhibited testicular p53 production in ATZ-treated rats, when compared to untreated ATZ group. Present study also showed that administration of water extract of AP roots largely ameliorated the activities of testicular biomarker enzymes (ACP, ALP, and γ-GT) in both serum and testis tissue. Such beneficial effect of AP extract could be attributed to its ability to attenuate ATZ-induced testicular oxidative stress and apoptosis by scavenging ROS, as well as its ability to enhance the antioxidant status.

In conclusion, current findings indicated that ATZ herbicide has reproductive metabolic disorders, as reflected by obtained alterations in the reproductive hormones, metabolic enzymes, proteins and lipid profile; and weights of whole body, testis and epididymis. Such hazards occurred due to ATZ induced oxidative stress and imbalance in pro-oxidant and antioxidant status of the testis. However, treatment with aqueous extract of AP roots can protect against ATZ-induced reproductive disorders in adult male rats via attenuation of the oxidative stress, inhibition of apoptosis and increase of antioxidants, which ultimately led to improve the testicular function.

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