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AMELIORATIVE EFFECT OF *HIBISCUS SABDARIFFA* LINN ON SUBCHRONIC CHLORPYRIFOS-INDUCED ALTERATIONS IN SEX AND THYROID HORMONES IN MALE WISTAR RATS

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ABSTRACT

Studies have shown that Chlorpyrifos (CPF), an Organophosphate (OP) insecticide alters both sex and thyroid hormones. Apart from inhibiting Acetylcholinesterase (AChE) activity, CPF has been shown to cause oxidative stress. The antioxidant potentials of many flavonoid-containing plants are increasingly being exploited in the therapy of many infectious and non-infectious diseases. *Hibiscus Sabdariffa* (HS) is one of the most widely used nutraceuticals that has been used traditionally to combat various illnesses due to its high flavonoid contents. The present study was therefore aimed at evaluating the ameliorative potentials of HS on subchronic chlorpyrifos-evoked alterations in sex and thyroid hormones in male Wistar rats. Forty-two (42) young adult male Wistar rats were divided at random into six groups containing seven (7) rats per group. Group I was administered distilled water (2 mL kg^{-1}) only while group II received soya oil (2 mL kg^{-1}), Group III was dosed with only aqueous extract of HS (500 mL kg^{-1} ~ $1/10^{th}$ of the LD_{50}), while group IV was given CPF (10.6 mL kg^{-1} ~ $1/8^{th}$ of the LD_{50}). Group V was pretreated with low dose of HS (250 mg kg^{-1} ~ $1/20^{th}$ of the LD_{50}) and then administered reconstituted CPF (10.6 mg kg^{-1}), 30 min later. Group VI was pretreated with high dose of the HS (500 mg kg^{-1}) and then administered CPF (10.6 mg kg^{-1}), 30 min later. The regimens were administered orally by gavage once daily for a period of 11 weeks. At the end of the treatment period, sera obtained from the blood samples were analyzed for the levels of Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH), testosterone, thyroid hormones (T_3, T_4 and TSH) and AChE. Thyroid and pituitary glands of each rat were also evaluated for Malondialdehyde (MDA) concentration. Results showed a non-significant ($p>0.05$) decrease in the concentrations of FSH, LH and testosterone in the CPF group relative to the other groups. There was significant decrease ($p<0.05$) in the concentrations of T_3, T_4 and an increase in TSH in the CPF group relative to the other groups. There were significant increases ($p<0.05$) in MDA concentrations in the thyroid and pituitary glands in the CPF group compared to the other groups. Pretreatment with aqueous extract of HS demonstrated a dose-dependent amelioration of CPF-induced alterations in the levels of testosterone, LH, FSH, AChE, T_3, T_4 and TSH in the serum and that of pituitary and thyroid glands MDA. This may be partly due to its high level of polyphenolic compounds that confer its antioxidant and possibly AChE restoration

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activities. It is therefore concluded that pretreatment of individuals who are occupationally exposed to CPF and probably other OPs with the extract of HS may result in protection from the insecticide-induced adverse reproductive health outcomes.

Keywords: Chlorpyrifos, Sex and Thyroid Hormone, *Hibiscus Sabdariffa*, Amelioration

1. INTRODUCTION

Global concern concerning the impairment of reproductive capacity of individuals due to endocrine disrupting chemicals is on the increase (Balanic *et al*., 2011) Chlorpyrifos (CPF), an Organophosphate insecticide (OP) that is widely used in agriculture, horticulture and public health has been shown to disrupt the activities of endocrine organs (De Angelis *et al*., 2009). CPF has been shown to pose a serious threat to the integrity of the male reproductive system (Viswanath *et al*., 2010; Shittu *et al*., 2012a, 2012b; 2013). Proper functioning of hypothalamo-pituitary-thyroid axis is indispensable in several body systems, including, that of reproduction. Despite the conservative nature of the thyroid system, its role in human and animal reproduction and development of the reproductive tract cannot be overemphasized (Singh *et al*., 2011). Apart from the inhibition of acetylcholinesterase, the induction of oxidative stress due to increase free radical generation and decrease systemic antioxidant potentials is one of the other mechanisms implicated in CPF toxicity (Shittu *et al*., 2012a; 2012b; 2013). Oxidative stress, attributed to cellular and subcellular alterations in prooxidant and antioxidant ratio in favour of the former has been linked with the pathogenesis of several diseases.

The antioxidant potentials of many flavonoid containing plants are increasingly being exploited in therapy (Ambali *et al*., 2012). HS is one of the most widely used nutraceuticals that has been used traditionally to combat various illnesses due to its high flavonoid contents. Therefore, the aim of the present study was to evaluate the ameliorative potentials of HS on subchronic chlorpyrifos-evoked alterations in sex and thyroid hormones in male Wistar rats.

2. MATERIALS AND METHODS

2.1. Experimental Animals

Forty two (42) young adult male Wistar rats (130-154g) were obtained from the Laboratory Animal House of the Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria. They were housed in metallic cages in the animal holding facility of the Department. The rats were fed standard rat diet (Vital Feeds, Nigeria) and water was provided *ad libitum*. The animals were allowed to acclimatize for two weeks prior to the commencement of the study. The procedures involved in the handling of Laboratory Animals were in consonant with the Guide for the Care and Use of Laboratory Animals by the Ahmadu Bello University Ethic Committee for Animal Experiments.

2.2. Plant Collection, Identification and Preparation

Dried calyces of HS was obtained from Samaru market in Zaria, Nigeria and were taxonomically identified and authenticated (voucher number 1802) in the Herbarium section of the Department of Biological Science, Ahmadu Bello University, Zaria and a voucher specimen deposited. The calyces were then sorted and ground to powder. Powdered calyces of HS was extracted in distilled water for 72 h using the cold extraction method and the resulting filtrate was concentrated using hot water bath in an evaporating dish to produce the residue known as the extract. The resulting extract was further air dried on the shelf to a constant weight and % yield determined. The resulting residue labeled aqueous extract of HS (AEHS), were stored in a refrigerator at -4°C until required for use.

2.3. Chemical Acquisition and Preparation

Commercial grade chlorpyrifos (20% E.C. TERMICOT® Sabero Organics, Gujarat, India), used for the study was reconstituted in soya oil (Grand Cereals Oil Mills Ltd., Jos, Nigeria) to a 10% stock solution. The extract was reconstituted in distilled water to a 10% stock solution prior to daily administration. The remaining chemicals used in the study were of analytical grades and were obtained from Sigma-Aldrich (Germany).

2.4. Subchronic Reproductive Toxicity Study

The 42 young adult male Wistar rats were divided at random into six groups containing seven (7) rats per group. Group I (DW) was administered distilled water (2 mL kg^{-1}) only, Group II (S/oil) received soya oil (2 mL kg^{-1}); Group III (AEHS) was dosed with aqueous extract of HS (500 mg kg^{-1} ~ $1/10^{th}$ of the LD_{50}); Group IV (CPF) was given CPF (10.6 mg kg^{-1} ~ $1/8^{th}$ of the LD_{50} (Ambali, 2009) reconstituted in soya oil; Group V (LAEHS+CPF) was pretreated with low dose of the extract of HS (250 mg kg^{-1} ~ $1/20^{th}$ of the LD_{50}) and then administered reconstituted CPF (10.6 mg kg^{-1} (Ambali, 2009), 30 min later; Group VI (HAEHS+CPF) was pretreated with high dose of the extract

of HS (500 mg kg^{-1}) and then administered reconstituted CPF (10.6 mg kg^{-1}), 30 min later. The regimens were administered orally by gavage once daily for a period of 11 weeks. At the end of the treatment period, the animals were sacrificed by jugular venesection after light chloroform anesthesia. Three millilitre (3 mL) of blood collected from each animal into a test tube were allowed to clot and then incubated on the shelve for 30 min. Thereafter, it was centrifuged at 6oo× g for 10 min to obtain serum. The sera samples were analyzed for the concentrations of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), testosterone and [thyroid hormones (Triiodothyronine (T_3), Thyroxine (T_4) and Thyroid Stimulating Hormones (TSH)] and the activity of acetylcholinesterase. Thyroid and pituitary glands of each rat were also analysed for Malondialdehyde (MDA) concentrations.

2.5. Evaluation of the Concentrations of FSH, LH and Testosterone

The concentrations of serum FSH, LH and testosterone were assayed using enzyme immunoassay ELISA kit (Syntron Bioresearch Inc., Carlsbad, U.S.A.) as stated by the manufacturer.

2.6. Evaluation of Thyroid and Pituitary Gland Malondealdehyde Concentrations

The level of thiobarbituric acid reactive substance, MDA as an index of lipid peroxidation was evaluated in the thyroid and pituitary gland tissues using the double heating method of Draper and Hadley (1990). The principle of the method was based on spectrophotometric measurement of the colour developed during reaction of Thiobarbituric Acid (TBA) with MDA. The thyroid and pituitary gland from each animal in all the groups were rapidly removed, weighed using a precision weighing scale and then homogenized in a known volume of ice-cold phosphate buffer to obtain a 10% homogenate, which was then centrifuged at 6,000× g for 10 min to obtain the supernatant. The supernatant was then used to assess the level of protein and MDA in the samples. Briefly, MDA concentration in the supernatant of thyroid and pituitary gland homogenates were evaluated as follows: 2.5 mL of 100 g L^{-1} trichloroacetic acid solution was added to 0.5 mL of supernatant from the thyroid and pituitary gland homogenates in a centrifuge tube and placed in a boiling water bath for 15 min. After cooling under tap water for 5 min, the mixtures were then centrifuged at 1000× g for 10 min. Thereafter, 2 mL of the supernatant was added to 1 mL of 6.7 g L^{-1} TBA solution in a test tube and placed in a boiling (100°C) water bath for 15 min. The solution was cooled under tap water and the absorbance measured using a UV spectrophotometer at 532 nm. The MDA concentration on the thyroid and pituitary gland was calculated by the absorbance coefficient of MDA-TBA complex 1.566×10^5/cm and expressed in nmoL/mg of protein. The protein contents of both samples were determined using the method of Lowry *et al*. (1951).

2.7. Evaluation of Thyroid Hormones Concentration

Thyroid hormones (T_3, T_4 and TSH) concentrations were assayed using enzyme immunoassay kit (Syntron Bioresearch Inc., Carlsbad, U.S.A.).

2.8. Evaluation of Serum Acetylcholinesterase Activity

Acetylcholinesterase activities of the pituitary gland and testes were determined using the method of Ellman *et al*. (1961), with acetylthiocholine iodide as substrate. The AChE activity was calculated based on the rate of colour change, using the extinction coefficient of 1.36×10^4 expressed as nanomoles/min/mg protein.

2.9. Statistical Analysis

Values obtained were expressed as mean ± SEM and then subjected to one-way Analysis of Variance (ANOVA) followed by Tukey post-hoc test. Values of $p<0.05$ were considered significant.

3. RESULTS

3.1. Concentrations of FSH, LH and Testosterone

There was no significant change ($p>0.05$) in the FSH concentration in between the groups. However, the CPF group had the lowest FSH concentration with it levels decreasing by 44.4, 31.452.1, 33.7 and 14.2% respectively relative to DW, S/oil, HAEHS, LAEHS + CPF and HAEHS + CPF groups (**Fig. 1**).

There was no significant change ($p>0.05$) in the LH concentration in between the groups. However, the LH concentration in the CPF group, was relatively lower by 22.0, 13.3, 33.3, 40.0 and 18.0%, respectively, relative to DW, S/oil, HAEHS, LAEHS + CPF and HAEHS + CPF groups (**Fig. 2**).

There was a significant ($p<0.05$) decrease in the serum testosterone concentration in CPF group relative to the S/oil and HAEHS group. Although not significant ($p>0.05$), the testosterone concentrations in the LAEHS + CPF and HAEHS + CPF were relatively higher, increasing by 35.5 and 41%, respectively when compared to the CPF group (**Fig. 3**).

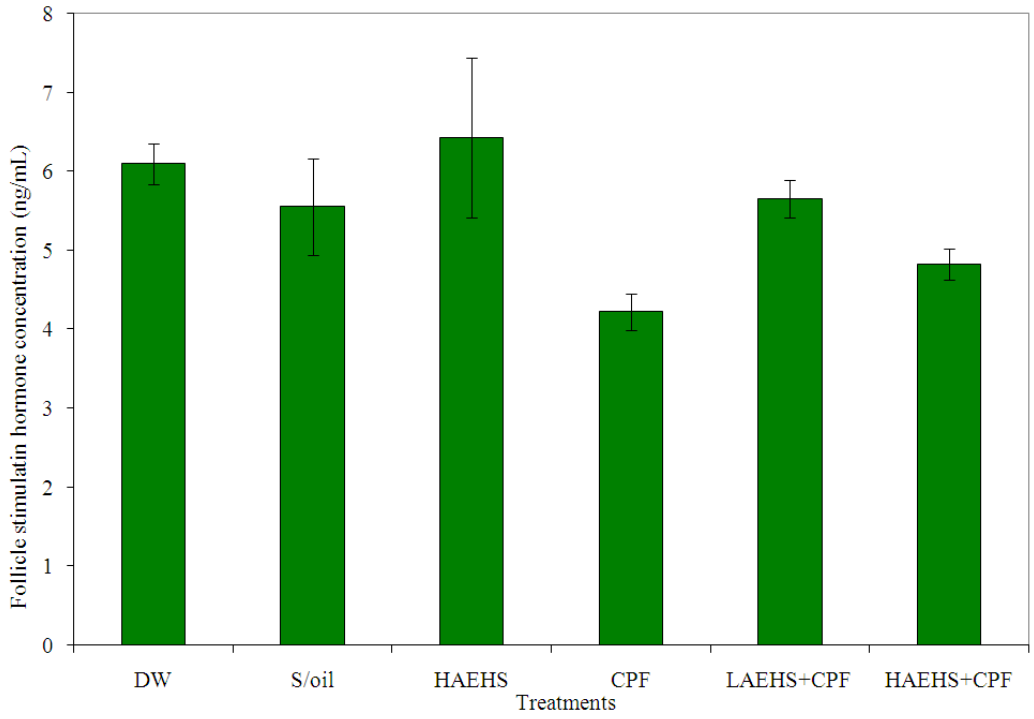

Fig. 1. Effect of subchronic exposure to Distil Water (DW), Soya oil (S/oil), Aqeous extract *H. sabdariffa* (HAEHS) and/or chlorpyrifos (CPF) on serum follicle stimulating hormone in adult male wistar rats

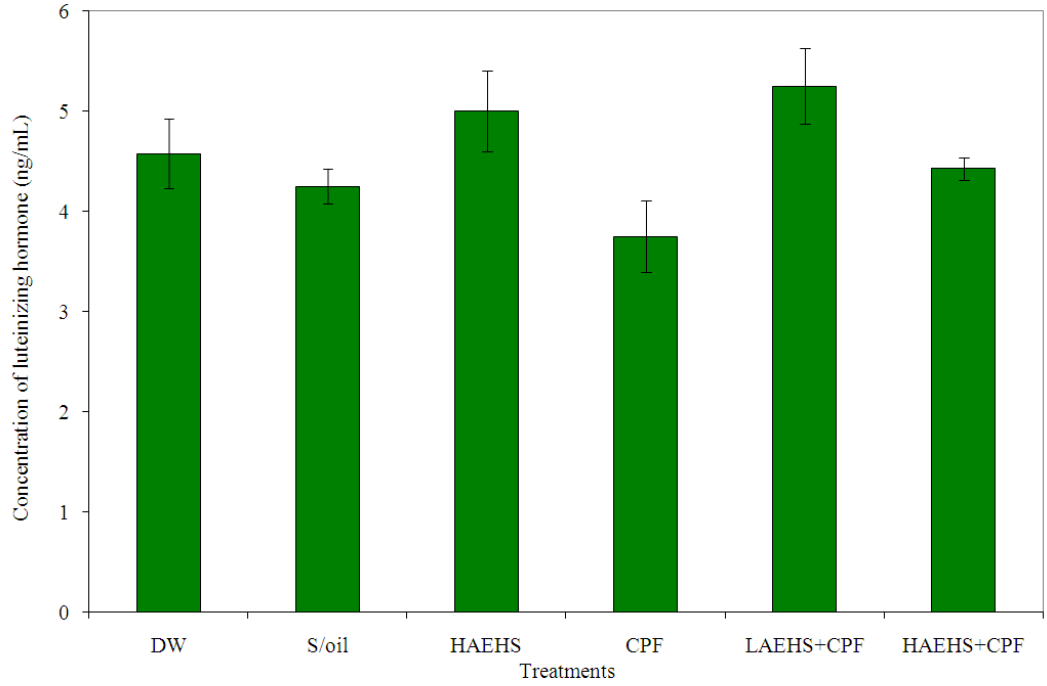

Fig. 2. Effect of subchronic exposure to Distil Water (DW), Soya oil (S/oil), aqeous extract *H. sabdariffa* (HAEHS) and /or Chlorpyrifos (CPF) on serum luteinizing hormone concentration in adult male wistar rats

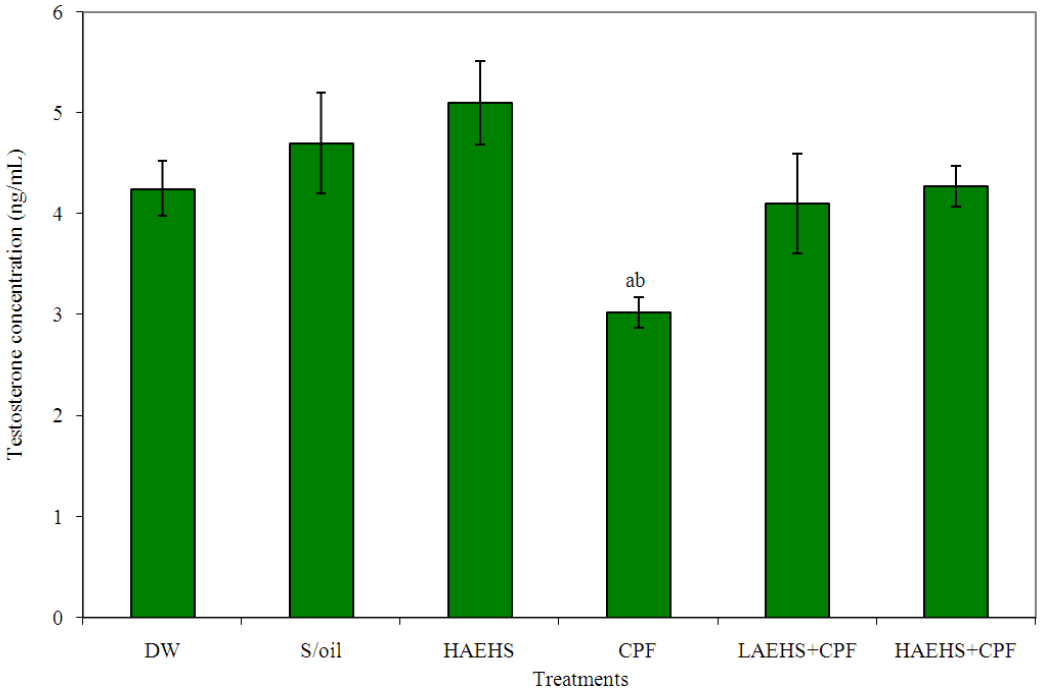

Fig. 3. Effect of subchronic exposure to Distil Water (DW), Soya oil (S/oil), aqeous extract of *H. sabdariffa* (HAEHS) and/or Chlorpyrifos (CPF) on serum testosterone concentration in adult male wistar rats. $^{ab}p<0.05$ Versus S/oil and HAEHS

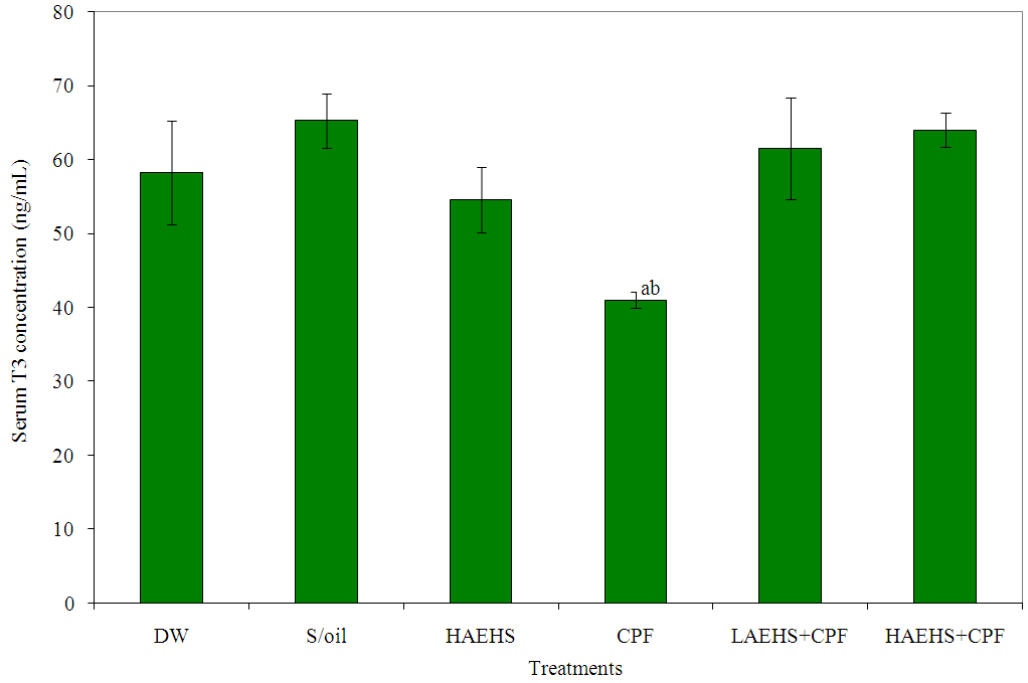

Fig. 4. Effect of subchronic exposure to Distil Water (DW), Soya oil (S/oil), aqeous extract *H. sabdariffa* (HAEHS) and /or Chlorpyrifos (CPF) on serum triiodothyronine (T_3) concentration in adult male wistar rats. $^{ab}p<0.05$ Versus S/oil, HAEHS and HAEHS+CPF

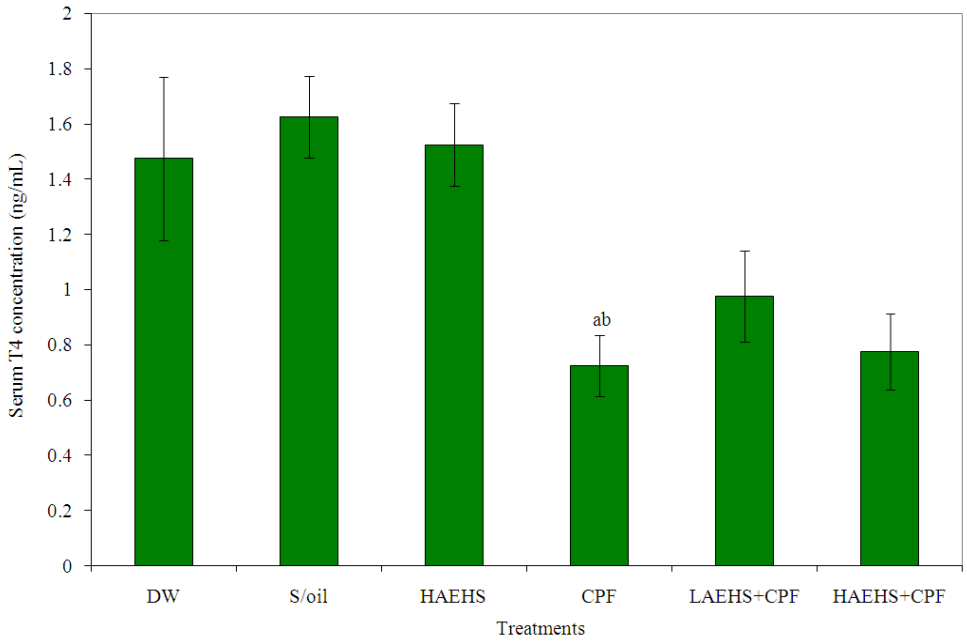

Fig. 5. Effect of subchronic exposure to Distil Water (DW), Soya oil (S/oil), aqeous extract *H. Sabdariffa* (HAEHS) and /or Chlorpyrifos (CPF) on serum thyroxine (T_4) concentration in adult male wistar rats. $^{ab}p<0.05$ Versus S/oil, LAEHS+CPF and HAEHS+CPF

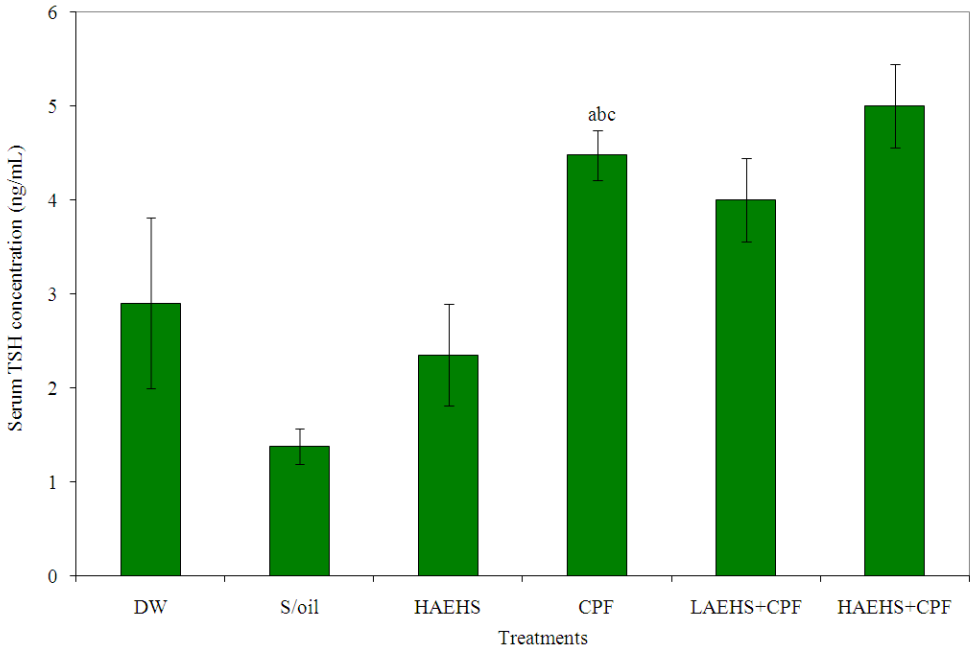

Fig. 6. Effect of subchronic exposure to Distil Water (DW), Soya oil (S/oil), aqeous extract of *H. Sabdariffa* (HAEHS) and/or Chlorpyrifos (CPF) on thyroid stimulating hormone (TSH) in adult male wistar rats. $^{ab}p<0.01$ Versus S/oil, HAEHS, LAEHS+CPF and HAEHS+CPF. $^{c}p<0.05$ Versus HAEHS

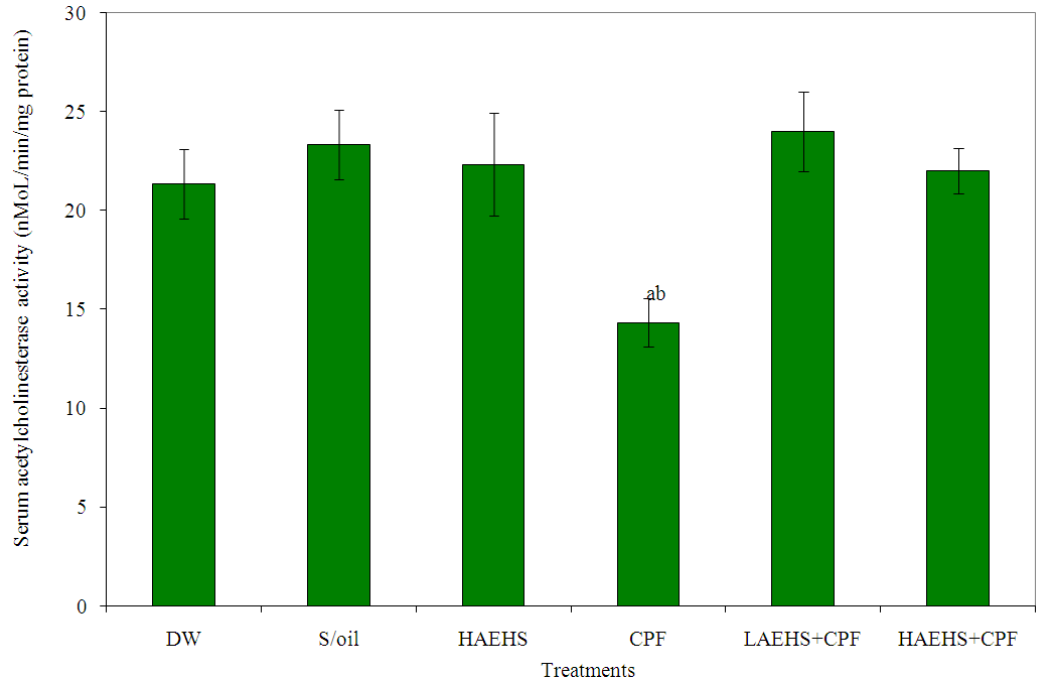

Fig. 7. Effect of subchronic exposure to Distil Water (DW), Soya oil (S/oil), aqeous extract of *H. Sabdariffa* (HAEHS) and /or Chlorpyrifos (CPF) on serum acetylcholinesterase activity in adult male wistar rats. [ab]p<0.05 Versus S/oil and LAEHS+CPF

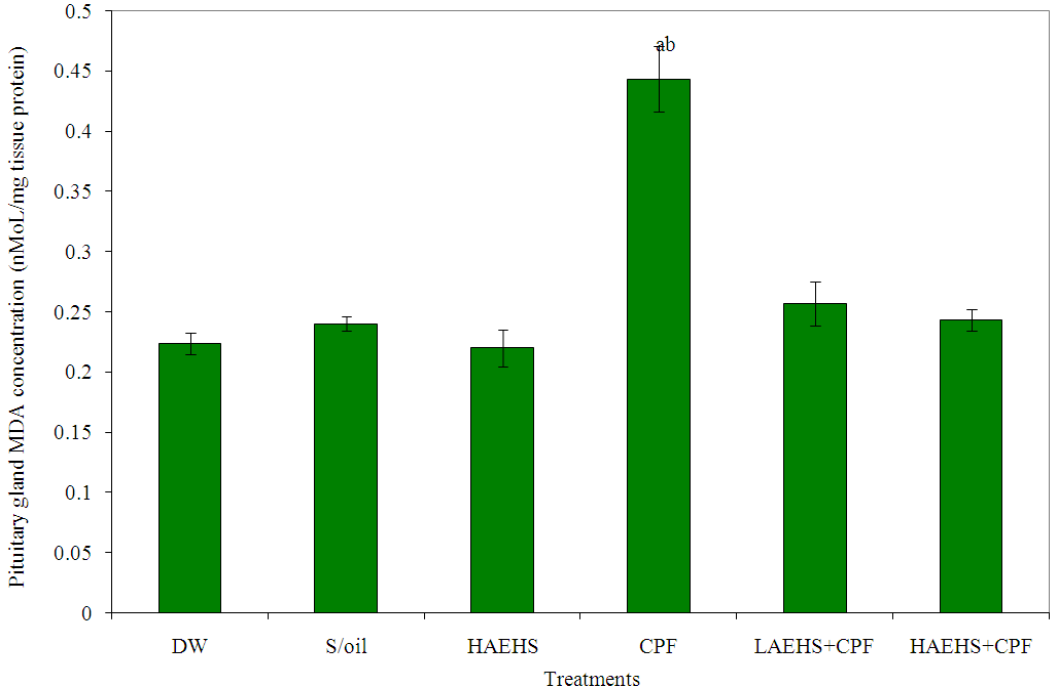

Fig. 8. Effect of subchronic exposure to Distil Water (DW), Soya oil (S/oil), aqeous extract of *H. Sabdariffa* (HAEHS) and /or Chlorpyrifos (CPF) on pituitary gland MDA concentration in adult male wistar rats. [ab]p<0.01 Versus DW, S/oil, HAEHS, LAEHS+CPF and HAEHS+CPF

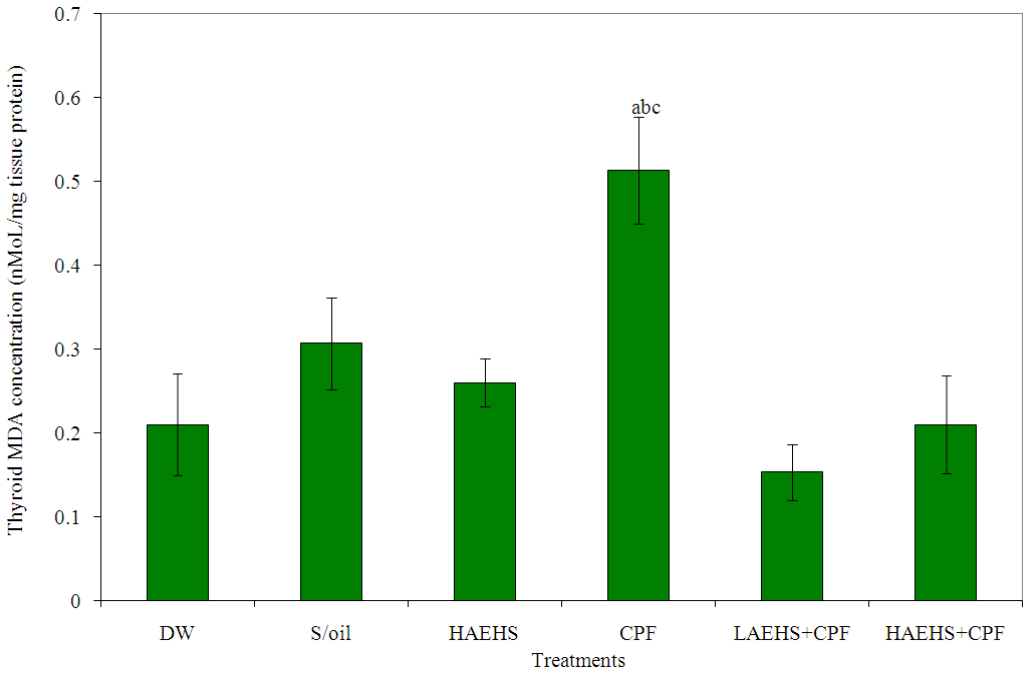

Fig. 9. Effect of subchronic exposure to Distil Water (DW), Soya oil (S/oil), aqeous extract of *H. Sabdariffa* (HAEHS) and/or Chlorpyrifos (CPF) on thyroid MDA concentration in adult male Wistar rats. [ab]p<0.05 versus DW, S/oil, HAEHS, LAEHS+CPF and HAEHS+CPF, [c]p<0.01 Versus HAEHS+CPF

3.2. Thyroid Hormones Concentrations

There was a significant ($p<0.05$) decrease in T_3 concentration in the CPF group, when compared to that obtained from the S/oil, HAEHS or HAEHS + CPF group (**Fig. 4**). There was a significant ($p<0.05$) decrease in T_4 concentration in the CPF group, when compared to that of S/oil, LAEHS +CPF and HAEHS +CPF groups, respectively (**Fig. 5**). There was significant ($p<0.01$) decrease in TSH concentration in the CPF group compared to the S/oil or HAEHS group. In addition, there was a significant ($p<0.01$) decrease in TSH concentration in S/oil group compared to that of LAEHS+CPF and HAEHS+CPF groups, respectively (**Fig. 6**).

3.3. Serum Acetylcholinesterase Activity

There were significant increases ($p<0.05$) in the AChE activity in the DW, HAEHS, LAEHS + CPF and HAEHS + CPF groups relative to that of the CPF group, respectively (**Fig. 7**).

3.4. Pituitary and Thyroid Glands Malondealdehyde Concentrations

The pituitary MDA concentration in the CPF group was significantly higher when compared to that of DW ($p<0.01$), S/oil ($p<0.01$), HAEHS ($p<0.01$), LAEHS+CPF ($p<0.05$) and HAEHS+CPF ($p<0.01$) groups, respectively (**Fig. 8**).

The thyroid gland MDA concentration of the CPF group was significantly higher compared to that of the DW ($p<0.05$), HAEHS ($p<0.05$), LAEHS+CPF ($p<0.01$) or HAEHS+CPF ($p<0.05$) group (**Fig. 9**).

4. DISCUSSION

In the present study, there was no significant difference in the FSH concentration between the groups when compared to one another. However, the CPF group had the lowest FSH concentration when compared to other groups. This observation agreed with the previous work that pesticides, including CPF lowered the FSH level (Zidan, 2009; Mandal and Das, 2011; Uslu *et al*., 2013; Shittu *et al*., 2013). FSH, a member of pituitary glycoprotein, plays a dual role in spermatogenesis by directly affecting the sertoli cells to stimulate and initiate germ cell number and indirectly, enhances androgen production by the Leydig cells (O'Shaughnessy *et al*., 2010). FSH and androgen act to stimulate and maintain spermatogenesis. The low FSH concentration in the CPF group, apparently, occurred as a result of

dysfunction in all the stages of hormonal regulation involving hormone receptor recognition and binding, hormone post-receptor activation, thyroid function and central nervous system (Mathur *et al*., 2010). OPs have been shown to disrupt the hypothalamo-pituitary endocrine function (Diamanti-Kandarakis *et al*., 2009; Fattahi *et al*., 2009), resulting in alteration in the levels of gonadotropins. Similarly, OPs have been reported to possess androgen receptor antagonist and suppress the gene responsible for gonadotropin synthesis (LH and FSH) or steroidogenesis (Liu *et al*., 2012).

The present study has shown that CPF increased the concentration of MDA in both the pituitary and thyroid glands. Previous studies have also shown increase in MDA concentration in various organs following CPF exposure (Ambali *et al*., 2010; Bas and Kalender, 2011; Shittu *et al*., 2012a). MDA is a by product of lipidperoxidation resulting from interaction of oxygen radicals with polyunsaturated fatty acids residues in membrane phospholipids that damages important biomolecular (Naudi *et al*., 2013). Oxidative damages has been reported to be a key factor in the subcellular damage resulting from pesticide exposure (Kumar *et al*., 2013). Thus the high MDA contents of both pituitary and thyroid tissues in the CPF group are indications of the level of lipoperoxidative changes, reflecting alteration in the structural and, consequently functional status of the organs. Furthermore, increase in lipid peroxidation in the pituitary might have resulted from failure of internal antioxidant system of the body to curtail the ROS being generated (Umosen *et al*., 2012) as a result of exposure to CPF and its ability to penetrate the blood-brain barrier (EL-Hossary *et al*., 2009). It is documented that the brain is most susceptible to lipid peroxidation due to its high oxygen utilization, relatively poor antioxidant system and the presence of easily oxidizable fatty acids (Guest and Grant, 2012). The increase in pituitary and thyroid MDA concentration which is indicative of participation of free radical-induced damage to the pituitary and thyroid glands may be responsible for the decrease in the concentration of FSH, LH, testosterone, T_3 and T_4 respectively observed in the CPF treated group.

The lipoperoxidative damage to the pituitary glands of the CPF group may have altered its structural integrity and functional status consequently affecting the synthesis and release of the gonadotropins. The improvement in the FSH concentration in the group pretreated with HS is a demonstration of the ameliorative potentials of HS on CPF –evoked pituitary lipoperoxidative changes.

The study showed a decrease in the FSH and LH concentrations in the CPF group when compared to the other groups. The reduced concentration of FSH and LH concentrations in the CPF group agreed with those recorded by previous workers following pesticide exposure (Zidan, 2009). This observation may be related to the inhibitory effect on genes, involved in gonadotropin synthesis, or interference with steroidogenesis (Liu *et al*., 2012). Similary, CPF has been reported to cause damage to the GnRH gene expression, hence reduction in LH and FSH (Sai *et al*., 2013) The AChE inhibition by CPF could lead to impulse blockade, which may have suppressed the brain synthesis and/or release of gonadotropins (LH and FSH) due to inhibition of their releasing hormone (Watts, 2012; Sai *et al*., 2013). Low levels of LH concentration have been shown to have long term effect on the Leydig cells involved in testosterone production in males (Pantalone and Faiman, 2012) while FSH plays a significant role in the maturational stages of spermatozoa. In the present study, pretreatment with Aqueous HS increased the FSH and LH concentrations. This indicates that oxidative damage to the hypothalamus and pituitary gland may have played a significant role in decreasing the FSH and LH concentrations recorded in the CPF group.

The decrease in the testosterone concentration recorded in the CPF group when compared to the other groups may be linked to the inhibitory effect of the pesticide on the secretion of pituitary gonadotropins (FSH and LH), which aids in testosterone biosynthesis (EL-Kashoury *et al*., 2010). Decrease in testosterone concentration may have occurred due to direct damage to the Leydig cells (Zidan, 2009). The increased MDA concentration indicates lipoperoxidative changes, hence possible oxidative damage to the testicular tissues, including the Leydig cells. These changes apparently impaired the synthesis of testosterone. Therefore, the low testosterone concentration in the CPF group can be linked to combined effects of oxidative stress induced lesions in the brain and testicular tissues. The aparent restoration of the testosterone concentration in the groups pretreated with aqueous HS indicates its antioxidative properties. The preservation of the integrity of testicular tissues, especially that of the Leydig cells due to the antioxidant effect of aqueous HS may have apparently enhanced the biological response to LH, which subsequently stimulated it to produce testosterone.

The increased T_3 and T_4 concentrations in the CPF group may be related to peroxidative damage to the thyroid glands as demonstrated by increased MDA concentration in this organ. Furthermore, the increased TSH concentration following CPF exposure reflects the response of this hormone to the lowered T_3 and T_4 concentrations. TSH stimulates the thyroid glands to increase the elaboration of T_3 and T_4. However, the failure

of the thyroid glands to respond to increased TSH stimulation for the elaboation of T_3 and T_4 in the CPF group may have been due to peroxidative damage to the gland. The effect of thyroid hormones in both male and female reproduction cannot be overemphasized. Apart from its effect on the gonadotropins, the damage to the thyroid gland may have been partly responsible for the altered reproductive status that was previously reported following CPF exposure (Shittu *et al*., 2013). Pretreatment with HS in the present study was shown to improve the concentrations of T_3 and T_4 and therefore moderated the CPF-evoked increase TSH concentration. This may have been due to the antioxidant property of HS, which protects the thyroid glands from the peroxidative damage.

The decreased activity of serum AChE observed in the CPF group can be linked to the inhibitory action of CPF on the enzyme, which subsequently led to accumulation of ACh at the post synaptic ganglion and cascade of dysfunctions in the peripheral and central pathways. Correlation between decreased AChE activity and increased MDA concentration has been previously reported (Rastogi *et al*., 2009). The significant increase in serum AChE activity seen in the groups pretreated with AEHS shows the AChE restoration potential of this plant, which can be ascribed to its antioxidant property.

5. CONCLUSION

In conclusion, the study has shown that subchronic exposure to CPF caused endocrine disruption, demonstrated by low FSH, LH, testosterone, T_3 and T_4 concentrations. Pituitary and thyroid glands lipoperoxidation and impairment of AChE activity have been shown to have contributed to the alteration in sex and thyroid hormones following subchronic CPF exposure. The improvement in sex and thyroid hormones concentration following pre-treatment with HS may have been partly due to its antioxidants and AChE restoration activities. Therefore, HS may be beneficial in providing protection to altered reproductive capacity in individuals occupationally exposed to CPF and by extension to other OP insecticides.

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