RESEARCH ARTICLE

Therapeutic Potential of Date Palm Pollen for Testicular Dysfunction Induced by Thyroid Disorders in Male Rats

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Abstract

Hyper- or hypothyroidism can impair testicular function leading to infertility. The present study was designed to examine the protective effect of date palm pollen (DPP) extract on thyroid disorder-induced testicular dysfunction. Rats were divided into six groups. Group I was normal control. Group II received oral DPP extract (150 mg kg⁻¹), group III (hyperthyroid group) received intraperitoneal injection of L-thyroxine (L-T4, 300 μg kg⁻¹; i.p.), group IV received L-T4 plus DPP extract, group V (hypothyroid group) received propylthiouracil (PTU, 10 mg kg⁻¹; i.p.) and group VI received PTU plus DPP extract. All treatments were given every day for 56 days. L-T4 or PTU lowered genital sex organs weight, sperm count and motility, serum levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone (T), testicular function markers and activities of testicular 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD). Moreover, L-T4 or PTU increased estradiol (E2) serum level, testicular oxidative stress, DNA damage and apoptotic markers. Morphometric and histopathologic studies backed these observations. Treatment with DPP extract prevented LT4- or PTU induced changes. In addition, supplementation of DPP extract to normal rats augmented sperm count and motility, serum levels of LH, T and E2 paralleled with increased activities of 3β-HSD and 17β-HSD as well as testicular antioxidant status. These results provide evidence that DPP extract may have potential protective effects on testicular dysfunction induced by altered thyroid hormones.

Introduction

Thyroid hormones (THs) have numerous actions including regulation of lipid and carbohydrate metabolism, oxygen consumption, normal growth and development. Alterations in THs
levels (hyper- or hypothyroidism) cause certain health problems e.g. Hashimoto’s and Graves’s diseases as well as cardiovascular and hepatocellular complications [1, 2]. For a long time, the gonads were believed to be unresponsive to THs; however, THs receptors were detected in human and rat testes throughout the life span [3]. Through these receptors, THs can regulate the maturation and growth of testis and control Sertoli cell and Leydig cell proliferation and differentiation during testicular development in mammals [4]. Moreover, studies have shown that thyroid dysfunction results in altered sex hormones levels, impaired testicular function and eventually infertility [5–7]. The mechanism underlying testicular dysfunction that results from thyroid disorders is still vague. However, observations from previous reports suggest oxidative stress, lipid peroxidation [6, 8] and cellular apoptosis [9, 10] as major culprits. THs modulate oxidative stress as they profoundly promote mitochondrial oxygen consumption [11]. Also, testes are rich in polyunsaturated fatty acids and poor in antioxidant defense; thus, they are more vulnerable to peroxidation injury than other tissues [12, 13]. Therefore, several antioxidant or anti-apoptotic agents have been used to ameliorate thyroid disorder-induced testicular dysfunction [5, 13].

The use of medicinal plants in the treatment of diseases and disorders dates back to ancient time and has considerably contributed to the development of pharmaceuticals where ~ 25% of modern drugs are derived from plants [14]. Date palm (Phoenix dactylifera L.) is considered native to the Middle East region as it has been cultivated since at least 6000 BC [15]. Various parts of date palm are widely used in traditional medicine for treating various disorders [16–18]. Date palm pollen (DPP) has been used by the early Egyptians and ancient Chinese as rejuvenating agent, especially as a cure for male infertility, and are used worldwide as dietary supplement [19, 20]. Moreover, DPP has been shown to have anti-inflammatory, aphrodisiac, anti-coccidial and anti-apoptotic activities [21–23]. Egyptian DPP has been shown to contain wide array of biochemically and nutritionally vital substances such as certain essential and non-essential amino acids, trace elements, fatty acid and vitamins as well as important flavonoids including rutin, quercetin, luteolin-7-O-β-D—glucoside, apigenin, isorhamnetin-3-O—glucoside and naringin. [20, 24, 25]. Furthermore, Egyptian DPP was reported to have estrogenic compounds e.g. estradiol (E2), estriol, and estrone that can alleviate infertility through their gonadotrophic activity in male rat [24, 26–28].

To the best of our knowledge, the role of DPP extract in ameliorating the testicular dysfunction induced by thyroid disorder has not been studied yet. Therefore, we designed the present study to determine whether DPP extract could attenuate thyroid disorder-induced abnormalities in testicular tissue, and to understand the possible biochemical and molecular mechanisms underlying the DPP effects.

**Materials and Methods**

2.1. Chemicals

Levo-thyroxine (L-T4), 6-n-propyl-2-thiouracil (PTU), 2,2’-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, quercetin, thiobarbituric acid, reduced glutathione (GSH), 5,5’-dithiobis (2-nitrobenzoic acid), sulfanilamide, N-(1-naphthyl) ethylenediamine, bovine serum albumin (BSA) and vanadium chloride were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest analytical grades commercially available.

2.2. Collection and extraction of date pollen sample

DPP was obtained from local herbal market in Met Ghamr, El-Dakahlia Governorate, Egypt (latitude: 30.743 and longitude: 31.43). It was collected in March 2013, air dried and kept at 20°C till extraction. About 2.5 Kg of DPP powder was extracted with 80% ethanol (3 ×5 liters)
by using Ultraturrax T25 homogeniser at temperature ≤ 25°C. The filtrate was collected three
times at 24 h intervals during a total extraction period of 72 h. The collected extract was evapo-
rated under reduced pressure, lyophilized to give 450 g of yellowish semisolid residue and kept
at 4°C protected from light until use [21].

2.3. Characterization of DPP extract

2.3.1. Minerals analysis, phenolic and flavonoid content. The minerals content of DPP
extract was determined according to AOAC [29]. Measurements were carried out using atomic
absorption spectrophotometer (Perkin Elmer 100). The data were calculated as μg/g extract.
The total phenolic content was assessed according to the method of Conde-Hernández and
Guerrero-Beltrán [30] using gallic acid as standard and calculated as mg gallic acid/g extract.
Also, total flavonoid content was assessed according to the method of Corpuze et al. [31] using
quercetin as a standard and calculated as mg quercetin /g extract.

2.3.2. Determination of antioxidant activity. The antioxidant potential of DPP extract
was determined by the DPPH radical scavenging assay according to the method of Brand-Williams et al. [32] with a minor modification. Increasing concentrations (14–400μg/ml) of the
DPP ethanolic extract in 1ml were mixed with equal volumes of 0.004% DPPH ethanolic solu-
tion. The mixture was shaken and incubated in the dark at room temperature. After 30 min the
reaction estimated by measuring the absorbance at 517nm. The radical scavenging activity was
calculated as a percentage of DPPH discoloration using the following equation:-

\[
\text{Radical Scavenging Activity \%} = \frac{A_0 - A_1}{A_0} \times 100
\]

Where \( A_0 \): absorbance of the control and \( A_1 \): absorbance of the test reaction. The extract con-
centration providing 50% inhibition (IC\(_{50}\)) was calculated from plotting inhibition percentage
against the corresponding extract concentration. All determinations were performed in tripli-
cate. The estimated IC\(_{50}\) represents the concentration of antioxidant required to scavenge 50%
of the radicals in the reaction mixture and is inversely related to the antioxidant activity.

2.4. Animal and experimental procedure

2.4.1 Ethics statement. The current investigation conforms to the standard ethical proce-
dures and policies approved by Ethical Committee for Animal Experimentation at Faculty of
Pharmacy, Cairo University and were approved by the Guide for the Care and Use of Labora-
tory Animals published by the US National Institutes of Health (NIH Publication No. 85–23,
revised 1996).

2.4.2 Animals and experimental design. Adult male Wistar rats weighing (250 ± 25g)
were obtained from the animal house of the National Organization for Drug Control and
Research, Egypt. They were allowed two weeks for accommodation to the new environment
before starting the experiment. The rats were housed at 23 ± 2°C and 55 ± 5% humidity with
12 h light/dark cycle and were given standard rodent chow and drinking water ad libitum.
Sixty rats were randomized into six groups (ten per group). Group I: (normal control
group), animals received distilled water orally (p.o.) and alkaline saline intraperitoneally (i.p.).
Group II: animals received DPP extract (150 mg kg\(^{-1}\), p.o.) [21]. Group III: (hyperthyroid

group), animals received L-T4 (300μg kg\(^{-1}\), i.p.) dissolved in alkaline saline [6]. Group IV:
(L-T4+DPP group), animals received DPP extract (150 mg kg\(^{-1}\), p.o.) and L-T4 (300μg kg\(^{-1}\),
i.p.). Group V: (hypothyroid group), animals received PTU (10 mg kg\(^{-1}\), i.p.) dissolved in alka-
line saline [33]. Group VI: (PTU+DPP), animals received DPP extract (150 mg kg\(^{-1}\), p.o.) and
PTU (10 mg kg\(^{-1}\), i.p.). The animals received the indicated treatments every day for 56 days.
that was chosen based on the time necessary to complete a spermatogenic cycle in rats [34]. The rats were weighed daily and the body weight was averaged for each week.

2.5. Blood collection and sample analysis

After completion of the treatment schedule, blood samples were collected from the retro-orbital sinus, under mild ether anesthesia and serum was separated, aliquoted and stored at -20°C till determination of thyroid hormones, sex hormones and total cholesterol. Serum level of free T3 (fT3), free T4 (fT4), thyroid stimulating hormone (TSH), testosterone (T), luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (E2) were determined by ELISA kits supplied from Monobind Inc. (USA) according to the manufacturer’s instructions.

2.6. Tissue preparation and sample analysis

After blood collection, the rats were immediately sacrificed by decapitation under mild ether anesthesia. Pairs of testes and epididymis, seminal vesicles (without the coagulating and full of secretion) and prostate gland were removed, cleared from adhering tissue, washed in ice-cold 1.15% KCL, blot dried and weighed. The right epididymis was used for estimating sperm count, while the left one was used for assessing sperm motility. The right testis was kept in 10% formal saline for the histopathological examination, while the left testis was homogenized in ice-cold 1.15% KCL to make 10% homogenate. An aliquot of homogenate was used for estimating malondialdehyde (MDA) level and the other aliquot was centrifuged at 10,000 rpm at 4°C for 30 min and used for measuring the rest of the biochemical parameters.

2.6.1. Evaluation of testicular marker enzymes. An aliquot of supernatant was used for determining the activities of acid phosphatase (ACP), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) using kits supplied from Quimica Clinica Aplicada S.A. (Spain), lactate dehydrogenase (LDH) by a kit supplied from Reactivos GPL (Spain) and glucose-6-phosphate dehydrogenase (G6PD) using a kit supplied from Worthington Biochemical Corporation, all according to the manufacturer’s instructions. In addition, sorbitol dehydrogenase (SDH) was measured according to the method of Chauncey et al. [35]. The protein content was determined according to Lowry et al. [36].

2.6.2. Determination of testicular 3β-hydroxyysteriod dehydrogenase (3β-HSD) and 17β-hydroxyysteriod dehydrogenase (17β-HSD) activities. Testicular 3β-HSD and 17β-HSD activities were measured according to the methods of Talalay [37] and Jarabak et al [38], respectively. The reaction mixture in a total volume of 2 ml contained 100 μmol sodium pyrophosphate buffer (pH 8.9), 0.5 μmol NAD+ for 3β-HSD and NADP+ for 17β-HSD, 0.14 μmol dehydroepiandrosterone for 3β-HSD and 0.5 μmol testosterone for 17β-HSD and 20 μg equivalent of microsomal protein as enzyme source. The reactions were carried out in a quartz cuvette at 25°C. The change in absorbance was measured at 340 nm for 3 min. Protein content in the enzyme source was estimated by the method of Lowry et al. [36] using bovine serum albumin as standard.

2.6.3. Oxidative stress parameters. Lipid peroxidation was quantified as MDA according to Uchiyama and Mihara [39], and expressed as nmol/g tissue. Nitric oxide (NO) content was quantified indirectly as nitrite according to the method of Miranda et al. [40], which depends on reduction of nitrate to nitrite by vanadium chloride. The released nitrites were colorimetrically detected by Griess reagent and NO levels were expressed as mmol/g tissue.

GSH content was evaluated by using the method of Van Doorn [41], which is based on the development of a stable yellow color when 5,5′-dithiobis (2-nitrobenzoic acid) is added to sulfhydryl compounds. Superoxide dismutase (SOD) activity was determined by measuring the inhibition of pyrogallol auto-oxidation at pH 8.5 according to Nandi and Chatterjee [42].
Catalase (CAT) activity was estimated from the ability of the tissue to decompose H₂O₂, whose concentration can be followed at 240 nm [43]. Glutathione peroxidase (GPx) activity was assayed by measuring oxidation rate of NADPH in the presence of H₂O₂, GSH and GR Glutathione reductase [44]. GR activity was assayed according to Long and Carson [45].

2.7. Epididymal sperm concentration and motility
Spermatozoa in the epididymis were counted by a modified method of Yokoi et al. [46]. Briefly, the epididymis was minced with anatomical scissors in 5 ml saline, placed in a rocker for 10 min, and tissue debris was allowed to settle for 2 min. The supernatant was diluted 1:100 with a solution containing 5 g sodium bicarbonate, 1 ml formalin (35%) and 25 mg eosin per 100 ml of distilled water. Approximately 10μl of the diluted sperm suspension were transferred to a hemocytometer and total sperm number was determined. Motility was evaluated according to the method described by Sonmez et al. [47]. Briefly, fluid was obtained from the caudal epididymis with a pipette and diluted to 2 ml with buffered sodium citrate solution (2.9%) pre-warmed to 37°C. Motility was visually evaluated at 400× magnification within 2–4 min of sperm isolation from the cauda. Estimations were performed from three different fields in each sample. The mean was used as the final motility score and data were expressed as percentages.

2.8. The comet assay
A piece of the left testis was used to prepare a cell suspension containing primarily Sertoli cells and spermatogonia that were isolated with the multiple digestion steps. The comet assay was done according to Singh et al. [48] with slight modification. Briefly, fully frosted slides were pre-coated on each end with 100 μl of 0.8% agarose in phosphate buffered saline, covered with a 22-mm × 22-mm glass coverslip and left at room temperature for 20 minutes. About 10000 cells were mixed with 70 μl of 1% low-melting-point agarose in phosphate buffered saline. The mixture was immediately spread onto each end of a pre-coated slide and covered with a fresh glass coverslip. After lysis, denaturation, electrophoresis, neutralization and staining, the slides were examined with a fluorescence microscope. For each slide, 100 cells were counted at least twice. The comets were captured with an Olympus fluorescent microscope equipped with a CCD camera, and the images were quantitatively evaluated for the percentage of DNA tail, tail length (μm), and tail moment using CASP software (Comet Assay Software Project 1.2.2).

2.9. Histological studies
The testes were removed and kept in 10% formol saline for 24 h, dehydrated in ethanol and embedded in paraffin. Sections were cut at 4 μm thicknesses, mounted on slides and stained with hematoxylin and eosin [49]. Some sections were mounted on positive charged slides for immunohistochemical studies. All histopathologic processing and assessment of specimens were performed by an experienced histologist blinded to the identity of the sample being examined to avoid any bias.

2.9.1. Immunohistochemistry staining for Fas-L and caspase-3. Tissue sections were deparaffinized, rehydrated and treated with 10% H₂O₂ to reduce endogenous peroxidase. For antigen retrieval, tissue sections were heated in citrate buffer pH 6.0 then cooled. The slides were blocked with 5% BSA in Tris buffered saline (TBS). The sections were immunostained with polyclonal rabbit anti-Fas L or anti-caspase-3 (Neo Markers Laboratories, Westinghouse, USA) in TBS containing 5% BSA. The slides were washed with TBS and incubated with peroxidase conjugated goat anti-rabbit immunoglobulin antibody. Finally, the sections were washed with TBS and incubated in diaminobenzidine HCl containing H₂O₂ that revealed the sites of
the reaction as brown areas. Counterstaining was performed using Meyer’s hematoxylin and the slides were visualized under light microscope.

2.9.2. Morphometric measurements. Using the computer assisted software Leica Qwin 500 LTD image analyzer; the following parameters were evaluated in 10 non-overlapping fields at a magnification 200x for each specimen:

1. Seminiferous tubules mean diameter and epithelial heights as well as Leydig cells number in hematoxylin and eosin stained sections.
2. Mean area percent of Fas-L and caspase-3 immunoreactivities in immunostained sections. They were measured in relation to a standard measuring frame.

2.10. Statistical analysis
Quantitative data were expressed as mean ± SEM. One way analysis of variance (ANOVA) was used for comparing different groups. Pair wise comparisons were done using Tukey-HSD test. All analyses were performed using SPSS 18 for Windows (SPSS Inc., Chicago, USA) and differences were considered statistically significant at \( p<0.05 \) for all tests.

Results
3.1 Body and genital sex organ weights
All weights are provided in Table 1. Treatment with L-T4 or PTU caused a significant decrease in body weight by 22.3% and 24.1%, respectively in comparison with normal control group. DPP extract ameliorated L-T4-induced decrease in body weight, however; had no effect on the body weight of PTU-treated or normal rats. Also, L-T4 or PTU induced a remarkable decrease in the weights of testes (13% and 14.6%, respectively), epididymis (29.7% and 33.3%, respectively), and prostate gland (52.3% and 61.5%, respectively) as compared to the normal control group. These changes were ameliorated by DPP extract co-administration. Additionally, administration of DPP extract to normal rats increased the weight of epididymis (13.1%), prostate gland (13.9%) and seminal vesicle (19%) over normal control animals.

| Weight (g)         | Cont.  | Ext    | L-T4   | L-T4 + Ext | PTU    | PTU + Ext |
|--------------------|--------|--------|--------|------------|--------|-----------|
| Final body         | 330.5 ± 5.33 | 338.4 ± 7.27 | 256.9 ± 2.06 **a**b**c** | 285.2 ± 3.22 **a**b**c**d**e**f** | 250.8 ± 5.11 **a**b**c**d**e**f** | 258.7 ± 4.51 **a**b**c**d**e**f** |
| Testes             | 3.22 ± 0.06 | 3.34 ± 0.06 | 2.80 ± 0.06 **a**b**c**d**e**f** | 3.01 ± 0.07 **a**b**c**d**e**f** | 2.75 ± 0.05 **a**b**c**d**e**f** | 2.96 ± 0.03 **a**b**c**d**e**f** |
| Epididymis         | 0.435± 0.012 | 0.492 ± 0.013 | 0.306 ± 0.010 **a**b**c**d**e**f** | 0.376 ± 0.019 **a**b**c**d**e**f** | 0.290 ± 0.010 **a**b**c**d**e**f** | 0.356 ± 0.009 **a**b**c**d**e**f** |
| Prostate gland     | 0.65± 0.01 | 0.74 ± 0.03 **a** | 0.31 ± 0.02 **a**b**c**d**e**f** | 0.50 ± 0.03 **a**b**c**d**e**f** | 0.25 ± 0.02 **a**b**c**d**e**f** | 0.49 ± 0.03 **a**b**c**d**e**f** |
| Seminal vesicle    | 0.79 ± 0.01 | 0.94 ± 0.05 **a** | 0.47 ± 0.02 **a**b**c**d**e**f** | 0.61 ± 0.04 **a**b**c**d**e**f** | 0.41 ± 0.02 **a**b**c**d**e**f** | 0.63 ± 0.03 **a**b**c**d**e**f** |

The data are presented as mean ± SEM (\( n = 10 \) rats/gp). The symbols
* and ** represent statistical significant at \( p<0.05 \) and \( p<0.01 \), respectively.
**a**: significant difference from normal control values,
**b**: significant difference from Ext values,
**c**: significant difference from L-T4 values and
**d**: significant difference from PTU values.

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3.2 Effect on thyroid hormones and TSH

The alteration in thyroid functional state was confirmed by assessing serum thyroid hormones and TSH levels. Hyperthyroidism was established by the observed increase in fT3 and fT4 level by 230.3% and 106.9%, respectively, and decrease in TSH by 29.4% in L-T4 treated group as compared to normal control group. On the other hand, PTU induced hypothyroidism as demonstrated by the significant decrease in fT3 (49.8%) and fT4 (59.9%) levels and increase in TSH (25.5%) level in comparison with normal control group (Fig 1). Co-administration of DPP extract with L-T4 triggered a substantial decrease in fT3 (44.2%) and fT4 (49.4%) and increase in TSH (25%) as compared to L-T4 treated group. Whereas, co-treatment of PTU group with DPP extract significantly enhanced the decline in fT3 and fT4 level by 10.4% and 19.1%.

Fig 1. Effect of DPP extract (Ext) on serum levels of: (A) fT3, (B) fT4 and (C) TSH in normal, L-T4- and PTU-treated rats. The data are presented as mean ± SEM (n = 10 rats/gp). The symbols a**, b**, c**, d** represent statistical significant at p<0.05 and p<0.01, respectively. a: significant difference from normal control values, b: significant difference from Ext values, c: significant difference from L-T4 values and d: significant difference from PTU values.

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Table 2. Effect of DPP extract (Ext) on serum levels of LH, FSH, T and E2 as well as on T/E2 and T/LH ratios in normal, L-T4- and PTU-treated rats.

| Parameters       | Experimental groups       |
|------------------|---------------------------|
|                  | Cont.  | Ext.     | L-T4 | L-T4 + Ext | PTU | PTU + Ext |
| LH (µg/L)        | 1.11 ± 0.037  | 1.32 ± 0.051 b* | 0.89 ± 0.018 a** | 1.14 ± 0.035 b** | 0.83 ± 0.029 b** | 1.18 ± 0.081 b** |
| FSH (IU/L)       | 7.48 ± 0.27  | 7.44 ± 0.23 | 5.74 ± 0.25 b** | 7.30 ± 0.43 b** | 6.20 ± 0.12 b** | 7.02 ± 0.14 a** |
| T (ng/ml)        | 2.03 ± 0.10  | 2.72 ± 0.10 a** | 1.30 ± 0.06 b** | 1.91 ± 0.09 b** | 0.85 ± 0.07 b** | 1.82 ± 0.14 b** |
| E2 (pmol/L)      | 240.67 ± 11.43 a** | 199.76 ± 7.04 b** | 232.76 ± 8.34 b** | 193.44 ± 10.36 b** |
| T/E2 × (10^−3)   | 12.78 ± 0.75  | 13.77 ± 0.95 | 5.46 ± 0.24 b** | 9.62 ± 0.55 b** | 3.76 ± 0.24 b** | 9.35 ± 0.45 a** |
| T/LH             | 1.82 ± 0.05  | 2.04 ± 0.06 a* | 1.46 ± 0.04 b** | 1.67 ± 0.04 b** | 1.01 ± 0.06 b** | 1.54 ± 0.06 a** |

The data are presented as mean ± SEM (n = 10 rats/gp). The symbols 
* and 
** represent statistical significant at p < 0.05 and p < 0.01, respectively.
b: significant difference from normal control values,
c: significant difference from Ext. values,
d: significant difference from L-T4 values and
e: significant difference from PTU values.
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respectively, and caused non-significant elevation in TSH level as compared to PTU treated group (Fig 1). Interestingly, administration of DPP extract to normal rats reduced fT3 and fT4 by 19% and 17.9%, respectively as compared to normal control group.

3.3 Effects on sex hormones and testicular steroidogenic enzymes

The values obtained for serum sex hormones are shown in Table 2. Administration of L-T4 or PTU was associated with considerable reduction in LH (19.8% and 25.2%, respectively), FSH (23.3% and 17.1%, respectively) and T (36% and 58.1%, respectively) serum levels, as well as in T/E2 ratio, marker for enhanced aromatase activity, (57.3% and 70.6%, respectively) and T/LH ratio, marker for reduced Leydig cell function, (19.8% and 44.5%, respectively) along with a significant increase in E2 serum level (50.4% and 45.5%, respectively) as compared to the normal control group. To determine the potential mechanism for the diminished T production following the induction of hyper- or hypothyroidism, the activity of testicular pivotal androgenic enzymes (3β-HSD and 17β-HSD) were investigated. Induction of hyper-or hypothyroidism produced a marked decline in the activities of 3β-HSD (39.9% and 47.2%, respectively) and 17β-HSD (33.9% and 40%, respectively) as compared to the euthyroid rats (Fig 2). Co-administration of DPP extract reversed the changes induced by L-T4 or PTU in sex hormones levels as well as the activities of 3β-HSD and 17β-HSD. Such observations point to the possible protective role of DPP against testicular androgenic disorders induced by thyroid abnormalities. Moreover, administration of DPP extract to normal rats increased LH, T and E2 serum level by 18.9%, 34% and 25%, respectively and T/LH ratio (12.1%) together with enhanced activities of 3β-HSD (22%) and 17β-HSD (23.1%) as compared to the euthyroid rats.

3.4 Testicular marker enzymes

All activities are shown in Table 3. Injection of L-T4 or PTU reduced testicular activity of SDH (30.2% and 23.4%, respectively), LDH (25.2% and 18.7%, respectively), ALP (49.4% and 59.7%, respectively), ACP (31.3% and 36%, respectively) and G6PD (25.9% and 40.8%, respectively), but increased activity of AST (58.5% and 51.1%, respectively) and ALT (35.7% and 26.6%, respectively), as compared to the normal control group. Co-administration of DPP extract
normalized the activities of these testicular marker enzymes except for ALP. Furthermore, among all of the studied testicular marker enzymes, SDH and LDH activities were significantly elevated (18.8% and 20.3%, respectively) in normal rats by administration of DPP extract as compared to the normal control group.

Table 3. Effect of DPP extract (Ext) on testicular markers in normal, L-T4- and PTU-treated rats.

| Parameters (U/mg protein) | Cont. | Ext | L-T4 | L-T4 + Ext | PTU | PTU + Ext |
|---------------------------|-------|-----|------|------------|-----|-----------|
| SDH                       |       |     |      |            |     |           |
|                           | 7.61 ± 0.19 | 9.04 ± 0.35 | 5.31 ± 0.31 | 8.08 ± 0.40 | 5.83 ± 0.37 | 9.81 ± 0.65 |
| LDH                       | 1.23 ± 0.05 | 1.48 ± 0.04 | 0.92 ± 0.04 | 1.34 ± 0.09 | 1.00 ± 0.05 | 1.28 ± 0.06 |
| ALP                       | 44.88 ± 3.85 | 45.40 ± 3.07 | 22.70 ± 1.16 | 33.10 ± 2.54 | 18.08 ± 1.17 | 24.31 ± 1.43 |
| ACP                       | 24.96 ± 1.27 | 25.12 ± 1.49 | 17.15 ± 1.13 | 22.72 ± 1.75 | 15.98 ± 1.08 | 25.65 ± 1.35 |
| AST                       | 20.95 ± 1.29 | 20.64 ± 0.77 | 33.21 ± 2.15 | 21.87 ± 1.25 | 31.66 ± 1.93 | 20.13 ± 1.00 |
| ALT                       | 11.10 ± 0.55 | 11.33 ± 0.40 | 15.06 ± 0.64 | 11.51 ± 0.71 | 14.05 ± 0.64 | 11.63 ± 0.57 |
| G6PD                      | 10.92 ± 0.44 | 10.40 ± 0.43 | 8.09 ± 0.50 | 9.97 ± 0.29 | 6.47 ± 0.32 | 10.51 ± 0.45 |

The data are presented as mean ± SEM (n = 10 rats/gp). The symbols * and ** represent statistical significant at p<0.05 and p<0.01, respectively. a: significant difference from normal control values, b: significant difference from Ext values, c: significant difference from L-T4 values and d: significant difference from PTU values.

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3.5 Oxidative stress parameters

As noticed from Fig 3, induction of hyper- or hypothyroidism augmented the level of MDA (34.5% and 44.2%, respectively) and NO (25% and 17.1%, respectively); but suppressed the activity of CAT (23.5% and 47.8%, respectively), SOD (32.1% and 26.1%, respectively), GPx (35.4% and 24.5%, respectively) and GR (35.7% and 22.2%, respectively), as well as the level of GSH (37.6% and 22.2%, respectively), with respect to the normal control group. Co-administration of the DPP extract prevented these alterations. Similarly, supplementation of the DPP extract to normal rats reduced MDA (14.4%) and NO (10.7%); but elevated CAT, SOD, GPx and GR activities by 15.4%, 30.3%, 13.4% and 18.5%, respectively and GSH level (18.5%) as compared to the normal control rats (Fig 3).

3.6 Sperm analysis

Parallel to the observed decline in testicular and steroidogenesis markers as well as the increase in oxidative stress parameters, sperm count and motility decreased by injection of L-T4 (30.3% and 29.9%, respectively) or PTU (38.8% and 34.4%, respectively) in comparison with the normal control group (Fig 4). DPP extract co-administration normalized both sperm count and motility in L-T4 or PTU-treated rats. Moreover, testicular function enhancing effect of the DPP extract was observed in normal rats as evidenced by the improved sperm number (37.7%) and motility (14.3%) as compared to the normal control rats.

3.7 DNA damage of testicular cells

Comet assay demonstrated considerable DNA damage in hyper and hypothyroid rats’ testes. Administration of L-T4 or PTU caused substantial increase in % DNA in tail (7.6 and 6.2 fold, respectively), tail length (8.1 and 5.9 fold, respectively) and tail moment (62.5 and 36.7 fold, respectively), as compared to normal control group. Co-administration of DPP extract counteracted the observed DNA damage (Fig 5 and Table 4). Moreover, normal animals supplemented with DPP extract demonstrated enhanced DNA integrity as evidenced by decreased tail length and tail moment by 28.6% and 33.7%, respectively as compared to the normal control group.

3.8 Histological studies

Testicular sections of the control and DPP extract-supplemented normal rats showed healthy architecture consisting of uniform, well organized seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue (Table 5 and Fig 6A and 6B). Testicular tissue of the L-T4- or PTU-treated rats showed histopathological changes characterized by degenerated, necrotic, shrunken and disorganized seminiferous tubules with irregular basement membrane, incomplete spermatogenesis, increased interstitial space area, wide lumens with and few sperms (Table 5 and Fig 6C and 6D). Testes form rats treated with DPP extract plus L-T4 or PTU revealed less prominent histopathological changes when compared with those from the L-T4 or PTU group. In these specimens, the seminiferous tubules restored their normal shape and diameter with complete spermatogenic series (Table 5 and Fig 6E and 6F).

3.8.1. Apoptotic markers expression in testicular cells

Immunohistochemical findings revealed that administration of L-T4 or PTU induced the expression of caspase-3 (20.7% and 22.9%, respectively) and Fas-L (27.3% and 30.3%, respectively), compared to the normal control group (Figs 7C and 7D, 8C and 8D, 9A and 9B). Co-administration of DPP extract reduced the L-T4 or PTU-induced overexpression of these pro-apoptotic markers to normal level (Figs 7E and 7F, 8E and 8F, 9A and 9B).
Fig 3. Effect of DPP extract (Ext) on testicular level of oxidative stress parameters: (A) MDA, (B) NO, (C) CAT, (D) SOD, (E) GPx, (F) GR and (G) GSH in normal, L-T4- and PTU- treated rats. The symbols * and ** represent statistical significant at $p<0.05$ and $p<0.01$, respectively. \(^{a}\): significant difference from normal control values, \(^{b}\): significant difference from Ext values, \(^{c}\): significant difference from L-T4 values and \(^{d}\): significant difference from PTU values.

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3.8.2. Morphometric measurements. L-T4 or PTU induced marked decline in seminiferous tubules diameter (12.2% and 27.5%, respectively) and epithelial heights (18.5% and 40.6%, respectively) in comparison with the normal control rats (Fig 10A and 10B). However, the Leydig cells count increased in L-T4-treated rats (18.7%) and decreased in PTU-treated rats (29.7%) as compared to the normal control rats (Fig 10C). Co-administration of DPP extract counteracted these changes (Fig 10A–10C). Additionally, administration of DPP extract substantially augmented seminiferous tubules epithelial heights in normal rats by 9.4% as compared to the normal control ones (Fig 10B).

Discussion

The present study demonstrated that hyper- or hypothyroidism induced abnormalities in sex hormones levels, sperm qualities, testicular marker enzymes activities and eventually testicular dysfunction that was greatly improved by DPP extract supplementation. Also, our investigation suggests that the DPP therapeutic effect may be through the prevention of apoptosis, DNA damage or oxidative stress insult.

Hyperthyroidism is well recognized for inducing body weight reduction due to the overwhelming catabolic activity of the elevated THs levels [1]. However, it is noteworthy that hypothyroidism also caused a decrease in the body weight, which may be due to loss of the growth-stimulating effect of the THs or to PTU-induced loss of appetite [5, 50]. The observed depressing effect of DPP extract on THs level in normal rats may be due to presence of phenolic and flavonoid compounds that may reduce both the thyroid iodide uptake and thyroid peroxidase activity [51].

Our study is the first to describe a novel effect of thyroid disorder or DPP extract on testicular marker enzymes: SDH and LDH are involved in the maturation and energy metabolism of spermatogenic cells and spermatozoa [52], ALP plays critical role in spermatogenic cells glucose uptake and division [53], ACP is one of the markers of testicular steroidogenesis [54] and G6PD provides reducing equivalents for steroidogenesis [52, 55]. Thus, hyper or hypothyroidism induced reduction in these enzymes activity indicated deterioration in testicular physiology.
that was ameliorated by DPP extract. On the other hand, DPP normalized the elevation of AST and ALT activities that is usually associated with reduced integrity of spermatozoa membrane and frequency of intact acrosome spermatozoa [56]. Accordingly, our data suggests that the DPP extract protected spermatozoa and improved the ability of testis to cope with stress.

Leydig cells synthesize and secrete T in response to LH [57]. Therefore, the decrease in T level in response to thyroid dysfunction may be attributed to decrease secretion of LH and/or compromised Leydig cell function as indicated by the lower T/LH ratio [58].

Fig 5. Effect of DPP extract (Ext) on DNA damage of testicular cells in normal, L-T4- and PTU-treated rats. The testicular DNA damage was measured by comet assay. (A) Control (Cont.) and (B) Ext groups show most of cells with intact DNA, (C) L-T4 and (D) PTU groups show cells with high degree of damaged DNA as well as (E) Ext+L-T4 and (F) Ext+PTU groups show cells with low degree of damaged DNA.

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decrease in the activities of the key testicular androgenesis enzymes, the 3β-HSD and 17β-HSD [59], may explain the machinery underlying the suppressed T production in hyper or hypothyroid rats. Our report is the first to describe novel stimulatory effect of DPP extract on these key steroidogenic testicular enzymes. Additionally, T biosynthesis may be adversely affected by the observed decrease in GPx activity [50]. The observed reciprocal relationship between T and E2 levels in hyper- and hypothyroid rats may be due to increased conversion of T to E2 by the enhanced aromatase activity as reflected in the marked decreased in T/E2 ratio [58].

Egyptian DPP was reported to have high contents of gonadotropin-like substances and estrogenic materials; thus, improving male fertility and augmenting serum levels of T and E2 [19, 22, 24, 27, 28] that is consistent with our findings. Also, the positive influence of DPP extract on normal serum level of T may be due to direct stimulatory effect of DPP extract on pituitary gland increasing LH release and on Leydig cell enhancing 3β-HSD and 17β-HSD activities. Furthermore, we and Hassan, [20] found the DPP to contain Zinc (S1 Table) that is required for T synthesis.

It has been reported that testicular oxidative stress is believed to be one of the causal factors for male infertility [11]. The harmful effects of hyper- or hypothyroidism may result from enhanced generation of free radicals and suppressed antioxidant enzymes in the testes [8, 13]. The elevated MDA level may be partly attributed to the high content of polyunsaturated fatty acids in the male germ cells membranes [13], thus rendering them particularly prone to lipid peroxidation. NO may cause spermatozoal dysfunction and infertility by inhibiting testicular T secretion, suppressing antioxidant enzyme activities [60] and generating peroxynitrite that is a more powerful oxidant [6]. GSH plays a pivotal role in the proliferation and differentiation of

Table 4. Effect of DPP extract (Ext) on DNA damage of testicular cells in normal, L-T4- and PTU-treated rats.

| Parameters                  | Cont.       | Ext         | L-T4        | L-T4 + Ext   | PTU         | PTU + Ext   |
|-----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Tail length (μm)            | 1.61 ± 0.12 | 1.15 ± 0.06a| 13.00 ± 0.89b | 5.01 ± 0.16c| 9.50 ± 0.62d| 7.40 ± 0.31e|
| Tail DNA %                  | 1.29 ± 0.06 | 1.19 ± 0.04 | 9.86 ± 0.51d | 4.55 ± 0.35e| 7.96 ± 0.40f| 5.93 ± 0.34g|
| Tail moment                 | 2.05 ± 0.14 | 1.36 ± 0.06a| 128.10 ± 10.82b| 22.55 ± 1.21c| 75.31 ± 5.37d| 43.51 ± 1.86e|

The data are presented as mean ± SEM (n = 10 rats/gp). The symbols * and ** represent statistical significant at p < 0.05 and p < 0.01, respectively.

†: significant difference from normal control values,
‡: significant difference from Ext values,
§: significant difference from L-T4 values and
d: significant difference from PTU values.

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Table 5. The severity of histopathological alteration in testicular tissue of different experimental groups.

| Histopathological alterations                                      | Cont. | Ext | L-T4 | L-T4+Ext | PTU | PTU+Ext |
|-------------------------------------------------------------------|-------|-----|------|----------|-----|---------|
| Degeneration and necrosis with lose of spermatogenesis in some of seminiferous tubules | —     | —   | +++  | —        | ++  | —       |
| Proliferation of interstitial Leydig cell                        | —     | —   | ++   | —        | —   | —       |
| Lose of spermatogenic series                                     | —     | —   | +++  | —        | ++  | —       |

+++ Sever, ++ Moderate, + Mild,—Nil.

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The decreased GSH level in hyper or hypothyroid rats may be due to the observed reduction in G6PD activity that is directly involved in GSH metabolism [52, 55] and/or to depression in GR activity that replenishes cellular level of GSH [50]. Consistent with our findings, the DPP extract was reported to have antioxidant effects [24, 26].

spermatogenic cells [50]. The decreased GSH level in hyper or hypothyroid rats may be due to the observed reduction in G6PD activity that is directly involved in GSH metabolism [52, 55] and/or to depression in GR activity that replenishes cellular level of GSH [50]. Consistent with our findings, the DPP extract was reported to have antioxidant effects [24, 26]. We
demonstrated that the extract has potent radical scavenging activity (S1 Fig) that may be due to its content of phenolic, flavonoid compounds, and trace minerals (S1 Table) as well as the reported presence of certain amino acids, and vitamins (A, E and C) [20, 25]. Also, the extract was reported to reduce nitric oxide synthase activity, which is consistent with the observed lowering NO level [23].
The observed imbalance in testicular oxidant-antioxidant status induced by thyroid disorder may result in considerable testicular DNA damage and apoptosis that may trigger further testicular complications [9, 10]. A significant negative correlation was reported between the level of sperm oxidative DNA damage and total sperm number [55, 61]. Fas system has been implicated as a possible regulator of germ cell apoptosis in the rat testis especially under certain pathological conditions such as hormone deprivation [52]. The protective effect of DPP extract
on testicular DNA damage and apoptosis may be attributed to the antioxidant action of extract through direct scavenging of ROS or interfering with free radical generation.

The reductions in weight of sex organs as well as morphometric measurements in hyper and hypothyroid rats are excellent indicators of gonadal toxicity [62] and may be due to the observed decrease in serum T, LH and FSH levels, increase in testicular oxidant insult, and/or enhanced testicular apoptosis and DNA damage. Testicular T is essential for the normal spermatogenesis as well as for the maintenance of the normal structural morphology and physiology of seminiferous tubule [63]. Additionally, reduced FSH level was shown to impair spermatogenesis process [64]. DPP extract ameliorated these alterations, which may be through the observed augmentation of T, LH level, antioxidant properties, protection against apoptosis and DNA damage. Such positive action of DPP extract were also demonstrated by Bahmanpour et al.[19]. It is also possible that the estrogenic content of DPP played an effective role in mediating the observed positive effects on testicular functions [65]. Estrogen regulates the reabsorption of luminal fluid in the head of epididymis causing sperm to enter cauda epididymis in concentrated form [66]. Furthermore, feeding mouse small amounts of estrogen in soy-based diet, was found to be useful for testicular function and improved germ cell development independent of gonadotropins [67]. However, adult rats fed high phytoestrogen diet displayed impaired spermatogenesis and increased germ cell apoptosis [68]. Therefore, a delicate balance between androgens and estrogens within the testicular milieu is crucial for maintenance of normal testicular physiology and reproductive function [69].

The current study indicates that alteration in thyroid hormone level adversely affected the physiological function of adult testes by suppressing steroidogenesis, spermatogenesis, as well as induction of oxidative stress and apoptosis. Treatment with DPP extract effectively prevented the deleterious effects of altered thyroid hormone levels. The fertility improving effect of DPP extract may be a result of the antioxidant properties that antagonized oxidative stress and apoptosis in testes. Moreover, the enhancement effect of DPP extract on hypothalamic—pituitary—testicular axis preserved steroidogenesis that in turn maintained normal testicular

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**Fig 9. Effect of DPP extract (Ext) on: (A) Caspase-3 and (B) Fas-L immunoreactivity of testicular cells in normal, L-T4- and PTU-treated rats.** The data are presented as mean ± SEM (n = 10 rats/gp). The symbols * and ** represent statistical significant at p<0.05 and p<0.01, respectively. a: significant difference from normal control values, b: significant difference from Ext values, c: significant difference from L-T4 values and d: significant difference from PTU values.

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functions. Consequently, DPP extract supplementation may present a potential therapeutic approach for testicular dysfunction resulting from thyroid disorders.

Supporting Information

**S1 Fig. DPPH radical scavenging activity (RSA) of DPP ethanolic extract.** All tests were conducted in triplicate and the results were expressed as mean ± SEM value. (TIF)

**S1 Table. Characterization of DPP extract (Ext).** Ethanolic extraction yield (EY) (%), 50% inhibition concentration (μg/ml), total phenolic content (mg gallic/g extract), total flavonoid content (mg quercetin/g extract) and different minerals (μg/g extract) measured in ethanolic extract of DPP. All tests were conducted in triplicate and the results were expressed as mean ± SEM value. (DOCX)
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Author Contributions
Conceived and designed the experiments: AME WAH SMR. Performed the experiments: AME WAH MMN. Analyzed the data: AME WAH MMN SMR. Contributed reagents/materials/analysis tools: AME WAH MMN. Wrote the paper: AME WAH MMN SMR.

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