INTRODUCTION

Treatment of disease began long ago with the use of herbs [1]. *Taraxacum officinale* (L.) Weber ex F.H. Wigg. (Compositae) is used in folk medicine as an anti-inflammatory, anti-oxidative, anti-carcinogenic and anti-coagulatory remedy [2]. Other health-promoting activities such as diuretic, laxative, cholagogue, anti-rheumatic, choleric and hypoglycaemic have been attributed to the use of *T. officinale* [2].

In Jordan it is also used to enhance male fertility by enhancing sperm parameters such as concentration, motility and normal morphology. However, it has been proven recently that *T. officinale* whole plant or leaves aqueous extracts decrease male fertility instead of improving it by...
causing an imbalance between spermatogonia self-renewal and differentiation [3,4].

Spermatogonial stem cells (SSCs), which are located near the basement membrane of the seminiferous tubule [5], are responsible for the maintenance of spermatogenesis in males and are capable of producing mature sperms [6].

SSC self-renewal and differentiation are controlled by several markers [7]. Glial Cell-Derived Neurotrophic Factor (GDNF) is essential for self-renewal and proliferation of SSCs [8]. GDNF works through a multi-component receptor complex composed of GDNF family receptor alpha 1 (GFRα-1) and Ret (tyrosine kinase transmembrane protein) [9].

Promyelocytic Leukaemia Zinc-Finger (PLZF) expression is restricted to the undifferentiated spermatogonia and it prevents SSCs differentiation [10]. Macrophage Colony-Stimulating Factor (MCSF) is another stem cell marker secreted by the interstitial Leydig and myoid cells; its effect is restricted to undifferentiated spermatogonia [11].

The current work was conducted to investigate if *T. officinale* root aqueous extract has anti-spermatogenic activity similar to that of the whole plant. In addition, the work aimed at studying the effects of the root aqueous extract on GFRα-1, PLZF and MCSF spermatogonial stem cells (SSC) markers.

**EXPERIMENTAL**

**Plant collection and extract preparation**

*T. officinale* was collected during the flowering season (February - October) of 2014 from Jubaiha region, Amman, Jordan. The plant was authenticated by a plant taxonomist at Raja’a Abu Eidah; The Hashemite University) and a voucher specimen (no. HU-42741) was deposited in the herbarium of the Department of Biology and Biotechnology, The Hashemite University, Zarqa, Jordan. The aqueous extract was prepared by soaking 100 g of air dried whole plant or root in 1 L of distilled water for two days at 45 °C. Afterwards, the mixture was filtrated by Whatman filter paper and then lyophilized [3,12].

**Screening of chemical constituents of *T. officinale***

The determination of the chemical composition of *T. officinale* was performed by high performance liquid chromatography coupled with mass spectrometer (HPLC-MS). Briefly, 0.1 mg of each extract was dissolved in 1 ml of 20 % acetonitrile in water. Prepared samples were homogenized and centrifuged to avoid column blocking. Samples were introduced to HPLC-MS and photodiode array detector (Finigan Surveyor PDA Plus Detector, Thermo Scientific, USA). RP/C18 column (150 mm × 4.6 mm) at a flow rate of 1 ml/min was used. HPLC-MS spectra were collected for the extracts and compared to the data found in literature. As such, assignment of the chemical composition was possible only for those compounds which were already extracted and characterized for *T. officinale*.

**Animal treatment**

Fifty adult Wistar male rats (*Rattus norvegicus*) weighing between 150 and 200 g were randomly selected and housed individually in cages. The animals were maintained under standard conditions; tap water and feed were provided ad libitum. After one week of acclimatization [12], the rats were randomly divided into five different groups, each of ten animals. The animals of the first and second groups were gavaged with 1/20 and 1/10 of *T. officinale* whole plant aqueous extract LD₅₀ and were considered the low dose whole plant receiving-group (LDWP) and the high dose whole plant-receiving-group (HDWP), respectively [3]. Rats of the third and fourth groups received 1/20 (3.68 g/kg) and 1/20 (1.84 g/kg) of the root aqueous extract LD₅₀ and were considered the low dose root-receiving group (LDR) and the high dose root-receiving group (HDR), respectively. The fifth group of animals received distilled water and was considered the control group. The doses were given orally using a gavage needle for 60 consecutive days [12].

**Fertility test**

On the 55th day of treatment, each male rat was cohabitated with two adult pro-estrus female rats. The presence of a typical mating plug and/or sperms in the vaginal smear were considered indicators of successful mating. The number, weight and sex of the offspring were determined [12].

**Male rat sacrifice**

Male rats were sacrificed by cervical dislocation, and the body weight was recorded. Rats were dissected and liver, testes, kidneys and seminal vesicles were removed and weighed. Relative organ weight was calculated by dividing the absolute organ weight (g) by the body weight of rat on sacrifice day (g) multiplied by 100. Blood was collected by heart puncture in a plain blood tube and centrifuged at 2000 rpm for 10 min.
Sperm collection

Sperm samples were collected from cauda epididymis based on the method of Soleimanzadeh and Saberivand [13]. The samples were used to study sperm count, motility and morphology [14]. In addition, epididymal sperm samples were used to study chromatin integrity by acridine orange staining [15] and agarose gel electrophoresis. For acridine orange staining, two slides were prepared for each rat and at least two hundred sperms for each slide were counted [15].

Testosterone assay

Serum testosterone level was evaluated using the Immulite 1000 immunoassay system (Siemens, Germany). Briefly, serum from control or treated rats and alkaline phosphatase-conjugated testosterone were incubated in a test tube containing polystyrene beads coated with testosterone-specific antibodies. Serum testosterone competed with the enzyme-labeled testosterone for the limited number of antibody binding sites on the beads. A chemiluminescence substrate was added to the test tube where it was hydrolyzed by the alkaline phosphatase and the absorbance was measured spectrophotometrically (Siemens, Germany).

Histological studies

One of the testes was cut into small pieces (5 × 5 mm) and fixed in Bouin’s fixative. The samples were then dehydrated in ascending alcohol series and cleared in xylene. The tissues were imbedded in pure paraffin wax and serial sections of 6 µm were made and stained with hematoxylin and eosin. Finally, the sections were cleared and mounted in DPX and then were examined by Nikon Eclipse 50i microscope.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

For quantifying mRNA level of each SSC marker [11,16], quantitative real-time polymerase chain reaction (qRT-PCR) on Line Gene 9680 BioGR thermal cycler (Bior Technology, USA) was carried out [17]. The SV Total RNA Isolation System (Promega, USA) was used for testicular RNA extraction and cDNA was prepared using Power cDNA synthesis kit (iNIRON Biotechnology). The quantification of the SSC markers (mcsf, gfra1, plzf) and β-actin (as a house keeping gene) was performed using the 2x Universal KAPA SYBR FAST qPCR Kit Master Mix (KAPA BIOSYSTEMS, USA). Each qRT-PCR was run as triplicate for each primer.

Ethical approval

The study was approved by the Institute Review Board (IRB) of The Hashemite University (AM/16/10/222/140115). Animal care, handling, and the animal experiments performed were approved by The Hashemite University Institutional Animal Care and Use Committee which conforms to the guidelines of National Research Council of the National Academics [18].

Statistical analysis

Statistical analysis of data was performed using STATISTICA 7 analysis program (StatSoft Inc., OK, USA). In order to determine differences between three or more means, one-way ANOVA with Fisher’s LSD for multiple comparisons post-tests were performed. Data are presented as mean ± SD and the level of significance was set at p < 0.05.

RESULTS

Phytochemical constituents of T. officinale

HPLC-MS was used to detect the chemical compounds present in T. officinale aqueous extracts. Various compounds were detected in the root aqueous extract including phenols (chlorogenic acid and chicoric acid), sesquiterpenes (tetracyhydrodendritin B, taraxacolide-O-β glucosepyranoside, ainsiloside and 11β-13-dihydro lactucin), triterpene (taraxasterol and α and β amyrin) and coumarins (scoptolitin and esculetin). The whole plant aqueous extract contained all of the above mentioned compounds in addition to hydroxycinnamic acid (caffeic acid) and monocafeoyltartaric acid (phenols), luteolin 7-O-glucoside, quercetin 7-O-glucoside, luteolin 7-O-rutinoside and luteolin 7-diglucosides (flavonoid glycosides), ixerin D, taraxinic acid β-D-glucopyranoside and 11,13-dihydotaraxinic acid (sesquiterpenes), and cichorin and aesculin (coumarins).

Body and organ weight of male rats

A significant decrease in body weight of the HDR-receiving rats was observed when compared to the control and whole plant-receiving rats (Table 1). In addition, there was a significant decrease in testis relative weight of the HDWP-; LDR- and HDR-receiving rats when compared to the control rats, and of the root-receiving rats when compared to the whole plant-receiving rats (Table 1). Similarly, the relative weight of the seminal vesicle showed a
significant decrease in all of the treated groups when compared to control group, and in the root-receiving groups when compared to the LDWP-receiving group (Table 1). The relative weights of the liver and the kidney did not show a significant change in any of the treated groups as compared to the control (Table 1).

**Serum testosterone level**

*T. officinale* aqueous extract administration caused a significant decrease in serum testosterone level in all of the treated groups [LDWP: 1.6 ± 0.4 ng/mL (p < 0.05); HDWP: 1.4 ± 0.3 ng/mL (p < 0.05); LDR: 0.9 ± 0.1 ng/mL (p < 0.01); HDL: 0.3 ± 0.1 ng/mL (p < 0.001)] when compared to the control group (3.7 ± 1.2 ng/mL), in the root-receiving groups when compared to the whole plant-receiving groups (p < 0.05 for LDR and p < 0.01 for HDR), and in HDR-receiving group when compared to LDR-receiving group (p < 0.05).

**Sperm concentration, motility and morphology**

Sperm concentration and progressive motility in all of *T. officinale*-treated groups decreased significantly when compared to the control, and in the root-receiving groups as compared to the whole plant-receiving groups (Table 2). A rat sperm with normal morphology has an elongated tail composed of midpiece, principal piece and end piece, with intact hook-shaped head (Figure 1a, b). The percentage of sperm morphological abnormalities increased significantly in the treated groups when compared to the control group, and in the root-receiving groups when compared to the whole plant-receiving groups (Table 2).

**Sperm chromatin integrity**

Sperm samples from cauda epididymis of the control and treated rats were stained with acridine orange. The results did not show DNA damage in either the control or treated samples as all sperms fluoresced green after excitation (Figure 1c). A single intact band was observed in all of the lanes (control and treated groups), while the ladder pattern of DNA, which is an indication of fragmented DNA, was not observed (Figure 1d).

**Effect of *T. officinale* on pregnancy parameters**

A significant decrease was observed in the pregnancy rate and the average number of fetuses/pregnant females in all of the treated groups as compared to the control group, and in the root-receiving groups when compared to the whole plant-receiving groups (Table 2). None of the female rats that were mated with the HDR-receiving males delivered fetuses after the regular pregnancy period (21 days) (Table 2).

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**Table 1:** Body weight gain (g) and relative organ weight (g) of control and treated rats (mean ± SD, n = 10)

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight gain (g)</th>
<th>Testes</th>
<th>Seminal vesicle</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>76.6 ± 19.8</td>
<td>0.69</td>
<td>0.32</td>
<td>4.7</td>
<td>0.37</td>
</tr>
<tr>
<td>LDWP</td>
<td>97.9 ± 13.5</td>
<td>0.70</td>
<td>0.23ab</td>
<td>4.7</td>
<td>0.35</td>
</tr>
<tr>
<td>HDWP</td>
<td>80.6 ± 28.6</td>
<td>0.66ab</td>
<td>0.19ax</td>
<td>4.6</td>
<td>0.34</td>
</tr>
<tr>
<td>LDR</td>
<td>73.7 ± 28.7</td>
<td>0.51a2bc</td>
<td>0.11a2b2</td>
<td>4.6</td>
<td>0.32</td>
</tr>
<tr>
<td>HDR</td>
<td>46.3 ± 23.9</td>
<td>0.5a2b2c</td>
<td>0.10a3b3</td>
<td>4.6</td>
<td>0.33</td>
</tr>
</tbody>
</table>

a p < 0.05; a2 p < 0.01; a2b p < 0.001 versus control; b p < 0.05; b2 p < 0.01; b2p < 0.001 versus LDWP; c p < 0.05 versus HDWP

**Table 2:** Effect of *T. officinale* treatment on sperm parameters (mean ± SD, n = 10)

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (10^6/ml)</th>
<th>Motility (%)</th>
<th>Morphological abnormality (%)</th>
<th>Pregnancy rate (%)</th>
<th>No. of fetus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>81.0 ± 8.9</td>
<td>67.6 ± 8.7</td>
<td>2.7 ± 0.9</td>
<td>90.0</td>
<td>8.3</td>
</tr>
<tr>
<td>LDWP</td>
<td>40.8 ± 8.3b2</td>
<td>26.7 ± 4.4aabc</td>
<td>19.7 ± 5.2abcde</td>
<td>75.0b</td>
<td>6.7aαe3</td>
</tr>
<tr>
<td>HDWP</td>
<td>40.9 ± 4.4abcde</td>
<td>30.9 ± 12.0abc</td>
<td>38.6 ± 8.9abcde</td>
<td>60.0abcde</td>
<td>5.9abcdef</td>
</tr>
<tr>
<td>LDR</td>
<td>25.6 ± 4.0abcde</td>
<td>15.4 ± 3.6abc</td>
<td>41.7± 9.0abcde</td>
<td>45.0abcde</td>
<td>3.8abc</td>
</tr>
<tr>
<td>HDR</td>
<td>14.7 ± 8.1abcde</td>
<td>7.8 ± 5.6abcde</td>
<td>60.9± 13.7abcde</td>
<td>40.0abcde</td>
<td>0abcde</td>
</tr>
</tbody>
</table>

a p < 0.05; a2 p < 0.01; a2b p < 0.001 versus control; b p < 0.05; b2 p < 0.01; b2p < 0.001 versus LDWP; c p < 0.05; c2 p < 0.01; c3 p < 0.001 versus HDWP; d p < 0.05; d2 p < 0.01; d3 p < 0.001 versus LDR; e p < 0.05; e3 p < 0.001 versus HDR

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Figure 1: Effect of *T. officinale* on sperm parameters. (a) Normal rat sperm showing the hooked head and tail; (b) Sperm head showing the acrosome (A) and nucleus (N); (c) Evaluation of chromatin integrity by acridine orange staining. (d) Representative agarose gel electrophoresis analysis of sperm DNA of both the control and treated rats. Lane 1: DNA molecular weight marker; lane 2: Control; lane 3: LDWP; lane 4: HDWP; lane 5: LDR; lane 6: HDR

Effect of *T. officinale* on rat testicular histology

Microscopic examination of testicular cross sections of control rats showed normal histology (Figure 2a, b). The germinal epithelium (GE) was intact and the lumen (L) was wide and full of sperms (Figure 2a). In addition, different stages of sperm development were noticed (Figure 2b). Cells of the interstitial tissue such as Leydig cells (LC), myoid cells (MC) and fibroblasts (F) were also observed (Figure 2b).

However, testicular sections of LDWP-receiving group showed late maturation arrest (spermatid stage) (Figure 2c, d; arrow) in most of the seminiferous tubules (ST) with a decreased number of sperms in the lumen and reduction in interstitial tissues (dashed arrow). HDWP-receiving group testicular sections showed reduction in ST numbers, most of which showed late maturation arrest, disorganized germinal epithelium and a reduction in the interstitial tissue (dashed arrow) (Figure 2e, f). The LDR-receiving group ST cross sections showed late maturation arrest (arrows) with a reduction in germ cell number (reduced germinal epithelium thickness) and interstitial cells (dashed arrows) (Figure 3c, d). On the other hand, cross sections prepared from seminiferous tubules of HDR-receiving rats showed sever disruption in ST architecture and large spaces between germ cells. In addition, early maturation arrest (spermatocyte stage) and germ cell hypoplasia were observed with almost a complete absence of interstitial tissue (dashed arrows). One striking observation in the HDR-receiving group was the absence of a clear lumen (Figure 3e, f).

Gene expression of SSC markers

Three spermatogonial stem cell (SSC) self-renewal and proliferation genes were analyzed by qRT-PCR (Figure 4a). The mRNA levels of these markers (*gfra1, mcsf* and *plzf*) were normalized to β-actin. The level of *gfra1* mRNA increased significantly in all the treated groups when compared to the control, and in the HD-receiving groups when compared to the LD-receiving groups (Figure 4b). *plzf* mRNA level increased significantly in the HD-receiving groups when compared to the control and the LD-receiving groups, and in the HDWP-receiving group when compared to the HDR-receiving group (Figure 4b). On the other hand, *mcsf* mRNA level decreased significantly in all treated groups (except the LDWP-receiving group) when compared to the control group, and in the root-receiving groups when compared to the whole plant-receiving groups (Figure 4b).
Figure 2: Cross-sections of seminiferous tubules (ST) of whole plant-receiving rats. (a-b) Cross sections of ST of control rats showing intact spermatogenic (germinal) epithelium (GE) and lumen (L). Different spermatogenic cells: spermatogonia (SG), spermatocyte (SC), spermatid (SD) and sperm (S) were observed. Different cells of the interstitial tissue were also evident: myoid cells (MC), Leydig cells (LC) and fibroblasts (F). (c-d) Cross sections of ST of LDWP-receiving rats showing late maturation arrest (spermatid stage; arrows), reduction in lumen diameter and interstitial tissue (dashed arrow). (e-f) Cross sections of ST of HDWP-receiving rats showing disruption of GE organization, late maturation arrest (arrows) and reduction in interstitial tissue (dashed arrow). Scale bar = 50 μm

Figure 3: Cross-sections of seminiferous tubules (ST) of root-receiving rats. (a-b) Cross sections of ST of control rats. (c-d) Cross sections of ST of LDR-receiving rats showing reduction in germinal epithelium (GE) and interstitial tissue (dashed arrow), and late maturation arrest. (e-f) Cross sections of ST of HDR-receiving rats showing severe disorganization in the architecture of the GE, the interstitial tissue was almost absent from all sections (dashed arrow), several spaces scattered among the spermatogenic cells were observed, and early maturation arrest was evident with large distribution of spermatogonia in ST. Scale bar = 50 μm
DISCUSSION

Taraxacum officinale has been used in folk medicine for its different medicinal properties [2]. In addition to its known health benefits [1,2], T. officinale whole plant decoction has been traditionally used in Jordan to enhance sperm parameters such as concentration and motility [13]. However, recent studies have proved that the whole plant or leaves aqueous extracts decrease male fertility instead of improving it [3,4]. Thus, we aimed to investigate the effects T. officinale root aqueous extract on fertility parameters and spermatogonial stem cell (SSC) markers by comparing it to the whole plant aqueous extract.

T. officinale treatment caused a significant decrease in testis and seminal vesicle relative weights in treated groups (Table 1). Similar results were observed when male rats were treated with T. officinale whole plant or leaves aqueous extracts [3,4] and Echinops echinatus (Compositae) roots [19]. The decrease observed was attributed to a reduction in testosterone level.

Testosterone regulates spermatogenesis, sperm differentiation and induces the development of male accessory reproductive organs [20]. Thus, any irregularities in testosterone production and concentration would have detrimental effects on spermatogenesis. T. officinale aqueous extract caused a significant decrease in serum testosterone level in all the treated groups when compared to the control group and in the root-receiving rats when compared to the whole plant-receiving rats. This is correlated with the decrease in the interstitial tissue (the source of testosterone) observed in testicular sections of whole plant- or root-treated groups.

In our study, cauda epididymal sperm concentration, motility and normal morphology showed a significant decrease in all of T. officinale-treated groups when compared to the control group (Table 2). This result is in agreement with the results of Padashetty and Mishra [19]; their explanation was a reduction in serum testosterone level which is similar to our results.

The testicular histology of treated rats could provide another explanation for the reduction in
sperm concentration observed. Testicular cross sections showed lesions in the seminiferous tubules, disorganized germinal epithelium, spermatogenesis maturation arrest and reduction in the interstitial tissue (Figure 2 and Figure 3). The reduction in testosterone level disrupts spermatogenesis and reduces fertility parameters [21]. These results were similar to the results obtained by others [22].

Sperms with rapid swimming abilities and normal morphology have a fertilizing advantage over slower abnormal sperms [23]. T. officinale treatment reduced sperm parameters causing a reduction in the fertility rate and the number of fetuses/pregnant female which is in agreement with previously published results of Calendula officinalis (Compositae) administration [24].

The current study measured the mRNA levels of three spermatogonial stem cell (SSC) markers: GDNF family receptor alpha 1 (gfra1), macrophage colony-stimulating factor (mcsf) and promyelocytic leukaemia zinc-finger (plzf) by qRT-PCR (Figure 4). These markers induce self-renewal and proliferation of undifferentiated spermatogonia rather than differentiation [7]. GDNF and its receptor GFRα1 are necessary for the maintenance and proliferation of undifferentiated spermatogonia. Over-expression of these markers led to accumulation of spermatogonia that are unable to differentiate in addition to testicular atrophy [25]. Hofmann [26] suggested that mice over-expressing gfra1 are infertile, develop testicular tumors and accumulate spermatogonia.

PLZF exerts its effects on undifferentiated spermatogonia only and it interacts with GDNF to block spermatogonia differentiation [27]. Costoya et al. [28] showed that testes lacking PLZF have an increased level of spermatogonia apoptosis. A nonsense mutation in plzf gene showed defective spermatogenic self-renewal [29]. Silvan et al.[30] reported that MCSF interacts with GDNF and acts specifically on undifferentiated SSCs and that its effect causes spermatogonia self-renewal rather than differentiation.

The reduced expression of mcsf (Figure 4) could be explained by the histology of the seminiferous tubules of the T. officinale-treated rats - particularly the high dose root-receiving animals, as there was a lack of interstitial tissue (the source of MCSF) from the treated animals (Figure 3). On the other hand, the over-expression of gfra1 and plzf (Figure 4) might have led to enhanced self-renewal of spermatogonia as was noticed in the testicular sections of HD-treated groups (Figure 3). This enhancement of self-renewal was at the expense of differentiation induction and sperm production. In turn, this might have contributed to the disruption in spermatogenesis and the maturation arrest observed.

_Taraxacum officinale_ is rich in phytochemicals. Nevertheless, few studies investigated the effects of individual compounds identified in _T. officinale_ extracts on spermatogenesis. Previous studies have shown that flavonoids and amyrin inhibited spermatogenesis [31-33]. On the other hand, chlorogenic acid was found to stimulate spermatogenesis by inhibiting testosterone degradation [34]. However, the difference in the effects of the two extracts (root and whole plant) cannot be explained based on these compounds since these compounds were found in both the root and whole plant aqueous extracts. Studying the effects of individual phytochemical constituent of _T. officinale_ aqueous extract on spermatogenesis is beyond the scope of this study.

**CONCLUSION**

_T. officinale_ root and whole plant aqueous extracts have been shown to exert inhibitory effects on spermatogenesis. More studies should be conducted to identify the specific anti-spermatogenic active ingredient(s) and whether the anti-fertility actions are reversible or not. Furthermore, additional studies are advised to investigate the specific effect of _T. officinale_ aqueous extracts on spermatogonial stem cell markers’ regulatory pathways. These studies could lead to the identification of specific ingredient(s) in _T. officinale_ that might be used as male contraceptive.

**DECLARATIONS**

**Acknowledgement**

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**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. LTH conceived and designed the study and helped to draft the
manuscript, RNA; RAA and ZAA collected and analysed the data, SRY and SIA participated in the design of the study and coordination, AZE participated in the design and performed the statistical analysis. All authors read and approved the final manuscript for publication.

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