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Ameliorative effect of methanolic extract of *Tribulus terrestris* L. on nicotine and lead-induced degeneration of sperm quality in male rats

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ABSTRACT

Ethnopharmacological relevance: The use of herbal and medicinal plants to treat male infertility is well known in history. *Tribulus terrestris* L. (TT) belongs to the Zygophyllaceae family and it is used in folk medicine to vitalize and also improve both physical performance and sexual function in men in addition to the protective effect of the gross saponins of TT against ischemic stroke and its clinical anti-inflammatory property.

Aim of the study: This study aimed to investigate the effects of methanol extract of *T. terrestris* on nicotine hydrogen tartrate and lead-induced degeneration of sperm quality in male rats and to identify the volatile bioactive non-polar compounds thought to be responsible for its activity using gas chromatography-mass spectrometry (GC-MS).

Materials and methods: The effect of *T. terrestris* on nicotine hydrogen tartrate and lead-induced infertility was evaluated in male rats. Fifty-four mature male albino rats weighing 220–250 g body weight were used. The rats were randomly divided into 9 equal groups (n = 6). Infertility was induced by administering nicotine hydrogen tartrate (0.50 mg/kg) through peritoneal injection (i.p.) or lead acetate (1.5 g/L) orally with drinking water for sixty days. Two doses (50 and 100 mg/kg body weight of the animal) of *T. terrestris* were also used. At the end of the experimental period, the rats were anesthetized and sacrificed. Blood samples were collected. Hormonal analyses were carried out on the serum. The testicle, epididymis, and accessory sex organs (seminal vesical and prostates) were removed for histopathological analysis. Gas chromatography-mass spectrometry (GC-MS) analysis of the methanol extract was also carried out to identify major volatile compounds in *T. terrestris methanol* extract.

Results: Nicotine and lead toxicity caused a significant ($p < 0.05$) decrease in the number of sperm, motility, and an increase in the sperm abnormalities such as the reduction in weight and size of sexual organs (testis, epididymis, and accessory sex glands), reduction of diameter and length of seminiferous tubules. The administration of *T. terrestris* methanol extract, however, improved the semen quantity and quality, sexual organ weights, and fertility of male rats and, thus, ameliorated the adverse effects of nicotine and lead. Ten major compounds were found from the GC-MS analysis of the extract of *T. terrestris* methanol extract.

Conclusion: Findings showed that *T. terrestris* plant methanolic extracts ameliorated nicotine hydrogen tartrate and lead-induced degeneration of sperm quality in male rats. The GC-MS analysis of the *T. terrestris* plant methanolic extracts revealed the presence of several important bioactive compounds which were thought to be responsible for the ameliorative effect. Further isolation and evaluation of the individual components would provide relevant lead to finding new drugs.

Abbreviations: Gas chromatography-mass spectrometry, GC-MS; *Tribulus terrestris* L, TT; Mass Selective Detector, MSD; Enzyme-Linked ImmunoSorbent Assay, ELISA; Luteinizing hormone, LH; intraperitoneal injection, i.p; red blood cells, RBCs; One-way analysis of variance, ANOVA; Statistical Analysis System, SAS.

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1. Introduction

Numerous medicinal plants have been investigated for male infertility improvement. Thus, a large number of plants are being traditionally explored to treat it (Sharma et al., 2017). The use of herbal plants that are rich in antioxidants has gained global attention in recent times (Ahmed et al., 2019, 2020; Ahmed, 2021; Singh et al., 2021). *Tribulus terrestris* L. (TT) has also been studied for the development of natural antioxidant formulations in the areas of medicine and nutrition, and specifically for male fertility (Zheleva et al., 2012; Khaleghi et al., 2017; Nasir et al., 2020). Haghmorad et al. (2019) also reported that the administration of *T. terrestris* had a higher improvement on several sexual parameters such as epithelial thickness, an increase of Leydig, spermatogonia, and Sertoli cell numbers. In folk medicine, TT herb is reportedly used not only to energize and vitalize but to also improve both physical performance and sexual function in men, such as sperm viability and motility viability (Asadmobini et al., 2017; Nasir et al., 2020; Tian et al., 2019). Nevertheless, available data show that the mode of action of TT, its sexual function efficacy, and androgen enhancing properties are controversial or rather inconclusive (Neychev and Mitev, 2005, 2016). The protective effect of the gross saponins of TT against ischemic stroke and its clinical anti-inflammatory property, however, have been well studied and established (Tian et al., 2021; Wang et al., 2021).

*T. terrestris*is an annual plant of the Zygophyllaceae family. It is generally known as Tribulus and mainly grows in sub-tropical and the countries around the Mediterranean Sea. It is considered a small plant; its height is between 10 and 60 cm. It can be a prostrate, hirsute, or silky hairy shrub. Its leaves are paripinnate, opposite, unequal, oblong or an elliptical lanceolate, and pinnate from 5 to 8 pairs. The TT fruits are 3–6 mm long and ax-shaped; they are 7–12 mm in diameter, arranged radially, and have a firm texture. its root is slender, cylindrical, fibrous, and branched frequently. Its root bears several small rootlets and they are light brown colored (Chhatre et al., 2014). The TT plant's fruits and roots have been used by the Chinese for thousands of years as folk medicine. TT plant has been documented in Asia and Europe to treat sexual dysfunctions and for other pharmaceutical activities such as enhancing sexual function, cardiac protection, and providing antitumor, anti-urolithic, antidiabetic, anti-inflammatory, and antioxidants effects. It is composed of multiple biologically active compounds like vitamins, alkaloids, saponins, flavonoids, steroids, tannins, unsaturated fatty acids, and more (Adewoyin et al., 2017; Khaleghi et al., 2017).

One of the main active substances found in *T. terrestris* are saponins from furostanol type have been widely used for many disease treatments, like libido and infertility disorders in both sexes as well as urinary and cardiovascular disorders (Kostova and Dinchev, 2005). According to the literature, the administration of *T. terrestris* extract in both humans and animals improves libido and spermatogenesis (Martino-Andrade et al., 2010). TT positively affects the fertility potential of oligozoospermia patients, reproductive parameters, and sperm quality in humans. It also improves sexual activity, spermatogenesis, and erection of experimental animals (Roaiah et al., 2017). TT extract enhanced sperm concentration and motility and also decreased abnormal morphology in experimental mice (Adaay and Mattar, 2012). *T. terrestris* may be used as a safe therapeutic alternative to current modalities for the management of motility dysfunction in males (Zheleva et al., 2012). The high antioxidant content and the inhibition of lipid peroxidation activity of TT make it beneficial for infertility therapy.

T. terrestris is commonly used in Chinese and Indian medicine to improve sex functions, treat heart and circulatory system diseases, and has also been used as a treatment for diabetes, antioxidant, anti-aging, and anti-tumors (Zhu et al., 2017). Roaiah et al. (2017) reported that TT extract has a significant effect on the viability and motility of human sperm with no effect on DNA fragmentation of human sperm *in vitro*. Khaleghi et al. (2017) also demonstrated that *T. terrestris* extract has a noticeable effect on improving the total motile spermatozoa and also improving the progressive motility. Nassar et al. (1998) stated that a noteworthy stimulatory effect on human sperm motility could have a relation with trace elements, specifically Ca^{2+} which is known in TT extract. Ca^{2+} inhibits the enzyme phosphate diesterase, which could prevent cyclic adenosine monophosphate degradation and in addition to improving sperm motility levels (Keshtmand et al., 2015). TT extract is also rich in zinc, thus leading to improvement in sperm motility due to its involvement in protein synthesis and nuclear chromatin stabilization (Sellandi et al., 2012).

Notable volatile bioactive compounds that might be responsible for its fertility enhancement property are, however, yet to be comprehensively studied. Therefore, this study assessed the effects of methanol extract of *T. terrestris* on nicotine and lead-induced infertility in male rats and identified its volatile bioactive non-polar compounds thought to be responsible for its activity using gas chromatography-mass spectrometry (GC-MS).

2. Materials and Methods

Nicotine hydrogen tartrate ($C_{14}H_{20}N_2O_6$: 2,3- Dihydroxybutanedioic acid; 3-(1-methylpyrrolidin-2yl) pyridine, nicotine hydrogen tartrate salt, B-Lead acetate ($Pb(CH_3COO)_2$), and sodium bicarbonate were from Sigma–Aldrich (Chemie, Steinheim, Germany) and Merck (Darmstadt, Germany) while all solvents used were either analytical or chromatographic grade.

3. Animals

The research was approved during the ethical committee meeting of 1444–1443(1/2) under the project "5726-CAVM-2019-2-2-I" and conducted following the European Community guidelines (EEC Directive of 1986; 86/609/EEC). Fifty-four mature male albino rats weighing 220–250 g body weight were obtained from the Faculty of Pharmacy – King Saud University, Riyadh, Kingdom Saudia Arabia They were housed in cages and fed on laboratory animal feed pellets [containing crude protein (20.0%), crude fat (4.00%), crude fiber (3.50%), ash (6.00%), salt (0.50%), calcium (1.00%), phosphorus (0.60%), vitamin A (20.00 μg), vitamin D (2.20 μg), vitamin E (70.00 IU), energy (ME Kcalkg 2850.00)] and water *ad libitum* for a period of 2-weeks before the beginning of the experiment to ensure that only healthy animals were used and the emaciated ones were discarded.

3.1. Plants collection

T. terrestris whole plant samples (shoots, leaves, flowers, and roots) were collected during the flowering season (February–May 2020), after the rainy season, from the natural pastures and lands surrounding the farms in the Qassim region in the Kingdom of Saudi Arabia, specifically in Khob Al- Muradisiyah in Buraidah. The plant was authenticated and deposited in the herbarium of the College of Agriculture and Veterinary Medicine, Qassim University, Saudi Arabia by Prof Abdulrahman Alsugair, with a voucher specimen no. Sp. PI:387 (1753).

3.2. Extract preparation

Plant samples were washed properly with running water. They were properly dried in the air. Then, all parts of the plant (paper, roots, sticks, and flowers) were ground using a grinding machine to obtain a very fine powder. About 200 g of the powder was extracted with 2 L of methanol (99.9%) under mild agitation for 72 h (Ahmed et al., 2015). Methanol was selected for the extraction following the literature (Selvamangai and Bhaskar, 2012; Kanthal et al., 2014; Shalaby and Hammouda, 2014; Chauhdary et al., 2019; Batiha et al., 2020). The extract was filtered and concentrated using a rotary evaporator (Buchi Labortechnik AG, Switzerland). The crude extract was stored in the refrigerator at −12 °C before use.

3.3. Animal grouping, extract doses, and chemical preparations

The rats were randomly divided into 9 equal groups (n = 6) for appropriate treatments (Table 1).

The *T. terrestris* extracts and drugs were administered once daily and orally for 60 days covering the period of the rat spermatogenic cycle (Shalaby and Hammouda, 2014). The extracts were prepared according to the literature (Akomolafe and Oboh, 2017; Hasanzadeh et al., 2017) with modifications. In the acute study of *T. terrestris* extracts, the reported LD_{50} was >200 mg/kg (Chauhdary et al., 2019). Thus, only two doses (50 and 100 mg/kg body weight of the animal) of *T. terrestris* were prepared and administered daily for 60 days. Nicotine hydrogen tartrate (0.50 mg/kg) was freshly prepared with normal saline solution (0.9%) and given by peritoneal injection (i.p.) to induce fertility deficiency (Salahipour et al., 2017). Lead acetate was prepared (1.5 g/L) with drinking water (Ramah et al., 2019).

3.4. Samples collection and processing

At the end of the experimental period, blood samples were collected using microcapillary tubes by puncture of retro-orbital plexus of veins in the eye. The rats were anesthetized by prolonged exposure to ether inside the anesthetic box. Then, animals were sacrificed and dissected. The testicle, epididymis, and accessory sex organs (seminal vesical and prostates) were extracted, grossly examined, and weighed accurately. Sperm samples were taken from the epididymis, mixed on a clean warm slide with a drop of normal saline, and examined under a microscope to evaluate movement (this process was done quickly and in ideal conditions to preserve the life of sperm as possible). Histological analysis was carried out using both Eosin and Nigrosin stains to evaluate sperm abnormality.

3.5. Preparation of serum

Blood samples were left to clot at room temperature and then centrifuged for about 15 min at × 3000 r.p.m. to obtain clear serum. The serum was stored in a deep freezer at – 70 °C until just before other analyses.

3.6. Hormonal analysis

The serum was used for the estimation of testosterone levels and luteinizing hormone (LH) levels. The serum testosterone level was determined by the ELISA method using a testosterone kit (DRG Instruments, GmbH, Germany) according to the kit manufacturer's instructions. ELISA kits for serum LH were purchased from Genzyme (Cambridge, USA).

Table 1
Animal grouping description.

Control group	G1	Normal drinking water and foods.
Negative Control group (vehicle)	G2	Normal saline: 1 mL i.p. 1 mL pf 1% gum solution 1% orally by gavage
	G3	*T. terrestris*extract (100 mg/kg/day) by gavage
	G4	*T. terrestris* (50 mg/kg/day) + nicotine 0.50 mg/kg/day (i.p)
	G5	*T. terrestris* (100 mg/kg/day) + nicotine 0.50 mg/kg/day(i.p)
	G6	*T. terrestris* (50 mg/kg/day) + lead acetate (1.5%) with drinking water.
	G7	*T. terrestris* (100 mg/kg/day) + lead acetate (1.5%) with drinking water.
Positive group	G8	Nicotine hydrogen tartrate 0.50 mg/kg body weight intraperitoneal injection (i.p) per day
	G9	Lead acetate 1.5 g/L per day in drinking water

3.7. Progressive motility

A clean dry slide was placed on a heated microscope stage and allowed to warm at a temperature of 38 °C. A drop of semen from the vasa deferentia was mixed with a drop of saline 0.9% on the slide using a small 0.9% on the slide using a small pipette. The progressively motile sperm percentage was determined and recorded according to the method described by Bearden and Fuquay (1984).

3.8. Epididymal sperm counting

A hemocytometer and pipette of RBCs estimation were used for counting epididymal sperm. One epididymis was mixed with 5 mL sodium bicarbonate solution 5% of each control and treated rats of each group. After mixing the epididymis with the sodium bicarbonate solution, the mixture was filtered. The filtrate was withdrawn up to the mark of 0.5 and the pipette was then filled up to the mark by sodium bicarbonate solution of 5% (Bearden and Fuquay, 1984). The content of the pipette was mixed by holding the ends of the pipette between the thumb and the index finger and shaking it vigorously. A few drops of fluid were blown out, then a small amount of the diluted sperm suspension was placed at the edge of the cover slide. The semen was drawn from the cover slide by the capillary action and the spermatozoa were counted using the high power objective lens, according to the method described by Bearden and Fequary (1980).

$$\text{Sperm count} = n \times 1000 \times 10$$

N: number of spermatozoa in 25 large squares 1000: dilution
10: depth

3.9. Sperm cell abnormalities percentage

A drop of saline was placed on a clean dry slide. Then, a drop of undiluted semen content from vas deferens was put on the saline solution and mixed carefully. Then, drops of Eosin and Nigrosin stains were added and the film was spread on the slide. One hundred sperm were observed at random per slide under 40 × (objective lens) and 16 × (eyepiece) of the microscope and the percentage of abnormal sperm (coiled tail and detached head) were recorded.

3.10. Histopathological examination

The rats were sacrificed after the end of the drug administration. A macroscopic examination was performed. Then, fresh specimens from the testes, epididymis, seminal vesicle, and prostate gland were removed and fixed in a 10% buffered formalin solution. Slides were prepared to study the histological changes in the testes, epididymis, seminal vesicle, and prostate gland. The histomorphometric analysis of the diameter and thickness of the seminiferous tubules of the spermatogenic layer was performed using Image J analysis software (National Institutes of Health, MD, USA). Whereas the average of each parameter was determined by the assessment of ten fields at × 100 magnification power for each animal.

3.11. Gas chromatography-mass spectrometry (GC-MS) analysis

The methanolic extracts of various natural products and phytochemicals have been analyzed using GC-MS (Batiha et al., 2020; Kanthal et al., 2014; Selvamangai and Bhaskar, 2012). In the current study, about 20 g of the powdered plant was soaked in 100 mL methanol (99.9%) for 24 h. The methanol extract was filtered using Whatman No.1 filter paper and through sodium sulphate (to remove the traces of moisture). The methanol extracts were analyzed using Agilent Gas Chromatography (6890 N Model) coupled to 5973 Mass Selective Detector (MSD) USA. The results obtained were compared with an in-built

main library (C:/Database/NIST08.L) to confirm the presence of some phytochemicals present in the plant and a Gas chromatograph interfaced to a mass spectrophotometer equipped with Elite -5MS (5% diphenyl/95% dimethylpolysiloxane), 30 × 0.25 mm × 0.25 μm df. For GC/MS detection, an electron ionization system with ionization energy of 70Ev was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min at an injection volume of 2 μL (split ratio 10:1). Injector temperature was programmed at 250 °C; ion source temperature was maintained at 200 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C/min, to 200 °C, then 5 °C/min to 280 °C, ending with 9 min' isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan-interval of 0.5 s and a fragment from 45 to 450 Da. The total GC running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The software adapted to handle mass spectra and chromatograms was Turbo Mass Ver 5.2 (Soumya et al., 2014).

3.12. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the mean difference between the various rat groups. Duncan's new multiple range tests were performed for the post hoc analysis to access the significance of different mean values of the animal groups. All the calculations and analyses were carried out using SAS (2004).

4. Results

4.1. Effect of T. terrestris L., nicotine, and lead on organs' weights

The administration of nicotine hydrogen tartrate (0.50 mg/kg body weight through intraperitoneal injection) and lead acetate (1.5 g/L) for 60 days induced a significant decrease ($p < 0.05$) in the weights of the testis, epididymis, accessory gland (Table 2) compared to all other groups. The co-administration of *T. terrestris* extracts with nicotine hydrogen tartrate and lead acetate, however, ameliorated the adverse effect of nicotine hydrogen tartrate and lead acetate on the organs' weights.

4.2. Sperm characteristics

The results showed that nicotine hydrogen tartrate and lead acetate administration induced a significant decrease ($p < 0.05$) in the sperm number, an increase in the sperm abnormalities, and a decrease in the motility (Table 3) compared to all other groups. The co-administration of nicotine hydrogen tartrate and lead acetate with *T. terrestris* extract, however, significantly ($p < 0.05$) alleviated the adverse effect of nicotine hydrogen tartrate and lead acetate. The influence of the plant extracts was dose-dependent.

4.3. Hormones level, seminiferous tubules, and spermatogenic cell layer

The administration of nicotine hydrogen tartrate and lead acetate for two months induced a significant decrease ($p < 0.05$) in the level of the hormone (LH and testosterone), the diameter of seminiferous tubules, and the length of the spermatogenic cell layer (Table 4) compared to all other groups while the co-administration of nicotine hydrogen tartrate and lead acetate with the *T. terrestris* extract significantly ($p < 0.05$) alleviated the decrease in the hormones, the diameter of seminiferous tubules, and the length of the spermatogenic cell layer as compared to the effect of nicotine hydrogen tartrate and lead acetate alone. The influence of the plant extract was dose-dependent in the protection against nicotine hydrogen tartrate and lead acetate intoxication.

4.4. Histopathological examination

No histopathological abnormalities were found in the examined organs of the normal control group given normal drinking water and rat pellets only (G1). Similar results were obtained in the negative control group given vehicle only (G2). The testis showed normal histological criteria of seminiferous tubules with active spermatogenesis (Fig. 1aandb). The epididymis showed the normal histological appearance of their tubules with an accumulation of spermatozoa in the lumen. The seminal vesicles and prostate gland also had a normal structure of the acini with the presence of eosinophilic secretion in the lumen (data not shown). The testis of the experimental group administered 100 mg/kg T. *terrestris*extract (G3) only revealed a normal histological appearance of seminiferous tubules (Fig. 1c). The epididymis showed normal histological criteria of their tubules and mild interstitial edema admixed with small numbers of lymphocytes (Fig. 2a). The seminal vesicles revealed the normal histological appearance of their glands and muscular wall. The prostate gland revealed also a normal structure of the acini and mild interstitial edema (data not shown).

The testis of the experimental groups co-administered *T. terrestris* extracts (50 and 100 mg/kg) and nicotine hydrogen tartrate (G4 and G5, respectively) showed normal histological criteria of seminiferous tubules with active spermatogenesis (Fig. 1d and e, respectively). The epididymis of the G4 group showed congested blood vessels, and the interstitium was markedly expanded by focal homogenous eosinophilic material (edema) admixed with small numbers of lymphocytes while the epididymis, seminal vesicles, and prostate gland of the G5 group revealed the normal histological appearance of their tubules and acini

Table 2
Effect of *T. terrestris*, nicotine and lead on organs' weights.

Animal Groups	Organs' Weight (g/100 g b.wt) (N = 6)		
	Testis	Epididymis	Accessory gland
Control normal	0.99 ± 0.02^{A}	0.48 ± 0.01^{B}	0.76 ± 0.03^{A}
Control vehicle	0.97 ± 0.01^{A}	0.44 ± 0.03^{B}	0.76 ± 0.04^{A}
TT100	1.02 ± 0.05^{A}	0.55 ± 0.02^{A}	0.79 ± 0.02^{A}
Nicotine	0.73 ± 0.05^{C}	0.32 ± 0.01^{C}	0.65 ± 0.03^{C}
TT50+ nicotine	0.94 ± 0.03^{AB}	0.44 ± 0.01^{B}	0.71 ± 0.04^{B}
TT100+ nicotine	0.97 ± 0.03^{A}	0.49 ± 0.02^{B}	0.73 ± 0.03^{B}
Lead	0.79 ± 0.04^{B}	0.36 ± 0.01^{C}	0.64 ± 0.02^{C}
TT50+lead	0.94 ± 0.03^{A}	0.48 ± 0.03^{B}	0.68 ± 0.05^{B}
TT100+lead	0.96 ± 0.06^{A}	0.48 ± 0.02^{B}	0.70 ± 0.03^{B}

Values are means ± standard error (n = 6). The results of all the various groups were analyzed using a one-way analysis of variance (ANOVA). Values within each column not sharing a common superscript letter (A, B, C, etc.) are significantly different ($p < 0.05$) using Duncan's new multiple range post hoc tests. *T. terrestris* (TT).

Table 3
Effect of *T. terrestris*, nicotine, and lead on sperm characteristics.

Animal Groups	Sperm characteristics (N = 6)		
	Sperm number (× 10^6/mL)	Sperm abnormalities (%)	Sperm motility (%)
Control normal	25.00 ± 2.25^{A}	5.21 ± 0.33^{C}	70.00 ± 2.58^{B}
Control vehicle	24.00 ± 2.56^{AB}	5.32 ± 0.31^{C}	69.17 ± 2.39^{B}
TT100	26.00 ± 1.81^{A}	4.92 ± 0.85^{D}	75.83 ± 2.71^{A}
Nicotine	13.33 ± 1.54^{D}	11.31 ± 0.48^{A}	18.33 ± 2.47^{D}
TT50+ nicotine	20.17 ± 2.33^{C}	6.20 ± 0.63^{B}	53.33 ± 4.94^{C}
TT100+ nicotine	23.33 ± 3.20^{B}	5.93 ± 0.49^{B}	56.67 ± 4.41^{C}
Lead	16.33 ± 2.23^{D}	8.21 ± 0.48^{A}	15.00 ± 4.08^{D}
TT50+lead	19.12 ± 3.50^{C}	5.41 ± 0.48^{C}	52.55 ± 6.76^{C}
TT100+lead	23.17 ± 2.90^{B}	5.13 ± 0.82^{C}	55.00 ± 4.08^{C}

Values are means ± standard error (n = 6). The results of all the various groups were analyzed using a one-way analysis of variance (ANOVA). Values within each column not sharing a common superscript letter (A, B, C, etc.) are significantly different ($p < 0.05$) using Duncan's new multiple range post hoc tests. *T. terrestris* (TT).

Table 4
Effect of *T. terrestris*, nicotine and lead on hormones level, seminiferous tubules, and spermatogenic cell layer.

Animal Groups	Hormones level		Seminiferous tubules and spermatogenic cell layer	
	Luteinizing hormone (IU/mL)	Testosterone (ng/mL)	Seminiferous tubules (μm)	Spermatogenic cell layer (μm)
Control normal	3.97 ± 0.18^{A}	4.80 ± 0.15^{B}	147.75 ± 2.80^{B}	48.94 ± 4.20^{B}
Control vehicle	3.60 ± 0.21^{C}	4.75 ± 0.15^{C}	145.08 ± 5.15^{B}	46.20 ± 3.17^{C}
TT100	3.72 ± 0.29^{B}	4.93 ± 0.09^{A}	165.98 ± 8.45^{A}	57.34 ± 4.59^{A}
Nicotine	2.55 ± 0.23^{F}	2.55 ± 0.23^{E}	87.21 ± 3.27^{F}	10.20 ± 0.86^{H}
TT50+ nicotine	3.35 ± 0.40^{D}	3.27 ± 0.44^{D}	131.28 ± 6.00^{D}	36.14 ± 1.76^{E}
TT100+ nicotine	3.53 ± 0.31^{C}	3.53 ± 0.31^{D}	154.41 ± 4.57^{B}	44.85 ± 1.83^{D}
Lead	2.72 ± 0.24^{F}	1.90 ± 0.07^{F}	102.59 ± 6.50^{E}	21.36 ± 2.52^{G}
TT50+lead	3.13 ± 0.28^{E}	3.42 ± 0.29^{D}	137.94 ± 6.16^{C}	34.21 ± 1.88^{F}
TT100+lead	3.30 ± 0.20^{D}	3.72 ± 0.23^{D}	130.43 ± 6.45^{D}	37.83 ± 2.10^{D}

Values are means ± standard error (n = 6). The results of all the various groups were analyzed using a one-way analysis of variance (ANOVA). Values within each column not sharing a common superscript letter (A, B, C, etc.) are significantly different ($p < 0.05$) using Duncan's new multiple range post hoc tests. *T. terrestris* (TT).

while (Fig. 2b). Necrotic debris admixed with degenerated spermatozoa, and small numbers of inflammatory cells were also found in the lumen of epididymal tubules. The seminal vesicles revealed hyperplasia of the lining epithelium. The prostate gland revealed mild interstitial edema and some acini were ectatic and lined by attenuated epithelium (supplementary data).

Similarly, the testis of the experimental groups co-administered *T. terrestris* extracts (50 and 100 mg/kg) and lead acetate (G6 and G7, respectively) revealed congestion of the testicular blood vessels and interstitial edema with normal histological appearance of the majority of seminiferous tubules (Fig. 1f and g, respectively). The seminal vesicles and prostate gland of the G6 group revealed a normal histological appearance of their glands (data not shown). The seminal vesicles of the G6 group, however, revealed inflammatory cells infiltrating the muscular wall mainly neutrophils and the prostate gland revealed congested blood vessels, mild interstitial edema, and normal structure of the acini (supplementary data). The epididymis of the G7 group showed normal histological criteria of their tubules with little accumulation of degenerated spermatozoa in the lumen admixed with few inflammatory cells (Fig. 2c).

There was marked testicular degeneration of large numbers of seminiferous tubules (Fig. 1h and i) in the positive control groups given nicotine hydrogen tartrate and lead acetate only (G8 and G9, respectively). The degenerated tubules were lined by one or two layers of the vacuolated germinal epithelium with reduced spermatogenesis and the absence of spermatozoa in the lumen. The degenerated tubules had an undulant basement membrane and their lumen contained exfoliated epithelium admixed with necrotic debris (supplementary data). The testicular interstitium was expanded by focal homogenous eosinophilic material (edema) admixed with few lymphocytes. The epididymis showed congested blood vessels and interstitial edema and there was necrotic debris admixed with degenerated spermatozoa, spermatogenic cells and a small infiltrate of inflammatory cells in the lumen of epididymal tubules (Fig. 2dande, respectively). The interstitium of seminal vesicles was expanded by homogenous eosinophilic material (edema); the lumen of seminal glands contained eosinophilic secretions admixed with inflammatory aggregates mainly plasma cells and fewer

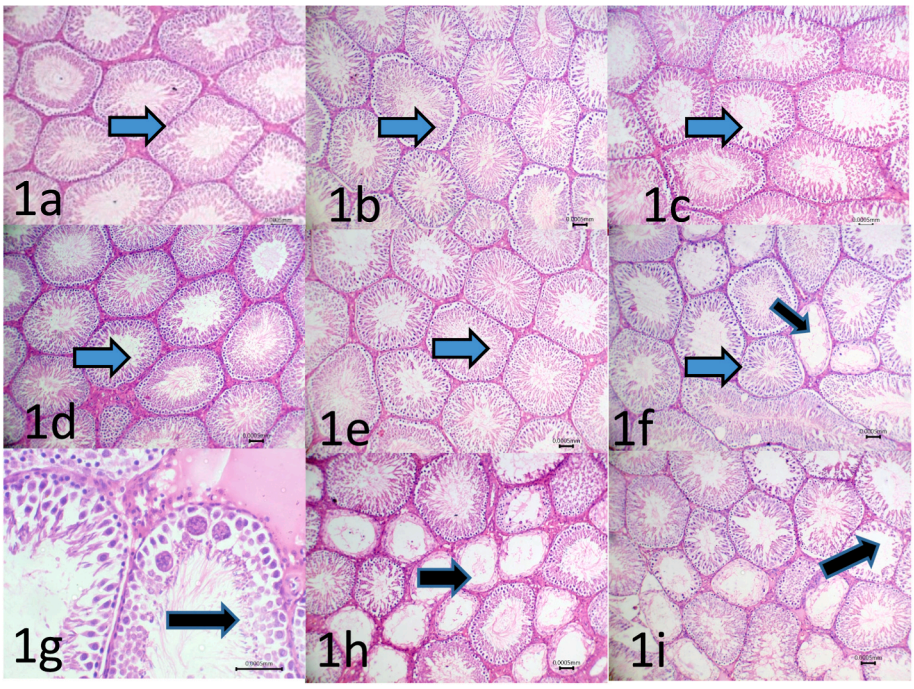


Fig. 1. (a) G1 (normal drinking water). Testis showing normal histological criteria of seminiferous tubules with active spermatogenesis (b) G2 (normal saline). Testis showing normal histological criteria of seminiferous tubules with active spermatogenesis and accumulation of spermatozoa in the lumen (c) G3 (TT100 mg/kg/day). Testis of rat showing a normal histological appearance of seminiferous tubules (d) G4 (TT50 mg/kg/day + nicotine 0.50 mg/kg/day). Testis showing normal histological criteria of seminiferous tubules with active spermatogenesis (e) G5 (TT100 mg/kg/day + nicotine 0.50 mg/kg/day). Testis showing normal histological criteria of seminiferous tubules with active spermatogenesis (f) G6 (TT50 mg/kg/day + 1.5% lead acetate). Testis showing congestion of the testicular blood vessels and interstitial edema with normal histological appearance of the majority of seminiferous tubules (g) G7 (TT100 mg/kg/day + 1.5% lead acetate). Testis showing degeneration of individual tubules characterized by vacuolization of germ cells and small numbers of spermatogenic multinucleated giant cells (h) G8 (nicotine 0.50 mg/kg/day). Testis showing marked testicular degeneration of large numbers of seminiferous tubules with the degenerated tubules lined by one or two layers of the vacuolated germinal epithelium with reduced spermatogenesis and absence of spermatozoa in the lumen (i) G9 (1.5% lead acetate). Testis showing marked testicular degeneration of large numbers of seminiferous tubules with the degenerated tubules lined by one or two layers of the vacuolated germinal epithelium with reduced spermatogenesis and absence of spermatozoa in the lumen. The Blue arrows show seminiferous tubules with active spermatogenesis while black arrows show degenerative changes and depletion of cells in seminiferous tubules. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

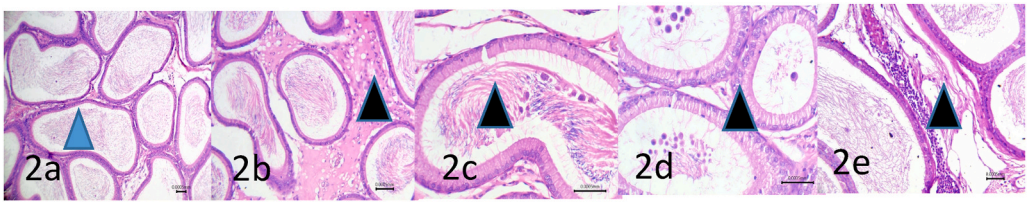


Fig. 2. (a)G3 (TT100 mg/kg/day). Epididymis showed normal histological criteria of their tubules and mild interstitial edema admixed with small numbers of lymphocytes (b) G4 (TT50 mg/kg/day + nicotine 0.50 mg/kg/day). Epididymis showed congested blood vessels and the interstitium was markedly expanded by focal homogenous eosinophilic material (edema) admixed with small numbers of lymphocytes (c) G7 (TT100 mg/kg/day + 1.5% lead acetate). Epididymis showing normal histological criteria of their tubules with an accumulation of degenerated spermatozoa in the lumen admixed with few inflammatory cells (d) G8 (nicotine 0.50 mg/kg/day). Epididymis showing congested blood vessels and interstitial edema with necrotic debris admixed with degenerated spermatozoa, spermatogenic cells, and a small infiltrate of inflammatory cells in the lumen of epididymal tubules (e) G9 (1.5% lead acetate). Epididymis showed congested blood vessels and interstitial edema with necrotic debris admixed with degenerated spermatozoa, spermatogenic cells, and a small infiltrate of inflammatory cells in the lumen of epididymal tubules. The Blue triangles show epididymis with normal histological criteria of their tubules while black triangles show degenerative changes such as congested blood vessels and interstitial edema with necrotic debris. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

macrophages and lymphocytes. The prostate gland revealed interstitial edema and some acini were ectatic and lined by attenuated epithelium (supplementary data).

Fig. 3 (a). G3. Epididymis of rat showing normal histological criteria of tubules and mild interstitial edema admixed with small numbers of lymphocytes.

4.5. GC-MS analysis of Tribulus terrestris L

The GC-MS analysis of the methanol extract of *T. terrestris* showed the presence of ten major compounds (Table 5). The compounds are 3-O-methyl-d-glucose (23.48), n-hexadecanoic acid (3.95), 9,12,15-octadecatrienoic acid, (z,z,z)- (3.57), hexadecanoic acid, methyl ester (3.22), phytol (2.66) while the minor compounds are 1- hexadecanol, 2-methyl (1.42), tricyclo[4.4.0.0(2,7)]dec-8-ene-3- methanol, α,α,6,8-tetramethyl-, diosgenin (1.35), stereoisomer (1.23), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (1.03), squalene (0.73). There, were, however, other unidentified but minor compounds as shown by the chromatogram (Fig. 3).

5. Discussion

The current work is unique in studying and unraveling the notable volatile bioactive compounds in the *T. terrestris* methanolic extract that might be responsible for its fertility enhancement property. The results showed that the administration of nicotine hydrogen tartrate or lead acetate for sixty days induced a significant ($p < 0.05$) decrease in the weights of the sexual organs, namely testis, epididymis, and accessory sex glands. According to the literature, environmental pesticides and heavy metals can cause DNA fragmentation, especially during spermatogenesis, resulting in male infertility or low fertility (Adewoyin et al., 2017). The co-administration of nicotine hydrogen tartrate or lead acetate with TT extracts, however, significantly ($p < 0.05$) alleviated the adverse effects of nicotine hydrogen tartrate or lead acetate-induced infertility. The improvements were comparable to the normal and negative groups. The results are in agreement with the findings of Soldin et al. (2011) who reported that nicotine and lead had negative effects on the potential of fertility in albino rats, reduced the weights, disorganized the histology of some of their reproductive organs, and affected the male

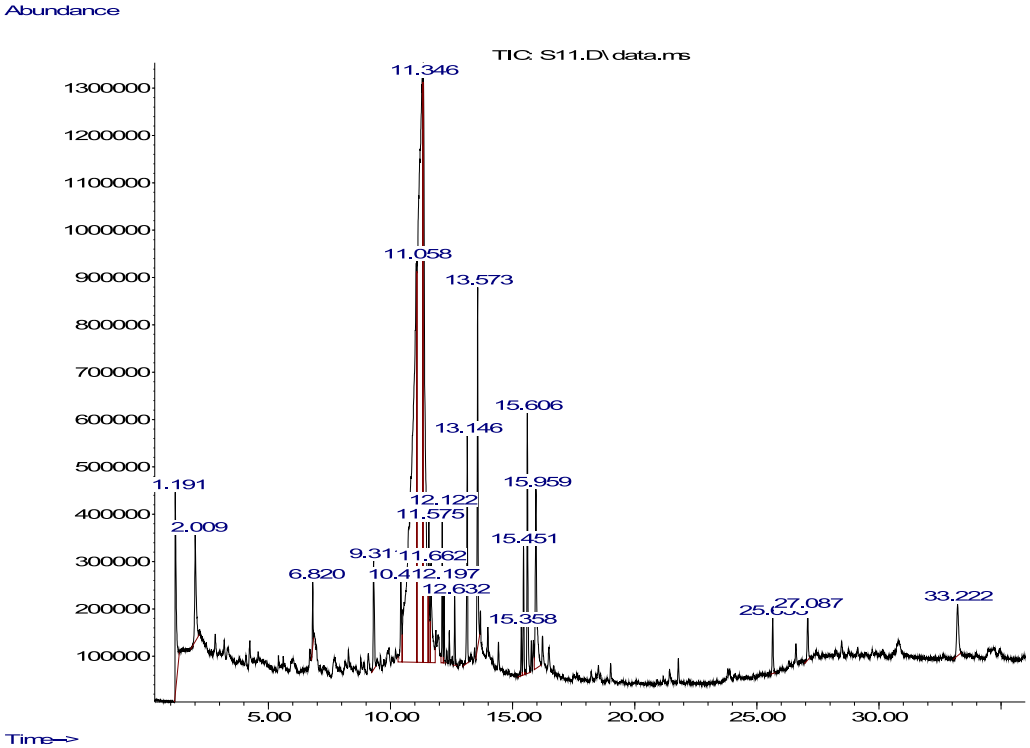
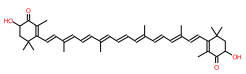
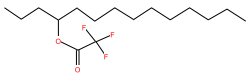
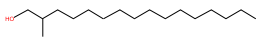
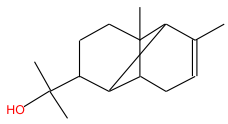
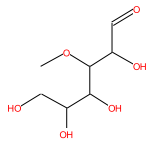
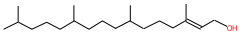
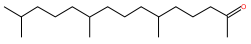
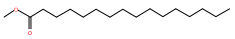
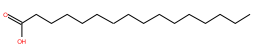
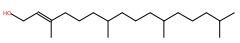
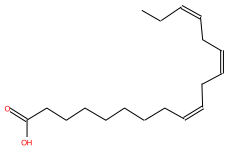
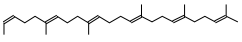
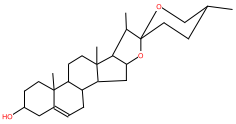


Fig. 3. Chromatogram from the GC-MS analysis of *T. terrestris* methanol extract.

Table 5
Major compounds from the GC-MS analysis of methanol extract of *T. terrestris*.

Retention Time	Peak % area	Name	Molecular formula	MW	Chemical structure
2.009	0.61	Astaxanthin	$C_{40}H_{52}O_4$	596	
6.820	0.61	4-Trifluoroacetoxytetradecane	$C_{16}H_{29}F_3O_2$	310	
9.313	1.42	1-Hexadecanol, 2-methyl	$C_{17}H_{36}O$	256	
10.423	1.23	Tricyclo[4.4.0.0(2,7)]dec-8-ene-3-methanol, α,α,6,8-tetramethyl-, stereoisomer	$C_{15}H_{24}O$	220	
11.058	23.48	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	194	
12.122	1.03	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	
12.197	0.54	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	268	
13.147	3.22	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	
13.576	3.95	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	
15.606	2.66	Phytol	$C_{20}H_{40}O$	296	
15.959	3.57	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278	
25.653	0.73	Squalene	$C_{30}H_{50}$	410	
33.222	1.35	Diosgenin	$C_{27}H_{42}O_3$	414	

fertility indices in addition to subsequent effects of cessation on these reproductive parameters.

TT methanol extracts reportedly improved semen quantity and quality, dose-dependently (Shalaby and Hammouda, 2014). Moreover, Graça et al. (2004) stated that common changes in the macroscopic level of the accessory sex organs of experimental animals include the diminished testis weight, epididymis, seminal vesicles, and ventral prostate. Similarly, Wang et al. (2008) stated that rats exposed to lead-acetate-intoxication for fifty-five days had a decrease in the mean weight of the testis, prostate gland, epididymis, vas deferens, and seminal vesicle.

The improvement of sexual organ weights with the co-administration of the plant extracts might be attributed to their active constituents and antioxidant potentials (El-Wakf et al., 2015; Kumari and Singh, 2015). Additionally, *T. terrestris* possesses aphrodisiac and pro-sexual activities because of its capability of increasing the testosterone and testosterone precursor levels which enhance the weight and size of sexual organs including the testes, epididymis, and accessory gland (Neychev and Mitev, 2016). The weight, size, and secretory function of the testis, epididymis, accessory sex organs (seminal vesicles, ventral prostate), and vasa differentia are strictly controlled by androgens (Aladakatti et al., 2011). Male fertility is correlated with the testis weight because the increase in daily sperm output, sperm production, and Sertoli cell numbers have been associated with large testis weight while poor fertility is related to small testis due to a decrease in the number of sperm production, diminished spermatogenesis, reduction in the length of the seminiferous tubule, and a decrease in reproductive activity (Johnson et al., 2008; Oyedeji et al., 2013).

The results of the current study on the sperm characters clearly showed that the sperm abnormalities including the decrease in LH and testosterone levels observed in the positive groups administered nicotine hydrogen tartrate or lead acetate alone were significantly ($p < 0.05$) and dose-dependently alleviated in the experimental groups co-administered with TT extracts and nicotine hydrogen tartrate or lead acetate. The normal and negative control groups had the highest levels of LH and testosterone hormones, followed by the high-dose experimental group (TT100). The result is in agreement with the work of Shalaby and Hammouda (2014), who reported an increase in the levels of FSH, LH, and testosterone following the administration of TT methanol extracts to rats. The decrease in the level of LH and testosterone hormones in the positive control rat groups might be attributed to Leydig cell apoptosis that inhibits androgen biosynthesis in rat Leydig cells. The results are comparable to the reports of Zhao et al. (2018) wherein nicotine

impaired the male reproductive hormone system through the inhibition of androgen biosynthesis and induction of Leydig cell apoptosis. Exposure to nicotine is a known cause of alteration to the male reproductive hormones (Oyeyipo et al., 2014).

The positive control groups administered nicotine hydrogen tartrate or lead acetate only showed marked testicular degeneration of large numbers of seminiferous tubules with marked cytoplasmic vacuolization of germ cells having only reduced spermatogenesis, absence of spermatozoa in the lumen, and one or two layers of germinal epithelium.

The epididymis showed congested blood vessels and interstitial edema admixed with aggregates of lymphocytes. The interstitium of the seminal vesicles showed an expanded homogenous eosinophilic material (edema) admixed with inflammatory infiltrate comprising lymphocytes, macrophages, and neutrophils. The prostate gland also revealed interstitial edema admixed with a similar inflammatory infiltrate. The results are in agreement with the literature (Cederroth et al., 2010; Condorelli et al., 2013; Jeng et al., 2014) wherein spermatogenesis was greatly inhibited by nicotine exposure. The sperm function has also been reportedly affected by exposure to lead (Vigeh et al., 2011; Ali et al., 2018; Hassan et al., 2019; Ommati et al., 2019). The improvement of sperm characters and reproductive system enhancement in the experimental group treated with TT might be attributed to their antioxidant activity, which has been reported in the literature (Kumari and Singh, 2015).

Nicotine and lead toxicity caused a significant decrease in the number of sperm, motility, and an increase in the sperm abnormalities such as the reduction in weight and size of sexual organs (testis, epididymis, and accessory sex glands), reduction of diameter and length of seminiferous tubules and negative changes to the tissues of the reproductive organs.

The pre-treatment and co-administration of methanol extract of *T. terrestris* with nicotine and lead demonstrated the antioxidant and protective effects of TT in intoxicated rats. The effect may also be due to the TT protective effect of *T. terrestris* against testicular toxicity owing to the increased release of FSH, LH, and testosterone as well as the enhanced tissue antioxidant capacity (Shalaby and Hammouda, 2014). The results of the histological analysis of the testis of the TT-treated rats revealed the normal histological appearance of seminiferous and epididymis tubules though with mild interstitial edema admixed with small numbers of lymphocytes. There was also a normal histological appearance of the seminal vesicle glands and the muscular wall while the prostate gland revealed the normal structure of the acini and mild interstitial edema. The highest seminiferous tubules diameter was observed in TT100, followed by the control group, the control vehicle, while the lowest diameter was found in the positive groups administered nicotine hydrogen tartrate or lead acetate only.

Spermatozoa are highly susceptible to peroxidative damage owing to the high concentration of polyunsaturated fatty acids involved in the regulation of spermatogenesis, sperm maturation, and capacitation. Such peroxidation of sperm lipids does not only destroy the structure of the lipid matrix in the membranes of spermatozoa but also leads to a rapid loss of intracellular ATP leading, a decreased sperm viability or in extreme cases complete inhibition of spermatogenesis (Türk et al., 2008).

The results are in line with the literature (Vigeh et al., 2006; Cederroth et al., 2010; Nesseim et al., 2011). The methanol extracts of *T. terrestris* reportedly affected a partial amelioration of rat histopathological lesions (Shalaby and Hammouda, 2014). The hexane and aqueous soluble fractions in the methanol fractions of TT have also been reported to promote changes in the intertubular compartment because they increased the nuclear volume, cytoplasmic volume, and individual volume of Leydig cells in male Wistar rats (Oliveira et al., 2015).

The novelty of the current study is the identification of relevant novel and important volatile bioactive compounds through the GC/MS analysis. The result is in agreement with the work of Khaleghi et al. (2017) who reported that *T. terrestris* extracts contain a variety of components including phenolic acids and flavonols which are highly associated with antioxidant activity. These antioxidant activities could be responsible for the improvement in sperm motility among the experimental groups. Oxidative stress harms sperm function and impairs male fertility by affecting sperm viability (Agarwal et al., 2014; Dutta et al., 2019).

Semen cryopreservation produces noteworthy amounts of reactive oxygen species (ROS), which might lead to impairment of sperm morphology and ultimately function. TT protective effects, however, improve human sperm viability and motility owing to its antioxidant properties (Asadmobini et al., 2017). In the current study, ten volatile bioactive compounds were identified and thought to play some roles in the ameliorative effect of TT. Chauhdary et al. (2019) had previously reported only eight compounds. One of the major compounds found in the methanolic extract of *T. terrestris*, in the current study, was 3-O-methyl-d-glucose which is a metabolically inactive glucose derivative with a significant protective effect on dried mouse sperm (Liu et al., 2012). It is readily uptaken by the tissues of the seminiferous epithelium and epididymal epithelium (Turner et al., 1983). Other important compounds identified from the methanolic extract of *T. terrestris* were n-hexadecanoic acid, 9,12,15-octadecatrienoic acid, (z,z,z), and hexadecanoic acid. The role of fatty acids (FA) and their esterified form (phospholipids) as structural components of the cell membrane are well known. FAs are not only an energy source but also precursors of bioactive lipid mediators which strongly influence cellular functions and responses. Furthermore, membrane flexibility increases with phospholipids with a high amount of polyunsaturated fatty acids (PUFA) due to the presence of multiple double bonds. Thus, PUFAs incorporation has an impact on semen quality and spermatogenesis (Collodel et al., 2020; Hu et al., 2018).

Other minor compounds such as 1- hexadecanol, 2-methyl, tricyclo [4.4.0.0(2,7)]dec-8-ene-3- methanol, α,α,6,8-tetramethyl-, diosgenin and squalene were also identified. Diosgenin is a well-known natural steroidal saponin that reportedly ameliorates testicular and male infertility through partial restoration of mitochondrial integrity and partial suppression of apoptosis, inflammation, oxidative stress, and neutrophil infiltration (Khosravi et al., 2019; Wu et al., 2015).

6. Conclusion

Nicotine and lead toxicity caused a significant decrease in the number of sperm, motility, and an increase in the sperm abnormalities such as the reduction in weight and size of sexual organs (testis, epididymis, and accessory sex glands), reduction of diameter and length of seminiferous tubules and negative changes to the tissues of the reproductive organs. The administration of methanol extract of *T. terrestris* to rats, however, improved the semen quantity and quality, sexual organ weights, and fertility of male rats and, thus, ameliorated the adverse effects of lead and nicotine. The GC-MS analysis of the *T. terrestris* plant methanolic extracts revealed the presence of several important bioactive compounds. *T. terrestris* is, thus, suggested as a plant of high phytopharmaceutical importance. Further isolation of the individual components would provide relevant lead to finding new drugs and thus could assist in the development of novel drug molecules with new drug targets.

CRediT authorship contribution statement

Wael Ammar Aldaddou: carried out the, Project administration. **Abdullah S.M. Aljohani:** Project administration, Supervision, was involved in the, Project administration, and project supervision. **Idris Adewale Ahmed:** were involved in the, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, and the first draft of the manuscript. All authors were then involved in the first review and subsequent completion of the critical review, final review, editing, and submission of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115337.

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