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Evaluation of Toxicity and Fertility Effects of *Inula viscosa* Aerial Parts Extract in Male Rats

Manal A. Abbas*, Nagham A. Hameed, Ekbal H. Al-Khateeb and Rana M-N. Al Haj

Faculty of Pharmacy and Medical Sciences, Al-Ahliyya Amman University, Amman 19328, Jordan

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Abstract

Inula viscosa (Asteraceae) is a herbaceous perennial Mediterranean plant. Despite its very common use in folk medicine, few studies have evaluated its toxicity. This study was conducted to investigate the toxic effects of this plant on male rats especially on the reproductive system. The flavonoid-rich acetone extract of *I. viscosa* was administered intraperitoneally to mature male rats for 60 days. In addition, a one generation fertility study was performed in order to detect the teratogenic effects of this plant. No statistically significant difference was found in the sperms count, sperms morphology, total serum testosterone level, or number and weight of newborns. Also, no gross morphological defects were observed in newborns of treated and control groups. A histological study demonstrated normal spermatogenesis. In addition, a normal architecture of prostate, liver and kidney was observed. However, some morphological alterations were detected in seminal vesicles. Furthermore, liver and kidney tests were normal. In conclusion, this study suggests that the *I. viscosa* extract has no toxic effects in male rats.

Keywords *Inula viscosa*, Toxicity, Male reproductive system, Fertility, Teratogenic effect, Folk medicine

1. Introduction

Herbal drug therapy is a common practice adopted in folk and alternative medicine and has been used in the treatment of various disorders since ancient times (Nabavizadeh *et al*., 2009). According to Ekor (2013), about 80% of individuals from developing countries use traditional medicine for their primary healthcare needs. Jordan is a country rich in flora regarding the number of plant species (Oran, 2014). It was recorded that 20% of the total flora of Jordan is medicinal plants (Oran and Al-Eisawi, 2014) which are used in folk medicine, and can be used in pharmaceutical industry. Despite the deficiency in the evidence-based safety and efficacy of herbal medicine, herbal drug utilization has been increasing in the developing countries, including Jordan (Akour *et al*., 2016). According to a survey conducted by Bardaweel (2014), 92% of males with infertility problems in Jordan resort to herbalists to treat their problems.

Inula viscosa (syn. *Dittrichia viscosa* (L.) Greuter/ Asteraceae) is a herbaceous perennial Mediterranean plant. It is known as Taioon in Arabic (Hudaib *et al*., 2008), and it can found in ruderal environments (along roads) (Parolin *et al*., 2014). In traditional medicine, *I. viscosa* is used externally as muscle relaxant (Hudaib *et al*., 2008), sedative, antiseptic, for wound healing, treating women infertility, antirheumatic, expectorant and for haemorrhoids (Ali-Shtayeh *et al*., 1998). Internally, *I. viscosa* is used as antipyretic, general tonic, antidiabetic, diuretic, antispasmodic, antihelmintic (Ali-Shtayeh *et al*., 1998; Seca *et al*., 2014), chronic coughing, and tumors (Oran and Al-Eisawi, 2015). Recently the *I. viscosa* plant has drawn the attention because of its pharmacological activities, such as its antioxidant activity (Assi *et al*., 2015), antimicrobial, anti-inflammatory (Assaf *et al*., 2016), antihypertensive and vasodilator effects (Hakkou *et al*., 2017). According to three studies conducted *in vitro*, *I. viscosa* extracts exhibited cytotoxic effects on cancer cell lines, but their effect on normal cells was not tested (Merghoub *et al*., 2009; Merghoub *et al*., 2016; Messaoudi *et al*., 2016). Similarly, *I. viscosa* extracts were effective against cancer lines with variable toxicity against the non-cancerous Vero cell line (Talib and Mahasneh, 2011). The common use of this plant in folk medicine necessitates testing its toxicity *in vivo*.

In local folk medicine, a water bath of *I. viscosa* leaves is used to induce abortion (Akour et al., 2016). Scientific research has revealed that *I. viscosa* extracts has anti-implantation and progesterone lowering activities in pregnant rats (Al-Dissi *et al*., 2001). The wide distribution and the very common use of *I. viscosa* in traditional medicine (Oran and Al-Eisawi, 2015) entailed the investigation of the effects of this plant *in vivo*. Also, long-

* Corresponding author. e-mail: manabbas@yahoo.com.

term assessment studies of its toxicity are lacking especially its effects on the male reproductive system. Therefore, the present study was conducted to shed more light on both areas; toxicity and male rat fertility.

2. Materials and Methods

2.1. Plant Collection and Preparation

The *Inula viscosa* aerial parts were collected from Al Subaihi/Jordan during May-June, 2015. The plant was authenticated by a taxonomist, Prof. Dawud Al-Eisawi of the Department of Biological Sciences at the University of Jordan. The aerial parts were dried at room temperature away from direct sunlight, coarsely grinded, and extracted by maceration in acetone since this extract is reported to be rich in flavonoids (Grande *et al*., 1985). The resulting extract was evaporated to dryness under reduced pressure using rotary evaporator. The dried extract was stored at -20 ºC until used.

2.2. Animals

Six week old Wistar rats weighing (180-200gm) were maintained under standard animal room conditions (23 $\pm$ 2 ºC) with 12 hrs light/12 hrs dark cycle. Pelleted food and tap water were available *ad-libitum*. The animals were acclimatized for one week before being used in the experiment. The experimental animals of this research were handled and treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Determination of the Median Lethal Dose (LD50)

The toxicity of the *I. viscosa* extract was evaluated by the calculation of intraperitoneal (i.p) LD_{50} which determines the dose that kills 50% of animals. The LD_{50} in rats was determined to evaluate the proper treatment dose to be used in this study. For the LD_{50} determination, BALB/c male mice (weight 20-25 g) were obtained from the Animal House of Al-Ahliyya Amman University. The LD_{50} for the *I. viscosa* acetone extract was determined according to the method of Alawi and Jeryes (1982). Mice were divided into eight groups (ten mice each). The doses of the plant extract ranged from 200 mg/kg to 1000 mg/kg, and were given i.p. Animal behavior was carefully observed for two hours, and the number of dead mice was counted in the experimental groups after twenty-four hours.

2.4. Toxicity and Fertility Study

In this part of the study, nine-week male Wistar rats were used. A total of twenty-four rats were randomly divided into three groups (eight rats each): Group I (high dose group) received 82.95 mg/kg (1/10 of the LD_{50} of the acetone extract of *I. viscosa*). Group II (low dose group) rats received 41.475 mg/kg (1/20 of the LD_{50} of the acetone extract). Group III (control group) received the vehicle of the acetone extract (100 µL of Dimethyl sulfoxide (DMSO) per rat daily). Rats received their I.P treatments once a day, and the treatment period lasted for sixty consecutive days in accordance with the WHO protocol (1983).

On the last day of the treatment, the body weight of each rat was recorded. Kidney, liver, epididymis, prostate and seminal vesicles were carefully removed from each animal and weighed quickly using a sensitive 6-digit analytical balance (Shimadzu, Japan). Tissues were fixed in 10% formalin fluid, dehydrated using graded concentrations of alcohol, and embedded in paraffin. The paraffin sections were then cut at five µM thickness using Leica microtome (Germany), stained with hematoxylin-eosin stain to be used later for the histopathological examination. Sections were examined by two histo-pathologists who were blind to the treatments, namely, Dr Iqbal Al-Qubtan of the The Hashemite University-Zarqa, and Dr. Nasrat Babouq of Biolab, Amman, Jordan. The diameter of seminiferous tubules was measured using an MC 170 HD Leica Camera, Switzerland, and the LAS EZ software.

Blood samples were collected from retro-orbital plexus for biochemical analyses, centrifuged at 2500 rpm for ten min, and stored at -20 ºC until used. The total testosterone level was performed at the Specialty Hospital (Amman–Jordan) using Chemiluminescent Microparticle Immunoassay technology (ARCHITECT i 1000 Testosterone *assay*, Abbott). Total protein**,** alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen and serum creatinine were measured using ARCHITECT c 4000 kit, *Abbott.*

For sperm count and morphology, the right cauda epididymis was removed and opened by a surgical blade in 4 ml of Hank's balanced salts solution (HBSS) to exude epididymal contents. Using a haemocytometer, two hundred sperms were counted per specimen. Sperm morphology was examined at 400X magnification and one hundred sperms were counted per specimen. Abnormalities in sperm head, mid-piece or tail were recorded.

Twenty four untreated virgin Wistar female rats weighing (160-180 g) were used for mating the treated males. On the 55th day of the treatment, each male rat was caged separately with one untreated adult female for five days. All males were sacrificed under ether anesthesia at the end of the mating period. Mated females were housed individually after being examined for vaginal sperms. After birth, the number of viable and dead newborns was recorded. The pups were weighed and examined for signs of gross physical deformity.

2.5. Statistical Analysis

All data were expressed as mean± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 7. Organ weights, sperm count, percentage of deformation in sperm morphology, number and weight of newborns, total serum testosterone, total protein**,** ALP, ALT, AST, urea and serum creatinine were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. Differences between means were considered statistically significant at $P < 0.05$.

3. Results

3.1. Determination of the Median Lethal Dose (LD50)

The administration of high doses of *I. viscosa* showed convulsions. The LD_{50} of acetone extract was found to be 829.5 mg/kg.

3.2. Toxicity Study

During this study, animals did not show any change in their general behavior, skin changes, including hair loss, defecation, or other abnormal physiological signs. No statistically-significant difference in organ weight was found between *I. viscosa* treated and control groups except for a higher testis weight in the *I. viscosa*-low dose group (Figure 1). Normal histological architecture of testis was observed (Figure 2 A, B). Cross sections of seminiferous tubule of both *I. viscosa* treated and untreated groups, showed discrete layers of germ cells. Spermatogonia were present on the basal lamina and spermatocytes were arranged inward. No retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen were observed. Furthermore, the diameter of seminiferous tubules was not statistically different between the control group (89.4±23.3 µm) and the *I. viscosa*-high dose group (93.1±21.1 µm). The epididymis had normal appearance. It did not contain desquamated germ cells or cell debris (Figure 2 C, D). Also, no changes in prostate were observed (Figure 2 E, F). On the other hand, it was noted that the height of mucosal folds in seminal vesicles were shorter in the *I. viscosa*-treated groups (Figure 2 G, H).

Data in Figure 3 also indicates that the administration of acetone extract of *I. viscosa* for sixty days did not alter serum hepatic enzymes activity, serum total protein and urea in rats belonging to both treated groups in comparison with the control group. Creatinine was normal in the group that received a high dose of *I. viscose*, but lower than that of the control group in the low dose group. Furthermore, the histological examination of liver and kidney sections for all the groups involved in the study revealed a lack of any abnormal appearance in these organs (Figure 3 A,B,C,D).

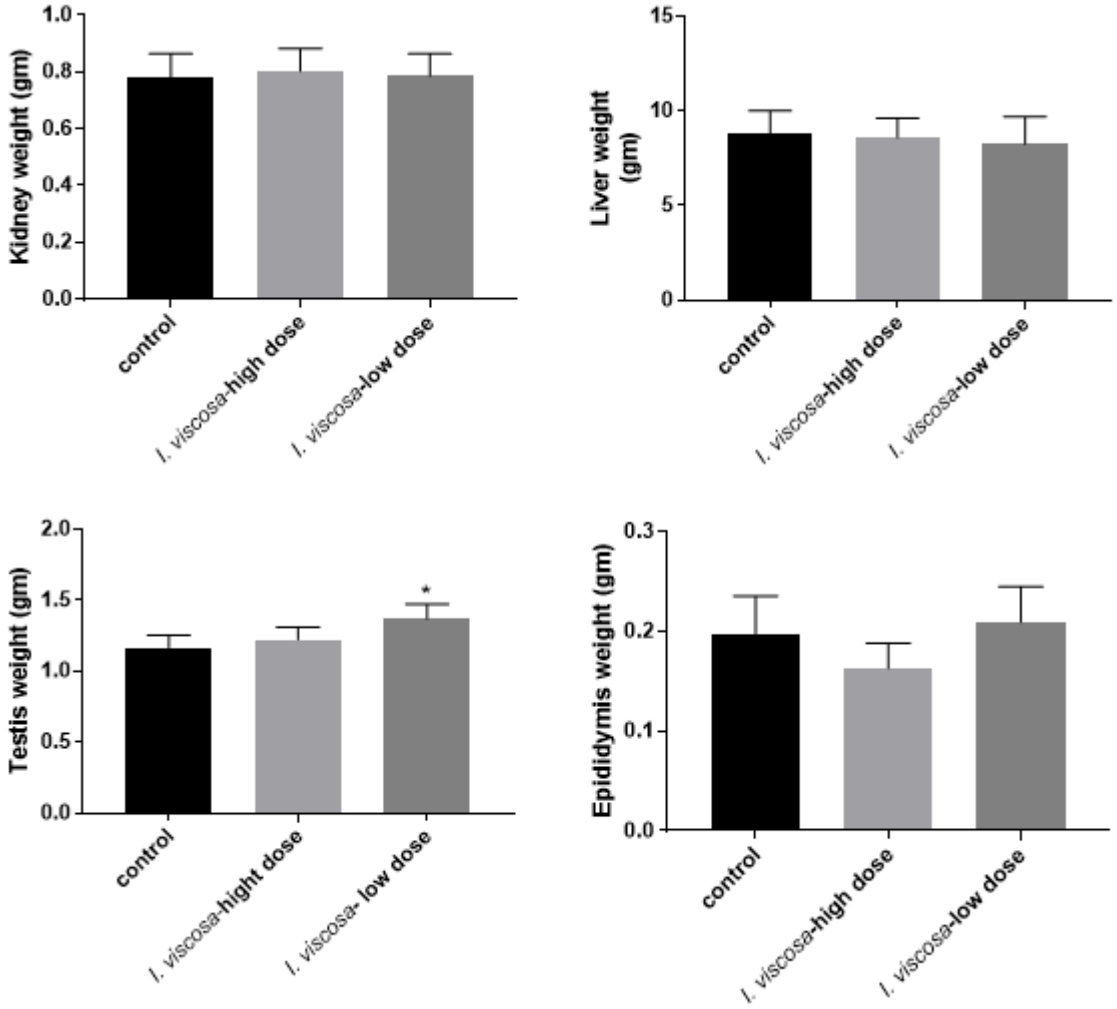

Figure 1. Effect of acetone extract of *I. viscosa* on the organ weight.

* Statistically significant difference at $p<0.05$

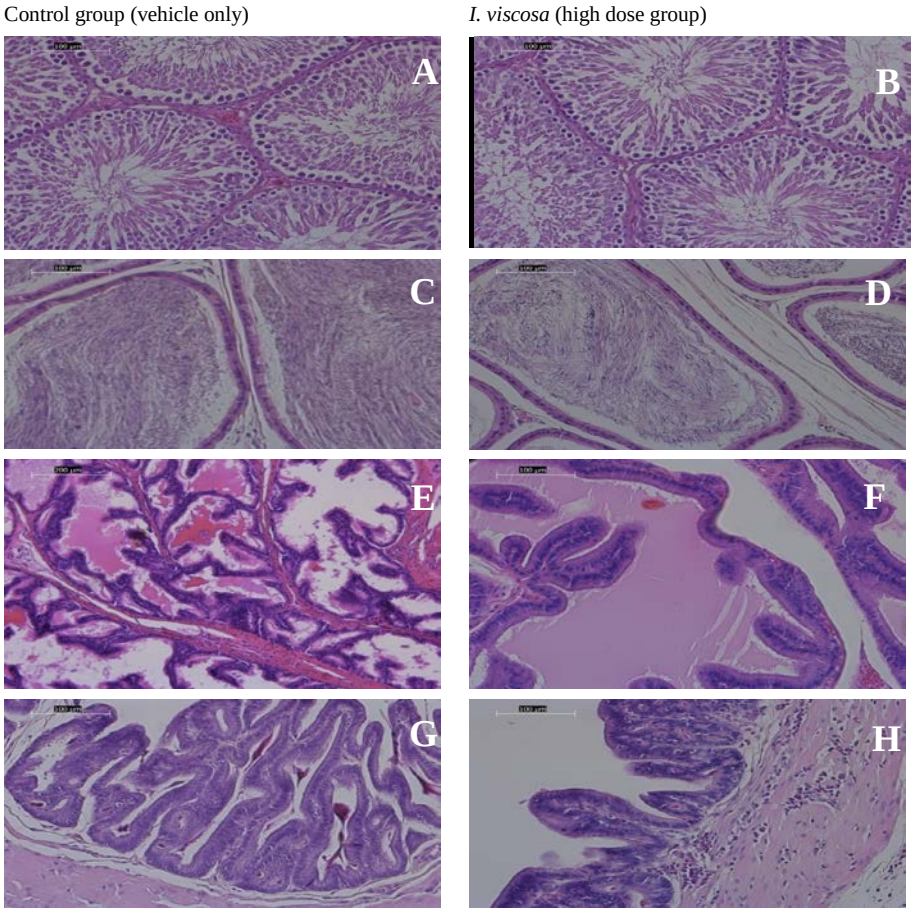

Figure 2. Cross-sections of control and *I. viscosa*-treated groups. (A,B) testis, (C,D) epididymis, (E,F) prostate, and seminal vesicle (G,H). Haematoxylin and eosin stain.

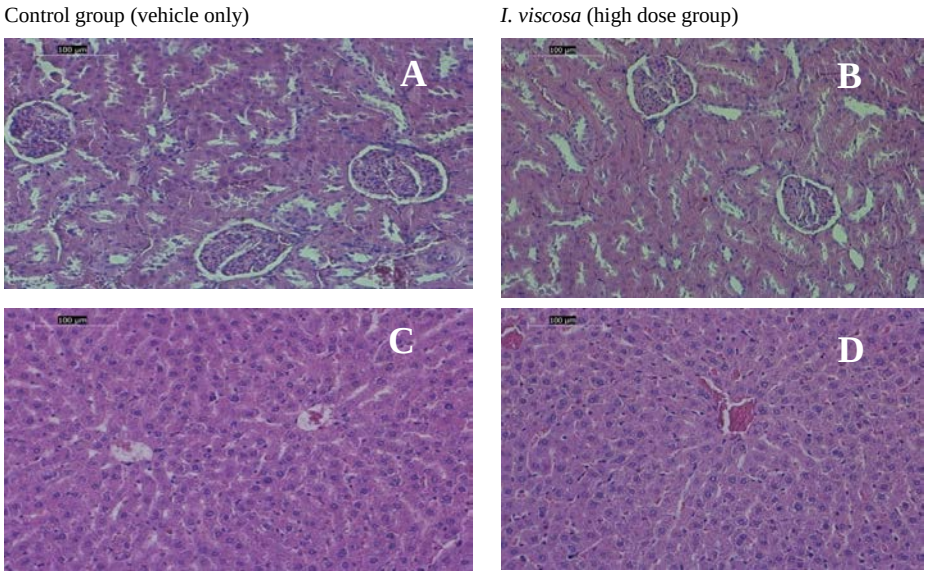

Figure 3. Cross-sections of control and *I. viscosa*-treated groups. (A,B) Kidney, (C,D) liver. Haematoxylin and eosin stain

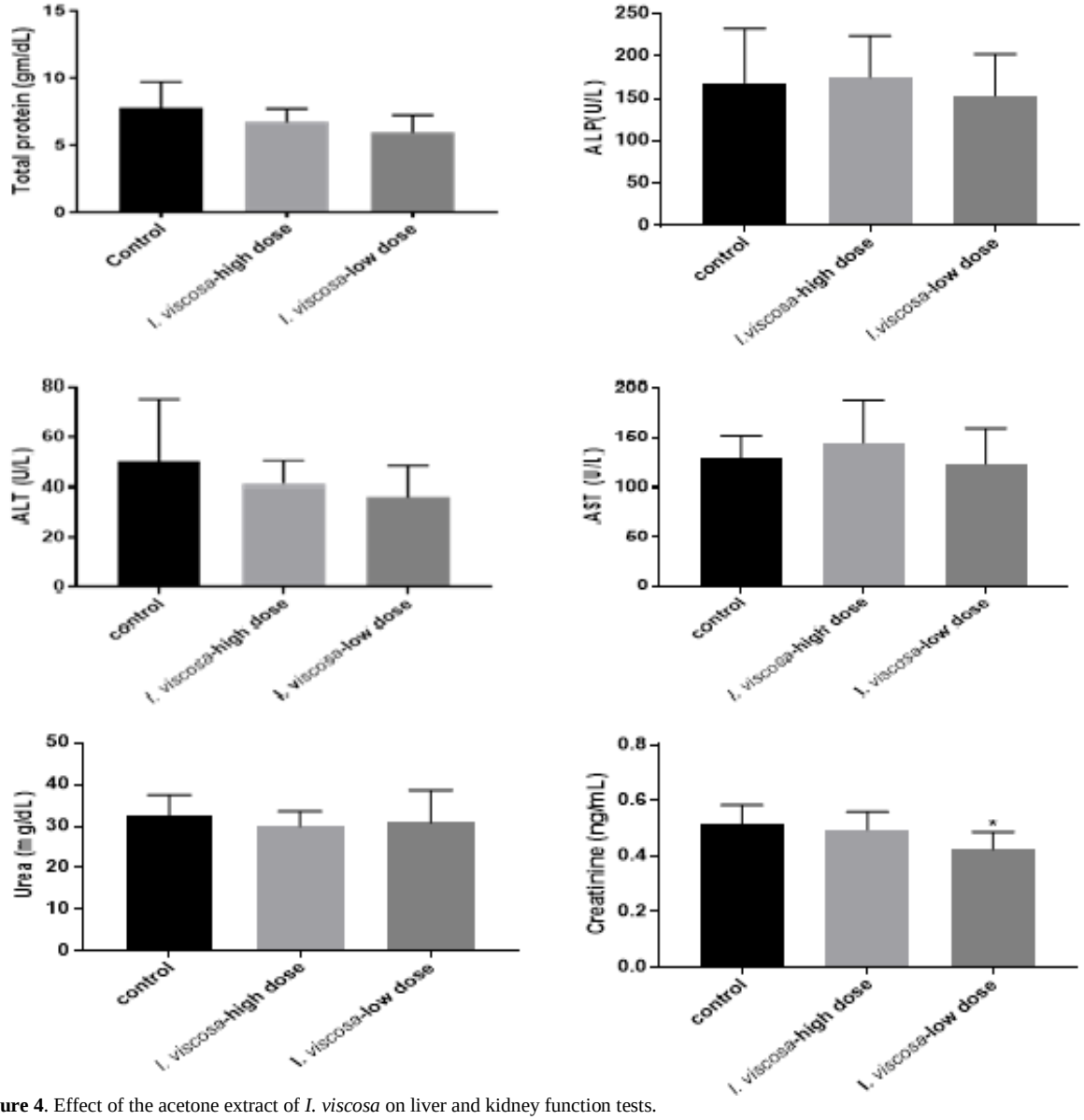

Figure 4. Effect of the acetone extract of *I. viscosa* on liver and kidney function tests.

* Statistically significant difference at $p<0.05$

3.3. Fertility Experiment

The normal sperm count, sperm morphology and the total testosterone level after the administration of various doses of *I. viscosa* acetone extract for sixty days were recorded in comparison with the control group as shown in (Table 1). In the fertility study, the number and weight of newborns in the *I. viscosa* high and low dose-treated groups were not statistically different from those of the control group (Table 1). Furthermore, no gross deformations were observed in newborns.

Table 1. Effect of Acetone extract of *I. viscosa* on sperm count, total testosterone level, number and weight of newborns. Data are represented as mean ± SEM.

Group tested	Sperm count ($x10^6$ sperms/mL)	Sperm abnormalities (% abnormal)	Total testosterone (ng/mL)	Average n° of living newborns	Weight of newborns (gm)
Control group (vehicle)	56.67±5.99	3.02±0.98	3.02±0.98	9.0±0.28	4.96±0.18
High dose acetone extract of *I. viscosa*	52.83±9.80	2.37±0.76	2.37±0.76	11.0±0.45	5.67±0.30
Low dose acetone extract of *I. viscosa*	57.40±2.21	3.23±1.00	1.24±0.34	9.0±1.21	4.96±0.17

* Statistically significant difference at $p<0.05$

4. Discussion

Interest in studying the pharmacological activity of medicinal plants has increased recently. In fact, reports of efficacy are, by far, more numerous than those on toxicity. Therefore, there is an urgent need to study the toxicity of plant extracts (Bello *et al*., 2016; Ghadirkhomi *et al*., 2016). In this study, no significant differences in kidney, liver and epididymis weights were found upon the administration of the *I. viscosa* aerial part extract. This is an indication that the extract did not alter the size and weight of the organs. As for the control group, the measured values for kidney and liver function tests were found almost normal compared to the normal range published by Charles River laboratories in 2008 (Giknis and Cifford, 2008). Even though, many researchers have already reported factors that affect the serum clinical pathology values in rats such as availability of food before and at the time of bleeding, type of anaesthesia, site of blood sampling, storage conditions, differences in the analytical methods used (Matsuzawa *et al*., 1997), in addition to the environmental conditions, gender, age, origin of breeding system, and feeding (Teixeira *et al*., 2000).

Hepatic enzyme activity was studied to detect any liver malfunction. These enzymatic values went up in acute hepatotoxicity but declined with intoxication due to the damage in the liver (Obi *et al*., 2004). In case of tissue damage, enzymes specific to that tissue are released into circulation. This led to an increase in the activity of these enzymes in the serum (Aliyu *et al*., 2006). Depending on the results clarified in Figure 4, there was no significant difference in the serum ALT, AST, ALP activities between groups receiving different doses of the *I. viscosa* extract and the control group during the treatment period. This indicates that the extract of *I. viscosa* was not harmful to the liver.

The results of the total serum protein and serum urea levels obtained in this study in the control group were within the limits, and are somehow close to the results obtained by other Jordanian researchers (Khouri and Daradka, 2012). The total serum protein may be altered due to liver and/or kidney damage (Fox, 2016). In this experimental study, no significant change in total protein and urea levels was observed between the control and the treated groups. Also, the creatinine level was not raised during the sixty-day treatment with the *I. viscosa* extract. On the contrary, the low dose of the *I. viscosa* extract has significantly lowered the creatinine level. This is an indication that the *I. viscosa* extract was not toxic to the kidney. It is well-known that creatinine is the main catabolic metabolite of the muscle, excreted by the kidneys, and that it is a good indicator of a renal failure (Aliyu *et al*., 2006).

In our sixty-day study, the normal histological morphology of the liver and kidney supports our biochemical results which indicate that the *I. viscosa* extract has no hepatic or renal toxicity. Similar results were obtained by Zaza (2005) who found no change in the liver-enzyme levels (AST, ALT) when aqueous, methanolic and petroleum ether extracts of *I. viscosa* were administered to rats for one month. Atoom (2002) reported that the ethanolic extract of this plant has a slight hepatoprotective effect. In fact, a soft drink of *I. viscosa* is commercially available, and is used to treat non-alcoholic steatohepatitis and other non-alcoholic fatty liver diseases (Assy *et al*., 2012). All these results can be explained by the presence of active constituents in the *I. viscosa* extract, including flavonoids, which are endowed with an antioxidant activity (Assi *et al*., 2015).

An aqueous extract of *I. viscosa* was found to have progesterone lowering effect in pregnant rats (Al-Dissi *et al*., 2001). In our study, no effect of the *I. viscosa* extract on testosterone level was found in male rats. The effect of the *I. viscosa* extract results from the complex interactions between different components that have synergistic and/or antagonistic effects. The acetone extract of *I. viscosa* aerial parts is rich in biologically- active flavonoid aglycones (Grande *et al*., 1985). From this fraction the flavones apigenin, scutellarein 6-methyl ether (hispidulin) and luteolin, 6-methoxyluteolin (nepetin) were isolated (Wollenweber *et al*., 1991). Apigenin was reported to have adverse effects on the male reproductive system in mice (Li *et al*., 2010). On the other hand, luteolin was reported to possess phosphodiesterase type five (PDE5) inhibitory activity (Ko *et al*., 2004) indicating beneficial effects on erection.

Regulatory guidelines for reproductive and fertility toxicity studies consider histopathology as a sensitive and early indicator of spermatogenic disturbances (Creasy, 1997). There are four targets for testicular toxicants. These are: the Sertoli cell, the Leydig cell, the germ cells (each has its own specific chemical sensitivity) and the vascular endothelium. In this study, no change in the histological architecture of the testis and epididymis was found. Also, there was no change in the seminiferous tubule diameter. In addition, the number of epididymal sperms was comparable to that of the control group. Furthermore, no change in testosterone level was found between the control group and the *I. viscosa* treated groups. Therefore, it can be concluded that the *I. viscosa* extract is unlikely to have a harmful effect on testis. A higher testicular weight was found in the *I. viscosa*-low-dose group compared to the control group while no change in epididymis weight was observed between the control and the *I. viscosa*-treated groups (Figure 1). According to Creasy (1997) if a significant decrease in testis weight was found, it is expected to find an obvious germ cell loss. On the other hand, if there is no significant reduction in testis and epididymis weights, it is unlikely to have a clear change in testicular morphology.

Since accessory sex glands are androgen-dependent, they may reflect changes in the endocrine status and testicular function (Campion *et al*., 2012). Seminal vesicle mucosal fold height was lower in the *I. viscosa* treated groups. Further research is needed to investigate this aspect. To our knowledge, the results of this study are the first to demonstrate the effect of the *I. viscosa* acetone extract on male rat fertility.

5. Conclusion

In relation to the experiments performed in this study, one can conclude that the administration of *I. viscosa* acetone extract for sixty days at the doses investigated had no toxic effect on male fertility and organ toxicity in rats.

This may support the safety of using this plant in Jordanian folk medicine. However, further studies are needed either to ascertain its safety or to investigate the effects of its active constituents and pure compounds (after isolation) in humans.

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Conflict of interest

The authors declare no conflict of interest.

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