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*Full Length Research Paper*

# Mesquite (*Prosopis juliflora*) pod extract decreases fertility in female but not male rats

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**The administration of Mesquite pod extract (containing mesquitol, daidzein and genistein) to female and male rats disrupts reproductive variables. However, its effect on fertility is not known. This study evaluated fertility in male and female rats treated with mesquite pod extract, comparing its effects with those of daidzein and estradiol. The following treatments were given for 30 days to groups of female and male rats: vehicle, mesquite pod extract, DAI and $E_2$. Treatments were administered subcutaneously for 30 days. These extract disrupted both the female and male sexual behavior in a similar way to DAI, but less than $E_2$. Mesquite pod extract increased the number of days in estrus and decreased lordosis intensity during proestrus. Mesquite pod extract-treated males showed lower testicular and glandular weights, as well as decreased sperm motility, viability and count. In females treated with mesquite pod extract, the number of pups was lower than in control females, and 10 to 20% of pups were dead. These effects were similar to those with DAI-treatment. Despite the lower sperm quality, the fertility of mesquite pod extract- and DAI-treated males seem not to be disrupted, as they could impregnate control females. These results show that mesquite pod extract can disrupt female but not male fertility**.

**Key words:** Mesquite pod extract, daidzein, fertility, offspring, sexual behavior, phytoestrogens.

## INTRODUCTION

Discovery of clover disease in ewes (Bennetts et al., 1946) consumption of feed containing large amounts of plant oestrogens has been suspected to cause temporary or permanent fertility problems in ruminants. It

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has been reported that low intake of food with high content of isoflavones causes temporary infertility, and prolonged consumption can cause permanent infertility (Marshall, 1973; Adams, 1990; Adams, 1995).

There are very few studies on the effects of fodder with high phytoestrogen concentration on livestock fertility. A recent study evaluated the effects on conception and early gestation of nulliparous ewes fed for five months before gestation with red clover rich in the phytoestrogen formononetin. Although fecundity was not reduced, lower levels of progesterone were observed as well as increased amount of fetal fluids, which can increase the risk of vaginal prolapse before term (Mustonen et al., 2014). Heifers fed clover had a lesser conception rate, and a greater percentage of heifers returning to estrus than silage-fed heifers. This indicates that isoflavone content in clover disturbs hormonal balance during early pregnancy, leading to a reduction in the fertility of heifers (Hashem et al., 2016).

In women with problems becoming pregnant, a high dietary isoflavone intake has been associated with a higher risk of nulligravidity, that is a higher risk of never becoming pregnant or never giving birth to a live child. Indeed, when isoflavone intake exceeds 40 mg/day, the overall lifetime risk of never becoming pregnant increases 13% (Jacobsen et al., 2014).

In animal models, several plant extracts have been reported to have antifertility activity. The methanolic extract of *Artemisa vulgaris* leaves which is a plant containing flavonoids, possesses estrogenic activity at doses 300 or 600 mg/kg when administered orally from day 1 to 10 of pregnancy causes major implantation failure (100%) in female rats (Shaik et al., 2004). Similarly, the aqueous extract of *Pouzolzia mixta* (a plant native of Africa and used by women as a post-coital contraceptive) administered orally to female rats at a dose of 300 mg/kg for 7 days followed by mating and given an additional treatment for 10 days post-conception, inhibits both implantation and fertility (Sewani-Rusike, 2013). Flower extracts of *Tabernaemontana divaricata*, a plant native of Asia, which also contains flavonoids, is used by women as a traditional medicine for family planning. This possesses estrogenic, anti-implantation and early abortive activity, when administered in doses of 500 mg/kg to female rats (Mukhram et al., 2012). This experimental evidence reinforces the fact that any plant with estrogenic activity can also have potential anti-fertility activity in females.

In men, it has been hypothesized that compounds with endocrine disrupting effects such as phytoestrogens may be associated with decreased semen quality (Xia et al., 2013). Although the evidence is not conclusive, epidemiological studies suggest that phytoestrogen consumption in Chinese and Japanese men can be related to erectile dysfunction (Bai et al., 2004),and lower sperm concentration (Xia et al., 2013; Giwercman, 2011; Iwamoto et al., 2007; Phillips and Tanphaichitr, 2008), causing idiopathic male infertility mainly in Chinese and Japanese men. A similar effect has been observed in American men through the intake of soy based foods (Chavarro et al., 2008).

Mosquite (*Prosopis sp*) is a widespread legume in arid and semi-arid areas in Mexico, Africa and Asia. This leguminous plant is widely used to feed several livestock species due to its high content of protein, carbohydrates, fiber (Kathirvel and Kumudha 2011), minerals and vitamins (Choge et al., 2007; Freyre et al., 2010). In addition, mesquite pod is a source of food for human consumption as bread (Cattaneo et al., 2016; Cruz, 1999), cakes or porridge (Freyre et al., 2010), syrup and beverages (Cruz, 1986), desserts, and as a coffee substitute (Azevedo et al., 1987), which possesses antioxidant activity (Karim and Azlan, 2012). Mesquite contains high amounts of some phytoestrogens such as the flavanol mesquitol (in concentration of 6.4 µg/g) (Sirmah et al., 2009), flavonols such as quercetin, luteolin, and isoharmnetin, the flavone vitexin (Gianinetto et al., 1975), and isoflavones genistein (60.25 µg/g) and daidzein (5.27 µg/g) (González et al., 2015). All these phytoestrogens may contribute to the estrogenic effects of mesquite in livestock and other animals, including human beings. The only reports about the effects of mesquite pod extract on female and male reproduction are from our laboratory. Mesquite pod extract alters estrous cyclicity, decreases lordotic quotient and intensity of lordosis in intact rats. In ovariectomized rats, mesquite pod extract induce vaginal estrus, increased vaginal epithelium height and lordosis; all these effects are similar to those caused by daidzein and genistein (Retana-Márquez et al., 2012). In male rats, mesquite pod extract disrupts sexual behavior, increases testicular germ cell apoptosis, decreases sperm quality and plasma testosterone concentrations in a similar way than genistein and daidzein (Retana-Márquez et al., 2016).

To date, there are no studies about the possible undesirable effects of mesquite pod on fertility when used to feed livestock or human beings. Considering that mesquite pod extract disrupts some reproductive variables in female and male rats, we hypothesize that mesquite pod extract can decrease fertility in female and male rats. Therefore, the aim of this study was to evaluate fertility in rats treated with mesquite pod extract. The percentages of pregnant females observed and the number of offspring per litter were evaluated. The effects were compared with those of daidzein and estradiol used as controls.

## MATERIALS AND METHODS

### Animals

All experimental procedures used in this study were approved by the Universidad Autónoma Metropolitana's Institutional Animal Care and Use Committee, in accordance with the National Institute

of Health's Guide for the Care and Use of Laboratory Animals, and Mexican Official Regulation (NOM-062-ZOO-1999).

Adult female (250 to 300 g) and male (300 to 350g) Wistar rats (three months of age) were housed five per cage (50×30×20 cm), under standard vivarium conditions. The colony room was maintained on a 12:12 reversed light cycle (lights off: 10:00) and under controlled temperature (23 ± 1°C). Food and water were available ad libitum throughout the experiments. The rodent diet used was "2018 Teklad global" from Harlan Laboratories. Although this diet contains phytoestrogens DAI and GEN (range from 15.0 to 25.0 µg/g, www.harlan.com), there are no reports confirming any estrogenic effects of this rodent diet (Naciff et al., 2004). This amount is far below the experiment given to the animals in this study. Moreover, all animals in this study were exposed to the same diet ad libitum regardless of their treatment group. The effects of mesquite extract, daidzein (DAI), and estradiol ($E_2$) were tested in intact rats.

**Treatments**

The following treatments were given to the experimental groups of females (n=30/group, to have 10 females copulating with males from all the groups) and males (n=10/group. This number was enough to impregnate all females): 1) negative control group: rats received only vehicle injections (corn oil, 0.2 ml); 2) mesquite pod extract group: rats received mesquite pod concentrated extract (3.5 g/kg/day wet pod weight); 3) DAI group: rats received DAI (reference D7802, Sigma, purity >97%; 5 mg/kg/day); 4) positive control group: rats received $E_2$ (reference E1024-1G, Sigma, purity >98%; 30 µg/kg/day). All the treatments, except mesquite extract were dissolved in corn oil and administered subcutaneously (sc) in the dorsal region of the neck, daily for 30 consecutive days. The volume of injection was 0.2 ml in all cases. Treatment administration was made in the same vivarium room every day.

The dose of mesquite pod extract was selected according to daily consumption in ewes (140 g/day), which is equivalent to 3.5 g/kg/day and corresponds to 1g/0.3Kg/day in the rat. DAI dose was selected according to those used to elicit physiological responses in males, such as reduced reproductive hormone levels and erectile responses in male rats (Retana-Márquez et al., 2016), as well as disrupted estrous cyclicity, reduced body weight, ovarian hypertrophy, and some non-lasting effects on socio-sexual behavior in female rats (Retana-Márquez et al., 2012; Henry and Witt, 2002). The $E_2$ dose used in this study is known to induce testicular atrophy in males (Ikegawa et al., 1995), and receptive behavior in female rats (Retana-Márquez et al., 2003).

**Mesquite pod extract preparation**

Only mature pods from mesquite (*Prosopis juliflora*) were used. They were collected in summer from the arid Hidalgo region of central Mexico.

The Mesquite pods were dried in an airflow oven at 60°C for 24 h. It was then ground (pod and seeds) in a Thomas-Willey cutting mill with 5 mm diameter sieves and then with 2 and 1 mm diameter sieves. The extract was obtained from 2 kg of powdered fruit by a Shoxhlet extractor to depletion with ethanol-water (90:10 v/v) for 18 h. Ethanol was eliminated by distillation at 78 to 87°C. The final concentration of extract was 1.76 g/ml. The concentrated aqueous extract was administered in a dose of 1g/0.2 ml.

Mesquite pod extract contains: mesquitol in concentration of 6.4 µg/g (Sirmah et al., 1987), isoflavones genistein (60.25 µg/g) and daidzein (5.27 µg/g) (González et al., 2015). Other components of the extract are: crude protein (26.69 to 29.84%), crude lipid (11.89 to 13.75%), total crude fiber (8.78 to 9.89%), ash (3.99 - 4.95%) and carbohydrates (42.45 to 46.37%). The range of anti-nutritional factors reported are as follows: total free phenolics 4.93 to 8.58%, tannins 6.81 to 9.15%, L-DOPA 2.21 to 4.52%, phytic acid 0.33 to 0.89 g100g-1, and trypsin inhibitor activity 40.4 to 48.2 TIU mg-1 protein (Kathirvel and Kumudha, 2011). The treatments were administered to males and females for 30 days during which, the estrous cycle was monitored in females.

**Female measurements**

***Vaginal cytology and estrous cyclicity***

Estrous cycles were monitored by daily evaluation of vaginal smears which were stained with hematoxilin-eosin and evaluated with an optic microscope (Olympus, model CX41RF). Vaginal smears were obtained two hours after the onset of the dark period, under red light (40 Watts). Estrous cycles were classified as follows: (a) 3-day cycle, an irregular shortened cycle usually resulting from a condensed or absent diestrus period; (b) 4-day cycle, a normal length cycle consisting of full estrus, metestrus, diestrus, and proestrus periods; (c) 4 to 5-day cycle, also call normal length cycle that includes an additional 24 h of diestrus, called diestrus II; (d) constant estrus, an irregular estrous cycle defined by the persistence of cornified cells beyond 2 days (Henry and Witt, 2002).

***Behavioral testing***

Female sexual behavior was assessed in a Plexiglas arena (40x40x50 cm) with males. Testing was done during the first three hours of the dark phase, under red light. Female behavior was assessed through receptivity and proceptivity. Receptivity for each female was determined as a lordosis quotient

$$[LQ = (\text{number of lordosis}/10 \text{ mounts}) \times 100]$$

The intensity of lordosis (extent of dorsiflexion) was quantified according to the lordosis score proposed by Lehman and Erskine (2004). The rating of lordosis intensity (LR) was established based on the degree of spinal dorsiflexion and the extent to which the sagittal ridge of the head lined up in a vertical plane. The rating was based on a scale of no vertebral dorsiflexion (0), slight dorsiflexion coupled with slight movement of the head toward the vertical plane (1), moderate dorsiflexion coupled with vertical movement of the head (2), extreme dorsiflexion coupled with vertical movement of the head (3).

Proceptivity was evaluated by determining the incidence of hopping, darting, and ear-wiggling across the whole receptivity test (Madlafousek and Hlinak, 1977). Females were considered to be proceptive when two of these behaviors are showed during the testing period. Female sexual behavior was assessed in females in Proestrus stage, from day 30 of treatment.

**Male measurements**

***Behavioral testing***

Male sexual behavior was assessed three times, one assessment per week. Behavioral testing was performed under dim red lights, 3 h after the onset of the dark phase of the light/dark cycle. Male sexual behavior was assessed by placing the male in a Plexiglas arena (40 x 40 x 50 cm) 5 min before a stimulus receptive female was presented. Female rats were brought into sexual receptivity by

administering estradiol benzoate (Sigma Chemical, Co. St. Louis MO., Purity 98%, 10 µg/100 µl oil, SC) 44 h before sexual tests.

Progesterone (Sigma Chemical, Co. St. Louis MO., purity 99%, 1 mg/200 µl oil, SC) was administered 4 h prior to testing. After presentation of the female, tests were lasted for 30min. Upon presentation of the female, the following variables of male sexual behavior were recorded: latency to the first mount, latency to the first intromission, and latency to the first ejaculation; number of mounts (mounts with pelvic thrusting) and intromissions (mounts with pelvic thrusting and penile insertion) of the first copulatory series. In addition, ejaculation frequency (number of ejaculations during 30 min of recording), and post-ejaculatory interval (time between ejaculation and subsequent intromission) were recorded. The full description of male sexual behavior variables has been detailed elsewhere (Hull et al., 2006; Meisel and Sachs, 1994).

### *Sperm analysis*

The right cauda epididymis was stored with 1 ml of saline at 37°C. Each cauda epididymis was cut using fine tip dissection scissors to release the sperm stored in the cauda.

### *Sperm viability*

Sperm viability was assessed by using one-step eosin-nigrosin (5% nigrosin, 1% eosin and sodium citrate dissolved in distilled water) staining technique (Lucio et al., 2009). A sample of epididymal sperm suspension (10 µl) was mixed with the colorant solution (10 µl) and analyzed on a pre-warmed slide with a cover slip.

Slides were observed under an optical microscope (Olympus Light Microscope CX 41) 40x objective lens. Unstained spermatozoa were counted as viable whereas stained spermatozoa were counted as dead. Different fields were analyzed randomly until two hundred spermatozoa were counted. Sperm viability was reported as the percentage of viable spermatozoa out of the total count.

### Sperm motility

Sperm motility was assessed by counting motile and non-motile sperm from a total of 200 spermatozoa. Sperm motility was expressed as a percentage of motile spermatozoa out of the total spermatozoa counted.

### Sperm count

Epididymal sperm count was performed using Neubauer haemocytometer. Epididymal sperm suspension (25 µl) was taken to 500 µl final volume with distilled water. The sperm suspension (10 µl) was placed on the Neubauer haemocytometer and counted in eight different spots. Count discrimination criteria were set using the spermatozoa head position inside the chamber squares that were being analyzed. Formula count was resolved as follows:

Sperm count X # chamber squares (8) X dilution factor (21) X 10, 000 / 2.

Final sperm count was expressed in millions per milliliter.

### Fertility evaluation

At the end of 30 days of treatment, females were allowed to copulate with males of all the groups, according to the scheme showed in Figure 1.

Males were allowed to ejaculate twice with each female in order to ensure pregnancy. This was considered at day 0 of pregnancy. Treatments continued for all females during gestation period till day 22. Litter size was evaluated after birth.

### Statistical analyses

Lordosis quotient, intensity of lordosis, body and glandular weights, as well as male sexual behavior variables were, analyzed by one-way ANOVA, followed by Newman-Keuls post hoc test.

Percentages of females with normal or abnormal cycles, percentages of females presenting lordosis, receptivity and proceptivity, percentages of pregnant females and percentage of dead pups, as well as spermatic variables in males were analyzed by Chi-square test followed by Dunn's multiple comparisons.

The number of offspring was analyzed by a two way ANOVA with treatments of the males in which females are copulated as factors, followed by Newman-Keuls post hoc test. The level of significance was considered with $p<0.05$. Data are reported as mean ± S.E.M. All analyses were performed with GB-Stat School Pack.

## RESULTS

### Females vaginal cytology and estrous cyclicity

Vaginal smears showed the treatment-dependent changes in estrous cycles. Normal progression of all stages was observed in the estrous cycle of vehicle females. Mesquite pod extract administration to females caused irregular estrous cycles in 95% of subjects ($p<0.01$), with an increase in the number of days in estrus (2 to 3) and some 3-day cycles throughout the treatment.

DAI administration also caused an increase in the number of days in estrus (3 to 4) in 87% ($p<0.01$) of females, and a decrease of days in diestrus. All females treated with $E_2$ showed shortened cycles (absence of metestrus and diestrus) from the second day of treatment, and constant estrus from day 12 of treatment. Representative estrous cycles of females from the different experimental groups are shown in Figure 2.

### Female sexual behavior

The percentage of sexually receptive females exhibiting lordosis behavior throughout the tests in the treated groups (Mesquite, DAI, $E_2$) was significantly different from the control group. In fact, all females from control and $E_2$ groups exhibited lordosis behavior. In contrast, mesquite pod extract-treated females (77%) or DAI-treated females (85%) displaying lordosis in proestrus were fewer than control and $E_2$-females ($p<0.05$). The lordosis quotient (LQ) was also different among groups.

Indeed, LQ in DAI- and $E_2$-treated females was similar to control females. In contrast, females treated with

| | ♂ | ♂ | ♂ | ♂ |
|---|---|---|---|---|
| ♀ | CON/CON | MES/CON | DAI/CON | $E_2$/CON |
| ♀ | CON/MES | MES/MES | DAI/MES | $E_2$/MES |
| ♀ | CON/DAI | MES/DAI | DAI/DAI | $E_2$/DAI |
| ♀ | CON/$E_2$ | MES/ $E_2$ | DAI/ $E_2$ | $E_2$/$E_2$ |

**Figure 1.** Representative estrous cycles of females treated with vehicle (CONTROL), mesquite pod extract (EXT), estradiol (E2) or daidzein (DAI). Control females had normal 4–5 day cycles. Mesquite pod extract, estradiol, and daidzein induced some 3-day cycles, with absence of diestrus periods, as well as an increase in the days in estrus. (E) Estrus; (M) Metestrus; (D) Diestrus; (P) Proestrus.

**Table 1.** Female sexual behavior in control and experimental females during proestrus. Percentage of females presenting proceptive and receptive behaviors. Lordotic quotient and intensity were evaluated after 30 days of treatment.

| Group | Proceptive behavior (%) | Sexual receptivity (%) | Lordotic quotient | Lordosis Intensity |
|---|---|---|---|---|
| Control | 100[a] | 100[a] | 100 ± 0[a] | 2.75 ± 0.04[a] |
| Extract | 43[b] | 77[b] | 86.5 ± 5.9[b] | 1.83 ± 0.10[b] |
| Daidzein | 48[b] | 85[b] | 98.5 ± 1.5[a] | 2.35 ± 0.21[b] |
| Estradiol | 100[a] | 100[a] | 100 ± 0[a] | 3.00 ± 0[a] |

[b] Different from control group.

mesquite pod extract group showed lower LQ (86.5; $p<0.01$; Table 1). The intensity of lordosis (extent of dorsiflexion), was also different depending on the treatment. Maximum lordosis intensity was observed in all control females during proestrus, and this variable did not differ from $E_2$-treated females. In contrast, females in natural proestrus treated with mesquite extract, besides showing a lower lordosis quotient, had a lower intensity of lordosis (1.83) than control and $E_2$ females ($p<0.01$). Low lordosis intensity was also observed in DAI-treated females (2.35) compared to control and $E_2$ females ($p<0.05$).

Finally, only 43% of females from the mesquite extract group and 48% of DAI-treated females displayed proceptive behavior (hopping, darting and ear wiggling) during proestrus ($p<0.01$; Table 1). Since females were copulated with males in order to become pregnant, body and glandular weights were registered in females after weaning. Body weight in females are from mesquite extract, DAI and $E_2$ groups were lower than in those of the control group ($p<0.05$). $E_2$-treated females showed the lowest body weight ($p<0.01$). Ovaries and uterus weights in females are from mesquite pod extract, DAI and $E_2$ were not different from controls, but vagina weight increased in all experimental groups compared to the control group ($p<0.05$). No data on females treated with $E_2$ are shown, since all females were in estrus due to the hormonal treatment (Table 2).

## Males

### Body and glandular weights

At the end of the 30 days of treatments, body weight of males treated with mesquite pod extract and $E_2$ was lower than that of control males ($p<0.01$; Table 3). No difference was observed in males from DAI group compared to control males. Testicular weight in males from all the experimental groups was lower than that of

**Table 2.** Body and glandular weights in control and treated females after weaning.

| Groups | Body Weight (g) | Ovaries weight (mg) | Uterus weigth (mg) | Vagina weigth (mg) |
|---|---|---|---|---|
| Control-Proestrus | 301.8 ± 11.5[a] | 71.2 ± 5.1[a] | 630.0 ± 97.7[a] | 97.5 ± 12.5[a] |
| Mesquite pod extract – Proestrus | 265.9 ± 6.9[b] | 75.6 ± 2.4[a] | 778.7 ± 113.5[a] | 155.7 ± 7.1[b] |
| Daidzein – Proestrus | 255.6 ± 6.7[b] | 78.0 ± 2.4[a] | 792.0 ± 60.4[a] | 168.0 ± 22.4[b] |
| Estradiol – Estrus | 225.5 ± 5.7[c] | 31.4 ± 1.4[c] | 720.0 ± 41.7[a] | 143.0 ± 9.2[b] |
| | | | | |
| Control – Diestrus | 284.6 ± 3.1[a] | 70.0 ± 9.1[a] | 647.5 ± 177.2[a] | 122.5 ± 13.1[a] |
| Mesquite pod extract - Diestrus | 251.0 ± 16.8[b] | 63.7 ± 2.6[a] | 860.0 ± 110.0[a] | 160.0 ± 20.0[a] |
| Daidzein - Diestrus | 238.3 ± 10.2[b] | 83.0 ± 6.3[a] | 744.0 ± 60.7[a] | 142.0 ± 15.6[a] |

[a] Not different from control group; [b] different from control group, $p<0.05$; [c] different from all the groups, $p<0.01$

**Table 3.** Body and glandular weights in control and treated males after 30 days of treatment.

| Group | Body weigth (g) | Testes weight (g) | Epididymis (mg) | Seminal gland (g) | Prostate (mg) |
|---|---|---|---|---|---|
| Control | 404.94 ± 8.26[a] | 1.88 ± 0.03[a] | 758 ± 8.13[a] | 2.05 ± 0.09[a] | 685 ± 31.13[a] |
| Extract | 352.45 ± 14.11[b] | 1.68 ± 0.03[b] | 692 ± 20.91[b] | 1.55 ± 1.13[b] | 553 ± 31.02[b] |
| Daidzein | 393.58 ± 14.31[a] | 1.64 ± 0.03[b] | 611.11 ± 30.75[b] | 1.74 ± 1.09[b] | 603 ± 51.98[a] |
| Estradiol | 312.13 ± 7.44[b] | 0.36 ± 0.01[c] | 0.24 ± 0.02[c] | 0.17 ± 0.008[c] | 0.13 ± 0.01[c] |

[b] different from the control group, $p<0.01$; [c] different from all the groups, $p<0.01$.

**Table 4.** Sperm total motility, viability, sperm concentration, and percentage of abnormalities observed in control and treated males with 30 days of treatment.

| Group | Motility (%) | Viability (%) | Count x $10^6$ (Mean ± SEM) | Abnormalities (%) |
|---|---|---|---|---|
| Control | 89.25 ± 0.85[a] | 92.2 ± 1.11[a] | 173.64 ± 3.33[a] | 0.9 ± 0.14[a] |
| Extract | 55.75 ± 5.89[b] | 68.5 ± 3.77[b] | 105.98 ± 14.27[b] | 2.75 ± 0.78[b] |
| Daidzein | 70.33 ± 2.45[b] | 72.72 ± 2.48[b] | 95.65 ± 9.71[b] | 3.72 ± 0.78[b] |
| Estradiol | 25.5 ± 7.31[c] | 29.33 ± 3.01[c] | 10.64 ± 5.61[c] | 5.17 ± 0.18[c] |

[b] different from the control group, $p<0.01$; [c] different from all the groups, $p<0.01$.

Prostate, seminal glands and epididymis weights from males of the mesquite extract, DAI and E2 groups were lower than those of control males ($p<0.05$). Atrophic glands and epididymis were observed only in males from E2 group ($p<0.01$; Table 3).

## Sperm parameters

In males treated with mesquite pod extract, DAI or E2, the values of total sperm motility, sperm viability and sperm count were lower than in control males ($p<001$; Table 3). $E_2$-treatment caused the most disruptive effects, and only few spermatozoa were obtained from atrophic epididymi of males after 30 days of treatment. In males from mesquite pod extract and DAI groups, these values were lower compared with those of control males; values registered in mesquite pod extract- and DAI-treated males did not differ significantly between them.

Finally, the percentage of sperm abnormalities increased in males from all experimental groups, with the highest percentage in the $E_2$ group ($p<0.01$). Abnormalities consist of tailless heads, head and flagellum errors, as well as double head spermatozoa. The percentage of sperm abnormalities observed in mesquite extract and DAI groups did not differ between them (Table 4).

## Male sexual behavior

All males from experimental groups copulated, and all males from the control and mesquite extract groups ejaculated; 93% of DAI-treated males and 20% of $E_2$-

**Table 5.** Male sexual behavior variables (X ± S.E.M.). Males were treated during 30 consecutive days.

| Variables | Control | Extract | Daidzein | Estradiol |
|---|---|---|---|---|
| Mount Latency (s) | 7.2 ± 1.04[a] | 54.5 ± 21.04[b] | 14.5 ± 2.72[b] | 145.5 ± 45.99[b] |
| Intromission Latency (s) | 9.9 ± 1.03[a] | 102.9 ± 30.49[b] | 49.25 ± 15.34[b] | 211.11 ± 74.75[b] |
| Ejaculation latency (s) | 283.1 ± 25.58[a] | 519.2 ± 84.35[b] | 422.33 ± 18.30[b] | 992.0 ± 162.0[b] |
| Number of mounts | 3.6 ± 0.49[a] | 12.5 ± 3.74[b] | 8.66 ± 0.91[b] | 16.0 ± 6.0[b] |
| Number of ejaculations | 3.0 ± 0[a] | 2.0 ± 0[b] | 1.7 ± 0.21[b] | 0.2 ± 0.13[b] |
| Postejaculatory interval | 286.5 ± 26.40[a] | 441.8 ± 62.14[b] | 409.0 ± 75.32[b] | 684.5 ± 278.5[b] |

[b] Different from control group.

**Table 6.** Percentages of females pregnant after copulating with males from control and experimental groups after 30 days of treatment.

| Group | Pregnant females (%) | Dead pups (%) |
|---|---|---|
| C-C | 100 | 0 |
| C-M | 100 | 0 |
| C-D | 100 | 0 |
| C-$E_2$ | 0 | - |
| M-C | 90* | 20* |
| M-M | 100 | 10 |
| M-D | 100 | 10 |
| M-$E_2$ | 0 | - |
| D-C | 90* | 10 |
| D-M | 70* | 0 |
| D-D | 90* | 20* |
| D-$E_2$ | 0 | - |
| $E_2$-C | 0 | - |
| $E_2$-M | 0 | - |
| $E_2$-D | 0 | - |
| $E_2$-$E_2$ | 0 | - |

*p<0.05 compared with C-C group. C-C: control female-control male; C-M: control female-mesquite male; C-D: control female daidzein male; C-$E_2$: control female-$E_2$ male; M-C: mesquite female-control male; M-M: mesquite female-mesquite male; M-D: mesquite female-daidzein male; M-$E_2$: Mesquite female-$E_2$ male; D-C: daidzein female-control male; D-M: daidzein female-mesquite male; D-D: daidzein female-daidzein male; D-$E_2$: Daidzein female-$E_2$ male; $E_2$-C: $E_2$ female-control male; $E_2$-M: $E_2$ female-mesquite male; $E_2$-D: $E_2$ female-daidzein male; $E_2$-$E_2$: $E_2$ female-$E_2$ male.

treated males ejaculated. Male sexual behavior was disrupted in all experimental subjects: males treated with mesquite pod extract, DAI or $E_2$ displayed longer mount, intromission and ejaculation latencies than control males ($p<0.05$). The number of mounts in males treated with mesquite extract, DAI or $E_2$ was higher than in the control group ($p<0.05$). The number of ejaculations was lower in males treated with mosquite, DAI and $E_2$ ($p<0.05$).

The post ejaculatory interval was also higher in experiment than in control males (Table 5).

### Fertility: number of pups per litter

Regarding fertility, all control females became pregnant when copulating with males from vehicle, mesquite extract or DAI groups (Table 6). No dead pups were observed after birth.

Most females treated with mesquite pod extract (90%) became pregnant when copulating with control males. All of the mesquite extract-treated females became pregnant after copulating with mesquite extract- or DAI-treated males. In the case of DAI-treated females, only 70% of those copulating with males treated with mesquite pod extract got pregnant ($p<0.05$). None of the $E_2$-treated females became pregnant and none of the females copulating with $E_2$-treated males got pregnant (Table 6).

The number of litters was different depending on the treatment. Significantly reduced litters were observed in mesquite extract-treated females copulating with control, mesquite extract- or DAI-treated males ($p<0.05$). The same was observed in DAI-treated females copulating with mesquite pod extract- and DAI-treated males (Table 7). The mortality in pups born from females treated with mesquite pod extract or DAI increased in 10 to 20% compared with control females ($p<0.05$). No litter was obtained from $E_2$-treated females and females copulating with $E_2$-treated males (Table 6).

## DISCUSSION

Results of this study show that mesquite pod extract disrupts sexual behavior and reproduction in female and male rats. In females, mesquite pod extract induces an increase in the number of days in estrus and a decrease in the intensity of lordosis during proestrus, as well as proceptivity and receptivity. Body weight decreased while vaginal weight increased in all experimental

**Table 7.** Average number of litters born from control and experimental females copulating with control or experimental males. Females and males received treatments during 30 consecutive days.

| Groups | C-C | C-M | C-D | M-C | M-M | M-D | D-C | D-M | D-D |
|---|---|---|---|---|---|---|---|---|---|
| Litters | 9.25 ± 0.45[a] | 9.55 ± 0.74[a] | 8.33 ± 0.64[a] | 5.12 ± 0.91[b] | 6.62 ± 0.70[b] | 5.00 ± 0.75[b] | 6.57 ± 1.35[a] | 6.42 ± 1.08[b] | 5.28 ± 1.20[b] |

[b] Different from control group.C-C: control female-control male; C-M: control female-mesquite male; C-D: control female-daidzein male; M-C: mesquite female-control male; M-M: mesquite female-mesquite male; M-D: mesquite female-daidzein male; D-C: daidzein female-control male; D-M: daidzein female-mesquite male; D-D; daidzein female-daidzein male.

females compared with controls.

In addition, the number of pups born from females treated with mesquite pod extract was lower than in control females, and the mortality of pups increased between 10 and 20%. In males, mesquite pod extract causes a decrease of body, testes and glandular weights, as well as decreased sperm motility, viability and count after 30 days of treatment. The effects of mesquite pod extract on male sexual and reproductive variables were similar to those observed in animals treated with DAI, but less pronounced than in animals treated with $E_2$. Altogether, these findings support our initial hypothesis which states that, mesquite pod extract decreases fertility in female and male rats.

The decrease in body weight observed in experimental females and males can be attributed to the estrogenic effects of mesquite pod extract and DAI, although the most pronounced effects were observed in subjects treated with estradiol. Estradiol has the ability to control energy balance, food intake, and body fat distribution which may be mediated through its interaction with orexigenic and anorexigenic hormones.

In rats and mice, estrogen exerts a tonic inhibitory effect on meal size and daily food intake throughout the ovarian cycle and a cyclic inhibitory effect during the peri-ovulatory phase. Estradiol acts via the estrogen receptors (ERs) in the hypothalamus to reduce feeding which may mediate its anorectic effects by decreasing the expression or releasing orexigenic neuropeptides such as neuropeptide Y (NPY) and Ghrelin, at the same time can increase the central sensitivity to anorexigenic peptides such as Leptine and Cholecystokinin, and increasing the neurotransmission of Serotonin (Brown and Clegg, 2010).

Considering these effects of estradiol and to a lesser extent those of DAI and phytoestrogen content (mesquitol (6.4 µg/g), Genistein (60.25 µg/g) and Daidzein (5.27 µg/g) in mesquite pod extract, it is possible that the reduced overall condition of both females and males prior to mating could decrease their fertility, with a negative effect on reproductive performance. Although reproductive organs in females did not decrease due to the treatments, it is possible that the changes in the female reproductive system caused by low body weight can lead to reduction or even suppression of ovulation and other disorders generated by a complex hormonal balance of the hypothalamic pituitary-ovarian system, with consequent reduction in the levels of hormones involved in fertility, such as GnRH, LH, FSH, and estrogen. This can be evaluated in other studies. All these changes are able to modify ovarian function, preventing the development of appropriate conditions for ovulation to occur. Furthermore, the nutritional status of the mother is of great importance for the survival of embryos and the health of offspring. Considering the decrease in body weight data in Table 2 (females) as well as body and glandular data in Table 3 (males), it seems necessary to evaluate the nutritional status of animals treated with mesquite pod extract, DAI and $E_2$.

The alterations in estrous cycle and female sexual behavior due to mesquite pod extract could be explained by its content of phytoestrogens and their disruptive effects on the gonadal axis. In the hypothalamus, phytoestrogens can disrupt the control of GnRH neurons by Kisspeptin (Patisaul and Adewale, 2009; Patisaul and Jefferson, 2010; Jefferson et al., 2005), leading to a decrease in GnRH, in the same way that estradiol does (Ördög and Knobil, 1995). Phytoestrogens can also attenuate the preovulatory surge of LH and FSH by suppressing circulating estrogen (Hooper et al., 2009; Trock et al., 2006), decreasing ovarian steroidogenesis and foliculogenesis stimulation.

In ovary, phytoestrogens can cause an absence of corpora lutea, large antral-like follicles with degenerating or no oocytes and ovarian cysts (Kouki et al., 2003). All these effects contribute to female infertility.

Regarding female sexual behavior, this is mediated by ERα subtype (Krege et al., 1998), located in the hypothalamic ventromedial nucleus (VMN) (Chang et al., 2008; Kuiper et al., 1997), which is an important structure for the control of that behavior (Rubin and Barfield, 1980). The fact that neither mesquite pod extract nor DAI stimulate female sexual behavior, decreasing lordosis quotient and intensity might be explained by their higher affinity for ERβ (30 times higher) than ERα (Goldberg et al., 1996). Estradiol induces a stimulatory effect on female sexual behavior through its binding to both estrogen receptor subtypes α and β (Pfaff et al., 2006). Therefore, no inhibitory effect was observed in this respect.

The current study also shows that mesquite pod

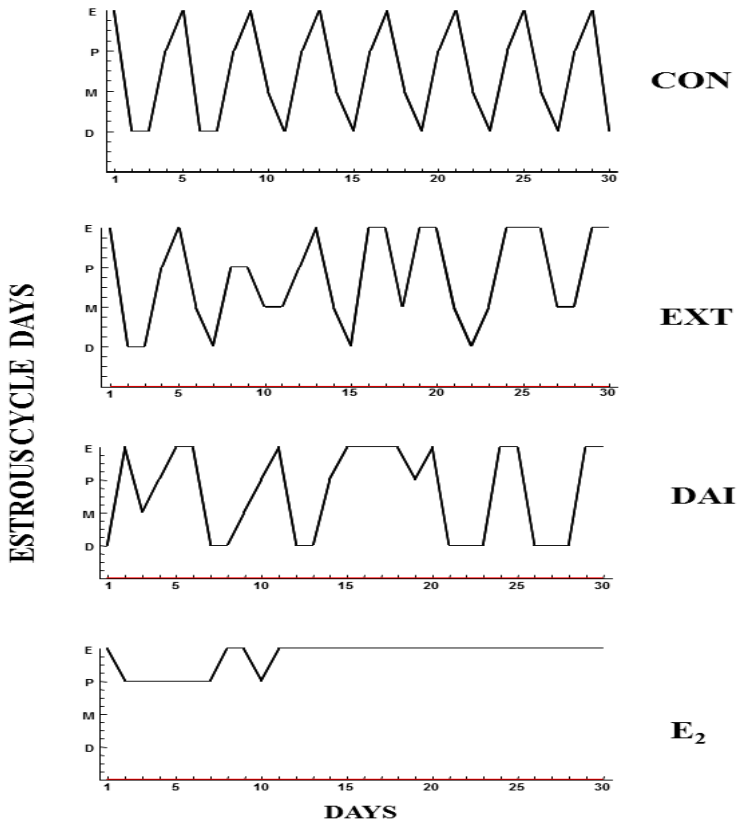


**Figure 2.** Representative estrous cycles of females treated with vehicle (CONTROL), mesquite pod extract (EXT), estradiol (E2) or daidzein (DAI). Control females had normal 4–5 day cycles. Mesquite pod extract, estradiol, and daidzein induced some 3-day cycles, with absence of diestrus periods, as well as an increase in the days in estrus. (E) Estrus; (M) Metestrus; (D) Diestrus; (P) Proestrus.

extract adversely affects pregnancy outcome, although the extract did not impair pregnancy. Females exposed to the extract, delivered fewer pups than control and DAI treated females, with 10 to 20% of mortality in born pups.

In regard to DAI-treated females, disruption of female sexual behavior and a reduction in the percentage of pregnant females, decreased numbers of pups where higher number of dead pups was also observed. Isoflavone intake can attenuate lordosis and decrease proceptive behavior in female rats (Patisaul and Jefferson, 2010). Additionally, it has been reported that neonatal treatment with genistein in female mice causes a significant decrease in the number of live pups (Jefferson et al., 2005). Taken together, the results obtained in this study suggest that both mesquite extract and DAI could have anti-zygotic, blasto-cytotoxic, or anti-implantation activity, causing a decrease in the number of implantation sites in the uterus, as has been observed in females treated with genistein (Jefferson, et al., 2005) and with extracts of other plants. The fact that $E_2$ treatment suppresses estrous cyclicity in females due to its overstimulation of vaginal epithelia, causes persistent vaginal cornification, as has been demonstrated earlier (Retana-Márquez et al., 2012). The fact that estradiol-treated females did not become pregnant is due to, the inhibition of the gonadotropin peak caused by suppressing the Gonadotropin releasing hormone (GnRH) pulse due to estradiol overdoses (Ördog and Knobil, 1995), thus suppressing ovulation.

Concerning male's sexual behavior, glandular weights and sperm quality, they decreased mesquite pod extract in a similar way as DAI treatment. These results confirm that mesquite pod extract disrupts reproduction in male rats. The effects of mesquite pod extract and DAI in male reproductive variables should be considered to explain the lower number of pups and/or dead pups, since the quality of sperms is also important for the progression of the zygote to blastocyst (Casillas et al., 2016). The results of this work show that 100% of control females copulating with control, mesquite pod extract- or DAI-treated males became pregnant, the number of

pups was the highest and all pups were alive. This indicates that despite the reduction in sperm viability, motility and count, the treatments did not disrupt the reproductive ability of males. However, when the females receiving mesquite pod extract or DAI copulated with control, mesquite extract or DAI-treated males, the number of pregnant females and the number of pups decreased. These results indicate that female reproduction seems more vulnerable to the effect of mesquite pod extract and DAI than in males.

Nonetheless, males treated with mesquite pod extract impregnated only 70% of females treated with DAI, although no pups were dead. This result could suggest that mesquite extract might contribute to decrease in male fertility. Concerning the effects of estradiol in male reproduction, large doses of estradiol induce testicular germ and Leydig cell apoptosis, causing a loss of almost all cellular types, which induces severe testicular atrophy and undetectable plasma levels of testosterone (Retana-Márquez et al., 2016), as was corroborated in this study. The consequent absence of testosterone and its metabolite, 5-Hydroxytestosterone, necessary for penile erection (Manzo et al., 1999), causes erectile dysfunction, thus increasing mount latency and the number of mounts, which leads to lack of intromission and ejaculation (Meisel and Sachs, 1994). On the other hand, the absence of sperm caused by great doses of estradiol prevents the possibility of oocyte fertilization and therefore no pregnancy occurs.

## Conclusions

Mesquite pod extract can disturb the estrous cycle, sexual behavior and fertility of female rats, decreasing the rate of pregnancy and the number of pups, as well as increasing the number of dead pups at birth. In males, despite mesquite pod extract and DAI decreasing sexual behavior and sperm quality, these effects seems not to disturb fertility, since they can fertilize control females. However, mesquite extract or DAI treatments in males could also contribute to decreased pregnancy rates and the number of live pups. Considering that mesquite is used to feed livestock and also for human consumption, the findings of this study could be taken into account for the possible side effects on reproduction and fertility in males and females.

It is important to evaluate the nutritional status of animals fed mesquite because its content of mesquitol, daidzein and genistein can account for a decrease in body weight, thus altering their reproductive performance.

## Conflict of Interests

The authors have not declared any conflict of interests.

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