

Effect of *Agave sisalana* Perrine extract on the ovarian & uterine tissues and fetal parameters: Comparative Interventional Study

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Abstract— *Agave sisalana* (Agavaceae), commonly known as sisal plant which is an anti-inflammatory, analgesic, anthelmintic, bactericidal and insecticidal activities. Its toxic effects on female reproduction are unknown. This comparative interventional study aimed to assess the ovarian and uterine tissues and fetal parameters of rats treated with the extract of sisal waste (100 mg kg⁻¹ body weight), obtained from decortications of the *A. sisalana*. The control received distilled water. The treatments were performed orally for 30 consecutive days. The results showed that the estrous cycle and ovarian tissue were not affected by plant extract. There was an significantly increase ($p < 0.05$) in the thickness of perimetrium in females treated with *A. sisalana*. The placental and litter weights also increased significantly whereas the litter size significantly decreased ($p < 0.05$) in the group receiving the extract. In conclusion, *A. sisalana* showed no pathological changes in the ovaries and uterine endometrium, but had a negative effect on the litter size and weight.

Keywords: Sisal, Gonadotoxicity, Reproduction Fetotoxicity.

I. INTRODUCTION

Agave sisalana commonly known as sisal which is a monocotyledonous plant from the Agavaceae family. Originally from Mexico, it is related to a great economic interest because of its tough fibers. Brazil is the largest producer and exporter of sisal fibers with 69% of global production.¹ Only 4% of the leaves decortications produce fibers, and the remaining material (waste) is commonly discarded by farms and can become an important factor of environmental contamination.²

The sisal waste consists of water, parenchymatous tissue, and cellulose, fibers of various sizes, inorganic compounds and components related to primary and secondary metabolism. This waste is rarely used, despite its indication for use as a supplement in ruminant feed² and a raw material for the production of drugs.³

The biological activities of this specie is mainly related to the presence of steroidal saponins in all parts of the plant.⁴ Studies have shown that this secondary metabolite is responsible for different biological properties, such as hemolytic,⁵ anti-inflammatory,⁶ antifungal,⁷ and antibacterial.^{6,8} However, few studies reporting phytotoxicity of these compounds have been described⁹ and the toxic effects of *A. sisalana* on female reproduction remain unknown.

So it is important to assess the reproductive toxicity of herbal extracts and secondary compounds in order to assess their gonadotoxicity and effects on fertility to ensure their medicinal use and discovery of new drugs.¹⁰

Then, considering the different pharmacological activities of *A. sisalana*, its high content of steroidal saponins, and its use in popular medicine and as animal feed, this study evaluated for the first time the

ovarian and uterine tissues and fetal parameters of rats treated with the extract of sisal waste obtained from decortications of the *A. sisalana* leaves.

II. METHODOLOGY

A comparative interventional study was carried out on rats to study the effect of *A. sisalana* on ovarian & uterine tissues and fetal parameters at Department of Biothecnology, São Paulo State University – UNESP, School of Sciences, Humanities and Languages, Av. Dom Antonio 2100, CEP 19806-170, Assis, São Paulo, Brazil.

2.1 Animals

Adult female Wistar rats (n=26), 12-weeks-old, weighing approximately 260 g, were obtained from the Universidade Estadual Paulista– UNESP (Botucatu, SP, Brazil) and kept at the Faculty of Sciences and Letters – UNESP (Assis, SP, Brazil). The females were maintained under controlled conditions of temperature and luminosity (22 ± 2 °C, 12 h light/dark photoperiod, respectively) and received water and commercial feed (Nuvital™, Colombo, PR, Brazil) *ad libitum*. The experimental protocol followed the Ethical Principles in Animal Research adopted by the Brazilian Society of Science in Laboratory Animals and was approved by the Ethical Committee for Animals Use – CEUA (Protocol Number 005/2013).

2.2 Obtaining and preparation of *A. sisalana* extract

The sisal waste was obtained by decortications of the leaves. This plant material was provided by sisal producers from Valente city (11° 24' 43" S, 39° 27' 43" W) in Bahia State, Brazil.

Sisal waste was submitted to the acid hydrolysis with sulfuric acid (pH 0.4 at 0.8) in high temperature (127 °C). The formed crystals after centrifugation were dried at 70 °C until constant weight and were separated as described by Gouveia et al. (2009),¹¹ with modifications. Finally, the extract was diluted in distilled water at a concentration of 100 mg kg⁻¹ body weight (BW).

2.3 Experimental groups

Females with regular estrous cycle were weighed and randomly distributed into two groups (n= 13/group): control group (distilled water) and the group treated with sisal extract acid hydrolysate (100 mg kg⁻¹ BW), orally (gavage) for 30 consecutive days. Preliminary tests showed that the 100 mg kg⁻¹ BW concentration of *A. sisalana* extract presented anti-inflammatory property (Unpublishing results). Then, this concentration was selected for the study.

The beginning of treatment occurred when females were in estrous phase of reproductive cycle. The females were weighed every second day for maintenance of dose level administered during the treatment.

2.4 Estrous cycle

The estrous cycle was monitored daily by vaginal swabs and cytological examination was performed under a light microscope. The cycle phases were identified as: (a) proestrus, consisting of nucleated basal cells; (b) estrus, with predominance of enucleated cornified cells; (c) metaestrus, consisting of leukocytes in combination with cornified cells; (d) diestrus, with predominance of leukocytes.¹²

2.5 Euthanasia of females and tissue preparation

At the end of 30 days, five females of each group were weighed and euthanized with an intraperitoneal overdose of anaesthetic (Thiopentax™, Cristalia, São Paulo, Brazil). The ovaries, uterus and vital organs of interest for toxicological evaluation (heart, liver and kidneys) were collected and weighed, and absolute (g) and relative (g/100 g BW; g%) weights were obtained. Ovaries and uterine horns were fixed in Bouin's solution and embedded in Paraplast (Labware – Oxford, St. Louis, MO, USA). The 5 µm thick sections were stained with hematoxylin and eosin (HE) for histomorphometric analysis in light microscopy.

2.6 Ovarian and uterine analysis

The identification of follicular types was based on the classification proposed by Pedersen and Peters (1968).¹³ The criterion for the identification of atretic follicles was based on the degree of regression of antral follicles, according to a classification described by Osman (1985).¹⁴ This classification considers: overall shrinkage of the granulosa and degenerative changes (Stage IA); granulosa wall degeneration and many nuclear fragments in the periphery of the antrum (Stage IB); degenerative oocyte surrounded by an envelope of degenerating cumulus cells or their remnants (Stage IIA); and, oocyte in the antrum, with granulosa wall usually constituted by a distinct inner lining and macrophages usually present in the antrum (Stage IIB).

In each ovary (5 sections/female/group), the growing, antral and atretic follicles were counted. The mean diameter of each healthy follicle was obtained for follicular type identification in the following classes:¹⁵ a) Class I: small preantral follicles (<90 µm); b) Class II: large preantral follicles (91–260 µm); c) Class III: small antral follicles (261–350 µm); d) Class IV: antral follicles of mean size (351–430 µm); e) Class V: large antral follicles (431–490 µm); f) Class VI: mature follicles or Graafian(>491 µm).

The number of corpora lutea and atretic follicles, as well as measurements of corpora lutea area and healthy follicles diameter and uterine morphometric parameters (luminal epithelium height and thickness of endometrial stroma, myometrium and perimetrium), were obtained using a Zeiss Scope A1–Axio microscope (Carl Zeiss, Jena, Germany) connected to an AxioCam ICc3 camera. The digitalized images were obtained by the image analyser Axio Vision version 4.7.2.

2.7 Fetal parameters

Eight females from each group were individually housed with males untreated for 5 consecutive days at the end of the experimental period. Vaginal swabs were performed daily and observed in microscope. The presence of sperm in the slides was designated as gestation day 1 (GD). Pregnant females were kept in individual cages and the body weight was recorded on GD1, GD7, GD14 and GD19.

On the GD19, the females were previously anesthetized with a lethal intraperitoneal dose of sodium thiopental (Thiopentax™, Cristalia, São Paulo, Brazil). The uterine horns were collected and weighed. The following records were obtained: number of implantations, number of gravid corpora lutea, litter size, litter weight, number of resorptions and placental weight.

The copulation rate [(number of females with sperm in the smear/number of mated females) × 100], fertility rate [(number of pregnant females/number of copulated females) × 100], pre-implantation loss rate [(number of corpora lutea – number of implantations/number of corpora lutea) × 100], and post-

implantation loss rate [(number of implants – number of fetuses/number of implants) × 100] were calculated.

Evaluation of fetotoxicity was performed through analysis of fetal external morphology under a stereomicroscopy (Citoval 2, Carl Zeiss, Germany).

2.8 Statistical analysis

In the presence of normality, we analyzed the data by the ANOVA complemented by t-Student test, and expressed results as mean ± standard deviation (SD). In the absence of normality, we analyzed data by nonparametric Kruskal-Wallis test complemented by Mann-Whitney, and expressed results as median ± interquartile deviation. Statistical analysis was conducted on GraphPad Prism software, version 5.00. Significance was set at $p < 0.05$.

III. RESULTS

In this present study observations regarding various parameters were found as follows:-

3.1 Estrous cycle

The estrous cycle duration and the number of days in the estrus phase were without significant difference ($p > 0.05$) in both groups during the experimental period (Table 1).

Table1
Effect of sisal extract on the reproductive cycle

S. No.	Parameters	Control Group (N=13)	Study Group (N=13)
1	Cycle duration (days) ^a	4.43 ± 0.64	4.36 ± 0.41
2	Number of estrus ^b	7 ± 1	8 ± 1

No significant difference between the groups, $p > 0.05$. ^aValues are expressed as median ± interquartile deviation. ^bValues are expressed as mean ± SD. Test for significance is Mann-Whitney test for ^aValues and Student t-test for ^bValues.

3.2 Reproductive and non-reproductive organs weight

Administration of sisal extract at 100 mg kg⁻¹ BW dose increased ($p < 0.05$) the absolute weight of the ovaries, but relative weight was similar to that found in control group (Table 2). Treatment with sisal extract did not alter absolute and relative weights of uterus ($p > 0.05$). There was no significant effect of sisal extract on the weight of the heart, liver and kidneys. (Table 2)

Table 2
Effect of sisal extract on the Organ weight

S. No.	Organs	Parameters	Control Group (N=13)	Study Group (N=13)
1	Ovaries	Absolute weight (g)	0.11 ± 0.01	0.14 ± 0.03*
		Relative weight (g%)	0.04 ± 0.005	0.04 ± 0.01
2	Uterus	Absolute weight (g)	0.41 ± 0.09	0.43 ± 0.07
		Relative weight (g%)	0.16 ± 0.04	0.10 ± 0.09
3	Heart	Absolute weight (g)	0.84 ± 0.06	0.89 ± 0.06
		Relative weight (g%)	0.33 ± 0.04	0.31 ± 0.03
4	Liver	Absolute weight (g)	9.42 ± 0.70	10.88 ± 2.20
		Relative weight (g%)	3.74 ± 0.38	3.75 ± 0.17
5	Kidneys	Absolute weight (g)	1.88 ± 0.07	1.93 ± 0.30
		Relative weight (g%)	0.75 ± 0.08	0.67 ± 0.10

*Statistical significance compared to Control group, $p < 0.05$. Values expressed as mean ± SD. Test of significance is Student t-test.

3.3 Analysis of ovarian tissue

The gonad tissue of experimental groups (Figure 1) exhibited cortical and medullar regions well characterized, constituted respectively by a fibrocellular tissue with follicles in various stages of development and degeneration, and a loose connective tissue richly vascularized. Corpora lutea was present in the ovarian stroma of control and sisal-treated groups.

Figure 1

Photomicrographs of ovaries in the rats of control (A) and sisal (B) groups. Observe the morphological integrity of the tissue in both groups. Follicular units (F), corpora lutea (CL), medula (M), cortex (C). Highlights: primordial (C), growth (D), antral (E) and atretic (F) follicles

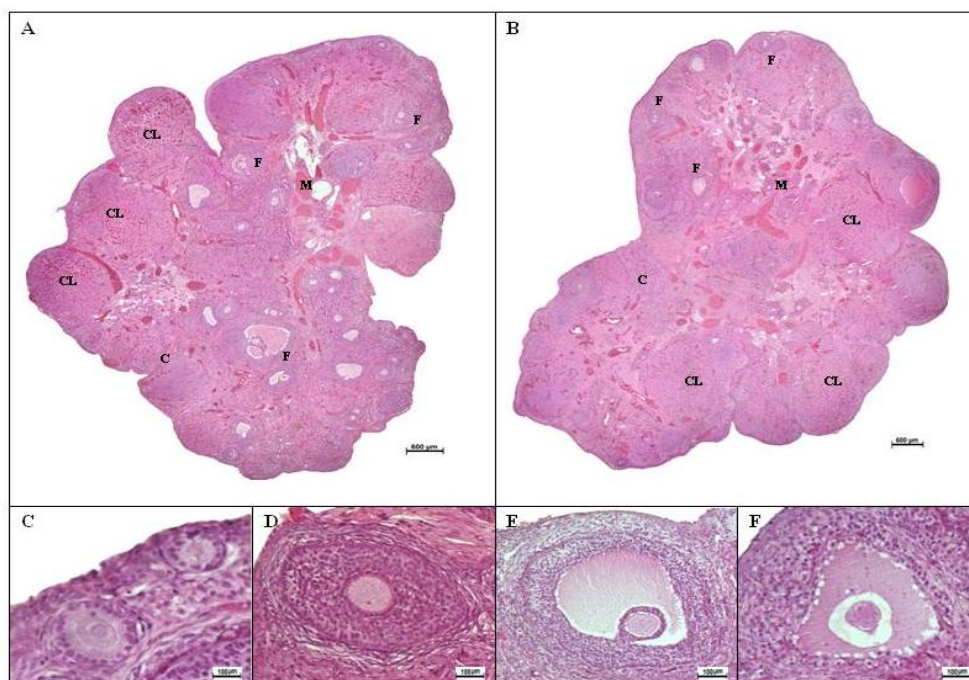
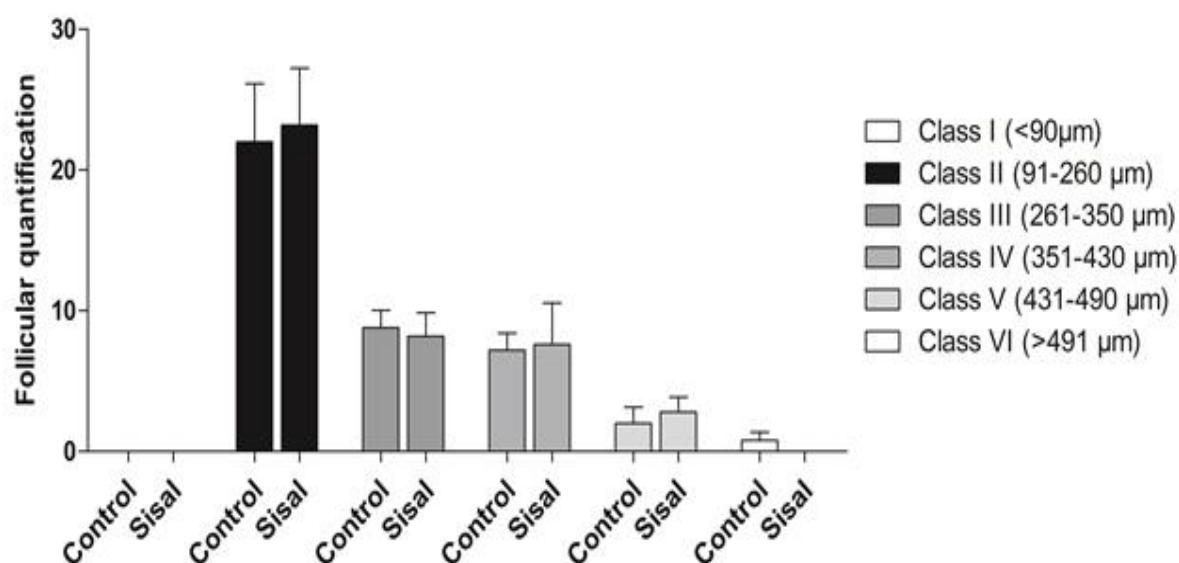


Figure 2

Quantification of healthy follicles in each class, in the control and sisal groups (n= 5 sections/ ovary/ female/ group)



The Figure 2 shows that there was no significant difference ($p>0.05$) between groups in the number of healthy follicles in each class of development. There was a predominance of large pre-antral follicles (Class II). Follicles of Class VI ($>491\ \mu\text{m}$) were present only in the control group. The number of atretic follicles in different stages of degeneration was similar ($p>0.05$) in both groups (Figure 3), having predominantly follicles in the most advanced stage of atresia. The number and area of corpora lutea in ovaries were not significantly affected ($p>0.05$) by treatment with sisal (Table 3).

Figure 3

Quantification of atretic follicles in different stages of degeneration, in the control and sisal groups (n= 5 sections/ ovary/ female/ group)

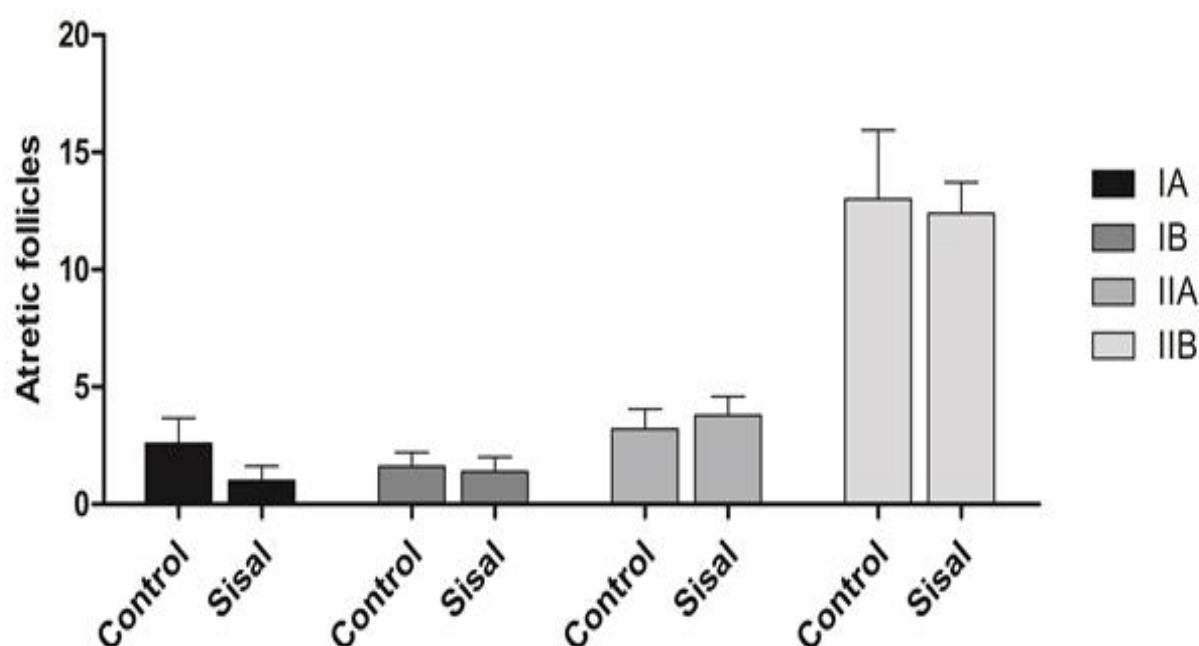


Table 3

Effect of sisal extract on the number and area (mm^2) of corpora lutea

S. No.	Parameters	Control Group (N=5)	Study Group (N=5)
1	Number of corpora lutea ^a	10.00 $\pm$ 3.87	12.50 $\pm$ 4.92
2	Area of corpora lutea ^b	290.10 $\pm$ 249.66	282.44 $\pm$ 253.59

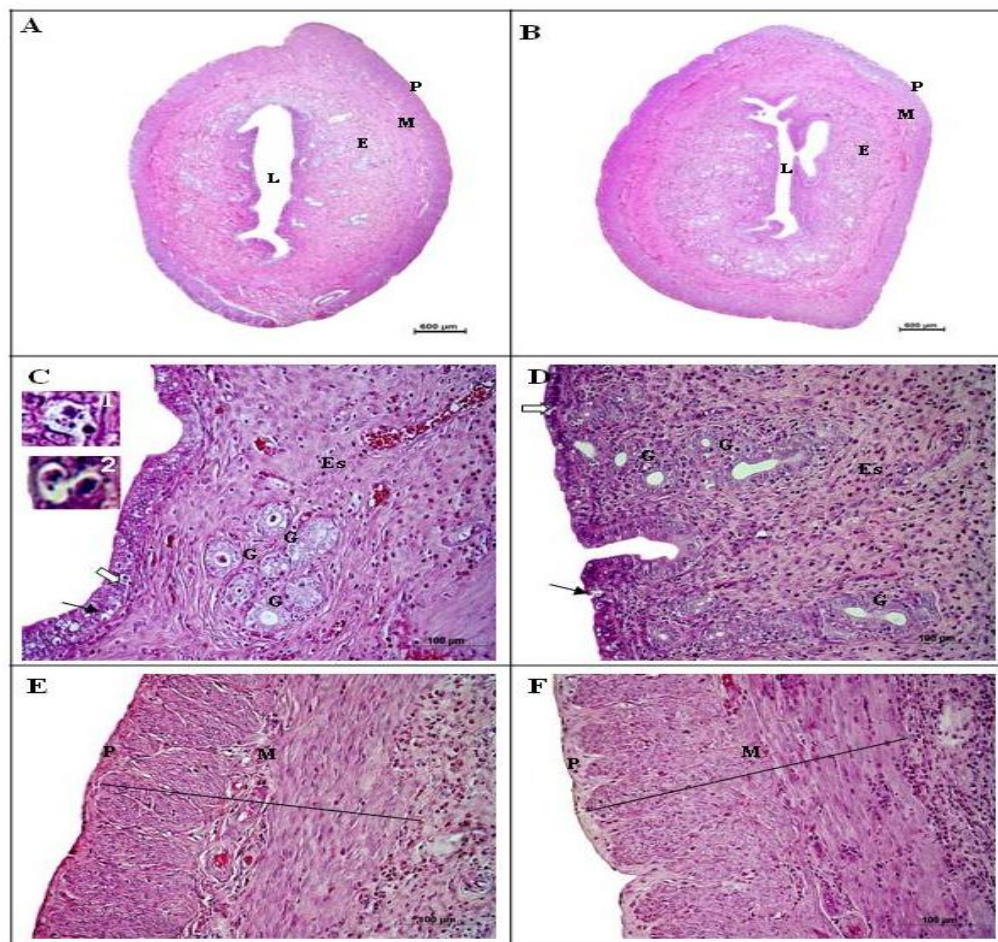
No significant difference between the groups, $p>0.05$. ^aValues expressed as median $\pm$ interquartile deviation. ^bValues expressed as mean $\pm$ SD. Test for significance is Mann-Whitney test for ^aValues and Student t-test for ^bValues.

3.4 Analysis of uterine tissue

Uterine tissue showed similar characteristics to endometrium and myometrium in both groups (Figure 4A & B). The endometrium presented columnar luminal lining, with secretory vacuoles, accompanied by some areas of cellular degeneration/necrosis (Figure 4C & D). Endometrial stroma, formed by loose connective tissue exhibited leukocytes dispersed all over tissue and presented glands without secretory activity, occasionally with epithelial cells in degeneration. Myometrium showed well-defined cytoarchitecture, with arrangement of fibers in outer longitudinal and internal circular layers (Figure 4E & F). No morphometric change were observed in endometrium and myometrium of females receiving sisal extract (Table 4). However, there was an increase ($p<0.05$) in thickness of perimetrium in sisal-treated group, compared to the control group (Fig. 4E & F and Table 4).

Figure 4

Photomicrographs of uterine cross-sections in the control (A, C, E) and sisal (B, D, F) groups. In both groups, endometrium and myometrium showed similar aspect. Luminal cavity (L), endometrium (E), myometrium (M), perimetrium (P), endometrial stroma (Es), glands (G), secretory vacuoles (black arrow, highlight C1), degenerating cells (white arrow, highlight C2). Hematoxylin–eosin; Bars= 600 μ m (A, B), 100 μ m (C-F)

**Table 4**

Effect of sisal extract on uterine morphometric parameters (μ m)

S. No.	Parameters	Control Group (N=5)	Study Group (N=5)
1	Luminal epithelium height ^b	34.78 $\pm$ 7.87	36.48 $\pm$ 7.16
2	Endometrial stroma thickness ^a	686.03 $\pm$ 236.19	707.75 $\pm$ 173.92
3	Myometrium thickness ^a	361.12 $\pm$ 149.44	373.56 $\pm$ 82.02
4	Perimetrium thickness ^a	27.65 $\pm$ 5.00	30.17 $\pm$ 5.38*

*Statistical significance compared to Control group, $p < 0.05$. ^aValues expressed as median $\pm$ interquartile deviation. Mann-Whitney test. ^bValues expressed as mean $\pm$ SD. Student t-test.

3.5 Fetal parameters

Copulation and fertility rates were similar in both groups (Table 5). No significant difference ($p > 0.05$) was observed in body weight of dams, gravid uterine weight and pre- and post-implantation losses in comparison between experimental groups. Placental weight and litter weight increased significantly ($p < 0.05$) in the group receiving the sisal extract. However, litter size was decreased ($p < 0.05$). We observed no change in fetal external morphology in the group receiving the sisal extract treatment.

Table 5
Effect of sisal extract on maternal and fetal parameters

S. No.	Parameters	Control Group (N=8)	Study Group (N=8)
1	Copulation rate (%)	100	100
2	Fertility rate (%)	100	100
3	Body weight of dams (g)^b		
	GD1	277.00 ± 17.00	265.50 ± 32.50
	GD7	298.50 ± 13.25	288.00 ± 36.25
	GD14	336.62 ± 33.07	317.00 ± 22.95
	GD19	379.00 ± 34.78	359.75 ± 36.38
5	Gravid uterine weight (g)^b	41.22 ± 3.72	37.93 ± 4.68
6	Placental weight (g)^a	0.45 ± 0.07	0.47 ± 0.11 [*]
7	Littersize^a	12 ± 0.50	10 ± 1.25 [*]
8	Pre-implantation loss (%)^b	0	0
9	Post-implantation loss (%)^b	0 ± 7.27	0 ± 9.31

^{*}Statistical significance compared to Control group, $p < 0.05$. ^aValues expressed as median ± interquartile deviation. ^bValues expressed as mean ± SD. Test for significance is Mann-Whitney test for ^aValues and Student t-test for ^bValues.

IV. DISCUSSION

A previous study conducted in our laboratory¹⁶ showed that *Agave sisalana* extract presented gonadotoxic effect when administered to male rats. Thus, the present study evaluated for the first time the possible toxic effects of sisal extract on ovaries, uterus and fetal parameters of rats.

In toxicity studies, any change in weight of reproductive or non-reproductive organs may be a good indicative of toxicity promoted by one or more phytochemical compounds from medicinal plants. *A. sisalana* is rich in steroidal saponins and also contains flavonoids and homoisoflavonoids.¹⁷ In this study, the ovarian absolute weight presented a slight increase, but relative weight was unaltered in sisal group. Similarly, in studies with plants rich in steroidal saponins, *Korean ginseng*¹⁸ or *Panax ginseng*,¹⁹ ovarian weight did not change. The uterus, heart, liver and kidneys weights were not affected by *A. sisalana* extract. Furthermore, the plant did not cause mortality in experimental group and did not promote signs of toxicity such as changes in behavior, ataxia, salivation, vomiting, diarrhea, polyphagia, fever, weakness, tremors and convulsion.²⁰ Interestingly, male rats treated with dry precipitate or hexane extract of sisal¹⁶ presented an increase in weight of testicles, epididymis, seminal glands and prostate, while the extract obtained by acid hydrolysis only promoted an increase in seminal glands weight. The increase in testicular weight in groups treated with sisal extracts was attributed to histological changes observed in this organ.

In this study, the ovaries of females treated with sisal waste extract at a 100 mg kg⁻¹ BW dose presented morphological characteristics and quantification of healthy and atretic follicles similar to control group. Mendes et al. (2016)¹⁶ described notable histopathological changes in testicles of animals that received sisal extract, characterized by depletion of seminiferous epithelium, detachment of immature germ cells, intraepithelial vacuolization, interstitial hemorrhage, vascular congestion and scarcity of spermatozoa in tubular lumen. It is possible that the ovarian tissue is less susceptible than testicular tissue to the effects

of steroidal saponins present in *A. sisalana*, once the physiological processes that occur in female gonad are not as dynamic as those taking place daily in testicles.

According to Francis et al. (2002),²¹ the effects of saponins appear to be related to interactions with steroid receptors since the basic structures of saponins are similar to steroidal hormones, important for reproductive function. In addition, it is reported that any extract containing saponins has the potential to cause hemolysis in red blood cells.⁸ In the present study, the reproductive cycle of females was regular in sisal group, having similar cycle duration and number of estrus compared to the control group. Neither ovarian tissue nor uterine tissue showed clinical condition of hemorrhage as observed previously in testicular¹⁶ or liver tissue.⁸

The endometrium is the layer that undergoes morphological and physiological changes during different phases of the estrous cycle, and therefore may be more susceptible to drug effects. Phytochemical constituents of *A. sisalana* did not interfere in endometrium and myometrium structure, but there was an increase in thickness of perimetrium due to predominance of fibrous tissue. Generally, this histological layer is not susceptible to morphological changes promoted by synthetic or natural endogenous substances. Some studies performed with plants rich in steroidal saponins such as *Cortex albiziae*²² or *African aspilia*²³ showed that there was an increase in luminal epithelium height, disorders in endometrial stroma cells or loss of epithelial lining, suggesting a possible relationship of saponins with uterine tissue. This relationship should be further investigated in order to assess the effects of saponins on uterine layers.

The estrous cyclicity in females treated with sisal and the presence of corpus luteum in ovary demonstrated that ovulatory process occurred due to regular balance between the hypothalamus-pituitary-gonad axis. Copulation and fertility rates were similar in both groups. Treatment with sisal extract did not compromise the reproductive capacity of females. Similar results were observed in other vegetal plants rich in saponins.¹⁹

Treatment of females in pre-gestational period influenced the litter size as well as the placental and litter weights. Litter size decreased but we did not observe any significant change in pre- and post-implantation losses. The placenta plays a fundamental role in fetal nutrition.²⁴ According to Brolio et al. (2010),²⁵ an increase of blood flow in the uteroplacental unit during gestational period can contribute to an increase in placental weight and consequently increase fetal weight -as observed in this study on the group treated with sisal extract. The authors reported that saponins present in *Yucca schidigera* decreased intestinal ammonia levels, leading to a reduction of oxygen demand on tissue and an increase of oxygen flow to the fetus until birth. This report points out that the saponins present in *A. sisalana* exhibited the same effect.

V. CONCLUSION

In conclusion, administration of *Agave sisalana* extract did not cause toxic effect on the ovarian tissue and uterine endometrium, but had a negative effect on the litter size and weight. The use of this medicinal plant by pregnant female population should be with caution due to its effects on pregnancy.

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CONFLICT OF INTEREST

None declared till now.

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