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Article

Effect of *Dioscorea villosa* extract and the phytoestrogen diosgenin on ovariectomized mice with zymosan-induced arthritis

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Humans are exposed to natural compounds such as phytoestrogens primarily through diet and supplements. These compounds promote health by alleviating the symptoms and illnesses associated with menopause and arthritis. Diosgenin (DSG) occurs naturally in plants such as *Dioscorea villosa* (DV) and binds to estrogen receptors, so it may have similar effects to this hormone, including against arthritis. Thus, we investigated the effect of chronic treatment with dry extract of DV and its phytoestrogen DSG on ovariectomized mice with arthritis. We found that dry extract of *Dioscorea villosa* (DV) contains the phytoestrogen diosgenin (DSG) in its composition. Furthermore, arthritic mice treated with DV and DSG showed reduced neutrophil accumulation in the articular cartilage. Also, the dry extract of DV administered orally (v.o) did not alter the leukocyte count in the joints or promote changes in the reproductive tract. However, DSG altered these parameters, with possible beneficial effects by reducing symptoms related to reproductive aging. Thus, oral treatment with dry extract of DV and subcutaneous (s.c) treatment with DSG showed promise by acting against inflammation caused by arthritis and reducing symptoms in the reproductive tract due to menopause.

Keywords: Estrogen. Menopause. Natural product. Rheumatoid arthritis. Osteoarthritis.

INTRODUCTION

The nutritional and therapeutic use of phytoestrogens from plants, such as soybean (*Glycine max*), red clover (*Trifolium pratense*) and Mexican yam (*Dioscorea villosa*), by women during menopause has been widely reported in the literature. These medicinal plants have improved the quality of life to these women, by decreasing the chances of developing symptoms associated with the final stages of reproductive aging (Bedell, Nachtigall, Naftolin, 2014; Chen, Lin, Liu, 2015).

The medicinal plant *Dioscorea villosa* (DV), also called wild yam or Mexican yam, belongs to the Dioscoreacea family, which is composed of approximately 660 species and found in temperate and tropical regions (Li *et al*., 2020). The nutraceutical and pharmacological activities attributed to this plant can be explained by the presence of saponins in its composition (Dong *et al*., 2012). Saponins are secondary metabolites that can be classified into non-steroidal saponins and triterpene saponins, depending on the structure of the genin that composes them up (Sidana, Singh, Sharma, 2016). DV has high levels of diosgenin (DSG) in its roots and rhizomes. DSG is classified as a sapogenin with a tetracyclic

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structure, containing 27 carbons. This tetracyclic genin is contained in the structure of several steroidal saponins, such as dioscinin. Thus, DV is a source of DSG that is widely used by the pharmaceutical industry for the production of steroid hormones (Dong *et al*., 2012; Nazir *et al*., 2021; Manda *et al*., 2013).

DSG can also be classified as a phytoestrogen, along with other compounds present in the plant species mentioned above. Examples are genistein and daidzein which are found in greater amounts in soybean (*Glycine max*), while biochanin is widely found in red clover (*Trifolium pratense*) (Bedell, Nachtigall, Naftolin, 2014; Chen, Lin, Liu, 2015; Nazir *et al*., 2021). Phytoestrogens are compounds derived from plants that have a structure and/or function similar to estrogen (E), which can interact with the different subtypes of estrogen receptors (ERα, ERβ, GPR30), promoting pharmacological activity mainly in those women during the menopause period (Bedell, Nachtigall, Naftolin, 2014).

Due to the increase in average global longevity, millions of women will spend a third or more of their lives after menopause. As the population ages, the incidence of many chronic diseases increases, including rheumatoid arthritis (RA) (Depypere, Comhaire, 2014; Martín-Millán, Castañeda, 2013). According to the literature, 1 to 2% of the world population is affected by RA (Talsania, Scofield, 2017). Women are affected about 6 times more than men and its prevalence increases with age (Barragán-Martínez *et al*., 2012). According to epidemiological studies, most white women are diagnosed with RA during the menopause transition, when their symptoms usually appear at around 45 years of age (Banas *et al*., 2016).

The pharmacotherapy recommended for arthritis typically has strong side effects in women who have developed RA and osteoarthritis in the menopause to post-menopausal transition period. An example is the loss of bone mass due to the use of glucocorticoids, which in turn further aggravates the osteoporosis commonly present in these women (Ruiz-Miyazawa *et al*., 2018). Therefore, hormone replacement therapy (HRT), especially with estrogen, has been indicated as part of the prevention plan for chronic diseases related to menopause, such as arthritis. However, this therapy is associated with an increased risk of developing thromboembolism, breast cancer and heart attack (Lobo *et al*., 2014; Bolton, 2016).

Therefore, administration of phytoestrogens has been shown to be safer therapeutic alternative in pre-clinical trials (Solopov *et al*., 2021). In addition, there is interest in investigating the potentially beneficial effects on the heart, bones and breasts during menopause (Xiao *et al*., 2016). Thus, investigation of the safety and efficacy of both natural products and their isolated compounds can generate relevant information to support their safe and rational use. Analysis of these compounds can expand their pharmacotherapy to other health problems associated with female reproductive aging and reduce the side effects of conventional therapies. Therefore, we investigated the effect of experimental arthritis induced by zymosan (Zym) in ovariectomized (OVX) mice, along with the possible prophylactic effect of dry extract and DSG phytoestrogen on women during the transition from menopause to post-menopause.

MATERIAL AND METHODS

Phytochemical Analysis

Dioscorea vilosa dry root extract was obtained from Shaanxi Meih Biochemics Co., Ltd., China (Lot number 17A03-B006-013243) and the diosgenin phytoestrogen was purchased from the Sigma-Aldrich (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) was performed with a Shimadzu® liquid chromatography system consisting of a DGU-20A3 degasser, two LC-20AD pumps, an SIL-20A HT auto injector, a CTO-20A column oven, an SPDM20Avp photodiode array detector (DAD) and a CBM-20A system (Kyoto, Japan, model CTO-20A), based on the method of Lima *et al*. (2013) with some modifications. Briefly, the DV extract was solubilized in methanol: ultra-purified water (50:50, v / v) and the DSG only in methanol. Samples were sonicated for 60 minutes for complete dissolution and then filtered through a 0.45 µm polytetrafluoroethylene (PTFE) membrane.

The separation of the chemical constituents was carried out with a Phenomenex C-18 column using

gradient elution at 1.0 mL / min with a mobile phase consisting of water (A) and acetonitrile (Synth, Brazil) (B). The exploratory gradient of 30 to 100% (B) was performed in 46 minutes, as suggested by Snyder *at al*. (1997), with some modifications. After optimization, the gradient elution condition used was: 30-40% (B) for 5 minutes; 40-68% (B) for 18 minutes; 68-100% (B) for 21 minutes and maintained at 100% (B) for 10 minutes. The return to the initial chromatographic conditions (100-30% B) was performed in 10 minutes, followed by conditioning the column for 10 minutes.

In vivo assays

Animals and Ovariectomy

Adult female Swiss mice (weighing 25 to 30 g) from the Central Animal Facility of Federal University of Sergipe (UFS) were used. The animals were divided into groups (n = 5-7), and remained in standard housing conditions, with 12-hour light/dark cycle and controlled temperature (22 ± 1ºC), with water and feed supplied *ad libitum*. The experimental protocols were carried out in accordance with the Animal Research Ethics Committee (CEPA) of Federal University of Sergipe, under the protocol number 71/2018.

The mice were submitted to bilateral ovariectomy through a dorsal incision, under anesthesia with ketamine (100 mg / kg) and xylazine (10 mg / kg) via i.p. Before surgery, the animals received pentabiotics (benzathine benzylpenicillin, procaine, potassium, dihydrostreptomycin and streptomycin) at a dose of 11.2 mg/kg via i.m., and the non-steroidal anti-inflammatory sodium diclofenac at 5 mg/kg via i.p. The experiments were carried out for 2 weeks, necessary for surgical recovery and reduction of endogenous estrogen (Silva *et al*., 2017).

Pretreatments and Experimental Design

Fifteen days after ovariectomy, the reproductive parameters were evaluated in order to ensure the reduction of endogenous estrogen concentrations. Then the animals were divided into 12 experimental groups with daily pretreatments for 20 days, except for the indomethacin group (I), which was pretreated only on the last day, 30 minutes before arthritis induction.

Negative control groups 1 and 2 received pretreatment with Tween 80 (TW, 0.2%) orally, and sesame oil (SO, 0.2% ethanol) (Sigma, St. Louis, MO, USA), subcutaneously (s.c), respectively. On the 20th day, both groups received intra-articular injection with 0.9% saline. Positive control groups 3 and 4 were pretreated with vehicles TW (0.2%), v.o., and SO (0.2% ethanol), s.c., respectively. On the 20th day, the animals received zymosan A (Zym, Sigma, St. Louis, MO, USA) at 100 µg/well in 10 µL of 0.9% saline, i.a, in the femorotibial joint. The other groups (5-11) were also pretreated daily for 20 days. Groups 5, 6 and 7 received pretreatment with the dry extract of DV in doses of 1, 10 and 100 mg/kg dissolved in TW at 0.2%. Groups 8, 9 and 10 were pretreated with DSG at doses of 1, 5 and 50 mg/kg dissolved in SO containing 0.2% ethanol, sc. Group 11 received HRT with 17 ß-estradiol (estrogen; E). (Sigma, St. Louis, MO, USA) at a dose of 50 µg / kg dissolved in SO containing 0.2% ethanol, s.c, Group 12 received pretreatment with a dose of 5 mg/kg dissolved in TW at 0.2%, i.p. The experimental design is represented in Figure 1.

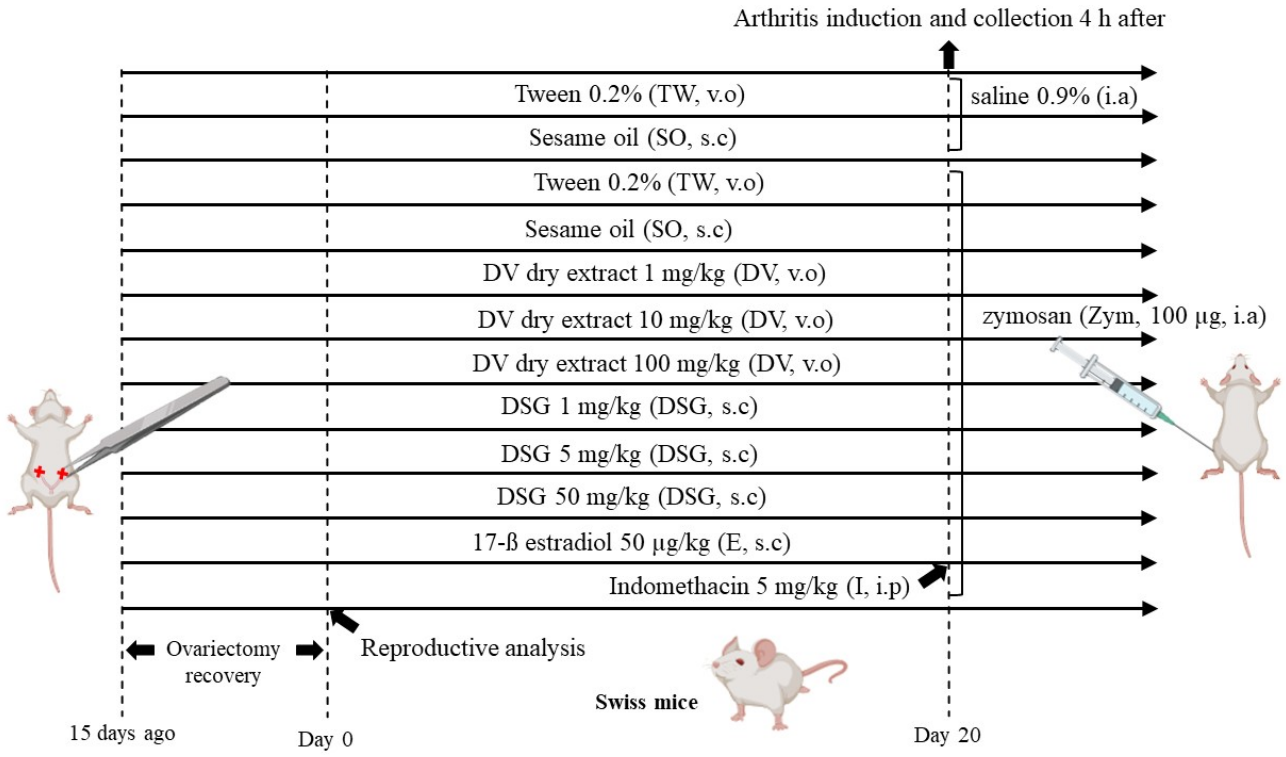

FIGURE 1 - Experimental design with treatment scheme for each group. Tween 80 (TW), sesame oil (SO), zymosan A (Zym), Dioscorea villosa (DV), Diosgenin (DSG), 17 ß-estradiol (E), and indomethacin (I).

Zym-induced arthritis model

Arthritis induction was performed after the last day of pretreatment (20th day) at different times according to the administration route (30 min, s.c. and 1 hour, v.o. before treatment). The arthritis was induced by injection of Zym, i.a, at a dose of 100 µg/cavity, dissolved in 10 µL of sterile saline in the right femorotibial joint, while the control group received only 10 µL of sterile saline, i.a. Four or 24 hours after induction, the animals were euthanized by excess isoflurane (2 to 3%) to perform the subsequent experimental protocols.

Leukocyte migration

The leukocyte migration was analyzed according to Silva *et al*. (2017). The knee joint was washed and diluted in PBS/EDTA. The total number of leukocytes was recorded as the leukocyte number per articular cavity. Differential cell counts were performed after smears were obtained by cytocentrifuge and stained with Leishman dye for characterization of leukocyte types.

Histopathology

Femorotibial joints were immersed in 4% paraformaldehyde for more 24 hours. Afterwards, the joints were decalcified in 0.8% nitric oxide solution for 48 hours. Next, the joints were dehydrated and embedded in paraffin and 6 µm thick slices were placed on microscopic slides. The histological slides were stained with hematoxylin and eosin (H&E) and analyzed with an optical microscope with 40, 100 and 400 x magnification.

Reproductive analysis

For the vaginal analysis, the animals were positioned at the same distance from the camera (Dsc h200, Sony) on an

illuminated white background. The size of the total vaginal area was measured using the ImageJ software (Fiji version).

To analyze the estrous cycle, a drop of saline was inserted into the vagina of the mouse with the aid of a Pasteur pipette, and quickly suctioned. Then the morphology of the epithelial cells was analyzed with the optical microscope at 100x magnification and classified in the different phases of the cycle: metestrus (Met), diestrus (Diest), proestrus (Pro) and estrus (Est) (Silva *et al*., 2017).

Finally, for analysis of the wet uterus mass, the animals were euthanized 4 and 24 hours after arthritis induction and the uterus was collected and weighed.

Statistical Analysis

The results were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism version 8.4.3. The significant differences observed were provided through analysis of variance (ANOVA) followed by the Tukey post-test, for comparison of three or more groups. Values of $P<0.05$ were considered significant.

RESULTS

Identification of the phytoestrogen diosgenin in the dry extract of *Dioscorea villosa*

Figure 2 shows the chromatogram of dry extract of DV (A) and phytoestrogen DSG (B) obtained by the modified method of Lima *et al*. (2013). The chromatogram of the dry extract of DV (Figure 2A) indicated the presence of the phytoestrogen DSG, a peak like that found in Figure 2B depicts the chromatogram of the DSG standard. Both peaks were identified at the same wavelength ($\lambda = 205$ nm) and at retention time of 28 minutes +/- 1.

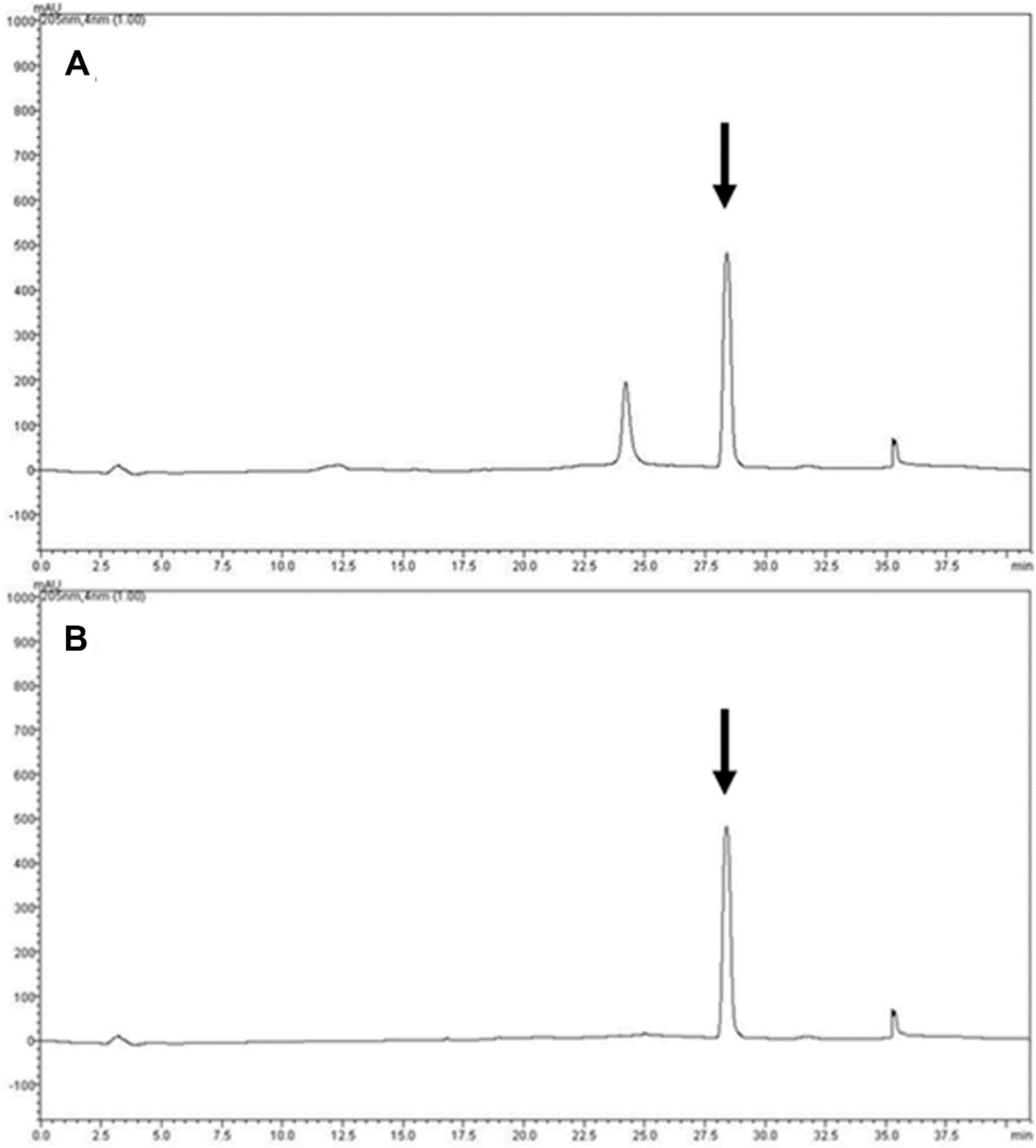

FIGURE 2 - HPLC-DAD chromatogram of the dry extract of *Dioscorea villosa* (DV; A) and the phytoestrogen diosgenin (DSG; B), λ 205 nm. Retention time 28 min (+/- 1).

Effect of dry extract of *Dioscorea villosa* and its phytoestrogen diosgenin in the zymosan-induced arthritis model.

The effects of dry DV extract and its phytoestrogen DSG on Zym-induced arthritis in animals previously affected by OVX are shown in Figures 3 and 4, as the result of the analysis of leukocyte migration and histology of the femorotibial joint, respectively.

In leukocyte migration, we observed that 95% to 97% of the infiltrate consisted of neutrophils 4 hours after Zym-induced arthritis (data not shown). Figure 3 shows that the positive controls groups, composed of animals that received vehicles as pretreatment (Tween at 0.2% (TW, v.o.) in A and Sesame oil (S.O, s.c.) in B for 20 days) and on the 20 day received injection of Zym (100µg/10µl, i.a.), had a significant increase in neutrophil migration to the femorotibial joint (TW, 26.45 ± 8.65; SO, 29.69 ± 3.37 neutrophils x10^4/joint cavity, 3A and 3B, respectively) when compared with the negative control groups that also received pretreatment with vehicle for 20 days, but that on the last day of treatment received only intra-articular injection of saline (TW, 2.01 ± 1.37; SO, 2.91 ± 1.11 neutrophils x 10^4 / joint cavity, 3A and 3B, respectively).

Moreover, the groups that received dry DV extract as pretreatment for 20 days at doses of 1, 10 and 100 mg/kg v.o. (Figure 3A) and DSG at doses of 1, 5, 50 mg/kg s.c. (Figure 3B) showed a decrease in neutrophil migration to the joint cavity (DV, 9.28 ± 2.26, 10.36 ± 3.95, 9.9 ± 4.55 neutrophils x 10^4/joint cavity; DSG, 12.16 ± 4.52, 4.92 ± 1.48 and 4.74 ± 2.54 neutrophils x 10^4/joint cavity, 3A and 3B, respectively) in comparison with the positive control group without treatment. The same result occurred for the group that received HRT with 17 ß-estradiol at a dose of 50 µg/kg (estrogen; E, 8.02 ± 2.56 neutrophils x 10^4 / joint cavity, 3 A; E, 6.54 ± 0.47 neutrophils x 10^4 / joint cavity, 2B) and with the group that received treatment with I, i.p., at a dose of 5 mg/kg (I, Figures 2A and B; 3.76 ± 0.35, 3.76 ± 0.34 neutrophils x 10^4/ joint cavity, respectively).

Initially, a pilot experiment 4, 12 and 24 hours after induction was performed to define the best time for histological analysis (data not shown). The most appropriate time for analysis was 24 hours after the induction of arthritis, where we observed the greatest morphological alterations, such as leukocyte infiltrate and edema.

The photomicrographs of the histological slides of the femorotibial joint 24 hours after the start of the inflammatory process in Zym-induced arthritis are depicted in Figure 2C, arranged from left to right in increasing magnification of 40, 100 and 400X, where each line of this graph represents an experimental group, or more groups in the case of controls. The negative control group, composed of animals pretreated with TW or SO, are represented in Figures 3C- A, B and C, where mild interstitial edema and subtle presence of leukocytes are observed. The same does not occur in the images representing the positive controls, which were also pretreated with TW and SO (Figures 3C- D, E and F). Instead, these images show the presence of intense leukocyte infiltrate, accompanied by edema and destruction of the femorotibial joint. However, in the images that represent both the group pretreated with a dose of 10 mg/kg of dry extract of DV (Figures 3C- G, H, I) and the group that received pretreatment with a dose of 5 mg/kg of DSG (Figures 3C- J, K and L), intermediate doses selected according to the previous experiment, had decreased leukocyte infiltrate in comparison with the positive control group. Likewise, Figures 3C- M, N and O represent group E, pretreated with HRT, which only developed moderate interstitial edema, with the presence of some leukocytes.

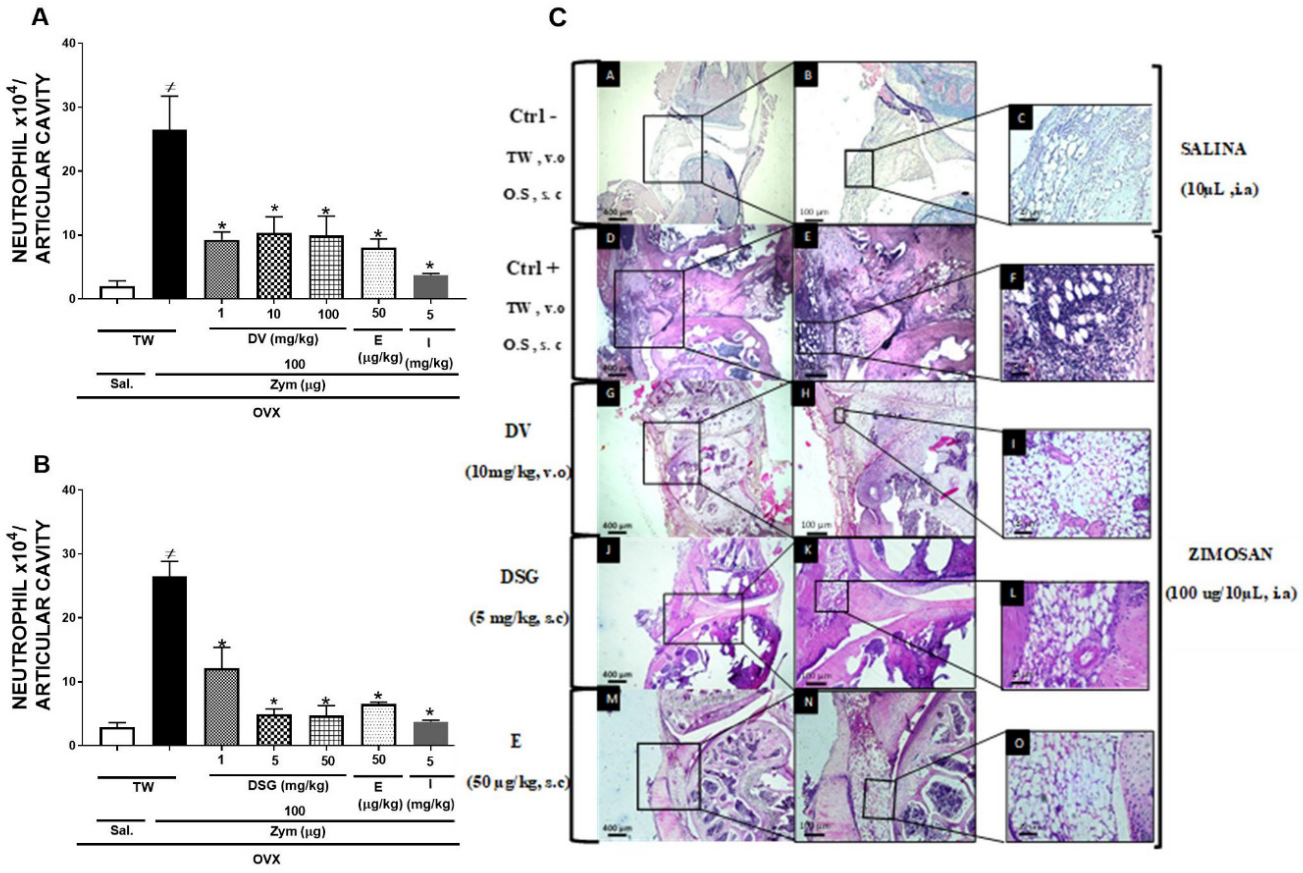

FIGURE 3 - Effect on neutrophil migration in Zym-induced arthritis in ovariectomized (OVX) mice. DV denotes dry extract (A), DSG represents phytoestrogen (B) and controls received estrogen (E) or indomethacin (I). After surgical recovery of two weeks, treatments were administered for 20 days. On the 20th day, the animals received intra-articular injections (i.a.) of Zym (100ug/cavity). After 4 hours, the femorotibial lavage was collected to assess leukocyte migration by total and differential counts. The results are expressed as mean ± SEM of two independent experiments (n = 7 / group), ANOVA followed by the Tukey test. ≠ $p < 0.05$ when compared to the Sal group; * $p < 0.05$ when compared to the (Crtl +) group (Zym). The femorotibial joint photomicrographs are presented in 40x, 100x and 400x magnification from left to right (C). Figures A, B and C represent the negative control (Crtl-) groups (TW, SO). The positive controls, (Crtl +) (TW, SO), pretreated with vehicle, received i.a. injections of Zym (100 µg/10 µL) and are represented in Figures D, E and F The DV groups (10 mg/kg) are shown in Figures G, H and I, while the DSG groups (5 mg/kg) are represented in Figures J, K and L. Finally, the animals that received hormone therapy (E, 50 µg/kg) are represented in Figures M, N and O. Data obtained from two independent experiments (n = 7/group).

Effect of dry extract of *Dioscorea villosa* and diosgenin on reproductive parameters

Figures 4A and 4B show the control groups pretreated with TW (Ctrl+, 0.13 ± 0.05g; Ctrl-,0.12± 0.04g, 4A) and with SO (Ctrl+, 0.1 ± 0.05g; Ctrl-, 0.06 ± 0.01g, 4B), revealing that Zym i.a. injection had no influence on the wet uterus mass. However, the animals that received treatment with HRT had increased wet uterus mass (E, 0.45 ± 0.07g, 4A; 0.47 ± 0.06g) in comparison with the animals of the negative (Crtl +) groups that received the vehicles separately (TW and SO) as treatment. In addition, it can be observed in Figure 4A that the groups pretreated with dry DV extract at doses of 1, 10 and 100 mg/kg did not have higher wet uterus mass (0.04 ± 0.01g; 0.03 ± 0.00g; 0.4 ± 0.00g, respectively, 4A) when compared to the control groups. The results were similar for the group treated with the lowest doses of DSG (1 mg/kg, 0.14 ± 0.03g and 5 mg/kg, 0.17 ± 0.06g) (Figure 4B). However, like the group treated with HRT, the group

pretreated at a dose of 50 mg/kg for 20 days showed a significant increase of wet uterus mass (0.32 ± 0.07g) when compared to the control groups.

Figures 4C to 4E show that both treatments with DV and DSG influenced the estrus cycle of OVX females. Before starting pretreatments, the estrous cycle of 79% of OVX females was in the metestrus phase, and another 18% were in the diestrus phase (Figure 4C). Animals that did not undergo these two phases of the cycle were not used in the formation of experimental groups. After 20 days of pretreatment, estrous cycle analysis was performed again (Figures 4D and 4E). The control groups that received pretreatment with TW (metestrus-75%, diestrus-25%) and SO (metestrus- 80%, diestrus-20%) were similar regarding the estrous cycle on day 0 (Figure 4C). In the groups that received HRT (E), the estrous cycle was modified in 100% of the animals (Figure 4D) and 93% (Figure 4E), and the other 7% were in the proestrus phase.

In the group that received pretreatment with DV, none of the animals developed the estrus phase of the cycle, but 34% of the animals treated with the dose of 10 mg/kg of dry extract of DV were in the proestrus phase (Figure 4D). The other animals in this experimental group were in the metestrus (50%) and diestrus (16%) phases (Figure 4D). Regarding the other doses, 90% of the animals in the group that received the dose of 1 mg/kg were in the metestrus phase of the cycle, while 10% of the animals were classified in the diestrus cycle. The proportion of cycles found in the highest dose group (100 mg/kg) was like that found in the control group, 79% / metestrus and 21% diestrus (Figure 4D).

Figure 4F shows that the group that received HRT (E) and DSG (50 mg/kg) as pretreatment had a significant increase in the total area of the vagina in comparison with the control groups. However, the evaluated parameters and doses did not present statistical differences for the groups that received DV compared to the control group. This did not occur with the DSG group, which reacted like HRT group, with greater uterus, modified estrous cycle and increased total vaginal area.

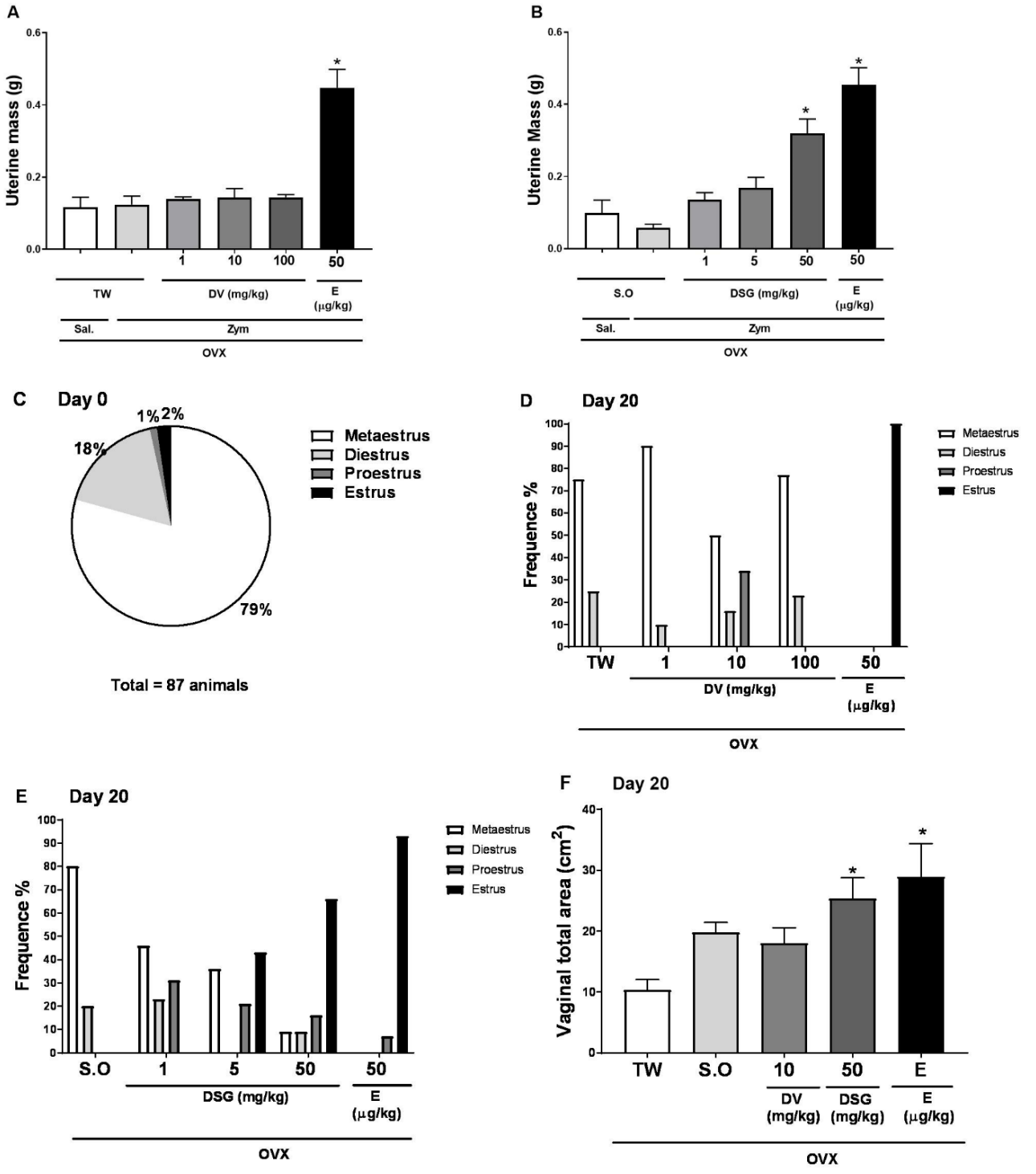

FIGURE 4 - Effect on the uterine mass of ovariectomized mice. Dry extract of DV and DSG (A and B). The estrous cycle was analyzed and classified in according to morphological changes in vaginal lavage cells (metestrus, diestrus, proestrus and estrus) on Day 0 (C) and the same analysis was repeated on the 20th day after two weeks of treatment (Day 20, D and E). On the same 20th day, the vaginas were photographed and analyzed by the Image J software (Fiji version): total area (F). The results are expressed as mean ± SEM of 3 independent experiments (n = 7/group). * $p < 0.0001$ when compared to the TW or SO group (one-way ANOVA followed by the Tukey post-test).

DISCUSSION

The phytoestrogen DSG was the major compound detected in the HPLC analysis. DV as a source of DSG has also been reported by other authors. Avula *et al*. (2014), when characterizing samples regarding DV by means of ultra-performance liquid chromatography together with mass spectrometry, identified the presence of DSG in its composition. It was noteworthy that two of the samples contained the DSG as a single component. Furthermore, in the same study, a chromatographic profile similar to DV was identified in 4 of the 5 food supplements analyzed, identified by the following numbers: 10210, 10211, 10212, 10213 and 10215 (Liang *et al*., 2016). Furthermore, Lima *et al*. (2013) had previously identified the presence of DSG as the main compound in the dry extract of DV.

Preclinical studies have shown other effects of DV. Its dry extract promoted analgesia in an experimental model of nociception, in addition to an anti-inflammatory effect by inhibiting the migration of leukocytes in carrageenan-induced peritonitis in mice (Lima *et al*., 2013). However, there is no evidence in the literature of the use of this species for the treatment of arthritis, although there are studies that demonstrate the effect of plants belonging to the same genus on this disease. According to Liang *et al*. (2016), the antiangiogenic effect of *Dioscorea nipponica* was observed in a model of collagen-induced arthritis in rats. Another study evaluated the effect of *Dioscorea zingiberensis* on Freund's adjuvant-induced arthritis, also in rats, where it promoted a decrease in the concentration of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), suppression of oxidative stress and reduced production of eicosanoids (PGE and LTB4) (Zhang *et al*., 2014).

Studies carried out by our research group using the same experimental model have shown that OVX increases the migration of neutrophils to the intra-articular cavity when compared to a control group, with preserved endogenous estrogen production and treatment with sesame oil vehicle (Silva *et al*., 2017). In the same work, both treatment for 5 days with HRT (in the same dose and route used in the present work) and different doses of tamoxifen, a selective estrogen receptor modulator (SERM), reduced the neutrophil migration into the joint.

The effect of DV and DSG on the reproductive parameters, in addition to providing clues about the safety of this treatment, also suggests a mechanism by which these compounds may be acting. In addition, the effects on the reproductive tract can alleviate the symptoms related to urogenital alterations of women during the transition from menopause to post-menopause, changes that are related to hypoestrogenism in these women (Bolton, 2016; Koebele, Bimonte-Nelson, 2016).

Although DSG is the major compound present in the dry extract of DV (Lima *et al*., 2013), it has low bioavailability when administered orally (Liu *et al*., 2017; Okawara *et al*., 2014). This can explain its milder effect on changes in the reproductive tract. In order to confirm this hypothesis, a study evaluated the effect of only DSG, administered orally for three days at doses of 20, 100 and 200 mg/kg on the uterus of immature rats. The authors reported that DSG had no effect on the wet uterus mass (Medigović *et al*., 2014). However, another study using DSG in OVX animals at doses of 10, 50, and 100 mg/kg found increased concentrations of metalloproteinases 2 and 9, which in turn acted on the expression of gelatinase and on collagen metabolism in the uterus (Chang *et al*., 2011). In the present work, changes in the reproductive tract were observed, mainly in the group that received pretreatment with E (50 mg/kg, s.c.).

Regarding morphological changes during the estrous cycle of intact animals, the proestrus and estrus phases were established due to the increase in endogenous estrogen concentrations. In the present work, like in other studies, it was evident that the animals submitted to ovariectomy were mostly in the metestrus phase of the estrous cycle (Mullen *et al*., 2012). Even so, HRT caused a change in the estrous cycle from the metestrus/diestrus phase to the proestrus/estrus phases in OVX animals (Zhang *et al*., 2019).

Also in previous studies by our group, we observed that like estrogen, tamoxifen modified the estrous cycle of these animals after five days of treatment (Silva *et al*., 2019). In addition, in a study with another phytoestrogen (the isoflavone biochanin A) at a dose of 9 mg/kg, s.c. for 15 days, milder activity of this compound was observed when compared to the HRT group (Felix *et al*., 2020), but its activity was similar to that of the dry extract of

DV at the dose of 10 mg/kg and DSG at the dose of 1 mg/kg demonstrated in the present work.

CONCLUSION

According to the results of this work, the dry extract of *Dioscorea villosa* (DV) contains the phytoestrogen diosgenin (DSG) in its composition. Pretreatments with these natural products, as well as hormone replacement therapy with 17 β-estradiol and indomethacin, were effective in reducing the severe inflammatory synovitis generated by the zymosan-induced arthritis model in OVX animals. The dry extract of DV v.o. did not alter the leukocyte count in the joint, while DSG, s.c. altered these parameters, but they remained within the established reference values for intact Swiss mice. The doses of 1 and 100 mg/kg of dry DV extract did not influence the reproductive tract, but the intermediate dose of 10 mg/kg had a slight influence only on the estrous cycle, as did the lowest dose of the phytoestrogen DSG (1 mg/kg). These effects may be beneficial in reducing symptoms related to reproductive aging. More studies are needed in this regard. Furthermore, doses of 5 and 50 mg/kg of DSG caused significant changes in the reproductive tract as well as the HRT. Therefore, these doses and routes of DV and DSG can be recommended for studies that aiming to observe their mechanisms and effects in this system.

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