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original research article

Effect of amygdalin on the treatment and recurrence of endometriosis in an experimental rat study

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Abstract

Background: *Endometriosis is an aggressive disorder and associated with infertility, pelvic pain and intra-abdominal adhesions in women of reproductive age. Women with endometriosis has the potential risk of recurrence ranging from 21.5% in two years to 50% in five years after recovery period.* ***Therefore, there is a certain requirement for new drugs as an alternative therapy to the current ones.***

Aim: *The aim of the present study is to compare the effects of amygdalin and leuprolide acetate on endometriosis development and recurrence in rats.*

Study Design: *Animal experiment*

Methods: *A total of 30 adult female rats were enrolled. Induction of endometriosis was performed by implanting endometriotic focci on the peritoneal side of the abdominal wall. Before amygdalin or leuprolide acetate treatment one of the implant was removed for histopathological analysis, and rats were randomly divided into three groups. Saline (Group 1), amygdalin (Group 2), and leuprolide acetate (Group 3) were administered for three weeks. After treatment, one of the remaining three implants was excised for histopathological evaluation, and all treatments were terminated. Estradiol was given after the estradiol induction for the recurrence of endometriosis. Rest of the implanted tissues were removed, then all rats were euthanised. The implant volumes, histopathological injury and fibrosis levels were observed.*

Results: *The endometriotic foci volumes in Group 2 and Group 3 were significantly lower than in Group 1 ($p = 0.001$, $p = 0.002$, respectively). The histopathological injury scores and fibrosis levels were not significantly different among the groups ($p > 0.05$).*

Conclusion: *The present study showed that amygdalin has an evident effect in the treatment of endometriosis.*

INTRODUCTION

Endometriosis is one of the most frequently gynecological disease of the reproductive tract. It is an estrogen-dependent disorder characterized by the implantation and survival of endometrial tissue formed by endometrial stromal and epithelial cells at ectopic sites, frequently in the peritoneal cavity *(1)*. Additionally, it is an aggressive disorder associated with infertility, pelvic pain and intra-abdominal adhesions in women of reproductive age *(2)*. In contrast to the amount of comprehensive studies, the pathogenic mechanism underlying the development of this condition remains unknown *(3,4)*.

Management of endometriosis consists of surgical or medical therapy. The primary goals of these interventions are the excision of ectopic endometrial tissues, restoration of normal anatomical structures, restriction of the disease progression, and relief of the symptoms *(5,6)*. Medical treatments including oral contraceptives, progestins, androgens, aromatase inhibitors, gonadotropin releasing hormone (GnRH) agonists, anti-tumor necrosis factor (TNF), and selective estrogen receptor modulators are widely used; however, no significant differences have been found from one to another *(7)*. Women with endometriosis has the potential risk of recurrence ranging from 21.5% in two years to 50% in five years after recovery period *(8)*. Therefore, there is a certain requirement for new drugs as an alternative therapy to the current ones.

Leuprolide acetate is a synthetic form of GnRH receptor agonists and is clinically used in the treatment of endometriosis. It suppresses the production of luteinizing hormone and follicle-stimulating hormone that subsequently lowers gonadal sex steroid production *(9)*. Furthermore, Amygdalin [D-mandelonitrile-β-D-gentiobioside; vitamin B17 ($C_{20}H_{27}NO_{11}$), also called Laetrile] is a cyanogenic glycoside, extracted from Semen Persicae, and derived from the aromatic amino acid phenylalanine. It was purified in 1830 from the kernels of the bitter almond (Prunus amygdalus) by the French chemists named Robiquet and Boutron-Charlard, and is intensely contained in the seeds of apricots, peaches, and plums. Besides its anti-cancer activity, it has also been administered as an alternative treatment for asthma, bronchitis, emphysema, leprosy, diabetes, and preventing and treating of migraine, hypertension. It has been thought that amygdalin is also an anti-inflammatory agent *(10)*. A recent study by Jiagang et al. showed the influence of amygdalin on the regulation of immune system cells during the development of atherosclerosis *(11)*. It has been proposed that amygdalin could affect the local activity of immune cells which are crucial pathogenic factors in the development and progression of endometriosis *(12)*.

We hypothesized that amygdalin can suppress the endometriotic implants because of its anti-inflammatory effect. A systematic search of the literature revealed that there has been no study conducted to show the effects of amygdalin on endometriosis. In this context, the aim of this study was to evaluate the effects of amygdalin and leuprolide acetate on the treatment and recurrence of endometriosis in a rat model.

MATERIALS AND METHODS

Chemicals

Amygdalin and chloral hydrate were purchased from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany. Estradiol depot was obtained from Jenapharm, Jena, Germany. Cefazolin sodium was purchased from Eczacıbaşı Pharmaceutical, Istanbul, Turkey. Ketamine and xylazine were obtained from Alfasan International B.V. Haematoxylin Harris and Eosin Y 1% Alcoholic were purchased from Atom Scientific Ltd (Manchester, UK). Masson's trichrome was obtained from GBL, Istanbul, Turkey. Leuprolide acetate was purchased from Abbott Laboratories, Abbott Park, IL, USA.

Animals

This prospective, randomized, and controlled experimental study was initiated after approval from Animal Experimentations Local Ethics Board (2014-HADY-EK-09). A total of 30 adult female Wistar-Albino rats (90 days-old, nulligravid, 250-300 grams) were obtained from Experimental Medicine Research Unit. Animals were kept in a room maintained at 20 – 24 °C with a 12-h light-dark cycle (lights on at 06:00 to 18:00) and a constant humidity of 40 – 50%. All rats were housed in polycarbonate cages and tap water ad libitum. Before the endometriosis procedures, the estrus cycle of all animals were hormonally synchronized to avoid the possible effects of steroid release, cell adhesion and growth. The estrus cycle was described in the following order: proestrus period (several number of epithelial cells with centric nucleolus), estrus period (cornified epithelial cells with no nuclei), metestrus period (leukocytes, mucus, and a small number of cornified cells), and diestrus period (multiple epithelial cells, mucus, and leukocytes) *(13)*. Rats were followed up until successfully complete at least two 4-day estrus cycles by analyzing vaginal smears periodically.

Endometriosis procedure

Induction of endometriosis

All rats were anesthetized using chloral hydrate 300 mg/kg intraperitoneally. Endometriosis was induced surgically using the method defined by Lebovic and Uygur et al *(14,15)*. A 3 cm midline vertical incision was performed to enter the abdominal cavity. The right uterine horn was moved away from the cervix up to a location approximately 1 cm far from the ovary. The uterine horn was incised longitudinally and divided into four pieces (0.4 X 0.4cm) consisting of both endometrium and myometrium. Pieces were placed in saline solution at 37°C and then attached, via the surgical auto-transplantation technique, to the peritoneum with their myometrial side (endometrial side faced to the peritoneal cavity) by using 5-0 Vicryl (polyglactin 910, Ethicon, Somerville, NJ, USA) on the right and left wall of the ventral abdominal side next to an artery. Midline abdominal incision was repaired in a continuous interlocking manner using 3-0 Vicryl (polyglactin 910, Ethicon, Somerville, NJ, USA). Cefazolin sodium 50

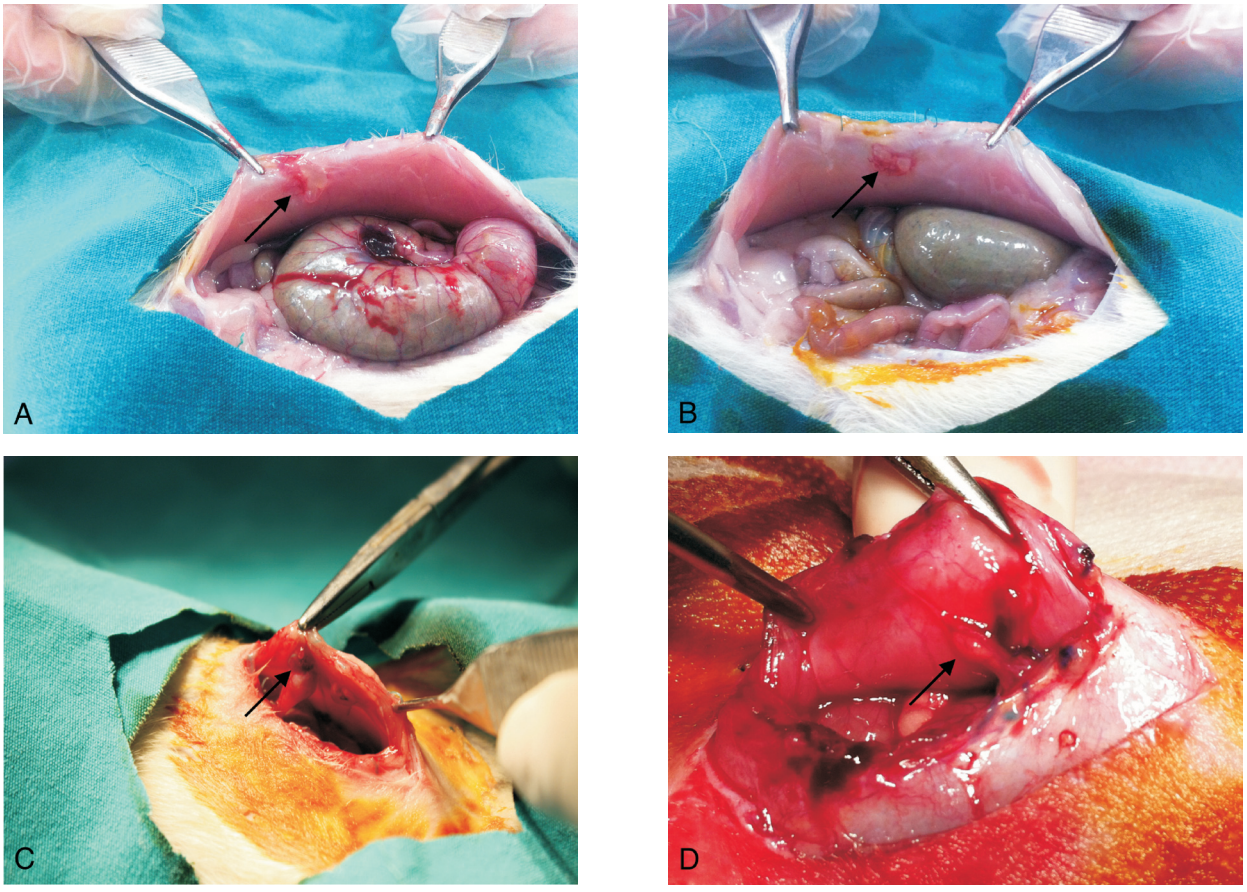


Figure 1. *Macroscopic images of endometriotic implant:* **A** *– before amydalin or LA treatment;* **B** *– after treatment;* **C** *– before amydalin or LA treatment;* **D** *– after treatment.*

mg/kg for three days and estradiol depot 50 mg/kg IM twice a week were administered to all rats in the postoperative period to reduce the duration of endometriosis induction and increase the efficiency *(16).*

Before amygdalin or LA treatment

This procedure was initiated 21 days after the induction of endometriosis. The implanted tissues were documented, and the height, length and width of the tissues were measured by a micrometer (Figure 1). The implant volume was computed using the formula of ellipsoid volume ($\pi/6 \times$ length $\times$ width $\times$ height). One of the four implants was excised for histopathological analysis. After no significant difference was found between removed tissue volumes following excision the rats were randomly divided into three groups as follows: Group 1 received 1 ml of saline 0.9% (Adeka, Istanbul, Turkey) intraperitoneally once a week, Group 2 received amygdalin 5 mg/kg intraperitoneally once a week, and Group 3 received leuprolide acetate 0.0375 mg/kg subcutaneously once a week. The doses of amygdalin and leuprolide acetate were used as indicated in the study conducted by Guo et al. and Dolapciogu et al., respectively *(17,18).*

After treatment

This procedure was performed 21 days after the previous one. The height, length and width of the tissues were measured by a micrometer, and one of the rest three implanted tissues was removed for histopathological evaluation. Thereafter, all medications were terminated and remaining estradiol injections for recurrence of endometriosis (estradiol depot 50 mg/kg IM twice a week) were given until the last operation.

After the estradiol induction for the recurrence of endometriosis

After 21 days of estradiol treatment, all rats were evaluated for visible signs of recurrent growth under chloral hydrate anesthesia, and remaining implanted tissues on the abdominal wall were removed. All rats were euthanized by administering ketamine 50 mg/kg and xylazine 10 mg/kg intraperitoneally.

Histopathological evaluation

The tissue samples harvested from all animals were fixed in 10% neutral buffered formalin solution for 24 hours, quickly upon collection. Samples were dehydrated

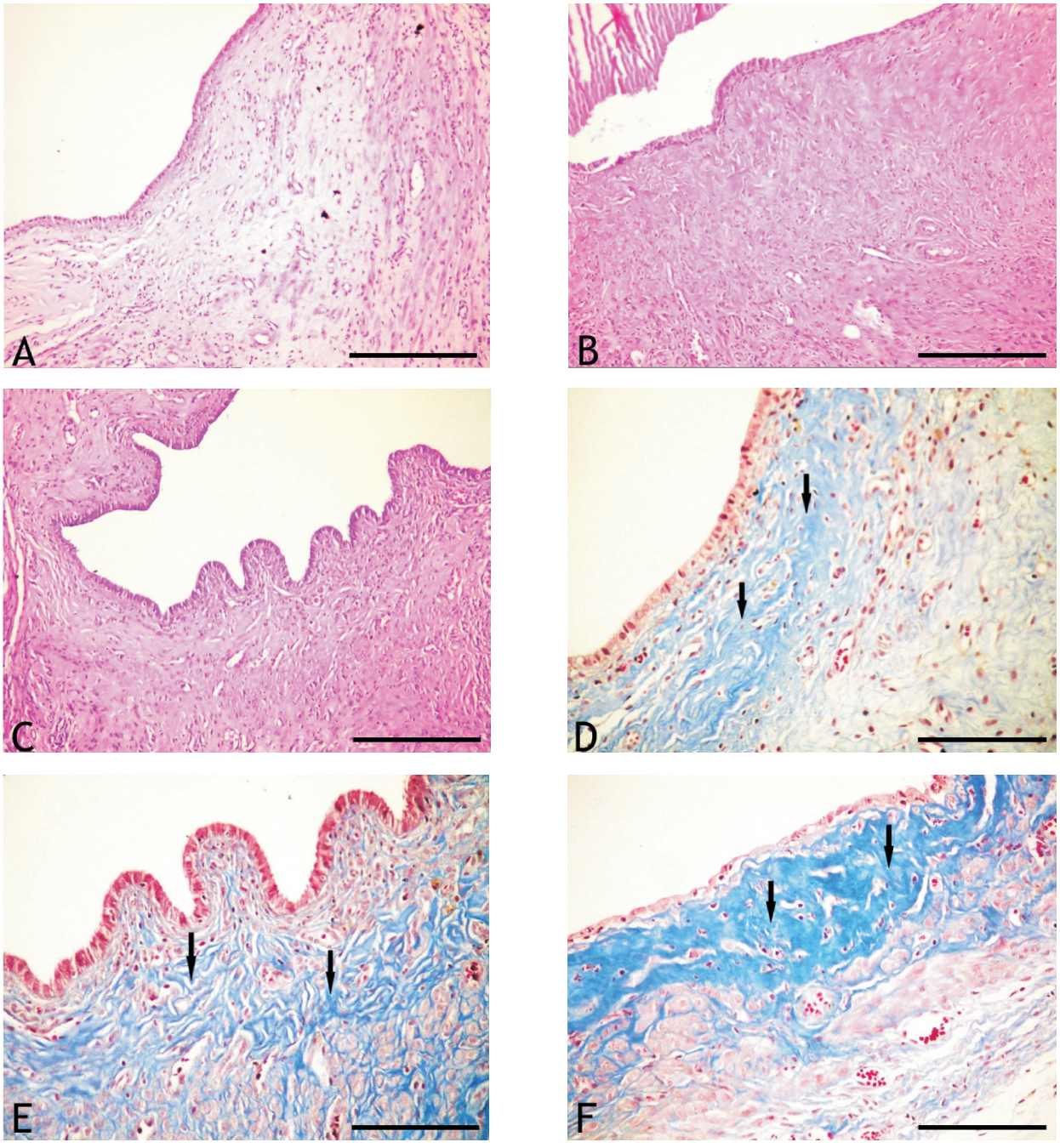
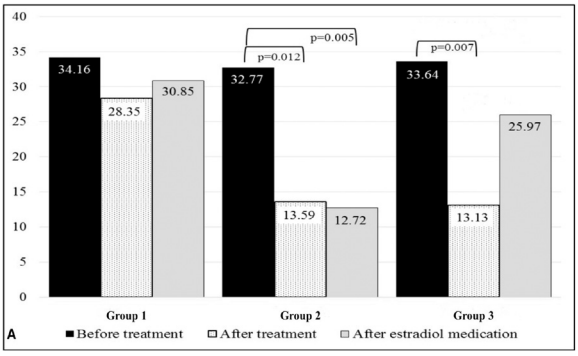
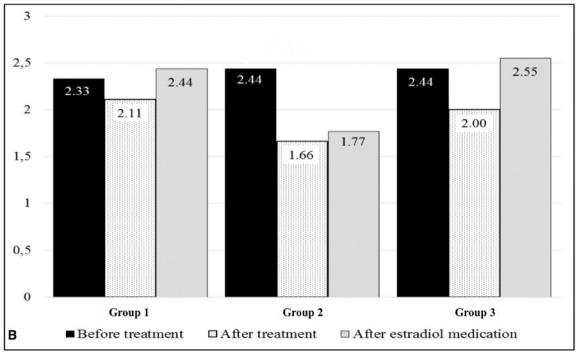


Figure 2. *Histopathological evaluation of endometriotic implant.* **A** *– Well-preserved superficial epithelium and elevated stromal vessel density (Group 1);* **B** *– Evident stromal fibrosis, mild leukocyte infiltration and moderate preserved epithelium (Group 2);* **C** *– Congestion in sub-epithelial stroma and mild fibrosis (Group 3);* **D** *– [+] fibrosis in Group 1;* **E** *– [++] fibrosis in Group 3;* **F** *– [+++] fibrosis in sub-epithelial stroma of Group 2.*

A-C: H&E staining, Magnification: ×100, Scale bar: 200 micrometer; **D-F***: Masson's Trichrome staining, Magnification: ×200, Scale bar: 100 micrometer.*

and embedded in paraffin for histopathological evaluation. Five µm thick sections were prepared from paraffin-embedded samples and mounted on glass slides. For histopathological assessment under light microscope (Nikon Eclipse E600W, Japan), the mounted sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome by a pathologist blinded to the study groups. A grading scale for the structure of epithelial cells in the implants described by Keenan et al. was used to determine the histopathological score of the sample tis-





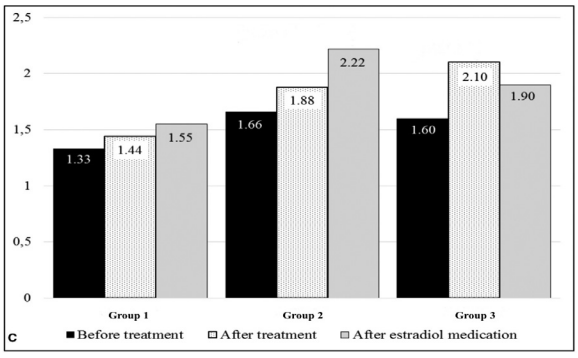


Figure 3. **A** – *Comparison of endometriotic implant volumes (Y axis represents the implant volumes in mm³);* **B** – *Comparison of histopathological scores among medication steps (Y axis represents the histopathological scores);* **C** – *Comparison of fibrosis levels among medication steps (Y axis represents the fibrosis level).*

Group 1; Control group, Group 2; Amygdalin group, Group 3; Leuprolide acetate group.

Data are given as means.

All other p values among medication steps into each group are >0.05.

Table 1. *Histopathologic comparison of the endometriotic implants among the groups*

Histopathologic features of implants	Group 1 (n=9)	Group 2 (n=10)	Group 3 (n=10)	p1	p2	p3
Endometriotic foci volumes (mm³)						
Before treatment	34.16±4.55	32.77±6.85	33.64±7.81	0.561	0.963	0.677
After treatment	28.35±7.47	13.59±7.75	13.13±6.64	**0.002**	**0.001**	0.813
After estradiol medication	30.85±8.15	12.72±8.56	25.97±7.65	**0.002**	0.173	**0.007**
Histopathological scores						
Before treatment	2.33±0.86	2.44±0.72	2.44±1.01	0.964	0.783	0.734
After treatment	2.11±1.05	1.66±1.05	2.00±1.00	0.156	0.729	0.33
After estradiol medication	2.44±0.52	1.77±0.83	2.55±0.52	0.072	0.647	0.042
Fibrosis levels						
Before treatment	1.33±0.50	1.66±0.70	1.60±0.69	0.172	0.399	0.565
After treatment	1.44±0.52	1.88±0.78	2.10±0.73	0.121	0.05	0.776
After estradiol medication	1.55±0.72	2.22±0.83	1.90±0.73	0.091	0.288	0.36

* Multiple comparisons were completed using one-way ANOVA test, posthoc analysis were conducted by Tukey's HSD test.

Bonferrroni corrected p value was used for significance as $p < 0.016$. Group 1; Control group, Group 2; Amygdalin group, Group 3; Leuprolide acetate group. Data are given as means ± standard deviation.

The significance between the groups:

p1; group 1 – group 2, p2; group 1 – group 3, p3; group 2 – group 3

sue. Well-preserved epithelium, Grade 3; leukocyte infiltration and moderately protected epithelium, Grade 2; slightly preserved, very infrequently observed epithelium, Grade 1; disappearance of epithelial line, Grade 0 *(16).* According to this scoring system, lower scores shows impaired histological structure. Sections stained by Masson's trichrome for observing the fibrosis intensity were assessed by the method described by Erdemir et al *(19).* The collagen density in the samples obtained from implanted tissues was assessed and graded on scales

ranging from 1+, the lowest density collagen, to 4+, the highest density collagen ratios under light microscopy (×400).

Statistical analysis

All analyses were performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) version 20.0 program. Multiple comparisons were conducted by one-way ANOVA test and posthoc analysis using Tukey's HSD test. Continuous variables were expressed as means ± standard deviation. Bonferrroni corrected p value was used for all analysis as $p < 0.016$.

RESULTS

Endometriosis was successfully induced in all groups. The mean (initial) weight of the rats after grouping was 272.8 grams and 274.7 grams in Group 2 and 3, and 269.2 grams in Group 1 ($p > 0.05$). No complications were observed among rats; however, one rat in Group 1 died during the study period.

The endometriotic implant volumes were presented in Table 1. Microscopic evaluation of the sections among groups was shown in Figure 2 (A, B, C, D, E, F). After amygdalin or leuprolide acetate treatment, the endometriotic implant volumes in Group 1, Group 2 and Group 3 were 28.35mm^3, 13.59mm^3 and 13.13mm^3, respectively. The endometriotic implant volumes after amygdalin or leuprolide acetate treatment in Group 2 and Group 3 were significantly lower than in Group 1 ($p = 0.002$ and $p = 0.001$, respectively). After estradiol medication, the implant volume in Group 2 was significantly lower compared to Group 1 and Group 3 ($p=0.002$ and $p=0.007$, respectively). The implant volumes in Group 2 progressively decreased from the beginning of the amygdalin treatment to the end of the estradiol medication ($p =0.012$, $p = 0.005$; Figure 3A). The endometriotic implant volumes in Group 3 were significantly lowered after leuprolide treatment compared to the beginning of the treatment ($p = 0.007$; Figure 3A). The mean histopathological scores were presented in Table 1. The fibrosis levels were not significantly different among the groups ($p > 0.05$; Table 1).

DISCUSSION

The present study revealed that amygdalin apparently lowered the volumes of the endometriotic implant compared to leuprolide acetate after estradiol medication. Additionally, a clinically evident decrease in histopathological score was observed by amygdalin compared to leuprolide acetate treatment after estradiol medication. Several drugs have been used for the endometriosis therapy in the clinical setting *(8).* However, none could provide a stable long-term benefit and recurrence rates have been found to be high after ceasing of treatment. Simsek et al. investigated atosiban (an oxytocin receptor blocking agent) and found that atosiban treatment decreased the endometriotic implant volumes *(13)*. Recently, Dolapcioglu et al. compared the effects of theranekron, medroxyprogesterone acetate and leuprolide acetate in a rat endometriosis model, and showed that theranekron led to improvement in endometriosis and exhibited a declined ratio of recurrence *(17)*. Nevertheless, current studies also indicated that amygdalin might be helpful for the treatment of fibrosis in the liver and kidney *(18)*. Yang et al. reported that amygdalin can suppress fibroblast proliferation and disrupts the process of renal interstitial fibrosis, and they concluded that the possible mechanism behind this cascade of events could be the increase in the type I collagenase secretion, which restricts fibroblast proliferation, induction of apoptosis, and suppression of type I collagenase by amygdalin *(20).*

In this context, the present study revealed that amygdalin is superior to leuprolide acetate in reducing the implant volumes and maintaining its consistency by displaying no visible signs of recurrent growth after the treatment ended. It is reported that amygdalin exhibits its effect by excreting cyanide through susceptible cells, resulting in cell death by cyanide toxicity. The toxicity of cyanide might occur in tissue containing glucosidase enzymes, which splits off glucose from amygdalin and prunasin *(21)*. In relation, a clinical trial by Brandelli et al. showed that women with endometriosis have an increased activity of glucosidases in the peritoneal fluid *(22)*. Amygdalin also leads to slightly increased but not significant fibrosis levels compared to initial, and provided this level after the end of amygdalin treatment that considered as diminishing of endometriotic implant with no visible signs of recurrent growth. A possible mechanism making a connection with the previous data is that endometrial tissue in the implants could be deteriorated by the cyanide toxicity of the endometriotic cells not the components of the connective tissue such as fibroblasts due to the enzymatic metabolization of amygdalin by glucosidases. Cyanide induced the production of reactive oxygen species provided the activation of p38 mitogen-activated protein kinase and nuclear accumulation of hypoxia-inducible factor-1-alpha those lead to apoptosis *(23).* To the extent of our knowledge, this is the first experimental study conducted to determine the effects of amygdalin on endometriosis treatment and recurrence, and the first time such an observation has been demonstrated in an endometriosis model. Amygdalin is a cyanogenic glycoside, wherein cyanide is the main reason of toxicity. The lethal dose is estimated to be between 50 – 300 mg for humans while taking orally, or a blood level over 3 microg/ml. However, oral administration is associated with higher incidence of toxicity than intravenous, intraperitoneal, or intramuscular injection because the human intestinal microflora possesses enzymes capable of effecting cyanide release. Recent research suggests an oral dose of 0.6 – 1.0 gr can

avoid toxicity in humans *(24)*. In contrast, there is no data available on the dosage of amygdalin in patients with endometriosis.

It is well-demonstrated that three drugs for the treatment of endometriosis including progestins, GnRH agonists and androgenic agents prevent the proliferation of the implants and provide diminished adhesion formation. Vercellini et al. reported that the use of GnRH analogs prevents the risk of recurrence after surgery in patients with moderate or severe endometriosis *(25)*. In contrast, some studies have been unsuccessful to show any evidence about the effects of post-operative medication in reducing endometriosis recurrence *(26-39)*. Recent findings demonstrated that post-surgical hormonal therapy for endometriosis has no improvement on pain relief or pregnancy rates, but a substantial decrease in endometriosis recurrence rate solely *(30)*. However, there has been no satisfactory evidence found to confirm the effects of hormonal suppression therapy and surgical approach on endometriosis. Therefore, the outcomes of the present study may provide additional information about the development and recurrence of this disease.

There are some limitations in the present study. First, this is an experimental study with restricted numbers of rats per group. This was related to our ethical rules regarding keeping to the 'principle of reduction' in animal experiments; however, studies with larger populations would be required to find minimal histopathological differences among groups. Second, a subjective scoring system was performed to assess the histopathological changes of the endometriotic implant. A standardized image-analysis program providing an automated interpretation of histopathological changes would be better to meet with a more accurate result, which currently is not available.

In conclusion, the present study demonstrated that amygdalin can suppress the progression of endometriosis and its recurrence. Amygdalin might be more beneficial both in the treatment and the prevention of visible signs of recurrent lesions than leuprolide acetate.

Ethics Committee Approval: Ethics committee approval was received for this study from the Animal Experimentations Local Ethics Board (2014-HADYEK-09).

Informed Consent: N/A.

Author contributions: Concept – H.Y.D.., Ç.K.İ.; Design – H.Y.D.; Supervision – H.Y.D., B.Ç., A.A.; Resource – H.Y.D., A.A.; Materials – H.Y.D.; Data Collection and/or Processing – H.Y.D., İ.B.D.; Analysis and/or Interpretation – H.Y.D., A.A.; Literature Search – H.Y.D., A.Z.Ö.; Writing – H.Y.D., B.Ç., İ.B.D.; Critical Reviews – H.Y.D., Ç.K.İ., A.A., A.Z.Ö., İ.B.D., B.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

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