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Investigating the Therapeutic Effects of *Echium amoenum* Extract on Anxiety, Perceived Stress, and Menstrual Cycle Regulation in Women with Delayed Menstruation: A Triple-Blind Randomized Clinical Trial Protocol

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ABSTRACT

Aims Except in pregnancy, functional hypothalamic amenorrhea (FHA) and polycystic ovary syndrome (PCOS) are the most prevalent causes of secondary amenorrhea. *Echium amoenum* (EA) Fisch & C.A.Mey. (Iranian Gol-e-Gavzaban or Iranian borage) possesses several notable properties, including anti-stress and anxiolytic effects, the capacity to elevate sex hormones, and the presence of prostaglandin precursor fatty acids. As a result, EA is being considered as a potential treatment for FHA induced by mental stress and PCOS, which may lead to the resumption of ovarian activity. This study aimed to determine the effect of thequeous extract of EA on the level of anxiety, perceived stress, and occurrence of menstruation in women with menstrual postponement referring to public comprehensive health centers and private gynecology clinics in Gonabad, Iran.

Materials & Methods This triple-blind randomized clinical trial was conducted on 82 non-pregnant women with menstruation postponement due to hypothalamic functional stress amenorrhea and polycystic ovarian syndrome. The women were referred to public comprehensive health centers and private gynecology clinics in Gonabad, Iran, in 2023. The subjects were randomly assigned to two groups: the test group (EA aqueous extract) and the control group (placebo) using the convenience sampling method and permutation blocks of size 2 and 4. The test group received 500 mg oral capsules containing the aqueous extract of EA, while the control group received capsules containing cornstarch as a placebo for 6 weeks, taking one capsule every day, 30 minutes after breakfast. Data were collected using demographic and obstetric questionnaires, the Cohen Perceived Stress Scale, the Hamilton Anxiety Rating Scale, a drug consumption form, a menstrual cycle registration questionnaire, and a food plan registration questionnaire. The data were analyzed using SPSS (version 21). The generalized estimating equations model was applied at a significance level of 0.05.

Conclusion If the findings indicate a favorable impact of utilizing EA aqueous extract for managing stress-induced FHA and PCOS, the administration of this herbal remedy may be recommended as a safe intervention with no adverse effects for these patient cohorts.

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Introduction

Secondary amenorrhea is defined as the cessation of menstruation for 6 months [1]. In addition, oligomenorrhea is characterized by prolonged menstrual cycles ranging from 40 days to 3 months [2]. The prevalence of secondary amenorrhea is 3%, and oligomenorrhea is 10.2% [1]. The most common form of secondary amenorrhea is functional hypothalamic amenorrhea (FHA), caused by mental stress with no evidence of endocrine causal factors [1]. Stress is an adaptive response of the body, mediated by its homeostatic systems, to internal and external stimuli that activate specific and nonspecific physiological pathways [3]. Another common form of secondary amenorrhea, especially in girls with symptoms of hyperandrogenism, is polycystic ovary syndrome (PCOS), whose timely diagnosis helps manage irregular, prolonged, or heavy bleeding and reduce metabolic complications. Oligomenorrhea, especially PCOS, can lead to various complications, such as infertility, cardiovascular and metabolic diseases, and mental disorders, such as depression and anxiety. Hormone therapy is the main treatment [4]. However, although oral contraceptive pills induce withdrawal bleeding, they do not restore normal hypothalamic–pituitary–ovarian axis function and thus may not support the resumption of natural cycles [5]. Therefore, studies are needed to identify safe and effective herbal compounds [6].

Echium amoenum L. (Boraginaceae) (EA) is an annual plant that grows to a height of 60–100 cm. Its flowers are purple after drying. All parts of the plant, except the root, are pharmacologically active [6]. According to a review in 2019, its chemical compounds include phenolic compounds, fatty acids, rosmarinic acid, anthocyanidins, and flavonoids [7]. Various studies have shown increased levels of pro-inflammatory cytokines and inhibition of anti-inflammatory cytokines under stress. Stress and anxiety have been linked to reduced activity of the inhibitory neurotransmitter ɣ-aminobutyric acid (GABA). GABA receptor A regulates excitatory messages of glutamate transmission and exerts neuroinhibitory effects in the brain. Structural disorders of GABA receptors play a role in controlling emotional states and lead to behavioral changes such as anxiety. Evidence shows that high levels of corticosteroids in chronic stress, with increased levels of *t*umor necrosis factor alpha, may interfere with GABAergic functions. Farajdokht et al. confirmed the involvement of A-GABA receptors in the protective effects of EA against anxiety, neuroinflammation, and hyperactivation of the HPA axis. Several studies have confirmed the sedative and anti-anxiety effects of EA, which are mainly attributed to its flavonoids and rosmarinic acid [8, 9]. On the other hand, plant hormonal effects have also been investigated. In Al-Saeidi et al.'s study, the impact of EA on the growth and differentiation of mammary glands was examined, and results showed that progesterone and prolactin were significantly increased in virgin rats [10]. In another study conducted by Fakher et al., EA was found to elevate sex hormone levels and enhance sperm count in male mice [11]. Therefore, since menstrual occurrence is primarily regulated by fluctuation of ovarian hormones under hypothalamus control [12], and given that EA contains prostaglandin precursor fatty acids [13], this plant may potentially contribute to the restoration of ovarian activity and resumption of menstruation in patients with stress-induced FHA and PCOS. Human studies have not reported any serious adverse effects of EA. A clinical trial comparing the use of EA extract with fluoxetine in menopausal women has shown this plant to be safe, with no documented adverse effects [14]. A study conducted by Sayyah et al. on patients with generalized anxiety disorder also reported no serious side effects [15]. Therefore, this study was designed to determine the effect of an aqueous extract of EA on anxiety, perceived stress, and the occurrence of menstruation in women with menstrual cycle postponement referring to public comprehensive health centers and private gynecology clinics in Gonabad, Iran.

Objectives

General Objective

This study aimed to determine the effect of the aqueous extract of EA on the level of anxiety, perceived stress, and occurrence of menstruation in women with menstrual cycle postponement

Specific Objectives:

- To determine and compare the mean anxiety score of women with delayed menstrual cycles before and after the intervention between the control and test groups
- To determine and compare the mean perceived stress score of women with delayed menstrual cycles before and after the intervention in two control and experimental groups
- To determine and compare the frequency of regular menstruation in women with delayed menstrual cycles before and after the intervention between the control and experimental groups

Study Hypothesis

1. The mean anxiety and perceived stress scores of women with delayed menstrual cycles after the intervention differed between the control and intervention groups.
2. The frequency of regular menstruation in women with delayed menstrual cycles after the intervention differed between the control and test groups.

Study Justification

FHA induced by stress and polycystic ovarian syndrome are prevalent contributors to secondary amenorrhea. Several studies have confirmed that EA possesses anti-stress, anxiolytic, and antidepressant properties [7-9, 13, 16],

highlighting the importance of managing irregular menstrual cycles stemming from stress-induced FHA. Additionally, various studies have reported the effects of EA on increasing sex hormones and antioxidants [10, 11, 17], suggesting its potential use as a supplement for patients with polycystic ovaries. It can reduce stress, improve quality of life, and enhance hormonal effects in PCOS patients, thereby regularizing the menstrual cycle in both groups. The results of this study guide treatment strategies regarding the inclusion of the aqueous extract of EA in standard care protocols, particularly given its demonstrated safety profile [10, 14-16].

Trial Design

This study outlines the protocol for a randomized, controlled, triple-blinded, parallel-group (with a 1:1 allocation ratio) clinical trial. This study focuses on women with secondary amenorrhea/oligomenorrhea attributed to FHA due to stress, as well as women with secondary amenorrhea/oligomenorrhea associated with PCOS.

Materials and Methods

This study has been registered at the Iranian Registry of Clinical Trials with the number IRCT20230417057944 N1.

Study Setting

This study was conducted at public comprehensive health centers and private gynecology clinics in Gonabad, Iran.

Eligibility Criteria

Each participant must sign an informed consent form indicating that they understand the purpose of and the procedures required for the study and are willing to participate.

Inclusion Criteria

The inclusion criteria included women of reproductive age between 17-45 years, FSH less than 20 units/liter, literacy for reading and writing, willingness and informed consent to participate in research, negative pregnancy test, menstrual cycle delay according to the average period of each person's menstrual cycle in the last six months, history of postponing menstrual bleeding for intervals of 36 days to 6 months, not using cigarettes, drugs and alcohol, absence of underlying medical diseases (pituitary disorders and adrenocorticotropic hormone-secreting tumors or Cushing's disease, thyroid disorders and hyperprolactinemia) and disorders related to the reproductive system (uterine anatomical abnormalities, neoplasia, Asherman syndrome, Müllerian agenesis, Gynecological neoplasia, hermaphrodism, genetic ovarian failure, premature ovarian failure, based on medical records or clinical evaluation, body mass index more than 18, absence of lactating amenorrhea, not suffering from known mental disorders other than stress and anxiety based on the person's statements and the scores obtained from the Hamilton Anxiety Rating Scale (HARS) and Cohen's perceived stress questionnaires, insensitivity to EA, not doing sports professionally, absence of nutritional disorders, such as bulimia nervosa and anorexia nervosa or malnutrition based on the person's statements, not consuming herbal, psychoactive, and antihypertensive drugs, not suffering from primary amenorrhea.

Exclusion Criteria

The exclusion criteria included unwillingness to continue cooperation, use of herbal, psychoactive, and antihypertensive drugs during the study, occurrence of unforeseen events (e.g., death of a relative, accident) that prevent continued participation, occurrence of side effects related to the consumption of borage during the study, failure to complete or incomplete completion of questionnaires during the study, occurrence of pregnancy during the study, not taking medication for three consecutive or alternating days during the 6 weeks, reduction of systolic blood pressure by at least 20 mmHg or reduction of diastolic blood pressure by at least 10 mmHg from the normal level (120/80-115/75) in weekly measurements.

Who Obtained Informed Consent?

After identifying a potential participant, the research team began the consent process. A thorough review of the informed consent document was conducted with individuals, during which they were introduced to the study's title, objectives, and research team. The potential risks and benefits associated with the use of the aqueous extract of EA were carefully explained, along with details about the study procedures and the participant's right to decline participation or withdraw without impacting their access to healthcare services. Participants were also assured that their data were kept confidential. They had the opportunity to ask questions and seek clarification before providing their consent through a signature.

Interventions

Explanation for the Choice of Comparators

Research indicates that stress and anxiety play significant roles in menstrual cessation and infertility among women with FHA induced by stress [18]. Additionally, stress, anxiety, and depression levels are elevated in women with PCOS, negatively impacting their quality of life [19]. The primary treatment for these menstrual disorders, such as secondary amenorrhea and oligomenorrhea, typically involves the use of pulsatile gonadotropin-releasing hormones (GnRH). However, the unavailability of a commercial GnRH pump in most countries limits this treatment option, leaving gonadotropin therapy as the main recourse in many cases [20]. Unfortunately, this treatment may induce withdrawal bleeding but does not restore normal ovarian function [21]. Given these challenges, this study aimed to compare anxiety, perceived stress, and the onset and regularity of menstruation among women with stress-induced FHA and PCOS who consume EA with those who consume a

placebo. By utilizing flour capsules as a placebo, researchers can assess the specific effects of the aqueous extract of EA compared to an inert substance. This approach helps control for potential confounding variables and enables a more accurate evaluation of the intervention's true efficacy.

Intervention Description

Following the research objectives, the research unit selection form was provided to eligible individuals to explain and secure informed consent.

This triple-blind randomized clinical trial focused on women experiencing oligomenorrhea (a prolonged type of menstrual cycle in which the cycle lasts from 40 days to three months [2]) or secondary amenorrhea (cessation or delay of menstrual bleeding occurring at intervals of 36 days to 6 months or longer without pregnancy [22]). These definitions may overlap [1]. The study utilized the Rotterdam diagnostic criteria for PCOS, which is characterized by the presence of at least two of the following features: oligomenorrhea/amenorrhea, hyperandrogenism, and polycystic ovaries observed on ultrasound [23, 24]. Endocrine manifestations of PCOS include elevated luteinizing hormone (LH) levels, which are strongly linked to infertility and miscarriage, as well as increased fasting glucose levels, occurring in 31% of women with PCOS even at normal weight [25]. Hyperandrogenism is defined as excessive clinical or biochemical androgen levels, with clinical indicators, such as acne, hirsutism, male-pattern hair loss (alopecia), and biochemical markers, including elevated plasma androgen concentrations [26].

Before the intervention, physical and gynecological examinations, ultrasound of the uterus and ovaries, and measurement of laboratory parameters according to the routine for patients with oligomenorrhea and secondary amenorrhea (thyroid-stimulating hormone, prolactin, FSH, LH, and total testosterone) were performed. After ruling out pregnancy, hyperprolactinemia and thyroid disorders, congenital adrenal hyperplasia and disorders related to the reproductive system were considered given that in other studies that utilized herbal medicines to alleviate anxiety and stress, the minimum duration of use was 6 weeks [27], and one month of using EA in the Sayah study [28]; 42 capsules (drug and placebo) were provided to them for 6 weeks. In the EA group, capsules were prescribed at a dose of 500 mg [29], once a day, 30 minutes after breakfast. Considering ethical considerations and previous studies demonstrating no complications at a 375 mg dose in humans and no impact on anxiety [16], and based on Sayah et al.'s study using 500 mg of aqueous extract of EA on humans with obsessive compulsive disorder for one month with no complications [28], a dose of 500 mg/day was adopted for this trial.

If menstruation occurs, participants should pause medication intake for one week. Once menstruation ends, participants should resume taking the medication until the full 6-week course is completed.

In the control group, the intervention was administered the same way as the experimental group, with the only difference being the use of 500 mg corn flour capsules instead of EA capsules.

Menstrual bleeding was defined as any vaginal bleeding consistent with normal menstrual flow (in terms of timing and pattern) and reported by participants. The primary outcome was the change in mean scores of stress and anxiety from baseline to six weeks after the intervention. Any menstrual bleeding occurring 3–7 days after starting treatment, during the treatment, and within four weeks after completion was documented. The secondary outcome was menstrual regularity, which was assessed across two consecutive cycles using the daily menstruation recording checklist.

Patients in both groups were monitored weekly to check their blood pressure and received phone calls after taking the medication. Both groups were followed up until the onset of the second menstrual period (up to 4 weeks) to assess menstrual regularity after the medication. During the study, research units were checked for exclusion criteria, and anyone meeting these criteria was excluded from the study. Figure 1 shows the flowchart of the study process. Table 1 summarizes the enrolment, intervention, and evaluation program. This protocol was designed according to the SPIRIT 2013 checklist.

Plant Material, Extract Preparation, and Quality Control

In this clinical trial, the EA extract was prepared by a knowledge-based company according to a predefined, standardized protocol. The plant material was obtained from dried flowers of EA, and its botanical identity was confirmed by a pharmacognosy specialist. To minimize the potential risk of toxicity associated with pyrrolizidine alkaloids, which are predominantly present in the leaves of the Iranian variety of this plant, only the petals were used for extract preparation.

Extraction was performed using an aqueous method. Briefly, a predetermined amount of dried petals was boiled in water, cooled, and then filtered. The resulting filtrate was concentrated and dried under controlled conditions. The final extract was formulated into capsules with a predefined dose for use in the intervention.

To ensure batch-to-batch consistency, quality, and reproducibility of the herbal extract, quality control and standardization were conducted by the manufacturing company. Standardization was based on the determination of total flavonoid content using a spectrophotometric method based on aluminum chloride complex formation, with results expressed as milligrams of quercetin equivalents per gram of dried extract. Each production batch was accompanied by a certificate of analysis, including physicochemical characteristics and acceptable ranges of active constituents. The final product was stored under standard conditions, protected from light and moisture, to preserve the stability of the active compounds until administration.

Table 1. Schedule of Enrollment, Interventions, and Assessment

Time point	Enrollment	Allocation	Post-allocation									
	Prior to study	W 0	W1	W2	W 3	W4	W5	W6	W5	W6	W7	W8
Enrollment												
Eligibility screen	X											
Informed consent	X											
Allocation	X											
Interventions												
Intervention group (EA)		X	X	X	X	X	X	X				
control group (corn flour)		X	X	X	X	X	X	X				
Assessments												
Evaluation of stress		X	X	X	X	X	X	X				
Evaluation of anxiety		X	X	X	X	X	X	X				
Evaluation of menstruation		X	X	X	X	X	X	X	X	X	X	X

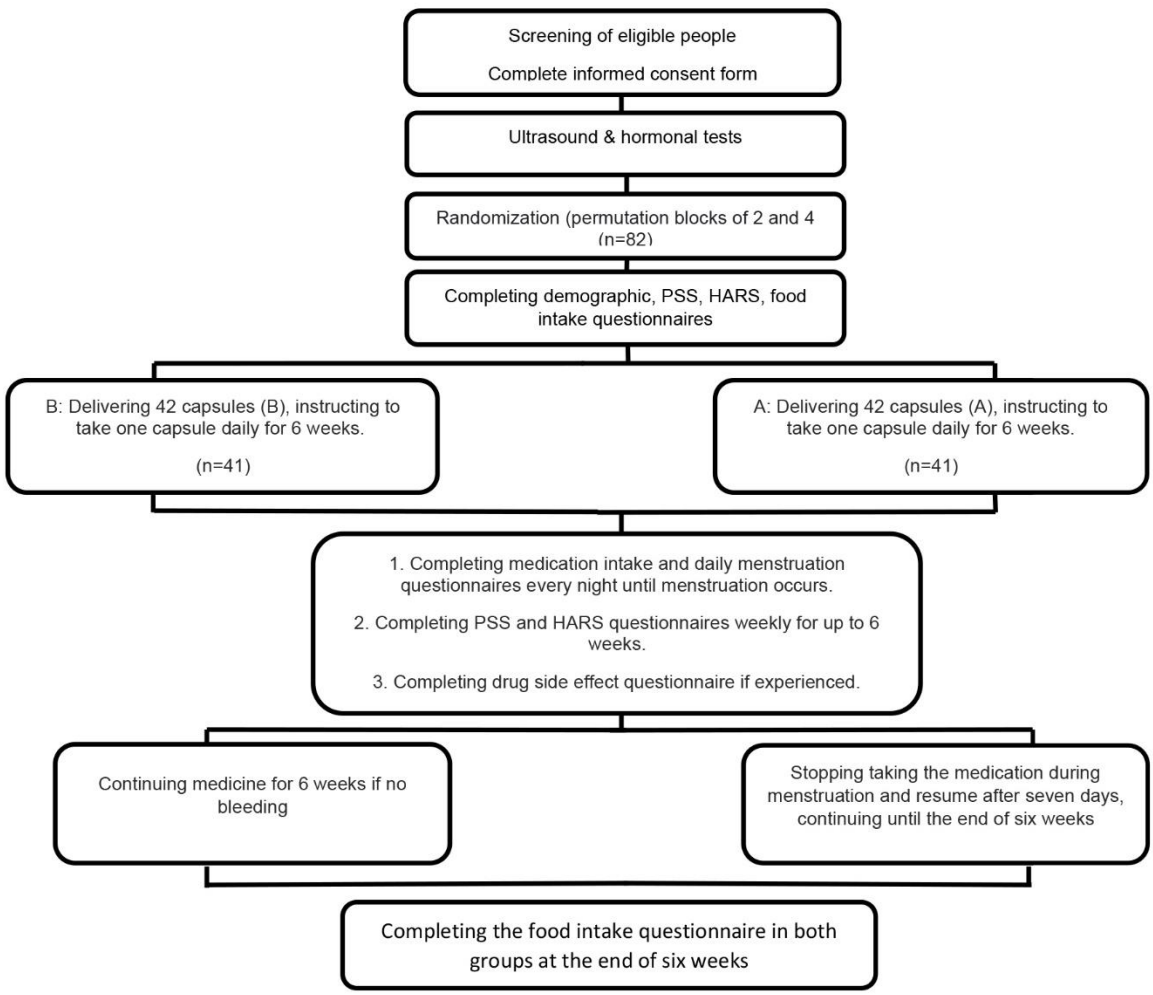

Figure 1. Representation of the Study Flowchart

Criteria for Discontinuing or Modifying Allocated Intervention

Consenting to enrollment in a trial implies agreement with trial treatments, trial follow-up, and data collection. However, a participant may discontinue treatment prematurely or be terminated from the study for any of the following reasons:

• If investigators determine that a participant's withdrawal is necessary based on clinical judgment, i.e., that participation in the study could potentially harm them, the study itself, or compromise the integrity of the data.

• A subject became ineligible to continue the intervention following hypersensitivity to the drug.

• Withdrawal of consent by the participant

Participation in this study was entirely voluntary.

Participants may withdraw from the study at any time. Those who discontinued the intervention continued to receive standard care and monitoring until their official withdrawal. While providing a reason for withdrawal was not mandatory, efforts were made to understand their decision respectfully and to ensure that participants' rights were protected.

Any adverse events were managed under the supervision of the relevant specialist, and participants were monitored until the issue was fully resolved or stabilized.

Strategies to Improve Adherence to Interventions

To facilitate communication with participants, their contact numbers were collected during recruitment. The researcher made weekly phone calls to remind each participant of their upcoming visit. Throughout the study, participants' blood pressure was monitored weekly, and they were asked about any potential complaints. Participants who had missed a visit in the past were scheduled for the earliest possible appointment.

Relevant Concomitant Care Permitted or Prohibited During the Trial

Participants had 24/7 access to the researcher via mobile phone for any issues that may arise during the study. The researcher and relevant specialist provided consultations as necessary. Participants were prohibited from using any herbal or pharmacological antidepressants or engaging in intensive physical activity during the study.

Provisions for Post-trial Care

In case of any issues, the relevant specialist implemented necessary measures, and the researcher oversaw the process until the issue was fully resolved.

Outcomes

Primary Outcomes

Primary Outcome 1: Perceived stress level

The first primary outcome was the change in mean stress scores from baseline to the end of the 6-week intervention. Stress levels were assessed using the Perceived Stress Scale-10 (PSS-10) at baseline and weekly during the 6-week intervention. This scale is utilized to determine the level of stress an individual perceives in their life situations. The Cohen Perceived Stress Scale was employed to gauge the severity of stress experienced by participants. The scale was completed by the participant before the intervention and every week afterward for up to six weeks. The researcher guided how to complete the scale. Participants responded to each item using a 5-point Likert scale ranging from 0 (never) to 4 (very often), with higher total scores indicating greater perceived stress [30].

The total score on this scale ranges from 0 to 56, and higher scores with a cut-off point of 21.8 indicate higher perceived stress. The Persian translation of the PSS-10 has demonstrated good reliability and validity in Iranian populations, exhibiting strong psychometric properties [31]. Previous research has shown high internal consistency (Cronbach's alpha = 0.82-0.87) and satisfactory test-retest reliability (intraclass correlation coefficients = 0.70-0.75).

Primary Outcome 2: Anxiety level

The second primary outcome was changes in mean anxiety scores from baseline to the end of the 6-week intervention, assessed using the HARS at baseline and weekly during the 6-week intervention. HARS can be used to evaluate the treatment process and patient's recovery rate and examine the change in the patient's score in intervals between two tests. It included 14 items, each related to a specific symptom of anxiety. In this test, each item was worth five marks, scored from 0 to 4 according to the severity of the symptoms. Zero indicated the absence of that symptom, and four indicated its presence in the patient. Each item was scored on a five-point scale. The overall score of the test showed the intensity of anxiety. The maximum score for each item was four, and the maximum overall score was 56 [32]. According to similar studies, a score of 0-17 indicated mild anxiety, a score of 18-24 indicated moderate anxiety, 25-30 indicated severe anxiety, and 30 and above indicated very severe anxiety.

Also, the reliability and validity of Hamilton's anxiety questionnaire were examined and modified by psychology professors according to the culture and characteristics of Iranian people in the study by Moghadamnia et al. The reliability coefficient of this test was reported by Leentjens et al. as 0.9 based on retesting. In Iran, in the Gharai study, the retest reliability coefficient for 30 people over 2 weeks was 0.85.

The HARS has been shown to possess strong validity and reliability within Iranian populations. Evidence for convergent validity includes significant correlations with the Beck Depression Inventory ($r = 0.73$), the Symptom Checklist-90 ($r = 0.71$), and clinician-based assessments ($r = 0.77$) [33]. The test-retest reliability was reported as $r = 0.50$ by Haghshenas [33], Alipour et al. [34] demonstrated high internal consistency, with a Cronbach's α of 0.81. Anxiety was assessed using the HARS by a single trained clinical assessor. The assessor received standardized training for administering HARS prior to the start of the study and followed clinical assessment guidelines. Since only one assessor was involved, inter-rater reliability could not be evaluated.

Secondary outcome

Secondary Outcome 1: Menstrual status

The study's secondary outcome was the regularity of the menstrual status, which was assessed using the Daily State of Menstruation Recording Checklist. This checklist, created by researchers, was used to record the dates of menstruation (start and end) and the status of menstruation. The first day of menstrual bleeding marks the start of the menstrual cycle, and the start date and duration of menstruation for the previous six cycles are recorded in the table. These sections are left blank if menstruation is delayed or absent in any month. Additionally, any bleeding 3-7 days after starting the treatment, while taking the

medication, and 4 weeks after stopping the medication was recorded using the checklist. The regularity of the menstrual bleeding pattern over two cycles of the study was determined. This checklist was designed to monitor participants' menstrual status for two months during the study. This period was calculated from the time they started taking the medication, while they were using it, and for 4 weeks after they stopped taking it (which marks the beginning of the second menstrual cycle). Regularity was defined as the occurrence of two cycles with a 21–35 day interval between the first days of bleeding.

Participant Timeline

Each woman was followed from the time of study enrollment until the completion of six weeks (end of medication), and then until the occurrence of the second menstrual cycle. In other words, the study followed each participant for two menstrual cycles from the beginning.

Response and Non-response

All eligible participants who provided informed consent were enrolled in the study. Participants who withdrew, did not complete the intervention, or failed to complete the questionnaires were considered non-responders. The rate of non-response was calculated and reported, along with reasons for withdrawal or missed assessments, to identify potential patterns and biases. Strategies to minimize non-response included regular reminders, flexible scheduling for follow-up assessments, and maintaining contact via phone or email. Only participants who completed the study according to the protocol were included in the final data analysis (Per-protocol analysis).

Sample Size

The sample size was calculated using the two-sample t-test formula for comparing independent-group means. Based on preliminary data from a similar study, the baseline mean (SD) was 44.56 (11.91) [35], To achieve a large effect size (Cohen's d = 0.8), a target mean of 34.5 was selected, representing a difference of 10.06 units between groups. With $\alpha = 0.05$ and power = 0.80, the minimum required sample size was 35 participants per group. To account for a potential 15% dropout rate, the sample size was increased to 41 participants per group.

Recruitment

The recruitment of study participants began in May 2023 and is expected to be completed by the end of 2024, with data analysis anticipated in the first half of 2025.

Assignment of Interventions: Allocation

Sequence Generation

Eligible participants were recruited using convenience sampling from the gynecology clinics. A computer-generated randomization list was prepared in advance using a permuted block method (block sizes 2 and 4) by an independent statistician via the Sealed Envelope website (http://www.sealedenvelope.com). Upon enrollment, each participant received a unique study ID and was allocated to one of the study groups in the order of enrollment according to the pre-prepared list, ensuring allocation concealment and minimizing selection bias.

Concealment Mechanism

The generated randomization list was provided to the researcher in sealed, opaque envelopes and opened sequentially when participants were qualified to enter the study.

Implementation

The allocation sequence was generated by the data manager (analyst) and sent to the blind researcher, who enrolled the participants and allocated them to interventions.

Assignment of Interventions: Blinding

Who Was Blinded

The participants, outcome assessor, and data manager remained blinded to the complete treatment period of six weeks. Treatment allocations were only revealed at the end of the study. The placebo capsule is designed to look like the study drug in terms of color and size, and both were provided in similar packaging by a single company. Because of the similarity in appearance, participants could not distinguish between the two. The chief investigator (corresponding author) labeled the drug and placebo capsule packs as A and B and then provided them to the outcome assessor. The analyst also received coded information about the groups in the data file.

Procedure for Unblinding if Needed

Full unblinding of the study will occur once all data have been entered into SPSS and the analysis has been completed by the analyst. Only the corresponding author and the pharmaceutical company responsible for producing the capsules, which have no role in the study, will be unblinded. Unplanned or urgent unblinding will only be conducted to safeguard participant safety. This will occur in the event of a reported serious adverse event, which will be discussed and approved by the study's regulatory bodies.

Data Collection and Management

Plans for Assessment and Collection of Outcomes

Data were collected using questionnaires and entered into SPSS (version 21) by the researcher as each participant was recruited and during follow-up visits, so that no data were missed. Several steps were undertaken to ensure the accuracy and reliability of the data collected during this study, including a review of the protocol and study procedures by the researcher and the responsible study staff before undertaking any study-related activities. The accuracy and completeness of the study documents were reviewed by designated quality assurance and control personnel and the study monitor, as determined by the regional ethics committee. Any discrepancies were

resolved by the responsible study staff or, as appropriate by others.

Plans to Promote Participant Retention and Complete Follow-up

During the informed consent process, the participants were fully informed about the study visits, procedures, retention strategies, and efforts made to contact them. Their addresses were also included in the informed consent form. They were informed about the free medical consultation and care available during the study period. Upon enrollment confirmation, participants were provided with a 24-hour researcher phone contact for any help or clarifications. During each visit, the researcher checked the participant's blood pressure, reviewed study procedures, and inquired about any issues or complication. A day before each visit, the researcher made telephone calls to remind the participants and ensure they had taken their medication. Participants with missed visits were scheduled for the earliest possible date. Those unable to attend weekly visits were advised to visit the nearest clinic or health center to check their blood pressure and bring the results to the next visit.

Statistical Methods

After data collection and coding, the data were analyzed using the SPSS software (version 21). Following data entry, screening, and accuracy validation, the normality of quantitative variables was assessed using the Kolmogorov-Smirnov test. Regarding missing data, we plan to carefully examine the pattern and mechanism of missingness and apply an appropriate method for handling missing data.

Descriptive statistics, such as mean and standard deviation for normal quantitative variables and median with first and third quartiles for non-normal quantitative variables, were utilized. Qualitative variables were described using numbers and percentages.

Statistical analyses were performed using appropriate parametric and non-parametric tests. The independent *t*-test was applied to compare normally distributed quantitative variables, whereas the Mann-Whitney *U* test was used for non-normally distributed quantitative data. For categorical variables, the chi-square test was employed; however, when Cochran's assumptions were not met, Fisher's exact test was utilized to ensure analytical accuracy. Additionally, the generalized estimating equations method was used to compare the average scores for perceived stress and anxiety across two groups. This allows for the adjustment of potential confounders. The significance level was set at 5%. In the present study, the main analysis was conducted using a per-protocol approach, including only participants who completed the study as planned. An intention-to-treat analysis could also be performed as a sensitivity analysis if necessary.

Data Quality

Before the commencement of the sampling process, the chief researcher and data manager (analyst)conducted a comprehensive review of all questionnaires and forms. After this review, participants were required to complete the questionnaires and forms. Subsequently, the trained outcome assessor meticulously entered and stored the data of each participant daily. This meticulous process was diligently performed until the sampling phase concluded. Upon completion of the sampling, the data were transmitted to the data manager for standardization and normalization. The data manager conducted a meticulous review to ensure the accuracy of the data entry. Subsequently, the analyst meticulously scrutinized the data for potential errors and, upon their correction, proceeded with the data analysis.

Dissemination Policy

We are committed to transparent and timely dissemination through academic publications, reputable conferences, national and international clinical councils, social media, and reports to participants upon request. Copies of the report are available at the library of Gonabad University of Medical Sciences, Gonabad, Iran. Our primary objectives are to foster knowledge sharing among researchers and empower practitioners with evidence-based insights. We prioritize open access to our research outputs, including data sharing and publication in open-access and trustworthy journals. We adhere to ethical principles of accuracy, transparency, and respect for participant confidentiality in all dissemination activities. Access to data is restricted to the main researchers of the study, with access possibly granted to other researchers upon request, with all individual information anonymized.

Source Documents and Access to Source Data

Representatives authorized by the sponsor and the Ethics Committee are entitled to inspect all documents and records that are required to be maintained by the investigator.

Confidentiality

The research team ensured that the guarantees of confidentiality and anonymity for the research participants were strictly upheld. Adhering to the principle of confidentiality, the researchers did not disclose identifiable data to any third parties without the participants' explicit consent. Data were securely transferred between collaborators using only the unique study ID, which was not linked to patient-identifiable information.

Safety Endpoints

No serious adverse effects have been reported in previous human studies. However, in the event of potential complications, participants were directed to the relevant specialist. The investigator assessed the tolerability and safety of the extract administered during the study in accordance with clinical experience and standard procedures. The incidence of subjects presenting adverse events (AE), AEs leading to withdrawal, adverse drug reactions, and serious adverse events was documented for

each treatment group. Participants were required to promptly report any side effects within 24 hours. In the event of serious side effects, the participant was referred to a specialist, and appropriate measures were taken to ensure the patient's safety. If deemed necessary by the specialist, the participant was instructed to discontinue the medication and excluded from the study.

Oversight and Monitoring

Data Monitoring

This research project was conducted under the close supervision of knowledgeable faculty members and a supervisor chosen by the Ethics Committee of Gonabad University of Medical Sciences, Gonabad, Iran. Participant safety oversight was conducted by the research team and the principal investigator. All adverse events, including mild reactions, were recorded and followed up on. In the event of any serious adverse event, participants were referred to the relevant specialist for appropriate management.

Safety Monitoring

Participants visited the gynecology clinic at Bohlol Hospital or the doctor's office every week for blood pressure checks and to report any issues or medication side effects. They also provided the researcher with contact information to address any concerns.

Adverse Event Reporting and Harms

Safety Consideration

Patients are required to visit the gynecology clinic at Bohlol Hospital or the doctor's office every week for blood pressure checks and to report any issues or medication side effects. They also received the researcher's contact information to address any concerns. Patients experiencing allergies or serious complications were excluded from the study.

Patients were also provided with a form detailing the usage and potential side effects of the drug. This form allowed them to report any side effects experienced while taking the drug. The form included several questions, such as the following: - Have you experienced any complications from the drug so far? (yes/no) - If yes, how severe are the complications? (mild/moderate/severe) - Have you needed to see a doctor or go to the hospital due to a specific problem while using the drug? (yes/no). If yes, please specify the type of problem. - How satisfied are you with the drug? (very low/low/moderate/high/very high) - Would you recommend this drug to others? (yes/no)

Methods and Timing for Assessing and Recording Safety Parameters

All participants were asked to promptly report any adverse reactions, particularly symptoms indicative of allergic responses, such as rashes, pruritus, or other allergic manifestations. Subsequent to drug administration, each participant was instructed to complete a comprehensive drug usage and side effects form to report any adverse reactions. The health visitor/researcher was responsible for either conducting in-person visits or making calls to ensure the well-being of the participants, address any concerns they may have, and ascertain if they encountered any challenges in completing the requisite form.

Adverse Events (AEs)

An AE is any unfavorable medical occurrence in a subject who has been administered a pharmaceutical product, regardless of the dose. It does not necessarily have to be caused by the treatment and may include any unintended sign, symptom, or disease that occurs after use of an investigational product, whether or not it is considered related to the product. This also covers new illnesses or injuries, as well as the worsening of existing conditions. An unexpected adverse event is not listed in the investigator's brochure or the current summary of product characteristics, or an event that is more specific or severe than a listed event.

Methods and Timing for Assessing Adverse Events

The monitoring period for adverse events began when the study participant received the aqueous extract capsules and continued until the conclusion of the study. Any medical occurrence after the signing of the informed consent form, and after ingestion of the extract, and linked to a study procedure, was documented as an adverse event and recorded in the adverse events paper document. Conversely, any medical incident occurring before the signing of the informed consent form and before ingestion of the extract, and unrelated to a study procedure, was noted as a pre-existing condition and recorded in the medical history. All adverse events, irrespective of severity, were diligently monitored by the investigator until resolution.

Causality Assessment

The determination of whether an adverse event or severe adverse event can be attributed to the administration of the study drugs, or other causes, such as the natural progression of the underlying disease or concomitant therapy, was based on the frequency of the event that has been reported in similar types of interventions in the literature.

Plans for Communicating Crucial Protocol Amendments to Relevant Parties (e.g., Trial Participants, Ethical Committees)

Protocol amendments, which involve changes to the established procedures and guidelines, are promptly and effectively communicated to all relevant parties in real-time. It is crucial to note that any modifications to the protocol cannot be implemented until they have been reviewed and approved by all applicable regulatory bodies. In the event of protocol amendments, it may be necessary to obtain re-consent from participants to ensure that they are fully informed and have the opportunity to provide consent based on the updated protocol.

Discussion

FHA is a common type of secondary amenorrhea that

causes estrogen deficiency in young premenopausal women, although it is reversible. The causes of this disorder have been related to mental stress, intense exercise, eating disorders, or a combination of these factors, which suppress the hypothalamus-pituitary-ovary axis [5]. A study of adolescent girls with FHA identified stressors included common life events such as changing schools, new involvement in sexual activity, separation from a sexual partner, chronic illness of a family member, and death of a friend. Finally, 50% of the adolescents participating in the study described family conflicts. Also, patients with FHA compared to those with normal menstruation have been shown to be less able to cope with stress [such as the response of the autonomic system)[18]. The loss of estrogen has profound effects on many body systems, such as cardiac, skeletal, psychological, and reproductive systems [5]. FHA caused by emotional stress is diagnosed by ruling out other causes, and despite low estrogen levels, serum FSH concentration may be normal or low [36]. In addition to the fact that patients with FHA have increased cortisol levels, the γ-aminobutyric acid neurotransmitter is also related to GnRH suppression[18]. In the present study, the EA aqueous extract plays a role by improving the function of the GABA A receptor, reducing stress, and thus improving the function of the nervous system. Therefore, we hope that the data of this study can be useful in the treatment of stress-induced FHA. Another common form of secondary amenorrhea is ovarian disorders, often associated with polycystic ovarian syndrome. Polycystic ovarian syndrome is the most common endocrine disorder in women [1], and it is associated with symptoms of hyperandrogenism, such as moderate to severe acne and excessive hair growth. Its timely diagnosis and treatment manage irregular bleeding, prevent long or heavy menstrual bleeding, and reduce metabolic diseases caused by it. Patients with PCOS show reproductive abnormalities, symptomatic insulin resistance, increased risk of type 2 diabetes mellitus, coronary heart disease, atherogenic dyslipidemia, cerebrovascular complications, and anxiety and depression [37].

In addition to the many properties of the plant that have been investigated in various studies, EA also increases sex hormones, especially progesterone [10], which may be helpful in patients with PCOS. On the other hand, anxiety and depression are more common in women with PCOS than in the general population. These mood disorders can have significant effects on quality of life. Adolescents and women with PCOS must be screened for anxiety and depression. Also, these people should be encouraged to identify and pursue resources to optimize their mental health [19]. Therefore, these people may benefit from this medicinal supplement in addition to routine treatment.

Trial Status

Recruitment of study participants commenced in May 2023 and is ongoing.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of Gonabad University of Medical Sciences, Gonabad, Iran, under approval number IR.GMU.REC.1402.010. The researchers provided comprehensive explanations to the participants, and the participants provided written informed consent. This study was registered at the Iranian Registry of Clinical Trials under the number IRCT20230417057944 N1.

Written informed consent was obtained from all participants in the study.

Data Availability

The processed data and code used for statistical analysis are available from the corresponding author upon reasonable request.

Trial Registration

This trial was registered at irct.behdasht.gov.ir [ID: IRCT20230417057944N1) on May 15, 2023.

Consent for Publication

Not applicable.

Conflict of Interest

The authors declared no conflicts of interest.

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Authors' Contributions

SN, FA, FM, and EN were involved in creating and designing the protocol. SN and FA developed and revised the study protocol regarding its content. FM reviewed statistical methods and calculations. SN, FA, FM, and EN approved the final version of the article.

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