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Research Article

PREPARATION AND EVALUATION OF *GLYCINE MAX*, *TRIGONELLA FOENUM*, *GLYCYRRIZA GLABRA* AND *ALOE BARBADENSIS* EXTRACTS VAGINAL CREAM

MOHAMMAD EBRAHIM AZEMI[1], ZAHRA MORSHEDI[2], ESKANDAR MOGHIMIPOUR[2]*

[1]Medicinal Plant Research Center, Department of Pharmacognosy, Faculty of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
[2]Nanotechnology Research Center, Department of Pharmaceutics, Faculty of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Corresponding Author: moghimipour@yahoo.com

Abstract

Vaginal atrophy is one of the bothering problems of postmenopausal women. Phytoestrogens are one of the alternative treatments for vaginal atrophy. The aim of this study was to evaluate the physicochemical properties and stability of vaginal cream containing *Glycine max*, *Trigonella Foenum*, *Glycyrriza glabra* and *Aloe barbadensis* extracts. To choose the best formulation a general formula of oil in water emulsion was considered and then corrected. The pH of formulations was in the normal range of vaginal pH and it can be an effective parameter in normalizing increased vaginal pH in vaginal atrophy. Microbial challenge test showed formulation preservative had the effective ability to preserve the formula. Release study carried out according to the permeated amount total flavonoids. Determination of total flavonoids was carried out using Aluminum chloride colorimetric method. The best formulation was chosen with respect to desirable appearance, consistency, viscosity, and stability parameters. Final formulation containing 20% stearic acid, 6.5% spermaceti, 0.05 propyl paraben, 10% glycerin, 3% tween 80, 0.15 methyl paraben, 13% total extracts and water. This study suggests this formulation for the clinical trial on vaginal atrophy.

Keywords: vaginal atrophy, *Glycine max*, *Trigonella Foenum*, *Glycyrriza glabra*, *Aloe barbadensis,* vaginal cream.

Introduction

Vaginal atrophy is one of the consequences of menopause that is associated with decreasing in vaginal epithelium thickness, in vaginal pH, reducing of vaginal blood flow, decreasing of vaginal increasing texture elasticity, and vagina becomes pale, dry and inflamed. These happen because of decreased in estrogen level related to menopause and aging (North American Menopause Society, 2007).Also in premenopausal women vaginal atrophy may be occur, this problem can be caused by surgical removal of the ovaries, chemotherapy, radiation and side effects of anti-estrogen medications, such as tamoxifen, danazol, leuprolide and nafarelin (Kaunitz *et al*., 2001). Symptoms of atrophy are vaginal dryness, dyspareunia, itching, irritation, burning, discharge, urinary problem and vaginitis (Nachtigall *et al*., 2005) (North American Menopause Society, 2013). The North American Menopause Society (NAMS) estimated 10%-40% of post-menopausal women suffering from vaginal atrophy but only 25% of them seek medical attention for the condition (North American Menopause Society, 2007). Treatment mainly focuses on alleviation of symptoms and reduction vaginal alterations. Options for management include hormonal and non-hormonal treatments. Among the first-line therapies are non-hormonal remedies, lubricants and moisturizers. The most common hormonal treatment to relieving symptoms is estrogen therapy. Systemic and local estrogen therapy is the common route of delivery (North American Menopause Society, 2013). However estrogen therapy is a rational approach to alleviate bothering symptoms of vaginal atrophy, it's may be unacceptable for some reasons. For example estrogen therapy is contraindicated in some postmenopausal women because of a history of cancer or risk of thromboembolism (Alessandro *et al*., 2003). Also some

reports have indicated estrogen therapy increases the risk of breast and endometrial cancer (Beral, 2003. Beral, 2005).Due to this concerns more than 80% of women unable and unlike to treat with estrogen (Bedell *et al*., 2013). Thus There is a clear medical need to presence of non-steroidal estrogen mimetic alternative to the treatment of vaginal atrophy and improve the quality of life of most postmenopausal women. Phytoestrogens are natural estrogen-like compounds in plants that are safe and don't increase thromboembolic risk and endometrial hyperplasia (Bedell *et al*., 2013). The present study is designed to formulate and evaluate physicochemical properties of vaginal cream containing *Glycine max* (4%)*, Trigonella Foenum* (2%)*, Glycyrriza glabra*(2%) and*Aloe barbadensis*(5%) extracts. It has been previously reported that *Glycine max* (Fabaceae)*, Trigonella Foenum* (Fabaceae)*, Glycyrriza glabra* (Fabaceae), contain isoflavonoid one of the three classes of phytoestrogen (Murkies *et al*., 1998) (Mackey and Eden*, et al*., 1998) (Benedec *et al*., 2012) and *Aloe barbadensis* (Liliaceae) has moisturizing effect (Dal'belo *et al*., 2006).

Materials and Methods

Extraction

Ethanol extract of *Glycine max, Trigonella foenum* and *Glycyrriza glabra* was prepared by cold maceration process. The powdered plant (500 g) was macerated in 1500 ml ethanol (80%, v/v) at the room temperature (25 °C) for 3 days. After this time, the resultant extract was filtered, freeze dried with Operon freeze drier (FDCF-12012, Korea) and stored in refrigerator. Aloe vera extract was purchased from Pardis golvare novin (Tehran, Iran).

Preparation of creams

Stearic acid, spermaceti, glycerin and water were selected for base formulation. The oil phase including stearic acid and spermaceti were mixed and heated to be melted in 70 °C. Glycerin and water were mixed and heated to the same temperature, as the aqueous phase. As preservative, propyl paraben and methyl paraben were added to oil and aqueous phases, respectively. The aqueous phase was added to oily phase and mixed with continuous stirring until cooling. During mixing the required amounts of the herbal extracts (13%) were added with constantly stirring. Different formulations (F_1- F_{10}) were prepared by varying amounts of ingredients (Table 1). Amount of propyl paraben (0.05), methyl paraben (0.15) and extracts (13%) was constant in all of formulations.

Table 1. Amounts of ingredients (g) used in each formulations for a total of 100mg cream

Formulation	Ingredients				
	Stearic acid	Spermaceti	Glycerin	Tween 80	Water
1	15	5	10	1.4	68.5
2	15	5	15	1.4	63.5
3	20	5	10	1.4	63.5
4	30	5	10	1.4	53.5
5	25	5	10	1.4	58.5
6	15	10	10	1.4	63.5
7	20	6.5	10	1.4	62
8	18	8	10	3	61
9	20	6.5	10	3	60.5
10	20	8	10	3	59

Homogeneity test

For evaluating the homogeneity of formulations, microscopic samples were prepared by spreading a 0.5g of each formulation on a slide. Then the slides were observed in an Olympus optical microscope (BX10, Japan) (×10 and ×40) (Aulthon, 2002).

Creaming and Coalescence

The physical stability was determined by sampling of each formulation and storing them at room temperature for 3 months. Their physical stability was evaluated after 1 week, 1 and 3 months storage (Paul, 1993).

Centrifugation test

The centrifugation tests were performed at 25°C and at 2000 rpm for 5, 15, 30 and 60 min by placing a 10g of each formulation in a centrifuge tube having 1 cm diameter (Beckman, U.S.A). Then the samples were evaluated regarding any phase separation and/or solid sedimentation (Poucher, 1993).

Thermal cycle and thermal variation

The samples were stored at 5 °C for 48 hour (h) and then at 25 °C for 48 h. The process was repeated for 6 times and then the physical stability and appearance of samples were evaluated. For evaluation of thermal changes, three sets of 20g samples of each formulation were stored at different temperature conditions (4 °C, 25 °C and 50 °C). After 24 h, 1 and 3 months, and then the samples were checked regarding their appearance and physical stability (Poucher, 1993).

Freeze-thaw test

Physical stability was tested with storing a 20g sample of each formulation periodically at 45-50 °C and 4 °C for 48 h each. The procedure was repeated 6 times(Poucher, 1993).

Viscosity determination

The rheological behavior using a Brookfield viscometer (model DV-I with No. 6 spindle) of the samples were evaluated. Each sample was placed in a container and spindle velocity was raised gradually to maximum extent. Then the viscosity was determined at 0.3, 0.6, 3, 6 and 60 rpm (Martin, 1983).

Determination of pH

A suspension of each sample was prepared with dispersing 5g of the formulation in 95mlof water and measurements performed at 48 h, 1week, 1 and 3 months after preparation (Poucher, 1993).

Microbial challenge test

To evaluate the effectiveness of the preservative in the formulation, the single microbial challenge test was made in accordance with requirements of USP30 for non-sterile products. 0.1 ml of 10^8 cfu/ml of the test strains, *Staphylococcus aureus* (PTCC No. 1189), *Pseudomonas aeruginosa* (PTCC No. 1599) and *candidaalbicans* (PTCC No.5027) were added to 20ml pre-diluted sample of the formulation in sterile containers. Containers were incubated at 22.5±2.5 °C for bacteria and at 37°C for candida for 28 days. At appropriate time intervals according to the USP procedure (1, 7, 14, 21, and 28 days), 1 ml of each incubated sample was added to a plate according to the pour plate count procedure. After incubation for 48h, the viable colony-forming units (cfu) were counted. The number of cfu in each plate and changes in microbial numbers were recorded at above mentioned intervals (USP, 2002).

Release study

The release study was carried out in Franz diffusion cells and cellulosic micro membrane was used as diffusion membrane. The membrane was soaked in distilled water for 24 h and then was fitted between the chambers. The receptor chamber was filled with double distilled water and the temperature was maintained at 37°C. 0.5 gram of each formulation was placed on the membrane. At appropriate time intervals, a 2 ml samples were withdrawn from receptor phase and replaced with distilled water to maintain sink condition. The amount of total flavonoids was determined using aluminum chloride colorimetric method. Briefly, 0.5 ml of each sample was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After 30 min incubation in room temperature, absorbance of the each mixture was measured at 415nm with a Biochrom spectrophotometer (Biowave II). The amount was calculated with quercetin calibration curve at concentrations 3.125 to 100 µg/ml (Chang C*et al*., 2002).

Results

The resultant of extraction of *Glycine max, Trigonella Foenum* and*Glycyrriza glabra*was 4.45%, 7.42% and 7.29%. Ten formulations (F_1–F_{10}) with varying extent of oil phase and emulsifier were prepared according to Table 1. The base of formulation was white and glossy while the main formulations were yellow. Appearance and stability evaluation showed that among all of formulations, F_8, F_9 and F_{10} had the best physical characteristics; so the other control experiments were carried out on the selected formulations. During 3 months stability studies, creaming or coalescence didn't occur and no significant change in the appearance and physical properties due to centrifugation, thermal cycle, thermal change and freeze-thaw tests was observed in F_8, F_9 and F_{10} formulations. pH of the formulations were between 4.5 and 5, close to the pH of vagina. According to the results of microbial challenge, no growth was observed after one week and up to day 28. The number of *candida albicans* fungi was progressively

reduced within the first week and no increase in population was observed after four weeks of storage. Therefore, it was shown that antimicrobial preservation of the formulations satisfied the USP criteria. The results of release study are shown in Fig 1. According to the release profile, the amount of released drug was constant in the mentioned formulations. The released flavonoids after 8h for F_8, F_9 and F_{10} were 53.74%, 53.66% and 51.8%, respectively.

Table 2. Fluidity and stability of formulations

Formulation	Fluidity	Stability
1	Fluid	Unstable
2	Fluid	Unstable
3	Fluid	Unstable
4	Thick	Unstable
5	Thick	Unstable
6	Intermediate	Unstable
7	Intermediate	Unstable
8	Ideal	Stable
9	Ideal	Stable
10	Ideal	Stable

Table 3. Physical properties of F_8, F_9 and F_{10} formulations

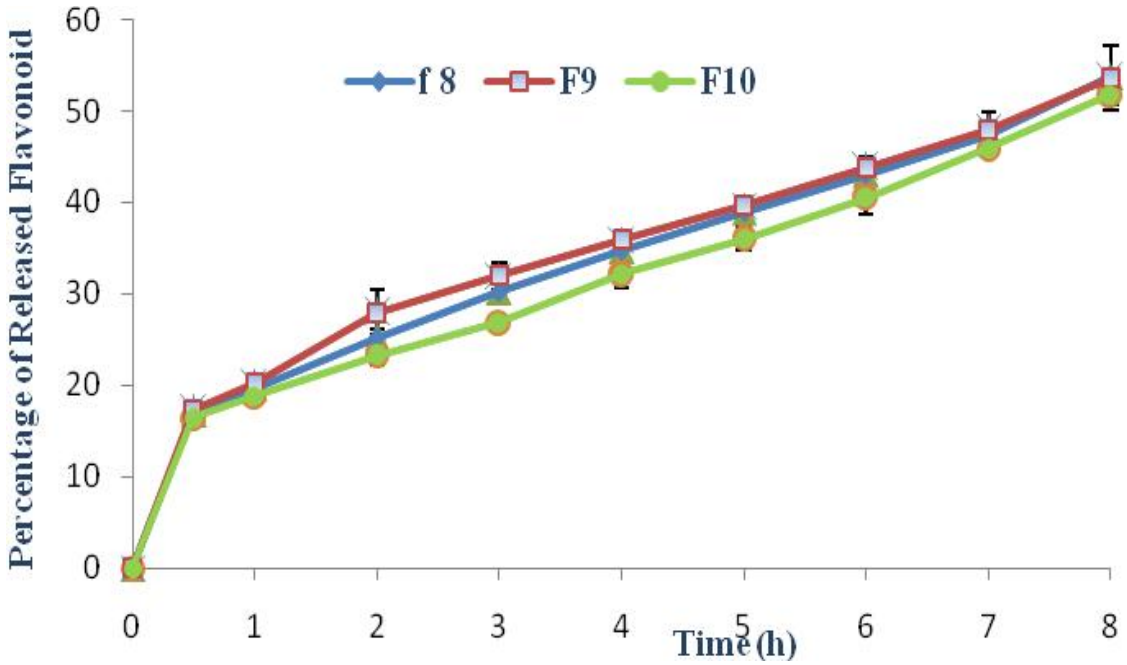
Parameter	F_8	F_9	F_{10}
Homogeneity	++	+++	++
Creaming and coalescence	not seen	not seen	not seen
Centrifugation test	stable	stable	stable
Thermal cycle and thermal variation	stable	stable	stable
pH	4.57±0.5	4.52 ± 0.5	4.63±0.5

Homogeneity: +++ = Excellent ++ = Very good + = Good

Table 4. Results of viscosity (cps) monitoring of F_8, F_9 and F_{10}

Formulation No.	Shear rate (rpm)				
	0.3	0.6	3	6	60
8	385.00 ± 0.02	141.28 ± 0.00	7.83 ± 0.01	2.56 ± 0.02	0.03 ± 0.00
9	381.10 ± 0.01	143.48 ± 0.03	8.26 ± 0.00	2.24 ± 0.00	0.04 ± 0.01
10	389.30 ± 0.05	150.14 ± 0.02	8.87 ± 0.03	2.89 ± 0.01	0.04 ± 0.01

Figure 1. Release behavior of selected formulations (F_8, F_9 and F_{10}).



Discussion

Vaginal atrophy symptoms affect quality of life. There are effective treatment options such as estrogen therapy but because of the risk of thromboembolism, breast and endometrial cancers, and some contraindications women don't willing to use it (Beral, 2003. Beral, 2005. Bedell *et al*., 2013). One of the alternative options is phytoestrogen. Among plants containing phytoestrogen, soy Beans (*Glycine max*), Fenugreek (*Trigonella foenum-graecum*) and Liqurice (*Glycyrrhiza glabra*) were chosen. Studies have shown that isoflavones of soy (non-steroid compounds that can weakly bind to estrogenic receptors) have estrogenic action (Petri Nahas *et al*., 2004). A (4%) soy vaginal gel of has been evaluated for treatment of vaginal atrophy and significant improvement was observed (Lima *et al*., 2013). Other investigations proved estrogenic activities of fenugreek seeds (Sreeja*et al*., 2010). The presence of anti-inflammatory isoflavones in Liqurice has been investigated by HPLC-MS analysis and it is concluded that the plant can be assumed as a rich source of phytoestrogens (Benedec *et al*., 2012). Also many studies showed that Liqurice have antioxidant and antibacterial effects (Makky *et al*., 2012). *Aloe vera* has good wound healing effect on skin and mucosal lining. Stimulatory effect of aloe components on cell proliferation has been reported but the mechanism of the influencing on cell proliferation is currently unknown (Boudreau and Beland *et al*., 2006). Also antimicrobial effect of aloe gel on genital pathogens has been evaluated and its antibacterial, antifungal and antiviral effect on vaginal infections is proved (Talwar *et al*., 2008).

At the beginning of the study, different formulations (F_1–F_{10}) were prepared. F_1, F_2 and F_3 formulations were very dilute and had low consistency. To overcome this problem, by increasing the amount of stearic acid, the amount of oily phase was increased (formulations F_4 and F_5). Although the addition of stearic acid increased consistency of the samples, but they didn't have proper flow properties and were broken after 48 h. In formulations F_6 and F_7, the amount of spermaceti was increased. These formulations had better appearance and flow properties but they were still unstable and phase separation phenomenon occurred after 48h. To enhance the stability in formulation F_8, F_9 and F_{10}, the amount of Tween 80 was increased that caused a proper appearance, consistency and flow properties.

During Creaming and coalescence, centrifugation, thermal cycle and thermal variation and also freeze-thaw tests, only F_8, F_9 and F_{10} had relatively stable emulsion characteristics and other formulations were not stable so these formulations were excluded. The formulations pH was in normal vaginal pH range that makes the preparation useful in regulating of increased vaginal pH in vaginal atrophy. Also, there

was no significant change in pH during 3 month of storage period, as well any chemical changes and reactions occurred during this time.

Since creams and other water containing dosage forms should be preserved from microbial contamination, a usual preservative mixture, methyl and propyl paraben (3:1 ratio) was added. As mentioned before, antimicrobial preservation of the formulations met the requirements of USP.

Finally, control experiments and stability control for F_8, F_9 and F_{10}formulations showed a homogenous appearance during 3 month storage and no creaming or coalescence occurred. However, F_9 was chosen as the best formulation due to its more acceptable homogeneity and clarity. The final compositions and amounts of ingredients in F_9 were: stearic acid 20%, spermaceti 6.5% and propyl paraben 0.05 as oily phase, and glycerin 10%, Tween 80 3%, methyl paraben 0.15% and total extracts 13% as aqueous phase.

Further clinical studies are needed to validate the efficacy of this herbal vaginal cream in alleviation of bothering vaginal atrophy symptoms. It may be suggested that F_9 formulation is a proper alternative for the currently available treatments for vaginal atrophy.

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