Original Article

Formulation of Herbal Gel of *Antirrhinum majus* Extract and Evaluation of its Anti-*Propionibacterium acne* Effects

Mohammad Ali Shahtalebi, Gholam Reza Asghari[1], Farideh Rahmani[2], Fatemeh Shafiee[3], Ali Jahanian-Najafabadi[3,4]

From the Departments of Pharmaceutics, [1]Pharmacognosy, [2]Students' Research Committee, and [3]Pharmaceutical Biotechnology and [4]Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: *Antirrhinum majus* contains aurone with excellent antibacterial and antifungal activities. In addition, visible light activates the endogenous porphyrins of *Propionibacterium acne*, which results in bacterial death. Therefore, considering the above-mentioned facts, the aim of the present study was to prepare a topical herbal gel of *A. majus* hydroalcoholic extract and to evaluate its antiacne effects with or without blue light combination as an activator of the porphyrins. **Materials and Methods:** Antibacterial activity of the shoot or petal extracts was evaluated by disc diffusion method and the minimum inhibitory concentration (MIC) was calculated. Various gel formulations were developed by the Experimental Design software. The obtained gel formulations were prepared and tested for pharmaceutical parameters including organoleptic features, pH, viscosity, drug content, and release studies. Finally, the antibacterial activity was evaluated against (*P. acnes*) with or without blue light. **Results:** The MIC of the extracts showed to be 0.25 μg/ml. Evaluation of the gel formulation showed acceptable properties of the best formulation in comparison to a gel in the market. Pharmaceutical parameters were also in accordance with the standard parameters of the marketed gel. Furthermore, statistical analyses showed significant antibacterial effect for gel when compared to negative control. However, combination of blue light with gel did not show any significant difference on the observed antibacterial effect. **Conclusion:** Because of the statistically significant *in vitro* antiacne effects of the formulated gel, further clinical studies for evaluation of the healing effects of the prepared gel formulation on acne lesions must be performed.

Keywords: *Acne, Antirrhinum majus, blue light, Propionibacterium acnes*

Introduction

Acne vulgaris is a skin disorder which affects virtually all people at least once during their life span, and its severity is directly related to production of sebum, which itself is stimulated by enlarged sebaceous glands subsequent to androgenic stimulants.[1,2]

Multiple physiological factors including follicular hyperproliferation and increased sebum production followed by follicles blockage and colonization of various microorganisms such as *Propionibacterium acnes* could be found in pathogenesis of acne.[1-3]

P. acne, a Gram-positive and anaerobic bacterium, plays an important role in acne pathogenesis. This organism is important in the development of inflammatory acne by its ability for metabolizing triglycerides of sebaceous into fatty acids, which chemotactically attracts neutrophils.[4,5]

Hormones and chemotherapeutic agents have extensively been used for acne treatment for many years. However, severe side effects and drug resistance are most concern with these drugs. Therefore, herbal remedies and photodynamic therapies with high antibacterial activity and without side effects have been widely studied as an alternative photo inactivation of various Gram-positive bacteria, including *P. acnes*.[5-7]

Antirrhinum majus (Snapdragon) is a member of *Scrophulariaceae* family. There are five flavones in *A. majus*, namely, apipnin, lutein, chrysoeriol, kampferol3-gliscoside, and kampferol 3,7diglucoside. A new aurone, bracteatin 6 glycoside, has also been found in this plant. *A. majus* seeds are a good source of oil (12.3%) too. The amounts of neutral lipids in the oil were the highest, followed by glycolipids and phospholipids. Linoleic and oleic acids accounted for 88% of the total fatty acids.[8-10]

Address for correspondence:
Dr. Ali Jahanian-Najafabadi, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Hezar Jarib Ave., Isfahan, Iran. E-mail: jahanian@pharm.mui.ac.ir

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Interestingly, most of the aurone analogs have shown excellent antibacterial and antifungal activities when compared with standard antibiotics and their minimum inhibitory concentrations (MICs) against Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, and *Clostridium tetani* and Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* and even against fungi such as *Candia albicans*, and *Aspergillus clavatus* has been determined.[11,12]

Recently, it has been revealed that visible light activates the endogenous porphyrins of *P. acnes*, which results in bacterial death due to photodynamic reactions. The absorption peak of bacterial porphyrins is at 415 nm, which falls into the blue light waveband.[13]

The wavelength of blue light that is effective in treatment of acne is between 405 and 470 nm. This is near the aurones' absorption wavelength (390–430 nm).[8,14] Therefore, it seems that simultaneous application of blue light exposure and the aurones could result in synergistic effects in their antibacterial effects. Hence, we prepared a topical gel formulation containing the *A. majus* extracts and evaluated its anti-*P. acne* antibacterial effects with or without simultaneous exposure to blue light, to search whether this combination shows a better antiacne effect or not.

Materials and Methods

Plant materials collection and authentication

A. majus was collected from the Flowers Garden of Isfahan in June 2014. The specimens were identified and the plant herbarium was kept in the Department of Pharmacognosy of Isfahan School of Pharmacy with the voucher No. 3319.

Preparation of plant extracts

The plant samples were dried in darkness and under ventilated hot air at 40°C to avoid destruction of main constituents. After drying, the obtained materials were subjected to determine the water content by loss on drying for being <2%. The dry powder crushed in blender (Hardstone)™, passed through meshes No. 14, and kept in air tight containers before use. The selected specimen was extracted by refluxing with 90% ethanol and concentrated under reduced pressure. It has been kept in freezer for 24 h before further study. To determine the extract density, about 1 ml of hydroalcoholic extract put on oven at 100°C until completely dried. This powder was weighed and considered as the petal or shoot density of extract.

The qualitative and quantitative analysis of active components was carried out by preparative paper chromatography with 50% acetic acid as developing solvent.[15-17]

Standardization of extract

Standardization of the extracts has been performed on the basis of their phenolic contents using the Folin–Ciocalteu method.[18]

Determination of the antibacterial activity of extract

To evaluate the antibacterial activity of the extracts, *P. acnes* (PTCC 6916) was obtained from Pasteur Institute of Iran (Tehran, Iran) and cultivated on brain–heart infusion (BHI) medium (Merck, Germany) supplemented with 1% glucose (BHI-Glu) and in an anaerobic jar.

All experiments were conducted on BHI-Glu-agar plates containing 1×10^8 colony forming unit (CFU)/ml of the bacteria as the seed layer, and cultivation was performed under anaerobic condition prepared using Anaerocult A® (Merck, Germany) gas packs in an anaerobic jar controlled with Anaerotest® indicator, for 72 h and at 37°C. Vancomycin 30 µg was used as the positive control.[19-21]

Determination of minimum inhibitory concentration of *Antirrhinum majus* extract

The MIC of the plant extract was determined by disk diffusion method. In this regard, sterile filter paper discs (with 6 mm diameter) were impregnated with 20 µl of different concentrations of *A. majus* extract, and the paper discs were aseptically placed on BHI-Glu agar plates seeded with the *P. acnes* bacteria, as described before. The plates then were left at ambient temperature for 30 min to allow prediffusion before incubation at 37°C for 72 h under anaerobic condition. Finally, MIC was determined by measuring the diameter of the zone of inhibition. All disk diffusion tests were performed in three independent experiments.[1,3]

Preparation of gel bases containing *Antirrhinum majus* extract

The amount of various materials required for preparation of gel bases were determined by Experimental Design® [Table 1]. To prepare each formulation, appropriate amount of potassium sorbate was dissolved in 5 ml of hot water. Subsequently, the given amount of carbopol 940 was

Table 1: Preparing a clear gel containing *Antirrhinum majus* extract

Ingredients/ formulations	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9
Carbopol (g)	1	0.25	0.5	0.75	0.4	0.6	0.5	1	0.5
Propylene glycol (ml)	5	5	5	5	5	5	5	5	5
Potassium sorbate (ml)	0.5	0.5	0.5	0.5	0.25	0.25	0.25	0.25	0.25
Isopropyl myristate (ml)	5	5	5	5	5	5	5	5	5
Antirrhinum majus extract (0.25 µg/ml) (ml)	2	2	2	2	2	2	2	2	2
Alcohol (ml)	25	25	25	25	17	17	17	26	17
Cremophor (g)	0	2	2	2	2	2	2	2	2
Tea 10% (ml)	10	20	15	10	8	8	8	1	5
Water q.s. to (ml)	100	100	100	100	100	100	100	100	100

dispersed in 50 ml deionized water with continuous stirring for 20 min. This dispersion was kept overnight for soaking. In another beaker, the required quantity of propylene glycol, isopropyl myristate, and cremophor were added to ethanol and deionized water. Required concentrations of *A. majus* extract diluted in ethanol 90% considering its MIC were added to the second beaker. Triethanolamine was added to the mixture for adjusting pH at 6.8–7.1.[1,22-24]

Evaluation of the gel formulations

Organoleptic evaluation

Organoleptic parameters and skin irritation test were performed on a group of volunteer women between 18 and 30 years, randomly chosen, and after informed consent, they were asked to use final formulation on their arm and let it remain for 24 h, and then, they gave their comments about the formulation. The organoleptic parameters were also checked by the following methods.

Visual appearance inspection

Physical appearance of the formulation was checked visually for color, consistency, greasiness, and odor.[1,25]

pH

pH of the prepared formulations was measured using a digital pH meter (Metrohm Switzerland) at room temperature in natural condition. After calibration, the determinations were carried out in triplicate and the average was calculated.[26]

Viscosity

The viscosity of formulated gels was determined using Brookfield viscometer at 50 rpm and 25°C.[1]

Drug content

About 10 g of the final gel was transferred to a flask containing 20 ml alcohol 90% and stirred for 30 min. Then, the content volume was increased up to 100 ml and filtered. One milliliter of this solution was 100X diluted with alcohol 90%. The absorbance of the final solution was measured by spectrophotometer at 410 nm. Drug content was calculated by the following formula:[27]

Drug content = (absorbance/slope) × dilution factor (1/1000)

In vitro diffusion studies

The *in vitro* diffusion studies for all formulations were done using the Franz diffusion cell. Twenty-five milligrams of the gel-containing *A. majus* extract was placed on the cellulose acetate membrane in phosphate buffer with pH 6.8, continuously stirred; temperature was maintained at 37°C ± 1°C. One milliliter aliquot was withdrawn from each system at time intervals of 15, 30, 60, 120, and 180 min, and 5 h and analyzed for drug release using ultraviolet (UV) spectrophotometer at 410 nm.[1,28]

Drug release kinetics

To study the release kinetics of the final formulation, the data obtained from *in vitro* release studies were plotted in various kinetic models.[28,29]

Antibacterial studies of the gel formulation

The antibacterial activity was evaluated by well diffusion method. *P. acnes* was incubated in BHI-Glu agar for 72 h under anaerobic conditions and adjusted to yield approximately 10^8 CFU/ml in BHI broth. The solidified agar plates were filled with 10 ml of seed layer containing 10^8 bacteria/ml. The equidistance wells were cut in the plates with the help of pastor pipet. In each of these wells, the gel formulation was placed and the plates were left at ambient temperature for 30 min to allow prediffusion before incubation at 37°C under anaerobic condition for 72 h. The antibacterial activity was estimated by measuring the diameter of zone of inhibition. All diffusion tests were performed in three independent experiments and antibacterial activity was expressed as mean ± standard deviation.[1,3]

Effect of blue light on antibacterial activity of *Antirrhinum majus* extract formulated gel

To evaluate the effect of blue light on antibacterial activity of *A. majus* extract alone or when gel formulated, various plate combinations were designed in duplicate, one as a sample exposing to blue light, and one control nonblue light exposing plate. According to the previously obtained MIC values for the free plant extracts, the similar concentrations of the plant extract were added into 50 mg of gel base, separately. The blue light effect on these gels has been determined from a 25-cm distance for 30 min.

Statistical analyses

SPSS Windows version 19 (SPSS Inc., USA) was used for statistical analyses by applying mean values using analysis of variance (ANOVA) with proper *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Antirrhinum majus contains aurones

The R_f value of about 4.2 for the *A. majus* extract showed the presence of aurones. In spectrophotometric identification of the extract, on the other hand, it was showed two significant bands at 260 and 406 nm, confirmed the release of extract from the plant. Average density of plant was 0.09 g/ml for shoots and 0.05 g/ml for petals. Total phenolic content of the extract showed to be 4.065 ± 0.014 (g of gallic acid/g of ethanolic extract).

The best formulation was selected according to the pharmaceutical properties

Nine gel formulations were prepared based on the obtained results from the Experimental Design™ software [Table 1].

Table 2: Pharmaceutical evaluation of the gel preparations

Parameter	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9
pH	6.12	9	7.23	6.7	6	5	6.2	6.02	6.12
Clarity	+	++	++	++	+++	+++	+++	+++	+++
Viscosity (mPa) At 25°C	Spdl: 74 5800	Spdl: 72 2.4	Spdl: 72 128	Spdl: 74 200	Spdl: 74 4500	Spdl: 74 4700	Spdl: 74 4600	Spdl: 74 5200	Spdl: 74 4600

+: Not clear, ++: Clear, +++: Very clear

The pH of most formulations ranged from 6.8 to 7.1, which was acceptable for topical formulations. Finally, the formulation F9 has been chosen for its desirable pharmaceutical properties. This formulation was translucent, produced smooth effect on skin, left nothing on skin, and showed good consistency and homogeneity [Table 2].

According to the results of ANOVA test, it was shown that there were no significant differences between the formulated and gel in the market in terms of spreadability [Table 3].

Analyzing of the data of pH in different times (1, 7, 30, and 120 days after the formulation) using ANOVA and Tukey *post hoc* test showed no significant changing in pH in comparison to the fresh formulation over the time ($P < 0.05$) [Table 4].

ANOVA and Tukey *post hoc* test showed no statistically significant reduction in terms of drug content, except after 3 months of formulation preparation at room temperature and normal condition ($P = 0.035$) [Table 5].

According to Figure 1, after the analysis of the extract release using UV spectrophotometer at 410 nm, at time intervals of 15, 30, 60, 120, and 180 min and 5 h, it was concluded that the most extract release from the membrane occurred after 5 h as 60.07% [Figure 1].

There were no significant differences between the zone of inhibition of final formulation with or without exposure to blue light

For both petal and shoot extracts formulated as gel, there were no significant differences between the zone of inhibition of gel with or without blue light combination from a 25-cm distance for 30 min in all final extract concentration ($P < 0.05$) [Table 6]. ANOVA and Tukey test, on the other hand, showed that there were no statistically significant differences between the effectiveness of the gel containing shoot or petal extract with or without light ($P > 0.05$) [Table 6].

Finally, the antibacterial effects of formulated gel and intact petal or shoot extract showed no statistical significant differences, so it is concluded that the formulation of the gel has no negative effects on the antibacterial activity of the *A. majus* extract [Table 6].

Discussion

Antimicrobial and antifungal properties of Snapdragon referred to aurones absorb wavelength between 240–270 and 390–430 nm, respectively, according to the study of Cerovic *et al*.[14]

Table 3: Spreadability of the gel formulation

Gram of the gel	Type of gel	Spreadability (mm/s), mean±SD
0.65	Formulated gel	10.15±0.63
0.65	Marketed gel	9.13±0.21

SD: Standard deviation

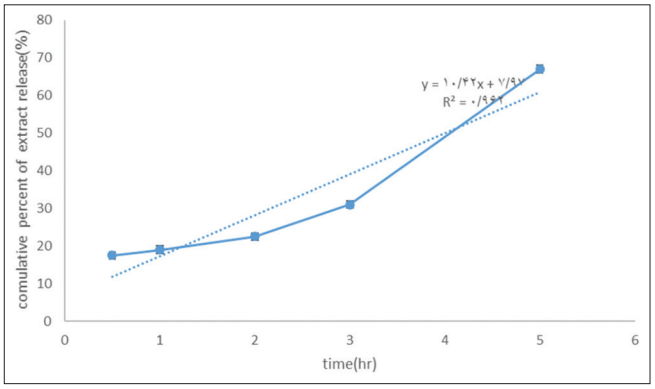

Figure 1: Cumulative percent of extract release toward the time

Jardosh and Patel. evaluated the antibacterial effects of synthetic aurones and reported significant antibacterial effects against *S. aureus*, *B. subtilis*, *C. tetani, E coli*, *P. aeruginosa*, and *Vibrio cholera* as well as antifungal effects against *C. albicans* and *A. clavatus*.[12] In addition, Riaz *et al*. approved the antibacterial and antifungal effect of Snapdragon extract against *Pasteurella multocida*, *Escherichia coli*, *B. subtilis*, *Aspergillus flavus*, *Aspergillus niger*, *Alternaria alternata*, and *Rhizopus solani*.[30]

In other studies, significant antimalaria, antimicrobial, antifungal, antivirus, anti-inflammatory, anticancer, and antiangiogenesis effects have been reported for aurones. Furthermore, the effects of some aurones in the treatment of skin pigmentation by inhibiting tyrosinase in the synthesis of melanin were reported.[31]

According to Jardosh and Patel. results, the MIC of aurones for *S. aureus*, *B. subtilis*, and *C. tetani* has been calculated as 12.5, 25, and 50 µg/ml, respectively. However, in our study, the estimated MIC of aurones in the shoot and petals was about 0.25 µg/ml. This higher antibacterial effects can be attributed to the higher sensitivity of *P. acnea* to the

Table 4: Changes in pH of formulation over the time

Time interval	Time 0	After 24 h	After 7 days	After 1 month	After 3 months
pH±SD	6.87±0.006	6.87±0.007	6.87±0.007	6.86±0.007	6.87±0.006

SD: Standard deviation

Table 5: Changes in drug content of formulation over the time

Time interval	After 24 h	After 7 days	After 1 month	After 3 months
Drug content percentage±SD	47.5±1.43	47.49±0.22	47.09±0.96	46±0.21

SD: Standard deviation

Table 6: Concentrations and zone of inhibition for shoot (Z1–Z4) and petal (X1–X4) extracts

Extract/blank/control	Concentration (μg/ml)	Zone of inhibition (mm), mean±SD
Z1	0.05	18.1±3.12
Z2	0.125	17.4±3.89
Z3	0.25	20.7±3.28
Z4	0.90	18.7±6.36
X1	0.05	15.7±1.66
X2	0.125	16.4±1.01
X3	0.25	22.0±1.92
X4	0.50	20.9±2.74
Vancomycin 30 μg	-	32.5±2.27

SD: Standard deviation

aurones in comparison to the investigated Gram-positive bacteria in the Jardosh and Patel study.[12]

Carbopol is one of the most ingredients used as the gel bases for the production of antiacne gels. In one study, this compound was used for the preparation a polyherbal antiacne gel, and the results showed that this gel had good consistency and spreadability.[32] Another study is the Mayank *et al*.'s project successfully used this polymer for the formulation a herbal antiacne gel-containing *Aloe vera* extract with suitable appearance, color, consistency, pH, and spreadability.[33] Moreover, this component did not interfere with the antimicrobial effect of final formulation, and the antiacne effects of the formulated gel were equal to the intact extract.

At the end of the study, we evaluated, for the first time, the effect of the blue light exposure on potentiating the antibacterial effect of the prepared gel formulation. While it was showed that the combination of *A. majus* gel with the blue light did not show any additive or synergistic effects in our study, according to Tzung *et al*.'s study, clinical application of blue light twice a week for 4 weeks from 15 cm distance with the power of 40 J/cm^2 is useful.[32] In another study, Dai *et al*. radiated blue light from 25 cm for 60 min on the microbial suspension, which reduced bacterial count by 15%–24%.[34,35] Furthermore, Ashkenazi *et al*. radiated blue light for 24 h from a distance of 10 cm to test tubes with the energy of 75 J/cm^2. Then, the results have been checked by electron microscopy and showed that the walls of many bacteria had been destroyed.[6]

Conclusion

In the present study, we prepared a topical gel formulation containing hydroalcoholic extracts of *A. majus*. It showed significant antibacterial effects against *P. acnes*. We also evaluated the effect of blue light exposure on its effectiveness on the observed antibacterial effects. However, it did not show any enhancement on the observed effects. In this regard, further clinical studies for the evaluation of the healing effects of the prepared gel formulation on acne lesions must be performed.

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Conflicts of interest

There are no conflicts of interest.

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