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Original article

Wound Healing Effect of a Topical Product from *Ajuga chamaecistus* ssp. *tomentella* in Pressure Ulcer: a Randomized Double-Blind Placebo-Controlled Clinical Trial

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Abstract

Background and objectives: *Ajuga* genus is used as wound healing in traditional Persian medicine**.** This study aimed to evaluate the effectiveness of *Ajuga chamaecistus* ssp. *tomentella* ointment on healing pressure ulcers in patients admitted to the intensive care unit. **Methods**: In this randomized, double-blind, placebo-controlled clinical trial, 131 patients with grade 1 or 2 pressure ulcers were randomly assigned into one of two groups through simple randomization. The study group received 3% Ajuga ointment, containing 17.26 µg/mL of 20-hydroxyecdysone (ecdysterone) as the main compound, while the control group received placebo twice a day for 14 days in addition to the standard care for pressure ulcers. Changes in the degree and size of wounds were considered as the primary outcomes of the study based on the 2-digit Stirling scale. **Results**: Forty patients in each group completed the research. Mean (95% confidence interval) difference values, for wound degree, between two groups on day 7 vs. day 0 was -0.88 (-1.01 to -0.76, p<0.001), and on day 14 vs. day 0 was -1.57 (-1.78 to -1.36, p<0.001). Mean (95% confidence interval) difference values, for wound area, between two groups on day 7 vs. day 0 was -1.730(-1.979 to -1.48, p<0.001), and on day 14 vs. day 0 was -3.142(-3.563 to -2.72, p<0.001).**Conclusion**: Topical application of Ajuga ointment significantly improved pressure ulcers on days 7 and 14 compared to placebo. Evaluation of the effects of this plant on a larger sample size, for a longer period of time and in different medical centers is recommended.

Keywords: *Ajuga*; ecdysterone; Persian medicine; pressure ulcer; randomized controlled trial

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Introduction

The skin is an important organ for keeping homeostasis in the body. Skin wound healing is one of the most complex biological processes, many of its pathophysiological mechanisms are still unknown [1]. Pressure ulcer is an injury to an area of the skin or underlying tissue located on

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bony prominences caused by pressure or pressure with an incision [2]. Pressure ulcers are severe and painful, which in the intensive care unit (ICU) of hospitals increase the duration of hospitalization as well as the mortality of patients along with the cost of treatment. The patients admitted to the ICU are at high risk of developing pressure ulcers, because in this ward they are confined to bed for a long time, receive sedative drugs, as well as mechanical ventilation, and are unable to change position; so the risk of pressure ulcers increases in ICU. The incidence of pressure ulcers in a hospital is 10 to 23%, while that of pressure ulcers in the ICU is 56%. Pressure is one of the most important factors in causing pressure ulcers. Diabetes, smoking, immunosuppression, vascular disease, and spinal cord injury are the main causes of pressure ulcers [3]. Meanwhile, poor nutrition, aging, metabolic problems, high blood pressure, decreased mobility, diminished sensory perception, sepsis, skin contamination with the urine and feces, moisture, friction, mechanical ventilation and long-term use of drugs such as anesthetics, sedatives, analgesics and muscle relaxants are other factors that lead to this disease [4].

Acute ulcers cause many problems for patients. The most important complications are sepsis and cellulitis osteomyelitis, as well as increasing patient mortality. In the elderly with pressure ulcers, the risk of death is increased by 60% one year after discharge from the hospital. The wound is also painful and can lead to depression [5]. Many methods and treatments such as various dressings as well as various topical agents for example collagenase ointment, foam dressings, basic wound contact dressings and polyvinyl pyrrolidone plus zinc oxide have been proposed to heal these wounds but none have shown to be superior to the others. In addition, the cost of treatment with these methods has increased significantly [3,6]. Herbal Medicines may help speed up the wound healing process. Studies of traditional Persian medicine (TPM) sources show that several plants are effective in wound healing, and new studies have proven the effects of these plants [7,8]. "Kamafitos" or "Khamanitos" (*Ajuga* spp) is one of the plants that have been introduced in Iranian traditional medicine books for wound healing. In "Makhzan al-Adviyeh" (one of the sources of TPM), this plant has been suggested for the treatment of joint pain and gout, menstruation, urinary retention (as a diuretic) and also wound healing. [9,10].

Ajuga (Lamiaceae) is also known as bugleweed, ground pine, carpet bugle, or just bugle. *Ajuga* species are grown in Europe, Asia (China, Korea, Japan, and Iran) and Africa, along with two species in southeastern Australia. *A. austro-iranica, A. chamaecistus, A. comate* (Syn.: *Ajuga chamaepitys* subsp. *chia*), *A. orientalis* as well as some subspecies of *A. chamaecistus* including *Ajuga chamaecistuss* ssp. *tomentella* are native to Iran [11,12].

In modern medicine, *Ajuga* spp. has proven to have various pharmacological effects such as, vasodilator [13], antioxidant [14,15], antibacterial [16] and anti-inflammatory [17,18] properties. The presence of various chemical compounds including flavonoids, iridoids, and phytoecdysteroids with biological effects have been reported in plants of the *Ajuga* genus [19]. Among phytoecdysteroids, 20-hydroxyecdysone (20-E) and cyasterone along with ajugalactone are the major constituents in various species of the genus *Ajuga* with anabolic, adaptogenic, anti-osteoporosis, and wound healing properties [10,20]. The aim of this study was to evaluate the effects topical ointment of 3% alcoholic extract of *Ajuga chamaecistus* ssp. *tomentella* on pressure ulcer healing in patients admitted to the intensive care unit (ICU) of the hospital.

Material and Methods

Ethical consideration

The study was approved by the ethics committee of the Pharmaceutical Sciences Research Center of Tehran University of Medical Sciences with the ethics code IR.TUMS.VCR.REC.1398.1007. Also, the study was registered at the Iranian Clinical Trial Registration Center under the number IRCT20191110045389N1. The consent form was completed by the patient or by his or her first-degree relatives. Their information was kept confidential. The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Chemicals

Ethanol (Nanosany Corporation, Iran), propylene glycol, beeswax, vaseline, and eucerin (Sepidaj Pharmaceutical Company, Iran), neutral green color, chlorophyll or E141, (Magnolia Company, Iran), 20-hydroxyecdyson (Sigma-Aldrich, Germany), methanol (Merck, Germany),

acetonitrile, HPLC grade (Merck, Germany) were used in the study.

Plant collection

Ajuga chamaecistus ssp. *tomentella* Ging. ex Benth is a native species of Iran, the plant was collected in May 2019 from the heights around Tehran. Herbarium specimen with number 6697-THE was kept at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Iran.

Extraction

The aerial parts of the plant were dried in the laboratory. One kilogram of dried plant was crushed, ground, and extracted three times with 70% ethanol with an interval of at least 3 days with maceration method. The extract was concentrated by rotary apparatus (40 °C) and finally completely dried by vacuum oven.

Preparation of topical formulation

Three percent topical ointment was prepared from propylene glycol (6%), beeswax (6%), vaseline (50%), and eucerin (33%) with the dry hydroalcoholic extract of the plant (3%). Placebo ointment was prepared from a base formulation (propylene glycol (6%), beeswax (6%), vaseline (50%), and eucerin (33%) without extracts of the *Ajuga* plant. Neutral green color (chlorophyll or E141 that approved by the FDA) was added to the placebo ointment to match the color of the placebo ointment with the *Ajuga* ointment. *Ajuga* and placebo ointments were prepared in exactly the same container with the same smell and color and special code.

Standardization and validation

Standardization was performed by determining the amount of 20-hydroxyecdyson (20-E) in topical ointment via HPLC method according to the previous validated study [20]. Standard solutions at five concentrations (100, 50, 25, 5, and 1 µg/mL) were prepared from standard material, 20-E, and methanol and injected three times into HPLC. The calibration curve for the standard sample according to the area under the curve (AUC) was drawn against concentration (µg/mL). The line equation was calculated as $y = ax + b$ and the correlation coefficient R^2. Limit of detection (LOD) and quantification (LOQ) were determined in accordance with the previous study [21].

Instrument

Waters, Milford, MA 2487 HPLC and 5 µm Perfectsil Target column (ODS-3 150×4.6 mm) (25 °C), was used. Detection was performed via a UV detector at a wavelength of 254 nm. The isocratic method was employed. The mobile phase utilized was a mixture of water and acetonitrile (75:25) and degassed before the operation; the volume of the injected sample was 10 µL. The flow rate of the mobile phase was 1 mL/min. The amount of 20-E in the unknown samples was calculated by comparing the AUC with the area under the standard peak.

Extraction from the ointment

Twenty-five mL of methanol was added to 1 g of ointment and shaken in an ultrasonic bath (40 °C) for 10 minutes. The supernatant was passed through a 0.45 mm filter and injected into the HPLC.

Ointment microbial quality control

Microbiological quality control of the ointment was performed based on WHO protocols [22].

Type of study

The present study was a randomized, double-blind, placebo-controlled clinical trial. It was performed in the general intensive care unit (ICU) of Shahid Sadoughi Hospital in Yazd, affiliated with Yazd University of Medical Sciences, from December 2020 to June 2021.

The patients (or patient companions) and nurses (treatment staff) were blind to the treatment.

Sample size determination

The sample size of the study was determined according to the sample size formula and data of previous studies as well as considering α of 0.05 and 80% power (b=0.8), [23,24] . Due to possible drop (10%) in patients, the sample size for each group of 40 patients was considered.

Inclusion criteria

Patients older than 18 years with grade 1 or 2 pressure ulcers (based on Two-digit Stirling Pressure Ulcer Severity Scale) and willingness to participate in the study with no local infection were enrolled.

Exclusion criteria

Exacerbation of grade 3 or 4 during treatment,

allergic reactions to components of the formulation (increased erythema and redness around the wound), pregnancy or lactation, reluctance to participate in the study, impossibility of monitoring treatment due to death, discharge or transfer to another ward and the presence of symptoms of wound infection. Wound infection was diagnosed with the appearance of abscesses formation, edema, erythema, warmth around the wound, discoloration of the wound and its margins, purulent discharge, brittle or bleeding granular tissue, abnormal and bad odor, bridge formation on the wound bed, either a change in pain or tenderness to the wound and opening of the wound.

The patients included in the study were randomly divided into two groups by using simple random sampling method and a random number table. In patients with multiple wounds, the wound with the highest grade and the largest area was examined. For all patients (placebo and *Ajuga*), wounds were washed with normal saline and zinc oxide ointment was applied 2 hours before initiating the treatment for patients. For the study group, 3% *Ajuga* ointment was prescribed twice a day and patients in the control group received placebo ointment twice a day. The ointment was used for both groups for 14 days. The amount of ointment was applied based on the wound surface, which was used by the nurses based on the fingertip unit (FTU) (approximately equal to 0.5 g of ointment) [25]. For all patients, in addition to the use of *Ajuga* ointments and placebo, supportive general wound care was performed. General wound care was also provided such as reducing wound-causing factors (pressure cutting / friction) and controlling general wound-related conditions (washing and cleaning the wound bed / dressing / patient nutritional support /treatment or control of comorbid conditions such as diabetes/COPD, renal and heart failure/ holding the relevant limb up to improve venous and lymphatic circulation) [26].

Wound assessment

The wounds of all patients were evaluated daily by the researcher. To evaluate the effectiveness according to the two-digit Stirling Pressure Ulcer Severity Scale, the degree of lesions was determined before the intervention as well as on days 7 and 14 of the study. In addition, at the baseline of the study and on days 7 and 14, the area of the wound was calculated based on the square centimeter by measuring the longest length and width of the area. The wound degree was determined based on the wound characteristics (wound color, wound surface, and wound discharge, etc) by ICU fellowship opinion according to two-digit Stirling comparison form. There are various scales for grading and classifying pressure ulcer pressure. The most common scales to evaluate wound are the Stirling scale in the United Kingdom, the original National Pressure Ulcer Advisory Panel (NPUAP) in the United States, and the original European Pressure Ulcer Advisory Panel (EPUAP) scale [27]. But there is not sufficient evidence to suggest the best pressure ulcers classification system in clinical practice [28,29]. Inter rater reliability and clinical utility of the 2-digit Stirling, 1-digit Stirling, and the EPUAP classification tools were compared in previous study [30]. The 2-digit Stirling was the preferred scale by raters and provided the highest level of inter-rater agreement. As more than 50% of pressure ulcers in the present study were detected within the intact skin without any exudates, so 2-digit Stirling scale was used in this study. This criterion is easy to use and has a wider classification for pressure ulcers, the wound is classified more accurately and a correct estimate of the damage is obtained [26].

Data collection

Demographic and clinical information of patients was collected including age, sex, vital signs, history of smoking, acute physiology and chronic health evaluation (APACHE) II score and sepsis-related organ failure assessment (SOFA) score, wound location, underlying diseases, reason for admission to ICU, laboratory data, and medications. Acute physiology and chronic health evaluation (APACHE) II score and sepsis-related organ failure assessment (SOFA) score are capable of predicting mortality in trauma patients hospitalized in the intensive care unit (ICU). The APACHE II score has a lot of use for evaluating various functions in ICU and its cost-effectiveness and appropriate power in displaying the quality of ICU care effectively. This system examines 12 physiological variables and calculates a maximum score of 71. The higher the score, the higher the risk of death. The SOFA scoring system widely evaluates the results related to the patient through the assessment of organ failure such as liver, lungs, blood platelets, heart and blood vessels, kidney and nerves and a

score between 0 and 4 is given. A higher score is associated with higher mortality [31,32].

To assess the nutritional status of the study participants, nutritional method (enteral or parenteral or a combination of both), total daily fluid intake and excretion, average daily caloric and protein intake as well as serum albumin level of each of them were recorded.

Statistical analysis

The software used for statistical analysis was SPSS version 25. The normal distribution of variables was examined by the Kolmogorov-Smirnov test. Discrete data were presented as percentages and Chi-square test was employed to examine the differences between the two groups. Continuous variables were expressed as mean ± standard deviation. Parametric and non-parametric variables were compared through independent sample t-test, paired- samples T test, and Mann-Whitney U test. Evaluation of changes in the degree and area of wounds during the study was performed using repeated-measures analysis of variance. In all analyses, p value less than 0.05 was considered statistically significant.

Results and Discussion

The extraction yield was 18%.The calibration curve of 20-E was linear (Y= 40.001X+46.426, R^2= 0.9995) within the concentration range of 1 to 100 µg/mL. By applying the standard curve, the amount of the main ingredient, 20-E, in *Ajuga* ointment was calculated to be 17.26 µg/g. The run time was considered 12 min; the peak of the active substance was drawn by the detector in 3.17 minutes (Figure 1). The LOD and LOQ for 20-E were calculated to be 0.76 and 2.3 µg/mL, respectively.

The microbial counts of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, molds and yeast in the product were in accordance with WHO protocol [22].

During the study period, 131 patients were screened, 100 of whom met the inclusion criteria. Twenty-seven patients were not eligible for inclusion in the study, of which 15 had grade 3 or 4 wounds, 5 had infections, 3 needed wound debridement, and 4 refused to sign the consent form and participate in the study. A total of 104 patients with the condition were divided into intervention and control groups according to Figure 2. Finally, 40 patients in each group completed the study.

The demographic and clinical information of the two groups were consistent (Table 1). The most common reason for ICU admission was surgery in both groups, and cardiovascular diseases showed the highest rate of underlying diseases.

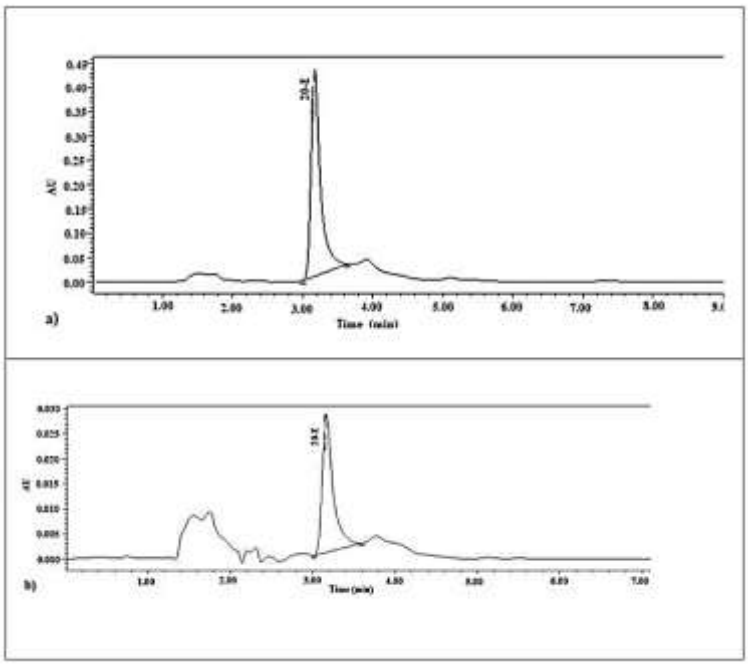

Figure 1. Chromatograms of 20-hydroxyecdysone (retention time 3.17 min); a) standard (100 µg/mL); b) ointment

The most common areas of ulcer in patients in both groups were the buttocks and upper back (between the scapula and thoracic vertebrae).

Tables 1 and 2 report that the two groups had no significant differences in terms of medication received (especially diabetic patients), physiological severity, and organ failure at baseline (APACHE II and SOFA scores), and other parameters. Table 2 shows that nutritional parameters were not significantly different between patients. Patients in the two groups were comparable in terms of fluid intake and nutritional factors affecting the wound healing process. The amount of fluid intake and nutritional factors were not significantly different between the two groups.

Mean score of severity (degree) and size of pressure ulcers in the study and control groups at the beginning of treatment were not significantly different (Table 2). Figures 3 and 4 indicate that the mean pressure ulcer score and size of wounds on days 7 and 14 in the *Ajuga* group were significantly more than the placebo group.

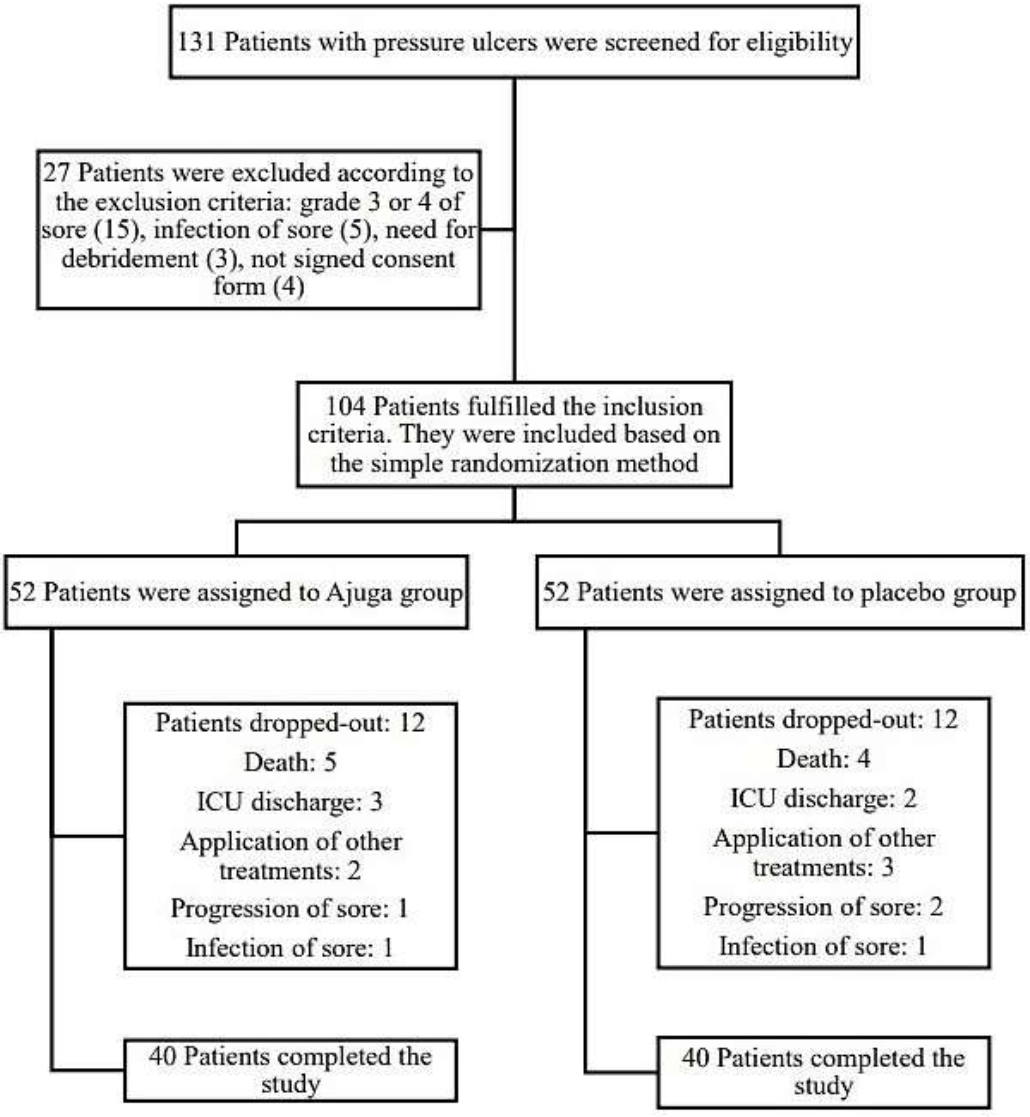

Figure 2. Flowchart of the randomized double-blind placebo-controlled clinical trial

Table 1. Clinical information of patients at the beginning of study

Variable[a]	Ajuga (n=40)	Placebo (n=40)	p-Value
Sex			
Male	21(52.5)	18(45)	0.22
Female	19(47.5)	22(55)	
History of smoking	2 (5)	3 (7.5)	0.12
APACHE II score	16.33 ± 5.25	14.89 ± 6.45	0.14
SOFA score	5.38 ± 2.66	4.67 ± 1.88	0.2
Baseline disease			
Cardiovascular diseases [b]	12 (53.3)	14 (35)	0.09
Diabetes mellitus	10 (25)	7 (17.5)	0.11
Gastrointestinal disorders	7 (17.5)	6 (15)	0.23
Neurologic disorders	2 (5)	3 (7.5)	0.09
Respiratory diseases	7 (17.5)	8 (20)	0.42
Malignancy	2 (5)	2 (5)	0.18
Admission diagnosis category			
Surgical	23 (57.5)	21 (52.5)	0.22
Medical	17 (42.5)	19 (47.5)	0.08
Wound location			
Buttock	13 (32.5)	11 (27.5)	0.31
Upper back	9 (22.5)	11 (27.5)	0.34
Around the hip bone	7 (17.5)	9 (22.5)	0.21
Sacrum	8 (20)	6 (15)	0.12
Others	3 (7.5)	3 (7.5)	0.07
Concomitant drugs			
Heparin	37 (92.5)	39 (97.5)	0.28
Pantoprazole	35 (87.5)	36 (90)	0.19
Vasopressors	12 (30)	11 (27.5)	0.35
Diuretics	10 (25)	11 (27.5)	0.31
Metoprolol	9 (22.5)	13 (32.5)	0.41
N-acetylcysteine	27 (67.5)	19 (47.5)	0.14
Captopril	5 (12.5)	4 (10)	0.25
Insulin	10 (25)	7 (17.5)	0.06
Statins	6 (15)	7 (17.5)	0.07
Antibiotics	26 (65)	24 (60)	0.16
Antiepileptics	8 (20)	9 (22.5)	0.41
Supplements[c]	16 (40)	17 (42.5)	0.41
Opioids	12 (30)	11 (27.5)	0.27
Amiodarone	4 (10)	3 (7.5)	0.16
Haloperidol	3 (7.5)	4 (10)	0.14
Bronchodilators	13 (32.5)	12 (30)	0.08
Pressure ulcers score[d]	1.75 (1.00-2.40)	1.71 (0.2-2.40)	0.619
Pressure ulcers surface (cm^2) area	3.36 (1.40-6.31)	3.23 (0.79-6.24)	0.276

APACHE: acute physiology and chronic health evaluation; SOFA: sepsis-related organ failure assessment; a: data are presented as Mean ± Standard deviation or No. (%); b: cardiovascular diseases defined as hypertension, myocardial infarction, ischemic heart disease, peripheral vascular disease, or cardiac arrhythmias; c: including vitamin A, vitamin B complex, vitamin C, vitamin D, vitamin E, vitamin K, Calcium, Copper, Zinc, Iron, and selenium at supplement doses; d: data are presented as mean (confidence interval)

The mean ± SD reduction in pressure ulcer score in the *Ajuga* group on the 7th day was -0.97±0.24 vs. -0.09±0.31 in the placebo group (p value <0.001); on the 14th day it was -1.66±0.49 in the *Ajuga* group vs. -0.085±0.4 in the placebo group (p value <0.001). Thus, the mean reduction of pressure ulcers score in *Ajuga* group was significantly greater than in placebo group on days 7 and 14 of study (Table 3).

Also, the mean ±SD reduction of pressure ulcer size in *Ajuga* group was significantly higher than placebo group on day 7 (-1.78± 0.77 vs. -0.048 ±0.17 and p value <0.001) and on day 14, respectively (-3.21± 1.26 vs. -0.666± 0.25 and p value <0.001) (Table 4, Supplementary figure).

During the study, no side effects such as swelling, rash and contact dermatitis were reported with the use of *Ajuga* ointment and placebo. This ointment causes dryness around the wound.

This study was performed in the ICU of Shahid Sadoughi Hospital in Yazd. During the study, 3% *Ajuga* topical ointment was used for 14 days, which reduced the size and severity of pressure ulcers compared to placebo in patients.

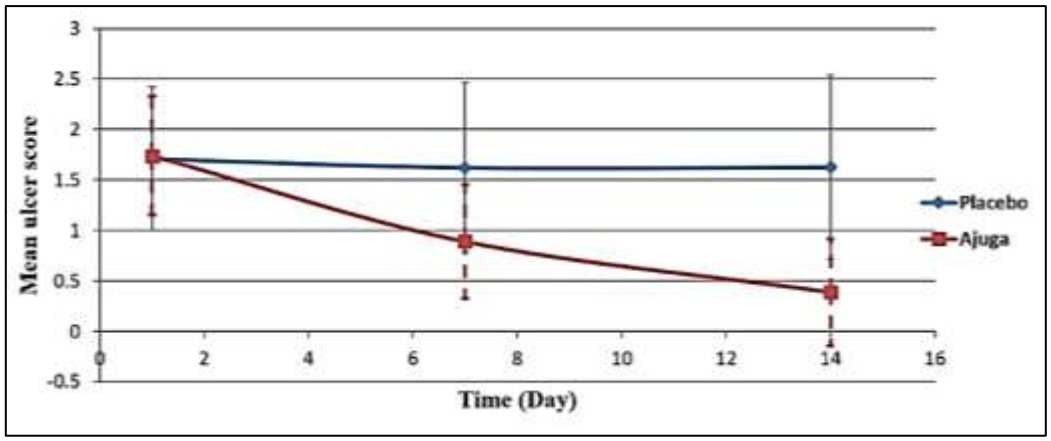

Figure 3. Changes in the mean ulcer score in study groups

Table 2. Vital signs and laboratory data of patients

Parameter[a]	Ajuga (n=40)	Placebo (n=40)	p-Value
T (°C)	37.35 ± 0.14	37.16 ± 0.23	0.18
HR (beats/minute)	97.40 ± 11.69	93.13 ± 12.23	0.49
RR (numbers/minute)	18.97 ± 1.72	18.46 ± 1.90	0.48
MAP (mmHg)	88.14 ± 5.67	89.23 ± 7.11	0.39
Ph	7.53 ± 0.02	7.41 ± 0.06	0.54
$PaCO_2$ (mmHg)	40.18 ± 6.73	40.45 ± 4.37	0.51
PaO_2 (mmHg)	90.21 ± 10.03	89.13 ± 9.11	0.57
O_2 saturation (%)	92.35 ± 11.60	93.55 ± 10.23	0.32
WBC (cells/ μL)	9307.24 ± 2756.53	9143.34 ± 2980.54	0.29
Hg (g/dL)	10.52 ± 0.98	9.21 ± 1.69	0.32
Plt (cells/ μL)	189652.21 ± 96535.45	209840.31 ± 140100.56	0.28
Na (meq/L)	134.67 ± 4.07	135.12 ± 3.11	0.31
K (meq/L)	3.87 ± 0.57	4.03 ± 0.25	0.21
Ca (mg/dL)	8.08 ± 0.41	8.29 ± 0.13	0.35
Mg (mg/dL)	1.65 ± 0.20	2.01 ± 0.13	0.46
P (mg/dL)	3.15 ± 1.32	3.21 ± 1.01	0.50
Blood sugar (mg/dL)	139.99 ± 38.83	143.21 ± 35.24	0.46
ESR (mm/h)	54.35 ± 19.92	54.99 ± 20.12	0.36
CRP (mg/L)	69.52 ± 22.08	70.21 ± 42.23	0.47
Scr (mg/dl)	1.09 ± 0.74	1.11 ± 0.51	0.30
BUN (mg/dl)	59.09 ± 42.22	58.78 ± 49.34	0.31
AST (IU/L)	23.45 ± 10.39	24.34 ± 9.34	0.41
ALT (IU/L)	14.66 ± 5.23	17.34 ± 5.64	0.33
ALP (IU/L)	193.01 ± 99.13	200.44 ± 99.91	0.55
Bilirubin (mg/dL)	1.40 ± 0.44	1.30 ± 0.13	0.24
INR	1.73 ± 0.29	1.69 ± 0.32	0.22
Weight(kg)	71.54±14.97	68.21±22.15	0.20
Enteral nutrition	27 (67.5)	25 (62.5)	0.16
Parenteral nutrition	4 (10.00)	7 (17.50)	0.10
Enteral + parenteral nutrition	9 (22.5)	8 (20.00)	0.30
Total intake (mL/day)	2865.32 ± 453.08	2938.11 ± 734.11	0.46
Total output (mL/day)	2724.19 ± 345.72	2870.23 ±634.34	0.33
Meancalorie intake (Kcal/day)	1788.60 ± 556.55	1894.15 ± 675.34	0.49
Mean protein intake (g/day)	79.15 ± 30.92	80.22 ± 21.56	0.62
Serum albumin (g/dL)	2.92 ± 0.51	2.80 ± 0.89	0.26

a: data are presented as Mean ± Standard deviation or No. (%); T: temperature; HR: heart rate; RR: respiratory rate; MAP: mean arterial pressure; $PaCO_2$: partial pressure of CO_2; PaO_2: partial pressure of O_2; WBC: white blood count; Hg: hemoglobin; Plt: platelet; Na: sodium; K: potassium; Ca: calcium; Mg: magnesium; P: phosphorus; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; Scr: serum creatinine; BUN: blood urea nitrogen; AST: aspartate aminotransferase; ALT: alanine transaminase; ALP: alkaline phosphatase; INR: international normalized ratio

Table 3. Changes in the degree of pressure ulcers during the study

Parameter	Day 7 vs. Day 0	Day 14 vs. Day 7	Day 14 vs. Day 0
Mean (95% confidence interval) difference values within placebo group [P value]	-0.09 (-0.19 to 0.13) [0.09]	0.002 (-0.06 to 0.07) [0.94]	-0.09 (-0.21 to 0.04) [0.188]
Mean (95% confidence interval) difference values within Ajuga group [P value]	-0.85 (-0.94 to -0.76) [<0.001]	-0.50 (-0.65 to -0.34) [<0.001]	-1.34 (-1.51 to -1.17) [<0.001]
Mean (95% confidence interval) difference values between placebo and Ajuga groups [P value]	-0.88 (-1.01 to -0.76) [<0.001]	-0.69 (-0.85 to -0.53 [<0.001]	-1.57 (-1.78 to -1.36) [<0.001]

Table 4. Changes in the area of pressure ulcers during the study

Parameter	Day 7 vs. Day 0	Day 14 vs. Day 7	Day 14 vs. Day 0
Mean (95% confidence interval) difference values within placebo group [P value]	-0.048 (-0.103 to .007) [0.087]	-.018 (-0.531 to 0.016) [0.286]	-0.066 (-0.146 to 0.013) [0.001]
Mean (95% confidence interval) difference values within Ajuga group [P value]	-1.32 (-1.54 to -1.09) [<0.001]	-1.04 (-1.28to -0.8) [0.001]	-2.36 (-2.77 to -1.95) [<0.001]
Mean (95% confidence interval) difference values between placebo and Ajuga groups [P value]	-1.730 (-1.979 to -1.48) [<0.001]	-1.411 (-1.654 to -1.168) [0.002]	-3.142 (-3.563 to -2.72) [<0.001]

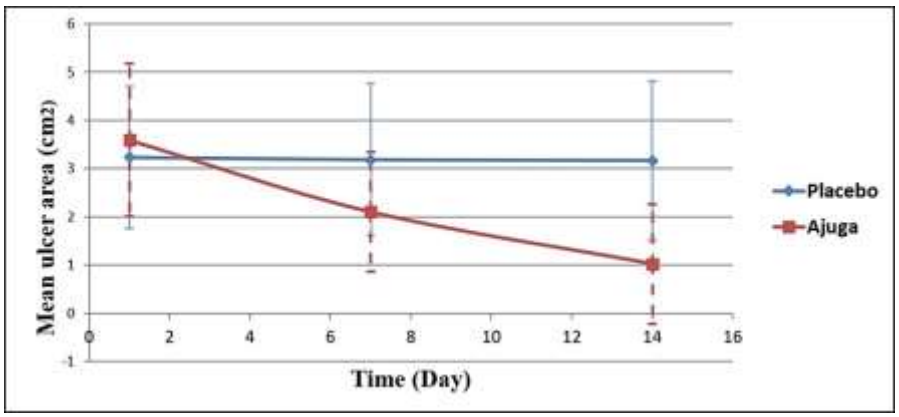

Figure 4. Changes in the mean ulcer area in study groups

In a previous clinical trial, the effect of *Ajuga chamaecistus* ssp. *tumentella*, a 3% topical cream, was shown to be more effective than nitrofurazone cream (0.2%) in healing second-degree burn wounds in terms of mean days of epithelialization onset, healing time, post-drug irritation and pain [33].

Since the control of grade 3 wounds and 4 is more difficult and costly [26], early diagnosis of pressure ulcers in the early stages and initiation of treatment can not only reduce the suffering of patients, but also reduce treatment costs, so this study was designed for grade 1 and 2 wounds.

In a recent research, the therapeutic effects of *Plantago major* topical formulation on the stage 1 pressure ulcer in patients was investigated by a randomized triple blind clinical trial on 130 patients in 14 days. The results showed a significant difference in healing of the ulcers between the intervention and control groups (96% and 73% improvement, respectively) [34]. In another study reported by Parizi et al., the healing effect of rosemary ointment on grade I pressure ulcer was studied in patients admitted to ICU in comparison with the control group that received routine care for seven days. Results indicated that the mean scores of pressure ulcer scale for healing decreased significantly in the rosemary group in comparison with the control group (p=0.001) one week after the intervention. Also, the ratio of complete ulcer healing displayed a significant difference between the two groups (p=0.004) [35]. In both studies, formulations containing extracts of medicinal plants were used to heal pressure ulcer. In these studies, the scale of pressure ulcers assessment, routine care in

control groups and the treatment time were different, however, the results showed the effectiveness of medicinal plants in healing grade I pressure ulcers.

Phytochemical analysis of *Ajuga chamaecistus* ssp. *tomentella* showed that it contains different compounds among which, 20-E is the major constituent [10,11]. Phytoecdysteroid compounds have shown wound healing effects by stimulating keratinocyte differentiation. Also oral administration of 20-E has improve rat bone fractures [36]. Due to the beneficial effects of these compounds and the presence of large amounts of phytoecdysteroids in this plant, the wound healing effect can be related to these compounds. Moreover, the antioxidant effects of different extracts of *A. chamaecistus* ssp. *tomentella* were investigated using various methods such as DPPH radical inhibition and FRAP iron ion reduction. It was found that methanol and aqueous extracts as well as the n-butane fraction containing compounds such as 20-E, and phenylethyl glycosides showed high antioxidant effects [37]. Two polyphenolic glycosides called teupolioside and verbascoside have been isolated from, *Ajuga reptans* and *Syringa vulgaris*, which have strong anti-inflammatory and wound healing properties. Besides, there is a significant amount of ecdysteroids (phytoecdysteroids) in *Ajuga reptans*, which have significant effects on wound healing. Wound healing effects of phenylpropanoid glycoside compounds isolated from different species of the genus *Ajuga* (*A. reptans, A. chia, A. orientalis, A. pseudoiva and A. turkestanika*) have been reported in several studies [38-40]. Anti-inflammatory effects of oral administration of various extracts of *A. chamaecistus* ssp. *tomentella* were examined in laboratory animals. It was found that different extracts show analgesic and inflammatory effects in the model of formalin-induced pain and inflammation [18].

The inflammatory phase is one of the essential steps in the wound healing process [41]. In this trial, the laboratory levels of ESR and CRP (inflammatory biomarkers) matched in both groups. Among the patients in the two groups, drugs that may affect the wound healing process, such as statins [41], phenytoin [42], insulin [43, 44], n-acetylcysteine [45], antibiotics [46], and heparin [47], were not significantly different. The potential benefits of topical *A. chamaecistus* ssp. *tomentella* in wound healing may be related to its anti-inflammatory, antioxidant, and collagen production effects. The limitation of this study was that it was limited to patients admitted to the ICU.

Conducting a study with a different percent of extract in the ointment, different dosage form and different trial duration should be investigated in future studies. Also the study in more centers with larger populations is necessary to achieve definitive results.

Conclusion

Topical application of 3% *Ajuga* ointment significantly improved pressure ulcers (grades 1 and 2) on days 7 and 14 compared to placebo. Nevertheless, investigation of the effects of this plant on a larger sample size for a longer period of time and across different medical centers is recommended.

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Author contributions

Mohsen Adib performed the study, data analysis and drafting of the manuscript; Mohammad Hossein Jarrahzadeh evaluated the pressure ulcer grade and wound size; Tayebeh Toliyat consulted in drug formulation; Laila Shirbeigi and Nafiseh Khosravi Dehaghi and Seyede Nargess Sadati Lamardi were involved in conception and design of the study and reviewed the manuscript. All authors approved the final version.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

ICU: intensive care unit; TPM: traditional Persian medicine; 20-E: 20-hydroxyecdyson;

HPLC: high performance liquid chromatography; LOD: limit of detection; LOQ: limit of quantification; WHO: World Health Organization; FTU: fingertip unit; COPD: chronic obstructive pulmonary disease; NPUAP: National Pressure Ulcer Advisory Panel; EPUAP: European Pressure Ulcer Advisory Panel; APACHE: acute physiology and chronic health evaluation; SOFA: sepsis-related organ failure assessment