Polyphenols profiles and wound healing activity of *Atriplex Halimus* leaf extract cream in Wistar rat

Perfis de polifenóis e atividade de cicatrização de feridas do creme de extrato de folhas de *Atriplex Halimus* em ratos Wistar

Perfiles de polifenoles y actividad cicatrizante de la crema de extracto de hoja de *Atriplex Halimus* en rata Wistar

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ABSTRACT
This study aimed to evaluate the wound healing effect of cream based on extract of *Atriplex halimus* collected from the Southwestern Algeria in rat Wistar. Polyphenols profiles and mineral elements were analyzed by HPLC and atomic absorption respectively. The impact of the cream was studied on the different phases of healing as well as its effect on wound contraction, wound closure time was also studied, and the rate of wound contraction was calculated. Results show the presence of flavonoids, phenolic acids, tannins, and minerals in aqueous extract of *A. halimus*. The wound healing results show that *A.halimus* cream accelerated wound contraction and reepithelialization . These results were confirmed by the histological study where the treated group showed significant wound healing and all damaged tissues were restored. This results obtained confirm that *A.halimus L* cream presents promising potential for the wound healing activity.

Keywords: *Atriplex Halimus*, wound healing, rat, cream, inflammation.

RESUMO
O objetivo deste estudo foi avaliar o efeito cicatrizante de um creme à base de extrato de Atriplex halimus coletado no sudoeste da Argélia em ratos Wistar. Os perfis de polifenóis e elementos minerais foram analisados por HPLC e absorção atômica, respectivamente. O impacto do creme foi estudado nas diferentes fases da cicatrização, bem como seu efeito na contração da ferida, o tempo de fechamento da ferida também foi estudado e a taxa de contração da ferida foi calculada. Os resultados mostram a presença de flavonoides, ácidos fenólicos, taninos e minerais no extrato aquoso de A. halimus. Os resultados da cicatrização da ferida mostram que o creme de A. halimus acelerou a contração e a reepitelização da ferida. Esses resultados foram confirmados pelo estudo histológico, no qual o grupo tratado apresentou cicatrização significativa da ferida e todos os tecidos danificados foram restaurados. Esses resultados obtidos confirmam que o creme de A.halimus L apresenta um potencial promissor para a atividade de cicatrização de feridas. O objetivo deste estudo foi avaliar o efeito cicatrizante de um creme à base de extrato de Atriplex halimus coletado no sudoeste da Argélia em ratos Wistar. Os perfis de polifenóis e elementos minerais foram analisados por HPLC e absorção atômica, respectivamente. O impacto do creme foi estudado nas diferentes fases da cicatrização, bem como seu efeito na contração da ferida, o tempo de fechamento da ferida também foi estudado e a taxa de contração da ferida foi calculada. Os resultados mostram a presença de flavonoides, ácidos fenólicos, taninos e minerais no extrato aquoso de A. halimus. Os resultados da cicatrização da ferida mostram que o creme de A. halimus acelerou a contração e a reepitelização da ferida. Esses resultados foram confirmados pelo estudo histológico, no qual o grupo tratado apresentou cicatrização significativa da ferida e todos os tecidos danificados foram restaurados. Esses resultados obtidos confirmam que o creme de A.halimus L apresenta um potencial promissor para a atividade de cicatrização de feridas.

Palavras-chave: Atriplex Halimus, cicatrização de feridas, rato, creme, inflamação.

RESUMEN
El objetivo de este estudio fue evaluar el efecto cicatrizante de una crema a base de extracto de Atriplex halimus recolectado en el suroeste de Argelia en ratas Wistar. Los perfiles de polifenoles y los elementos minerales se analizaron mediante HPLC y absorción atómica respectivamente. Se estudió el impacto de la crema en las diferentes fases de la cicatrización, así como su efecto en la contracción de la herida, también se estudió el tiempo de cierre de la herida y se calculó la tasa de contracción de la herida. Los resultados muestran la presencia de flavonoides, ácidos fenólicos, taninos y minerales en el extracto acuoso de A. halimus. Los resultados de la cicatrización de la herida muestran que la crema de A. halimus aceleró la contracción de la herida y la reepitelización. Estos resultados fueron confirmados por el estudio histológico en el que el grupo tratado mostró una cicatrización significativa de la herida y se restauraron todos los tejidos dañados. Los resultados obtenidos confirman que la crema de A.halimus L presenta un potencial prometedor para la actividad de cicatrización de heridas.

Palabras clave: Atriplex Halimus, cicatrización de heridas, rata, crema, inflamación.

1 INTRODUCTION

The wound is a destruction of the skin (epidermis and dermis) that can extend to the underlying tissues caused by physical, chemical, thermal, or microbial injury to the tissues (Sharifi et al. 2021). It is a pathology that can be extremely traumatic, sometimes fatal, and very often responsible for physical and psychological after-effects (Young & McNaught 2011). According to

WHO statistics, approximately 5 million people could die each year because of their wounds. Wound healing processes begins immediately after the skin injury, allow the reduction of its surface thanks to a centripetal movement of the edges of the wound(Shang 2021) . The healing of wounds consists of a cascade of overlapping phases; homeostasis, inflammation, proliferation/re-epithelialization and remodeling, carried out in a highly controlled manner to ensure successful wound repair (Velnar et al. 2009). This depends mainly on the repair capacity of the tissue, the type and extent of the damage, and the general health status of the tissues. The granulation tissue of the wound is composed of small new blood vessels, collagen, fibroblasts, edema. The undifferentiated mesenchymal cells from the wound margins are modulated into fibroblasts, which begin to migrate into the wound space with the fibrin strands. Collagen composed of amino acid (hydroxyproline) is the main component of the extracellular tissue, which provides strength and support. Wound therapy aims to restore skin to its pristine function, reestablishing damaged cells and skin tissue without scarring (Tottoli et al. 2020).

The healing activity of plants is recognized in all civilizations. This "natural pharmacy" has always been the subject of numerous scientific studies. The virtues and mechanisms of action of the bioactive molecules of these plants are now scientifically recognized and adopted by our contemporary medicine as well as by the cosmetics industry (Vitale et al. 2022). Recently, several studies have focused on using plants as beneficial agents in wound healing. Indeed, the wide availability, ease of administration, low cost, and reduction of side effects are the main advantages of these natural products (Berroukeche et al. 2024).

Atriplex halimus (Guettaf) is a shrub 1 to 3 m high, very branchy, forming clumps that can reach 1 to 3 m in diameter. The species is spontaneous within a relatively vast area encompassing the countries around the Mediterranean and the Middle East, from the Canary Islands to Iran (Algeria, Morocco, Libya, Tunisia, Syria, Jordan, Egypt, Saudi Arabia, France, Spain, Portugal, Italy, Greece, Albania, Malta). *A. halimus* is spontaneous in the semi-arid and arid bioclimatic areas (Nedjimi et al. 2013). The Arabian herbalists use the leaves to treat heart disease, diabetes, and rheumatism (Walker et al. 2014). In Western Sahara, the ashes of *Atriplex halimus L*, taken up by water, is used in the treatment of gastric acidity, the seeds are ingested as an emetic . Its use in traditional medicine is widely known for its hypoglycemic and hypolipidemic activities (Bounouar et al. 2022) (Chikhi et al. 2014). In folk medicine, the leaves of *A. halimus* are crushed and applied as a poultice to dry and heal wounds (Tahar et al. 2015). No scientific investigation was done before on the wound-healing activity of this plant, for this reason, the purpose of this study was to evaluate the wound healing activity of the cream made from an aqueous extract of *Atriplex Halimus* collected from the Algerian desert in rat Wistar.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

The aerial parts of *Atriplex halimus* plant were collected in March 2020, from the Bechar region (Southwestern Algeria). The plant was identified by Doctor Sakal F. Z Botanist from the University of Abdelhamid Ibn Badis Mostaganem (UMAB). A voucher specimen of the plant (H.S.02889) was deposited in the Herbarium of the University for future reference.

2.1.1 Preparation of the aqueous extract

50 g of *Atriplex halimus leaves* powder was added to 500 ml of distilled water, boiled for 10 min, the solution was filtered, lyophilized and stored at 4°C. The yield of this extraction was 20%.

2.1.2 Mineral Element Quantification

The analysis of mineral contents of leaves of *A. halimus* was performed using an atomic absorption spectrophotometer (AA-7000 SHIMADZU) with a C2H2-Air flame type. The mineralization of the aerial parts of the plant was carried out in a closed system by acid digestion using a mixture of nitric acid and perchloric acid (Lambert,1975). After dilution and filtration, the obtained solutions were used for the quantification of minerals such as Calcium (Ca), Magnesium (Mg), Iron (Fe), Zinc (Zn), Chromium (Cr), and Copper (Cu). The results were expressed in (ppm) milligrams per gram of dry matter using a calibration curve for each analyzed mineral.

2.1.3 Identification of A. halimus phenolic compounds by HPLC

The identification of *A.halimus* phenolic compounds was conducted on analyses by YL 9100 HPLC with YL-CLARITY software. The separation was carried out on an Agilent xdb eclipse C8 column (5 µm, 250 mm, and 4.6 mm; Merck Life Science, Germany). Water (A) and methanol (B) were used as mobile phases; both were acidified with 0.075 % of acetic acid and the chromatogram was extracted at $\lambda = 280$ nm. The identification was performed by comparison with standards spectra at each retention time. Stock reference solutions from 2 to 100 µg/mL concentration were used for the calibration curves and the external standard method was used to quantify each compound, and the results were expressed as mg g−1 dry weight ± SD.

2.2 EVALUATION OF THE HEALING ACTIVITY OF ATRIPLEX HALIMUS AQUEOUS EXTRACT

2.2.1 Preparation of the cream

The cream was prepared in two phases. The water phase was made up of 51,6% distilled

water and 0.4% xanthan gum. On the other hand, the oil phase was composed of 39,4 % sunflower oil and 8% emulsifying wax. The two phases were heated separately in two different beakers in a water bath. The water phase was poured little by little into the fatty phase and whisked vigorously for 20 minutes using an electric homogenizer until a stable emulsion. The cream obtained was left to cool at room temperature after that 0.6 g of conservative was incorporated. The cream concentrations were prepared in 5% (0.5 g of lyophilized extract of *A. halimus* was added to 9.5 g of cream), and 10 % (1g of lyophilized extract of *A. halimus* was added to 9 g of cream). The macroscopic properties of the cream were verified. Short and long-term stability were tested according to (Adejokun & Dodou 2020). The sensibility of the rat was tested by applying the cream to the rat skin and observing any modification after 48h.

2.2.2 Repartition of animals

Twenty healthy adult male Wistar rats weighing (270 to 300g). were obtained from the Algerian Pasteur Institute. Rats were kept in polyacrylic cages, Food was provided by dry pellets. Animal were maintained under standard housing conditions (room temperature at 25±5 °C with 12:12 light: dark cycles), and water *ad libitum*.. Animals were randomly divided into four groups of five rats each. The experimental study was carried out at University of Mostaganem following the Algerian Legislation (Law Number 17-457/2019) inherent to the protection of animals designed for experimental and other scientific purposes as well as with the guidelines of the Algerian Association of Experimental Animal Sciences (AASEA). Rats were randomly divided into five groups of five rats as follows:

Group C: Control group without any treatment. Group NC: Negative control, rats treated with 0.5 g of basic cream without extract. Group PC: Positive control, rats treated with 0.5g Madecassol (Cream indicated in the treatment of wound-healing purchased from the pharmacy). Group ATRIPLUS 5: rats treated with 0.5g of cream 5 % w/w *A. halimus*. Group ATRIPLUS 10: rats treated with 0.5g of cream 10 % w/w *A. halimus.*

2.2.3 Excision wound model

Intraperitoneal (i.p.) injection of ketamine (50 mg/kg) and diazepam (1 mg/kg) was used anesthetize rats. The dorsal part of the animals was shaved using a zero stretcher and disinfected using 70°C surgical alcohol and an antiseptic solution. A circular wound of 2 cm^2 in diameter was excised and removed from the back of rats using surgical scissors. The skin was then disinfected. Twenty-four hours after the wound creation, 0.5g of the preparation was applied daily simultaneously. Rats were housed individually avoiding any contact or intervention.

The effect of the extract was studied on the different phases of healing: The inflammatory phase, the proliferation and epithelialization phase, and its effect on wound contraction. Its effects on wound closure time were also studied, and the rate of wound contraction was calculated. The wounds were observed and photographed every 3 days under the same conditions until their complete closure. The effect of the extract on the inflammatory phase was studied by monitoring the time of appearance and disappearance of the signs of inflammation: redness, edema, and exudate in wounds. Then, during the proliferative phase, the time of appearance of the buds on the surface of the wounds treated with the extracts was recorded and compared with the control. During the epithelialization phase, the time of appearance of new epithelial tissue at the surface of the wounds compared to the control was noted. Following this phase, the wound area was measured by direct planimetry. To do this, a transparent graph paper was placed directly on the wound, and its outline was traced with a fine-tipped marker and the number of tiles in the outline was counted to determine the area of the wound (Balazs et al. 2001). Finally, the healing speed was studied by calculating the speed of contraction of the wound by the formula below:

V = ((Original wound surface - Surface of the healed wound) / Original wound surface) *100

2.2.4 Histological study

Skin biopsies 6 mm in diameter are taken immediately at the end of the experiments. Then fixed with 10% formalin. The histological study is carried out according to the protocol of (Iwanaga et al. 2016).

2.3 STATISTICAL ANALYSIS

The results obtained are expressed as mean ± standard deviation. They are processed by the XTSAT software. They are subjected to a one-way ANOVA analysis of variance followed by DUNETT's test. The value found can assert that the populations are different with a risk error p such that: $p > 0.05$ = the difference is not significant. $0.05 > p > 0.01$ = the difference is significant*. $0.05 > p > 0.001$ = the difference is highly significant**.$p < 0.001$ = the difference is very highly significant***

3 RESULTS

3.1 HPLC-DAD ANALYSIS OF ATRIPLEX HALIMUS

HPLC-DAD analysis of polyphenols extract revealed the presence of ten compounds (Table 1). The separated peaks were identified according to the comparison of the retention time and UV

spectra of these compounds with the standards. The chromatographic analysis of *A. halimus* L aqueous extract revealed the presence of the following phenolic compounds: Flavonols (Quercetin, Myricetin, Galangin, 5.Hydroxy Flavone), Flavanol (catechin) Flavanones (Hespertin), phenolic acids (gallic acid, vannillic acid), tannic acid and Alpha-tocopherol.

Table 1. Phenolic compounds analyzed par HPLC . Prepared by the authors (2024)

Classes of phenolic compounds	Compounds	Time of retention (min)
Hydroxybenzoic acids	Gallic acid	10,337
	Vanilic acid	24,727
Flavonoids	Myricetin	33,860
	Quercetin	5,660
	Hespertin	30,020
	Galangin	47,320
	Hydroxy flavones	54,432
	Catechin	17,870
Tannin	Tannic acid	6,637
Alpha-tocopherol	Alpha-tocopherol	11,427

3.2 MINERAL ANALYSIS

Based on the results obtained in this study (Table 2) it is clearly demonstrated that the leaves of the tested plant contains minerals such as calcium, magnesium, and iron, with high concentrations compared to the contents of the other elements, which were found to be present in small quantities.

Table 2. Mineral contents of the aerial parts of *A.halimus L.* Prepared by the authors (2024)

Element	Absorbance	Concentration (ppm)
Cr	0.0016	0.0678
Ca	2.0720	108.8443
Cu	0.0076	0.0187
Mg	2.1119	2.1365
Fe	0.7866	6.9881
Zn	0.2178	0.1346

3.3 ORGANOLEPTIC CHARACTERISTICS OF THE CREAM

The pH of the cream was approximately 4.5. Atriplex cream appear smooth and homogeneous with a yellow-green color, it has rapid absorption into the skin (Fig 1). Sensibility test of *A halimus* cream observed after application on the skin of rats indicated no signs of inflammation.

The test of short term stability studies on cream shows that after centrifugation Atriplex cream was intact, with no layers separation. Storing the cream at 50°C, 4°C, and 24°C for 24 h does not affect the cream. Cream stored at 24°C for 28 days showed a slight increase in pH with no changes in texture.

Figure 1. Image of water -in -oil cream formulation named ATRIPLUS. Prepared by the authors (2024)



3.4 WOUND CLOSURE RATE

Healing activity is expressed as a percentage of wound contraction in (Fig 2). Our results show that *A.halimus L* cream accelerates the healing process compared to the negative control group; progressive wound contraction percentages were observed in all groups (Control, negative and positive control, ATRIPLUS 5 and ATRIPLUS 10). However, the cream of *A halimus* shows a higher contraction percentage.

The morphological parameters of the wound surfaces were used to evaluate the healing effect of ATRIPLUS 5 and ATRIPLUS 10 . The presence of erythema and exudates was observed in all groups starting from day 0 , The speed of buds was rapid in the ATRIPLUS 5, ATRIPLUS 10, and positive control groups compared to the control, negative control groups, a reduction in the average surfaces of the wounds is observed in the five groups, but with different degrees . The results show no significant modification ($p > 0.05$) of the wound surface is observed during the first three days. However, The average surface area of wounds treated with ATRIPLUS 5% and ATRIPLUS 10% showed a contraction of 97.244% and 91.264% respectively at the end of the experiment (the 21st day). Highly significant reduction ($p < 0.05$) in the average surface area of the wounds treated with the Madecassol compared to NC and C groups. The evaluation of the macroscopic appearance of the wounds shows that they appear less inflamed as the days progress. Furthermore, nearly wound closure was observed in groups PC, ATRIPLUS 5, and ATRIPLUS 10 on day 21, while the wounds of untreated and treated with the basic cream animals were far behind (Fig 3).

Fig.2. Wound healing activity of *ATRIPLUS* cream on pourcentage (%) of wound contraction in exision model . Prepared by the authors (2024).Each value represents the mean ± standard deviation. (*) P<0.05; (**) P<0.01; (***) P<0.001: Significant difference compared to the control

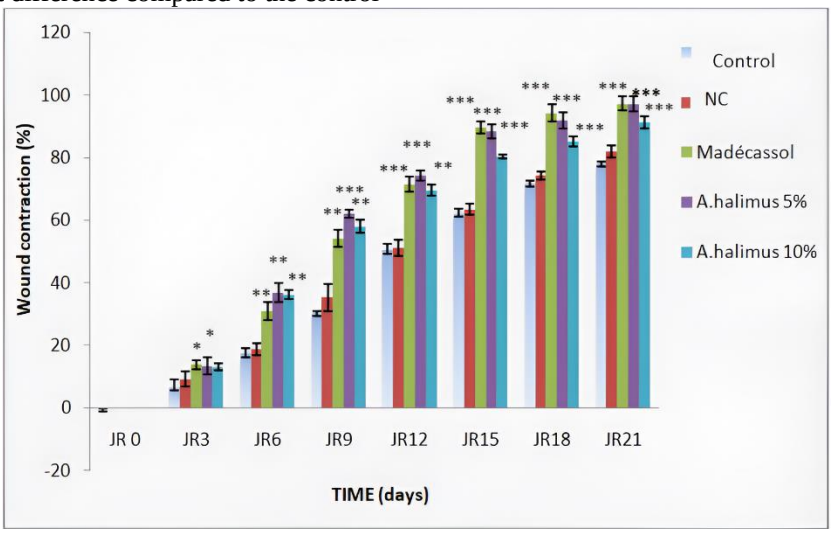
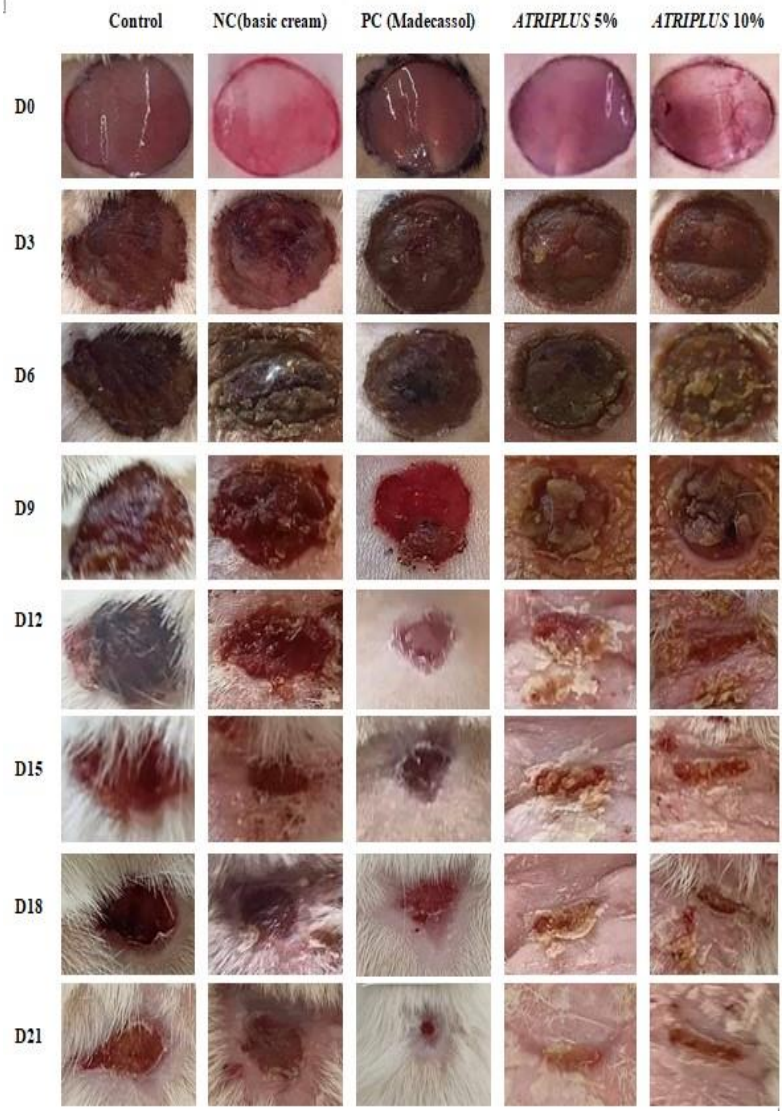


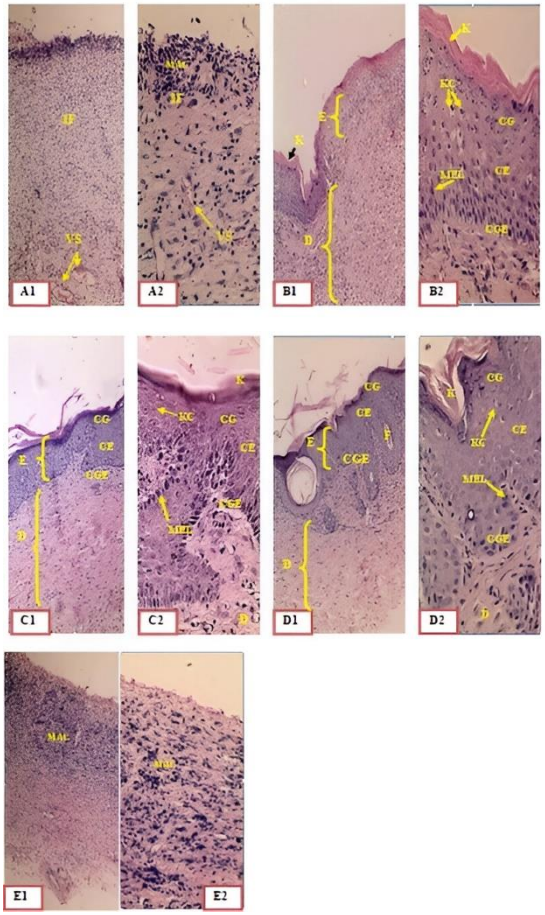
Figure 3. Wound contraction timeline over 21 days. Prepared by the authors (2024).



3.5 HISTOLOGICAL ANALYSIS

The histological results show destruction of the two layers of skin tissue (epidermis, dermis) and granulated tissue with aggregation of macrophages and broken collagen in the negative control and control groups, the reconstruction of the two layers in PC, ATRIPLUS 5 and ATRIPLUS 10 groups was observed with the reduction of macrophages numbers and an increase in collagen deposition, reorganization of skin structure and superficial healing with clear tissue remodeling.

Fig.4. Microscopic photograph of a histological section of the skin of rats (Staining with hematoxylin + eosin (A1; B1; C1; D1; E1): x10. (A2; B2; C2; D2; E2): x40). Prepared by the authors (2024).



A: microscopic appearance of a histological section of skin from control rats. B: microscopic appearance of a histological section of skin from rats treated with MADECASSOL. C: microscopic appearance of a histological section in rats treated with ATRIPLUS 10% cream D: microscopic appearance of a histological section in rats treated with ATRIPLUS 5% cream. E: microscopic appearance of a histological section in rats treated with base cream. CE: Spiny layer; CG: Granular layer; CGE: Germinal layer; MAC: macrophages; COLL: Collagen; D: Dermis; E: Epidermis; F:Hair follicle; G: Sebaceous gland; K: Keratin; MEL: Melanocytes.

4 DISCUSSION

A wound is a skin lesions represented by a disruption in the continuity of tissues and a breach of the skin barrier, requiring a complex dynamic process to be repaired or healed (Yazarlu et al. 2021). Many substances such as natural products (Dahanukar et al. 2000) (Bairy 2002), (Raina 2006), vitamins (Chow & Barbul 2014), and minerals (Barchitta et al. 2019) have been reported

by various researchers as having healing effects. Several drugs are used to stimulate the wound healing process originate from plants (Thakur et al. 2011), (Bairy, 2002) (Velnar et al. 2009) described 36 plants that different authors have studied in a theoretical study presenting a review on natural healing agents, the different phyto-constituents, the formulations of several plants, as well as the different nutraceutical products responsible for the healing activity were investigated. However, other plants, used in Algerian folk medicine with a good reputation in the healing process, such as *A. halimus L*, were to be studied in this area of research.

The wound contraction parameter remains a good indication for detecting potentially active extracts. Wound contraction indicates the rate of the healed area during the healing process, and a high rate of wound contraction indicates better treatment efficacy(Rahimi et al. 2013). In this study *Atriplex halimus L* cream showed a significant wound contraction. According to (Bahramsoltani et al. 2017) , natural healing products can act through one of the following mechanisms: antimicrobial, antioxidant effect, anti-inflammatory, or stimulation of collagen synthesis(Amari et al. 2021) . At this stage, we could postulate that the healing activity of the *A.halimus* leaf-based cream could be partly related its antioxidant, antibacterial and anti-inflammatory properties. A more comprehensive assessment of *A. halimus*, including its effect on collagen production, would be useful.

In this study polyphenols (flavonoids, phenolic acids, tannins and alpha-tocopherol) are identified in the leaves of *A. halimus L*, our results are in accord with(Tahar et al. 2015) which found that the leaves of *A. halimus L* are rich in tannins and flavonoids which explains their effectiveness against edema. Flavonoids have anti-inflammatory properties that modulate the functioning of the immune system(Zhao et al. 2017). They are likely to decrease histamine release from basophils and mast cells (Carvalho et al. 2021). A significant increase in rates of tissue remodeling and epithelialization, a decrease in macrophages in the proliferative phase, an acceleration in the rate of wound contraction and a promotion of angiogenesis and vasculogenesis have already been described as flavonoids pharmacology activities (Walid et al. 2024).

Tannic acid (TA) is a polyphenol present in abundance in the galls of Rhus and Quercus species, it is known for its antimicrobial, antioxidant and anti-inflammatory properties, the presence of tannins activate cell multiplication and regeneration, which contribute to increased healing rate (Su et al. 2017). A study carried out by (Orlowski et al. 2018) showed that treatment with TA could reduce the levels of IL-1 and IL-6, after skin lesions were applied to rats. During the inflammatory phase, TA had significant anti-inflammatory activity to protect the organism. Alpha-tocopherol has powerful antioxidant activity that protects membrane components from damage caused by free radicals (Stanizzi et al. 2014). Results show the richness of this plant in minerals elements such as calcium, magnesium, and iron. The mineral elements are involved in wound healing due to their

role as enzymes, structural factors, metalloenzymes, and antioxidants (Barchitta et al. 2019). Ca^{2+} uptake by mitochondria regulates keratinocyte differentiation *In- vitro*. Cu, Zn, and Mn significantly influence the activity of antioxidant enzymes and the redox balance (Wołonciej et al. 2016).

5 CONCLUSION

Based on the presented data, it can be concluded that the cream prepared with the plant *Atriplex halimus L* collected from the Algerian Sahara promotes wound healing activity. It can therefore be suggested for treating various types of wounds in humans. However, the preliminary results suggested that further evaluated for chronic toxicity should be done to ensure the safety of this plant. The aqueous extract of *A.halimus* L was able to reduce wounds healing in rats significantly. *A.halimus* L appears to be a promising plant for further preclinical trials on wounds healing activity. Hence, further research should be carried out to isolate and identify the bioactive compounds responsible for this pharmacological activity .

Competing interest: The authors declare no conflict of interest

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Declarations

Ethical approval and Consent to participate

The experimental study was carried out at UMAB following the Algerian Legislation (Law Number 17-457/2019) inherent to the protection of animals designed for experimental and other scientific purposes as well as with the guidelines of the Algerian Association of Experimental Animal Sciences (AASEA). this experiment was performed on albino rats. No human participants in the current study.

Declaration of generative AI in scientific writing

The AI tools were not to the used to analyze and draw insights from data as part of the research process.

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