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Research Article

Incorporating Preservatives And Additives On Aloe Vera Leaf Gel For Microbiological Stability And Oxidative Resistance During Storage: Wound Healing Assay In Vivo.

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Abstract

The therapeutic properties of Aloe vera gel are widely recognized. However, it should be stabilized immediately after extraction to avoid its rapid deterioration upon exposure to environmental conditions. In this study, the fresh gel was stabibized by incorporating benzoic acid (0.1 %), vitamin E (300 ppm) and xanthium gum (0.2 %) as preservatives and additives. Then, we monitored pH change, total antioxidant activity and microbial growth throughout the storage at 4°C and 25°C. At 4°C, the stabilized gel with preservatives showed the best antioxidative stability with maintaining the percentage DPPH inhibition up to 50 % after 30 days of storage Adding benzoic acid on fresh gel as an antimicrobial and maintaining an acidic pH (3.57-3.74) during storage were found to be effective against the development of spoilage micro-organisms. Unlike, the no stabilized gels which showed a microbial contamination with an increase in pH values. Furthermore, the healing effectiveness of the stable gel applied topically one daily to mice's skin wounds until wound closure was assessed by wound contraction every 2 days. Saline solution and Madécassol® cream are used as negative and positive controls respectively. The stable gel used at both concentration on mice groups showed a significant effect on wound closure rate and the shortened healing process compared to control groups. The wound healing began on the 2th post operative day with an increase in wound contraction from 70 and 75 % to 100 % on the 10th day. Thus, our stabilized gel maintained its wound healing efficacy.

Keywords: Aloe vera leaf gel; stabilization; antioxidative stability; microbiological stability; therapeutic efficacy; wound healing.

INTRODUCTION

The *Aloe vera* (A. Barbadensis Miller) is a succulent plant of Liliaceae family that grows in warm tropical and subtropical areas under drought conditions. Now, this plant is distributed all over the world for their therapeutic and cosmetics benefits [1]. The leaves were considered the most important part of the plant. A. vera gel is a mucilaginous jelly obtained from fresh leaves has been widely used in pharmaceutical, nutraceutical and cosmeceutical industries [2-5]. It is a potent source of numerous functional bioactive and nutrients compounds such as polysaccharides, vitamins, enzymes, minerals, sugars, amino-acids, glycoproteins, hormones, phytosterols and other chemicals compounds that were associated to its biological and functional activities [6-9]. Those includes wound healing, anti-inflammatory, anti-microbial, anti-oxidant, immune-boosting, anti-cancer, anti-diabetic, anti-aging and sunburn relief [10-19]. Therefore, A. vera gel has been used as wound healing agent [20-24] and as a natural antimicrobial [25-27] in a variety of wound care products and pharmaceutical formulations such as wound dressing, cotton fabric, hydrogel, alginate-based film, balm, ointment and cream. The gel can promote wound healing and prevent microbial growth in wounds without activating the immune response [24]. The several clinical studies of *A. vera* gel and derived products have reported a significant effect on wound repair mechanisms when they are used as a treatment [28-35]. Their therapeutic efficacy was associated with the improvement

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of skin hydration, oxygenation, nutrition, to stimulate epithelialization, fibroblast proliferation, collagen synthesis and to anti-inflammatory, antioxidant, antimicrobial and immunomodulatory effects. [22, 29, 36]. However, the limiting biomedical and therapeutic applications of A. vera gel include the quick oxidation, fast changing color, odor, viscosity and high enzymatic activity [37- 39]. Its stability is very influenced by the exposition of air, light, heat and microbes [37]. The oxidative phenomenon involves decomposition, a physical, chemical and biological deterioration which lead to the loss of the therapeutic activities [39 - 42]. Hence, *A. vera* gel should be processed by appropriate techniques to avoid change in physicochemical properties and to preserve the bioactive entities. There are various processing techniques applied for sterilizing and stabilizing the *A. vera* gel such as heating and incorporating of chemical preservatives and additives. The disadvantage of heating processes is to destroy the bioactive compounds of the gel with the physicochemical alterations [5, 43 - 45]. Other processing was applied to A. vera gel by adding chelating agent, antioxidants, antimicrobials, chemical stabilizers and natural preservatives to ensure its stability in the dark and cold conditions [39, 46, 47]. Nevertheless, these techniques can be performed with the addition of citric acid, benzoic acid, sodium benzoate, potassium sorbate as anti-microbials and ascorbic acid, vitamin E, vitamin C as antioxidants. Additionally, gums such as guar, xanthium and Arabic gum, being gelling agents dissolved and stables in acidic conditions to increase the viscosity of the gel and improve its texture. The objective of this study was to determine the effect of preservatives and additives incorporation (benzoic acid: 0.1%, vitamin E: 300 ppm and xanthium gum: 0.2%) and storage temperature (4°C and 25°C) in stabilizing fresh A. vera gel during 60 days. The pH change, total antioxidant activity and microbial growth were evaluated throughout storage period at temperature 4°C and 25°C. Furthermore, to asses the healing effectiveness of the stable gel applied topically and daily at 50% and 100% concentrations to mice's skin wounds by wound contraction and closure rate on the 2nd, 4th, 6th, 8th, 10th, 12th and 14th days. The present study was performed to demonstrate whether the processed *A. vera* gel still has the therapeutic properties as wound healing agent.

MATERIALS AND METHODS

Aloe vera gel samples preparation and treatment

Aloe vera leaves were obtained from "Arborescence" greenhouse in Marrakesh, Morocco, as the supplier. Species identification was conducted by Professor Lahcen Ouhmane from Cadi Ayyad University. The species has been classified as *A. barbadensis* Miller L. A voucher specimen is archived in laboratory pharmacology, neurobiology, anthropology and environment. Traditional hand filleted method was adopted to ovoid contamination by the yellow latex. The fresh leaves aged 3 and 4 years were selected and cleaned by dipping in the sodium hypochlorite solution (0.1%) and washing with distilled water. Then, they were cut in the base and leaved to drain vertically for two hours to remove the exudate. The epidermic was separated from the pulp, which was extracted, homogenized and filtered to remove the fibrous materials and to obtain a mucilaginous gel. The *A. vera* gels were immediately subjected to treatments with and without adding preservatives and stabilizers. These included benzoic acid (0.1%), vitamin E (α-tocopheryl acetate, 300 ppm) and xanthium gum (0.2%). The prepared *A. vera* gels samples codes are: GP4 and GP25 means A. vera gel treated with preservatives and stored at 4°C and 25°C respectively. GWP4 and GWP25 means A. vera gel without preservatives and stored at 4°C and 25°C respectively. All A. vera gels samples were stored on amber-colored glass bottles to prevent the effect light. The operations of gel extraction and stabilization were performed under sterile conditions. The analysis of pH, the total antioxidant activity and the microbial growth were carried out periodically every week throughout the storage at temperature 4°C and 25°C. The experiments were performed on all A. vera gel samples (with and without preservatives) and were done in triplicate.

Microbiological analysis and pH determination

The pH was directly measured using pH-meter (Instruments Hanna pH 211 with microprocessor). The microbiological determination was done using the method described by [48] with some modifications. The analysis was performed in triplicate on all *A. vera* gel samples with and without preservatives at different temperatures (4°C and 25°C), to assess the presence of mesophilic and lactic aerobic bacteria, yeasts and molds. One milliliter of each sample was obtained aseptically and homogenized with 9 mL of the physiological water (9‰). Further decimal dilutions were performed with the same diluent, and triplicates of at least three appropriate dilutions were seeded to suitable media. To count mesophilic and lactic aerobic bacteria, 100 µL of each dilution were pour-plated in the nutrient agar and in the MRS medium (Man, Rogosa, Sharpe) agar respectively. After incubation at 30°C for 48 h, mesophilic and lactic bacteria counts were performed. To count mold and yeast, 100 µL of the initial dilutions were inoculated by the same technique in a LB (Luria Basal) medium with an antibacterial agent (Chloramphenicol) 0.5%. The plates were then incubated at 25°C for 3 to 5 days. The microbiological data were transformed into logarithms of the number of units forming colonies (log cfu/ml). The limit of detection was 10 cfu/ml (1.0 log cfu/ml).

Diphenyl-1-1picrylhydrazyl (DPPH)° radical scavenging assay

Total antioxidant activity was determined in triplicate by using the DPPH method [49] based on the percentage DPPH radical inhibition. A. vera gel samples were filtered and diluted at different concentration and transferred into test tubes. 1 ml of DPPH radical prepared in Ethanol (0.006%) was added to test tubes with 1 ml of sample. The reaction mixture was mixed on a vortex for few seconds and left to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using the spectrophotometer that was calibrated with Ethanol. The control sample was prepared without addition of the sample. All solvent and reagents were purchased from Sigma Aldrich.

Total antioxidant activity was expressed at the percentage inhibition of the DPPH radical and determined by follow Equation: DPPH inhibition (%) = [1 – (Abs sample / Abs control) ⌡ x 100 Abs is absorbance at 517nm.

Wound Healing test

Twenty BALB/c adult female mice with weights of (20 to 25 g) were collected from the departmental animal house of the faculty of Sciences Semlalia, Marrakesh, Morocco and randomly divided into four groups: C^-, C^+, AVG 50 and AVG 100 with five animals per group. Group (C^-) serves as the negative control and received saline solution. Group (C^+) serves as positive control group and with a topical application of Madécassol® wound healing cream. **AVG 50** and **AVG 100** groups with a topical application of processed *A. vera* gel at 50% and 100% concentration respectively.

The mice were anesthetized with isoflurane under aseptic conditions. Then, the back of each animal was shaved using sterile blades and two skin wounds were made on both back sides of the median lines using a 6 mm skin biopsy forceps. The wounds of all animals were cleaned before receiving the first application of the *A. vera* gel (0.5 g) or the Madécassol® cream that was applied topically once a day on each wound until the 14th day. The animals were housed in normal conditions and received water and food ad libitum.

Evaluation of wound healing

The excisional cutaneous wounds were photographed regularly every two days and the wound contraction was calculated based on wound aeras (mm^2) measurements relative to the original dimensions [50] using the following equation:

$$\text{Wound contraction (\%)} = \frac{AW_1 - AW_2}{AW_1}$$

AW1 is the initial wound area and AW2 is the wound area after post operative days: 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th},12^{th} and 14^{th} days.

Statistical Analysis

The statistical analysis for all results were performed using Graph Pad Prism V10. Three ways ANOVA test (adding preservatives, storage time and storage temperature) were used to analyze results from experiments performed on A. vera gel samples, while results from at least experiment (Wound healing test in mice) were analyzed using Two ANOVA test (Treatment and Time), followed by Tukey's test for multiple comparisons. All values were expressed as mean ± SD and results were significant at $P < 0.05$.

RESULTS AND DISCUSSIONS

Change of pH change and microbial stability of A. vera gels during storage

The pH values of GP4 and of GP25 *A. vera* gels showed no significant difference during storage (**Fig. 1 A and B**). The values were between 3.66 ± 0.09, and 3.74 ± 0.13 for GP4, and between 3.60 ± 0.11 and 3.70 ± 0.05 for GP25. The pH of these gels was more acidic due to adding benzoic acid as microbial preservative. The acidulant pH allows to inhibit enzymes and improve the stability of the *A. vera* gel [2]. However, the pH values of untreated gels GWP4 and GWP25 stored at 4°C and 25°C respectively showed the significant differences ($p < 0,0001$) throughout storage (**Fig. 1 C and D**). At the beginning of storage, the pH of each gel was acidic with a value of 4.89 ± 0.075 for GWP4 and 4.46 ± 0.08 for GWP25 like the pH of fresh *A. vera* gel that was between 4 and 5. Then, the pH increased significantly ($p < 0,0001$) during storage from 4.89 ± 0.075 to 7.02 ± 0.050 for GWP4 and from 4.46 ± 0.08 to 7.14 ± 0.083 for GWP25. The high values of pH indicate the deterioration of *A. vera* gels due to the microorganism activity as shown in figure (**Fig.2**). An interaction effect between pH and storage period on total aerobic mesophilic bacteria (TAMB) was observed for GWP4 and GWP25 untreated gels, which was more important with an increase in pH values. In respect of storage period, the microbial growth increased gradually. The mean initial population of TAMB increased from 7.21 ± 0.20 to 19.6 ± 0.40 log cfu /ml for GWP4 gels and, from 8.08 ± 0.52 to 20.80 ± 0.2 log cfu /ml for GWP25 gels after 60 days of storage. Yet, no BL, yeasts and molds were detected at all storage period. At the first day of storage of untreated *A. vera* gel, the aerobic mesophilic bacteria count was higher than 2 log cfu /mL, which is considered as the upper acceptable levels according to Word Health Organization WHO (1999 [51]). Other researchers [52] have reported that the aerobic mesophilic bacteria count in no processed *A. vera* gel with high hydrostatic pression (HHP) ranging from 3.41 log cfu / ml to 8 log cfu /ml. The microbial population determined was dominated by aerobic mesophilic bacteria that consisted of gram negative and rod-shaped bacteria (Enterobacteriaceae) such as: *Rhanella aquatilis, Pantoea agglomerans, Enterobacter*

amnigenus, Serratia plymuthica and other isolates belonging to the genera Pseudomonas such as P. fluorescence and P. putida. Concerning microbiological analysis of our stabilized gels with preservatives GP4 and GP25, no microbials were detected during the whole storage period at both temperature 4°C and 25°C. This indicates the efficacy of benzoic acid (0.1%) incorporated to fresh *A. vera* gels as microbial preservative to avoid the growth of microorganisms and to improve the microbiological stability. Our result consisted in the study of [39] that showed the inhibition of natural microbial growth during storage temperature at 7°C and 25°C in A. vera gel stabilized by incorporating citric acid as microbial preservative. In addition, it reported that *A. vera* gel exhibited antimicrobial activity with lowering the growth of microorganisms during storage [53, 54, 10, 13, 46]. In our study, the adding of benzoic acid on fresh *A. vera* gel was effective for delaying microbial proliferation. The presence of antimicrobial compounds, the organic acid and the compounds with acidic properties in fresh *A. vera* gel act synergistically to make microorganisms inactive and to extend its microbiological shelf-life during storage [7, 55, 56].

Figure.1 pH change of all A. vera gels (A) GP4, (B) GP25, (C) GWP4 and (D) GWP25 during storage at 4°C and 25°C. GP4 and GP25: treated gels with preservatives and stored at 4°C and 25°C respectively, GWP4 and GWP25: untreated gels and stored at 4°C and 25°C respectively. Results are expressed as mean ± SD (n = 3). P values were calculated using three ways ANOVA followed with Tukey's test for multiple comparison (no = not significant, the significant differences: *p < 0.05, *** p < 0.001, **** p < 0.0001)

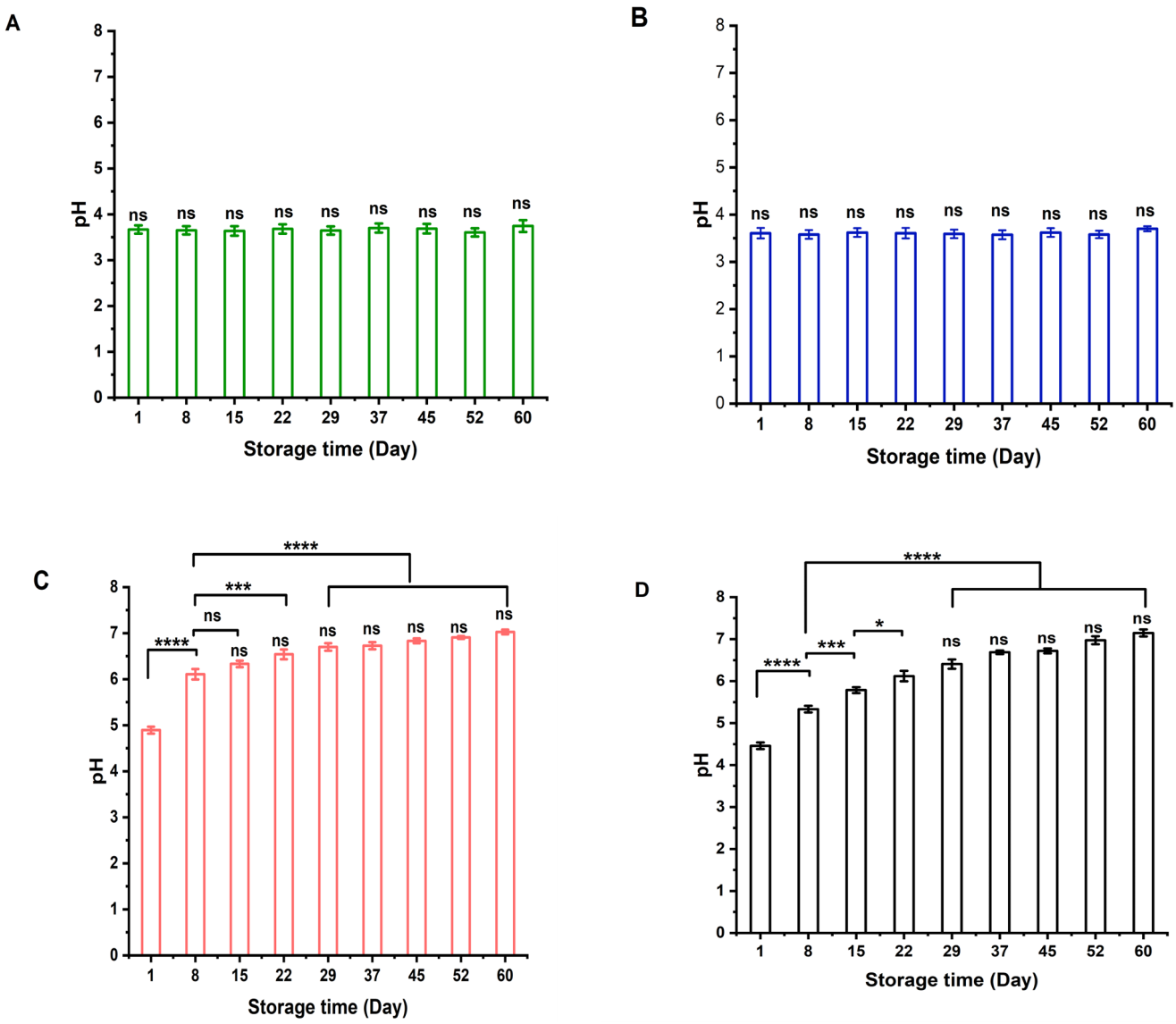
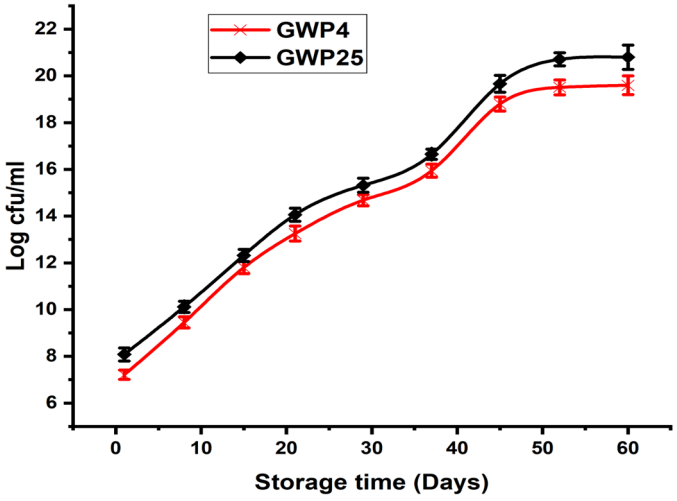


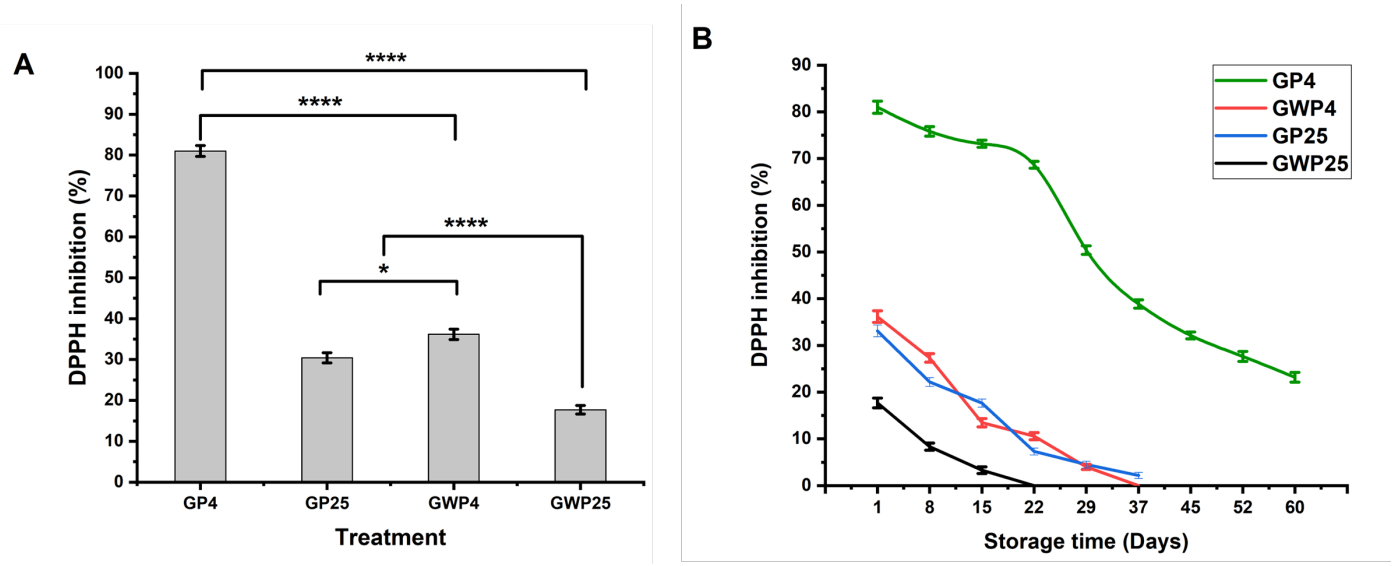
Figure.2 Growth evolution of total aerobic mesophilic bacteria during storage in untreated A. vera gels GWP4 and GWP25 stored at 4°C and 25°C respectively. Data are expressed as (mean ± SD) (n=3), the significant differences (P < 0,0001) at different storage time. Total aerobic mesophilic bacteria are not detected in GP4 and GP25 treated *A. vera* gels stored at 4°C and 25°C respectively, No BL, yeast and molds were detected at all storage time.



Total antioxidant activity of treated and untreated A. vera gels during storage

A decreasing trend on the total activity antioxidant of all treated and untreated gels with preservatives, stored at 4°C and 25°C as a result of the length of storage time (**Fig. 3 A and B**). At the first day of storage, the GP4 treated gel stored at 4°C showed the high significant antioxidant capacity ($p < 0.0001$) with 81.04 ± 1.32 % DPPH inhibitions compared to GWP4 untreated gel stored at the same temperature with 36.30 ± 1.26 % DPPH inhibitions and to GP25 treated and GWP25 untreated gels stored at 25°C with 30.42 ± 1.25 and 17.7 ± 1.05 % DPPH inhibitions respectively, The least untreated gel stored at 25°C showed a great decrease of antioxidant activities from 17.7 ± 1.05 to 6.78 ± 0.76 % DPPH inhibition after 8 days of storage with complete oxidation and deterioration. However, when we incorporated preservatives on the fresh gel and we stored it at the same temperature (25°C), the antioxidant capacity of GP25 treated gel decreased significantly ($p < 0.0001$) from 30.42 ± 1.26 to 7.32 ± 0.76 % DPPH inhibition after 30 days. Furthermore, our results showed that GWP4 untreated gel stored at 4°C a decrease in the antioxidant activities from 36.17 ± 1.26 to 8.97 ± 0.86 % DPPH inhibition after 21 days of storage. At the same storage temperature (4°C), the GP4 treated gel with preservatives showed a significant decrease ($p < 0.0001$) of antioxidant activities from 81 ± 1.32 to 22.50 ± 1.10 % DPPH inhibition after 60 days with maintaining up to 50 % during 30 days of storage. Our result was consisted in the study of [57], that have also reported the high initial total antioxidant capacity of 80.25 % DPPH inhibition in processed *A. vera* gel with high hydrostatic pressure and stored at 4°C. The degree of DPPH inhibition indicates the scavenging of antioxidant compounds of *A. vera* gel such as polysaccharides, β carotene, folic acid, E and C vitamins and B12 [13, 58, 59]. These compounds react with DPPH (free radical scavenging) reducing a number of DPPH molecules. Another study of [60] showed a decreasing trend of the antioxidant capacity of pasteurized *A. vera* gels during storage at 4°C and 25°C up to 30 days from 54 ± 0.85 to 23 ± 2.8 with loss of than 50 % DPPH inhibitions. The same study has also reported that the thermal treatment by pasteurization was the main cause of depletion of the natural antioxidants of *A. vera* gel such as vitamin C and glucomannan during storage at both temperature 4°C and 25°C with a great decrease at 25°C. Our results showed that vitamin E (300 ppm) incorporation on fresh gel as antioxidant can improve the oxidative resistance for our treated gels GP4 during storage particularly at 4°C. Besides this, the decreasing rate of antioxidant capacity was more considerable for GWP25 untreated gel stored at 25°C than the GWP4 untreated gel stored at 4°C due to direct influence of storage temperature. However, the storage at 4°C allowed to maintain the antioxidant capacity of GP4 treated gel up to 50 % of DPPH inhibition for 30 days, while the GP25 treated gel stored at 25°C loss 75 % of the antioxidant capacity. The study of [37] has also reported that the addition of BHT (750 ppm) as antioxidant on A. vera gel, with administrating nitrogen gas prevent the oxidation process and maintain the physicochemical properties a maximum 4 weeks.

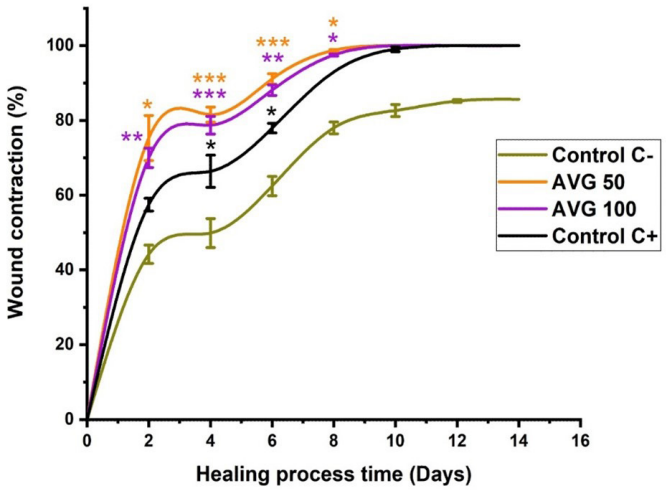
Figure. 3 Total activity antioxidant of all *A. vera* gels: GP4, GP25, GWP4 and GWP25 during storage at 4°C and 25°C after one day of storage (A) and at different storage time (B). GP4 and GP25 treated gels with preservatives and stored at 4°C and 25°C respectively. GWP4 and GWP25: untreated gels and stored at 4°C and 25°C respectively. The DPPH inhibition (%) are expressed as (mean ± SD) (n=3), P values were calculated using three ways ANOVA followed with Tukey's test for multiple comparison (significant differences at *p < 0.05, **** p < 0.0001).



Wound healing evaluation

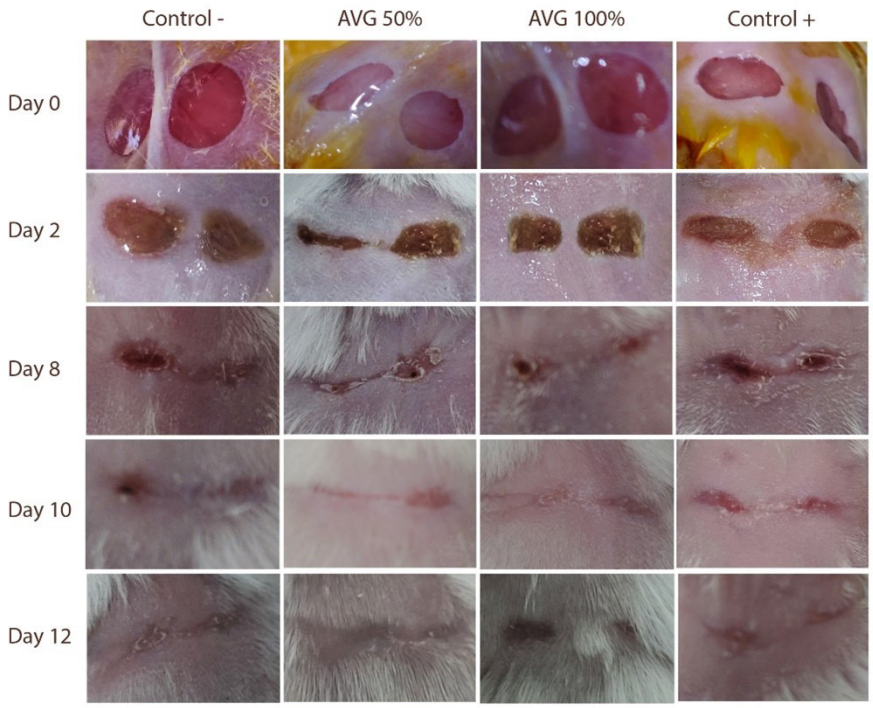
The two ways ANOVA analysis of the percentage of wound contraction during healing process time revealed significant differences among the treatment factors: time (F (1.258, 5.031) = 4628, p < 0.0001) and treatment (F (1.051, 4.203) = 186.7, p = 0.001), as well as the interaction between both factors (F (2.382, 9.529) = 45.31, p < 0.0001). The multiple comparison analysis confirmed that during healing process, an increasing trend of wound closure was observed on all mice groups: AVG 50, AVG 100, C+ and C- (**Fig. 4**). On the 2nd post-operative day, the wound contraction percentages of AVG 50 group that received a topical application of *A. vera* gel at 50% concentration, were significantly higher (75%) than control ones (C+) (57.5%) (p = 0.0057, q = 10.67) and (C-) (44.20%) (p = 0.0105, q = 9.049). Similar results were observed for the AVG 100 group, which showed significant results in comparison with the control groups (C+) (p = 0.0004, q = 20.66) and (C-) (p = 0.0019, q = 14.24).

Figure. 4 Wound contraction percentages of all mice groups during on 2^{th}, 4^{th}, 6^{th}, 8^{th},10^{th},12^{th} and 14^{th} days during healing process. The different treatments are described as follows: Group (C^-) serves as the negative control and received saline solution, Group (C^+) serves as positive control group with a topical application of Madécassol® wound healing cream, AVG 50 and AVG 100 groups with a topical application of processed *A. vera* gel at 50% and 100% concentration respectively. Results are expressed as (mean ± SD) (n = 5) and compared statistically between mice groups during healing process. P values were calculated using two ways ANOVA followed with Tukey's test for multiple comparison. The significant differences: *p < 0.05, ** p < 0.01, *** p < 0.001.



According to the results of figure 4 and to macroscopic wounds observation (**Fig. 5**), there was an improvement rate and an accelerated wound healing process in **AVG 50** and **AVG 100** mice groups. The healing of the last two groups during two post-operative days, showed a wound contraction of 75% and 70% respectively. On the 10th day, 100% wound contraction has completely occurred. However, the wound healing of positive control group (C^+), which received a topical application of Madécassol® cream was better than negative one (C^-). The stable *A. vera* gel used topically at both concentrations of 50% and 100% on wounded mice groups provide a good healing effect and more rapid wound closure. Several studies have also reported the promoting healing of topical application of *A. vera* gel on experimental animal models [61 - 66]. A study of [65] investigating the wound healing efficiency of *A. vera* gel in an excision wound model in rats has shown a faster wound healing. This process began to occur on 4th post-operative day and an improvement of the open wounds on 14th days with an increase in wound contraction rate. It also, reported an accelerating epithelization and fibrosis and with less inflammation and neovascularization during wound healing process. The *A. vera* gel could stimulate the expression of growth factor genes in the damaged skin of animals and play a crucial role in the wound healing process [65]. In our study, the stable *A. vera* gel action on wound cure indicates the shorter days of healing compared to the previous study. Another study of [66] related to A. vera gel action on excisional wounds in adult rat models has also shown an improved rate of wound healing in shorter days (9 days). Furthermore, an improvement occurred in the hematological blood profile. Based on our results and on the other studies, the stable *A. vera* gel applied topically in mice's wounds has a positive effect on wound healing with more rapid area contraction and can preserve its bioactive molecules. These can act synergically and exhibited the wound occlusive properties on cutaneous wounds of mice during wound healing process. The biochemicals compounds include acemannan, phytosterols, glycoprotein, brady-kinases, minerals, vitamins A, C and E, Amino-acids, saponins, gibberellins, stimulate formation of epidermal tissues, increase collagen and proteoglycan production and remodeling, while improving wound tensile and inhibiting inflammation [7, 13, 67, 68]. Additionally, the active molecules present in *A. vera* penetrate the tissue, to increase blood flow, to stimulate repair wound mechanisms and to prevent microbial growth during wound healing process. Because, *A. vera* gel has the excellent anti-microbial activities against bacteria, fungi and viruses [69 - 72], it can also be used as anti-microbial and as disinfecting agents to treat infections in wound's site [73].

Figure. 5 Progression of wound closure area of all mice groups **AVG 50, AVG 100**, Control C+ and Control C- on 2^{th},4^{th}, 8^{th}, 10^{th} and 12^{th} days during wound healing process. Photographed images of wounds created on the mice groups AVG 50, AVG100, Control C^+ and the Control C^- from day 1 till the day 12 of wound closure.



CONCLUSION

Based on this research, it can be concluded that the incorporation of benzoic acid, vitamin E and xanthium gum as stabilizers on fresh A. vera gel improved the oxidative resistance and ensured the microbiological stability during storage at temperature 4°C. The stable gel maintained the healing properties and provided a good effect on cutaneous wounds of mice. It can be used as an economic and an effective therapeutic option for wound healing as well. Further researches need to be done in order to assess the efficacy of topical application of A. vera gel on cutaneous wounds as a healing agent by histological, biochemical and microbiological approaches.

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Ethical Approval

All animal studies on wound healing followed the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama (Protocol # 18-08-1483) [74]. The study was also approved at national level by the Research Laboratory Council Committee of the Faculty of Sciences, Cadi Ayyad University, Marrakech, Morocco.

Consent to Participate

All research participants provided their informed consent freely and voluntarily to take part in the study.

Consent to Publish

All authors have provided their consent for publication in the journal "Applied Biochemistry and Biotechnology."

Authors Contributions

H. Chernane: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. M. **Sbahi**: Conceptualization, Methodology, Statistical Analyses, Writing – original draft. A. Choukri: Conceptualization, Methodology, Writing – original draft. **E. Darrag**: Conceptualization, Methodology. A. **Hammoumi**: Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

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Competing Interests

The authors declare no competing interests.

Availability of data and materials The data used to support the findings of this study are available from the corresponding author upon request.

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