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Article

Antibacterial and Wound Healing Activity In Vitro of Individual and Combined Extracts of *Tagetes nelsonii* Greenm, *Agave americana* and *Aloe vera*

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Abstract: Currently, there are various physical and mechanical agents that can cause skin wounds, which are still traditionally treated with plant extracts. It has been reported that the genus Tagetes has a wide range of biological properties, including antibacterial and wound healing activity. Likewise, *Agave americana* extract and *Aloe vera* gel have shown potential in the treatment of burn wounds and other skin conditions both in vitro and in vivo. In this study, the antibacterial and wound healing activities of each of these plants were investigated, as well as the possibility of enhancing these activities by combining them. First, the secondary metabolites of the extracts were quantified, the antibacterial activity was evaluated using the Kirby-Bauer method, and their cytotoxicity was measured in 3T3 and HaCaT cells using the sulforhodamine B assay. The results revealed that *Tagetes nelsonii* extract had a higher amount of secondary metabolites, which is why it exhibited antibacterial activity. Finally, the scratch assay showed that the individual extracts of *T. nelsonii* and *A. americana* demonstrated greater cell migration and proliferation starting from 12 h, as well as when using the combination of *A. americana* extract and *A. vera* gel, which almost completely closed the wound compared to the control.

Keywords: phytochemical profile; antibacterial activity; cytotoxicity; scratch assay; wound-healing activity

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1. Introduction

The human skin is susceptible to injuries or wounds caused by physical, mechanical, and chemical agents, which disrupt its integrity and induce systemic changes in health [1,2]. One of its characteristics is the ability to self-regenerate, which requires the involvement of specialized cells capable of participating in the healing process [3,4]. Indeed, although wound healing is a natural process, the presence of aerobic and anaerobic micro-organisms can lead to severe wound infections that require immediate attention [5–7]. In recent years, plants have been utilized in the pharmaceutical, cosmetic, and medicine industries due to their bioactive compounds with therapeutic properties. Thanks to this, they are a promising option for wound treatment and healing [8–10].

Tagetes nelsonii Greenm, commonly known as "*chik chawa*", is a plant belonging to the Asteraceae family, native to the state of Chiapas. It has been empirically used by various ethnic groups to treat colds, respiratory problems, and stomach disorders [11,12]. Although there is limited research on the therapeutic properties of these species, the Tagetes genus is known for its anti-inflammatory, insecticidal, antioxidant, antihypertensive, an-tileukemic, antimicrobial, and wound-healing activities [13–16]. In particular, for *T. nelsonii*, research on its therapeutic activities is limited, and its bactericidal activity has been reported against both Gram-positive and Gram-negative microorganisms [17,18]. Additionally, it has been noted that *T. nelsonii* contains volatile compounds such as (E)-Tagetone, (Z)-Tagetone, β-ocimene, α-copaene, β-caryophyllene, and eucalyptol, which exhibit anti-inflammatory and antioxidant activities, among others [19,20].

On the other hand, *Agave americana*, from the Agavaceae family, is native to Mexico, Arizona, and Texas, and it is traditionally used as an antiseptic, diuretic, and laxative due to its phenolic acids, flavonoid glycosides, homoisoflavonoids, and saponins [21–23]. It has been reported to have wound-healing and antibacterial properties that contribute to the healing of wounds [24–28]. Similarly, *Aloe vera* is another plant with remarkable wound-healing properties widely used for the treatment of wounds. The parenchymatous gel from its leaves is extensively studied due to the variety of its bioactive compounds such as polysaccharides, tannins, sterols, fatty, amino and organic acids, enzymes, saponins, vitamins, and minerals [29,30]. It has been shown that the fresh gel of *A. vera* has the ability to promote tissue repair in wounds due to its high water content, which helps keep the wound moist, enhances the migration of epithelial cells, and stimulates hyaluronic acid synthesis [31,32]. Additionally, it has demonstrated antibacterial activity against various pathogenic microorganisms [33,34].

Medicinal plants may exhibit individual biological activities; however, it is possible that their therapeutic properties can be enhanced in a synergistic context by combining them. Previous findings have demonstrated that a combination of plant extracts significantly enhances wound healing through cell proliferation and migration compared to the use of individual extracts [35–37]. In Mexico, there have been few studies on the combined use of plant extracts for wound healing, so the aim of this study was to analyze the in vitro antibacterial activity and wound-healing activity of individual and combined extracts of *T. nelsonii*, *A. americana*, and *A. vera* gel, plants that are traditionally used by the Mexican population.

2. Materials and Methods

2.1. Plant Material

The plants were collected between July and August 2021 from different locations in Chiapas: *Tagetes nelsonii* Greenm in the city of San Cristobal de las Casas (16°43′22.7″ N 92°37′05.6″ W), *Aloe vera* (L.) Burm. f. in the municipality of Ocozocuautla de Espinoza (16°37′37.6″ N 93°20′19.7″ O), and *Agave americana* (L.) in the municipality of Comitan de Dominguez (16°23′58.2″ N 92°12′46.6″ W). The plants were identified with the assistance of a professional botanist from the CHIP herbarium (voucher number: 56564, 55137, and 55136), which belongs to the Dr. Faustino Miranda Botanical Garden.

2.2. Preparation of Plant Extracts

The leaves of *Tagetes nelsonii* and *Agave americana* were washed and dried in an oven at 40 °C for 3 and 20 days, respectively. Subsequently, they were ground, and mixtures with methanol (1:10) were prepared, which were macerated for 48 h. After that, they were subjected to sonication (Cole-Parmer 08855-00, Vernon Hills, IL, USA) at 20 °C for 2 h [38]. Each extract was then filtered, centrifuged (HERMLE Labortechnik, Wehingen, Germany) at 3500 rpm for 15 min, and concentrated under reduced pressure using a rotary evaporator (BUCHI-R210, Labortechnik AG, Flawil, Switzerland) at 40 °C. The obtained extract was collected with sterile water in 50 mL conical tubes and frozen for lyophilization (Labconco, Kansas City, MO, USA). The lyophilized extract was stored in 50 mL conical tubes covered

with aluminum foil and frozen until further use [26,39–42]. For the extraction of *Aloe vera* gel, fresh healthy leaves were collected and washed, and then cut crosswise to remove the inner parenchymatous gel. Subsequently, the gel was homogenized in an electric blender (TAURUS, Oliana, Spain), centrifuged at 3500 rpm for 15 min. The gel was lyophilized and stored at 4 °C [29].

2.3. Phytochemical Analysis

The presence of sterols, terpenes, carbohydrates, and anthraquinones in the extracts was determined by previously reported protocols [43–46]. The qualitative analysis of flavonoids, tannins, saponins, and coumarins was performed using thin-layer chromatography (TLC) with silica gel 60 F_{254} plates, 10 × 10 cm (0.25 mm thick) (Merck®, Mexico City, Mexico), as a stationary phase. Then, 10 µL of each extract was applied to 1 cm from the lower limit. The methanolic extract sample was eluted in two movil phases: one with hexane-ethyl acetate-acetic acid (31:14:5) and one with methanol-chloroform-ammonium hydroxide (8.4:1.4:0.1). Chromatography plates were developed and revealed according to Wagner and Bladt [47].

The quantification of secondary metabolites was performed using UV-Visible spectrophotometry (HACH DR 5000, Loveland, CO, USA) through the following colorimetric methods: total phenolics were estimated using the Folin–Ciocalteu assay described by Singleton et al. [48]. This involved adding distilled water (1.5 mL), Folin–Ciocalteu reagent (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) (100 µL), and 20% sodium carbonate (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) (300 µL) to the sample. Gallic acid (1 mg mL^{-1}) was used as standard curve at a wavelength of 760 nm (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The results were expressed as milliequivalents of gallic acid per gram of dry extract.

The content of flavonoids was determined using the 2-aminoethylphenylborate method described by Ying and Wang [49]. It was added to the sample methanol (1 mL) and solution of 1% 2-aminoethyldiphenylborate (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) in methanol (50 µL). Rutin in methanol (0.1 mg mL^{-1}) was used as standard curve at a wavelength of 404 nm (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The results were expressed as milliequivalents of rutin per gram of dry extract.

The analysis of saponins was conducted according to the method described by Sim et al. [50] using a diosgenin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) standard curve in chloroform at a wavelength of 544 nm (J.T.Baker, Philadelphia, PA, USA) (1 mg mL^{-1}). It was added to the sample or dilution of 5% vanillin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) in methanol (300 µL) and 72% sulfuric acid (J.T.Baker, Philadelphia, PA, USA) in water (1.20 µL). The results were expressed as milliequivalents of diosgenin per gram of dry extract.

The quantitative estimation of tannins was carried out using the method described by Broadhurst and Jones [51] with modifications. This involved 8% vainillin in methanol (1 mL) and a solution of 72% sulfuric acid in methanol (1 mL) to the sample solution. Catechin in methanol (0.1 mg mL^{-1}) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used as standard curve at a wavelength of 500 nm. The results were expressed as milliequivalents of catechin per gram of dry extract.

The content of coumarins was determined using the method described by Akyar [52] with modifications. This involved adding 0.6 mL of 80% methanol, 0.9 mL of 5% lead acetate in methanol (J.T.Baker, Philadelphia, PA, USA), and 1.5 mL of HCl (J.T.Baker, Philadelphia, PA, USA) to the sample. Umbelliferone in methanol (0.1 mg mL^{-1}) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used as standard curve at a wavelength of 320 nm. The results are expressed as milliequivalents of umbelliferone per gram of dry extract.

2.4. Identification of Components in Extracts by Gas Chromatography Coupled to Mass Spectrophotometry

The composition of volatile compounds present in the different extracts were identified by coupled GC-MS (mass spectrometry) using an Agilent Technologies 7890 (Wilmington, NC, USA), fitted with gas chromatograph (MSD VL 5975 C), and the 8270D method [53]. The identification of the compounds was carried out by comparing the mass spectra obtained with those of the NIST 2.0 library.

2.5. Bacterial Strains

Microbial cultures of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Pseudomonas aeruginosa* (ATCC 27853) were used, which were maintained on trypticasein soy agar (MCD LAB, Mexico City, Mexico), incubated at 37 °C.

2.6. Determination of Antibacterial Activity Using the Disc Diffusion Method

The agar diffusion method with impregnated discs was employed based on the Kirby-Bauer method. For the preparation of the inoculum, two well-defined colonies were taken with a bacteriological loop and inoculated in 3 mL of distilled water, followed by incubation at 37 °C. The density was adjusted by comparing it with the standard turbidity of tube number 5 on the McFarland scale at 620 nm using spectrophotometry until a density of 1×10^8 CFU mL^{-1} was obtained [54,55]. All dry extracts were dissolved in dimethyl sulfoxide (DMSO) (MEYER, Mexico City, Mexico) at concentrations of 100, 400, and 800 mg mL^{-1}. Petri dishes with Mueller–Hinton medium (DIBICO, Mexico City, Mexico) were prepared, and 20 µL of the extracts were administered on each sterile Whatman No. 1 filter paper disc, which had a diameter of 6 mm. DMSO was used as the negative control and 10 µL of ciprofloxacin (5 mg mL^{-1}) as the positive control [26,55]. The Petri dishes were incubated at 37 °C for 24 h. The same procedure was followed for the antibacterial activity of the combined extracts. The extracts were added to the filter paper discs in the same quantities. The experiments were performed in triplicate. The inhibition zones were measured in millimeters. The results were reported as sensitive (S) ($\geq$18 mm), intermediate (I) (13–17 mm), or resistant (R) ($\leq$12 mm) [26,55–57].

2.7. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The broth microdilution method in tryptic soy casein broth (TS) (MCD LAB, Mexico City, Mexico) was performed according to the protocols established by the Clinical and Laboratory Standards Institute (CLSI) using 96-well microplates [58,59]. Each test was performed in triplicate for the three microorganisms and the three extracts at concentrations of 12.5, 25, 50, and 75 mg mL^{-1}. A positive control (antibiotic) and a negative control (DMSO) were also included. The microplates were incubated at 37 °C for 24 h. The absence of color change in the dilution indicated the MIC [60,61]. The determination of MBC was performed using the plate casting and drop plate methods. The concentration that did not produce any bacterial colony was considered as the MBC [38].

2.8. Cell Maintenance

The mouse embryonic fibroblast cell line (3T3) ATCC CRL-1658 was donated by PhD Leticia Rocha Zavaleta from Institute of Biomedical Research, UNAM [62], and the human keratinocyte cell line (HaCaT) was donated by PhD Nadia Jacobo Herrera from National Institute of Medical Sciences and Nutrition Salvador Zubiran [63]. In addition, 3T3 and HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM-F12) (Gibco, Thermo Fisher Scientific, Wilmington, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest LLC, Riverside, MO, USA) and 1% antibiotic-antimycotic (Caisson labs, Smithfield, UT, USA). They were then incubated (Steri-Cycle i160, Thermoscientific, Waltham, MA, USA) at 37 °C with 5% CO_2 for 24 h to allow 85% cell confluence. The cells were washed with 1 mL of phosphate-buffered saline (PBS) and treated with 800 µL

of 0.002% trypsin-EDTA (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), followed by incubation under the same conditions for 5 min. The cell pellets were collected and centrifuged (Labofuge 400, Thermoscientific, Wilmington, MA, USA) at 1500 rpm for 5 min. The supernatant was discarded, and the cell pellet was resuspended in fresh culture medium. The cell concentration was determined by counting in a Neubauer chamber using an inverted microscope (ECLIPSE TS100, NIKON, Tokyo, Japan) [33].

2.9. In Vitro Cytotoxicity Assay

The cytotoxicity of the crude extracts was evaluated using the sulforhodamine B (SRB) assay. First, 3T3 and HaCaT cells were seeded in 96-well plates at a concentration of 1×10^4 cells per well and incubated for 24 h to allow monolayer formation. Subsequently, the cells were treated with different concentrations of the extracts dissolved in DMEM-F12 medium with 2% FBS. The concentrations of the methanolic extract (dissolved in DMSO) used were as follows: *T. nelsonii*: 20, 50, 90, 120, 150, 250, 350, 450, 550, 650, 750 and 1000 µg mL^{-1}; *A. americana*: 1, 3, 7, 12.5, 25, 30, 40, 50, 70, 90, and 100 µg mL^{-1}; *A. vera* gel: 15, 30, 45, 60, 75, 90, and 100% *v*/*v*. Each microplate was photographed using an inverted microscope (TMS, NIKON, Tokyo, Japan) at 24 and 48 h. The cells were then fixed with 100 µL of 10% trichloroacetic acid (TCA) (J.T.Baker, Philadelphia, PA, USA) for 1 h at 4 °C, washed four times with tap water, and inverted to air dry for 24 h. Subsequently, the microplates were stained with 100 µL of 0.057% (*w*/*v*) SRB (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), incubated for 30 min at room temperature, and washed four times with 1% acetic acid to remove unbound dye. The plates were inverted and air dried for 24 h. Then, 200 µL of 10 mM Tris base solution was added to solubilize the bound dye, mixed on a rotary shaker (BIO-RAD Laboratories, S.A., Alcobendas, Spain) for 10 min, and the absorbance was measured at a wavelength of 510 nm using a plate reader (Agilent Epoch-Biotek, Santa Clara, CA, USA). The percentage of cell viability was calculated using Formula (1) [64].

$$Cell\ viability\ (\%) = \frac{OD\ treated\ cells}{OD\ control\ cells} \times 100 \quad (1)$$

Each assay was performed for the three extracts in triplicate, using the extract-free medium with the solvent as the negative control [64]. For the calculation of the LC_{50} values, the Hill function analysis of the dose-response curves was obtained through the application of the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA).

2.10. In Vitro Scratch Assay

3T3 and HaCaT cells were cultured in 6-well plates (5×10^5 cells per well) with DMEM culture medium supplemented with 2% FBS and incubated at 37 °C, 5% CO_2 for 24 h until reaching 90% cell confluence. After 24 h, a horizontal line was drawn at the bottom of each well, and two vertical scratches were made in each well using the tip of a 200 µL micropipette to simulate the wound. The cultures were washed with PBS to remove cellular debris. Then, 3T3 and HaCaT cells were treated with dilutions of 100 and 74 µg mL^{-1} of *T. nelsonii* extract, 20.53 and 15.5 µg mL^{-1} of *A. americana* extract, and 36.3 and 26.69% of *A. vera* gel. The combinations of the extracts were made according to the total volume of each well as follows:

Double combinations:

- 1.5 mL of *T. nelsonii* extract + 1.5 mL of *A. americana* extract
- 1.5 mL of *T. nelsonii* extract + 1.5 mL of *A. vera* gel
- 1.5 mL of *A. americana* extract + 1.5 mL of *A. vera* gel

Triple combination:

- 1 mL of *T. nelsonii* extract + 1 mL of *A. americana* extract + 1 mL of *A. vera* gel

Cells without treatment were used as the negative control. Immediately after scratching, photographs were taken with a phase contrast microscope (ECLIPSE TS100, NIKON,

Japan) using a 4 X objective to observe cell migration in the wound at 0, 6, 12, 24, and 48 h [64–66]. The open area of the wound was determined by analyzing the images using ImageJ software (version 1.50, Bethesda, MD, USA) [67,68]. The percentage of wound closure was calculated using Formula (2).

$$Wound\ closure\ (\%) = \frac{Ti - Tf}{Ti} \times 100 \quad (2)$$

where Ti = wound area at 0 h relative to the total analyzed area; Tf = wound area at 6, 12, 24, and 48 h of treatment relative to the total analyzed area.

2.11. Statistical Analysis

All experiments were performed in triplicate, and data were analyzed using GraphPad Prism™ software (version 5.01, La Jolla, CA, USA). Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Data are presented as mean ± standard deviation (SD), with a significance level of $p < 0.05$.

3. Results

3.1. Phytochemical Analysis

The characterization of the bioactive compounds in the extracts of *T. nelsonii*, *A. americana*, and *Aloe vera* gel was performed using colorimetric methods. Table 1 displays the qualitative colorimetric tests of the methanolic extracts, wherein the abundant presence of flavonoids, sterols, and terpenes can be observed in the *T. nelsonii* extract, and it is important to mention that these determinations are the first reported for the *T. nelsonii* species. The *A. vera* gel mainly showed the presence of carbohydrates and saponins, while *A. americana* exhibited a moderate content of saponins and tannins.

The highest content of phenols (2.220 ± 0.014 mg Eq gallic acid g^{-1}) and flavonoids (1.313 ± 0.006 mg Eq rutin g^{-1}) was determined in the *T. nelsonii* extract (Table 2). As for the *A. vera* gel and *A. americana* extract, these stood out for their saponins and tannins content. However, they showed a lower concentration of metabolites compared to *T. nelsonii*.

Table 1. Qualitative screening of phytochemical components in the methanolic extracts from *Tagetes nelsonii*, *Agave americana*, and *Aloe vera* gel.

Secondary Metabolites	*T. nelsonii*	*A. americana*	*A. vera*
Sterols	+++	+	+
Terpenes	+++	+	+
Anthraquinones	+	+	+
Carbohydrates	−	+	+++
Saponins	+	++	++
Flavonoids	+++	+	+
Tanins	+	++	+
Coumarins	+	+	+

The presence of phytochemicals is shown as abundant (+++), moderate (++), low (+), and absence (−) [34,35].

Table 2. Phytochemical quantification in the methanolic extracts from *Tagetes nelsonii*, *Agave americana*, and *Aloe vera* gel.

Secondary Metabolites	*T. nelsonii*	*A. americana*	*A. vera*
Total phenols	2.220 ± 0.014	0.102 ± 0.005	0.138 ± 0.007
Flavonoids	1.313 ± 0.006	0.026 ± 0.002	0.019 ± 0.001
Saponins	0.406 ± 0.018	1.009 ± 0.002	0.188 ± 0.014
Tannins	0.871 ± 0.011	0.518 ± 0.003	0.014 ± 0.002
Coumarins	0.103 ± 0.007	0.030 ± 0.001	0.020 ± 0.001

Values expressed in milligrams (equivalent of gallic acid, rutin, diosgenin, catechin, and umbeliferone as appropriate) per gram of dry extract (mg g^{-1}).

3.2. Antimicrobial Activity, Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) of the Plant Extracts

The inhibition zone values obtained with concentrations of 100, 400, and 800 mg mL^{-1} of the extracts are showed in Table 3. The *T. nelsonii* extract did not have a statistically significant difference concerning the extract concentration. However, the strains of *S. aureus* and *P. aeruginosa* were found to be sensitive to the 800 mg mL^{-1} concentration, with inhibition zones of 19.93 ± 0.11 mm and 18.00 ± 2.00 mm, respectively, while *E. coli* showed resistance. On the other hand, the *A. americana* extract and *A. vera* gel did not exhibit antibacterial activity against the tested microorganisms. On the contrary, the activity of the *T. nelsonii* extract was not affected when combined with the *A. americana* extract and *A. vera* gel against the strains *S. aureus* and *P. aeruginosa*.

Table 3. Antimicrobial activity of the methanolic extracts from *Tagetes nelsonii*, *Agave americana*, and *Aloe vera* gel.

Concentration	100 mg mL^{-1}			400 mg mL^{-1}			800 mg mL^{-1}		
	Inhibition Zone (mm)								
Microorganism	**P.a**	**E.c**	**S.a**	**P.a**	**E.c**	**S.a**	**P.a**	**E.c**	**S.a**
T. nelsonii	17.00 Aa	10.67 Aa	17.33 Aa	18.33 Aa	13.67 Aa	18.66 Aa	18.00 Aa	10.67 Aa	19.93 Aa
A. vera	WA	WA	WA	WA	WA	WA	WA	WA	WA
A. americana	6.83 B	6.33 B	WA	6.83 B	6.33 B	WA	6.83 B	6.33 B	WA

Mean values of zone of inhibition in millimeters. E.c., *Escherichia coli*; P.a., *Pseudomonas aeruginosa*; S.a., *Staphylococcus aureus*; WA., without activity. Values with the same letter are not significantly different according to the Tukey's multiple range test ($p < 0.05$). Capital letters indicate difference between plants and microorganism. Lowercase letters indicate difference with respect to concentration. Ciprofloxacin as positive control had inhibition zones of 28 (P.a), 25.33 (E.c), and 29.67 mm (S.a).

The minimum inhibitory concentration (MIC) with the *T. nelsonii* extract for all studied microorganisms was found to be 25 mg mL^{-1}, with a bactericidal concentration (MBC) of 50 mg mL^{-1} (Table 4). The casting plate and drop plate methods showed that the inhibition persisted for 72 h after inoculation. In contrast, for *A. americana* extract and *A. vera* gel, turbidity was observed at all tested concentrations for the three microorganisms, making it impossible to determine the MIC and MBC. This was consistent with the antimicrobial activity results, where *T. nelsonii* was the only plant that demonstrated inhibition against *P. aeruginosa* and *S. aureus.*

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of methanolic extract from *T. nelsonii*.

Microorganism	*T. nelsonii*		Ciprofloxacin	
	MIC mg mL^{-1}	**MBC mg mL^{-1}**	**MIC mg mL^{-1}**	**MBC mg mL^{-1}**
Escherichia coli	25	50	0.00016	0.00032
Pseudomonas aeruginosa	25	50	0.00032	0.00064
Staphylococcus aureus	25	50	0.00012	0.00025

3.3. Identification of Components by Gas Chromatography Coupled to Mass Spectrophotometry (GC-MS)

GC-MS analysis allowed the detection and identification of 15 compounds in total (Table 5). In *T. nelsonii* extract, the most abundant compounds identified included limonene, eugenol, phenol, 2,4-bis-(1,1-dimethylethyl), miristic acid, palmitic acid, 7-Hydroxy-2H-1-benzopyran-2-one, phytol, 11,14,17-Eicosatrienoic acid, docosane, and squalene.

Table 5. Metabolites of *T. nelsonii and A. americana* leaves identified by CG-MS.

Extract	R.T. (min)	Name	Class
T. nelsonii	5.839	Limonene	Terpenoids
	10.859	Eugenol	Terpenoids
	12.542	Phenol, 2,4-bis-(1,1-dimethylethyl)	Phenols
	15.578	Myristic acid	Fatty acid
	17.281	Palmitic acid	Fatty acid
	17.709	7-Hydroxy-2H-1-benzopyran-2-one	Coumarin
	19.124	Phytol	Terpenoids
	19.620	11,14,17-Eicosatrienoic acid	Fatty acid
	22.523	Docosane	Alkane
	24.923	Squalene	Terpenoids
A americana	12.540	Phenol, 2,4-bis-(1,1-dimethylethyl)	Phenols
	17.286	Palmitic acid	Fatty acid
	18.868	Heneicosane	Alkane
	19.002	8,11-Linoleic acid	Fatty acid
	19.043	Caprilic acid	Fatty acid
	19.081	Linolenic acid	Fatty acid
	19.124	Phytol	Terpenoids
	22.426	Eicosane	Alkane

For the *A. americana* extract, the most abundant compounds identified were phenol, 2,4-bis-(1,1-dimethylethyl), palmitic acid, heneicosane, 8,11-linoleic acid, caprilic acid, linolenic acid, phytol, and eicosane.

3.4. In Vitro Cytotoxicity of Plant Extracts

Cytotoxicity in cell culture is typically expressed as the LC_{50}, which means the concentration of a substance that is lethal to 50% of the cells. The values obtained of cytotoxic effect indicated that *T. nelsonii* extract does not cause cell damage in lines 3T3 and HaCaT at concentrations of 20–350 μg mL^{-1} for 48 h (Table 6). At higher concentrations (1 mg mL^{-1}), the extract caused a change in typical morphology and a reduction in size (Figure 1).

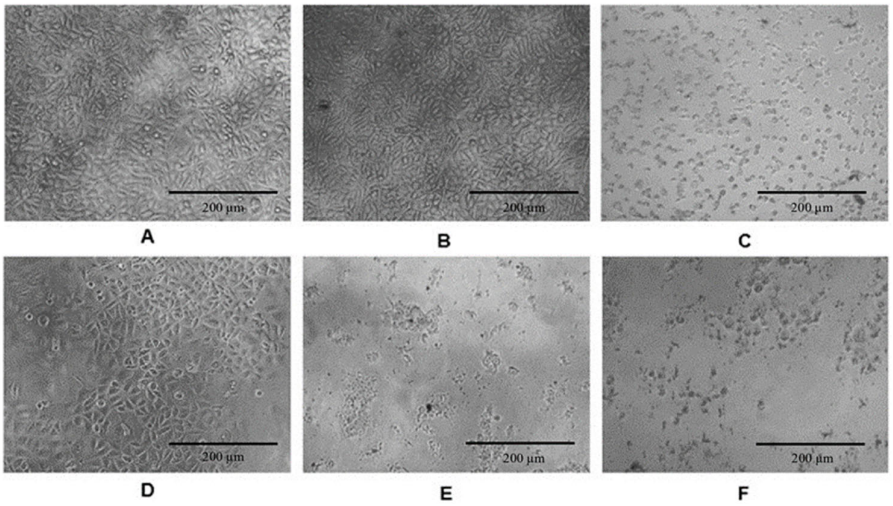

Figure 1. Morphological changes in 3T3 and HaCaT cells cultures exposed to different concentrations of *T. nelsonii* extract for 48 h. Images were taken using an inverted phase contrast microscope (NIKON TMS) at a X10× magnification. (**A**) 3T3 control cells; (**B**) 3T3 cells with 300 μg mL^{-1} without changes; (**C**) 3T3 cells with 1000 μg mL^{-1}; cell detachment; (**D**) HaCaT cells with 100 μg mL^{-1}; (**E**) HaCaT cells with 250 μg mL^{-1} cytoplasmic elongation; (**F**) HaCaT cells with 550 μg mL^{-1} formation of apoptotic bodies.

Table 6. Lethality concentration 50 (LC_{50}) of the methanolic extracts from *Tagetes nelsonii*, *Agave americana*, and *Aloe vera* gel against 3T3 and HaCaT cells cultures.

Extract	3T3	HaCaT
	LC_{50} μg mL^{-1}	LC_{50} μg mL^{-1}
T. nelsonii	346.19 ± 2.47	148.40 ± 1.60
A. americana	40.22 ± 2.43	31.19 ± 1.99
A. vera	64.71 ± 2.99	53.38 ± 4.17

Likewise, *A. americana* extract and *A. vera* gel did not exhibit cytotoxicity at ranges of 1–40 μg mL^{-1} and 15–60% in 3T3 cells cultures, respectively, and 1–31 μg mL^{-1} and 15–50% in HaCaT cells cultures, respectively. Figure 2 displays the effect on the concentration of the extract and gel on the morphology of 3T3 and HaCaT cells after 48 h of exposure. Therefore, cell viability was directly proportional to the extract concentration. Additionally, the analysis of the morphology of 3T3 and HaCaT cells revealed the appearance of characteristics similar to apoptosis at concentrations higher than 50 μg mL^{-1} of the *A. americana* extract (Figure 2A–F) and at concentrations higher than 45% v/v in the case of the *A. vera* gel (Figure 2G–L).

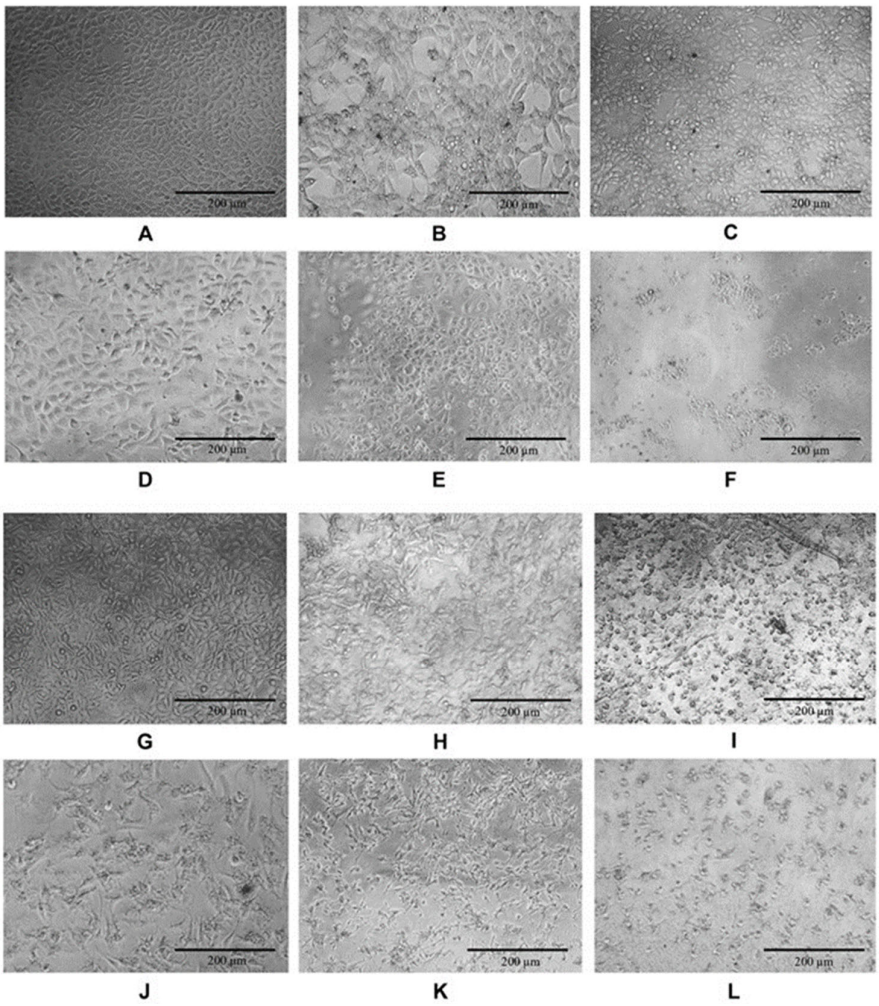

Figure 2. Morphological changes in 3T3 and HaCaT cells exposed for 48 h to different concentrations of *A. americana* extract (**A**–**F**) and *A. vera* gel (**G**–**L**). Images were taken using an inverted phase contrast microscope (NIKON TMS) at a X10× magnification. (**A**) 3T3 control; (**B**) 3T3 cells with 30 μg mL^{-1} formation of plasma membrane extensions; (**C**) 3T3 cells with 50 μg mL^{-1}; the cells exhibit intracellular

granules; (**D**) HaCaT control cells; (**E**) HaCaT cells with 7 μg mL^{-1}; formation of plasma membrane extensions; (**F**) HaCaT cells with 50 μg mL^{-1}; formation of apoptotic bodies; (**G**) 3T3 cells with 15% without changes; (**H**) 3T3 cells with 45% The cells begin to undergo morphological changes; (**I**) 3T3 cells with 75% cellular shrinkage; (**J**) HaCaT cells with 15% cellular condensation; (**K**) HaCaT cells with 30% cellular size reduction; (**L**) HaCaT cells with 60% cells becoming rounder and darker.

3.5. In Vitro Wound-Healing Activity with Scratch Assay of Individual Plant Extracts

Cell migration plays a crucial role in the wound-healing process; therefore, a scratch assay was performed to observe the closure of a simulated wound in response to individual and combined plant extracts, starting from non-cytotoxic concentrations. Figure 3 shows the wound closure percentages of the extracts on HaCaT and 3T3 cells from time 0 to 48 h. The statistical analysis revealed that the *A. americana* extract had a wound-healing effect starting from 6 h, reaching a closure of 89.74 ± 1.83% at 48 h. As seen in Figure 3A, there was no statistically significant difference between the control and the *A. americana* extract at 48 h, but it showed an advantage by having an effect starting from 6 h. Otherwise, for the HaCaT cell line, the statistical analysis showed that all treatments had a wound closure percentage above 70%, except for the *A. vera* gel at 48 h. However, it can be observed that the *T. nelsonii* extract showed a statistically significant difference compared to other treatments starting from 6 h (Figure 3B). Nevertheless, the *A. americana* and *T. nelsonii* extracts showed greater cell migration accelerating the reduction of the wound area compared to the *A. vera* gel (Figures 4 and 5).

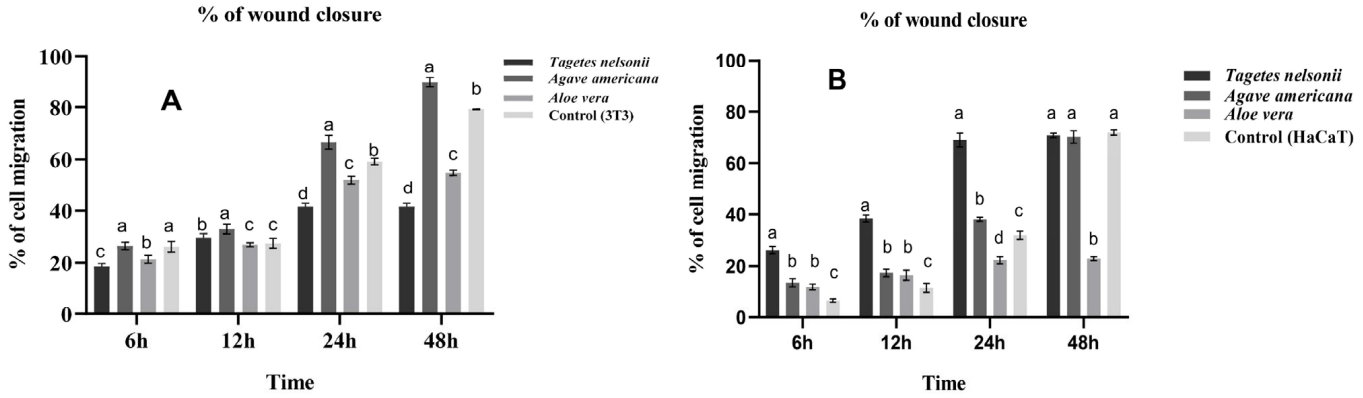

Figure 3. Effect of the extracts on wound closure expressed as percentage of cell migration. (**A**) on 3T3 cell culture with *T. nelsonii* extract (100 μg mL^{-1}), *A. americana* extract (20.53 μg mL^{-1}), *A. vera* gel (36.93% *v*/*v*), and control (untreated cells). (**B**) on HaCaT cell culture with *T. nelsonii* extract (74 μg mL^{-1}), *A. americana* extract (15.5 μg mL^{-1}), *A. vera* gel (26.69% *v*/*v*), and control (untreated cells). Different letters indicate statistically significant differences among the extracts with respect to each time according to the Tukey's multiple range test ($p < 0.05$).

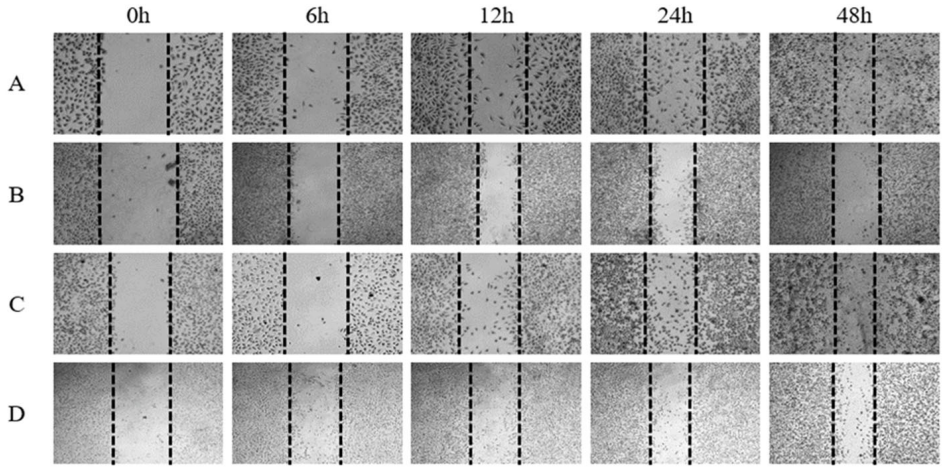

Figure 4. Effect of extracts on fibroblast (3T3) migration with the scratch assay from 0 h to 48 h of incubation: (**A**) untreated cells as control; (**B**) cells treated with *T. nelsonii* extract. No significant reduction in the wound area was observed in the evaluated time periods; (**C**) cells treated with *A. americana* extract. Cellular migration began at 12 h, and a reduction in the wound area was observed at 48 h; (**D**) cells treated with *A. vera* gel. Cellular migration began at 6 h, and a reduction in the wound area was observed at 48 h. Dotted lines delineate the wound edges. Fibroblast migration was photographed using an inverted microscope at a X10× magnification.

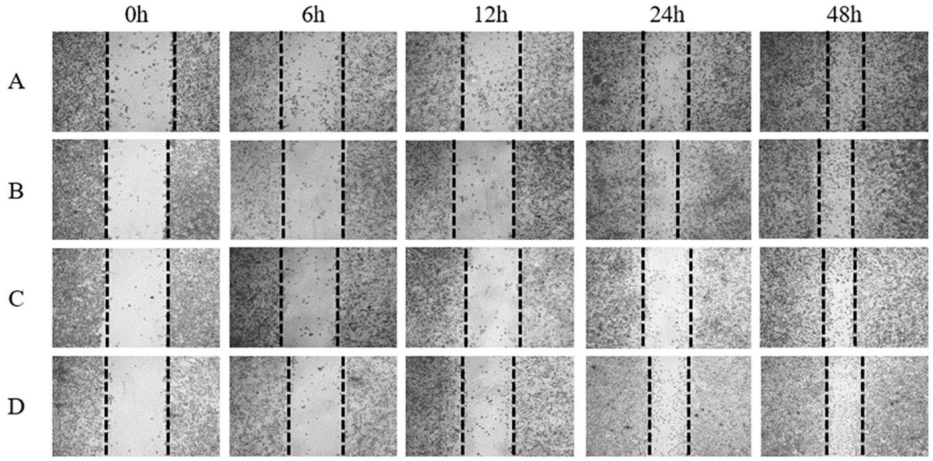

Figure 5. Effect of extracts on keratinocyte (HaCaT) migration with the scratch assay from 0 h to 48 h of incubation: (**A**) untreated cells as control; (**B**) cells treated with *T. nelsonii* extract. Cellular proliferation and migration started at 6 and 12 h, respectively; (**C**) cells treated with *A. americana* extract. The wound closure area at 48 h was the same for rows A and B; (**D**) cells treated with *A. vera* gel. Cellular migration was observed to begin at 12 h. Dotted lines delineate the wound edges. Keratinocyte migration was photographed using an inverted microscope at a X10× magnification.

3.6. In Vitro Wound-Healing Activity with Scratch Assay of Combined Plant Extracts

The wound closure percentages of the combined extracts on 3T3 and HaCaT cells culture are showed in Figure 6; this parameter was analyzed from time 0 to 48 h. The statistical analysis revealed that the best treatment in the 3T3 cell line was the combination of *A. americana* extract and *A. vera* gel, with a closure of 71.46 ± 2.37% and 96.66 ± 0.86% at 6 and 48 h, respectively, at concentrations of 20.53 μg mL^{-1} and 36.3%. These was demonstrated with the micrographs, where we observed an increased cell proliferation and migration that consequently leads to a significant reduction in the wound area in the scratch assay (Figure 7). Similarly, the combination of three plants showed a wound closure of 93.33 ± 0.46% at 48 h (Figure 6A) in 3T3 cell line, also showing a statistically significant difference compared to the control; however, both were above 90%. The other combinations

demonstrated wound closure above 80% at 48 h (Figure 6). In the HaCaT cell line, it was observed a decrease cell migration and proliferation when using the combination of all extracts at 48 h (Figure 8). Nevertheless, when mixes of two plants were tested, a significant difference in wound closure at 24 h was not found (Figure 6B).

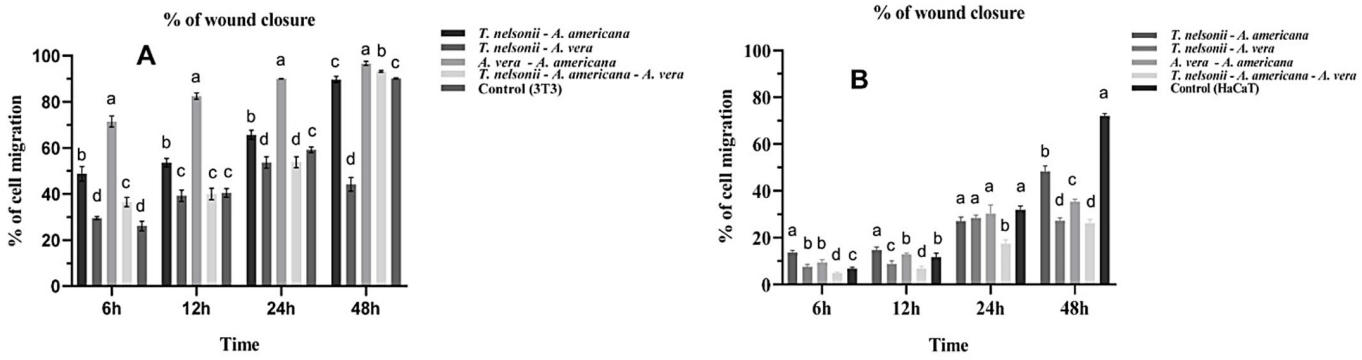

Figure 6. Effect of combined extracts on wound closure. (**A**) Percentage of wound closure relative to 3T3 cell migration with the combined extracts. (**B**) Percentage of wound closure relative to HaCaT cell migration with the combined extracts. Lowercase letters indicate statistically significant differences among the extracts with respect to each time according to Tukey's multiple range test ($p < 0.05$).

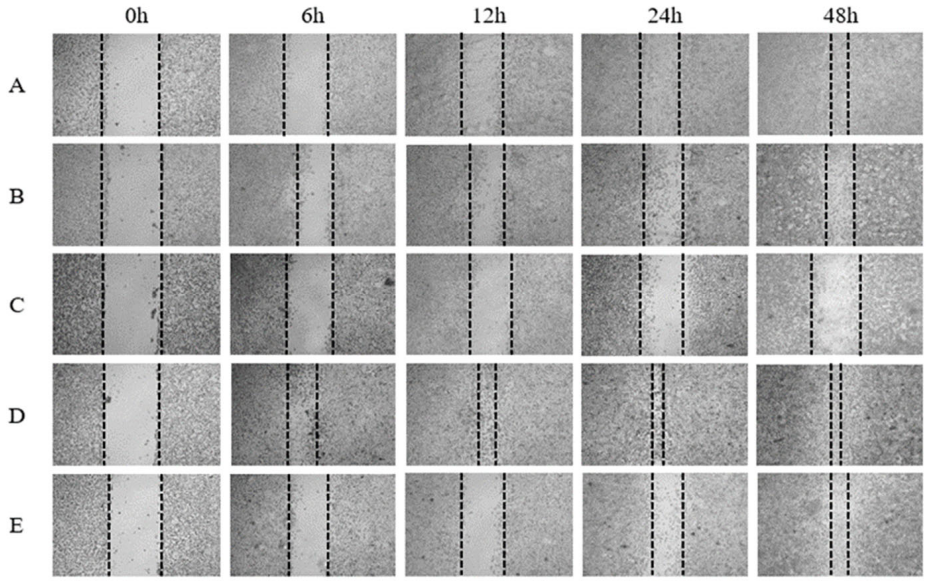

Figure 7. Wound-healing activity of combined vegetable extracts on fibroblast (3T3) migration with the scratch assay from 0 h to 48 h of incubation: (**A**) untreated cells as control; (**B**) cells treated with combined *T. nelsonii-A. americana* extract; (**C**) cells treated with combined *T. nelsonii-A. vera* extract. Cellular migration and proliferation were not observed; (**D**) cells treated with combined *A. americana-A. vera* gel extract. There was a reduction in the wound area starting from 6 h; (**E**) cells treated with combined extracts of all three plants. Cellular migration was observed starting at 24 h. Dotted lines delineate the wound edges. Fibroblast migration was photographed using an inverted microscope at a X10 magnification.

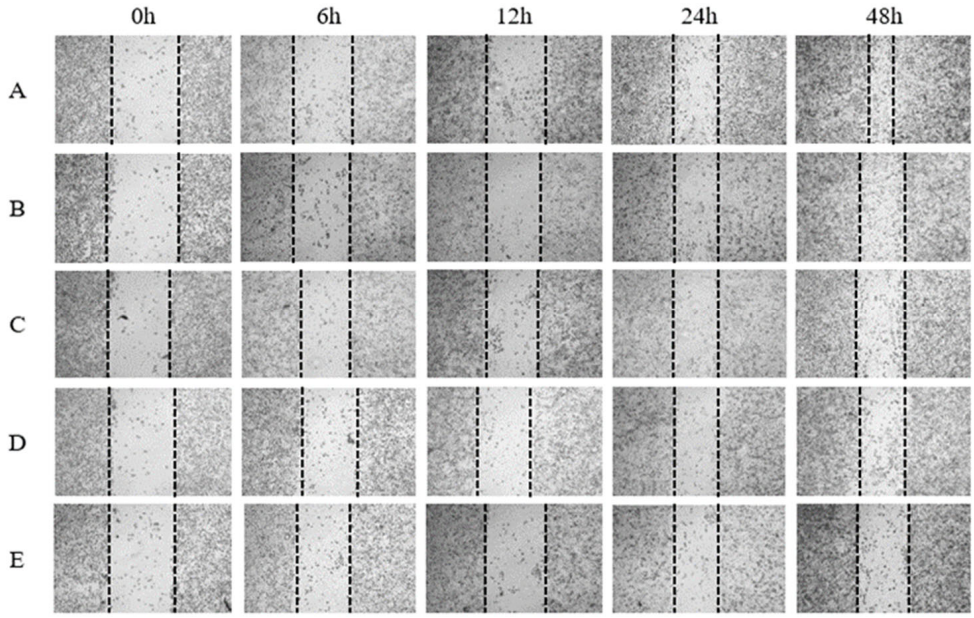

Figure 8. Wound-healing activity of combined vegetable extracts on keratinocyte (HaCaT) migration with the scratch assay from 0 h to 48 h of incubation: (**A**) untreated cells as control; (**B**) cells treated with combined *T. nelsonii-A. americana* extract. There was a reduction in the wound area at 24 h, but it was not greater than the control; (**C**) cells treated with combined *T. nelsonii-A. vera* extract; (**D**) cells treated with combined *A. americana-A. vera* gel extract. Cellular migration was observed at 24 h; (**E**) cells treated with combined extracts of all three plants. No significant reduction in the wound area was observed in the evaluated time periods. Dotted lines delineate the wound edges. Keratinocyte migration was photographed using an inverted microscope at a X10 magnification.

4. Discussion

4.1. Phytochemical Analysis

The chemical profile observed in the *T. nelsonii* extract coincides with what has been reported for other Tagetes species such as *T. minuta*, *T. erecta*, and *T. patula*, highlighting the presence of saponins, tannins, and coumarins. However, the differences in their content may vary according to environmental and geographical conditions [69,70]. In the *A. vera* gel, abundant carbohydrates and saponins were found, confirming what has been reported by Hamman [29], who mentioned that carbohydrates like galactomannan, glucomannan, glucose, and fructose, among others, mainly constitute the *A. vera* gel. Sterols and terpenes have also been reported in the gel, which are likely to be bound to the structures of some steroidal saponins or be part of the β-carotene structure, as reported in the gel's composition [71]. The phytochemical profile obtained from *A. americana* coincides with what has been reported for other Agave species, which also include flavonoids, alkaloids, sterols, and terpenes [69,72,73].

In this study, the contents of phenols and flavonoids in the *T. nelsonii* extract were higher than 53.92 mg g^{-1} reported in *T. patula* [21,73]. Sun and Shahrajabian [74] mentioned that these metabolites are the most widely found in the plant kingdom as they are essential for the functioning of plants and also precursors of other compounds such as coumarins, tannins, and anthocyanins, among others [75]. Burlec et al. [16] indicated that the phenolic compounds of the Tagetes genus, in synergy with terpenes and sterols, enhance the anti-inflammatory and antioxidant effects.

In the case of *A. vera* gel, various authors have stated that the parenchymatous gel contains 99.5% water, with the remainder being solids, which can explain the low concentration of metabolites compared to *T. nelsonii* (Table 2). The concentrations of phenols and flavonoids were lower compared to what was reported by Taukoorah and Mahomudally [76] and Tabatabaei et al., [77] who used a commercial gel. Nejatzadeh-Barandozi [78] also reported lower concentrations of these metabolites using different solvents.

The amount of phenols found in *A. americana* is consistent with other studies on Agave, where they reported a content ranging from 0.01 to 29 mg gallic acid per gram [69,72,79]. The high concentration of saponins in this genus is attributed to the effect of environmental factors such as the arid soil in which they grow, and it represents a defense mechanism against herbivores, pests, and insects [80].

Regarding the chromatographic analysis, it has been reported in the literature that the compounds found in this work present various biological activities. The 2,4-bis (1,1-dimethylethyl) [81] has been reported to have with antibacterial and antioxidant activity; terpenoids such as limonene [82], eugenol [83], squalene [84], and phytol [85] have antibacterial and antioxidant activity; fatty acids such as myristic acid [57], palmitic acid [86], 11,14, 17-eicosatrienoic acid [87], 8, 11- linoleic acid [86], caprilic acid [86], and linolenic acid [57] have anti-inflammatory, antimicrobial effects and wound-healing activity; and alkanes such as heneicosane [88] and eicosane [89] have antibacterial activity.

4.2. Antimicrobial Activity of Plant Extracts

The strains of *S. aureus* and *P. aeruginosa* showed inhibition in response to the *T. nelsonii* extract regardless of its concentration. *S. aureus*, a Gram-positive bacterium, possesses a single plasma membrane that is susceptible to different antimicrobial substances of both hydrophilic and lipophilic character. Several authors [90–92] have mentioned that the antimicrobial activity of the extracts obtained from plants is probably induced by the presence of phenolic compounds and sterols and terpenes, thanks to the presence of their hydroxyl groups. In this regard, *T. nelsonii* showed the highest concentration of phenols, mainly flavonoids, as well as the presence of terpenes such as limonene, eugenol, which, according to the literature, these compounds are capable of interacting with lipids and proteins in the membrane through hydrogen bonding, hydrophobic interactions, and electrostatic interactions, causing not only a change in the permeability and integrity of the plasma membrane but also inactivating microbial adhesins, enzymes, transport proteins in the cell wall, and alterations in energy metabolism [93,94]. The presence of tannins, saponins, anthraquinones, and coumarins was also observed, which, according to the literature, have different mechanisms of action that lead to bacterial death [91,92]. For instance, saponins are known to interact with hopanoids found in plasma membranes, causing changes in membrane fluidity; tannins, anthraquinones, and coumarins, possessing hydroxyl groups, can interact with the carbonyl group of amino acids and the oxygen atom in the head of membrane phospholipids, causing damage to the membrane, proteins, DNA, and essential functions of bacteria [79,80,90–92].

Otherwise, the resistance of Gram-negative bacteria like *E. coli* to different antimicrobial substances has already been reported [93]. These bacteria are characterized by having an outer and inner membrane composed of phospholipids, lipopolysaccharides, efflux pumps, and membrane protein structures such as porins. The latter serve as channels for entry into the bacterium, allowing certain antimicrobial substances like antibiotics to enter the bacterial cytoplasm. However, their effectiveness is influenced by various factors such as the size of the compound, its electric charge, and its hydrophobicity. Moreover, bacteria have the ability to generate mutations in porins, altering their structure and preventing the entry of substances [95].

The lack of antibacterial activity of the *A. Americana* extract and the *A. vera* gel on the microorganisms evaluated could be explained by their low content of secondary metabolites compared to the *T. nelsonii* extract. Additionally, the *A. vera* gel contains 99.5% water in its composition [29]. These results contradict other findings that highlight the antimicrobial activity of the ethanolic extract of the gel [26,94,96].

4.3. In Vitro Citotoxicity and Wound-Healing Activity

The non-toxicity of the Tagetes genus in the 3T3 and HaCaT cell lines is consistent with what has been reported by Burlec et al. [16], who evaluated the cytotoxicity of the ethanolic extract of *T. erecta* L. flowers in vitro on mouse fibroblastic cells and found that

this species was not cytotoxic at concentrations ranging from 0.25 to 1.00 μg mL^{-1}, with cell viability at 80%. On the other hand, this study observed that the minimum inhibitory concentration (25 mg mL^{-1}) of the *T. nelsonii* extract was higher than the LC_{50} found for both cell lines, making its application as an antibacterial agent in various industries feasible; however, its use as a wound-healing agent should be at concentrations lower than the LC_{50}.

The use of *A. americana* for wound healing has not been tested in vitro; there are studies demonstrating its in vivo wound-healing activity [24,25,97]. In this study, it was observed that the *A. Americana* extract presented compounds with healing and anti-inflammatory activity. To our knowledge, there are no reports on the use of *T. nelsonii* for wound healing, but rather the genus [98–102]; however, the presence of compounds with anti-inflammatory, antioxidant, and healing activity was observed. The presence of various bioactive compounds in plant extracts analyzed can contribute to wound closure through two mechanisms: (1) Antioxidant activity: reactive oxygen species (ROS) are produced when a wound occurs, which have the ability to cause damage to the cell membrane, alter metabolic pathways, induce lipid peroxidation, and inhibit proteins and DNA [103]. The presence of hydroxyl groups in these bioactive compounds allows them to donate electrons to stabilize free radicals in stable molecules [104]. (2) Anti-inflammatory activity, due to phenolic compounds that can interact with the amino acids tyrosine and serine at the active site of the enzyme cyclooxygenase-2 (COX-2), leading to its inhibition [105,106]. Additionally, saponins have been found to suppress the activity of platelet cyclooxygenase-1 (COX-1), reducing the production of thromboxanes (TXB2), prostaglandins (PGD2, PGE2) and 11-HETE [107]. These anti-inflammatory effects can help in reducing inflammation at the wound site and promote the healing process.

The use of *A. vera* gel for wound healing is not new, and previous studies have shown its potential in promoting cell proliferation both in vitro and in vivo, as well as in combination with other extracts [32,37]. Some compounds present in the gel have been reported to potentially contribute to wound healing. For example, mannose 6-phosphate and acemannan, present in the gel, have been shown in in vivo studies to promote wound healing. Mannose 6-phosphate binds to growth factor receptors on the surface of fibroblasts to increase the synthesis of hyaluronic acid, hydroxyproline, and collagen, thus promoting tissue repair [108,109]. Acemannan significantly increases cell proliferation and collagen synthesis [110–112]. The antraquinones found in the gel may suppress inflammatory responses by blocking inducible nitric oxide synthase (iNOS) and COX-2 mRNA expression, as well as enhance cell migration through cytokine and growth factor phosphorylation [57]. Sterols and saponins in the gel also increase hyaluronic acid synthesis [32,113]. It is worth noting that this study identified the presence of carbohydrates, phenols, antraquinones, and saponins in the gel.

Similarly, in the HaCaT cell line, it is evident that the combination of extracts does not increase cell proliferation, so it may require a continuous dosage of the extracts at specific intervals to enhance or maintain its effect. Therefore, it is recommended to conduct further research to determine the timing of each dose and extend the duration of the assay.

5. Conclusions

The results suggest that the methanolic extract from *T. nelsonii* has antibacterial activity against *S. aureus* and *P. aeruginosa*, likely attributed to the presence of phenolic compounds, sterols, terpenes, tannins, and saponins. Furthermore, the antibacterial activity of the *T. nelsonii* extract did not decrease when combined with the other plants. Likewise, the extracts from *T. nelsonii*, *A. americana*, and *A. vera* gel were shown not to be toxic in the 3T3 and HaCaT cell lines, indicating they are safe for use.

On the other hand, the results suggest that wound healing occurred more rapidly when the *A. americana* extract was used on 3T3 cells and the *T. nelsonii* extract on HaCaT cells. However, further studies with higher concentrations than those used in this study are needed for a more in-depth analysis of the wound healing capabilities of the extracts. Additionally, extract concentrations above the LC_{50} that exhibit antibacterial

activity could potentially be useful in the food, pharmaceutical, cosmetic, agricultural, and other industries.

It is recommended to test other bacteria isolated from the skin to see if the extracts have a wide range of bacterial inhibition. Additionally, conducting an in vivo study to confirm the wound-healing activity of the extracts and considering possible clinical trials in humans are recommended.

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