UNIVERSIDADE VILA VELHA-ES
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

ATIVIDADE ANTIOXIDANTE, ANTIMICROBIANA E CICATRIZANTE *IN VITRO* DA *Struthanthusvulgaris* (Vell.) Mart.

CÁTIA VITTORAZZI

VILA VELHA
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Dissertação apresentada à Universidade Vila Velha, como pré-requisito do Programa de Pós-Graduação em Ciências Farmacêuticaspara a obtenção do titulo de Mestre em Ciências Farmacêuticas.

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Dedico esta Dissertação à minha família, que é a minha base e que jamais mediu esforços para que eu alcançasse meus sonhos. Todas as minhas vitórias também são de vocês.

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“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e venceobstáculos, no mínimo fará coisas admiráveis” (José de Alencar)

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

µg - Micrograma

µL - Microlitro

ºC- Grauscentígrados

AAI- Índice de atividade antioxidante

BHT- Butil-hidroxi-tolueno

CO_2 - Dióxido de carbono

DAPI-4,6-diamidino-2-fenilindol

DL_{50} - Dose letalmédia

DMEM- Meio de cultura modificado tipo DMEM

DMSO-Dimetilsulfóxido

DPPH- 2,2-difenil-1-picril-hidrazil

FBS-Soro fetal bovino

g - Grama

L - Litro

MIC –Concentração inibitória mínima

MG -Miligramas

mL - Mililitro

mm-Milímetros

MTT-brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio

PBS – Soluçãotampãosalina-fosfato

PDGF-Fator de crescimento derivado das plaquetas

ROS - Espécies reativas de oxigênio

SD-Desvio padrão

SEM-Desvio padrão da média

S. vulgaris- Struthanthusvulgaris

TPC-Teor de fenólicos totais

RESUMO

VITTORAZZI, Cátia, Farmacêutica, M.Sc., Universidade Vila Velha-ES. Maio de 2015**. ATIVIDADE ANTIOXIDANTE, ANTIMICROBIANA E CICATRIZANTE *IN VITRO* DA *Struthanthusvulgaris*(Vell.) Mart.** Orientador: Prof. Dr. Marcio Fronza

Struthanthusvulgaris(Vell.) Mart. (Loranthaceae), geralmente conhecida como *erva-de-passarinho*, tem sido utilizada na medicina popular em diversas enfermidades, principalmente para lavar feridas. O objetivo é investigar o efeito cicatrizante *in vitro*, a atividade antioxidante e antimicrobiana dos extratos etanólico e hexânico das folhas e ramos de *S. vulgaris.* Os efeitos dos extratos das folhas e dos ramos de *S. vulgaris*foram investigados quanto à sua capacidade de estimular a proliferação e migração de fibroblastos no *scratchassay*. A atividade antioxidante foi avaliada com o DPPH (2,2-difenil-1-picrilhidrazil) radical livre, e a antibacteriana foi testada contra bactérias Gram-positivas e Gram-negativas empregando o teste de microdiluição. O conteúdo total de fenólicos e flavonóides foram determinados por métodos colorimétricos. Os extratos das folhas e ramos da *S. vugaris*na concentração de 100 µg/ml estimularam a migração e proliferação de fibroblastos com um aumento no número de células por 56,2% e 18,6%, respectivamente. A atividade antioxidante exibiu um IC50 de 24,3 e 18,9 µg/ml para os extratos de folhas e ramos, respectivamente. O extrato etanólico das folhas apresentou atividade antimicrobiana contra as bactérias Gram-positivas como o *Staphylococusmutans*e *Staphylococcus aureus*, exibindo valores de concentração inibitória mínima de 125 e 500 µg/ml, respectivamente. Uma quantidade significativa de teor de fenólicos totais nas folhas (813,6 ± 2,7 mg/g) e ramos (462,8 ± 9,6 mg/g), e a concentração baixa de flavonóides nas folhas (13,3 ± 4,3 mg/g) e ramos (1,9 ± 0,2 mg/g), foram encontradas. A atividade antioxidante e antimicrobiana em conjunto com o efeito estimulatório sobre a proliferação e migração de fibroblastos *in vitro,* proporcionam evidências cientificas ao uso popular da S. *vulgaris*na medicina tradicional para o tratamento de feridas.

Palavras chaves: Produtos naturais, fibroblastos, migração celular, scratch assay

ABSTRACT

VITTORAZZI, Cátia, Farmacêutica, M.Sc., Universidade Vila Velha-ES. May, 2015. **ANTIOXIDANT ACTIVITY, ANTIMICROBIAL AND HEALING *OF IN VITRO Struthanthusvulgaris*(Vell.) Mart.** Orientador: Prof. Dr. Marcio Fronza

Struthanthusvulgaris(Vell.) Mart. (Loranthaceae), commonlyknown as *erva-de-passarinho*, hasbeenused in folk medicine tobathwounds. The objectiveofthisworkwastoinvestigatethe*in vitro* woundhealingeffects, togetherwiththeantioxidantandantimicrobialactivityof*S. vulgaris*leavesandbranchesextracts. The effectsofethanolleafandbranchextractof*S. vulgaris*wereinvestigated for theirabilitytostimulateproliferationandmigrationoffibroblasts in the*scratchassay*. Antioxidantactivitieswereinvestigatedusingthe DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavengingassay, andtheantibacterialactivitywasevaluatedagainstGram-positiveandGram-negativebacteriabythemicrodilution test. The total phenolicandflavonoidcontentsweredeterminedbycolorimetricmethods. *S. vulgaris*leafandbranchextractsat 100 µg/ml concentrationstimulatedmigrationandproliferationoffibroblastsandenhancedcellnumbersby 56.2% and 18.6%, respectively. Antioxidantactivityexhibitedan IC50 of 24.3 and 18.9 µg/ml for theleafandbranchextracts, respectively. The ethanolleafextractshowedantimicrobialactivityagainsttheGram-positiveStaphylococcusmutansandStaphylococcus aureus bacteria, exhibitingminimuminhibitoryconcentrationvaluesof 125 and 500 µg/ml, respectively. Anappreciable total phenoliccontent in theleaves (813.6 ± 2.7 mg/g) andbranches (462.8 ± 9.6 mg/g), andrelativelylowconcentrationofflavonoids in theleaves (13.3 ± 4.3 mg/g) andbranches (1.9 ± 0.2 mg/g), weredetected. The antioxidantandantibacterialactivitytogetherwiththestimulationproliferationandmigrationoffibroblasts in vitro provide some support for the use of*S. vulgaris*in traditional medicine for treatmentofwounds.

Keywords: Natural products, fibroblasts, cellular migration, scratch assay.

Artigocientífico

Antioxidant, antimicrobial and wound healing properties of *Struthanthus vulgaris*

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Running title:

Wound healing activity of *Struthanthus vulgaris*.

Keywords:

Natural products, fibroblasts, cellular migration, scratch assay.

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Abstract

Context:*Struthanthus vulgaris* (Vell.) Mart. (Loranthaceae)has been widely used in traditional medicine in Brazil to bathe wounds.

Objective: To investigate the *in vitro* wound healing effects, together with the antioxidant and antimicrobial activity of*S. vulgaris* leaf and branch extracts.

Material and methods: Ethanol leaf and branch extracts of *S. vulgaris* were investigated at 1 to 100 µg/ml concentrationsin the scratch assay after 14h.Antioxidant activitywasinvestigatedusing the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging assay, and the antibacterial activitywastested at concentrations up to 1,000µg/ml against Gram-positive and Gram-negative bacteriaby the microdilution testafter 24 h. The total phenolic and flavonoid contents were determined by colorimetric methods.

Results:*S. vulgaris* leaf and branch extracts at 100µg/ml concentration stimulated migration and proliferation of fibroblasts and enhanced cell numbers by 56.2% and 18.6%, respectively.Antioxidant activity exhibited an IC_{50} of 24.3 and 18.9 µg/ml forthe leaf and branch extracts, respectively.Ethanol leaf extract showed antimicrobial activity against the Gram-positive*Staphylococcus mutans*and*Staphylococcus aureus*bacteria, exhibiting minimum inhibitoryconcentrationvalues of 125 and 500µg/ml, respectively.An appreciable total phenolic content in the leaves (813.6 ± 2.7 mg/g) and branches (462.8 ± 9.6 mg/g), and relatively low concentration of flavonoids in the leaves (13.3 ± 4.3 mg/g) and branches (1.9 ± 0.2 mg/g), were detected.

Discussion and conclusion: The antioxidant and antibacterial activity, together with the strong ability to stimulate proliferation and migration of fibroblasts, provide some support for the traditional use of *S. vulgaris*.

Introduction

The hemiparasite*Struthanthus vulgaris* (Vell.) Mart. (Loranthaceae), popularly known as *erva-de-passarinho*, is widely used in Brazil folk medicine. The infusion prepared from the aerial parts of the plant is frequently used for stomach ailments, disorders of the respiratory system and in particular, to bathe cutaneous ulcers (Salatino et al., 1993; Vieira et al., 2005). The chemistry and biological activities of *S. vulgaris* are practically unknown. Phytochemical prospection of secondary metabolites has described positive reactions for flavonoids, condensed tannins (proanthocyanidins) and saponins from the fresh leaves of *S. vulgaris* (Vieira et al., 2005). The same author also demonstrated the antimicrobial activity of the hydroalcoholic extract of fresh leaves and fractions against *Bacillus cereus* (Gram positive bacteria) and *Pseudomonas aeruginosa* (Gram negative bacteria) (Vieira et al., 2005). The chemical and biological activities of other species of *Struthanthus*genus are reported in the literature. For example, the species *Struthanthusmarginatus* (Desr.)Blumecontains trace elements, including silicon, manganese, iron, copper, and zinc, which could explain its traditional use for wound healing (Freire et al., 2011; Leitão et al., 2013). In another work, rutin was extracted from *Struthanthussubtilis*Kujit and exhibited *in vitro* cytotoxic activity against human tumor cell lines (Cordero et al., 2003). Meanwhile, dose dependent hypotension and cardiotoxic effects were reported for the methanol extract of leaves of *Struthanthusvenetus* (Kunth) Blume (Lorenzana-Jiménez et al., 2006). Leaf extracts of *S. marginatus* exhibited *in vivo* protection against gastric lesions induced by ulcerogenic agents without any toxicity, which could justify its popular use for gastric disorders (Freire et al., 2011).

The wound-healing process of repair that follows skin injury is a dynamic, complex, and well-organized process that is divided into three overlapping phases: inflammation, proliferation and remodeling. These involve biochemical and physiological phenomena that behave in a harmonious way, in order to restore the integrity and normal functions of the skin (Gurtner et al., 2008; Reinke&Sorg, 2012). The healing process requires the interaction between various tissues and many cell types, including neutrophils, macrophages, keratinocytes, fibroblasts and endothelial cells (Barrientos et al., 2008; Behm et

al., 2012). Alterations in any of these steps can lead to a delay or inability in dermal proper wound healing, causing pathological manifestations such as keloids and hypertrophic scars (Gauglitz et al., 2011; Seifert&Mrowietz, 2009).

Cutaneous wounds are particularly susceptible to bacterial and other infections, and also provide an entry point for systemic infections. Consequently, the presence of microorganisms can seriously delay the healing process, resulting in the formation of unpleasant exudates and toxins and prolonging the inflammatory phase (Agyare et al., 2013; Houghton et al., 2005; Misic et al., 2014). Therefore, antimicrobial agents can be used to prevent wound infection and accelerate the wound healing process.

Neutrophils are considered the first type of cells to be recruited into the site of injury, and have an important role in antimicrobial defense and the debridement of devitalized tissue by the production of proteolytic enzymes and reactive oxygen species (ROS) (Fitzmaurice et al., 2011). ROS is produced in high amounts at the wound site, as a defense mechanism against invading bacteria (Süntar et al., 2012). However, at the same time, the presence of increased numbers of neutrophils and ROS in high concentrations can induce severe tissue damage and even lead to neoplastic transformation, decreasing the healing process due to damage to the cellular membranes, DNA, proteins and lipids. Fibroblasts and other cells may be killed, and skin lipids will be made less flexible by excess ROS (Annan and Houghton, 2008; Houghton et al., 2005; Mensah et al., 2001). Thus, antioxidant substances appear to be important for the successful treatment and management of wounds.

Given that antioxidant and antimicrobial activities have been found to have positive effects on wound healing (Adetutu et al., 2011; Annan&Houghton, 2008; Srinivas et al., 2008; Süntar et al., 2012), while fibroblast migration and proliferation play a crucial role in the healing process by initiating the proliferative phase of repair (Ebeling et al., 2014; Fronza et al., 2009; Mensah et al., 2001), our study aimed to evaluate the antioxidant, antimicrobial and wound healing activity of *S. vulgaris*.

Material and methods

Cell lines, chemicals and biochemicals

Swiss 3T3 albino mouse fibroblasts (ATCC® CCL-92™), murine hepatoma (Hepa 1c1c7) cells (ATTC® CRL-2026™) and human ovarian carcinoma cell line (OVCAR-3) (ATCC® HTB-161™) (Cell Line Service, Rio de Janeiro, Brazil) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin, at 37°C, in a humidified atmosphere containing 5% CO_2 (all Sigma-USA). Collagen solution type I from rat tail, Mitomycin C, platelet-derived growth factor-BB (PDGF) and Prolong Gold antifade reagent with 4,6-diamino-2-phenylindole (DAPI), 2,2-diphenyl-1-picryl-hidrazyl (DPPH), butylatedhydroxytoluene (BHT), and quercetin, Folin-Ciocalteau reagent were purchased from Sigma Chemical Co, MO, USA. All solvents and reagents were of analytical grade, and were purchased from Vetec (Rio de Janeiro, Brazil). Mueller-Hinton agar was purchased from Himedia Laboratories Pvt (Mumbai, India).

Plant material

Leaves and branches of the mistletoe *Struthanthus vulgaris* were collected in the municipality of Castelo, Southeastern Brazil, in March 2013. They were taxonomically identified by botanist MsSolange Schneider, and a voucher specimen (UVVES-2395) was deposited in the herbarium of the University Vila Velha, Brazil.

Preparation of plant extracts

The air-dried and ground leaves and branches (102 and 122 g, respectively) were defatted with hexane (1 L) in a Soxhlet apparatus for 6-8 h. After drying, the plant material was exhaustively extracted with ethanol (1 L) using a Soxhlet apparatus for 24-48 h (Sultana et al., 2009). Subsequently, the solvent was removed under vacuum at 40°C (Fisaton 801, São Paulo, Brazil) and a dry ethanol extract was obtained (12.3 g and 8.6 g, respectively). This dry extract was stored at -20°C until further use.

Determination of total phenolics

The spectrophotometric Folin-Ciocalteu method was used to measure the total phenolic content (TPC) in the extracts, as previously described by Krepsky et al. (2012) and Scherer and Godoy (2009). All the analyzes were performed in triplicate and the results expressed as mean ± standard deviation. The TPC was expressed in milligrams of pyogallol equivalents per gram of dry extract.

Estimation of total flavonoids

The aluminum chloride colorimetric method was used for the determination of total flavonoid concentration, as previously described by Krepsky et al. (2012). The content was calculated using the calibration curve of quercetin (1-12 µg/ml) and the results expressed in milligram of quercetin equivalents per gram of dry extract. All determinations were carried out in triplicate.

DPPH free radical scavenging activity

The antioxidant activity of the leaf and branch extracts was evaluated by monitoring their ability to quen ch the stable free radical DPPH, according to Scherer and Godoy (2009). The antioxidant activity was expressed as IC_{50} in µg/ml and by the antioxidant activity index (AAI). The assays were carried out in triplicate, and antioxidant activity was compared with the commonly used synthetic antioxidant butylatedhydroxyanisole (BHT) and tocopherol (vitamin E).

Antimicrobial activity

The antibacterial activity of the leaf and branch extracts was carried out by the agar di usion test against the Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ATCC 12386), *Streptococcus mutans* (ATCC 25175), and *Enterococcus faecalis* (ATCC 29212), and the Gram-negative bacteria *Escherichia coli* (ATCC 8739), as previously described by Agyare et al. (2013) and Valgas et al. (2007), with modifications. All strains were obtained from the American Type Culture Collection (ATCC). In summary, the microorganisms were seeded on Müller-Hinton plates and then incubated in a bacteriological oven at 37 ± 1 °C for 24 h. Bacterial suspension (inoculum) was diluted with sterile physiological solution to 10^8 CFU/ml (turbidity = McFarland

barium sulfate standard 0.5). Subsequently, 10 µl of leaf and branch extracts of *S. vulgaris*, at a final concentration of 10, 20 and 40 mg/ml, were added to the plates and the zones of growth inhibition were measured after 24 h incubation at 37°C. The antimicrobial activities of the extracts were compared with the activity of the antibiotic ampicillin. Experiments were carried out at least in triplicate, and the antibacterial activity was expressed as the mean of the inhibition diameters (mm) produced.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) of the extracts were tested against the Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ATCC 12386), *Streptococcus mutans* (ATCC 25175), and *Enterococcus faecalis* (ATCC 29212), and the Gram-negative bacteria *Escherichia coli* (ATCC 8739), by the standard NCCL method (NCCL, 2008), in a 96-well microtiter plate. The final concentration of cells was adjusted in a turbidimeter (930 NTU - Nephelometric Turbidity Units) 0.5 on the McFarland scale, in the order of 10^6 CFU/ml. 100 µl of culture medium (Mueller-Hinton agar 2.1%), sample or antibiotic, and the inoculum were added to each well. The final tested concentrations of the extracts were 1000.0, 500.0, 250.0, 125.0 and 62.5 µg/ml. After addition of the inoculum, the plates were incubated for 24 h. Afterwards, 100 µl oftriphenyltetrazolium chloride (TTC) (0.5% aqueous solution) was added. After 4 h incubation, the MIC was determined as the lowest concentration capable of inhibiting visible growth of cells, determined by TTC. The antimicrobial activities of the extracts were compared with the activity of the antibiotic ampicillin. In all plates, positive and negative controls (six wells of each) were included. The experiments were carried out at least in triplicate.

Assessment of *in vitro* cytotoxicity

The *in vitro* cytotoxic activity was evaluated in the colorimetric MTT assay according to Mosmann (1983). In summary, 3T3 fibroblasts, Hepa-1c1c7 and OVCAR-3 cells were plated in 96-well flat-bottomed tissue culture plates with 6 x 10^5 cells/ml, and after overnight incubation at 37ºC (5% CO_2 and 95% air), cells were incubated for an additional 24 h in the presence or absence of

increasing concentrations (1.0-200.0 µg/ml) of ethanol extract of the leaves and branches. Actinomycin D and camptothecin were used as positive controls (Ariyoshi-Kishino et al., 2010; Wu et al., 2011; Xu&Krystal, 2010). After incubation, 100 µl of 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/ml in PBS: Medium (1:3)) was added per well, and the plate incubated for 2 h to allow reaction of MTT by cellular mitochondrial dehydrogenases. The excess MTT was aspirated and the formazan crystals formed were dissolved with 100 µl of dimethyl sulfoxide (DMSO). Absorbance of purple formazan, proportional to the number of viable cells, was measured at 595 nm using a microplate reader (Molecular Devises, Spectra Max 190, USA). The experiments were carried out at least in triplicate.

In vitro cell migration assay

The proliferation and/or migration abilities of fibroblasts exposed to leaf and branch extracts were assessed using the *in vitro* scratch wound assay, which measures the expansion of a cell population on surfaces (Fronza et al., 2009; Liang et al., 2007). In summary, fibroblasts (2×10^6 cells/ml) were cultured in 24-well plates containing glass coverslips previously coated with collagen (40 µg/ml, 2 h at 37°C) to nearly confluent cell monolayers, then an artificial linear wound was introduced. The monolayers were then divided into groups and treated for 14 h with different concentrations of the extracts (1-100 µg/ml). PDGF was used as positive control. After treatment, the cells were fixed and stained with DAPI. Pictures were taken of the scratched areas, using a Samsung (SDC-415ND) camera coupled to Leica microscope (model DMLS) and connected to a computer, in which the images were processed and stored. Cell migration into the wound area was quantified using software CellC®, and the results were expressed as percentage of cells that migrate and/or proliferate into the wound area in comparison with the untreated control group.

Statistical Analysis

Statistical analyses were performed using GraphPad software (San Diego, CA, 176 USA). Data were expressed as mean ± standard error of mean (SEM) or standard deviation (SD). Statistical variations were determined using one or

two-way analysis of variance (ANOVA), where appropriate. Values of $p<0.05$ were considered significant.

Results

The quantitative analysis of total phenolic contents (TPC) and flavonoids in the leaf and branch extracts of *S. vulgaris* revealed significant amounts of TPC and a moderate concentration of total flavonoids, as shown in Table 1. The TPC was expressed as milligram equivalents of pyrogallol per gram of dry extract, while the total flavonoid content was calculated in milligrams equivalents of quercetin per gram of leaf and branch dry extracts. A higher amount of TPC was found in the leaves, with significant difference compared to the branches (813.6 ± 2.7 and 462.8 ± 9.6 mg/g of pyrogallol, respectively). Quantitative analysis revealed total flavonoids in the extracts ranging from 1.9 ± 0.2 to 13.3 ± 4.3 mg/g of dry extract of quercetin equivalents in the leaf and branch extracts, respectively (Table 1). Statistical analysis showed significant ($p<0.05$) differences in the total amount of flavonoids present in the leaves and branches.

The antioxidant activity of phenolic compounds are known to be mainly due to redox properties, which allow them to act as hydrogen donor reducing agents, singlet oxygen quenchers, heavy metal chelators, and hydroxyl radical quenchers (Al-Temimi&Choudhary, 2013). The radical scavenging activity was expressed as IC_{50} in µg/ml, and by the Antioxidant Activity Index (AAI) proposed by Scherer and Godoy (2009), which classifies the antioxidants as weak when AAI < 0.5, moderate when AAI is between 0.05 and 1.0, strong when AAI is between 1.0 and 2.0, and very strong when AAI > 2.0. The ethanol leaf extract (1.8 ± 0.5) exhibited strong activity and ethanol branch extract (2.3 ± 0.8) very strong activity. The AAI values of the extracts were similar to those of the commonly used synthetic antioxidant butylatedhydroxylanisole (BHT) (4.3 ± 0.7) and tocopherol (vitamin E) (3.2 ± 1.1) (Table 2).

Bacterial infections of different types of wounds present a challenge in the treatment of skin injuries and wound healing (Misic et al., 2014). Therefore, antibacterial activity of *S. vulgaris* was preliminary assayed by the agar diffusion method against some common bacteria present in the wounds (Table 3).

Ethanol leaf and branch extracts were found to be active against tested organisms, with varying mean zones of inhibition. *S. aureus*was found to be more susceptible to the extracts. The antibacterial activity was further investigated in the microdilution test, and the results were presented as minimum inhibitory concentration (MIC) in µg/ml (Table 4). Ethanol leaf and branch extracts of *S. vulgaris* exhibited prominent antimicrobial activity against the Gram-positive *S. mutans*and *S. aureus*bacteria, exhibiting MIC in the range of 125-500 µg/ml. Ampicillin, a standard antibiotic that was used as a positive control, exhibited MIC in the range of 250-500 µg/ml (Table 4).

Next, the safety of extracts was tested *in vitro* against one normal cell line (mouse fibroblasts) and two cancer cell lines; the human ovarian carcinoma cell line (OVCAR-3) and murine (Hepa1c1c7) hepatoma cells. No cytotoxic effects were detected in the leaf and branch extracts at concentrations ranging from 1.0 to 200.0 µg/ml (data not shown) in all tested cell lines.

Furthermore, the ability of ethanol leaf and branch extractsof*S. vulgaris* to stimulate fibroblast proliferation and/or migration was investigated using the scratch assay. Ethanol leaf extract increased cell numbers in the artificial wound site (Figure 1A) reaching the maximum stimulatory effect of 56.2 ± 7.4% at a concentration of 100 µg/ml, whereas the same concentration of the ethanol branch extract resulted in a much lower migration and proliferation activity, with a maximum stimulatory value of 18.6 ± 6.3% (Figure 1B). Comparing the results with the control group, a significant difference was observed at 50 and 100 µg/ml concentrations with the ethanol leaf extract. The significant activity of leaf extract from *S. vulgaris* in promoting healing was similar to that observed for PDGF at a concentration of 2 ng/ml concentration (48.8 ± 3.6%). Treatments of leaf extract at a concentration of 100 µg/ml restored the cells (closed the gap) to a confluent or near confluent state within 24 h, in contrast to the control cells (data not shown).

Increased cell numbers may be due to immigration and/or proliferation of cells. In order to distinguish between these two effects, the “wounded” monolayer of fibroblasts was cultivated together with mitomycin C (5 µg/ml), with either PDGF (2 ng/ml) or the plant extracts. The addition of mitomycin C prevents mitosis and

thereby proliferation, therefore the remaining increase in the number of cells was due solely to migration. The total cell number decreased to about 43.9 ± 1.9% and 10.3 ± 2.9% respectively, for the leaf and branch extracts tested, at a concentration of 100 µg/ml. A similar decrease was observed with PDGF (Figure 1A and B). Hence, the total cell numbers presented only a slightly decreased in the presence of mitomycin C for all tested concentrations. This indicates that the effect observed in the scratch assay is mainly due to migration, and only marginally to proliferation.

Discussion

Natural products, especially those derived from plants, have formed the basis of sophisticated traditional medicine systems that have been explored since thousands of years, and are still being used directly or indirectly against all classes of diseases (Cragg&Newman, 2013). In general, plant products are largely preferred because of their widespread availability, fewer side effects, and effectiveness as crude preparations (Agyare et al., 2009). The use of plant extracts or plant-derived compounds in the treatment and management of cutaneous wounds can, in most cases, be associated with effective antioxidant and antimicrobial properties (Süntar et al., 2012; Thakur et al., 2011; Annan&Houghton, 2008).

Phytochemical screening assay is considered a simple, quick, and inexpensive procedure that often used to characterize various types of secondary metabolites found in plants (Sasidharan et al., 2011). Phenolic compounds are well-known phytochemicals found in all plants. They consist of simple phenols, benzoic and cinnamic acid, coumarins, tannins, lignins, lignans and flavonoids (Khoddami et al., 2013). Leaf extract of *S. vulgaris* exhibited an expressive amount of phenolic compounds and a moderate concentration of total flavonoids. Phytochemical constituents of a plant are often correlated to and determine the physiological action on the human body, and phenolic compounds are well known molecules that have many biological properties, including antioxidant activity, that play an important role in wound healing by preventing and protecting oxidative damage from free radicals (Agyare et al., 2013; Fitzmaurice et al., 2011; Süntar et al., 2012). Using the 2,2-diphenyl-1-

picrylhydrazyl (DPPH) assay, which is a widely used technique for measuring the antioxidant activity of plant extracts due to its low cost and accuracy, and the fact that it is easy to perform (Alam et al., 2013), the leaf and branch extracts showed strong antioxidant activity, confirming their anti-radical properties. Thus, the antioxidant activity exhibited by the leaf extract, associated with the significant phenolic and flavonoid content, suggest a clear correlation of these constituents in the process of wound healing. Our results are in agreement with the current literature, which emphasizes the relationship between antioxidant activity and the presence of phenolic compounds, showing that they contribute to the antioxidant activity of the plant extracts (Al-Zoreky, 2009; Hossain et al., 2011; Saeed et al. 2012).

The presence of microorganisms in open wounds makes them susceptible to the development of infection-related complications which has harmful effects on patients, causing increased pain and discomfort and significantly impairing the processes of normal wound healing (Houghton et al., 2005; Misic et al., 2014). According to Duarte et al. (2005) and Adetutu et al. (2011) for a plant extract to have pharmacological relevance and to be considered potentially therapeutically useful, it must have MIC values MIC below 8.0 mg/ml and 2.0 mg/mL, respectively. Based on this classification, our data showed that *S. vulgaris* extracts exhibited significant antimicrobial activity against some of the most common bacterial pathogens usually found in cutaneous wounds, including *Staphylococcus aureus* (Han et al., 2011; Misic et al., 2014). Thus, it could be inferred that the extracts exhibited antimicrobial activity, and topical application may protect the cutaneous wounds from pathogenic bacteria and their harmful effects on wound healing.

Another important find of our study that provides a significant contribution to the cutaneous wound healing properties of *S. vulgaris* was the stimulatory effects on the migratory activity of fibroblasts. Fibroblasts are known to play an essential role in cutaneous wound healing, and cell migration is one of the most essential steps, during the proliferative phase, responsible for wound closure (Barrientos et al., 2008; Gurtner et al., 2008; Reinke&Sorg, 2012). Using the scratch assay, a convenient and suitable *in vitro* test that gives robust and reproducible results for the migration of fibroblasts in an artificial wounded area

(Ebeling et al., 2014; Liang et al., 2007), the leaf and branch extracts mainly influenced the migration of the fibroblasts, which may contribute to the rebuilding of new granulation tissue and thus restore the normal functions of the skin.

Conclusion

The evidence reported in our study indicates the relevant contribution of *S. vulgaris* ethanol branch and leaf extracts to the treatment of cutaneous wounds. The *in vitro* results demonstrated that, specially the leaf extract stimulated migration of fibroblasts, and exhibited antioxidant and antimicrobial activities, which may be due to the presence of phenolic and flavonoid compounds. In conclusion, the results are obtained prompt further research, seeking to identify the active molecule that induces the *in vitro* cell migration and proliferation response, and thereby promote a better understanding of the wound healing potential effects observed in this study with *S. vulgaris.*

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Tablesand Figure

Table 1. Quantification of total phenolics and flavonoid contents in dry plant material of *S. vulgaris.*

Plant material	**Total flavonoids* (mg/g)**	**Total phenolics** (mg/g)**
Leaves	13.3 ± 4.3 [a]	813.6 ± 2.7[a]
Branches	1.9 ± 0.2 [b]	462.8 ± 9.6[b]

*results expressed in mg of quercetin equivalents per gram of dry extract; ** results expressed in mg of pyrogallol equivalents per gram of dry extract. Different letters in the same column correspond to significant differences ($p < 0.05$). The tests was performed in triplicate, and the results are expressed as mean ± standard error.

Table 2. Radical scavenging activity of leaf and branch ethanol extract of *S. vulgaris*.

	DPPH scavenging activity *	
Sample	**IC_{50} µg/ml**	**Antioxidant activity index (AAI)**
Ethanol leaf extract	24.3 ± 7.6	1.8 ± 0.5
Ethanol branch extract	18.9 ± 5.4	2.3 ± 0.8
Butylatedhydroxyanisole	9.4 ± 4.3	4.3 ± 0.7
Tocopherol (vitamin E)	11.8 ± 6.3	3.2 ± 1.1

*Tests were performed in triplicate, and the results are expressed as mean ± standard deviation.

Table 3. Antibacterial activity of ethanol leaves and branches extract of *S. vulgaris* by the agar diffusion method.

Microorganisms	**Inhibition zone diameter (mm)**				
	Leaves (mg/ml)		**Branches (mg/ml)**		**Ampicillin (µg)**
	20	**40**	**20**	**40**	**10**
Streptococcus agalactiae	*ne*	9.7 ± 1.1	8.0 ± 1.1	9.0 ± 1.1	19.2 ± 1.3
Enterococcus faecalis	*ne*	8.0 ± 1.1	9.0 ± 1.1	8.3 ± 1.5	20.0 ± 2.1
Staphylococcus mutans	*ne*	7.7 ± 0.6	*Ne*	7.6 ± 0.5	39.2 ± 3.5
Staphylococcus aureus	*ne*	11.0 ± 3.5	*Ne*	17.0 ± 1.0	22.0 ± 2.0

ne: not effective

Table 4. Minimum inhibitory concentrations (MICs) of ethanol leaf and branch extracts of *S.vulgaris*determined by the microdilution method. The experiments were repeated two times.

Extract	MIC (µg/ml)				
	S. agalactiae	*E. faecalis*	*S. mutans*	*S. aureus*	*E. coli*
Leaves	> 1000	> 1000	125	500	1000
Branches	> 1000	> 1000	500	1000	1000
Ampicillin	500	500	250	250	250

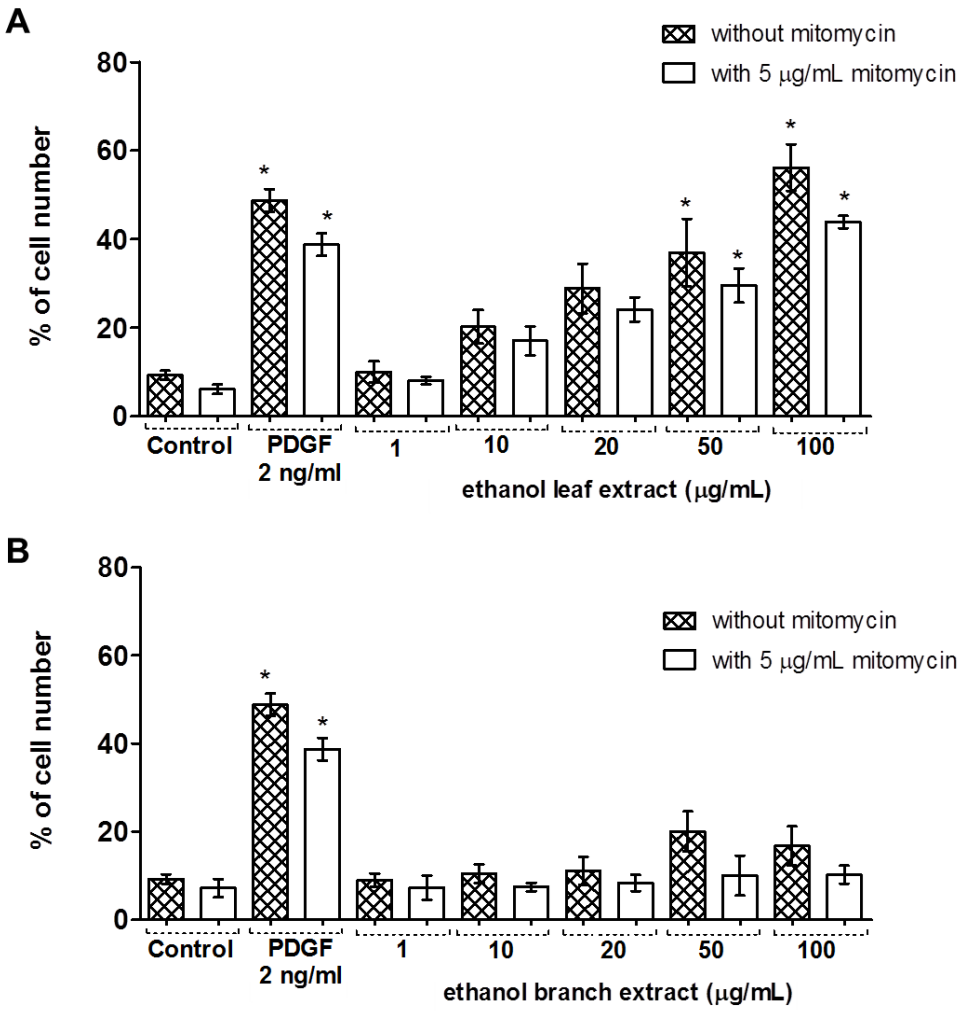

Figure 1. Effect of ethanol leaf and branch extract from *S. vulgaris* on the migratory and proliferative activities of 3T3 mouse fibroblasts in the scratch assay. The experiments were performed in the absence (dashed bars) or presence (open bars) of 5 µg/ml of antimitotic mitomycin C after 14 h incubation (37 °C, 5% CO_2) in DMEM medium supplemented with 10% fetal bovine serum. *S. vulgaris* extracts were tested at concentrations ranging from 1.0 to 100 µg/ml. PDGF was used as positive control at a concentration of 2 ng/ml. Data are expressed as percentage of cell number in the injured area, compared to the control group (DMEM medium only). Bars represent the mean ± SD of three independent experiments, *P<0.05, compared to the control group, by two-way-ANOVA.