Original Article

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Healing and cytotoxic effects of *Psidium guajava* (Myrtaceae) leaf extracts

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Abstract

Aim: The aims of this study were to evaluate the wound healing potential *in vivo* and the cytotoxic effects *in vitro* of *Psidium guajava* (*Myrtaceae*) leaf extract and commonly used corticosteroids. **Methods:** The healing effect was studied *in vivo* by the clinical and histological evaluation of traumatic lesions in the oral mucosa of rats treated with these substances. Each rat received 2 daily applications of the medicine tested and the animals were sacrificed after 1, 3, 5, 7 and 14 days. Tissue sections stained with hematoxylin & eosin were analyzed. The histological evaluation involved a 5-point scoring system based on the degree of healing, ranging from 1 (total repair of epithelium and connective tissue) to 5 (epithelial ulcer and acute inflammatory infiltrate). The Kruskal-Wallis test was used for statistical analysis of the histological scores. For the *in vitro* toxicity assay, each substance was applied to mucosa fibroblast cell cultures in conditioned media. The media were conditioned by placing the substances in contact with fresh culture medium for 24 h. The cytotoxicity analysis was performed using the MTT assay. Data were analyzed statistically by ANOVA and Tukey's test at 5% significance level. **Results:** *In vitro,* the guava extract caused a decrease in the cell viability and growth when compared to the control and corticosteroids. *In vivo*, guava extract caused accelerated wound healing from the 3rd day, whereas the corticosteroids delayed tissue repair and were associated with bacterial surface colonization, the presence of micro-abscesses and intense inflammatory infiltrate in the submucosa. **Conclusions:** Although in a short-term cytotoxicity analysis, the guava extract reduced the cell population *in vitro*, while *in vivo,* the extract accelerated wound healing.

Keywords: *Psidium guajava*, wound healing, cell culture, guava leaves, cytotoxicity.

Introduction

Medicinal plants contribute significantly to primary health care in many countries and serve as the starting point for several semi-synthetic analogs. Numerous plants and plant components have demonstrated antiinflammatory and wound healing properties as well as cytotoxic activity, which illustrates the potential for novel agents to be identified from uncharacterized natural plant resources[1-5].

The guava tree, *Psidium guajava* Linnaeus (Myrtaceae) (hereafter referred to as guava), is a tropical hardwood plant that can reach a height of 10 m. It is considered native of Mexico and extends throughout South America, Europe,

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Africa and Asia[6-8]. Guava is used medicinally in many parts of the world as an antiinflammatory and antiseptic as well as in the treatment of diabetes, hypertension, pain, fever, respiratory disorders, gastroenteritis, diarrhea and dysentery. The leaves are applied to wounds, ulcers and joints (for the relief of rheumatic pain) and are also chewed to relieve toothache[8].

The wound healing properties of a methanolic leaf extract of *Psidium guajava* have been determined in only one paper using the excision wound model. More than 90% wound healing was observed after 14 days post-surgery, compared to 72% healing in the group treated with distilled water[9]. The wound healing process involves a variety of events (inflammation, cell proliferation and contraction of the collagen lattice) and may be hampered by the presence of oxygen free radicals or microbial infection. The aims of this study were to evaluate the wound healing potential in vivo and *in vitro* cytotoxic effects of a *Psidium guajava* (*Myrtaceae*) leaf extract and commonly used corticosteroids.

Material and methods

Plant Collection and Preparation

Guava leaves were collected from the city of Valinhos in the state of São Paulo, Brazil and registered at the Herbarium of the Bioscience Institute, São Paulo, Brazil. The leaves were dried for 96 h in an oven at 40° C and ground. The hydroalcoholic extract was prepared with 100 g of leaves through fractionated percolation (7 days by fraction), using 70% ethanol at room temperature, obtaining 100 mL of final extract. The extract was stored in a refrigerator at 5° C until use.

Leaves of *Psidium guajava L.* were submitted a phytochemical analysis of the main groups of active compounds using the following methods:

• Tannins – Dry leaves were boiled in water (decoction), filtered and tested with proteins (gelatin), heavy metals (iron, lead and copper) and alkaloids (pilocarpine hydrochloride) in precipitate reactions.

• Flavonoids – Dry leaves were extracted with 70% alcohol, cooled and filtered. The extract was tested through colorimetric reactions with hydrochloric acid, magnesium metal, ferric chloride III, sodium hydroxide and UV light (366 nm).

• Essential oil – The oil of the leaves was extracted through hydrodistillation (Clevenger method).

• Saponnins – The dry leaves were boiled in water, cooled and filtered. Ten mL were shaken for 15 s and set to rest for 15 min. The 1-cm layer of foam was analyzed.

• Free anthraquinones – The dry leaves were shaken with hexane. The supernatant was removed and tested with ammonium hydroxide. Anthraquinones glycosides – The dry leaves were boiled in 10% hydrochloric acid and iron chloride, filtered and tested with ammonium hydroxide.

• Cardiac glycosides – A hydroalcoholic extract was purified by precipitation with lead acetate, filtered and extracted with chloroform. The chloroform fraction was evaporated. Keller Killiani, Baljet, Kedde, Lieberman-Burchard reactions were tested colorimetric methods.

• Alkaloids – Dry leaves were boiled in 10% hydrochloric acid, cooled, filtered, basified, extracted with chloroform, dried and dissolved again with 10% hydrochloric acid. Bertrand, Mayer, Bouchardat and Dragendorff precipitation reactions were tested.

The quantitative analysis of total polyphenols (taninns) was carried out by precipitation with dry skin powder. The results were detected by absorbance in a spectrophotometer (SP 22, Biospectro, São Paulo, Brazil) with UV light l 715 nm.

Experimental Groups

For both the in vitro and in vivo studies, four groups were tested: Group I (GI): Control (no drugs); Group II (GII): guava tree leaf hydroalcoholic extract (10%) in orabase formulation; Group III (GIII): topical triamcinolone acetonide (Omcilon A, Bristol Myers Squibb, Sao Paulo, SP, Brazil); Group IV (GIV): 0.05% clobetasol propionate cream (Psorex®, Medley, Campinas, SP, Brazil)

Cytotoxicity analysis (in vitro study)

The toxicity of the guava leaf formulation and corticosteroids used in the topical treatment of oral ulcers was determined *in vitro*. The response of human gingival fibroblasts to substances leached from these drugs was analysed. Cell viability was determined using the MTT reduction test (short-term and long-term assays) following contact with the substances. Cell culture was chosen, as this method enables the control of bias by eliminating factors related to the subject reaction[5].

Cell culture

The cells were cultured as described elsewhere[5]. Briefly, the FMM1 cell line of human gingival fibroblasts was used. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil) and 1% anti-mycotic-anti-biotic solution (10,000 units of penicillin, 10 mg of streptomycin and 25 ¼g of amphotericin B per mL in 0.9% sodium chloride; Sigma Chemical Company, St. Louis, MO, USA). The cells were kept in an incubator at 37° C and a humidified 5% CO2 atmosphere. Cultures were supplied with fresh medium every other day. Cells between the 10th and 14th passages were used in all experimental procedures.

Culture medium conditioning

In order to obtain the conditioned media (e.g., media containing the substances dissolved or leached from the test materials), 50-mL centrifuge tubes containing the materials were filled with DMEM (Sigma). Conditioning was carried out for 24 h at 37° C, using 0.2 g of each substance per ml of fresh medium[5]. Conditioned media were applied to the cell cultures and cell viability was determined after 1, 3 and 5 days through an analysis of mitochondrial activity.

Experiment

For the determination of the effect of the substances on

cell viability, FMM1 cells were plated into 96-well microtitration plates (1.8 x 10^3 cells/well). The experimental cultures were grown under conditions of nutritional deficit (culture medium supplemented with 5% FBS). This *in vitro* situation produces an environmental situation that is potentially similar to *in vivo* stress conditions during wound healing[5]. Mitochondrial activity analysis was performed to infer cell viability and plot cell growth curves. All experiments performed with eight replicates.

Analysis of mitochondrial activity

Mitochondrial activity was analyzed using the MTT-based cytotoxicity assay. The MTT assay involves the conversion of the water soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into an insoluble formazan. The formazan is then solubilized and the concentration is determined by optical density at @570 nm. For such, an MTT reduction analysis kit (Vybrant MTT, Molecular Probes, Eugene, OR, USA) was used. Immediately after the end of the assay procedures, absorbance was read in a microplate reader (Biotrak II, Biochrom Ltd, Eugendorf, Austria) using a 562 nm filter. The absorbance data was used to plot the cell growth curves.

Statistical Analysis

The optical density data corresponding to cell viability obtained in eight replicates are presented as mean ± SEM. The data were compared using analysis of variance (ANOVA) complemented with Tukey's test. The level of significance was 5% ($p < 0.05$).

Wound Healing Evaluation (In Vivo Study)

Wistar rats (*Rattus novegicus albinus,* Rodentia; Mammalian) weighing 250 to 300 g were used in this study. The animals were fed before and during the experimental period with solid chow and water *ad libitum*. This study was conducted under the approval of the local Ethics Committee and is in accordance with national guidelines for the care and use of experimental animals that are in compliance with the National Institutes of Health guide for the care and use of laboratory animals.

Traumatic ulcers were made on the tongue using a circular scalpel with 3 mm of diameter. Then each animal received two daily applications of the medicine tested with swab at 12-h intervals throughout the experiment. After 1, 3, 5, 7 and 14 days, 5 animals from each experimental group were sacrificed by anesthetic overdose. After ether inhalation, the animals were anesthetized with administration of 1 mL/kg of 1% ketamine hydrochloride (Dopalen, Vetbrands, São Paulo, SP, Brazil and 2% xylazine hydrochloride (Anasedan, Vetbrands).

The tongue was removed and immersed in a 10% buffered formalin solution for 48 h. After washing with water, the specimens were dehydrated and embedded in paraffin. The tissue specimens were sectioned into 5-ìm-thick slices and stained with hematoxylin-eosin for routine histological analysis. The medial section of the ulcers was selected analysis under a light microscope at a magnification of x40.

Clinical evaluation

The state of the ulcer repair process was clinically analyzed after sacrifice of the rats. The status of the repair process was then classified as 'repaired' when no clinical signs of the ulcer were observed or 'not repaired', when clinical signs of the ulcer were observed.

Histological Evaluation

The evaluation of tissue responses was performed considering the microscopic aspects of the epithelium and connective tissue. For the epithelial tissue analysis, the width of the wound covered by epithelial cells was taken into account. For the connective tissue, the status of the inflammatory process (determined by the type and amount of inflammatory cells) and the presence or absence of fibrosis were analyzed. The histological evaluation was based on the degree of tissue healing using a 5-point scoring system ranging from Grade 1 (total repair of the epithelium and connective tissue) to Grade 5 (epithelium ulcer and acute inflammatory infiltrate). The microscopic characteristics of each grade were as follows:

Grade1: total epithelium repair with underlying connective tissue with fibrosis and absence or presence of few inflammatory cells;

Grade2: total epithelium repair with underlying connective tissue with fibrosis and moderate number of inflammatory cells, represented by macrophages, plasma cells and lymphocytes;

Grade3: presence of ulcer with two-thirds of its width covered by epithelium and the connective tissue showing mild fibrosis and moderate number of inflammatory cells, represented by macrophages, plasma cells and lymphocytes;

Grade4: presence of ulcer with one-third of its width covered by epithelium and the underlying connective tissue with a moderate degree of fibrosis and number of inflammatory cells represented by macrophages, plasma cells and lymphocytes;

Grade 5: less than one-third of epithelium width repaired and connective tissue with moderate or large number of inflammatory cells, represented by neutrophil granulocytes, macrophages, plasma and lymphocytes; presence of micro-abscesses.

Statistical analysis

The data on the scores obtained in 5 replicates *per* group at each experimental time are presented as mean ± SEM. The data were compared using the Kruskal-Wallis test, complemented with Dunn's test. The level of significance was set at 5% ($p < 0.05$).

Results

Phytochemical analysis

The results from phytochemical analysis are displayed on Table 1. Total polyphenols (tannins) in the leaves and hydroalcoholic extract was 11.91% (±0.85) and 8.53% (±

Table 1. Phytochemical analysis of the main groups of active compounds.

Active compound	Presence
Taninns	+
Flavonoids	+
Essential oil	+
Saponnins	+
Free antraquinones	-
Antraquinones glycosides	-
Cardiac glycosides	-
Alkaloids	-

0.64), respectively. The amount of tannins and the phytochemical screening of *Psidium guajava* leafs are in agreement with previous studies[7-8,10-12] showing that the extraction process was adequate.

In vitro analysis

Cell viability of the four experimental groups is displayed in Figure 1. All experimental groups exhibited positive cell viability at 24 h. The cultures treated with guava had the least cell viability among all groups ($p < 0.05$) throughout the entire experiment. Regardless of the drug formulation, the cultures treated with corticosteroids had similar cell viability to that of the control cultures at 24 h. On days 3 and 5 the viability of cells treated with corticosteroids was also significantly lesser than that in the control group.

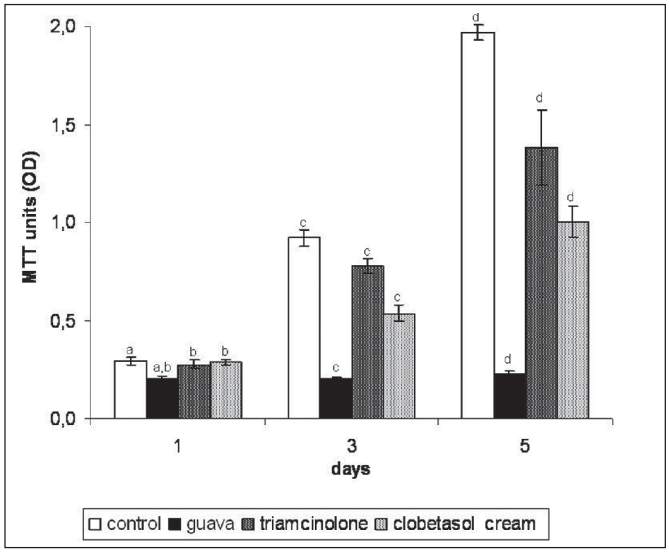

Fig. 1. Cell viability (MTT assay) in the four experimental groups. Same letters indicate statistically significant difference among groups.

In vivo analysis

The clinical evaluation revealed no detectable repair for the first five days in any group. On day 7, the guava group exhibited the highest percentage of repair of all groups. Total repair occurred only on day 14. The corticosteroid groups achieved the worst tissue repair results throughout the entire experiment (Figure 2).

The histological analysis (Figure 3) revealed that the animals treated with guava exhibited faster wound healing than the control and corticosteroid groups. On days 5 and 7, the guava group exhibited significantly better histological findings than corticosteroid groups comparable with Grade 1 (e.g., total epithelium repair with underlying connective tissue with fibrosis and either the absence or presence of few inflammatory cells). The experimental groups treated with corticosteroids reached this stage of tissue repair only after 14 days of treatment. Moreover, the wound healing in the corticosteroid groups was significantly slower than that of the control group ($p < 0.05$).

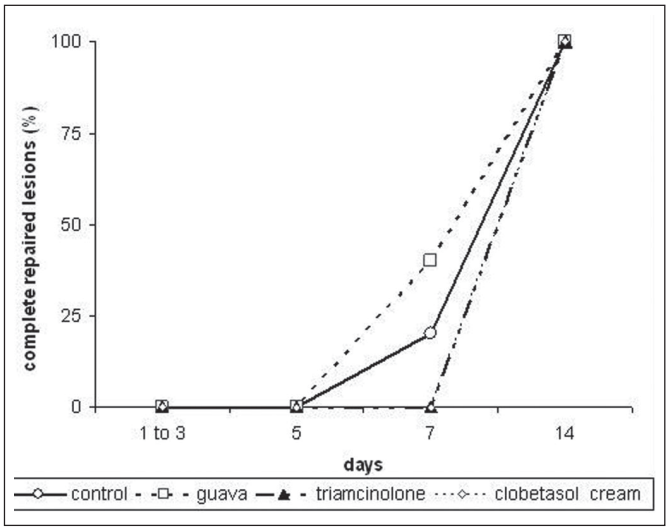

Fig. 2. Clinical evaluation (percentage of repair) of the four experimental groups.

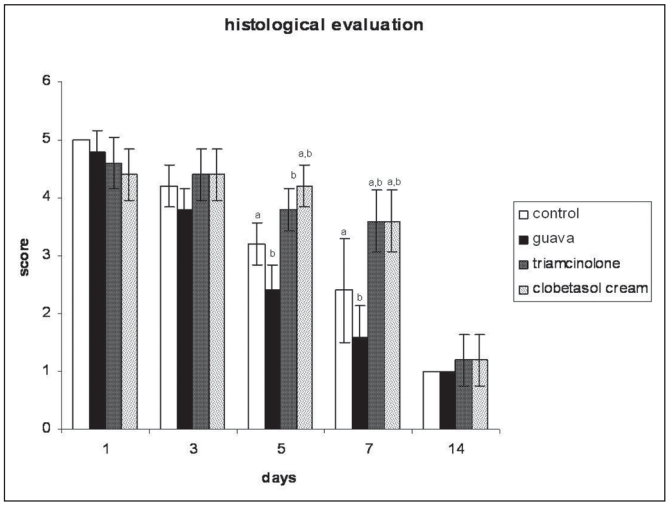

Fig. 3. Histological analysis (scores) of the four experimental groups. Same letters indicate statistically significant difference among groups.

Discussion

A wide range of therapies for promoting wound healing have been sugested, such as topical corticosteroids, antimicrobial agents, cyanoacrylate adhesives, chemical

cautery, phototherapy with low-power lasers and other antiinflammatory, immunosuppressive, and immunomodulatory agents[13,14]. Topical corticosteroids applied 4 times a day constitute the main armamentarium in controlling the symptoms of ulcerous and inflammatory diseases. However, the long-term and/or repeated application of corticosteroids could cause adrenal suppression[15]. The therapeutic use of medicinal plants would be of importance to avoid the undesirable side effects of corticosteroid therapy[5]. In the quest for drugs capable of replacing the most commonly used corticosteroids for improving wound healing, the present study analyzed guava, which has been proven to maintain cell survival (as shown by the continued metabolic activity, i.e., the reduction of the tetrazolium salt by cells) and was able to enhance the healing of experimental wounds in rats.

The different phases of the wound healing process overlap and, ideally, an herbal medication should affect at least two different processes before it can be said to have some scientific support for a traditional use[16]. After the initial rally of neutrophils to a wound site, several other types of cells are recruited to carry forward the repair processes. These include monocytes and, more importantly, fibroblasts, which are attracted into the site to initiate the proliferative phase of repair. An extract that stimulates the growth of fibroblasts can therefore be considered useful in helping a wound to heal. Fibroblasts can be grown in a culture and their proliferation is assessed by the metabolism of a colorful substance, such as MTT[5,16].

In the present study, although the viability of treated cells (cultured medium conditioned by the guava formulation) was significantly lesser than in all other groups, viable cells were still present in the culture plates after 5 days of contact with the drug. This result is in agreement with previous cytotoxic tests on guava leaf extracts[2,17-18].

Wound infection is a frequent occurrence in ulcerative lesions and the postoperative period, which could delay the wound healing process and lead to complications culminating in chronic non-healing wounds. A number of plant products, including those from guava, are known to have antibacterial activity, which enhances their medicinal value[1,4,6,8-9,12,19-23]. Based on the reduction in the cell population when in contact with the guava extract and the fact that the histological analysis revealed no formation of abscess on the ulcer surface, the results of the present study indicate that guava has antibacterial properties. In contrast, the corticosteroid groups exhibited no antibacterial activity, considering the extensive abscess formation and proliferation of cells in contact with the conditioned media, as observed in a previous study on chamomile[5].

The clinical and histological analyses revealed that rats treated with the guava leaf extract experienced faster healing than that in the groups treated with corticosteroids, thereby demonstrating the effectiveness of guava leaf extract in wound healing. This positive influence of guava leaf extract on wound healing has previously been documented[6,9] and may be explained by the fact that this plant contains many different substances, including tannins and flavonoids. Data suggest that these compounds have antiinflammatory, astringent, antibacterial and antifungal properties[11].

Natural products have provided and will continue to provide a unique element of molecular diversity and biological functionality in drug research and development. Based on the obtained results and within the limitations of the study (i.e., number of animals evaluated), we suggest that *Psidium guajava* is a phytotherapy that may contribute to tissue healing.

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