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A DECADE OF RESEARCH ON *Virola oleifera*: ETHNOPHARMACOLOGY BOTANY, PIIYTOCHEMISTRY, AND PHARMACOLOGICAL APPLICATIONS

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Tese apresentada à Universidade Vila Velha como pré-requisito do Programa de Pós-graduação em Ciências Farmacêuticas, para obtenção do grau de Doutora em Ciências Farmacêuticas.

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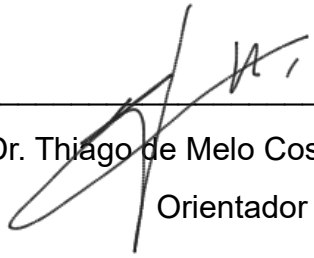

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SUMÁRIO

RESUMO

CARVALHO, GLAUCIMEIRE ROCHA., Dr.: Universidade Vila Velha -ES; Julho 2024; **Uma década de pesquisa sobre V*irola oleifera*: etnofarmacologia, botânica, fitoquímica e aplicações farmacológicas.** Orientador Thiago de Melo Costa Pereira

Virola oleifera (Schott) A. C. Smith é uma árvore endêmica das regiões de Mata Atlântica do Sul e Sudeste do Brasil e possui diversas propriedades farmacológicas. A resina de *Virola oleifera* (VO) desta planta tem sido amplamente utilizada na medicina popular para o tratamento de diversas doenças infecciosas crônicas e doenças hemorrágicas. Esta revisão representa a primeira abordagem bibliográfica abrangente sobre a espécie de VO destacando seus usos etnomedicinais, fitoquímicos e atividade biológica. Além disso, visa orientar futuras pesquisas com este extrato vegetal.

Palavras-chave: Bicuíba, Antioxidante, Farmacologia, Compostos Fenólicos e Medicina Tradicional

ABSTRACT

CARVALHO, GLAUCIMEIRE ROCHA., Dr. Vila Velha University -ES; July 2024; **A decade of research on *Virola oleifera*: ethnopharmacology, botany, phytochemistry and pharmacological applications.** Advisor Thiago de Melo Costa Pereira

Virola oleifera (Schott) A. C. Smith is a tree endemic to the Atlantic Forest regions of South and Southeast Brazil and has several pharmacological properties. The resin of *Virola oleifera* (VO) this plant has been widely used in folk medicine for the treatment of various chronic infectious diseases and hemorrhagic diseases. This review represents the first comprehensive bibliographical approach to the *Virola oleifera* species, highlighting its ethnomedicinal, phytochemical and biological activity uses. Furthermore, it aims to guide future research with this plant extract.

Keywords: Bicuíba, Antioxidant, Pharmacology, Phenolic Compounds and Traditional medicine

CAPÍTULO 1

1. *VIROLA OLEIFERA* AND ITS ETHNOPHARMACOLOGICAL TRADITIONS

Virola oleifera (Schott) A. C. Smith is a tree endemic to the Atlantic Forest regions of South and Southeast Brazil, exhibiting distinct pharmacological potential, as evidenced by various studies (Pereira et al., 2017; Quintanilha e Lobão, 2017; Francisco et al., 2021). Belonging to the Myristicaceae family, *Virola oleifera* (VO), also known as *Virola bicuhyba*, is part of a larger family comprising approximately 21 genera and nearly 500 species (Rodrigues, 1980; Aguilar et al., 2019). Within the Atlantic Forest biome, which encompasses 5 genera and 70 species (with 35 found in the Amazon), VO is specifically located in the Afonso Claudio region of the state of Espírito Santo, Brazil (Bôa et al., 2015; González-Rodríguez et al., 2021; Santamaría-Aguilar; Lagomarsino, 2022). The species is considered semi-deciduous, preferably grown on slopes, but it also develops in moist soil. It stands out in the woods for its wide canopy with sparse foliage and small clusters of capsule-type fruits with red seeds, reaching 35 m in height. The stems can grow up to one meter in diameter, and inside the bark contains reddish exudate (Rodrigues, 1980; Reitz and Klein, 1968; Bôa et al., 2015).

Virola has garnered attention for medicinal properties, derived from sap extraction through incisions in the trunk, commonly known in Brazil as 'bicuíba', 'blood-of-bicuíba', 'bicuíva', 'bocuva'. The term originates from the Tupi language, where "uku" refers to tallow, grease or fat, and "uba" denotes plant or tree, signifying a tree that produces a fatty substance (Reitz; Klein, 1968; Carvalho et al., 2022). Traditionally, substances extracted from the bark were used to address various conditions such as bleeding, diarrhea, dysentery, hemorrhoids, bronchial asthma, tumors in the joints, and intestinal worms (Pereira et al., 2017; Coutinho et al., 2017; González-Rodríguez et al., 2021).

This review on the species of VO aims to contribute to the scientific community by offering a comprehensive understanding of its ethnopharmacological uses, pharmacological activities, and the reproducibility of research on its biological properties. Moreover, it aims to support the safe

medicinal utilization of plant extracts and to guide future research towards the discovery of new molecules with pharmaceutical potential.

Fig. 1. A) Presence of *Virola oleifera* (VO) in the Afonso Cláudio region located in the state of Espírito Santo (ES-Brazil). B) Adult specimen, reaching approximately 30 meters in height, with a stem diameter of 105 centimeters. C) Use of aseptic container for resin extraction. D) VO seed fruits. E) Collection of the reddish resin from the tree stem.

2. DESCRIPTION OF THE CHEMICAL COMPOSITION

The chemical compounds present in VO resin belong to various classes, including polyphenols which can be found in both the leaf and resin (González-Rodríguez et al., 2021). Quantification of the VO resin using spectrophotometric and chromatography-mass spectrometry methods revealed a high content of polyphenols (~82%), such as phenolic acids and flavonoids (48.26 ± 28.27 mg quercetin equivalent/100 g of resin), as well as tannins (67.66 g/100 g resin), ferulic acid (22.6 µg/mg), and gallic acid (142.1 µg/mg) (Bôa et al., 2015; Pereira et al., 2017). These compounds have been the subject of investigations to determine their effectiveness in different animal models of oxidative stress-dependent diseases.

Studies demonstrate that the leaves of VO contain compounds such as (+) – aristolignin, galbacin, eupomatenoid-8 (Fernandes et al., 1993), verrucosin,

galbulin (Sartorelli et al., 1998), otobafenol in the arils, otobain, methylaustrobailignan-6 and arylalkanones in kernels (Sartorelli and Kato et al., 1997), oleiferin-A, B, C, D, E (Fernandes et al., 1993), F, G, H, 4-hydroxy-5,3',4'-trimethoxy-2, 7'-cyclolignan (Sartorelli et al., 1998). The analgesic activity of the methanolic extract of VO leaves was evaluated by the writhing test in mice, and these effects were attributed to flavonoids and oleiferin-C (astilbine and quercitrin) (Kuroshima et al., 2001).

3. PHARMACOLOGICAL INSIGHTS AND EXPERIMENTAL MODELS OF *VIROLA OLEIFERA*

Of all the investigations published to date (2024) in the global literature on the pharmacological effects of *Virola oleifera*, 62.5% of the articles originate from our research group. Since 2015, a range of pharmacological activities associated with VO resin has been documented in oxidative stress-dependent models, as outlined below.

3.1 Renoprotection

Bôa et al. (2015) reported that pre-treatment with a dose of 300 mg/kg of VO resin provided significant nephroprotection in all parameters when compared with the standard drug N-acetylcysteine (200 mg/kg) in an innovative radiocontrast-induced nephrotoxicity model in mice (developed by our lab group). VO preserved kidney function through its antioxidant and antiapoptotic effects, resulting in reduced renal dysfunction and morphological tubular injury, as well as decreased medulla and cortex renal cell damage in a dose-dependent manner.

3.2 Cardiovascular protection

The antiatherogenic activities of VO resin have been demonstrated by Coutinho et al. (2017) in the atherosclerotic $LDLr^{-/-}$ mouse model. Chronic oral administration of VO resin at a dose of 50 mg/kg exhibited significant antiatherogenic activity by reducing vascular lipid deposition, protecting vascular endothelium and smooth muscle cells against peroxide-induced cytotoxicity, and

decreasing nitric oxide production induced by LPS in macrophages. These results are partially attributed to systemic and local antioxidant and anti-inflammatory mechanisms, which demonstrate the resin's protective effect on lipid vascular deposition, independent of hypercholesterolemia. These antiatherogenic results with VO opened new avenues of research for this resin as a potential adjuvant to classical cardiovascular therapies (e.g., statins, ezetimibe), which solely aim to reduce LDL cholesterol levels without a direct impact on the inflammatory response or oxidative stress reduction.

3.3 Gastroprotection

The gastroprotective effect of VO has been elucidated by Pereira et al. (2017) in two experimental models of gastric ulcers dependent on oxidative stress. In this study, resin was administered at doses of 10 mg/kg and 100 mg/kg, 30 minutes before inducing gastric lesions, resulting in significantly reduced gastric mucosal damage comparable to the reference control, lansoprazole at 3 mg/kg (equivalent to approximately 20 mg in an adult, according to Regan-Shaw et al., 2007). The gastroprotective activity of VO resin could be attributed to its composition, which is rich in phenolic compounds. This gastroprotective response, through mechanisms distinct from PPIs (proton pump inhibitors), opens further investigations aiming to explore alternatives for reducing long-term consumption of molecules that increase gastric pH. Such an approach may help avoid a variety of nutritional disadvantages (such as reduced absorption of vitamin B12, calcium, iron) and prevent various drug interactions such as ketoconazole and itraconazole that require an acidic environment for better dissolution and consequently, absorption (Wang et al., 2015).

3.4 Healing activity

Carvalho et al. (2022) developed an innovative formulation containing 5% VO to evaluate its wound-healing efficacy in rats. Through topical application of the cream VO 5% (incorporated with 10% Compritol ATO 888 (a non-irritant lipid excipient composed of a blend of different esters of behenic acid with glycerol)), to incisions, superior tissue healing was observed compared to the Dersani®

((linoleic, capric, caproic and caprylic acids, sunflower oil, soy lecithin, vitamin A and E), reference treatment. Histological analysis confirmed the presence of regular borders and crusts, indicative of improved healing. Furthermore, the VO treatments exhibited a significant decrease in protein oxidation and lipid peroxidation levels. These results strongly indicate the wound healing effect of the VO cream, which can be attributed, at least in part, to its antioxidant mechanism, which contributes to the re-epithelization response. Despite VO having been investigated for its popular reports of significant wound healing and antihemorrhagic effects, this was the first study to demonstrate relevant topical effects of the resin. These findings also indicate VO as a promising ingredient in dermocosmetics for wound healing and even in future anti-aging therapies.

3.5 Immunomodulator activity

VO is popularly used to treat rheumatic pain and joint tumors (Rodrigues in 1980; González-Rodríguez et al., 2012). To evaluate the results of this traditional practice, Francisco et al. (2021) analyzed the therapeutic potential of this resin from VO in the treatment of joint and bone diseases. The results demonstrated that VO has no significant effects on nitric oxide production induced by lipopolysaccharides in chondrocytes. Coutinho et al., (2017) demonstrated different results. The stimulation of macrophages with LPS followed by RV treatment resulted in a significant reduction in nitric oxide production in a concentration-dependent manner. These results could be justified by the use of different cellular models and the tolerance of cells to high concentrations of VO. Interestingly, the VO treatment reduced cell viability in multiple myeloma dose dependent manner, while osteosarcoma and chondrosarcoma were affected only by the highest dose. Additionally, in the multiple myeloma cell line, VO caused cell cycle arrest in the G2/M phase.

The evaluation of the potential of VO as an adjuvant therapy revealed its ability to synergize with dexamethasone, increasing cellular toxicity. In the case of bortezomib activity (an inhibitor of the degradation of various pro-apoptotic factors with consequent activation of programmed cell death), the resin neutralized the activation of the ERK1/2, Bax and caspase-3 signaling pathways.

These results confirm the efficacy of the resin in the treatment of bone and joint diseases. Although it has not shown notable advantages for inflammatory or catabolic processes linked to arthritis, VO can be used as adjuvant therapy to increase the effectiveness of chemotherapy drugs. However, potential interactions between herbs and medications should be carefully considered before clinical implementation

3.6 Toxicity

In addition to all the protective effects of VO already demonstrated, it is worth noting that its potential toxicity has also been investigated. *In vitro* investigations demonstrated that VO resin does not have cytotoxic effects on fibroblasts (Coutinho et al., 2017). Regarding acute toxicity, a study reported by Pereira et al. (2017) showed that two animals died after receiving the resin at a dose of 2000 mg/kg, while other animals did not show macroscopic changes. The results indicate that the VO resin would be classified in category 5, with an estimated LD50 (median lethal oral dose) of 2500 mg/kg. The cytotoxicity of gold nanoparticles from VO resin was also assessed through the Alamar Blue assay for cellular viability analysis. No significant signs of cytotoxicity were observed, as reflected by the high percentage of cell survival in the J774A cell line (Dos Santos et al., 2018).

3.7 *Virola oleifera* – capped gold nanoparticles

Milaneze et al. (2016) reports the reproducible synthesis of gold nanocrystals using VO resin. These gold nanoparticles (AuNPs) are characterized by their uniformity in size and shape, remaining stable in solution. Due to the reducing and capping properties of the bioactive present in the VO aqueous extract, a layer forms around the gold ions of the stable biofunctionalized AuNPs, highlighting the presence of organic compounds that can potentially provide significant biological activity to these nanoparticles. Thus, this consolidates a new and promising approach in the production of gold nanoparticles with bioactive properties.

Investigations into the antioxidant activity and cytotoxicity of gold nanoparticles reveal that VO resin acts as a protective and functionalizing agent. The gold nanoparticles derived from VO resin showed enhanced antioxidant properties compared to raw exudate and exhibited minimal cytotoxicity, as demonstrated by Dos Santos et al. (2018). These results consolidate an innovative and promising approach in the production of gold nanoparticles with bioactive properties.

4 Challenges and Futures perspectives

4.1 Challenges in the translational area

Natural products are often used in both basic and translational research in Brazil. This practice benefits from the country's rich biodiversity, the pharmacological properties inherent to these compounds, as well as low rates of side effects and reduced production costs (Pinto et al., 2002). However, research involving the utilization of natural compounds, such as VO resin, encounter several challenges that demand effective approaches and strategies.

Although research involving the use of natural compounds, such as VO resin, faces challenges such as variability in the chemical composition of natural products, there are ways of improving the soil that can favor and reduce these impacts. The difficulty associated with large-scale production of VO resin, due to the reduction in raw materials, is directly impacted by deforestation. In this context, reforestation strategies and the implementation of sustainable practices in collection and cultivation emerge as beneficial measures that can significantly contribute (Pinto et al., 2002).

The discreet incentives granted by funding agencies and the distance between academic institutions and the private sector have an impact on the innovative capacity of translational research. This is evidenced by the allocation of resources to clinical trials, which, in turn, are notoriously expensive. Therefore, overcoming this financial gap is crucial to driving significant advances in national scientific research, strengthening competitiveness and fostering academic excellence.

It is worth mentioning that we have a product analogous to *Virola oleifera*, Pycnogenol (PYC), an antioxidant extracted from the bark of the French maritime pine. Successful global experiences, such as the notable case of PYC, highlight the success associated with tree resins, demonstrating significant impacts on health (Rohdewald, 2002; Robertson et al., 2012; Zhang et al., 2018;). This emphasizes the importance of preserving, reforesting, and sustainably exploiting VO, which has a similar potential impact to PYC. Additionally, financial incentives for continued research and appropriate allocation of natural resources are essential to enable substantial advances in health and medicine.

4.2 Extinction and preservation of *Virola oleifera*

The Brazilian Atlantic Forest, acknowledged as a biodiversity hotspot, is currently confronting one of the greatest threats among the biomes of Brazil, such as deforestation and climate change (Myers et al., 2000; Joly et al., 2014; Romanelli et al., 2022). Over 500 years, the Atlantic Forest has been a target of exploitation and destruction, resulting in a mere 8% of its original forest cover remaining (Rodrigues et al., 2020). In this context, VO is one of the most impacted species, suffering a significant reduction in its area (Colombo; Joly, 2010). This situation underscores the urgent need for the implementation of effective conservation and preservation measures to reverse this alarming trend. The sustainable use of plant resources is a fundamental priority for human subsistence and the promotion of forest biodiversity maintenance.

4.3 Futures perspectives

Comprehensively, in-depth studies on translational investigations are needed to substantiate the previously reported therapeutic potential of VO resin. Although several ethnopharmacological effects of VO resin have been reported, there are still several research gaps to be explored such as in the areas of food and dermocosmetics. Previous studies demonstrate the use of plant extracts with high antioxidant content as nutraceuticals, being applied for both food and medicinal purposes (McCubrey et al., 2017; Rahman et al., 2022; Obregón-La et

al., 2023). In this context, VO resin can be a promising alternative due to its properties.

Furthermore, research into experimental diabetic models is essential due to the challenge of treating diabetic wounds. In addition to investigating the real healing capacity of VO in more vulnerable models, since treating wounds in diabetics is still a major challenge in the dermocosmetic area. Although research demonstrates that the crude extract VO has antioxidant activity, the understanding of the specific mechanisms of action of the resin are not completely elucidated, thus requiring additional molecular biological investigations.

Preliminary studies, not yet published, has pointed to new potential therapeutic applications of VO. As example, we demonstrated that a cream prepared from VO (1%, applied twice a day for 10 days) showed an excellent healing effect in a domestic animal (cat). In addition, unpublished data reveal that a pre-treatment with VO resin at a dose of 10 mg/kg has nephroprotective, hepatoprotective, antimutagenic and antigenotoxic effects in a paracetamol toxicity model. More recently, we also observed the pro-aggregating effects of VO resin in an *ex vivo* experimental model, contributing to the understanding of the mechanisms of action.

5 CONCLUSION

In traditional medicine, plants are widely used due to their phytochemical properties, accessibility, and probably, limited side effects. Our review highlights resin as a promising therapeutic agent, demonstrating its role in preventing and treating various health conditions. The research focuses on its diverse biological activities, showcasing its efficacy in both *in vivo* and *in vitro* experimental models, notably its powerful antioxidant activity. The findings reveal significant benefits including renoprotective, gastroprotective, cardiovascular protective effects, and wound healing properties, all observed at therapeutic doses without notable adverse effects. However, challenges like deforestation, weather conditions, and financial incentives hinder research and commercial resin production. This emphasizes the importance of promoting Brazilian research initiatives to further investigate the resin's mechanisms, fostering scientific advancement,

environmental preservation, and sustainable medical innovations based on plant resources.

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CAPÍTULO 2

Virola oleifera: A Promising Alternative Treatment for Feline Dermatitis – A Case Report

Abstract

Virola oleifera (Schott) A.C. Smith, is commonly used in Brazilian traditional medicine to treat inflammation and heal mucous tissues. Recently, our group have shown the healing capacity of a formulation prepared with its resin through antioxidant mechanisms that contribute to re-epithelialization and reduction of oxidative stress. Other studies conducted by our group with *Virola oleifera* resin (VO) have also demonstrated an abundance of antioxidant molecules in its chemical composition, as well as various pharmacological activities in several models related to oxidative stress. Therefore, VO presents a promise as a natural therapeutic agent for the treatment of wound healing and inflammatory conditions in both veterinary and human fields. Here, we report the case of a 2-year-old Sphynx domestic cat with a 3-month history of skin injury on the neck refractory to conventional treatments. Our aim was to test the efficacy of VO resin for the treatment of this animal. A cream was prepared from VO (1%) and applied twice a day for 10 days. After 1 week, there was an improvement in tissue coloration and a macroscopic reduction of the lesion, with successful wound healing within 10 days of treatment. This is the first report demonstrating the excellent healing effect of the antioxidant VO in a domestic animal.

Keywords: antioxidant, Bicuíba, cats, wound

INTRODUCTION

The use of medicinal plants is common in traditional practices across various cultures worldwide. Over the past few decades, numerous species have been investigated for their wound-healing, anti-inflammatory, and antioxidant activities ‘Beling et al., 2014; Hoffmann; Griffiths, 2018`. In this context, *Virola oleifera* (Schott) A.C. Smith, a tree belonging to the Myristicaceae family in the Atlantic Forest, has garnered significant attention due to its rich composition of phenolic, flavonoid, and tannin compounds, which exhibit potent antioxidant effects, as well as anti-inflammatory and antiapoptotic properties ´BÔA et al., 2015; Pereira et al., 2017`.

In Brazil, *Virola oleifera* (VO), commonly known as "Uccuúba," "Bocuva," "Bicuíba," "Bicuíva," or "Candeia-do-caboclo" ´Rodrigues, 1980`, has been widely used to stop bleeding, promote wound healing, alleviate skin diseases, arthritis, and inflammatory conditions ´ Rodrigues, 1980; Coutinho et al., 2017; Carvalho et al., 2022`. In the last decade, scientific reports from our research group have highlighted the therapeutic potential of crude resin, demonstrating its beneficial effects on atherosclerosis progression, gastroprotection, and protection against renal dysfunction ´Bôa et al., 2015; Coutinho et al., 2017; Pereira et al., 2017`. Specifically, a recent development involving a 5% VO cream formulation for wound care showed healing effects accompanied by antioxidant properties ´Carvalho et al., 2022`.

In this context, our case report investigates the possible healing potential of a formulation containing extracts of VO at a lower concentration (1%) for the treatment of resistant wound healing in cats.

CASE REPORT

A 2-year-old female domestic Sphynx cat was presented with a three-month history of a superficial lesion on the right side of the neck refractory to conventional treatments. During the clinical history and physical examination, the cat exhibited persistent cutaneous bleeding and pruritus, which were

exacerbated by skin itching. These symptoms are indicative of eosinophilic granuloma complex (EGC) (Figure 1A and 1B), a condition characterized by eosinophilic inflammation.

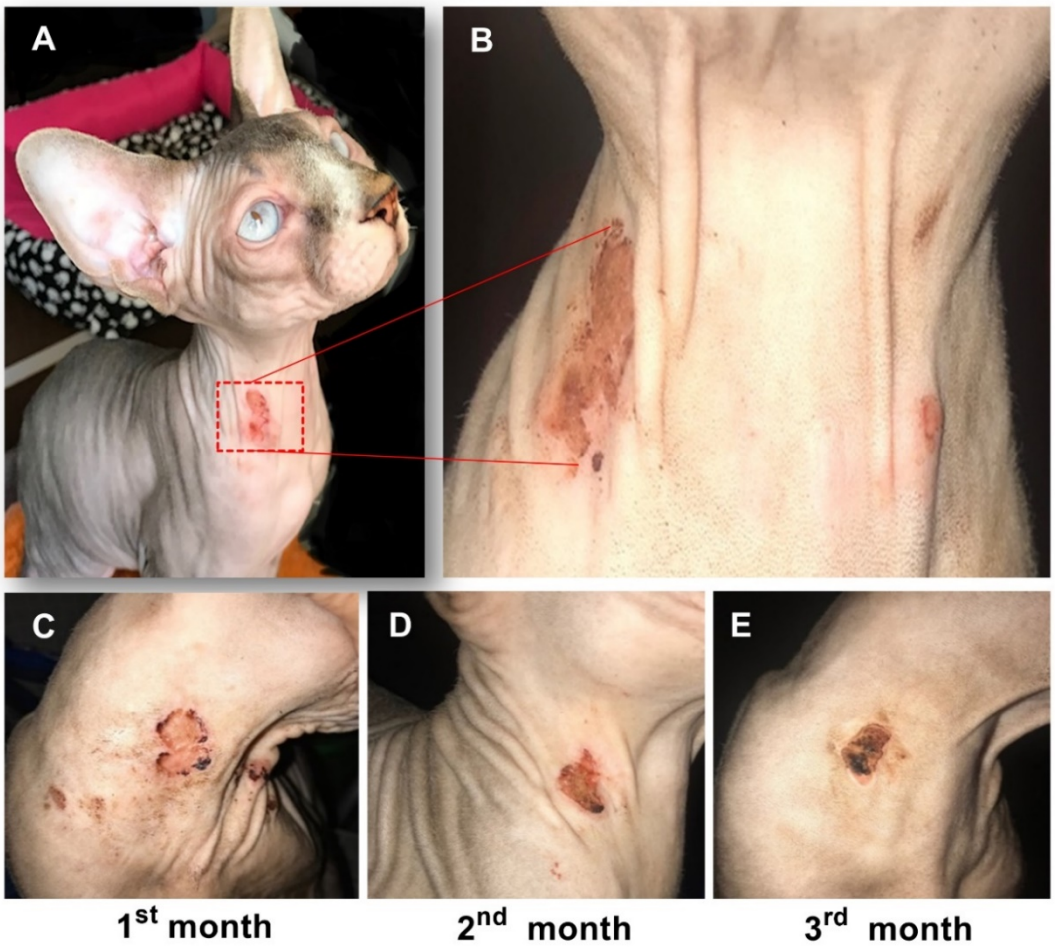


Fig 1. Images of cat skin injury in neck. Skin injury before therapeutic drugs (A - B). Skin injury after 1st (C), 2nd (D) and 3rd (E) months of the classical pharmacotherapy.

The bacterial septic suppurative inflammatory process was confirmed through cytology smear by our group. Additionally, the bacterial culture findings indicated sensitivity and resistance to various microorganisms, including Coagulase-negative *Staphylococcus* and *Acinetobacter baumannii*/*calcoaceticus*. Hematological and serum biochemical inflammatory parameters did not show significant changes. Initially, therapeutic approaches were conducted using the

classical Cefalexin (HMC Chemical Technology Co., Ltd., Beijing, China) at a dosage of 100 mg/kg for 10 days, along with Vetaglós® cream (Vitapan, Anápolis-GO, Brazil) containing retinol palmitate (5000 IU/g), vitamin D3 (900 IU/g), and zinc oxide (15%). Despite one month of treatment, the lesion did not show any improvement. Therefore, Ketoconazole cream (HMC Chemical Technology Co., Ltd., Beijing, China) at a dosage of 10 mg/kg was added (Figure 1C). Subsequently, the medication was switched to Alcort® (CEPAV Pharma, São Paulo-SP, Brazil), a prednisolone formulation, at a dosage of 2.5 mg/kg for 5 days. In the second month, Doxycycline (HMC Chemical Technology Co., Ltd., Beijing, China) at a dosage of 20 mg/kg for 10 days and Trok-N® cream (Eurofarma, Itapevi-SP, Brazil) containing ketoconazole (2%), betamethasone dipropionate (6.4%), and neomycin sulfate (0.25%) were used (Figure 1D). Despite one month of ineffective therapy, prednisolone (4.5 mg/kg for 12 days) in combination with Quadriderm® cream (Schering-Plough, Kenilworth, New Jersey, USA) containing betamethasone (6.1%), clioquinol (1%), tolnaftate (1%), and gentamicin (0.16%) was administered. However, at the end of the treatment, there was no improvement in the magnitude and quality of the lesion, and bleeding and itching persisted (Figure 1E).

Considering the need for alternative or complementary treatments to the traditional therapeutic approach, the use of VO cream was suggested based on contemporary data by Carvalho et al. (2022) in rats. Interestingly, this study from our lab demonstrated enhanced wound contraction and reduced levels of protein oxidation and lipid peroxidation. Thus, the healing capacity of VO may be attributed, at least in part, to its antioxidant properties, which can contribute to reepithelialization, supporting our hypothesis that VO is a promising approach for the treatment of superficial lesions.

MATERIALS E METHODS

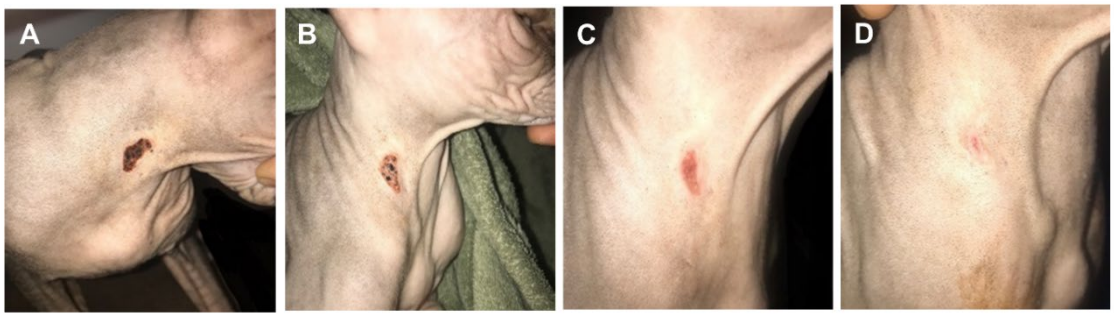
Resin Material, Cream Preparation and Administration

The VO was collected in November 2019 from the Fazenda Guandu district (Afonso Claudio-ES, Brazil; coordinates: S20°13490'W041°06692') under proper official authorization (IEMA 629/09 and Resolution29-12/06/2007). A voucher

specimen (VIES 19648) was deposited at the Department of Botany, Federal University of Espirito Santo. The fluid exudate was obtained from 0.5 cm incisions made in the tree trunk. The fluid (90 ml) was collected in an aseptic amber glass container and stored at 4°C until complete drying. Subsequently, the exudate was dried at 40°C for 75 hours, resulting in 36.5g of dried granulated resin 'Bôa et al., 2015; Pereira et al., 2017'. For the cream preparation, 10 mg of the granulated resin was incorporated into 1g of the base cream. The cream was applied twice daily for 10 days.

RESULTS

On the 3rd day of treatment, we observed that VO was able to reduce the wound size, bleeding, and itching. By the 7th day, there was an improvement in tissue coloration and a noticeable reduction in the size of the lesion, indicating successful wound healing within the 10th day treatment period (Figure 2). It is important to highlight that no adverse effects were reported by the owner. Interestingly, this feline did not experience a recurrence of EGC even 6 months after the treatment, demonstrating only the presence of a discrete scar.



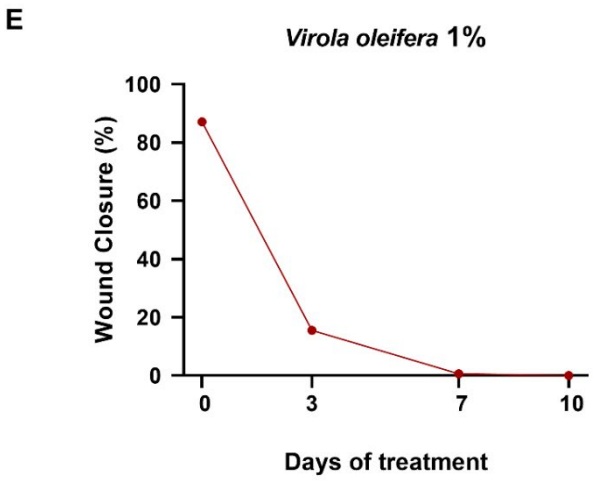


Fig 2. Images of cat skin injury in neck under treatment of VO cream (1%). Start (A), 3rd (B), 7th (C) and 10th (D) days of treatment. Quantitative analysis of wound contraction (E).

DISCUSSION

This is the first case report describing an innovative and effective therapeutic approach for dermatitis disorders using a low-concentration cream obtained from the resin of the VO tree found in the Atlantic Forest. Our findings corroborate previous research from our team, which demonstrated a significant healing effect of VO cream in rats ´Carvalho et al., 2022`. These results further support our hypothesis that VO holds great promise as an approach for treating superficial lesions.

Virola oleifera is commonly used in traditional medicine due to its notable responses to inflammatory conditions ´Ebeling et al., 2014`. Within the tree trunk, a reddish exudate is produced, which possesses antioxidant and anti-inflammatory properties (González-Rodríguez et al., 2021). Its composition comprises a combination of antioxidants such as polyphenols, flavonoids, ferulic acid, and gallic acid, which contribute to its reparative effects, partly through antioxidant pathways ´Bôa et al., 2015; Pereira et al., 2017; Carvalho et al., 2022`. In parallel, the eosinophilic granuloma complex is characterized by an exaggerated inflammatory response, oxidative stress, hypersensitivity, cutaneous manifestations on the skin, pruritus, and pain ´Rocha et al., 2019; Ravens et al., 2014; Buckley, 2012; Ramirez et al., 2018`. These clinical manifestations align with the diagnosis of EGC in the present case, wherein a Sphynx domestic cat experienced an intense inflammatory process persisting for three months, accompanied by challenges in healing, bleeding, and itching.

The classical therapeutic options for EGC include topical and systemic approaches with antimicrobials and anti-inflammatory drugs ´Hopke et al., 2019; Forsythe, 2011`. However, in the current case, these treatments failed to heal the lesion or alleviate the symptoms of itching. In light of the unsuccessful therapeutic

outcomes, we decided to test the VO cream as a new therapy for this condition. Surprisingly, the 10-day treatment with VO ´at low dose) resulted in effective wound healing without any adverse effects. This finding can be supported by *in vivo* and *in vitro* studies that have demonstrated the potential of antioxidants to promote wound healing and regulate local oxidative stress ´Guidoni et al., 2019; Baek, Min-Geol, 2015; Fitzmaurice et al., 2011`. Numerous studies have provided strong evidence of the involvement of oxidative stress in the pathogenesis of various skin diseases ´Baek, Min-Geol, 2015; Fitzmaurice et al., 2011`. Furthermore, phenolic compounds such as ferulic and gallic acid have been shown to act as scavengers and/or inhibit enzymes involved in the production of oxidant factors. They also exhibit potential antioxidant, anti-inflammatory, and antibacterial activities, supporting pro-collagen synthesis ´Guidoni et al., 2019; Baek, Min-Geol, 2016; Carvalho et al., 2022`. Interestingly, previous *in vitro* ´Pereira et al., 2017` and *in vivo* ´BÔA et al., 2015; Coutinho et al., 2017; Pereira et al., 2017` experiments have demonstrated the potential antioxidant effects of VO resin. Therefore, our hypothesis regarding the healing effect of VO can be justified, at least in part, by its antioxidant properties. An additional mechanism of action of VO could be its proaggregating effect, which activates the GPIIb/IIIa pathway (data not yet published). This property may explain the observed reduction in bleeding in the wound of cat treated with VO. Furthermore, the low cytotoxicity of VO, as previously demonstrated in *in vitro* studies on fibroblasts ´Coutinho et al., 2017`, may also justify the favorable reepithelialization.

In conclusion, this report provides the first evidence of a notable healing effect achieved with a cream containing VO resin with antioxidant properties. These findings warrant further research in veterinary clinical practice, and probably, in clinical human studies.

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CAPÍTULO 3

The Role of *Virola oleifera* on Human Platelet Signaling Pathways

ABSTRACT

Ethnopharmacological relevance: *Virola oleifera* (Schott) A. C. Smith, a plant from the Myristicaceae family, has been used in traditional Brazilian folk medicine as a natural healing agent. Previous data from our lab demonstrated that trunk resin exhibits antioxidant properties in other models related to oxidative stress. However, its effects on platelet aggregation have not yet been investigated.

Aims of the study: To evaluate the potential effects of resin from *Virola oleifera* (VO) on human platelet aggregation, as well as the underlying key mechanisms involved.

Materials and methods: For the assays using human platelet aggregation, washed platelets were pre-incubated in an aggregometer with different concentrations of VO (ranging from 1 to 20 µg/mL in DMSO). Subsequent pre-incubations with apyrase and indomethacin were performed (to evaluate the participation of P2Y and thromboxane, respectively). Additionally, platelet toxicity was assessed using the Calcein viability assay.

Results: We demonstrated that VO resin increased platelet aggregation at doses of 10 µg/mL, attenuated by apyrase (28±11%, $p<0.05$), without affecting platelet viability at any of the evaluated doses (>90%).

Conclusions: In summary, our study reveals the pro-aggregation effect of VO extract (via P2Y), emphasizing its topical therapeutic potential for wound healing.

Keywords: Virola; bicuiba; platelet aggregation; Thromboxane A2; P2Y receptors

1. Introduction

Platelet aggregation, a complex process mediated by multiple signaling pathways, serves as a cornerstone in hemostasis, stopping of bleeding after to vascular injury [Xu et al., 2016]. This intricate process entails the adhesion of platelets to one another at the sites of vascular injury, a critical event for the formation of hemostatic plugs leading to wound healing and tissue repair [Jansen et al., 2021; Belyaev et al., 2023]. The initiation of the aggregation process is marked by the activation of receptors present on the platelet surface, which triggers intracellular signaling and finally activating integrin α IIb β 3 [Sang et al., 2021; Nouruzi et al., 2022].

Resin derived from *Virola oleifera* (Schott) AC Smith, a species native to the Atlantic Forest, has been traditionally employed to mitigate bleeding, treat inflammatory conditions, and heal injuries to the skin and mucous membranes [González-Rodríguez et al., 2021]. Recent *in vivo* and *in vitro* investigations have shown the potential of *Virola oleifera* (VO) in the wound healing process, attributing its efficacy to its significant antioxidant properties that reduce oxidative stress, thereby playing a crucial role in wound healing [Bôa et al., 2015; Pereira et al., 2016; Coutinho et al., 2017; Francisco et al., 2021; Carvalho et al., 2022]. However, its effects on platelets have never been studied. Therefore, our aim is to investigate the potential effects of VO on human platelet aggregation as well as the underlying key mechanisms involved.

2. Material and methods

2.1 Platelet isolation

Fresh whole blood was collected into 3.2% (w/w) trisodium citrate tubes (Vacuette®, Greiner Bio-One International GmbH, Kremsmünster, Austria) from consented healthy volunteers who had not received anti-platelet drugs for at least ten days. Washed platelets were obtained after serial centrifugations in presence of 1 µM prostacyclin and resuspended in modified Tyrode's buffer (MTB; 134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM

$MgCl_2$, 5 mM glucose, pH 7.3) to obtain a final concentration of 2.5 x 10^8platelets/mL.

2.2 Light Transmission Platelet Aggregation

To assess VO effect on platelet aggregation, 300 µL of washed platelets (2.5 x 10^8 platelet/mL) were incubated 4 minutes at 37ºC in the Chrono-Log® 490-X aggregometer (Chrono-log Corporation, Havertown, PA, USA), and 1 minute at 37ºC with constant stirring at 1200 rpm. The platelet aggregation was measured during 5 minutes after the addition of VO resuspended in 100% DMSO at different final concentrations (1 µg/mL, 3 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, and 20 µg/mL). For the assays using platelet aggregation inhibitors, washed platelets were pre-incubated for 5 minutes at RT with apyrase (final concentration of 2 Units/mL), indomethacin (final concentration of 10 µM) or both prior VO addition.

2.3 Calcein-AM Viability Assay

To measure potential VO platelet toxicity, a viability assay based on Calcein-AM binding was performed by flow cytometry. Washed platelets (2.5 x 10^6 platelets/mL) were incubated with VO resuspended in 100% DMSO at different concentrations or 0.2% DMSO as vehicle for 10 minutes at 37ºC. Then, 1 µL of Calcein-AM (0.1 mg/mL) (Sigma-Aldrich, Saint Louis, MO, USA) was added and incubated for 20 minutes at 37ºC. Calcein-AM binding was measured in triplicate by an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) and represented as the percentage of the total events. A positive control of platelets without VO and vehicle (DMSO) was used, and a lower 75% Calcein-AM binding was considered cytotoxic.

2.4 Statistical analysis

All data are expressed as the mean ± SEM (standard error of the mean). The statistical analysis was performed by 1-way analysis of variance (ANOVA) followed by post-hoc Tukey's test using Prism software (Prism 8.0, GraphPad

Software, Inc., San Diego, CA, USA). The differences were considered significant when $p<0.05$.

3. Results

3.1 VO *induces platelet aggregation through the purinergic P2Y receptors pathway*

To test the effect of VO on platelet aggregation, an assay was conducted in which VO was administered at different concentrations to washed platelets (2.5×10^8 platelets/mL) and aggregation was measured for 6 minutes. Interestingly, VO 10 µg/mL (43±10%) and 15 µg/mL (43±8%) significantly increased platelet aggregation compared to the two lowest concentrations used: 1 µg/mL (5.0±1.3) and 3 µg/mL (7.3±1.6%) (Fig. 1A), therefore presenting a dose-dependent effect.

To check platelet viability after VO addition, a Calcein-AM binding assay was employed. Calcein-AM binds to alive cells and fluorescence intensity is measured by flow cytometry. These results revealed that neither VO nor its vehicle (DMSO) significantly affected platelet viability compared to the control group of platelets stained with Calcein-AM (Fig. 1B).

To elucidate the platelet aggregation pathway triggered by VO administration, two platelet aggregation inhibitors were employed: indomethacin, a cyclooxygenase (COX) inhibitor that impedes the release of thromboxane A2 (TXA_2) (COX/TXA_2 pathway), and apyrase, an ATP/ADP scavenger (P2Y receptor pathway). The platelet aggregation instigated by VO was reduced with apyrase (28±11%, $p<0.05$) and apyrase in conjunction with indomethacin (19±7%, $p<0.05$) in comparison to the control group devoid of inhibitors (50±9%). Thus, VO induced platelet aggregation via P2Y receptors, as evidenced by the decrease in platelet aggregation following the addition of an inhibitor of this pathway (Fig. 1C and 1D).

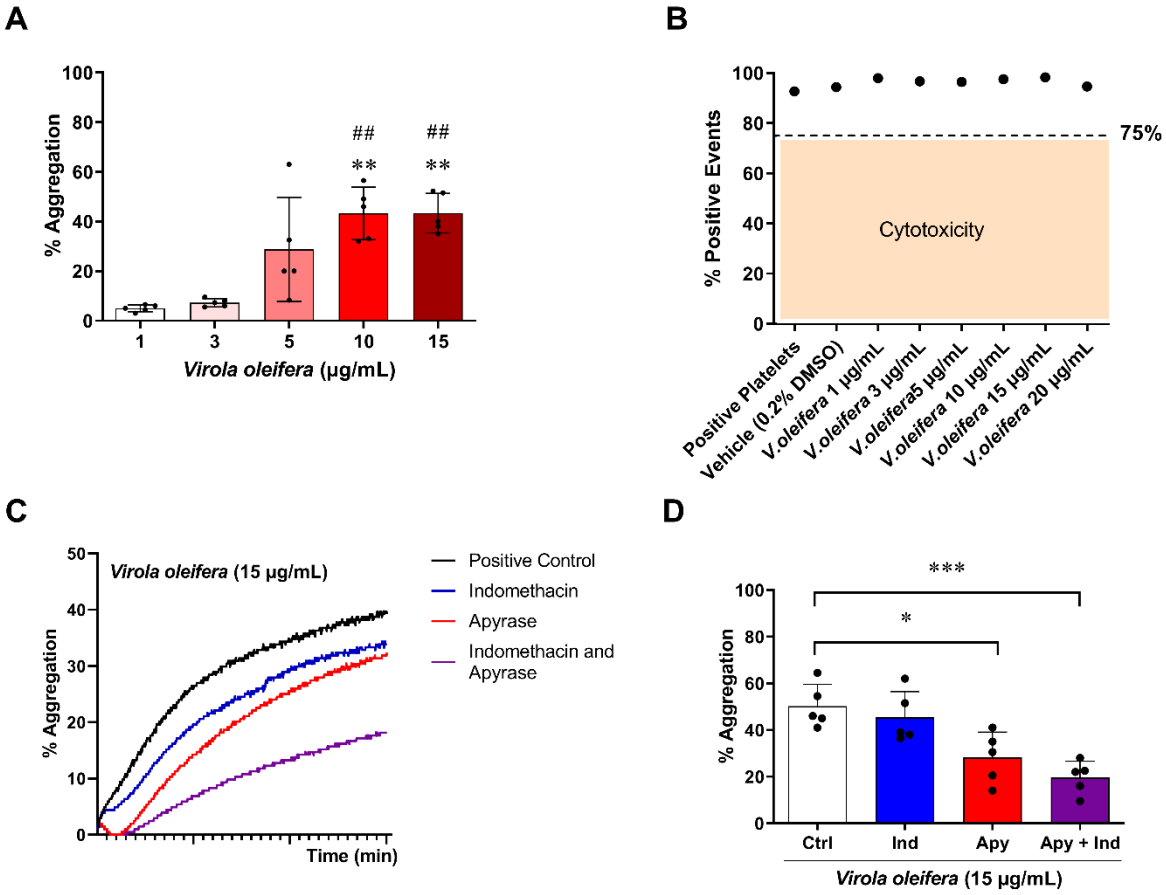

Fig 1. (A) Platelet aggregation profile induced by different concentrations of *Virola oleifera* (n=5) **p < 0.01 compared to 1 µg/mL VO concentration, ## p < 0.01 compared to 3 µg/mL VO final concentration. (B) Level of platelet viability after the treatment with vehicle at the maximum dose (0.2% DMSO) or VO at different concentrations (n=1). (C) Representative aggregation curves following pre-treatment of platelets with inhibitors indomethacin (Ind), apyrase (Apy), or both and subsequent aggregation induced by VO (final concentration 15 µg/mL). (D) Platelet aggregation profile induced by VO (final concentration 15 µg/mL) after the pre-treatment with platelet aggregation inhibitors (n=5) * p < 0.05, ** p < 0.001.

4. Discussion

Popular reports that VO could heal wounds and stop bleeding motivated our translational research group to examine these effects. In 2022, Carvalho et al. demonstrated in rats that a formulation containing 5% VO had superior in re-epithelialization compared to the commercially available product Dersani® (composed of essential fatty acids) also with antioxidant properties. However,

there was still a gap to explore whether VO could exert any effect on platelet aggregation, justifying this study.

Interestingly, our results revealed, for the first time, the pro-aggregatory role of VO on human platelets (in a dose-dependent effect manner), primarily via P2Y receptors but not via TXA_2 (observed by apyrase and/or indomethacin assays). Therefore, in addition to its antioxidant properties [Bôa et al., 2015; Pereira et al., 2016;] also accompanied by enhancement of NO bioavailability [Coutinho et al., 2017; Carvalho et al., 2022], the VO may aid in healing through complementary and synergistic mechanisms [Francisco et al., 2021; Carvalho et al., 2022; Li et al., 2023; Fadilah et al., 2023; Bordean et al., 2023; Wang et al., 2002; Choi et al., 2021].

Finally, it is important to highlight the translational feature of the *ex vivo* data obtained, since all samples used were from human blood, reinforcing the therapeutic potential of VO resin for humans. Furthermore, our study sheds light on the significance of ethnopharmacology in the 'discovery' of new bioactive compounds in Brazil. stimulating not only local/regional economic but also encouraging the preservation of *Virola oleifera* species.

5. Conclusion

In conclusion, we demonstrate the pro-aggregation effect of VO extract (via P2Y). These findings highlight the therapeutic promise of VO resin in wound healing scenarios. Future efforts involving the isolation and characterization of the active molecule are imperative to safely elucidate this therapeutic potential.

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