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RESEARCH ARTICLE OPEN ACCESS

CALOPHYLLUM BRASILIENSE CAMBESS (JACAREÚBA) EXTRACT: EFFECTS UNDER RATS SKIN WOUND HEALING

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ABSTRACT

Objective: To evaluate the healing activity of a crude extract of *C. brasiliense* on cutaneous wounds in rats. **Method**: adult male rats were wounded and randomized in three groups, where each one of those have received different topical treatment: 0.9% Saline Solution; Base Gel and Base Gel with crude extract of *Callophylum brasiliense* Cambess 2%. Morphometric and morphological analyses were performed at 7 and 14 days after topical treatment. **Results**: the animals topical treated with Base Gel with crude extract of *Callophylum brasiliense*Cambess 2% had improved the closure wound, angiogenesis, and the collagen fibers type I and III content. **Conclusion:** topical treatment with the base gel containing crude extract of C. Brasiliense 2% can modulate the different phases of the wound healing process, showing the high potential of this herbal medicinal as healing.

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INTRODUCTION

Since the beginning until the nowadays, skin lesions are a serious huge health problem for humanity. Pain, immobility, disability, psycho-emotional changes that are related to self-esteem and self-image, lower overall quality of life, hospitalization, and withdrawal from social life, someone of the situations and conditions involving the impaired wound healing (MUSTOE *et al.,*2006; BOATENG, CATANZANO, 2015; JÄRBRINK *et al.,*2016). Lesions skin usually result in tissue loss, reaching the skin layers, as the epidermis and dermis, in superficial lesions, or in some cases these lesions are deeper, and can extend of the adipose tissue (MANDELBAUM *et al.,*2003; KOMAKECH *et al.,*2019).

The healing process is divided into three phases: inflammatory or initial, proliferative or fibroplasia, and maturation or remodeling (KUMAR, 2006). Each phase is characterized by the presence of cell infiltrates at specific sites, which occurs in an integrated manner and is coordinated by chemical mediators to optimize repair. The dressing and wound care techniques have constantly improved. Currently, numerous treatments are available for wound healing, with various brands and different routes of administration (FRANCO, GONÇALVES, 2008; BUDOVSKY *et al.,*2015; JARIĆ *et al.,*2018). Newly, the Brazilian Ministry of Health has stimulated the insertion of complementary care practices into the official healthcare system. The National Politic on Medicinal Plants and Phytotherapy (BRASIL, 2006) and National Policy on Integrative and Complementary

Practices (BRASIL, 2006) aim to stimulate access to complementary practices and medicinal plants for healthcare, both safely and effectively. *Calophyllum brasiliense* Cambess, commonly known as Jacareúba, is a tree that belongs the Clusiaceae family (REYES-CHILPA*et al.,* 2004). It is distributed mainly in the Atlantic Forest and Brazilian Cerrado regions. Many species are being used in traditional medicine to treat pain, infections, inflammation, and ulcers (DHARMARATNE *et al.,* 1999). Previous studies have shown that extracts and oils of the genus *Calophyllum* have antimicrobial and antifungal activity (MOREL *et al.,* 2000). Considering the popular use of *C. brasiliense* and the results of studies on biological effects of this plant, the present study, we evaluated the healing activity of the crude extract (CE) of *C. brasiliense* on cutaneous wounds in rats.

MATERIALS AND METHODS

Source of C. Brasiliense waste: The leaves of *C. brasiliense* were collected in Parque Estadual da Ilha do Cardoso in Cananéia, located on the southern coast of São Paulo state, Brazil. The plant was identified by PhD Maria CM Young, and a voucher specimen was deposited (SP 363818) in the herbarium of the Botanical Institute of São Paulo, Brazil. The leaves were weighed, dried in circulating-air greenhouses (Quimiss Q-31, Diadema SP, Brazil) at 35 °C, milled in a knife mill (TecnalMarconis TE048), and were stored in a dry dark place.

Preparation of crude extract from C. brasiliense: The comminuted sample was underwent cold maceration in ethanol: water (9:1 v/v), and successive extractions until all active compounds were retrieved (PRISTA*et al.,* 2006). Thereafter, the macerate was filtered and evaporated *in vacuo* in a rotary evaporator at 35-40 °C until complete elimination of the organic solvent. The product from that evaporation (i.e., a dark green residue) was dissolved in dichloromethane. The organic solvent was removed by evaporation at room temperature for obtained the crude hydroalcoholic extract (CE; ≈75.0 g), and it was stored at −10 °C, protected from light until the use (CARDOSO *et al.,* 2017).

Hydrogel formulation: The topical treatment was performed using two different polymers, F-127® triblock copolymer and Carbopol 934P® gel, to make the hydrogel without (BG) or with the extract CE 2%. The topical formulations were stored at 4-8 °C and were applied during topical treatment. These formulations were prepared before starting the experiment and were used throughout the treatment period.

Statement of Ethics and Study Design: The research protocol was approved by the Committee on Ethics in the Use of Animals (CEUA) of the State University of Western Paraná (Unioeste); see appendixA. Thirty male Wistar rats *(Rattus norvegicus albinus*), with an average weight of 250±30 g, were obtained from the Unioeste Central Biotherm. The animals were individually housed in polyethylene cages under controlled environmental conditions (23±2 °C, 50-70% relative air humidity, and 12/12 h light/dark cycle). Animals were fed with standard chow (Nuvilab, Curitiba, Brazil) and water *ad libitum*. The animals were given a 7-day acclimatization period before the experiments began. The animals were randomly assigned for three groups (n = 10/group): negative control group with 0.9% saline solution (Sal 0.9%), hidrogel with 2%, control group with hydrogel base (BG) and CE (CE 2%), and each group was analyzed at 7 and 14 days post-wounded. And each group was randomly divided according to the treatment period (7 and 14 days; n = 5/group). For the induction of skin lesions, the animals were intraperitoneally anesthetized with 2% Xilazin® (10 mg/kg) and 10% Cetamin® (75 mg/kg) (FLECKNELL, 2009; FISH *et al.,* 2008) and positioned in a ventral decubitus position for depilation of the dorsocostal region. Skin lesions were made in a 1cm^2 area that was demarcated with a metallic punch, removing the epidermis and dermis (LOPES *et al.,* 2005; SANTOS*et al.,* 2006; SPERONI*et al.,* 2002). After 24 h, daily treatment of the skin lesions began. After 7 and 14 days of treatment, the animals were intraperitoneally euthanized with a lethal dose of 120 mg/kg thiopentax®.

Wound closure: The cutaneous lesions were evaluated macroscopically the percentage of closure, based on measurements in two directions (length × width) using a digital caliper, and the area (cm^2) was calculated. The percentage of closure was calculated using the following formula: (A0 - AI) / A0) × 100, where A0 is the initial area of the lesion, and AI is the area on the day of measurement (7 and 14 days) (ARUNACHALAM, PARIMELAZHAGAN, 2013).

Histological analysis: Wound specimens (including the edges) were removed, fixed in 4% formaldehyde for 8 hours at room temperature (RT), processed and embedded in Paraplast®. Five μm thick sections were positioned in glass slides and routinelystained with hematoxylin & eosin (H&E).

Reepithelialization: We estimated wound reepithelialization by morphometric analysis of wound sections stained with hematoxylin/eosin (H&E), using the formula (distance traversed by epithelium)/(distance between wound edges). We analyzed 10 randomly selected fields per animal from digital images obtained using a Stemi SV6 Zeiss Germany microscope – Go¨ttingen, Deutschland with a 1.2_ magnification, a Canon power shot A640 10MP digital camera, and Axion vision 4.8 (06-2009) software. The results are expressed as the percentage of the wound area that had reepithelialized.

Collagen fibers: In all groups, we viewed histological sections stained using the Picrosirius 1% technique (Sirius red in saturated solution of picric acid) (DAYAN*et al.,* 1989) under polarized light to evaluate Types I and III collagen fibers. Fibers were identified by their birefringence pattern (Type I: red, orange, and yellow and Type III: green). We visualized 10 random fields (250_ magnification) per group using a binocular microscope with Axiocam ICc5 camera (Zeiss, West Germany). The density of Types I and III collagen fibers (per square micrometer) was determined using the Image-Pro Plus 4.1 software (Media Cybernetics, L.P., Silver Springs, MD, USA).

Analysis of blood vessels: Digital photomicrographs were obtained using a LeitzAristoplan optical microscope (Leica) with a 20x objective and a Nikon (DS-Ril) camera. The NIS-Elements software was employed for image capturing. Only the dermal wound region, just below the crust, was photographed (2–5 pictures per animal, 3–4 animals per group). The Image J public software (NIH, Bethesda, US) was used for morphometric analysis using the grid plugin. A grid of 130 points was used in each photograph and the number of points observed in the interior of small blood vessels was counted and expressed as percentage of the total points, representing the area occupied by vessels.

Statistical analysis: The results were analyzed using GraphPad Prism 5.0 software. The data are expressed as mean ± SD. Difference between groups were determined by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test when appropriate. The level of significance that was used to reject the null hypothesis was 5% (p< 0.05).

RESULTS

Wound closure: The topical treatment with the CE 2% was able to accelerate the wound closure (Fig1). On the day 7, animals treated with the topical CE 2% showed 33% more wound area closure when compared with Sal 0.9% and BG (12% and 12%, respectively; p< 0.05) groups (Fig. 1A). On the 14 days post wounding, all the topical treatments no demonstrated significantly results between the groups.

Re-epithelialization: Fig. 2A shows the re-epithelialized surface on the different days of treatment. Fig. 2B shows the length at 7 days and thickness at 14 days of the re-epithelialized surface. In both periods there were no significant differences between groups.

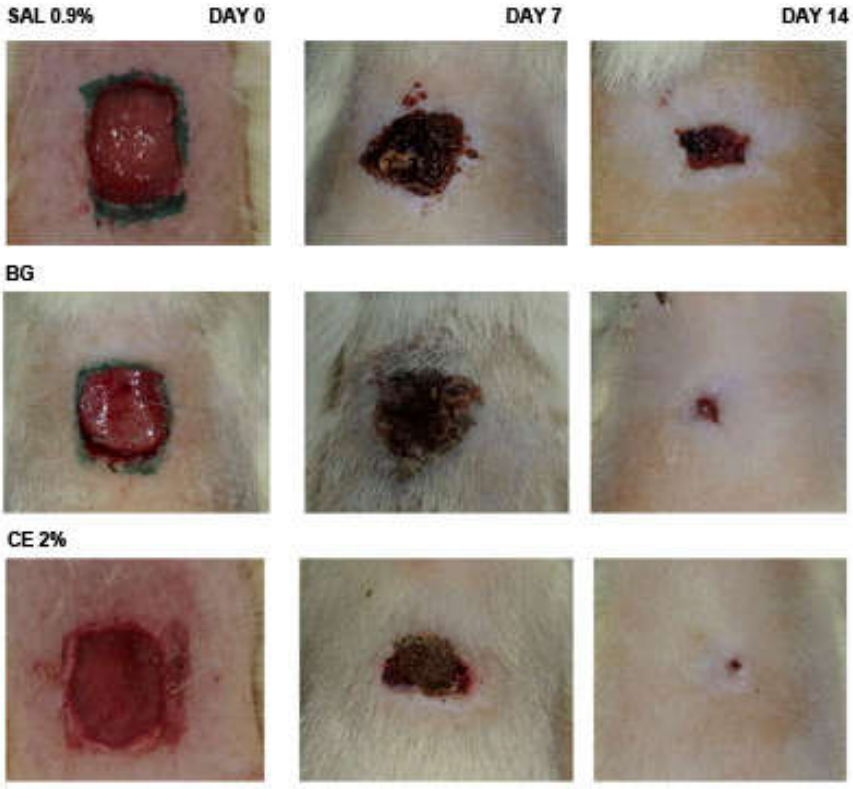

Figure 1. Photographic representation of cutaneous wounds in rats at 0, 7, and 14 days of treatment with the Hidrogel containing 2% CE (CE 2%), Hidrogel (BG), and 0.9% saline solution (Sal 0.9%)

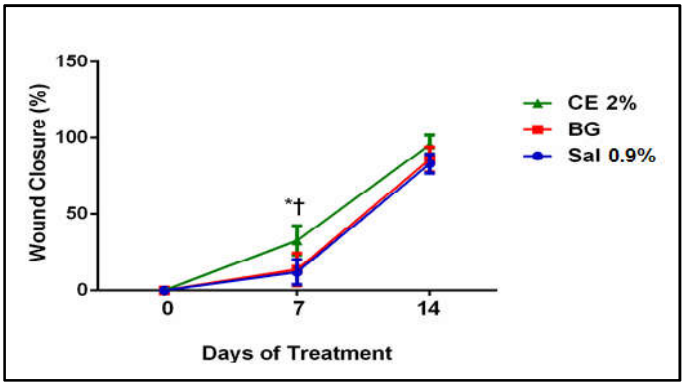

Figure 1 A. Cutaneous lesion closure in animals treated with the Hidrogel containing the 2% CE (CE 2%), Hidrogel (BG) and 0.9% saline solution (Sal 0.9%). The data are expressed as mean ± SD. *n* = 10. *,†*p*< 0.05, compared with Sal 0.9% and BG groups. (7 and 14 days; *n* = 5/group).

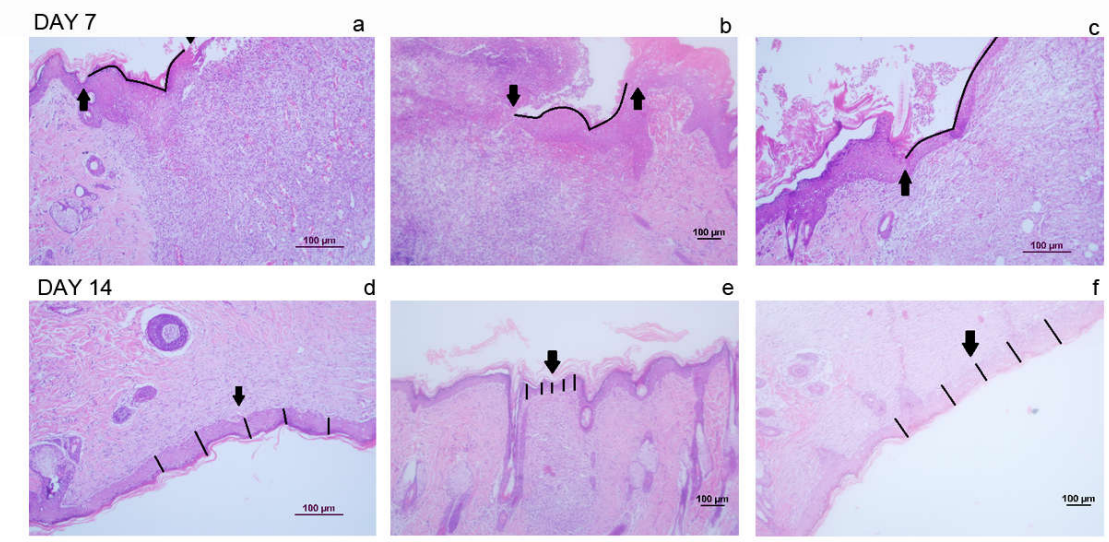

Figure 2 A. Histological sections, length and thickness of the re-epithelialized area of one side (day 7) and the center (day 14), respectively, of the lesions treated with: Hidrogel containing 2% CE (CE 2%, a;d), Hidrogel (BG; b;e), and 0.9% saline solution (Sal 0.9%, c;f). (—) Re-epithelialized surface length; (▌) surface thickness of re-epithelialization. The arrows indicate the beginning and the end re-epithelialized area at 7 days and the center of the area at 14 days (10× magnification)

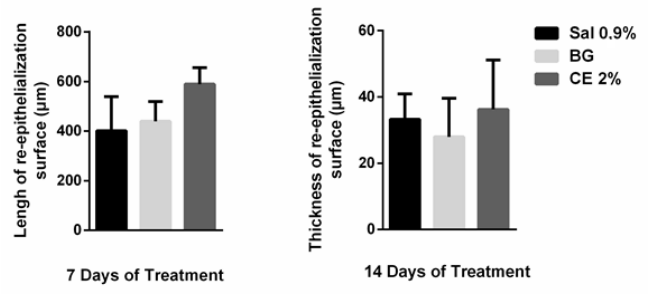

Figure 2 B. Length (µm) at 7 days and thickness (µm) of the re-epithelialized surface of cutaneous lesions at 14 days of treatment with the Hidrogel containing 2% CE (CE 2%), Hidrogel (BG), and 0.9% saline solution (Sal 0.9%). The results are expressed as mean ± SD. *n* = 10. No different significant statistic was observed

Neovascularization: The morphometric analysis of the lesions at 7 days showed a significant increase in the number of blood vessels in wounds that animals treated with the CE 2% comparing to other groups ($p< 0.01$). This significant increase was maintained at 14 days but only comparing to Sal 0.9% group ($p< 0.05$; Fig. 3).

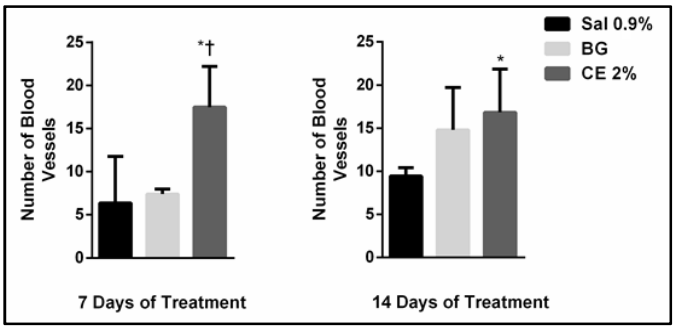

Figure 3. Number of blood vessels in skin lesions treated with the Hidrogel containing 2% CE (CE 2%), Hidrogel (BG), and 0.9% saline solution (Sal 0.9%) at 7 and 14 days. The data are expressed as mean ± SD. *n* = 10. *, †*p*< 0.01, compared with Sal 0.9% and BG groups; **p*< 0.05, compared with Sal 0.9% group

Collagen: The percentage of type III collagen fibers at 7 days was higher in the CE 2% group compared with the BG ($p< 0.05$) and Sal 0.9% ($p< 0.01$) groups (Fig. 4 A). After 14 days, there were no significant differences between groups. The percentage of type I collagen at 7 days was higher in the 2% CE group, which was significantly different from the Sal 0.9% group ($p< 0.05$) (Fig. 4 B). After 14 days, there were no significant differences between groups.

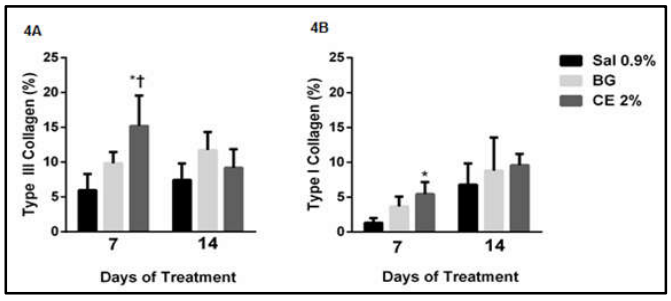

Figure 4. Percentage of collagen type III (immature) and collagen type I (mature) fibers in cutaneous lesions treated with the Hidrogel containing 2% CE (CE 2%), Hidrogel (BG), and 0.9% saline solution (Sal 0.9%) on days 7 and 14. The data are expressed as mean ± SD. *n* = 10. Type III collagen: †*p*< 0.05, compared with BG group; **p*< 0.01, compared with Sal 0.9% group. Type I collagen: **p*< 0.05, compared with Sal 0.9% group

DISCUSSION

The topical treatment with the crude extract of *Calophyllum brasiliense* Camb (CE 2%) showed accelerated the wound closure and increase the collagen contend in the proliferative phase of wound healing process (7 day); even as increased the vessel numbers in both phases analyzed. *Calophyllum brasiliense* Cambess, is a rich source of bioactive compounds such as coumarins, xanthones, steroids, triterpenes and bioflavonoids (ANDRADE *et al*, 2011). Among the chemical component found in leaves of the plant is cumarina. The leaves contain mammea-type coumarin derivatives, as mammea A/BA, A/BB, B/BA, and B/BB, isomammeigine, and mammea C/AO, C/OB, B/BA cycle F, and B/BB cycle F, and other). This class of substances is characterized by the presence of the 5,7-dioxygenated coumarin skeleton that has a C-4-linked phenyl, or alkyl radical, and acyl or prenyl substituents attached to C-6 or C-8 (FRANCO, GONÇALVES, 2008). Ethnopharmacological studies have already reported the use of this species against bronchitis, gastritis, hepatites (Reyes-Chilpa*et al*, 1997), pain (Sartori *et al*, 1999), inflammations, diabetes, hypertension (Lewis, 1977), diarrhea (Duke,Martinez, 1994) and have antimicrobial and antifungal activity (REYES-CHILPa *et al*, 2004). The coumarin (-) mammea A/BB that was isolated from leaves of *C. brasiliense* was shown to have potent leishmanicidal activity against *L. amazonensis* and *L. braziliensis* (VASQUEZ, 1990; TIUMAN *et al,* 2012; HONDA *et al*, 2010; BRENZAN*et al* 2008). Previously, an extract of *C. brasiliense*, tested as cream showed healing effects on cutaneous lesions in rats (BRENZAN*et al*, 2007). The present study was broader and used a different vehicle from the previous study, which suggests that this plant can be developed as an effective natural product for the treatment of cutaneous lesions. The topicaltreatment with CE 2% showed healing properties, as lesion closure. Our results showed that this treatment has a beneficial effect on the healing of lesions, interacts directly with the crusts, appearing to severe a temporary barrier to cover a wound until the underlying epidermis fully epithelializes, terminally differentiates, and restores a permanent skin barrier (LORDANI *et al*, 2016). Wound closure is characterized by decreased wound area in which centripetal contraction of the edges accelerates closure of the lesion and determines the period of re-epithelialization (USUI *et al*, 2013).

In the present study, most of the observed lesions were considered macroscopically cured after 14 days of treatment, but the proliferation phase of healing may take up to 3 months to become complete (Upadhyay *et al*, 2013), and the remodeling phase will continue for several months (Pham*et al*, 2015) after closure of the lesion. Another important factor during healing is angiogenesis. The angiogenesis has a dual function: providing necessary essential nutrients and oxygen to the injured site and promoting the formation of granulation tissue (MERCANDETTI, MOLNAR, 2011; SCHREML *et al*, 2010). Angiogenesis occurs at the proliferative stage, which begins approximately on the fourth day after the wound occurs (Singer, Clark, 1999) and the treatment with the CE 2% increased angiogenesis, histologically indicated by an increase in the number of blood vessels in the lesion (Fig. 3). After 14 days, the animals in the CE 2% group exhibited a small reduction of the number of new blood vessels, it is characteristic of the late period of the proliferative stage (BROUGHTON, JANIS, 2006). The angiogenic activity during the cicatricial process can be attributable to control of the inflammatory response that was induced by the CE 2%. The proliferative phase begins with re-epithelialization, which involves the extracellular matrix and collagen production (NAUTA *et al*, 2011). Re-epithelialization completes wound closure through reorganization of the cytoskeleton and the migration and proliferation of keratinocytes at its borders (GANTWERKER, HOM, 2011). As wound closure progresses, epidermal re-epithelialization can be determined by thickness (14 days) and re-epithelialized layer length (7 days) (Santoro, Gaudino, 2005).

In the present study, the type III collagen synthesis was higher in lesions of animals that were treated with the CE 2%, with a progressive reduction after 14 days. Also, the amount of type I collagen fibers was higher in lesions that were treated with the CE 2%. After proliferation, the remodeling phase begins 7 days after injury. In this step, the collagen composition changes, and its disposition becomes more oriented. Type III collagen, which is initially more abundant than type I collagen, is degraded over time,

whereas type I collagen is increased by fibroblasts (BUENO *et al*, 2016). Type I collagen is also essential during the maturation period (ISAAC*et al*, 2010). The remodeling phase is the last stage in the healing process, characterized by greater tissue resistance that is attributable to the substitution of granulating tissue that is rich in type III collagen with stronger tissue that is rich in type I collagen (GONÇALVES*et al*, 2010).

CONCLUSION

The hydrogel containing 2% of crude extract of *C. brasiliense* promoted the accelerate wound healing in rats, demonstrating beneficial effects on various phases of the wound healing process and cutaneous permeation. This preparation may be a promising herbal medicine with wound healing activity. Further studies that test different doses, vehicles, and treatment times are needed to better understand the complete mechanism of healing and the extract's effects in humans. The Authors declares that there is no conflict of interest.

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