Use of Ointment or Aqueous Extract of Neem (Azadirachta indica) for the Repair of Experimental Skin Lesions in Sheep

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Abstract

The skin is considered the largest organ in the body and this protective barrier can be broken in some situations. The wound healing process is a complex mechanism. Based on the difficulties encountered for treatment, alternatives are sought and one of the options may be phytotherapy. Azadirachta indica, known as Nim, is perhaps one of the most widely known and used medicinal plants. This research aimed to evaluate the viability and effectiveness of therapy with Neem, in the form of ointment or aqueous leaf extract. Eighteen sheep were randomly divided into three groups and subjected to the creation of a 7 × 7 cm excision wound on the chest wall. After, treatment was started according to each group. Animals in G1 did not receive any treatment; in G2, the ointment of Neem was applied and animals in G3 were treated with the preparation of an aqueous solution of Neem leaves. Photographic images were recorded, in addition to blood collection for complete blood count and tissue biopsy. The wound area measurements showed no statistically significant difference between the groups (p > 0.05). Fibrinogen levels were not different, and there was no difference in platelet levels. In the histopathological evaluation, G2 registered a greater presence of ulceration, when compared to G3, on day 7. On day 14, G2 still had a greater amount of ulceration, differentiating from G1. G2 also registered a greater presence of crust and a higher score of granulation tissue, in the last evaluation period, compared to G3. Analyzing the results obtained in this research and taking into account the conditions in which the study was carried out, it is possible to
conclude that the use of Neem was not effective in accelerating the rate of wound healing in sheep.

**Keywords**

Phytotherapy, Sheep, Skin, Wound

1. Introduction

Formed by two layers, dermis and epidermis, the skin is considered the largest organ in the body [1]. Its functions are to protect the body from complications from the external environment, preventing dehydration, in addition to regulating body temperature, capturing sensations and synthesizing vitamin D [2]. This barrier, however, can be broken in some situations, such as when a wound occurs [3]. Divided into two large groups, the wounds can be classified as open or closed [4].

Wounds are caused by a disorder of the cellular system that results in a solution of continuity and can occur due to traumas of different origins, such as physical, chemical, thermal, microbial or immunological, compromising well-being and quality of life of affected patients [5] [6].

The wound healing process is a complex mechanism, which aims to restore the anatomy and functionality of the skin that has undergone a continuity solution [7]. Considered a complex and dynamic process, in which the devitalized and absent cell structures in the tissue layers are being replaced, wound healing is characterized by a set of biochemical events represented in a well-organized cascade to repair damaged tissues [8].

This mechanism of wound healing is generally divided into three phases: inflammatory, proliferative and remodeling [9]. The first phase occurs right after the trauma and lasts between 24 and 48 hours. At this time, hemostatic mechanisms are initiated in order to immediately stop blood loss, by vasoconstriction and platelet aggregation that induces blood clotting and subsequently vasodilation and phagocytosis, triggering inflammation at the lesion site. In the clinical evaluation, cardinal signs characteristic of inflammation observed are flushing, heat, tumor, pain, and loss of function [10].

In the second phase of wound healing, the proliferative phase (fibroplastic) will start after 2 days and last for up to 3 weeks after inflammation. This phase is divided into three other stages: granulation, contraction, and epithelialization. In the granulation stage, fibroblasts will form a collagen bed and new capillaries will be produced. In the second moment, during contraction, the edges of the wound begin to come together to decrease the area of the injury and in the third act, epithelial tissues are formed over the wound site [11]. The remodeling phase lasts from 3 weeks to 2 years. In this phase, the production of new collagen occurs and the tissue resistance to tension is increased, with the scar flattening and
the tissues recovering 80% of their original tension strength [12] [13].

Wounds are often a source of serious concern for both the patient and the physician, especially chronic wounds, which affect a large number of patients and seriously reduce their quality of life [14]. The proposed treatments for these injuries include the administration of medications topically or systemically, to assist in the repair of wounds [15]. The most commonly used topical agents include antibiotics and antiseptics [16], also agents for debridement [17] and wound healing promoters [18] can be used.

Based on the difficulties found for the treatment of skin lesions, alternatives are sought, aiming at greater practicality, better results, less treatment time and lower cost. One of these alternatives may be phytotherapy. It is estimated that between 70% and 80% of the world population has confidence in non-conventional medicine, mainly related to herbs, according to data from the World Health Organization (WHO). This condition is more observed especially in the case of developing countries, where the costs of medical or veterinary consultations and the price of conventional medicines are beyond the financial possibilities of most people [19].

_Azadirachta indica_, popularly known as Nim, is perhaps the most widely known and used traditional medicinal plant in India and is also grown in other tropical areas of the world [19]. The Nim or Amargosa is a leafy tree originally from India and it belongs to the Meliaceae family, the same as the Santa Bárbara (Cinamomo), Cedro or Mahogany trees. Although it is native to Burma and the arid regions of the Indian subcontinent, where there are approximately 18 million trees, it is currently grown in the United States, Australia, countries in Africa and Central America [20].

Well known in India for over two thousand years, because it is supposed to be one of the medicinal plants with a broad spectrum of biological activity, each part of the tree was used as a natural medication against various diseases. Several studies have been carried out, demonstrating possible antipyretic, immunostimulating, antiulcer, antioxidant, hypoglycemic and hepatoprotective activity of the plant [21].

Currently, Neem is considered to have several medicinal properties. The oil derived from this plant contains margosic acid, fatty acid glycerides, butyric acid and traces of valeric acid. Several other active ingredients would also be present in Nim, such as nimbidin, nimbidial, azadirachtina, nimbina, azadirina, gedunina, salanina, being that they would present diverse medicinal activities. Neem oil would be especially beneficial for curing skin conditions such as ulcers, eczema, as well as ringworm and mange in dogs. Also considered a powerful insect repellent, antibacterial, antifungal, antiviral, anti-inflammatory, in addition to strengthening the body’s general immune responses [18].

Additionally, Neem oil also contains fatty acids that contribute to the formation of collagen, promoting wound healing and maintaining skin elasticity. It’s speculated that the oil’s active ingredient could help in the wound healing
process, maintaining the suppleness of the skin, while this process occurs. Due to its high content of essential fatty acids, the oil keeps the lesion site moist and allows the establishment of an adequate texture for the skin during the healing process. Furthermore, both leaf extract and seed oil have a proven antimicrobial effect and can keep any wound or injury free from secondary infections by microorganisms. Other clinical studies also suggest that Neem inhibits inflammation as effectively as cortisone acetate, further accelerating wound healing [18].

Thus, this research aimed to evaluate the viability and efficacy of therapy with Neem, in the forms of ointment or aqueous extract of commercially available leaves, for the treatment of skin wounds experimentally induced in sheep, evaluating the rate of contraction of wounds, macroscopic behavior of the lesions and their microscopic aspects.

2. Material and Methods

2.1. Animals

This study was approved by the Ethics Committee on the Use of Animals at the University of Rio Verde (Rio Verde/Goiás-Brazil), under protocol number 04/16. Eighteen adult Santa Inês sheep (Ovis aries), weighing approximately 30 kg, were used.

The sheep were subjected to clinical and laboratory tests, with blood count analysis. For this purpose, 3 mL of blood was collected from the external jugular vein of each animal; for analysis of the red blood cells (He), hemoglobin (Hb), hematocrit (Ht), leukocyte (Leu), plasma proteins (PP) and fibrinogen (Fg).

After discarding any clinical changes, the animals were identified individually, kept in a potter with a masonry shed and with food and water at will, for more than 15 days for their adaptation. Throughout the experiment, the animals were kept in these conditions.

Respecting the adaptability period, the sheep were randomly divided into three groups with six animals each. G1 was the control group and did not receive any treatment. In G2, the sheep were treated with Neem ointment and the animals submitted to treatment with aqueous Neem extract preparation belonged to G3.

2.2. Surgical Procedures

To perform the surgical procedure, the animals were fasted for 36 h and water for 12 h before the intervention. The anesthetic medication used was constituted by atropine sulfate subcutaneously, at a dose of 0.02 mg·kg⁻¹, after 15 minutes, it was administered intramuscularly 0.1 mg·kg⁻¹ of xylazine hydrochloride associated with 8 mg·kg⁻¹ of ketamine hydrochloride. For intravenous fluid therapy, Ringer’s Lactate solution was used, through cannulation of the brachycephalic vein during the entire surgical process, in 10 - 15 ml·kg⁻¹·h⁻¹ venous drip.

After preparing the surgical team and the animal, the surgeon demarcated on
the skin, with the help of a dermatological pencil, the excised area of 7 × 7 cm in
the region located on the last rib. The skin incision was made with the scalpel
blade, as it causes less tissue trauma than the scissors, electric scalpel or laser.
The cutaneous edges were manipulated in an atraumatic manner and the blood
vessels were identified for hemostasis. The fat was divulsed and completely ex-
cised (Figures 1(a)-(f)). To perform the surgical procedures, the animals have
fasted to solid food for 36 h and 12 h for water before the intervention. The
anesthetic medication used was constituted by atropine sulfate subcutaneously,
at a dose of 0.02 mg·kg−1. After 15 minutes, 0.1 mg·kg−1 of xylazine hydrochlo-
ride associated with 8 mg·kg−1 of ketamine hydrochloride were administered
intramuscularly. Ringer’s Lactate solution was used for intravenous fluid ther-
apy, using cannulation of the brachycephalic vein during the entire surgical process,
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**Figure 1.** Measurement of the surgical wound, with the aid of a digital caliper (a); applica-
tion of local anesthesia, on the skin to be excised (b); beginning of the surgical wound
incision (c); development (d); conclusion (e) and final aspect of the surgical wound (f).
blade, as it causes less tissue trauma than the scissors, electric scalpel or laser. The cutaneous edges were manipulated in an atraumatic manner and the blood vessels were identified for hemostasis. The fat was divulsed and completely excised (Figures 1(a)-(f)).

Right after the creation of the experimental lesion, treatment was started according to each group.

2.3. Postoperative

In the immediate postoperative period, the sheep were monitored until the complete restoration of consciousness and only after being recovered were they released to remain in the foal. The animals underwent daily cleaning of the surgical wound, dressing change and were clinically evaluated for physiological standards. Cutaneous viability was also assessed clinically, through visual perception of color, warmth, pain and/or bleeding.

2.4. Evaluation

Photographic images were recorded both on the day of the procedure (D0) and on the evaluation period, on days 7, 14, 21 and 28 after the surgical procedure, to assess skin regeneration. At these same times, the wounds were measured with the aid of a caliper to measure their area.

At the end of the first week, the animals were submitted to a new blood collection from the external jugular vein to perform the complete blood count and to evaluate the postoperative concentrations of hematocrit, platelets, and fibrinogen.

To perform tissue biopsy, the animals were also submitted to anesthesia according to the protocol described above and submitted to an aseptic surgical procedure on days 7, 14, 21 and 28 after the defect was created. At this moment, the surgeon, with an 8 mm punch, performed a skin biopsy, removing a skin fragment that addressed both the created lesion and the skin that did not suffer any damage (Figure 2(b)).

Figure 2. Topical daily application of Neem ointment on the wounds of animals in G2 (a); collection of skin sample for histopathological analysis, with 8 mm punch (b).
2.5. Histopathological Analysis

The fragments were fixed in a 10% buffered-formaldehyde solution, routinely processed for histopathological examination, and stained by hematoxylin and eosin. Samples were evaluated for the presence of immature granulation tissue (dermal immature fibrovascular tissue), mature granulation tissue (dermal mature fibrovascular tissue), lymphoplasmacytic superficial perivascular dermatitis, heterophilic perivascular deep dermatitis, and interstitial granulomatous dermatitis. Moreover, fibrosis, mineralization, acanthosis, ulceration, and crust were also part of the evaluation. Each wound was scored as one (discrete), two (moderate) or three (severe) for each of the categories.

2.6. Statistical Analysis

The results of the three groups were analyzed using the ANOVA test complemented by the Kruskal-Wallis test to compare wound areas and the ANOVA test complemented by Tukey’s test to compare the results obtained in the histopathological evaluation. These statistical analyzes were performed using the Bioestat 5.0 software. For all comparisons, the level of significance was set at $\alpha = 0.05$.

3. Results

Surgical procedures were performed without complications and all animals showed adequate recovery during the monitoring period after surgery. The evaluations carried out during the post-surgical follow-up indicated the absence of inflammation or secretion worthy of note, except for two animals from G3 that had myiasis infestation and underwent conventional complementary treatment to eliminate the parasites.

3.1. Results of Measuring Wound Areas

All wounds were measured daily using a digital caliper, registering the width and height of the lesions, keeping the animals always in the same quadrupedal position. The measurements were duly noted and subsequently, the area of each of the wounds was calculated (Table 1). For comparison between the three groups, measurements of the area of wounds were taken into account on the 7th, 14th, 21st, and 28th days post-surgery.

| Day | Injury area (cm$^2$) | Standard error | p value |
|-----|---------------------|----------------|---------|
|     | G1                  | G2                  | G3                  |
| 0   | 68.87 (6/6)         | 66.56 (6/6)         | 63.59 (6/6)         | 41.002   | 0.5946    |
| 7   | 41.90 (6/6)         | 52.87 (6/6)         | 44.17 (6/6)         | 59.249   | 0.0905    |
| 14  | 18.52 (6/6)         | 15.91 (6/6)         | 16.47 (6/6)         | 22.862   | 0.6216    |
| 21  | 7.82 (6/6)          | 6.55 (6/6)          | 7.18 (6/6)          | 6.024    | 0.6782    |
| 28  | 2.66 (5/6)          | 1.80 (2/6)          | 1.20 (3/6)          | 4.745    | 0.5211    |
21st and 28th postoperative days, with none of the groups showing statistically significant differences when compared to each other (p > 0.05).

Although there was no significant difference, the two treated groups presented smaller wound area measurements than the control group, starting on day 14. Furthermore, it should be noted that by day 28, 50% of animals in G2 and G3 showed complete healing, while only 16.66% of the animals in G1 obtained the same condition. Also, at the end of the evaluation period, the injuries were smaller in the treated groups, and the G3 analysis resulted in a smaller injury area compared to the other groups. The graph in Figure 3 shows the rate of reduction of the wound area of the groups, in each evaluation period.

3.2. Blood Count Results

Three blood samples were taken, on the day of the procedures (D0), seven days (D7) and thirty days (D30) after the surgery. After obtaining the complete blood count of all animals, the following parameters were evaluated: fibrinogen and platelets.

Considered an important indicator of inflammation, fibrinogen is a precursor to fibrin, which plays an important role in blood clotting. Therefore, any type of inflammation or tissue damage suffered may cause elevated plasma fibrinogen levels. On day 0, the fibrinogen levels of treated animals were lower (by up to 50%, p < 0.001) than that of untreated animals, with no difference in values on days 7 and 30 (Table 2).

![Figure 3](image)

**Figure 3.** Rate of reduction of wound areas in the postoperative period, from day 0 to day 28.

**Table 2.** Fibrinogen levels of sheep that did not receive treatment for the lesions (G1) and those that were treated with commercial ointment (G2) or aqueous extract of Neem leaves (G3).

| Fibrinogen (g/dL) | G1   | G2   | G3   | Standard error | p-value |
|-------------------|------|------|------|----------------|---------|
| DIA 0             | 0.40a| 0.20b| 0.27b| 0.02           | 0.001   |
| DIA 7             | 0.60 | 0.53 | 0.53 | 0.07           | 0.736   |
| DIA 30            | 0.40 | 0.40 | 0.47 | 0.06           | 0.705   |

*Medias followed by different letters in the lines differ by the student’s t-test at 5% probability.*
Platelets perform physiological activities related to hemostasis, however, for this to happen, the number of platelets needs to stabilize at appropriate values, so that they can aggregate and form the initial hemostasis [22]. There was no difference (p > 0.05) in platelet levels as a result of treatments (Table 3).

3.3. Histopathological Analyzes Results

There was no statistically significant difference for most of the variables analyzed by histopathology, except for the presence of ulcers in the wounds, crust, and granulation tissue (GT). On day 7, G2 registered a greater presence of ulcer in the wounds, when compared with G3, differing statistically. On the 14th, G2 still had a greater amount of ulcer, but this time significantly differentiating from G1.

The G2 also showed a greater formation of crust on the wounds, in the last evaluation period, when compared with the G3. Finally, also on the 28th, G2 differentiated itself again from G3, this time showing a higher concentration of GT. The values that showed a statistically significant difference are listed in the table below (Table 4).

4. Discussion

In vivo evaluations are extremely necessary to confirm the efficacy of plants with potential benefits for human or animal health, especially when it comes to wound healing. Thus, the present study aimed to determine the healing rate of

Table 3. Platelet levels of sheep that did not receive treatment for the lesions (G1) and those that were treated with commercial ointment (G2) or aqueous extract of Neem leaves (G3).

| Platelets (10^3/μL) | G1        | G2        | G3        | Standard error | p-value |
|---------------------|-----------|-----------|-----------|----------------|---------|
| DIA 0               | 548.33    | 610.00    | 588.33    | 41.20          | 0.573   |
| DIA 7               | 725.00    | 706.67    | 665.00    | 32.66          | 0.432   |
| DIA 30              | 690.00    | 718.33    | 611.67    | 30.41          | 0.065   |

Table 4. Histopathological analysis of the lesions, showing the occurrence of a statistically significant difference for the parameters ulcer, crust, and granulation tissue.

| Ulcer              | Dia | G1   | G2   | G3   | Standard error | p-value |
|--------------------|-----|------|------|------|----------------|---------|
| Ulcer              | 7   | 1.167| 2.5000a| 0.8333b| 1.144          | <0.05   |
| Ulcer              | 14  | 0.3333a| 2.3333b| 0.8333| 1.167          | <0.05   |

| Crust              | Dia | G1   | G2   | G3   | Standard error | p-value |
|--------------------|-----|------|------|------|----------------|---------|
| Crust              | 28  | 0.5000| 1.500a| 0b   | 0.500          | <0.05   |

| Granulation Tissue (GT) | Dia | G1   | G2   | G3   | Standard error | p-value |
|-------------------------|-----|------|------|------|----------------|---------|
| Granulation Tissue (GT) | 28  | 0.1667| 0.7500a| 0b   | 0.122          | <0.05   |

*Medias followed by different letters in the lines differ by the Student t-test at 5% probability.
wounds submitted to treatment with the ointment or aqueous extract of Neem, as well as to compare the macro and microscopic behavior of the wounds.

Neem oil, together with other plants with supposedly medicinal effects, were evaluated in a 2009 study, regarding its action for wound healing, in excision and incision models in albino rats, compared with a control group and framycetin sulfate group as standard medicine [23]. The formulation showed a significantly higher contraction rate and reduced epithelialization period in both models. In the excision model, healing was 99% on the 16th day, compared with 85% and 75% of framycetin sulfate cream and control, respectively. In the incision wound, there was a significant increase in tensile strength. Thus, the authors concluded that the formulation had potential for wound healing activity, for both models, justifying its use [23].

Another research was carried out to evaluate the healing activity of the ethanolic extract of the Neem leaf, in the wound experimentally induced in rats. For this, the curative effect supposedly produced by the Neem extract was evaluated by the rate of wound contraction and resistance to skin rupture, using as models of excision and incision wounds in albino Wister rats, comparing the treated group with the control (soft white paraffin) and standard (ointment 1% framycetin sulfate). The ethanolic extract of Neem leaves showed a high pro-healing effect, almost equivalent to the standard medicine, which may be partially due to the anti-inflammatory action, proliferation of fibrocollagenic tissue and angiogenesis properties. Therefore, it could be used as a healing agent for wounds, as long as it is confronted by clinical trials, according to the researchers [24].

In a study carried out in 2016, researchers tested the ethanol extract of Neem tree bark topically, comparing it with povidone-iodine, for wound healing by excision in Wistar rats and were successful with the proposed treatment. The animals were treated daily and followed up for 15 days and the authors concluded that the application of the ethanol extract from the bark proved to be faster and with better wound closure and contraction, when compared to povidone-iodine ointment, suggesting that such response it could occur due to the presence of several phytochemicals present in the skin, although they have recommended further studies to isolate the possible component responsible for generating wound healing activity [25].

The results obtained in the present research demonstrated that the rate of wound contraction did not differ statistically between the two treated groups and the control group, in any of the periods in which the evaluations were performed, contrary to the studies previously reported. Moreover, it is possible to notice that at the time of the first evaluation, at the end of the first week of treatment, the G2 had the worst rate of wound healing, suggesting that the vehicle in which the ointment was made available, was not the most appropriate. On the other hand, an important fact to be considered was the observation that until the 28th, 50% of the animals in G2 and G3 showed complete healing, while only 16.66% of animals in G1 will manifest the same situation.
Although several studies have already aimed to evaluate the curative activity of Neem leaves, the results are very varied. In a study carried out in Indonesia, in 2012, 27 rats were randomly grouped into 3 groups and subjected to the creation of a 1.5 cm excision wound. The control group was treated with a topical application of saline solution, the treatment group with a topical application of Neem leaf extract and the positive control group was treated with a topical application of povidone-iodine for 15 days. At the end of the evaluation, the authors concluded that both Neem leaf extract and povidone-iodine similarly accelerated the rate of wound healing. Thus, the authors recommend Neem leaf extract as it is a natural product. They emphasize, however, that the study had limitations since histological examinations of the wound were not performed to verify the inflammatory effect of Neem [26].

For this present study, in the analysis of the fibrinogen variable, considered an important indicator of inflammation, it was found that there was no significant difference between the three groups at the end of the first week of treatment and, also later, at the end of the evaluation period occurred to 30 days. In this way, it cannot be said that Neem has contributed to possible anti-inflammatory potential.

In another study, carried out in 2010, the researchers evaluated the healing activity of methanol extracts from Neem and Tinospora cordifolia leaves in models of excision and incision wounds in Sprague Dawley rats. The possible curative effect produced by the extract of each plant was evaluated by the rate of wound contraction, skin rupture force, and healing histopathology. As a conclusion of this study, the authors indicate that the healing potential of Neem was better than that of Tinospora cordifolia, evidenced by the higher percentage of wound contraction and greater resistance to the traction of scar tissue. Histopathologically it can also be confirmed by the absence of polymorphonuclear leukocytes in the excision wound, at 21 days, providing scientific justification for the traditional use of these plants in wound management [19].

Unlike what was found by the authors previously reported, in this study, the histopathological analysis of the samples demonstrated a greater presence of ulcers in G2, differing significantly from G3 in the first week, and G1 in the second. This microscopic finding, especially regarding the evaluation of the first week, is in line with the macroscopic evaluation, as it occurred just at the time when G2 presented the worst rate of wound contraction, reinforcing the suspicion that the vehicle used in the commercial ointment could have caused some irritation at the site of the lesion, or it did not allow adequate tissue oxygenation, a situation that does not occur when using the aqueous extract. It is noteworthy that the basic principles for optimal wound healing are to minimize damage and provide perfusion and oxygenation of tissues, appropriate nutrition, and a moist environment so that the anatomical and functional restoration of the affected region occurs [27].

In a study carried out in 2017, researchers used the dog as an animal model.
Fourteen healthy animals of this species were divided into two groups and subjected to the creation of a wound on the skin of the right flank through a surgical procedure, such as the present research reported here. One group underwent treatment with Neem, while the control group received treatment with Neosporin ointment, both twice a day, for 5 days, when the animals were evaluated for wound healing. Also like the present study, at that time, the authors found that the healing score was not significantly different ($p > 0.05$) between Neosporin ($4.17 \pm 0.79$) and Neem ($3.97 \pm 0.53$), and the levels of fibrinogen, neutrophils, and monocytes for the groups were also not significantly different ($p > 0.05$), again according to what was presented in the present study. Still, those researchers considered Neem to be a good alternative for the treatment of wounds in dogs and as effective as commercial antibiotics, such as Neosporin [28].

On the other hand, it has already been reported that medicinal plants would be very beneficial in the treatment of wounds, as they could promote a rapid rate of healing, resulting in minimal scarring for the patient [29]. In this sense, the present study conforms, because the animals of the two groups treated, both with the ointment and with the aqueous extract, present more aesthetically acceptable scars, at the end of the evaluation period, at 28 days.

5. Conclusion

Analyzing the results obtained in this research and taking into account the conditions in which the study was carried out, it is possible to conclude that the use of Neem, both in the form of ointment and aqueous extract, was not effective in accelerating the healing rate of wounds in sheep. Nor can it be said that they have contributed to lower inflammation rates in wounds. However, a greater number of animals in the treated groups showed complete healing of the wounds before the end of the evaluation period, in addition to resulting in more aesthetically acceptable scars. For all these reasons, the use of Neem can be considered as a therapeutic option, mainly because it is a natural product.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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