Effect of Ointments of *Sulphur* or *Pyrogenium* on Larvae of *Cochliomyia hominivorax* (Diptera: Calliphoridae)

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Abstract

Background Myiasis by *Cochliomyia hominivorax* (Diptera: Calliphoridae) is a serious problem in animal health in tropical and sub-tropical regions. Ointment-type preparations are a good option of formulation in cases of myiasis in farm and pet animals. *Sulphur* and *Pyrogenium* have already shown *in-vitro* efficacy on *C. hominivorax*. This article describes an *in-vitro* experiment to test the inhibition of development from exposing larvae of *C. hominivorax* to two homeopathic ointments (prepared individually with *Sulphur* or *Pyrogenium*).

Methods The homeopathic ointments were produced by mixing sterile lanolin, tocopherol and homeopathic medicine on a hydroalcoholic basis according to the Brazilian Homeopathic Pharmacopoeia. Larvae were obtained from naturally occurring myiases in sheep (wild larvae) or from a laboratory colony. The test consisted of exposing a group of 10 third-stage *C. hominivorax* wild larvae in contact with *Sulphur* or *Pyrogenium* ointment, or a group of 15 laboratory-propagated larvae in contact with the alcoholic vehicle of the ointment or homeopathic medicines prepared in sterile water (*Sulphur* or *Pyrogenium*), and observing the effect on the development, longevity and fertility of the blow-fly specimens.

Results The *C. hominivorax* larval inhibition rate was 90.0% for the *Sulphur* ointment group and was 86.0% for the *Pyrogenium* ointment group. The non-alcoholic vehicle and the alcoholic vehicle inhibited the development of 24.0% and 22.08% of the larvae respectively. *Sulphur* prepared in sterile water inhibited the development of 74.67% and *Pyrogenium* in sterile water inhibited 73.33% of larvae. Specimens that survived contact with homeopathic ointments had their longevity decreased and did not reproduce.

Conclusion Ointments of *Sulphur* or *Pyrogenium* were able to inhibit the development of *C. hominivorax* larvae. The ointment vehicle was harmless.

Keywords
- myiasis
- *in-vitro* inhibition test
- homeopathic ointment

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Introduction

Cochliomyia hominivorax (Diptera: Calliphoridae) is the main Diptera species able to cause cutaneous myiasis in animals in Brazil.[1–3] In myiasis caused by this blow-fly, the larvae need to parasitize the living tissues of their hosts to feed on their fluids and thus obtain the necessary nutrients to complete their development.[4] This process is painful and aggressive to the host, causing great damage to the health and comfort of the affected animals.[5] These myiases develop quickly. Because they are open and bloody wounds, the bacterial contamination requires specialized veterinary care and attention. Cases without proper treatment can result in septicemia and the death of the animal.[4]

The losses caused by myiasis in Brazilian cattle herds are about US $3.24 billion dollars per year.[6] The treatment of myiasis represents a great challenge for the veterinary profession, especially in organic systems of animal husbandry, because in this type of farm the use of chemical anti-parasitic is restricted and these products are currently the main form of available control. In Brazil, homeopathy is suggested for the health management of animals as a method to prevent and treat diseases in organic systems.[7]

Being a parasitic disease, the use of systemic anti-parasitic drugs is common. However, topical treatments are always preferable. In such circumstances, ointments are a great option as a pharmaceutical formulation for dispensing medicines considering the treatment of myiasis.[8] Despite few reports on their use in the homeopathic clinic, ointments offer an interesting way to dispense homeopathic medicines. The Brazilian Homeopathic Pharmacopoeia recommends its use and regulates the technical requirements for the preparation of homeopathic medicines dispensed in this dosage form.[9]

The clinical condition of a patient affected by myiasis caused by *C. hominivorax* shows some similarities with the pathogenesis of the homeopathic medicines *Pyrogenium* and *Sulphur*.[10] These medications have shown individually *in-vitro* effects on larvae of this Diptera.[11] However, the test performed in that study used the drugs in their hydroalcoholic form and used specimens of dipterans solely from a laboratory colony maintained under artificial conditions. Thus, the present study evaluated the effect of two homeopathic medicines (*Pyrogenium* or *Sulphur*), individually prepared in the pharmaceutical form of ointment, on wild larvae of *C. hominivorax* obtained from myiasis of sheep, to verify whether the effect would be repeated in a situation closer to the actual challenge of the treatment of myiasis. The alcoholic and the non-alcoholic vehicle of the ointments were also tested. To add important information about the understanding of this study, we performed an *in-vitro* test with the homeopathic medicines, handled only in sterile water without alcohol; this part of the experiment used laboratory-propagated larvae.

Materials and Methods

Ethical Approval

This study was approved by the Ethics Committee on Animal Research (CEUA) at the Federal University of Santa Catarina (UFSC), with the Protocol Number: 6324250619. All the processes and standards that guided the execution of this research are in accordance with the guidelines of the National Council for the Control of Animal Experimentation in Brazil and with the Directive 2010/63/EU. Information regarding this research project can be checked with open access at: http://ceua.sites.ufsc.br/ using the registration number above. The ethical approval certificate is exhibited in ►**Supplementary file S1**, available online only. Additionally, the complete ARRIVE 2.0 Checklist for this study is available in ►**Supplementary file S2**, available online only.

Cochliomyia hominivorax Larvae (Diptera: Calliphoridae)

The *C. hominivorax* larvae used in the tests of the groups "Ointment of *Sulphur* 12cH", "Ointment of *Pyrogenium* 12cH" and "non-alcoholic vehicle of ointments" are wild specimens and were obtained by manual collection of naturally occurring myiases in sheep belonging to the Agroecology Research and Outreach Center of the UFSC Experimental Farm. The collection of larvae is provided for in the research project mentioned above (►**Supplementary file S1**, available online only). The sheep were kept under rotated grazing management throughout the entire period of study, with tap water always available. The pasture is natural and the animals were supplemented with ovine-specific commercial feed and mineral salt *ad libitum*. During the experimental period the animals remained together with the general flock. Animals did not suffer any type of injury or damage to their health or well-being while participating in this research. The Agroecology Center UFSC possesses the organic certification seal of the Southern Biodynamic Association (ABDSul) and follows all international animal welfare standards.

The *C. hominivorax* larvae used in the tests of the groups "*Sulphur* 12cH prepared in sterile water", "*Pyrogenium* 12cH prepared in sterile water" and "alcoholic vehicle of ointments" were obtained from a laboratory colony, according to the methods explained by Barros et al.[11] The colony of *C. hominivorax* is sited in the Laboratory of Animal Parasitology of the UFSC, Florianópolis, Santa Catarina. The colony is composed of a group of approximately 300 adult insects in one cage of $35 \times 35\,cm^2$ in a room at controlled ambient temperature ($33 \pm 3°C$) and humidity ($60 \pm 5\%$). The diet of adult insects consisted of a mixture of water, organic honey and agar. The larval stage was kept in containers containing artificial diet in a bio-oxgen demand (B.O.D.) chamber with controlled temperature at $36 \pm 1°C$ and $60 \pm 10\%$ relative humidity. The larval artificial diet is composed of minced meat and artificial liquid diet (powdered blood, powdered eggs, powdered whole milk, water, and formaldehyde). The specimens that survived the test were kept at the Animal Parasitology Laboratory at UFSC until their natural death, at approximately 28 days. These specimens were handled following all the welfare guidelines for laboratory specimens in Directive 2010/63 / EU. The propagation occurred according to the methods for maintenance in the laboratory of dipterans of this species, presented by Barros et al.[11]

Homeopathic Ointments

The homeopathic ointments were developed and produced in partnership with Compounding Pharmacy Milligramm, Florianópolis, SC, Brazil (CNPJ: 15.392.920/0001–70). The production process complied with all Good Manufacturing Practices for medicines regulated by Resolution n° 17 of April 16, 2010, of the National Health Surveillance Agency (ANVISA).[12] The ointments were prepared according to the Brazilian Homeopathic Pharmacopoeia.[9]

As a vehicle for the formulation of the ointment, a mixture of sterile lanolin, vaseline and tocopherol (vitamin E) was used. This formulation is suggested[9] as an ideal vehicle for homeopathic medicines in the pharmaceutical form of ointment. This vehicle was impregnated with a homeopathic matrix of the respective homeopathic medicine individually (*Sulphur* or *Pyrogenium*) in a hydroalcoholic solution with 70% alcohol content (v/v) and dynamized to 12cH in the proportion of 1:10 (homeopathic matrix: vehicle), according to the technical recommendation described in the 3rd edition of the Brazilian Homeopathic Pharmacopoeia.[9] The incorporation of the homeopathic matrix in the vehicle was performed mechanically. The homeopathic ointments were dispensed in sterile tubes as a way to guarantee the physical and microbiological safety of the formulation before its use. The vehicle ointment mixed in alcohol 70% (v/v) was formulated in the proportion 1:10, with therefore a final concentration of 7% (v/v) alcohol in the ointment.

Homeopathic Medicines Prepared in Sterile Water

The homeopathic medicines prepared in sterile water were developed and produced in partnership with Compounding Pharmacy Milligramm, as above. *Sulphur* 12cH and *Pyrogenium* 12cH were produced according to the Hahnemannian centesimal method: 100 succussions were performed between each Hahnemann dilution up to potency 12cH, and water was used as the dispensing form of the final medicine. The medicines, whose dilution and dynamization methods are described in the Brazilian Homeopathic Pharmacopoeia, were prepared on the same day as the tests were performed.[9]

Contact Tests

With minor modifications, the tests performed in this study were based on the methods of Barros et al.[11] The contact test consisted of evaluating the effect of direct contact between medicines as ointment (homeopathic preparations) and healthy third-stage larvae (L3) of *C. hominivorax* on the emergence of adult insects. The L3 were deposited on filter paper in glass containers (9 cm height and 4 cm diameter), which received a group of 10 wild larvae or 15 laboratory larvae each. Experimental design was completely randomized. Each treatment and control group had five replicates. Treatment groups were: ointment of *Sulphur* 12cH, ointment of *Pyrogenium* 12cH, *Sulphur* 12cH prepared in sterile water, and *Pyrogenium* 12cH prepared in sterile water. There were two negative control groups: alcoholic and non-alcoholic vehicle of ointments.

One group of 10 L3 wild larvae or 15 L3 laboratory larvae of *C. hominivorax* was placed in direct contact with 5 g of the ointment, or with 1 mL of the homeopathic medicine prepared in sterile water, until the emergence of adult insects. Then, the larvae were covered with a layer of sterile vermiculite and the containers were sealed with tulle netting, tightened by a rubber band to allow gas exchange during the pupal stage. The flasks were kept in a B.O.D. chamber at 37°C ± 1°C and 60 ± 10% relative humidity, with controlled photoperiod and humidity. Different B.O.D. chambers were used in the tests: one for negative controls and another one for the tests involving homeopathic medicines prepared in sterile water or as ointment. The inhibition rate of larvae of *C. hominivorax* promoted by the tested substances was determined after 9 days of incubation. Diptera insects that were capable of completing metamorphosis and emergence were considered non-inhibited, whereas those that could not complete these processes were considered inhibited. After the experiment, the used specimens were subjected to cold narcosis at −22°C for 30 minutes before being discarded as biological residues, in accordance with the ethical approval.

Statistical Analysis

The statistical design used in this study was completely randomized and each experimental unit was composed of a group of larvae in a test flask with the tested substances. The response factor observed was the inhibition rate of the *C. hominivorax* larvae after contact with the ointments or medicines. For the analysis of life span and fertility of the specimens that survived the contact test, descriptive statistical methods were used. Emergence rate of adult insects, expressed as group mean ± standard deviation (SD), was evaluated by descriptive statistics and by analysis of variance (ANOVA). Homogeneity of variances was verified by F-test and group means were compared with Tukey's test ($p < 0.05$). The data were analyzed using IBM SPSS Statistics version 23 software.

Results

The raw data in this study are available with open access in the Mendeley Data repository http://dx.doi.org/10.17632/rd86256w2k.1.

The *C. hominivorax* inhibition of development rate observed in the groups that had been exposed to the *Sulphur* and *Pyrogenium* ointments were 90.0 ± 7.1% and 86.0 ± 8.9% respectively. The average inhibition rate observed in the *Sulphur* group did not differ statistically from the *Pyrogenium* group ($p > 0.05$).

The non-alcoholic vehicle used in the homeopathic ointments' preparation was inhibitory to 24.0 ± 11.4% of the larvae. The alcoholic vehicle limited the development of 22.08 ± 7.9% of the larvae. The average inhibition of development rate observed in the group of the alcoholic vehicle did not differ statistically from the group of non-alcoholic vehicle ($p > 0.05$). The two vehicles' rates differed meaningfully from the rates observed in groups of homeopathic ointments ($p < 0.05$). The *C. hominivorax* larval inhibition after contact

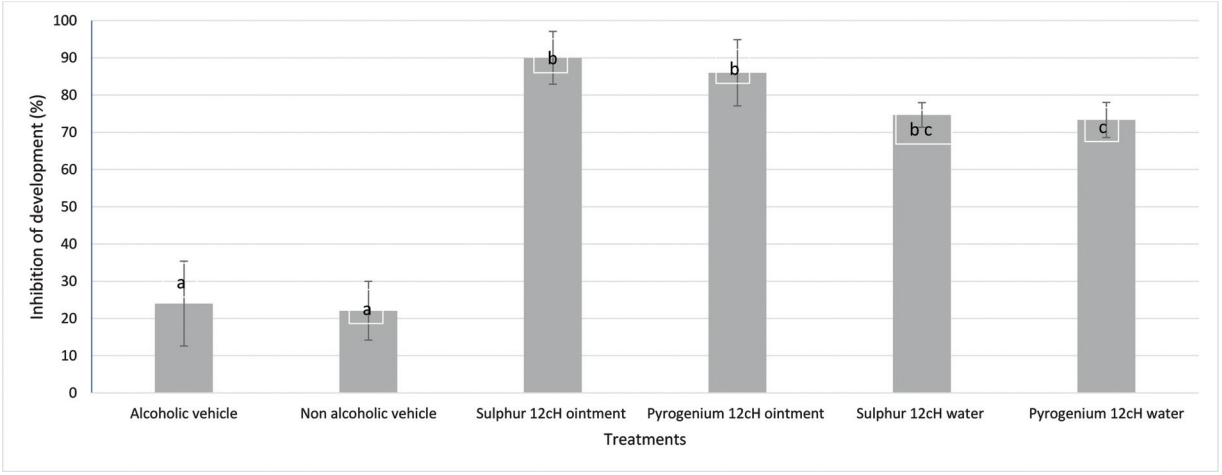

Fig. 1 Average rate of inhibition of development ± standard deviation (%) of two different homeopathic medicines (*Sulphur* 12cH or *Pyrogenium* 12cH) in ointment form, the alcoholic 70% (v/v) and non-alcoholic vehicle of the ointments and the same medicines prepared in sterile water on larvae of *Cochliomyia hominivorax* (Diptera: Calliphoridae) in tests *in vitro*. Different letters mean statistical differences ($p < 0.05$).

with the homeopathic ointments or with the alcoholic and non-alcoholic vehicle is shown in ►**Fig. 1**.

The medicines prepared only in sterile water and without alcohol showed an inhibitory effect on *C. hominivorax* larvae development. *Sulphur* prepared in sterile water hampered the development of 74.67 ± 3.3% and *Pyrogenium* prepared in sterile water hampered 73.33 ± 4.7% of larvae. The average inhibition rate observed in the two groups did not differ statistically from each other ($p > 0.05$). This information is shown in ►**Fig. 1**.

Those *C. hominivorax* specimens exposed to the homeopathic medicines and which still survived had a considerably shorter life span than the surviving specimens in the vehicle group. The natural death of the surviving specimens in the vehicle group occurred on average at 27.78 ± 6.2 days after their emergence from the pupae. In the *C. hominivorax* surviving specimens of the *Pyrogenium* group, this event occurred at 9.10 ± 3.9 days on average. For the *Sulphur* group, the natural death of the surviving specimens occurred on average at 11.33 ± 4.5 days of life. The means obtained for the *Sulphur* and *Pyrogenium* groups did not differ from each other ($p > 0.05$). However, both were shorter time intervals than in the vehicle group ($p < 0.05$). Besides this, the *C. hominivorax* specimens exposed to the homeopathic medications did not reproduce. The Diptera specimens exposed to the homeopathic ointments' vehicle reproduced normally and generated viable descendants. The longevity of the *C. hominivorax* specimens that participated in these tests and survived is shown in ►**Table 1**.

Discussion

Our initial hypothesis was that homeopathic ointments would have no direct effect on *C. hominivorax* larvae and would only be efficient when applied to myiasis conditions in animals. However, *in-vitro* tests using the third instar of pre-pupae larvae in contact with homeopathic medicines had their development inhibited and there was no emergence of insects from within the pupae.

The effect on larval development obtained in this trial was similar to that previously reported in our work involving laboratory specimens and homeopathic medicines in their hydroalcoholic form.[11] This is an important step in building the necessary knowledge to advance this line of research. Finding out if homeopathic medicines maintained their effectiveness, prepared in the pharmaceutical form of ointments in *C. hominivorax* wild larvae, adds security for clinical studies involving living beings affected by myiasis. Safety of the vehicle used in the preparation of homeopathic ointments on the *C. hominivorax* larvae was evident. The specimens exposed to the vehicle of the ointments performed their life cycle normally. The survival rates of this group, as well as their life span and reproductive capacity, was compatible with specimens propagated in the laboratory.[13] This showed that the effects observed on the inhibition of the development of *C. hominivorax* specimens were due to the action of the homeopathic medicines impregnated in the ointments.

The veterinary medical use of ointments has important advantages for the patient in the context of wound healing, as in the case of the treatment of myiasis by *C. hominivorax*. Recently, Santos et al[14] demonstrated the beneficial effect of an ointment composed of *Bellis perennis*, *Calendula officinalis* and *Myristica sebifera* on healing experimental wounds in laboratory mice. Besides, Sella et al[15] obtained better healing

Table 1 Average life duration (days) ± SD of wild *Cochliomyia hominivorax* (Diptera: Calliphoridae) flies surviving the *in-vitro* test with two different homeopathic ointments (*Sulphur* 12cH or *Pyrogenium* 12cH) and the non-alcoholic vehicle of the ointments. Different letters in the same column mean statistical differences ($p < 0.05$).

Experimental group	Average life duration (days)
Non-alcoholic vehicle	27.78 ± 6.2^{b}
Sulphur 12cH ointment	11.33 ± 4.5^{a}
Pyrogenium 12cH ointment	9.10 ± 3.9^{a}

results and higher speed of closing experimental equine wounds with the use of a complex homeopathic ointment formulated with the same base used in the present study. That study also showed less painful sensitivity in wounds treated with homeopathic ointment. These studies show there is great interest in investigating the use of homeopathic ointments on animal wound healing.

Studies with human patients and homeopathy are the basis of homeopathic clinical research. Pre-clinical studies involving homeopathic medicines have gained increasing attention from researchers[16]: there are studies with animal models for diseases,[17–19] as well as *in-vitro* research, that test the effects of homeopathic medicines directly on infectious agents or on cell cultures that exhibit reduced growth.[11,20–22] Special attention is required when analyzing pre-clinical studies with homeopathic medicines, especially regarding the interpretation of the effect of the vehicles used in their manipulation. Bonamin[23] suggests preparing the three last potencies only with sterile water. In this way, we performed a test with the medications dispensed in sterile water, without alcohol. The results of this test suggest that the homeopathic medicines had an inhibitory effect on *C. hominivorax* larval development, even though it was slightly less intense than the rates obtained when the medicine was handled in a hydroalcoholic solution at 30% (v/v).[11]

Any highly organized system reacts to stress, always producing the best possible response it is capable of at the time. In humans and vertebrate animals, this means that the defense mechanism offers the best possible response to the pathogenic stimulus, according to the current state of health and to the intensity of stress. When illness occurs, the first disturbance is said to take place in the body's electromagnetic field, which then triggers the defense mechanism.[24] This concept was announced as the basis of Samuel Hahnemann's homeopathic therapy. In the case of invertebrate organisms that undergo complete metamorphosis, such as *C. hominivorax*, the epithelial structures named disks are the driving force for the transformation of insects. After a larva has finished a series of moults, it enters the pupal stage. At this stage, digestive enzymes break down part of the larva's cellular structure, with the exception of the disks, forming an "insect soup", with the disks being arranged in pieces. The disks begin to form the outer structures of the future dipteran during this process. Working from outside in, these structures will continue to form organs, wings and eyes. Insect metamorphosis involves structural changes that are very different from vertebrates' different stages of development. The current authors make the inference that the homeopathic stimulus may have been very strong, causing a secondary effect of disorganization of this delicate process that is the metamorphosis. This would be like a pathogenetic illness and may have interrupted its development. An effect similar to that described is the study by Giesel et al,[25] where leaf-cutting ant colonies (*Acromyrmex* spp.) were treated with homeopathic medicines and this caused an overall decrease in insect activities for 20 days after its application. According to our theory, this demonstrates that there was an inhibition of insect development, as in these colonies the production of new individuals is intermittent. Additionally, the action of homeopathic substances on the "vital force" of insects may not follow the same logic as that used for more phylogenetically evolved beings.

In addition to all the biological effects of *Sulphur*, that have already been reported in the scientific literature, its action on *C. hominivorax* larvae has been reported in a previous *in-vitro* test with the medicine in 30% hydroalcoholic preparation[11] and now this result has been repeated with the use of *Sulphur* in the form of ointment. Therefore, a new therapeutic possibility for this important polychrest medicine may be emerging for the treatment of myiasis caused by *C. hominivorax* in animals.

Sulphur is one of the main medications prescribed in cases of skin disease. The use of *Sulphur* for chronic skin diseases (such as psoriasis and atopic dermatitis) is already widely described in the scientific literature.[26–28] Its positive effect on the inflammatory, as well as on the skin healing, process of human patients affected by wounds of varying degrees of severity has also been reported.[29]

Pyrogenium is a peculiar and traditional homeopathic medicine used in medical practice. It is an excellent medication to treat severe fevers in general, and for severe inflammatory conditions that can progress to septicemia. The fever is accompanied by intense mental anxiety. Its use is common in the treatment of serious infections that occur in the postoperative period and that accompany fetid and purulent secretions.

Evidence of its power to control acute and intense fevers,[30] which are characteristic events of myiasis caused by *C. hominivorax*, reinforces the potential of *Pyrogenium* for treating this clinical condition. There is also its anti-bacterial power,[31] which is of great interest in this veterinary clinical context, since bacterial contamination is always a serious risk to an animal's health because these are open and very bloody wounds. Many cases without adequate treatment culminate in septicemia and death.[4] *Pyrogenium*'s *in-vitro* effect on *C. hominivorax* larvae has been reported previously,[11] and that positive finding has been replicated in the present study. Thus, just as for *Sulphur*, a new therapeutic possibility for *Pyrogenium* may be emerging: the topical use of homeopathic ointments in animals to treat myiasis caused by *C. hominivorax*.

The use of homeopathic medicines in the pharmaceutical form of ointment is still not widespread among Brazilian homeopaths. However, ointments offer some benefits that are especially interesting in the context of the treatment of dermatological diseases, mainly due to their form of application.[32] The homeopathic ointment *Traumeel S* (Biologische Heilmittel Heel GmbH) is a traditionally used formulation for inflammatory and degenerative processes of the locomotor system and skin trauma in general, and several controlled studies have already been performed in which the positive effects of this formulation have been demonstrated.[33–35] Besides *Traumeel*, which is already a consolidated drug in the pharmaceutical market, several authors have reported the effectiveness of homeopathic ointments in the context of dermatology.[36–38]

To date, there are no published studies reporting the effect of homeopathic ointments on animals affected by myiasis. It is anticipated that the effects demonstrated on *C. hominivorax* larvae can be repeated in the more challenging *in-vivo* situation and that ointments based on *Sulphur* and *Pyrogenium* may be a new therapeutic option for the treatment of myiasis. To test this hypothesis further, direct studies would be necessary on animals affected by *C. hominivorax* myiasis.

Conclusion

Both the homeopathic ointments tested showed an evident biological effect on the development of *C. hominivorax* specimens. In addition to larval inhibition after exposure to the ointments, there was a notable negative effect on the development of the specimens that survived. The development of *in-vivo* studies may be a suitable next step for testing the possible clinical use of these homeopathic medicines on *C. hominivorax* myiasis in animals.

Highlights

- Each of the homeopathic medicines *Sulphur* and *Pyrogenium*, prepared in the form of ointment, is able to inhibit the development of wild larvae of the fly *Cochliomyia hominivorax* (Diptera: Calliphoridae), decrease the lifetime of adult flies of this species and prevent their reproduction.
- The standard vehicle used in the handling of homeopathic ointments (sterile lanolin, petroleum jelly and tocopherol) had no harmful effect on *C. hominivorax*.
- The homeopathic medicines *Sulphur* and *Pyrogenium* may be promising medicines for use in the treatment of myiasis caused by *C. hominivorax*.

Supplementary Material

Supplementary file S1. Certificate of approval by the Ethics Committee on Animal Research at the Federal University of Santa Catarina.
Supplementary file S2. ARRIVE 2.0 form.

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Conflict of Interest

Authors Giuliano Pereira de Barros, Patrizia Ana Bricarello and Maria Clara Huschel Hillmann declare that they have two patent applications pending on the intellectual property for the homeopathic ointments that are reported in this article. The registration of this patent is in co-authorship with the Federal University of Santa Catarina, in accordance with current legislation in Brazil.

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