Microbiology Research Journal International

Volume 34, Issue 10, Page 30-38, 2024; Article no.MRJI.123281
ISSN: 2456-7043, NLM ID: 101726596
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Evaluation of Antifungal Activity and Skin Toxicity of *Piliostigma thonningii* in Female Rabbits

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/mrji/2024/v34i101488

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/123281

Original Research Article

Received: 15/07/2024
Accepted: 17/09/2024
Published: 21/09/2024

ABSTRACT

The recurrent nature of dermatophytes, combined with the development of resistance to antifungal agents, makes the management of these infections more difficult. The search for plant-based compounds that can effectively combat dermatoses offers an ideal alternative in the African cultural context.

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Cite as: *Josette, Agré Don, Bolou Gbouhoury Eric-Kevin, KONAN Gbê Kouakou N'Dri Ange, Koné Check Hamed Baba, Kakou Marie Ange-Evelyne, and N'Guessan Jean David. 2024. "Evaluation of Antifungal Activity and Skin Toxicity of Piliostigma Thonningii in Female Rabbits". Microbiology Research Journal International 34 (10):30-38. https://doi.org/10.9734/mrji/2024/v34i101488.*

Aims: Fungal infections, such as those caused by *Candida albicans*, *Aspergillus fumigatus*, and *Trichophyton mentagrophytes*, pose an increasing challenge to public health. This study explores the antifungal efficacy and cutaneous toxicity of the hydroethanolic extract of *Piliostigma thonningii,* a medicinal plant used in Africa.

Methodology: The methodology involved two main components. First, antifungal tests were conducted to evaluate the activity of the extract against the three fungal species using the double dilution method in inclined tubes. The minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) were determined. Second, a cutaneous toxicity assessment was performed by applying the extract to the skin of female rabbits to observe reactions according to a modified Draize scale.

Results: The results showed that the *Piliostigma thonningii* extract inhibits the growth of all three fungal species in a dose-dependent manner. The MFC values were 100 mg/mL for *Aspergillus fumigatus* and above 100 mg/mL for Candida albicans and Trichophyton mentagrophytes. Antifungal assays calculated the inhibitory concentrations necessary to ensure the survival of 50% of the microorganisms, with IC_{50} values of 8.2 mg/mL for Candida albicans, 10.26 mg/mL for *Aspergillus fumigatus*, and 16.75 mg/mL for *Trichophyton mentagrophytes*. Regarding skin toxicity, the results indicated that the extract is slightly irritating but not corrosive, with reversible effects within 72 hours.

Conclusion: This suggests that *Piliostigma thonningii* is a promising therapeutic alternative. However, further research is needed to confirm its safety and clinical efficacy.

Keywords: Piliostigma thonningii; antifungal activity; cutaneous toxicity.

1. INTRODUCTION

Fungal infections represent a growing public health concern globally, affecting a wide range of tissues and organs, including the skin, nails, mucous membranes, and, in some cases, internal organs. Cutaneous mycoses stand out among these infections due to their high prevalence, affecting between 20% and 25% of the global population. The prevalence of these infections varies significantly by region, reaching up to 66% in Côte d'Ivoire for specific conditions such as onychomycosis [1]. These mycoses also include conditions like ringworm, athlete's foot, and cutaneous candidiasis, which manifest through various symptoms, including skin thickening, pruritic lesions, and changes in nail color and texture, leading to physical discomfort and significant psychological impact [2,3]. Conventional antifungal treatments, though effective in some cases, are increasingly limited by the emergence of pathogen resistance to drugs and associated side effects, including hepatic and renal toxicities [4]. Moreover, the prolonged duration of these treatments can compromise patient adherence, thereby increasing the risk of relapse [5]. Faced with these challenges, there has been a significant increase in interest in alternative therapeutic approaches, particularly the use of medicinal plants.

Piliostigma thonningii, a medicinal plant widely used in traditional African practices, has shown promise due to its diverse pharmacological properties. It is recognized for its antifungal, anti-inflammatory, and wound-healing effects, which are attributed to the presence of bioactive compounds such as flavonoids, saponins, and tannins. These compounds exert their action by disrupting the fungal cell wall, thus preventing their proliferation, and by facilitating the repair of damaged tissues [6,7,8]. Previous studies have also shown that *Piliostigma thonningii* possesses antimicrobial properties against a variety of pathogens, expanding its therapeutic potential beyond fungal infections [9].

However, the use of *Piliostigma thonningii* in medicine requires a thorough evaluation of its safety, particularly concerning its application on the skin. Skin toxicity remains a crucial concern, as it could lead to adverse reactions such as contact dermatitis, allergies, or severe irritations, compromising its therapeutic use [10]. Indeed, toxicological studies are essential to ensure that the topical application of this plant is safe for patients, especially in the context of prolonged or repeated treatments.

Thus, this study aims to evaluate the antifungal activity of *Piliostigma thonningii* while examining its safety on the skin of Hyplus rabbits. In addition to providing crucial information on the safety and efficacy of this plant, the results obtained could contribute to the development of new treatment strategies for cutaneous mycoses,

offering a viable alternative to conventional therapies [11,12].

2. MATERIALS AND METHODS

2.1 Plant Material

The plant used for the various tests, *Piliostigma thonningii*, was collected from the village of Gbena, 7 km from Séguéla, in the Worodougou region. Its identification was carried out at the National Center of Floristics (CNF) of Félix Houphouët-Boigny University (UFHB), where it is listed under number UCJ009480.

2.2 Animal Material

The animal material consisted of six (6) Hyplus rabbits, aged 3 to 4 months and weighing between 1.15 kg and 2.12 kg. They were acclimated for 2 weeks in the CNF's shaded area, housed in a 6-compartment cage equipped with a waste disposal system to ensure good hygiene by removing urine and feces. Each compartment had a feeder and a water bowl. The rabbits were marked according to the extract and concentration of the extracts used.

2.3 Preparation of the Hydroethanolic Extract of *Piliostigma thonningii*

The leaves of *Piliostigma thonningii* were cut into small pieces and then air-dried away from sunlight. They were subsequently ground into a fine powder using an electric grinder. Then, one hundred grams (100 g) of this fine powder were placed into an Erlenmeyer flask containing 1 liter of 70% hydroethanolic solution. Maceration was carried out for 30 minutes. The resulting macerate was ground and then filtered successively: first through a white cloth, three times through hydrophilic cotton, and finally, once more with Whatman No.1 filter paper. The different filtrates were then dried in an oven at 45°C for 48 hours [13,14].

2.4 Method for Studying the Skin Toxicity of the Hydroethanolic Extract of *Piliostigma thonningii*

The study was conducted according to OECD Guideline 404 [11].

2.4.1 *In vivo* test principle

The skin of animals selected for the experiment was treated with a single dose of *Piliostigma thonningii* extract, while untreated areas served as controls. The degree of irritation or corrosion was observed and evaluated according to a predefined scale at specified intervals, with detailed descriptions provided by the experimenter for a comprehensive analysis of the effects. The study duration was adjusted to assess the reversibility of observed effects. Animals showing persistent signs of distress or pain were euthanized, and these observations were included in the result evaluation.

2.4.2 Preparation for *In vivo* testing

2.4.2.1 Animal selection

The study was conducted using six (6) Hyplus rabbits, aged 3 to 4 months, nulliparous and non-pregnant. They were divided into four groups for each dose of *Piliostigma thonningii* extract. The Hyplus breed was chosen due to the lack of suitable albino rabbits.

2.4.2.2 Animal preparation

The rabbits were first weighed, and then the dorsal region of their trunk was shaved 24 hours before each test, ensuring no scratches were left on their skin. The animals were marked according to the doses administered.

2.4.2.3 Extract dose

The hydroethanolic extract of *Piliostigma thonningii* was administered at doses of 200 and 500 mg/kg body weight on the rabbits' skin.

2.4.3 Evaluation of the irritant and corrosive effect

The extract was applied to a 6 cm² area of the dorsal region of the trunk of the animals, using a vehicle consisting of alcohol diluted to 10%, considered non-aggressive to the skin. A volume of 0.5 mL of the solution was applied to the test areas.

2.4.3.1 Initial test

This test required one rabbit for each dose of the extract, making a total of two (2) rabbits. Each received three successive patches applied to shaved areas of the skin. One rabbit was exposed to a dose of 200 mg/kg body weight, while the other received 500 mg/kg. The extract was evenly distributed on compresses, which were then applied to the rabbits' skin. The

compresses were secured with a non-irritating adhesive tape. The first patch was removed after three (3) minutes, the second after one hour (1 h), and the last after four hours (4 h). At each removal, the presence or absence of skin reactions was carefully noted, with untreated areas serving as controls. The rabbits were observed for 14 days, and skin reactions were assessed at 24 h, 48 h, and 72 h after the removal of the last patch. At the end of this observation period, the animals were weighed.

2.4.3.2 Confirmatory test

For this test, four (4) rabbits were used, with two (2) rabbits for each dose of 200 and 500 mg/kg body weight. A single patch was applied to the rabbits' skin for 4 hours. Skin reactions were observed and scored one hour after patch removal, and at 24 h, 48 h, and 72 h during the observation period. The animals' weight was measured at the end of the study.

2.4.4 Evaluation and calculation of skin reactions

Reactions were evaluated using the arbitrary scoring scale for skin reactions. Scores for erythema and edema were recorded for each rabbit. The irritancy of each extract or mean skin irritation index (IIM) was calculated from the averages of the parameters (erythema and edema), and the tested products were classified according to the IIM classification (based on the modified Draize classification) [12].

$$ME = \frac{Sum\ of\ all\ erythema\ scores}{Total\ number\ of\ erythema\ scores};$$

$$MO = \frac{Sum\ of\ all\ oedema\ scores}{Total\ number\ of\ oedema\ scores};$$

$$IIM = \frac{ME+MO}{2},$$

ME: Mean erythema; MO: Mean oedema; IIM: irritation index Mean.

The scores obtained during the observation period for the initial trials were determined from the mean of the erythema or oedema scores obtained on the three (03) patches received (3 min, 1h and 4h).

2.5 Evaluation of the Antifungal Activity of *Piliostigma thonningii*

2.5.1 Preparation of the agar medium

The agar medium was prepared according to the manufacturer's instructions and distributed into various test tubes (3 cm x 12 cm).

2.5.2 Incorporation of extracts into the agar

Tests were conducted individually for each fungal species to determine antifungal parameter values. The double dilution method in inclined tubes was used to incorporate the extract into the agar. Pre-cooked agar was distributed into 10 numbered test tubes from 1 to 10, with 20 mL in tube no. 1 and 10 mL in each of the other tubes (no. 2 to no. 10) of each series. Among these 10 tubes, 8 contained plant extracts, while 2 were control tubes without extract: one served as a control for germ growth (GC) and the other, without germs, served as a control for culture medium sterility (SC). Generally, and according to the test series, the concentrations ranged from 1000 µg/mL to 0.38 µg/mL. For the 8 tubes in each series, the concentrations followed a geometric dilution factor of ½, from tube no. 1 to tube no. 8. After incorporating the extract, all 10 tubes in each series were sterilized at 121°C in an autoclave for 15 minutes, then inclined on a small base at room temperature to allow the agar to cool and solidify.

2.5.3 Preparation of the inoculum

For antifungal assays, samples were prepared individually from 48-hour-old cultures for *Candida albicans*, *Aspergillus fumigatus*, and 5-day-old cultures for *Trichophyton mentagrophytes* on slanted agar media. One or two isolated colonies were taken for each type of fungus using a 2 mm diameter loop and mixed in 10 mL of sterilized distilled water. This suspension resulted in a mother suspension labeled 10°, with a concentration of 10^6 cells/mL. From the 10° suspension, a 10^{-1} suspension was prepared by diluting 1 mL of the 10° suspension in 9 mL of sterilized distilled water to obtain a total of 10 mL, with 10^5 cells/mL [13].

2.5.4 Seeding the culture media

The fungal species *Candida albicans*, *Aspergillus fumigatus,* and *Trichophyton mentagrophytes* were cultured in all tubes of the series, except for the sterility control tube no. 10. Seeding was performed by streaking until exhaustion with 10 µL of the 10^-1 suspension (at a concentration of 10^5 cells/mL), equivalent to 1000 seeded cells. The cultures were incubated at 30°C in a MEMMERT incubator for 2 to 7 days, depending on the types of pathogenic fungi examined [13,14].

2.5.5 Counting the germs

After two days for *Candida albicans* and *Aspergillus fumigatus*, and 5 to 7 days for

Trichophyton mentagrophytes, the different fungal species were counted by performing a direct count of the colonies. Fungal growth was assessed in the experimental tubes of each series based on the survival rate, calculated in comparison with the growth control tube with a 100% survival rate, using the following formula:

S= (n/N)×100; With : S = Germ survival (expressed as a percentage); N = Number of colonies in the control tube; n = Number of colonies in the experimental tube.

Analysis of the experimental data led not only to the plotting of activity curves but also to the identification of the following antifungal parameters:

The Minimum Fungicidal Concentration (MFC) corresponds to the lowest concentration of extract that eliminates 99.99% of fungal species compared with the growth control, leaving a survival rate of 0.01%;

The MIC is the lowest concentration of extract above which no growth visible to the naked eye is observed.

The dose required to obtain 50% inhibition (IC_{50}). This corresponds to the quantity of extract that caused 50% inhibition of the growth of the fungal species. This value is obtained visually by analysing the sensitivity curve.

3. RESULTS

3.1 Cutaneous Toxicity

The scores obtained for *Piliostigma thonningii* in the initial test were 0.53 for 200 mg/kg body weight and 0.58 for 500 mg/kg body weight. According to the modified Draize classification, a score between 0.5 and 2 indicates that the substance is considered slightly irritating. Thus, it can be said that the raw extract of *Piliostigma thonningii* is slightly irritating to the skin but not corrosive, as the effects disappeared after 72 hours without causing irreversible damage.

Subsequent tests confirmed these results. For the two rabbits receiving the 200 mg/kg dose, the scores were 0.75 and 0.63, and for those receiving the 500 mg/kg dose, both scores were 0.75. The effects also disappeared after 72 hours without leaving any traces, reaffirming that this extract is not corrosive.

The rabbits showed some discomfort after the application of the adhesive tape but calmed down within a few minutes after adjusting to its presence. Additionally, hair regrowth in rabbits receiving 200 mg/kg body weight was 2 days ahead of those receiving 500 mg/kg body weight.

3.2 Antifungal Activity of the Hydroethanolic Extract of *Piliostigma thonningii*

The evaluation of the hydroethanolic extract of *Piliostigma thonningii* on the *in vitro* growth of *Candida albicans, Aspergillus fumigatus*, and *Trichophyton mentagrophytes* revealed a progressive decrease in the number of colonies in the experimental tubes compared to the control as the concentration of the extracts increased. This demonstrates that the studied extracts inhibit the *in vitro* growth of the three fungal species in a dose-dependent manner, allowing for the determination of minimum fungicidal concentrations (MFC) and minimum inhibitory concentrations (MIC).
In the presence of *Piliostigma thonningii:*

- MIC = MFC = 100 mg/mL for *Aspergillus fumigatus*
- MIC = 100 mg/mL and MFC > 100 mg/mL for *Candida albicans*
- MIC = 100 mg/mL and MFC > 100 mg/mL for *Trichophyton mentagrophytes*

Antifungal profiles obtained from colony counts in the 10 experimental tubes allowed for the evaluation of germ growth as a percentage of survival compared to the control tube, which represented 100% survival. These antifungal tests then graphically determined the inhibitory concentrations required to ensure 50% survival of microorganisms (IC_{50}).

In the presence of *Piliostigma thonningii:*
- IC_{50} = 8.2 mg/mL for *Candida albicans*
- IC_{50} = 10.26 mg/mL for *Aspergillus fumigatus*
- IC_{50} = 16.75 mg/mL for *Trichophyton mentagrophytes*

4. DISCUSSION

The results obtained for the hydroethanolic extract of *Piliostigma thonningii* indicate mild skin irritation, classified according to the modified Draize system, with scores of 0.53 and 0.58 for doses of 200 mg/kg and 500 mg/kg body weight (BW), respectively. These findings are consistent with observations by N'Guessan et al. [15], who

studied the skin effects of *Lippia multiflora* extract, a plant also rich in flavonoids and tannins, on animal models. They reported that these compounds can cause temporary irritation due to their interaction with epidermal cells but do not result in permanent damage. The effects observed, which disappeared within 72 hours without residual traces, align with studies by Macêdo et al. [16], who assessed skin irritation caused by the hydroethanolic extract of *Arnica montana*. They showed that, although slightly irritating, Arnica has reversible effects that do not cause persistent skin lesions, which is crucial for the acceptability of an extract for prolonged skin application. The secondary tests, with slightly higher scores (0.63 to 0.75), remain within the same category of mild irritation, which is comparable to results obtained by Ezeonwumelu et al. [17] in their evaluation of skin effects of *Azadirachta indica* (neem) extracts on animal models. They observed that variations in extract concentration could temporarily increase irritation without causing skin corrosion.

Comparison with the work of Radha et al. [18], who studied the skin effects of *Hamamelis virginiana* (witch hazel) and *Aloe vera*, shows that *Piliostigma thonningii* presents a similar irritation profile to these plants, which are classified as mildly irritating but widely used due to their therapeutic properties. The behavior of the rabbits, which adapted to the presence of the adhesive tape after brief discomfort, is consistent with observations by Shibasaki et al. [19], who studied the effects of skin devices on rats following the application of *Camellia sinensis* (green tea) extract. They noted that animals might initially react to devices applied to their skin but quickly adjust if the devices do not cause severe pain or discomfort. The difference in hair regrowth rates between the groups treated with 200 mg/kg and 500 mg/kg BW is reminiscent of observations by Barreto et al. [20], who studied the effect of *Calendula officinalis* (marigold) extract on tissue regeneration in mice. They reported that the concentration of the applied extract could influence the rate of tissue regeneration, although this minor difference warrants further investigation.

The results obtained in this study are supported by similar observations in the scientific literature on various medicinal plants, reinforcing the conclusion that the raw extract of *Piliostigma thonningii* is mildly irritating but not corrosive. This concordance with other studies suggests that *Piliostigma thonningii* could be a promising candidate for topical therapeutic applications, pending further testing to confirm its safety and efficacy.

The results obtained with the hydroethanol extract of *Piliostigma thonningii* on *Candida albicans*, *Aspergillus fumigatus* and *Trichophyton mentagrophytes* are in line with several previous studies highlighting the efficacy of plant extracts against fungal infections. A study by Akinpelu et al. [21] showed that *Ocimum gratissimum* extracts had a similar dose-dependent inhibition against *Candida albicans*, with a MIC of 80 mg/mL. These results support the idea that plant extracts can indeed inhibit the growth of *Candida albicans*, although the minimum concentrations required may vary from plant to plant. The MIC (100 mg/mL) obtained in this study is 10 times higher than the MIC (100 µg/mL) obtained by Ogundipe et al. [22] with the hydroethanol extract of the same plant. Various reasons could explain this discrepancy in results, notably the nature of the phytochemical composition and the profile of the *C. albicans* species studied. A MIC of 200 µg/mL and 250 µg/ was obtained with hydroethanolic extracts of *Piliostigma reticulatum* [23] and *Piliostigma tomentosa* [24] respectively. This further demonstrates the anticandidogenic potential of hydroethanol extracts from the *Piliostigma* genus.

Work by Ali et al [25] on *Azadirachta indica* extract revealed a MIC of 90 mg/mL for *Aspergillus fumigatus*, which is comparable to the MIC of 100 mg/mL observed for *Piliostigma thonningii*. This suggests *that Piliostigma thonningii* has a relatively high antifungal potential, similar to other plants known for their antimicrobial properties. With regard to *Trichophyton mentagrophytes*, a study by Nweze et al [26] on *Garcinia kola* extract found an MIC of 150 mg/mL, which is higher than that observed with *Piliostigma thonningii* (100 mg/mL). Ogundipe et al. showed 60-70% inhibition of micellian growth of *Trichophyton mentagrophytes* at 50 mg/mL with the aqueous extract of *Piliostigma thonningii* [22]. This difference may be attributed to the specific chemical composition of the extracts, suggesting that *Piliostigma thonningii* may be more effective than other plants against this fungal species. Its activity would also depend on the nature of the extraction solvent used.

It should also be noted that the IC_{50} values obtained in this study (8.2 mg/mL for *Candida albicans*, 10.26 mg/mL for *Aspergillus fumigatus*

and 16.75 mg/mL for *Trichophyton mentagrophytes*) demonstrate the relative efficacy of *Piliostigma thonningii* against these pathogens. In comparison, a study by Sukhikh et al [27] on *Eucalyptus globulus* extract reported an IC_{50} of 12 mg/mL for *Candida albicans*, showing similar efficacy to that observed with *Piliostigma thonningii*. This reinforces the idea that this plant has significant antifungal potential, which merits further exploration.

The results of this study confirm that *Piliostigma thonningii* possesses significant antifungal properties, comparable to those of other plant extracts studied. However, further research is needed to identify the specific bioactive compounds responsible for these effects and to optimize their therapeutic application. These findings pave the way for the potential use of *Piliostigma thonningii* as an alternative treatment in managing fungal infections, particularly in regions where access to conventional antifungals is limited.

5. CONCLUSION

The hydroethanolic extract of *Piliostigma thonningii* demonstrated not only a dose-dependent antifungal activity against *Candida albicans*, *Aspergillus fumigatus*, and *Trichophyton mentagrophytes* but also limited skin toxicity. The tests revealed that the extract, although slightly irritating, is not corrosive, and the observed effects were reversible after 72 hours without causing permanent damage. This combination of antifungal efficacy and low toxicity positions *P. thonningii* as a promising candidate for the development of alternative treatments against fungal infections. However, further research is needed to deepen the understanding of the active compounds responsible and to assess the safety of this extract in broader clinical applications.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ETHICAL APPROVAL

All authors hereby declare that the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised in 1985) were strictly followed, as well as all applicable national laws. Additionally, all experiments were reviewed and approved by the appropriate ethics committee.

ACKNOWLEDGEMENTS

We would like to express our gratitude to the traditional practitioners who introduced us to the benefits of this plant in the traditional environment. We also extend our thanks to all the members of the Biology and Health Laboratory for their active participation in this work. Additionally, we are grateful to the National Center of Floristics of Ivory Coast for their assistance in identifying this plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Available:https://doi.org/10.3390/plants11111441

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/123281