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Proposed Methodology

Three Experimental Animal Parasite Models for Assessing Anthelmintic Efficacy and Immunopotentiating Properties of Natural Products: Protocols and Applications

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ABSTRACT

Introduction: Helminthic infections, including soil-transmitted helminths (STHs) and intestinal tapeworms, pose a major global health challenge, classified as neglected tropical diseases (NTDs).

Objective: Current control efforts are hampered by expensive treatments, emerging drug resistance, and high reinfection rates. This necessitates the urgent discovery of new anthelmintic drugs and immunomodulatory compounds. Natural products are a promising source for these new therapies.

Materials and methods: This paper introduces three experimental animal parasite models specifically designed to evaluate the effectiveness and immune-boosting properties of natural products, namely *Hymenolepis diminuta* (tapeworm) in mice, *Ascaridia galli* (nematode) in chickens, and *Raillietina cesticillus* (tapeworm) in chickens.

Rationale: These models provide controlled environments for assessing potential therapeutic compounds and offer systematic methodologies for identifying effective and immunomodulatory natural products.

Conclusions: A significant challenge in treating helminth infections is targeting larval stages embedded in host tissues, such as those seen with *Rodentolepis* (*Hymenolepis*) *nana* in humans and hypobiotic trichostrongylid nematode larvae in ruminants. Therefore, demonstrating a natural product's effectiveness against *A. galli* larvae embedded in the intestinal mucosa is particularly important. Such efficacy would indicate that the product possesses valuable physicochemical and pharmacological properties, paving the way for developing novel treatments against hidden, tissue-dwelling parasites in both humans and animals.

KEYWORDS: INTESTINAL HELMINTHS; ANIMAL MODELS; NATURAL PRODUCTS; ANTHELMINTIC EFFICACY; IMMUNOPOTENTIATION

INTRODUCTION

Helminthic infections, caused by diverse multicellular worms, represent a significant global health burden, particularly in the context of neglected tropical diseases (NTDs). Among these, soil-transmitted helminths (STHs), including prominent nematode pathogens such as *Ascaris lumbricoides*, *Trichuris trichiura*, *Ancylostoma duodenale*, *Necator americanus*, and *Strongyloides stercoralis*, are highly prevalent. The life cycles of STHs involve the contamination of soil with parasite eggs or larvae from human faeces, especially in areas with inadequate sanitation, leading to human infection through ingestion or larval skin penetration. The World Health Organization (WHO) estimates that STH infections affect approximately 1.5 billion people, accounting for nearly 24% of the global population [1]. These chronic infections often result in impaired nutritional status, suppressed immune responses, and a range of debilitating conditions [2]. Beyond nematodes, cestode infections, such as those caused by *Taenia saginata*, *Taenia solium*, and *Diphyllobothrium latum*, also contribute to the global helminthic disease burden, typically acquired through the consumption of undercooked beef, pork or fish, respectively. Another serious pathogen is the dwarf tapeworm *Rodentolepis* (*Hymenolepis*) *nana*, the most common human tapeworm, a unique aspect of which is its ability to complete its life cycle within a single host, leading to potential autoinfection and a high parasite burden, particularly in immunocompromised individuals and children.

Despite advances in medical science, the comprehensive control of human intestinal helminth infections remains elusive. Challenges include the absence of effective vaccines, the high cost of existing treatments, the escalating issue of drug resistance, and rapid reinfection rates in endemic regions. These limitations underscore the urgent need for novel therapeutic strategies. Consequently, research efforts have increasingly turned to natural products, recognizing their historical and demonstrated efficacy as antiparasitic agents. Furthermore, there is growing interest in approaches that enhance the host's intrinsic immune responses to combat helminthic infections. The scientific community worldwide is actively investigating the immunomodulatory potential of various natural products, such as whole plants, their extracts, and isolated phytochemicals [3].

The development and application of robust experimental animal models are critical for the systematic evaluation of potential anthelmintic agents and immunopotentiating compounds derived from natural products. These models provide a controlled environment to assess efficacy, understand mechanisms of action, and inform the development of future therapeutic interventions. This paper aims to provide detailed protocols and applications of three experimental animal parasite models suitable for assessing both the anthelmintic efficacy and immunopotentiating properties of natural products. The outlined methodologies will serve as a valuable resource for researchers investigating novel anthelmintic and immunomodulatory compounds.

MATERIALS AND METHODS

MATERIALS

The parasite models

Three animal/parasite models are proposed, namely the tapeworm *Hymenolepis diminuta* (Rudolphi, 1819) in the laboratory mouse (*Mus musculus*), the nematode *Ascaridia galli* (Schrank, 1788) in chickens (*Gallus gallus domesticus*), and the tapeworm *Raillietina cesticillus* (Molin, 1858) in chickens.

Parasites source

H. diminuta is a common intestinal parasite found mainly in the small intestines of rats, mice, and hamsters, though it can occasionally infect other mammals, including humans. Adult worms are often isolated from wild rodents. Similarly, *R. cesticillus* and *A. galli* are prevalent intestinal parasites identifiable in local backyard and free-range chickens during post-mortem examinations.

Establishment of parasite lifecycles

In the insect intermediate host

The lifecycle of *H. diminuta* can be propagated using laboratory-reared insect intermediate hosts, such as the confused flour beetle *Tribolium confusum* and the red flour beetle *Tribolium castaneum*. Terminal gravid proglottids from rat worms are homogenized in water using a blender. The eggs in the homogenate are then purified through repeated cycles of settling and decanting the supernatant. The isolated eggs, which are contained in the sediment, are subsequently placed on moist filter paper for beetles that have been starved for 5 to 6 days. After 24 hours of feeding, the beetles are transferred to glass jars filled with whole wheat flour and incubated at 25°C in a humid environment, allowing for the development of infective cysticercoids (larvae) within the insects. *T. confusum* is a long-lived insect with a lifespan exceeding three years [4]. The entire life cycle, from egg to adult, typically takes 40 to 90 days. Therefore, it is essential to sift larvae and pupae from the stock on a monthly basis to prevent dilution of the infected colony. Cysticercoids can be recovered by mechanically disrupting infected beetles in modified Hanks' balanced salt solution.

For the establishment of *R. cesticillus*, beetles (*T. confusum* or *T. castaneum*) that have been starved for 5 to 6 days are fed shredded gravid proglottids obtained from patent worms (15 to 21 days old) from experimentally infected chickens or naturally infected birds over two or three consecutive days. Mature cysticercoids (over 15 days old) are then recovered through dissection of the insects.

A. galli has a direct life cycle, meaning it does not require an intermediate host.

In the definitive host

Infection of mice with *H. diminuta***:** The required number of cysticercoids will be administered via intragastric intubation while under light ether anesthesia or by using gavage needles as necessary. It is recommended to use larvae that are no more than 30 days old to minimize the risk of reduced infectivity associated with increasing age of the insect.

Infection of chickens with *R. cesticillus*: Chickens are infected with the parasite by feeding them larvae contained in gelatin capsules, a technique the author found superior to the common dropper method [5]. Results also indicate that the infectivity of *R. cesticillus* cysticercoids to chickens decreases with the age of the larvae; by approximately 7 months of age in the insect (*T. confusum*), the larvae become nearly non-infective.

Infection of chickens with *A. galli:* Embryonated *A. galli* eggs are collected by dissecting the uterus of adult female worms. These eggs are then cultured *in vitro* in 0.1N sulfuric acid at 18 ◦C for 30 days, at which point they become infective to chickens [6]. Infections can be established using one of two primary methods: direct oral administration of the infective eggs, or by feeding the eggs contained in gelatin capsules.

Animal treatment

Infected mice or chickens will receive oral treatment with the natural product under investigation, in accordance with the protocols established by the implementing institution.

Parasite recovery

H. diminuta is a non-invasive intestinal species, with a relatively weak scolex devoid of hooks. Tensile tests [7] show an attachment force of 0.021 ± 0.011 g to the mucosa which makes the parasite easily recoverable. Following treatment, infected mice in experimental and control groups are euthanized. Their intestines are opened and examined for worms using a technique described by Hopkins *et al.* [8]. This method effectively reveals “destrobilated worms” —those that consist solely of scolices (head) and the neck region. Bolla and Roberts [9] described the neck of *H. diminuta*, as a "germinative region" characterized by rapid cell replication and growth. This area is most active in DNA synthesis and cell division 2 to 4 days after infection, leading to the formation of the strobila through the continuous differentiation and budding of new segments, or proglottids. In this regard, the observation of destrobilated worms, as opposed to the complete elimination of the parasite, provides insight into an investigational product's possible mode of action by selectively targeting this region. The worms from each mouse are washed to remove debris, counted, dried at 95-100 °C for 24 hours, and then weighed to provide *biomass per mouse.*

In contrast*, R. cesticillus* has a large scolex with a wide rostellum armed with 400-500 hooklets. The scolex attaches firmly to the intestinal mucosa. To ensure accurate results when studying chickens infected with *R. cesticillus,* two separate experimental groups are necessary. One group should be treated and euthanized 24 hours later, while the other group should be treated and euthanized 14 days later. Evidence [10] indicates that the application of certain agents, such as niclosamide, can lead to the detachment of the strobila of *Raillietina tetragona*, a closely related intestinal tapeworm that affects chickens. This detachment can result in the presence of destrobilated worms, which may be easily overlooked during post-mortem examinations conducted shortly after treatment. Due to their broad rostellum and the large number of rostellar hooks, these destrobilated worms can remain firmly attached to the intestinal mucosa. In such cases, the opened intestine should be cut into segments and incubated in physiological saline at 37°C for at least one hour to facilitate the detachment and recovery of these worms. Delaying the examination for 14 days in one group increases the potential for any destrobilated parasites present to regrow and generate strobila, making them more easily identifiable.

After chickens ingest infective *A. galli* eggs, larvae hatch and penetrate the small intestine's mucosal lining. They undergo a histotrophic phase (tissue development) lasting approximately 17-18 days. Larvae then return to the intestinal lumen, maturing into adults around 28-30 days post-ingestion [11]. Therefore, experimental design must optimize the timing of necropsy following product treatment. This allows for assessing treatment effects on both the larval stage while in the mucosa, and the adult parasite in the intestinal lumen.

Assessment of the therapeutic efficacy of products

In both tapeworm and nematode infections, assessment of efficacy is conducted by counting the number of residual parasites present in the intestine of treated animals at necropsy, as well as measuring their *biomass* (in mg of dry weight) in comparison to untreated controls that received a similar number of infective larvae. Statistical analysis will be performed to evaluate the differences. For illustrative purposes, these recovery figures can be converted into percentages using a mean index calculated with the following formula:

$$\text{Percent efficacy} = \frac{(a - b)}{a} \times 100,$$

where (a) represents the mean number of parasites in the control group, and (b) denotes the mean number in the product-treated animals.

Assessment of the immunopotentiating properties of products

H. diminuta is one of the most extensively studied tapeworms [12] and an important model species in cestode biology [13]. Research consistently demonstrates that mice, as non-permissive hosts for *H. diminuta*, develop a robust Th2-mediated immune response that peaks around 8 days post-infection and leads to the expulsion of even low-dose (e.g., 5-6 cysticercoids) primary infections within 8-14 days, effectively rendering them immune [14-17]. There appears to be no minimum antigenic threshold in *H. diminuta* infections; even a single worm can elicit a protective immune response in the host [18]. Based on these findings, we propose an experimental design to evaluate a product's immuno-potentiating properties involving three distinct groups of mice:

- A group that had been receiving the test product over a specified period of time (experimental group).
- A group that received a primary 6-cysticercoid infection without any prior treatment with product (immune control group).
- A group that received neither cysticercoid infection nor product treatment (naïve control group).

Mice that receive the cysticercoid infection will be administered an appropriate anthelmintic treatment, such as praziquantel, on day 8 of the infection to eliminate any residual worms. By this time, they will have developed natural immunity against a homologous challenge and will be classified as immune.

The other two groups will also receive anthelmintic treatments to standardize the protocol. All three groups will undergo a challenge with *H. diminuta* through the surgical transplantation of single strobilate worms from cortisone-immunosuppressed mouse donors into the duodenum [19] or by the oral administration of 6 cysticercoids. When mice are subjected to surgical implantation of strobilate worms, they are killed five days after challenge. By this time, naïve control mice are not expected to reject the challenge worm, as a strobilate *H. diminuta* transplant is not rejected by naïve mice in less than seven days, compared to approximately four days in immune recipients [19]. Therefore, the disparity in rejection rates between naïve and immune mice regarding surgically transplanted worms should be evident by day five post-challenge, if not earlier. Consequently, for a natural product to be considered effective as an immuno-potentiator, the survival and growth patterns of transplanted worms in product-treated mice should diverge from those observed in naïve mice and align more closely with those seen in immune controls. The significance of this directional divergence is assessed statistically. Additionally, the inclusion of an immune control group facilitates a direct comparison between immunity enhanced by an administered natural product and that stimulated by a natural infection. In the author's experience, day eight post-challenge reveals the most pronounced difference in rejection rates between naïve and immune mice challenged with cysticercoids. Furthermore, using a multi-worm challenge with cysticercoids amplifies even small differences in the immune status of mice immunized by various procedures [20]. The assessment of the immunostimulant effect will be performed in terms of the following criteria:

- The product-induced expulsion (rejection) of the parasite is assessed by comparing the number of residual parasites remaining in the intestine of the experimental group to that of the immune and naïve control groups. According to Hopkins *et al.* [8], a destrobilated worm, measuring <0.1mm long and <0.1 mg dry wt., is in the process of being rejected and should be excluded from recovery figures.

- The product demonstrated a suppressive effect on the growth (*biomass*) of residual parasites remaining in the intestines of the experimental group, compared to both the immune and naïve control groups.

Both the nematode *A. galli* and the tapeworm *R. cesticillus* evoke a protective immune response in their avian host, the chicken [21-24]. A similar principle of experimental design applies when assessing the immunopotentiating properties of products in birds infected with either parasite.

For testing the therapeutic efficacy, infections with *R. cesticillus* can be initiated using low-level cysticercoid infections in young chicks. When evaluating the immune response, high doses of up to 50 cysticercoids can be administered to chickens aged 6 to 8 weeks, by which time they have typically achieved full immunological competence [25].

DISCUSSION

A key advantage of the models in this study is that the parasites used—tapeworm cysticercoid larvae or nematode larvae—don't multiply within the host. Each introduced larva develops into only one adult parasite. This eliminates variability inherent in systems with self-reproducing parasites, which can lead to unpredictable growth and unreliable assessment of results.

Using chickens in these experiments offers another significant advantage: they serve as an excellent model for studying both humoral and cell-mediated immunity, especially when testing products with potential immunopotentiating (immune-boosting) properties against intestinal parasites. Researchers can differentiate these two immune responses by selectively removing or disabling the *bursa Fabricii*, a hind-gut primary lymphoid organ unique to birds crucial for humoral immunogenesis. This can be done through surgical removal combined with irradiation of newly hatched chicks [26], prenatal *in ovo* hormonal treatments [27], or by using more advanced transgenic chicken models [28]. Consequently, these diverse chicken models provide valuable tools for investigating how natural products might enhance the immune response against intestinal worms.

Intestinal helminth infections often present a significant treatment challenge due to larval stages embedded within host tissues. These hidden stages are frequently less susceptible to anthelmintic drugs than adult worms. A prime example of this phenomenon is seen in human infections with the dwarf tapeworm, *R. nana*, which is the most common human tapeworm. The life cycle begins when ingested eggs release oncospheres that invade intestinal villi and mature into cysticercoid larvae. Upon rupture of the villi, these larvae are subsequently released back into the intestinal lumen, where they then develop into adult worms. The infection can intensify significantly through internal autoinfection, where eggs laid by these parasites hatch inside the same host. This leads to a build-up of a large number of cysticercoids, perpetuating the infection. Another critical instance of larval embedding that confers resistance to treatment is the phenomenon of arrested development, or hypobiosis. This survival strategy is observed in parasites, such as trichostrongylid nematodes in ruminants, where larvae embed in the intestinal mucosa. This embedded state often renders them resistant to conventional anthelmintics at doses lethal to adult worms. This underscores the critical need for treatments that can reach these hidden developmental stages. Therefore, demonstrating a natural product's proven efficacy against, for example, *A. galli* larvae embedded in the intestinal mucosa would be highly significant. Such efficacy would indicate that the product possesses valuable physicochemical and pharmacological properties, paving the way for developing novel therapeutic agents against hidden, tissue-dwelling parasites in both human and animal hosts.

A description of three models is presented in this study. Other models, such as *Raillietina tetragona*, an intestinal poultry tapeworm with a worldwide distribution, may also be utilized. The intermediate host of this

parasite is the ant *Pachycondyla sennaarensis* [29], currently reclassified as *Brachyponera sennaarensis* (Mayr, 1862), an insect with a wide distribution across Africa, the Arabian Peninsula, and Iran. This model has been successfully employed in our laboratory to screen the anticestodal activity of medicinal plants [30] and chemotherapeutic agents such as benzimidazoles [31], praziquantel [32], and niclosamide [10], using cysticercoid larvae from naturally infected ants. Researchers may also experiment with *Choanotaenia infundibulum*. Results indicate that infective cysticercoids of this parasite (Figure 1) can develop within a few weeks in the beetle *T. castaneum* and may be recovered from naturally infected larvae and adults of the tenebrionid beetle *Alphitobius diaperinus* [33].

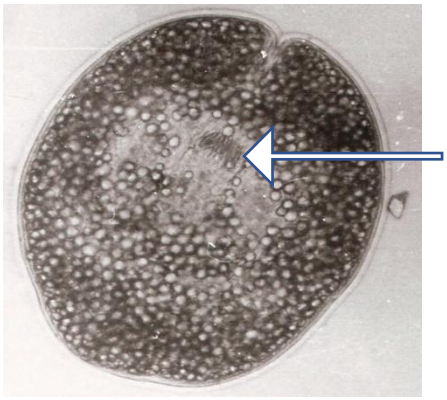

Figure 1: Cysticercoid of *Choanotaenia infundibulum* recovered from *Tribolium castaneum*
Note the rostellar hooklets (arrow) and the numerous calcareous corpuscles (400x)
Retrievable from fig share https :// doi: 10.6084/m9.figshare.29555105

CONCLUSIONS

The key conclusions drawn from this research are:

- **Model Suitability**: The helminth animal parasite models described in this study can be used as robust, cost-effective, and easy-to-maintain systems for high-throughput screening of natural products.
- **Unique Model Strengths:** The chicken model is particularly effective for assessing the immunopotentiating properties of natural products and delineating specific components of the immune response. The mouse model's unique ability to reject the tapeworm *Hymenolepis diminuta* was leveraged to study immunomodulatory effects of natural products against gut-dwelling tapeworms.
- **Addressing Treatment Challenges:** The use of the nematode *Ascaridia galli*, a parasite that undergoes a histotrophic tissue phase of development, provides a crucial opportunity to evaluate natural products against difficult-to-treat helminth infections, a key area for future therapy development.
- **Dual Relevance:** The use of these particular models has significant implications for both human health and veterinary medicine, highlighting the dual benefits for assessment of the therapeutic and immunostimulant properties of the natural products.
- **Global Applicability**: The core principles and methodologies employed in this study can be expanded and adapted globally to assess natural products against helminth intestinal infections.

- **Perspective:** We emphasize that this is a proposed methodology paper. Study-derived evidence of therapeutically effective and immunopotentiating natural products will pave the way for the development of effective, safe, and relatively low-cost anthelmintic drugs and immunostimulants.

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Conflict of interest

The author has no conflict of interest regarding this article.

Data Availability: All relevant data are within the paper.

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