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THERAPEUTIC EVALUATION OF PRAZIQUANTEL AND QUERCETIN IN ADULT WORMS: *S.MANSONI* REGARDING IN VITRO EXPERIMENTATION

Keylla Walesca da Silva Santiago
Fundação Oswaldo Cruz – Aggeu Magalhães Institute / IAM-PE, Brazil
Universidade de Pernambuco – UPE, Institute of Biological Sciences – Universidade de Pernambuco – ICB/UPE, Brazil
http://lattes.cnpq.br/3085176934451590

Rubens Emanoel Tavares da Rocha
Fundação Oswaldo Cruz – Aggeu Magalhães Institute / IAM-PE, Brazil
http://lattes.cnpq.br/0743864235096379

Gabriel Gazzoni Araújo Gonçalves
Universidade Federal de Pernambuco – UFPE, Brazil
http://lattes.cnpq.br/1891690680840129

Hallysson Douglas Andrade de Araújo
Universidade Federal de Pernambuco – UFPE, Brazil
http://lattes.cnpq.br/1006353889898512

Juliana Carla Serafim da Silva
Universidade de Pernambuco – UPE, Institute of Biological Sciences – Universidade de Pernambuco – ICB/UPE, Brazil
http://lattes.cnpq.br/5224553521824035

Carlos Ralph Batista Lins
Fundação Oswaldo Cruz – Aggeu Magalhães Institute / IAM-PE, Brazil
http://lattes.cnpq.br/9093726620207386

Rhaíssa Evelyn Moraes Ramos
Fundação Oswaldo Cruz – Aggeu Magalhães Institute / IAM-PE, Brazil
http://lattes.cnpq.br/8779806332972683

Luiz Carlos Alves
Universidade Federal de Pernambuco – UFPE, Brazil
http://lattes.cnpq.br/4951638470777523

Fábio André Brayner dos Santos
Universidade Federal de Pernambuco – UFPE, Brazil
http://lattes.cnpq.br/7464633588638483

Fábio Lopes de Melo
Fundação Oswaldo Cruz – Aggeu Magalhães Institute / IAM-PE, Brazil
http://lattes.cnpq.br/8455111843250834

Bruno de Melo Carvalho
Universidade de Pernambuco – UPE, Institute of Biological Sciences – Universidade de Pernambuco – ICB/UPE, Brazil
http://lattes.cnpq.br/6293444890871058

Abstract: Introduction: Schistosomiasis is an infectious parasitic disease caused by worms of the genus *Schistosoma.* In Brazil, its greatest endemicity is in the state of Pernambuco. Currently, treatment is performed with Praziquantel (PZQ), also the only drug of choice. However, as PZQ-resistant strains are expanding rapidly, the development of new therapeutic approaches is extremely important. Quercetin (QUER) is a flavonoid widely distributed in nature that provides the body with several benefits, such as: potent antioxidant, anti-inflammatory, immunomodulatory, gastro and hepatoprotective. This study aims to evaluate the therapeutic effects of PZQ and QUER isolated and/or associated in schistosomiasis in in vitro experimental models. **Methodology:** Twenty male Swiss mice from the vivarium of the Aggeu Magalhães Institution, 28 days old, were kept under a controlled cycle (12h light/12h dark) with diet and water ad libitum. The animals were infected by 125 cercariae/mL (per individual) of *S. mansoni* of the LE strain from the Reference Service in Schistosomiasis of the IAM. The adult worms were removed through cardiac perfusion, after 45 days, then maintained under RPMI 1640 medium supplemented with antibiotics. Subsequently, the plates were incubated at 37°C for 2 hours in an oven at 5% CO2 for adaptation, being monitored every 24 hours and observed under an inverted optical microscope. The groups were divided into: I) DMSO (negative control; 0.5 µg/mL); II) PZQ (positive control; 0.5 µg/mL); III) QUER (15µg/mL); IV) PZQ+QUER (0.5 µg/mL +15 µg/mL), different concentrations were tested, totaling a final volume of 2mL. This work was evaluated and approved by CEUA / IAM, under protocol number: 159/2020. **Results:** Groups III and IV showed 100% worm mortality in 24h; total absence of motor activity (score 0) and marked tegumentary damage. **Conclusion:** Quercetin

showed promising schistosomicidal activity against adult worms of S. mansoni, due to its direct action on the pathogen alone and/or associated with praziquantel.
Keywords: Quercetin. Praziquantel. Schistosoma mansoni. Polyphenolic compounds. Molecular biology.

INTRODUCTION

Schistosomiasis is an infectious parasitic disease caused by trematodes of the genus *Schistosoma*, which occurs endemically in several countries in the tropical and subtropical regions of the planet (SILVA; CHIEFFI; CARRILHO, 2005). According to the World Health Organization, it is estimated that about 240 million people are infected and approximately 120 million suffer from some type of associated clinical morbidity, characterizing an important public health problem (W.H.O., 2017).

In Brazil, about 1.5 million people live in endemic regions, in which the Southeast and Northeast regions are the most affected. existing municipalities, cases were registered in 102 of these, with the majority located in the Zona da Mata and Litoral regions (BRAZIL, 2014). However, new areas of transmission were found in regions close to the coast and in the Metropolitan Region of Recife, suggesting that the disease is in active expansion, with individuals infected by one of the different species of *Schistosoma* sp. (BARBOSA et al.,2015).

Currently, Praziquantel is the only drug administered in the treatment of schistosomiasis, this appeared more than four decades ago, however, it does not act on all evolutionary forms of the pathogen and does not prevent reinfections, so there is already evidence in the literature of the appearance of strains resistant as a result of their large-scale use in schistosomiasis control programs (DOENHOFF; CIOLI; UTZINGER, 2008; GREENBERG, 2013). These factors, combined with the difficulty of treating children and adults with swallowing problems, highlight the urgent need to discover new compounds for the treatment of schistosomiasis (SILVA, et al. 2021; KOKALIARIS, et al., 2022). There are many studies for this purpose, in which new natural compounds are tested (CARLOTO, ACM et al., 2019; SHARMA, S et al., 2007; MAHMOUD, MR.; EL-ABHAR, HS; SALEH, S, 2002 ; DE MELO et al., NI 2011) and drug association (BOTROS, S, BENNET, JL, 2007; SILVA et al., 2021). However, to date, no drug capable of replacing praziquantel in the treatment of schistosomiasis has been found.

Quercetin is a flavonoid chemically called 3,5,7,3'-4' pentahydroxyflavone, with molecular formula C15H10O7. It is presented in a golden-yellow color, with a density of 1.8 g/cm^3. It has a pungent characteristic and aromatic odor, similar to oregano, as well as low solubility in water. Commonly found in apples, onions and capers, it has important activities such as: potent antioxidant, antihypertensive, anti-inflammatory, antimicrobial and antiprotozoal, in addition to anthelmintic action (CARLOTO, ACM et al., 2019). In addition, quercetin was identified as a selective inhibitor of the oxidized nicotinamide adenine dinucleotide (NAD+) catabolic enzyme of S. mansoni (SmNACE), located in the tegument of the adult parasite and presumably involved in its survival by evading the host's immune system (CUNHA, NL et al., 2012). Furthermore, Kuhn, I (2010) observed that this mechanism has been attributed to two main processes: the induction of apoptosis of subpopulations of naive T lymphocytes induced by NAD+, and the formation of cyclic ADP-ribose, a messenger of mobilization of Ca2+ produced by NAD+ which is involved in many cellular pathways including chemotaxis of neutrophils and dendritic cells.

In line with the literature, flavonoids have evidenced anti-inflammatory and immunomodulatory functions, in which they constitute an important source in their use for the clinical treatment of numerous diseases. Thus, the present study aims to evaluate the therapeutic effects of praziquantel and quercetin alone and/or associated in adult worms of S. mansoni in in vitro experimentation.

METHODOLOGY

ANIMAL MODEL

Animals were identified by coding to minimize risk of bias by observers. The study was carried out with 4-week-old Swiss mice from the vivarium of the Instituto Aggeu Magalhães (IAM) of the Oswaldo Cruz Foundation (FIOCRUZ) and allocated in a controlled environment with a light:dark cycle (12h/12h), temperature controlled at about 22 °C, specific feed for rodents and water ad libitum.

ASSESSMENT OF SURVIVAL OF ADULT WORMS: *S.MANSONI*

Twenty mice previously infected (after 45 days) were used by immersion of the tail in a solution with approximately 125 cercariae/ mL of S. mansoni strain LE (Luis Evangelista). perfusion through the portal venous system to remove infective parasites. The worms were recovered in sterile cups, then transferred to the supplemented RPMI 1640 medium, where they were washed in the same medium and then quantified.

CRITERIA FOR EVALUATING THE SCHISTOSOMICIDAL EFFECT

Two pairs of adult worms were placed in each well of a 24-well culture plate containing RPMI 1640 medium supplemented with antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL; pH=7.5) and 10% fetal serum bovine (FBS), and submitted to different concentrations of quercetin (SIGMA-ALDRICH®) and praziquantel (SIGMA-ALDRICH®), isolated and associated to evaluate the viability of specimens of *S. mansoni*. Then, the plates were incubated at 37°C for 2 h in an oven at 5% CO2 for adaptation. The negative control contained DMSO (dimethyl sulfoxide PA) (SIGMA-ALDRICH®) together with the supplemented RPMI medium and was formed by adult worms incubated in the suspension vehicle (same volume). Praziquantel® (PZQ), being the positive control, was tested at 0.5 µg/mL. Afterwards, it was added to the medium in a complementary amount so that all wells obtained a final volume of 2mL. This test was performed in triplicate.

MOTILITY AND SURVIVAL

The motility and survival of adult S. mansoni worms were evaluated with the aid of an inverted microscope (Leica Microsystems, DM IL Wetzlar, Germany) at intervals of 24, 48, 72, 96 and 120 h of incubation and monitored in the Laboratory of Immunology (Aggeu Magalhães Institute) every 24 hours through inverted microscope, observing morphology, mortality and mobility proposed by Ramizes et al. (2007) adapted to the experiments, following the assigned scores: 0- for dead worms with total absence of movement; 1- very slow worms and occasional movements of the extremities or bowel (small sporadic spasms); 2-worms with head and tail movement, but with reduced mobility; 3- active worms, showing normal morphology and movement. Afterwards, the material was removed from the culture plate and added to the Karnovsky fixative solution and sent for scanning electron microscopy, for further observation of the structure of the parasite.

SCANNING ELECTRON MICROSCOPY – S.E.V.

With the conclusion of the observation, the groups with 100% mortality in 24h had the parasites removed from the plate, and then washed in a buffer solution (0.1M sodium cacodylate, pH 7.2), placed in eppendorf tubes for later fixation in 0.1M sodium cacodylate buffer, 2.5% glutaraldehyde and 4% PFA. Then, post-fixation was performed in 1% osmium tetroxide in 0.1M cacodylate buffer for 90 minutes protected from light. Afterwards, three washes were performed in 0.1M cacodylate buffer for subsequent dehydration of the worm specimens, using an increasing sequence of ethanol at 30%, 50%, 70%, 90% and 03 times at 100% for 10 minutes each wash. After the process of dehydration of the samples, the critical point phase was performed for the replacement of ethanol by carbon dioxide. The equipment used was the Critical Point Dryer, CPD 030, BAL-TEC), Technological Platforms Nucleus of the Aggeu Magalhães Institute – FIOCRUZ/PE. After this step, the dry material was removed from the equipment and sent to be mounted on metal stubs using double-sided carbon tape. Finally, the samples were metallized in an atmosphere of a thin layer of gold, for visualization and analysis in the scanning electron microscope (CARL ZEISS EVO MA10, ZEISS), from the Laboratory of Immunopathology Keizo Asami – LIKA/UFPE.

STATISTICAL ANALYSIS

Numerical data were analyzed using Graphpad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA) and are expressed as mean ± standard deviation (SD). Statistical differences were determined using one-way analysis of variance (ANOVA) in conjunction with Turkey's test for single-step multiple comparisons. Significant differences were defined as $p < 0.05$.

ETHICAL CONSIDERATIONS

The project was approved by the Institute's Ethics Committee for the Use of Animals: Instituto de Pesquisas Aggeu Magalhães/ Fundação Oswaldo Cruz (CEUA/IAM), filed under the number 159/2020 valid until February 2023.

RESULTS

IN VITRO EFFECTS OF QUERCETIN ON ADULT WORMS OF *S.MANSONI*

Table 1 expresses the motility results of the quercetin schistosomicidal assay against the adult worms of *S.mansoni* after 24 hours of incubation. The results show that quercetin (QUER: 15 µg/ml) alone was capable of preventing the motility of adult worms (score 0), as well as when associated with praziquantel (PZQ: 0.5 µg/ml +QUER: 15 µg/ml) causing the same effect. In the negative control group (DMSO: 0.5 µg/ml) 100% of the worms remained alive during the monitoring period (score 3).

GROUPS	24h	48h	72h
P1)DMSO (negative control)	100% V Score 3	100 % V Score 3	100 % V Score 3
P2)PZQ (positive control)	75 % M; 25% V Score 1	100% M Score 0	-
P3)QUER	100% M Score 0	-	-
P4)PZQ+QUER	100% M Score 0	-	-

Scoring Criteria: ***Score 3***- active worms with normal body movements**;** ***Score 2***- slow worms with delayed body movements, may only show movements at the extremities of the anterior and posterior region; ***Score 1***- very slow worms, with occasional movement of the extremities (head and tail) or intestine, but with reduced mobility (sporadic small spasms); ***Score 0***- dead worms with total absence of movement **(Ramizes et al., 2007; Silva et al., 2018).**

Table 1 - Motility score of adult worms from *S. mansoni*, treated with Praziquantel (PQZ-0.5 µg/ml) and quercetin (QUER15 µg/ml) every 24 h of incubation

ULTRASTRUCTURAL ANALYSIS OF INTEGUMENTARY CHANGES IN WORMS OF: *S. MANSONI*

Figure 1A shows the preserved morphology of the adult worms incubated with the RPMI 1640 medium supplemented with DMSO (negative control) and figures 1B and 1C, the characteristics of the in vitro treatment with praziquantel. The effect of PZQ on male worms of S. mansoni after 24 h of exposure showed a worm with dorsoventral curvature, in the anterior region desquamation was observed, while in the median - dorsal region areas with extensive swelling, displacement and agglomeration of tubercles were clearly evident, in some tubercles causing exposure of the subtegumentary tissue and their eruption (Figures 1B-1C). Figure 1D shows an adult male worm treated with 15 μg/ml of QUER for 24 hours and indicates severe tegumentary damage such as desquamation, tegument erosion, destruction of papillae and tubercles, plus the presence of blisters along the entire tegument of the parasite (figure 1E). Figures 1F and 1G show the in vitro exposure of the effects of PZQ (0.5 μg/ml)+QUER (15 μg/ml) on the male worm, highlighting desquamation, severe loss of spicules, total destruction of tubercles, integumentary eruptions, plus of severe damage to the oral (I) and ventral (II) suckers (contorted).

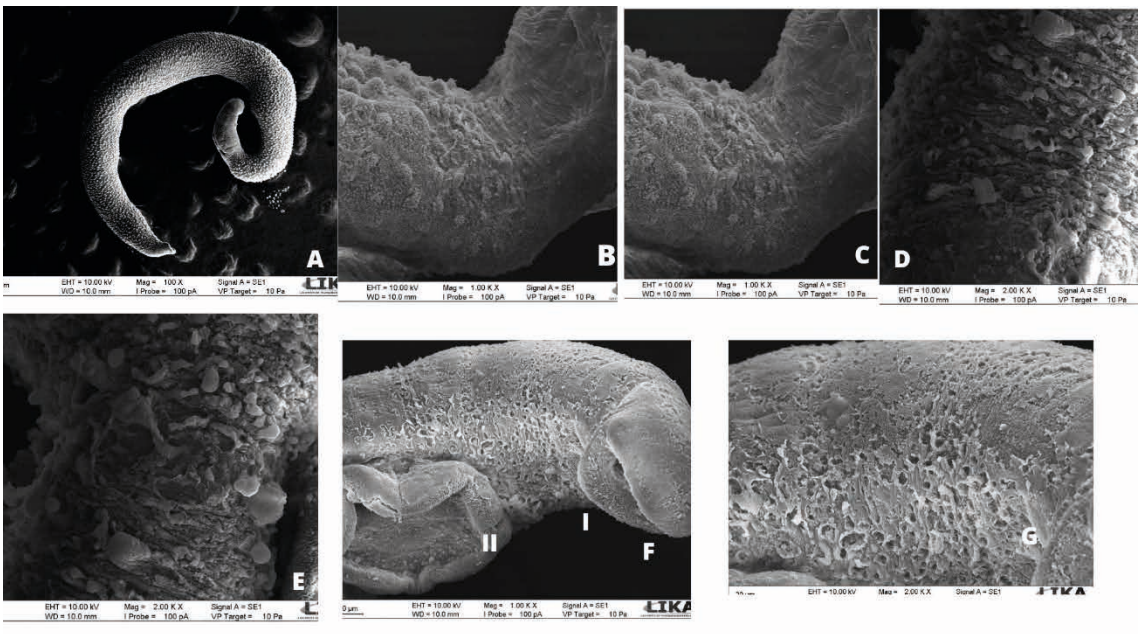

FIGURE 1: Electron micrographs of *S. mansoni* from the DMSO group – negative control (A). (B-C). Electromicrographs of S. mansoni from the positive control group – praziquantel (D-E) Group treated with QUER (15 μg/ml). (F-G) PZQ (0.5 μg/ml) + QUER (15 μg/ml) treated group. I) oral suction cup. II) ventral sucker

DISCUSSION

The search for new bioactive compounds that can be used as an antiparasitic for neglected diseases has received greater attention due to the limited scientific and governmental investments to remedy these problems (WENIGER et al., 2006; BOU et al., 2014; EFFERTH et al., 2015). Several in vitro studies were performed to search for new active compounds against species of:

Schistosoma sp. (CASTRO et al., 2013; 2015; MORAES, 2015; YAO et al., 2016). These desires meet the initiative to discover new schistosomicidal compounds demanded by the Special Program for Research and Training in Tropical Diseases (NWAKA & HUDSON, 2006).

The outermost membrane of male worms (tegument) of the *S. mansoni* proved to be an important target in the development of new schistosomicidal drugs, taking into account that the integument makes the first contact with the drugs (MELMAN et al., 2009; SILVA et al., 2021). Among the various vital functions carried out by the integument, important functions in the different metabolic processes of the worm stand out, such as: development, absorption of nutrients, excretion, metabolization of lipids and cholesterol, synthesis of some proteins, immune modulation, playing a fundamental role in the protection against the attack of the host's immune system (SKELLY, PJ et al., 2014; SOTILLO, J. et al., 2015). Analyzes observed through S.E.V revealed severe and progressive tegumentary changes that occurred after exposure to quercetin alone (WER: 15 µg/ml) and/or associated with praziquantel (PZQ: 0.5 µg/ml +QUER: 15 µg/ml) within 24 hours. In this study, the results obtained in vitro showed that QUER was active against adult worms of *S. mansoni.* These integumentary alterations in the worms will probably not allow their tissue repair, the damage was highly extensive and may also show antigens on the surface of the worm, this profile both favors the host's immune response and complements the activity of the drug praziquantel (SOTILLO J et al., 2015). Some in vitro studies on natural products have reported that male worms of *S. mansoni* are often more susceptible than female worms (DE MELO et al., 2011; SANDERSON et al., 2002). However, in this study, all QUER concentrations studied in vitro showed reduced worm motility, worm folding, severe tegument damage, including loss of spicules, destruction of tubercles, depression, desquamation, tegumentary erosion and formation of blisters on adult worms.

Some studies carried out using flavonoid polyphenols, including quercetin, confer antioxidant potential by reducing free radicals present in cells, which can lead to beneficial changes in metabolic pathways such as protein and enzyme synthesis, generally improving the important homeostatic system of the body. organism (KANDASWAMI, C; MIDDLETON, EJR, 1994; BOOTS, AW et al., 2008; UEDA, S et al., 2002).

With regard to PZQ, the drug is effective on species that infect humans, causing integumentary alterations, characterized by vacuolation, peeling of the integument, tubercles and spines, increased permeability of the membrane and influx of Ca2+, causing excessive muscle contraction, followed by paralysis and death of the *S. mansoni* (XIAO et al, 2007). Despite several works reporting a good schistosomicidal activity over decades, there are many efforts to deepen the mechanism of action of this drug. The tegumentary alterations observed in the S.E.V. corroborate our results.

CONCLUSION

In this study, the tests carried out in vitro with the polyphenolic compound quercetin showed a potential effect on the motility and mortality of adult worms of *Schistosoma mansoni,* in addition to causing relevant ultrastructural alterations in the parasite's integument.

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