Methanol extract from *Psidium sartorianum* (arrayán) berries induces *in vitro* damage on *Taenia crassiceps* WFU cysticerci

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Abstract

*Taenia crassiceps* is an asexually proliferating cestode, may give rise to a high number of cysts in the experimental conditions and become a suitable model organism for drug screening in the absence *T. solium* cysts, responsible for human and swine cysticercosis. Although there is no conclusive evidence on resistant cestodes, alternative treatments are relevant for the human and animal health. A methanol extract from *Psidium sartorianum* (O. Berg) Nied. Berries (AME) was used to evaluate anti-parasitic effect on *T. crassiceps* cysticerci. Metacestodes were exposed to 25, 50 or 100 mg/ml of AME for up to 72 h. The tissue impairment at the tegumentary and subtegmentary levels was seen after 2.5 h of treatment with 100 mg/ml or 24 h after when treated with 50 mg/ml of AME. Here we report denuded microtriches and blebbing on the surface of cysts. The results indicate that high concentrations of AME must be employed in order to achieve relevant tissue damage in the short time. Therefore active components must be characterized for their anti-parasitic effect.

Keywords: Plant extracts, *Psidium sartorianum*, parasitic helminth, *Taenia crassiceps* WFU, ultrastructural damage

1. Introduction

Human and swine parasitic cestode *Taenia solium* is one of the six helminths, which cause the neglected tropical diseases (NTDs). State-of-the art given by WHO/TDR in 2012 for NTDs points out also that the social and economic conditions, which favor the maintenance of the life-cycle of *T. solium*, are not likely to be changed in the short term [1]. Thereby, control of this helminthiasis requires tools such as pig vaccination and concomitant chemotherapy based on known or developing drugs. ABZ is widely used in mass drug administration (MDA) by the WHO program to control the soil-transmitted helminths and lymphatic filariasis, as well as large-scale initiative for cestodes. Human *Taenia* species are spread mainly in Latin America, Asia and Africa, affecting around 40 million people [2]. Infection caused by *T. solium* is important due to neurocysticercosis. In addition, recent cases of human infected by *T. asiatica* [3], *T. saginata* [4], *T. crassiceps* [5-7] and *T. multiceps* [8-10] should not be underestimated in poverty conditions or globalized world. Both, humans and animals infected with larval stages of taeniids and treated with either ABZ or praziquantel have shown variable responses due to differences in drug absorption after oral administration [11-13]. Although there are no reports related to ABZ resistance in cestodes, positive outcome of treated cysticercosis also depends on the number, type and location of the cysts [14, 15].

Plant extracts and its purified active components may become relevant natural substitutes of chemically designed drugs and optimize existing methods to combat infectious diseases [16]. *Psidium* species are commonly known as “guava” and have been used in worldwide traditional medicine. Oil or water extract from leaves, bark, roots or fruits from the different species of *Psidium* have been recommended to treat a number of human and animal diseases. Extract or purified active components from *Psidium* sp. are known to be effective against the pathogenic fungi, bacteria, protozoa, and also have an anti-oxidant, anti-inflammatory, cardio-active, hepato-protective and anti-diabetic properties [17]. *Taenia crassiceps* WFU is an asexually proliferating cestode that grows in the peritoneal cavity of experimentally infected mice, and may give rise to many hundreds of cysts after serial passages [18]. This model has been frequently used for *in vitro* drug screening [19, 20], in substitution of *T. solium* cysticerci, which must be found and then resected from the infected...
porcine host. The present study reports damage in the
tegment of T. crassiceps WFU cysticerci, for the first time, the
blebbing together with denuded subsequent layers, under in
vitro effect of methanol extract from P. sartorianum.

2. Material and Methods

2.1 Cysticerci of Taenia crassiceps WFU

Taenia crassiceps WFU cysticerci were maintained in female
BALB/c mice by intra-peritoneal passages [21]. Parasites were
obtained from the peritoneum of infected host, after cervical
dislocation. For drug assay six cysts with 2 mm long
invaginated scolex and transparent contractile bladder were
selected. Cysts were rinsed in PBS containing 0.2% antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA) and
sterilized with 0.5% peptone (Bioxon, Mexico) and 0.2% antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA) for the subsequent steps.

2.2 Psidium sartorianum (arrayán) methanol extracts
(AME)

Psidium sartorianum “arrayán” is a native to México and
Central America myrtaceae plant, and is used in the
traditional medicine to treat diarrhea, dysentery, scabies and
intestinal parasites. The arrayán tree can reach a height of 20
m in the warm and temperate climates, 0-1600 m.a.s.l. The
ripening season occurs mainly from September to March.

2.3 Treatment of T. crassiceps cysts with AME, ABZ or
nitazoxanide (NTZ)

Six cysts were pre-incubated in 2 ml of Hank’s Balanced Salt
Solution (HBSS; Sigma-Aldrich, MO, USA) complemented with 0.5% of peptone (Bioxon, Mexico) and 0.2% antibiotic-
antimycotic for 24 h at 37 °C in 5% CO2. AME was diluted to
0.5 g/ml of HBSS, adjusted to pH 7.4 and insoluble particles
eliminated by centrifugation for 2 min at 2500 rpm/min.
Cysticerci of T. crassiceps were treated with the AME at 25,
50 or 100 mg/ml. Anti-Helminthics ABZ (~97%) and NTZ were kindly donated by Dr. Rafael Castillo, Facultad de Química, UNAM, and
used as a damage-control drugs. ABZ and NTZ were dissolved in 0.05% DMSO (Sigma-Aldrich, St. Louis, MO,
USA) in HBSS at concentration of 0.08 µg/ml or 0.13µg/ml,
respectively. Viability of the cysts based on the motility and
morphometric parameters, was monitored by light microscopy
between 2-72 h post-administration. Experiments were carried
out in triplicate. Procedures were performed in accordance
with the guidelines established by the Comité de Ética e
Investigación de la Facultad de Medicina, UNAM, and
animals were manipulated according to the Mexican Official

2.4 Transmission (TEM) and scanning electron
microscopy (SEM)

Samples were prepared for TEM and SEM [22]. Briefly,
parasites were rinsed in HBSS, fixed for 24 h in Karnovsky
solution [24], washed in 0.15 M cacodylate buffer for further
24 h and post-fixed in 1% osmium tetroxide during 2 h at 4°C.
Fixed parasites were included in poly form (Polysciences,
Warrington, PA, USA) 1 µm thick sections were stained with
0.5% toluidine and examined by light microscopy [23].

3. Results

3.1 Effect of AME on cysts viability

Taenia crassiceps WFU cysticerci showed 90% evagination
after one-day pre-incubation in supplemented HBSS. Both
untreated or treated only with 0.05% DMSO parasites,
showed characteristic bladder contractions and evagination-
invagination scolex movements during 72 h of experimental
period. Under light microscopy, cysticerci became completely
paralyzed after 2.5 h of treatment with 100 mg/ml of AME.
In the presence of 50 mg/ml of AME, movements of 95% of
cysts were reduced after 2.5 h and were absent in 100% of
taenid cysts after 24 h of incubation. Treatments with 25
mg/ml of extract, as well as 0.08 µg/ml of ABZ or 0.13 µg/ml
of NTZ, had slowed the movement of all parasites during 72 h
of assay.

3.2 SEM and TEM findings

Cysticerci incubated in HBSS, as well as in the solution
supplemented with DMSO only, had an intact tegument and
microtriches seen by SEM (Fig. 1A and B). Microvilli and
tegment with dense granules and mitochondria (Fig. 1C) and
parenchymal tissue with organized myofibrils (Fig. 1D) were
also seen by TEM. After 2.5 h of incubation with 100 mg/ml
of AME, randomly selected and submitted to TEM cysts
showed blebs and collapsed tegument microtriches (Fig. 2A
and B). Cysts under TEM had a brush border partially
substituted by blebs of different size, protruding from the
membrane surface (Fig. 2C and D). Blebs were also seen in
the close proximity of an apparently intact microtriches (Fig.
2C). The lack of tegumentary organization, a swollen and
thickened basal matrix enclosing the parenchymal tissue, as
well as dispersed mitochondria without typical crests, were
seen in the Fig. 2D. Bundles of myofibers were normally
situates below the basal matrix, while compacted glycogen
particles were organized in sacs in the proximity of tegumentary
myocytes in untreated cysticerci (Fig. 3A). Distended and
swollen sacs with less compacted material were found in the
parenchyma of cysts treated with 100 mg/ml of AME (Fig.
3B). Cysts incubated for 24 h in 50 mg/ml of AME presented
damaged tegument sections as well, characterized by a partial
loss of microtriches and blebbing of the membranous material on the surface (not shown). Cysts treated with 0.13 µg/ml NTZ or 0.08 µg/ml of ABZ and examined by light microscopy had an apparently intact tegument with normal microvilli and parenchymal organization (not shown).

Fig 1: Intact tegument (A) and microtriches (B) of untreated *T. crassiceps* WFU cysts seen by SEM, and dense granules together with microtriches in distal cytoplasm (C) and organized longitudinal myofibrils (D; double head arrow) documented by TEM. T, tegument; P, parenchyma; mt, microtriches.

Fig 2: Scanned micrographs of cysts surface after treatment with 100 mg/ml AME with partially obliterated and balloon shaped tegumentary microtriches (A; arrow) and bundles of protruded myofibrils in denuded tegument (B; double head arrow). Electron micrographs treated with AME cysts showing apparently normal microtriches intercalated with disarranged distal cytoplasm, swollen mitochondria and blebbing surface (C; arrowhead). Tissue fragment (D) with absent tegument and dispersed above basal membrane (filled arrow) mitochondria (double head arrow) and blebs (arrowhead). T, tegument; P, parenchyma; mt, microtriches.
Similar to other traditional remedies, AME may contain more sartorianum content of vitamin C [28]. Complete chemical definition and vesicular fluid and the tegument damage with a parenchymal cysticerci resulted in slowed contractions, blurred aspect of microscopic examination, exposed to AME completely rigid also at physiological temperature. At calcified individuals slow down their movements or are scolex intussusception movements. Dying or partially contractions were considered as viability criterions, including *in vitro* with apparently similar in scolex size were selected for phenotypically similar individuals. After extraction, cysts experimentally infected mice, yielding in a hundreds of and therefore, become a suitable for drug screening model *Mesocricetus auratus* tapeworms in experimental hamsters (*T. crassiceps in vivo*). A transparent bladder and the body and pathogenic fungi [27], AME obtained from *P. sartorianum* is now in process of patenting by UNAM. Similar to other traditional remedies, AME may contain more than one compound with therapeutic values; known for *P. sartorianum* berries are tannins, phytic acid and a high content of vitamin C [28]. Complete chemical definition and lack of rigorous toxicity testing may complicate any patenting process; however efforts to evaluate and develop botanical preparations with a medicinal value should not be diminished [29].

The absence of helmint-cell lines, difficulties for *in vitro* helminthes culturing, as well as lack of complete egg-to-egg cycle, are considered a major problem for drug screening [30]. The continuous maintenance of *T. crassiceps in vivo* may represent a good whole-organism-based screening helminthiasis model. Besides developing metacestodes in mice, a scolex-presenting WFU strain may give rise to adult tapeworms in experimental hamsters (*Mesocricetus auratus*) and therefore, become a suitable for drug screening model well. Cysticerci proliferate in the peritoneum of the experimentally infected mice, yielding in a hundreds of phenotypically similar individuals. After extraction, cysts with apparently similar in scolex size were selected for *in vitro* AME treatment. A transparent bladder and the body contractions were considered as viability criterions, including scolex intussusception movements. Dying or partially calcified individuals slow down their movements or are completely rigid also at physiological temperature. At microscopic examination, exposed to AME *T. crassiceps* cysticerci resulted in slowed contractions, blurred aspect of vesicular fluid and the tegument damage with a parenchymal disorganization. After 2.5 h of treatment with 100 mg/ml of AME, parasites were dead. Anti-helminthics such are ABZ or ABZ-sulfoxide, NTZ or tizoxanide are known to cause death linked to the relevant tissue damage of these metacestodes [31]. While those authors have used a multiple doses for *in vitro* cyst’s treatment, only a single dose of ABZ or NTZ was used during 72 h of our experimental design, which apparently did not affect the tegument integrity, when compared to the single dose of AME. Although drug-related damage has been documented for *T. crassiceps*, this parasite has high proliferation properties and, any regenerative loci situated on the tegument, may give rise to the new buds [32, 33]. A deeply demuded parasite surface, with a damage seen at the subtegumentary level and relevant membranous blebbing, caused by the highest concentration of AME, suggest good anti-helminthic effect on this experimental model. In the absence of 100% efficacy of existing anti-helminthic drugs, AME must show also potent cysticidal effect on *T. solium* cysts, which lack of asexual proliferation. *Taenia solium* metacestodes are expected to present similar to *T. crassiceps* damage, if cultured in the presence of AME, whose effective concentration must be previously determined. Moreover, active components of AME must be characterized in the future in order to reduce high concentrations of extract. *In vivo* evaluation of cysticidal properties of arrayán extract represents the most important challenge in porcine cesticercosis. Mexican backyard pigs are heavily exposed to the *T. solium* eggs, and 3-25% of rural porcine population is reported as positive for cesticercosis [33]. Treatment of infected *T. solium* pigs is based on oxfendazole, but also development of TSOL18 [34] and S3Pvac [35] vaccines to control this zoonotic disease in concomitant way. Other *Psidium* species, such as *Psidium guajava* commonly known as “guava”, can be a part of the natural diet in omnivorous animals from *Suinae* family [36]. The hog industry in some pork-meat demanding countries, use a “guava” to treat diarrheic diseases [37, 38]. If AME cysticidal effect on *T. solium* cysts is maintained also *in vivo*, then pigs fed with processed arrayán, may have a reduced risk of cesticercosis. This strategy together with a better sanitation and health education may give, as states WHO, a promising result on eradication taeniasis-cesticercosis.

4. Discussion

Anti-helminthic compounds are known to have a partial efficacy due to the: a) applications of single-dose treatment, b) grade of endemicity of certain parasites, c) a low bioavailability of the used drug and, c) eventual development of resistance toward the treatment. Recent politics in health care have been emphasizing on the development of alternative drugs, especially in and for the countries whose population also relies on the traditional medicine. Mexican plant locally called “arrayán” is a member of the Myrtaceae family and belongs to the compendium of the worldwide medicinal plants. Based on anti-parasitic effect on the *Hymenolepis nana* and pathogenic fungi [27], AME obtained from *P. sartorianum* is now in process of patenting by UNAM. Similar to other traditional remedies, AME may contain more than one compound with therapeutic values; known for *P. sartorianum* berries are tannins, phytic acid and a high content of vitamin C [28]. Complete chemical definition and lack of rigorous toxicity testing may complicate any patenting process; however efforts to evaluate and develop botanical preparations with a medicinal value should not be diminished [29].

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5. Conclusions
A myrtaceae plant *Psidium sartorianum* has been used to evaluate cysticidal effect on *Taenia crassiceps*. Metacestodes of this Taeniidae parasite have been used as a model organism for anthelmintic drug screening. Methanol extract obtained from *P. sartorianum* at concentration of 100 mg/ml resulted in the death of *T. crassiceps* after 2.5 h of *in vitro* incubation. Scanning and transmission electron microscopy revealed an extensive tegumentary and sub-tegumentary damage in tissue organization of parasite. The cystidial components of AME and *in vitro*/*in vivo* assays on cestodes, may provide an alternative anti-helminthic treatment.

5.1 Conflict of interest
The authors declare no conflict of interest.

6. Acknowledgements
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7. References
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