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ANTI-CESTODAL EFFECT OF *CALOTROPIS PROCERA* LEAF EXTRACT ON EXPERIMENTAL *HYMENOLEPIS NANA* INFECTION

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Abstract

Hymenolepis nana is the most widely recognized cause for cestodal helminthic infections worldwide. Infection prevalence rate increases in tropical areas especially among children. Praziquantel (PZQ) is the drug of choice; nonetheless, several instances of treatment resistance have been reported. This study evaluated the effect of crude aqueous extract *Calotropis procera* (CAE) and crude methanolic extract (CME) leaves, separately, and in combination with half dose of PZQ on *H. nana* in infected albino mice, in comparison to full-dose PZQ. Albino mice (180) were divided into two main groups to evaluate the effect on adult and cysticercoid stages. Each group comprised 6 sub-groups: infected not-treated, infected treated with PZQ, CAE, CME, PZQ+CAE and PZQ+CME. Results showed significant reduction of worm burden, total egg output and cysticercoids in all PZQ treated groups either single or combination. As regards plant extracts, methanolic extract treated groups showed higher reduction rate of adult worm burden, total egg count and number of cysticercoids in comparison with aqueous extract group. Adults showed marked tegumental changes and abnormalities with all treatments, but, more rapid with praziquantel. Also, both treatments and their combinations caused significant reduction in histopathological cysticercoid stage count.

Key words: *Hymenolepis nana*, Praziquantel, *Calotropis procera*, Scanning Electron Microscope

Introduction

Hymenolepis nana and *H. diminuta* are globally widespread zoonotic cestodes, but endemic to Asia, Southern and Eastern Europe, Central and South America, and Africa (Thompson, 2015). In tropical and subtropical crowded areas of poor sanitary conditions, with high percentage of children was susceptible to infection (Al-Mekhlafi, 2020). Human can be infected directly through the ingestion of *H. nana* eggs in contaminated food or drinks, or accidently by ingesting fleas and flour-eating beetles containing *H. nana* cysticercoid. Humans and rats are *H. nana* definitive hosts with worms inhabiting small intestine (Cox *et al*, 2005).

Praziquatil is the current treatment of choice, with a single dose of 25mg/kg to be repeated after one week. However, its resistance indicated need for alternatives treatment (Rashed *et al*, 2018; Mohammed *et al*, 2023).

Nowadays, herbal extracts are worldwide used as natural-safe anthelminthic (El-Wakil *et al*, 2022; El‑Sayad *et al*, 2023; Ghareeb *et al*, 2023). The medicinal plants contain cysteine proteinases (Sharma *et al,* 2012). *Calotropis procera* has powerful *in vitro* and *in vivo* anthelminthic effects (Mansur *et al,* 2014). Also, its leaves have an antidote for snake bite, sinus fistula, rheumatism, mumps, burn injuries, and body pain (Mehta and Sashindran, 2011), larvicidal activities against house fly (Morsy *et al,* 2001), and mosquito-vectors larvae (Elimam *et al,* 2009)

This study aimed to evaluate the effect of *Calotropis procera* leaves aqueous and methanolic extracts compared to praziquatil®, (PZQ) or combined with both *Calotropis* extracts on *H. nana* worms in laboratory-infected mice.

Material and Methods

Experimental animals: Swiss Albino mice male (180) parasitic-free of age 6-8 weeks and weight between 20-25gm were used.

Experimental infection with *H. nana* eggs: Stool samples were collected from Abo-Rish

Children Hospital infected patients and examined microscopically using direct wet smears for *H. nana* eggs. Heavily infected samples with more than 3eggs/field were used to infect mice. Egg viability was confirmed prior to infection by using Chausov's method (Chausov, 1964). Each mouse received a dose of about 200eggs orally by using an oesophageal syringe (Shady *et al*, 2014).

Regimens: 1- *C. procera* extract: Leaves were morning collected from trees in El-Orman Garden, Giza, and kindly identification by Mrs. Therese Labib, Consultant of Botany at the National Gene Bank. The prepared of crude methanolic extract (CME) and water extract (CAE) were orally given per mouse in a dose of 500mg/kg for 3 consecutive days. 2- PZQ tablets (Distocide®, EIPICO, 10th of Ramadan, Egypt) were given orally in a single dose of 25mg/kg by a small stainless steel oral cannula.

Experimental design: Mice were divided into two main groups. GI: 108 mice to assess the effect of the used extracts and PZQ on adult worms. 14th days post infection (P. I.), faecal pellets were collected and examined to confirm infection. *C. procera* leaves extracts was given on 3 consecutive days and PZQ was given in a single dose. Mice were sacrificed on 2hr, 1st, 3rd days post treatment. GII: consisted of 72 mice to assess the effect of extracts and PZQ on cysticercoid stages, then *C. procera* extracts treatment started on the 2nd day P.I. for three consecutive days, but PZQ was given in a single dose. Half of treated mice were sacrificed between 90 to 96hours P.I., the highest time of cysticercoids concentration (Campos *et al*, 1984). The second half of mice was left for 20th day P.I. for the adults' development**.**

Each group was subdivided into six subgroups of 6 mice each, as infected not treated (positive control), infected PZQ treated, infected CAE treated, infected CME treated, infected treated with PZQ & CAE and infected treated PZQ & CME (PZQ a half dose in each).

Parasitological assessment: Mice were anesthetized and killed by neck dislocation. The small intestines were dissected out, and cleaned with saline to get rid of all intestinal contents. Worms began to be free from intestinal mucosa after a few minutes, and the recovered worms were counted and gathered for microscopic study.

Egg output: Mice fecal pellets from each group were collected and centrifuging for about 2 minutes at a speed of 600rpm after being diluted in a specific amount of saline. Eggs per gram were expressed as egg counts. All samples were inspected on the same day to prevent desiccation of the eggs**.**

Egg viability: Egg count and viability were assessed using a vital staining with Acid fuchsine aqueous solution (Victorov *et al*, 2000). Stained smears were microscopically examined, living eggs were yellow in color while dead ones red or magenta.

Cysticercoid study: Mice were sacrificed on the 4th day post infection (P.I.) and small intestines were dissected out into small pieces, opened and carefully adjusted between two slides avoiding crushing cysticercoids.

SEM: Adults were collected on 2hr, 1st, 3rd day post treatment (P.T.) in tubes of 2% glutaraldehyde and 0.2M sodium cacodylate buffer, and processed for ultrastructural study using Environmental SEM (Inspect S: FEI, Holland).

Histopathological study: Intestines were dissected out on 4th day P.I., stained in hematoxyline and eosin and microscopically examined for pathological changes of cysticercoids, which were photomicrographed.

Statistical analysis: Data were collected, computerised and analysed using statistical package for the Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA). Data were given using mean ±standard deviation. Comparisons between quantitative variables were done using non-parametric Kruskal-Wallis and Mann-Whitney tests (Chan, 2003). P-values less than 0.05 were considered significant.

Ethical consideration: The study was approved by Cairo University (Approval no. CU,

III-F-22-18), which when with Helsinki Declarations (2008) on animals' model.

Results

The *C. procera* extracts treated infected mice showed a reduction in worm burden and total egg output on the 1st day after treatment with significant differences as compared to positive control. PZQ and combined treatment showed a reduction in worm burden and total egg output on 2 hours after treatment with significant differences as compared to positive control.

Examination of the stools at different times using Chausov's method, showed eggs with different colours due to viability degree. Viable eggs were not stained with a yellowish colour against a red background. Dead eggs took the stain a red colour, without the embryos six-hooked against a red background. Red-shelled eggs outer one was stained red with yellowish embryos, denoting the loss of viability as eggshell was fragile and permeable to stain but the embryo was still alive.

C. procera extracts treated mice showed a statistical increase in the percentage of red-shelled and dead eggs that started on the 3rd day after treatment when compared to infected control. But, PZQ and combined treated showed a significant increase in red-shelled and dead eggs 2 hours post-treatment (P.T.) as compared to positive control.

Ultrastructural changes of adults treated with PZQ and PZQ-extracts combinations showed severe tegumental damage within 2 hours P.T. as swelling of scolices, neck, segments and worm shrinkage with whitish vesicles, abnormal architecture and tegumental surfaces sloughing and worms complete eradication on 3rd day P.T.

Mice treated with *C. procera* CAE or CME showed gradual tegumental changes on 1st day P. T. as compared to positive control.

Viable egg was ovoid, with circle of hooks, between intestinal villi, or embedded at intestinal margin and cysticercoids were smaller and darker with an ill-defined circle of hooks in positive control. There was a significant reduction in cysticercoids number detected in intestines of positive control as co-mpared to treated ones.

There was a significant reduction of worm burden in PZQ and combined treatment as compared to positive control. But, total egg output showed a significant reduction in all treated group as compared to negative control. They showed a significant increase in red-shelled and dead eggs as compared to positive control.

Histopathological study on 4th day P.I.**:** The small intestinal sections of positive control showed multiple oval-shaped well-defined cysticercoids embedded within intestinal villi with submucosal infiltration of inflammatory lymphocytes and plasma cells. *C. procera* treated mice most cysticercoids were normal with well-defined shape, but few were ill-defined and darkly stained with few inflammatory cell infiltrations in submucosa. Most of cysticercoids were rarely detected in PZQ and combined treated mice as rare dark stained and rare restoration.

Details were given in tables (1, 2 & 3) and figures (1, 2, 3 & 4).

Table 1: Effect *C. procera* extracts and PZQ on *H. nana* burden and total egg output in mice 14-day P.I. at different time

	Worm burden (Mean ± SD)			Total Egg Output (Mean ± SD)		
	2hr	1st day	3rd day	2hr	1st day	3rd day
Control	9.5±5.21	8.67±2.42	6.33±1.86	1933.33±628.23	2640±480	2760±582.96
PZQ	0.33±0.52* (96.49%)	0.17±0.41* (98.08%)	0±0* (100%)	133.33±75.28* (93.10%)	80±65.73* (96.97%)	0±0* (100%)
CAE	6.5±3.27 (31.58%)	5.33±0.82* (38.46%)	2.67±1.37* (57.89%)	1866.66±413.11 (5.17%)	1800±178.88* (31.82%)	1800±178.88* (36.96%)
CME	6±1.09 (36.84%)	4,66±1.36* (46.15%)	2.33±1.21* (63.15%)	1800±456.07 (6.90%)	1666.66±516.39* (36.86%)	1600±357.77* (42%)
PZQ+CAE	1.17±0.75* (87.72%)	0.5±0.55* (94.23%)	0.33±0.52* (94.74%)	800±116.62* (58.62%)	640±247.87* (75.67%)	600±379.47* (78.26%)
PZQ+CME	1.33±0.52* (85.96%)	0.67±0.52* (92.31%)	0.33±0.52* (94.74%)	1000±252.98* (48.28%)	800±252.98* (69.70%)	500±134.16* (81.88%)

*P< 0.05 versus positive control.

Table 2: Effect *C. procera* extracts and PZQ on egg viability in mice treated 14-day P. I. at different times.

Mice group	Egg viability% 2h P.T			Egg viability% 1st day P.T			Egg viability% 3rd day P.T		
	Viable	Red shelled	Dead	Viable	Red shelled	Dead	Viable	Red shelled	Dead
Control	100	0	0	100	0	0	100	0	0
PZQ	37.5	25	37.5	0	50	50	0	0	100
CAE	89	6	5	72	16	12	61	25	14
CME	83	11	6	60	22	18	56	22	22
PZQ + CAE	69	12.5	18.5	53	16	31	34	33	33
PZQ + CME	70	10	20	25	25	50	20	30	50

Table 3: Effect *C. procera* extracts and PZQ on cysticercoids, worm burden, egg output & viability in mice treated 24 hours P.I.

Mice groups	Cysticercoids 4th day P.I.	Worms on 20th day P.I.	TEO on 20th day P.I.	Eggs viability on 20th day P.I.		
				Viable	Red-shelled	Dead
Control	9.67±3.39	8±2.76	1880±489.9	100	0	0
PZQ	0.33±0.52* (96.55%)	0.5±0.84* (93.75%)	80±65.73* (95.74%)	12.5	25	62.5
CAE	6±1.26* (38%)	6.33±1.97 (20%)	1333.33±500.67* (29%)	75	10	15
CME	5.33±1.21* (44.82%)	5.83±0.98 (27.08%)	1200±219.08* (36.17%)	76	8	16
PZQ+CAE	1.17±0.98* (87.93%)	1.33±1.21* (83.33%)	200±44.72* (89.36%)	35	15	50
PZQ+CME	0.67±0.82* (93.10%)	0.5±0.84* (93.75%)	80±65.73* (95.74%)	25	25	50

*P< 0.05 versus positive control.

Discussion

For many years control has entailed the use of just a single drug, praziquantel (at 40mg /kg body/wt), the only drug effective against the adult stage of all *Schistosoma* species (WHO, 2002). However, for prolonged use low cure rate and existence of PZQ resistance both in vivo and in vitro were reported in different endemic areas (Tesfiei *et al,* 2020).

In the present study, the effect of aqueous and methanolic extracts of *C. procera* leaves separately were tested on *H. nana* (dwarf tapeworm) infected albino mice, in combination with half dose of PZQ. The reduction in mice treated with CAE and CME (3rd day post-treatment) as regards worm burden were 57.89% and 63.15% and for total eggs/gm were 36.96% and 42%, respectively, as compared to control. This agreed with Shady *et al*. (2014), who found that the CAE of *Carica papaya* seeds on *H. nana* in albino mice caused significant reduction in the worm burden and in total eggs deposited on the 3rd day post-treatment. Also, Mansur *et al.* (2014) reported that cysteine proteinases in plants have *in vitro,* anti-cestodal action on young newly hatched scolices or mature adult worms of *H. diminuta* (rat tapeworm) and *H. microstoma* causing a significant motility ending worms' deaths. But, Mansur *et al.* (2016) *in vivo* the natural plant cysteine proteinases against *H. diminuta* gave neither significant differences on worm burden nor faecal egg output. This discrepancy could be due to the medicinal plants anti-parasitic activities or the solvent used in the extracted plant part, and the parasite-itself or concentrations or doses used

In the present study, mice treated with *C. procera* leaves extracts showed a significant difference in the number of viable eggs, red-shelled eggs and dead eggs starting from the 3rd day PT as compared to the control. This agreed with Shady *et al*, (2014), who reported that CAE of *C. papaya* seeds caused a significant difference in the number of viable eggs, red-shelled eggs, and dead eggs on the 3rd day P.T.

In the present study, the effect of PZQ caused significant reduction in number of adults and eggs output/gm within 2 hours PT. This agreed with Alhawiti *et al*. (2019), who reported that in PZQ (25mg/Kg) neither worms nor eggs were detected one day P.T. In the present study, PZQ (half dose) combined with *Calotropis* extracts showed a significant reduction against adults burden, and egg viability and/or eggs output/gm. This agreed with Rashed *et al*. (20180 and Thomas and Timson (2020), they reported the PZQ synergistic anthelminthic action together of medicinal plant leaves extracts, as a new combined treatment to minimise its

resistance and diminish adverse effects.

In the present study, mice treated with CAE and/or CME showed mild SEM changes at 2 hours PT, mild changes were 1st day PT, increased to marked changes on 3rd day PT. These were on tegument as vesiculations and oedema, swelling of scolices, loss of hooks and obliteration of suckers. These all agreed with Shady *et al*, (2014), who used *C. papaya* seed extract against *H. nana* adults within 2 hours PT with a maximum peak in 3 days PT.

The present PZQ-treated worms showed rapid morphological changes that appeared within 2 hours of treatment. This agreed with Beshay (2018), who showed that a single dose of PZQ (25mg/kg) caused severe tegumental damage, vesiculations with loss of normal morphology due to the release of endogenous Ca^{++} leading to spastic paralysis and vacuolations and tegumental rupture by leakage of glucose and amino acids.

In the present study, *H. nana* from mice treated with *C. procera* extracts and PZQ showed complete cuticle damage and worm death. The combined treatment induced synergistic activity against *H. nana* by incressing chloride ion conducted via worm muscle membrane causing hyperpolarizattion and reducing excitability ending in paralysis and death (Campelo *et al,* 2017).

In the present study, there was reduction in *H. nana* cysticercoids number on the 4th day post-infection, CAE and CME by 38% & 44.82% respectively. This agreed with Rashed *et al,* (2018) reporting a significant reduction in cysticercoids number on 4th day post-infection in mice treated with *Rosmarinus officinalis* oil. But, Shady *et al*, (2014) recorded no significant reduction in the number of cysticercoids, only a reduction in dimensions and morphological changes in mice treated with Cysteine proteinases derived from *C. papaya* seeds extract

In the present study, PZQ caused a signi ficant cysticercoids reduction in number on 4th day PI, compared to control. This agreed with Chai, (2013), who found that cysticercoid stages of *H. nana*, *H. diminuta*, and *H. microstoma* were all killed by PZQ in infected mice or rats.

Also, the present *C. procera* extracts combined with PZQ showed a significant reduction in cysticercoids' number of on 4th day PI and most of them were darkly stained lack ing recognized hooks. This agreed with Rashed *et al.* (2018), who reported that *H. nana* cysticercoids were complete killed on 4th day PI with combination of PZQ and *R. officinalis*.

In the present study, *C. procera* leaves CAE and CME on *H. nana* cysticercoids minimized inflammatory cell infiltrations in submucosa of treated mice compared to control. This may be explained by the anti-inflammatory, and antioxidant effect of *C. procera* leaves extracts (Rashed *et al*, 2018). Also, PZQ combined with *C. procera* leaves showed restoration of the normal intestinal architecture with rare inflammatory cell infiltrations in the submucosa.

Conclusion

The outcome results showed mice treated with *C. procera* CAE or CME alone and combined with PZQ (half dose) gave marked improvement in all parameters of assessment which is considered a safe, effective and promising alternative plant-based anthelminthic.

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Explanation of figures

Fig 1: Vital staining of *H. nana* eggs by Chausov's method showed (A) Viable egg with yellow colour against a red background, (B) Red-shelled egg against a red background, (C) Dead egg stained red against a red background (× 100).

Fig 2: SEM of adult *H. nana* worm showed (A) normal scolex with four well-defined suckers and normal rostellum with hooks, (B) More tegument damage with multiple blebs and vesicles in mice treated with PZQ, (C) loss of normal segmental architecture with mild oedema and minute small blebs in mice treated with CAE, (D) total loss of scolex shape & hooks, neck shrinkage, multiple whitish blebs of large size and shrinkage of immature segments with blebs in mice treated with CME, (E) shrinkage of segments and loss of normal architecture with marked scattered whitish blebs of different sizes with notches in mice treated with PZQ+CAE, (F) More tegument damage, sloughing and erosions with big sized whitish vesicles in mice treated with PZQ+CME.

Fig 3: cystecercoid stage at villious intestinal margin by conventional microscope on 4th day after treatment in (A) Untreated infected showed well defined margins, tapering posterior end without hooks' circle (black arrow), (B) Mice treated with PZQ showed smaller and darker distorted cysticercoid with absence circle of hooks, (C) Mice treated with CAE or CME showed darker cysticercoid with ill-defined margins and circle of hooks (black arrow), (D) Mice treated with PZQ+CAE or PZQ+CME showed dark shaped worm with an ill-defined circle of hooks (black arrow) (× 100).

Fig 4: T. S. small intestine on 4th day P.I. H & E stained: (A) Positive control showed well-defined multiple rounded *H. nana* cysticercoids embedded within villi (black arrows) (X100), (B) Mice treated with PZQ showed intact epithelial villous lining with ill-defined darkly stained cysticercoid in between intestinal villi (black arrow) (X200), (C) Mice treated with CAE or CME showed multiple moderately defined *H. nana* cysticercoids embedded within villi (black arrows) (X100), (D) Mice treated with PZQ+CAE or PZQ+CME showed ill-defined deformed darkly stained cysticercoid in between intestinal villi (black arrow) (X200).