In vitro Anticestode Activity of Painted Nettle Leaves Extract to *Hymenolepis microstoma*: Observation Using SEM

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Abstract

Study *in vitro* on the anticestodal activity of *Coleus blumei* leaves extracts against *Hymenolepis microstoma* was conducted. Leaves of *C. blumei* were collected and extracted with hexane, chloroform, ethanol and water. Anthelmintic activity was evaluated with an assay using *H. microstoma* in a serial microplate dilution method by determination of mortality time. The parasites were treated with varying concentrations, 10, 5, 2.5, 1, and 0.5 % of the plant extract. Scanning electron microscope analysis was performed to determine effect of painted nettle extract on the tegument and other superficial body changes of *H. microstoma*. The result of this study showed extracts fraction of semipolar solvent (ethanolic and cloroform extracts displayed strong anthelmintic activity with the highest activity belong to ethanolic extract. In general, ethanolic extract proved to be a more efficient extractant of biologically active compounds than either cloroform, hexane or aquous extract. On the assessment of the motility and mortality of the worms, a concentration-dependent activity of the plant extract was clearly discernible. In the scanning electron microscopy, extensive distortion and destruction on the surface fine topography of the tegument were evident. Thus, the present experiment provides the plausibility of *C. blumei* leaves as an anthelmintic agent. The promising activity displayed by ethanolic extracts has led to further investigation of the active compound, toxicity of the extract and *in vivo* efficacy.

Key words: *Coleus blumei*, anthelmintic, SEM, *Hymenolepis microstoma*

Introduction

Cestode or it is more known well as tape worm is important endoparasite that cause the serious disease in animals as well as human. The rate of animal cestodosis case particularly on poultry in Indonesia still about 20 to 100% (Sasmita, 1980; Inbandiah, 1995; Retnani *et al.*, 2007). Cestodosis can cause economical loss through decrease of growth, production (meat, milk, egg and wool), reproduction performance, unuseful carcass and mortality (Over *et al.*, 1992). Beside directly impact in animals, zoonotic tape worm infected animal such as pig, ruminant, dog, cat and rat are also very potency become infection source on human (Over *et al.*, 1992). There are four species of zoonotical cestode in human and to be serious problem of public health around the world included Indonesia those are *Taenia solium* (*pork tapeworm*), *T. saginata* (*beef tapeworm*), *Diphyllobothrium lattum* (*fish or broad tapeworm*), *Hymenolepis nana* (*dwarf tapeworm*) (Craig and Ito, 2007).

Generally, the control of helminthiasis included tape worm is very depended on frequency of anthelmintic administration routinely and regularly. Anthelmintic is needed in control of helminthiasis, not only expensive for poor farmer but also negative impact of residue in meat when duration of action of drug is still going on. The use of commercial anthelmintic can also cause problem of resistance of worm on that drug if the use is extensively and out of recommendation. Almost all kinds of present anthelmintic decrease their effectiveness is caused resistance nematode development on all of types of anthelmintic. On the other hand, Indonesia has so many natural botanical resources. The big natural resources prepare material resources for traditional medication practician to cure some diseases involve

parasite. Recently, herbal medicine still hold the important role in health especially in development countries, which there are gap between prize, stock and demand of market medicines (Akerele *et al*, 1998). Traditional medication has important role as source of material of antiparasite included effective anticestode for people, especially tropic development countries as Indonesia. Many traditional drugs are recognized as effective drug to eradicate cestode and they have good effectiveness for anticestode (He *et al.*, 1992; Lamtiur, 2000; Widdhiasmoro, 2000; Tangpu *et al.*, 2004; Temjenmongla and Yadav, 2005; Yadav and Tangpu, 2006; Roy *et al.*, 2008), and no effective anticestode anymore (He *et al.*, 1992; Kustiawan, 2001).

Coleus blumei is one of plant belong to 66 plants commodity of biopharmacy based on resolution of Indonesia agriculture ministry of No: 511/Kpts/PD. 310/9/2006 (Promosiana 2007). This plant is family Labiatae is found almost around Indonesia. Indonesian people use this plant for to cure various diseases included helminthiasis (De Padua, 1999). Scientifically, anthelmintic activity of *Coleus blumei* leaves has been proved by He *et al.*, investigation (1992). Standard of this research results was difficult because the preparation of extract. The concentration of extract active ingredient fluctuate depend on plant water concentration, it is influenced by season and places so that difficult to measure the right doses in g/kg body weight. Standard of preparation, extract method, the right kind diluent to get active ingredient that has anthelmintic activity is still needed exploration. This study was done to know the kind of *Coleus blumei* leaves that has anthelmentic activity on *Hymenolepis microstoma* model *in vitro*. The influence of *Coleus blumei* leaves extract on the *Hymenolepis microstoma* body was investigated particularly the part of tegument using Scanning Electron Microscope (SEM).

Materials and Methods

Preparation of *Coleus blumei* leaves extract

Used *Coleus blumei* leaves were obtained from around Bogor area. Determination of *Coleus blumei* species was done at Herbarium Bogoriensis LIPI, Bogor. Collected *Coleus blumei* leaves were cut small then dried under solar during 2 days. Dried *Coleus blumei* leaves was made to become powder using blender. Five hundreds grams of *Coleus blumei* leaves powder was extracted with four diluents, hexane, chloroform, ethanol and water using dipping method during 72 hours. Filtration was performed every day until to become pure filtrat. Obtained filtrat was co densed using rotation evaporator until to become gross extract of *Coleus blumei* leaves (four gross extracts, hexane, chloroform, ethanol and water). This gross extract was used for examination of anthelmintic activity.

Preparation of *Hymenolepis microstoma*

H. microstoma was obtained from *H. microstoma* infected mice. *H. microstoma* infected mice were killed using Nembutal. Cavum abdominal of mice was opened and intestine was separated from other organ. *H. microstoma* in intestine was taken, collected and kept up in 0.5% of NaCl until used.

Test of anthelmintic activity *in vitro*

Preparated *H. Microstoma* was put in microtitration mug which each already contained water extract solution, ethanol, cloroform or hexane *Coleus blumei* leaves in medium of 0.5 % of NaCl, in various of rate of concentration, 10, 5, 2.5, 1 and 0.5 %. The same treatment was performed with serial concentration of praziquantel as recommendation anthelmintic, and one group was incubated in medium of 0.5 % of NaCl as negative control. Every hole of microtitration mug was filled 3 tapes worm and examination repeated 3 times of replication. Activity of anticestode was observed through check of mortality time and observed every 0.5 hour. The worm was not shown physically moving after stimulating sensitive brush was taken and entered into warm NaCl physiologis believed death.

Observation using scanning electron microscope (SEM)

Death worm of each treatment was fixed with alcohol 70% and preparated for observing SEM. Preparation of worm for SEM observation that initiated with to clean specimen in buffer $CaCO_3$ during 2 hours. Then it sonicated by ultrasonic sonicator for 2 minutes. Then, fixation used 2 % of glutaral dehyde for overnight. After fixation, serial dipping was done by 2 % of tanic acid for 2-6 minutes, caccodylate buffer for 4 times and each time for 15 minutes, 1% of *osmium oxalat* for 2-4 hours and 2 times of distilled wáter, consecutively.

Dehydration process was done by series alcohol and t-buthanol, then to be continued using *freez dried*. After mounting on metal stubs, specimen of worm was coated with gold staining. Examination of tegument integrity of *H. microstoma* was performed with Jeol JSM 500 scanning electron microscope in acceleration electron 20kV.

Statistical analysis

Data of mortality time were presented as mean ± standard deviation. The differences of the mean of mortality time of worm around treatments were

analyzed using anova and to be continued with Duncan multiple range test and a p value below 0.05 was considered significant (Steel dan Torrie, 1980).

Results and Discussion

The Influence of exposure of *H. microstoma* on *Coleus blumei* leaves extract for mortality.

In vitro examination of this study used *H. microstoma* model to evaluate mechanism of drug action and/or efficacy of synthetic drug on *H. microstoma* (Becker *et al.*, 1980; Siles-Lucas and Hemphill, 2002) as well as herbal medicine (He *et al*,. 1992; Lamtiur, 2000; Widdhiasmoro, 2000; Tangpu *et al.*, 2004; Yadav and Tangpu, 2006). The use of *H. microstoma* model *in vitro* in order to selection of anticestoda drug can decrease test cost and the number experimental animals.

Anticestoda activity *in vitro* on *H. microstoma* of *Coleus blumei* leaves extract was seen in Table 1 and Figure 1. The mean of the mortality rate of incubated *H. microstoma* in control medium was 29 and 50%, respectively. Treatment group has mortality time faster than control group ($p<0.05$). Mortality time gradually decreased to follow the increase of extract concentration. The mean of *H. microstoma* mortality time most fast was ethanol extract, chloroform, hexane and water, consecutively.

The exposure of *H.microstoma* on ethanol extract and chloroform showed the equal influence between ethanol extract and cloroform with anticestoda standard, praziquantel. Needed time of both (ethanol extract and cloroform) and anticestoda standard for each concentration was not significantly different, while needed time of each concentration of hexane extract and water was longer than anticestoda standard (praziquantel) ($p<0.05$).

Various activities among kind of *Coleus blumei* leaves extract predicted to be cuased differences of proportion of component active ingredient that responsible on anthelmintic activity of every extract. Bioactive material activity of material from plant was depended on the type of extract and extract method (Eloff, 1998). The use of different diluent caused difference of composition of obtained chemical agent. This circumstance was caused chemical agent was more dissolvable in water and the other chemical agent was more dissolvable in ethanol, cloroform and/or hexane. Aktivitas anticestoda activity of *Coleus blumei* leaves in ethanol and cloroform has the same effect and/or similar with standard anticestoda. The result of this study showed that bioactive material of *Coleus blumei* leaves that has anthelmintic activity was contained higher in extract of semipolar diluent (i.e. ethanol and cloroform). This circumstance also can be seen in icrease extract concentration to quicken mortality and representation of the presence of concentration dependent activity.

The highest anthelmintic activity in ethanol extract and cloroform showed that chemical agent that has high anthelmintic activity in highest concentration is semipolar organic agent that capable more dissolved in ethanol and cloroform. Phytochemical qualitatively of *Coleus blumei* leaves showed it has secondary metabolized content, flavonoid, steroid, tanin and saponin (Ridwan and Ayunina, 2007). This secondary metabolized group was considered as source of chemical agent that reponsible for therapeutic activity of se eral herbal medicine. Several chemical agents of terpenoid and phenol such as lignan, chalcones and flavonoid, have been proved to has much antiparasite activity (Kayser *et al.*, 2003). Meanwhile so many reports reported plant that has much tanin capable to decrease the number of egg per g feces and the number of worm particularly nematoda (Athanasiadou *et al.*, 2001; Hoste *et al.*, 2006).

Table 1. Anticestode Activity *In Vitro* of *Coleus blumei* Leaves Extract in Various Diluents on Adult *Hymenolepis microstoma*

Groups	Mean ± standard deviation (X ± SD) of *H. microstoma* mortality time (hours)					
	0.00	0.50	1.00	2.50	5.00	10.00
Control	29.50 ± 0.97					
Praziquantel		7.72 ± 2.53^{ab}	6.00 ± 3.17^{ab}	4.78 ± 3.43^{ab}	3.11 ± 2.6^{a}	1.39 ± 0.05^{a}
Ethanol		6.61 ± 0.89^{a}	5.28 ± 0.87^{a}	4.11± 1.14^{a}	3.11± 1.6^{a}	2.00± 1.52^{a}
Cloroform		9.56± 2.42^{b}	8.00± 2.73^{b}	7.22± 1.91^{b}	5.33± 2.11^{a}	4.22± 0.79^{c}
Hexane		16.83± 2.18^{c}	16.06± 3.21^{c}	12.94± 2.46^{d}	10.39±2.26^{d}	8.72± 3.93^{d}
Water		19.50± 1.87^{c}	17.50± 3.71^{c}	16.17± 4.09^{d}	13.06± 5.9^{d}	8.06± 3.78^{d}

Notes: Different superscript in the same column showed significantly differences ($p<0.05$)

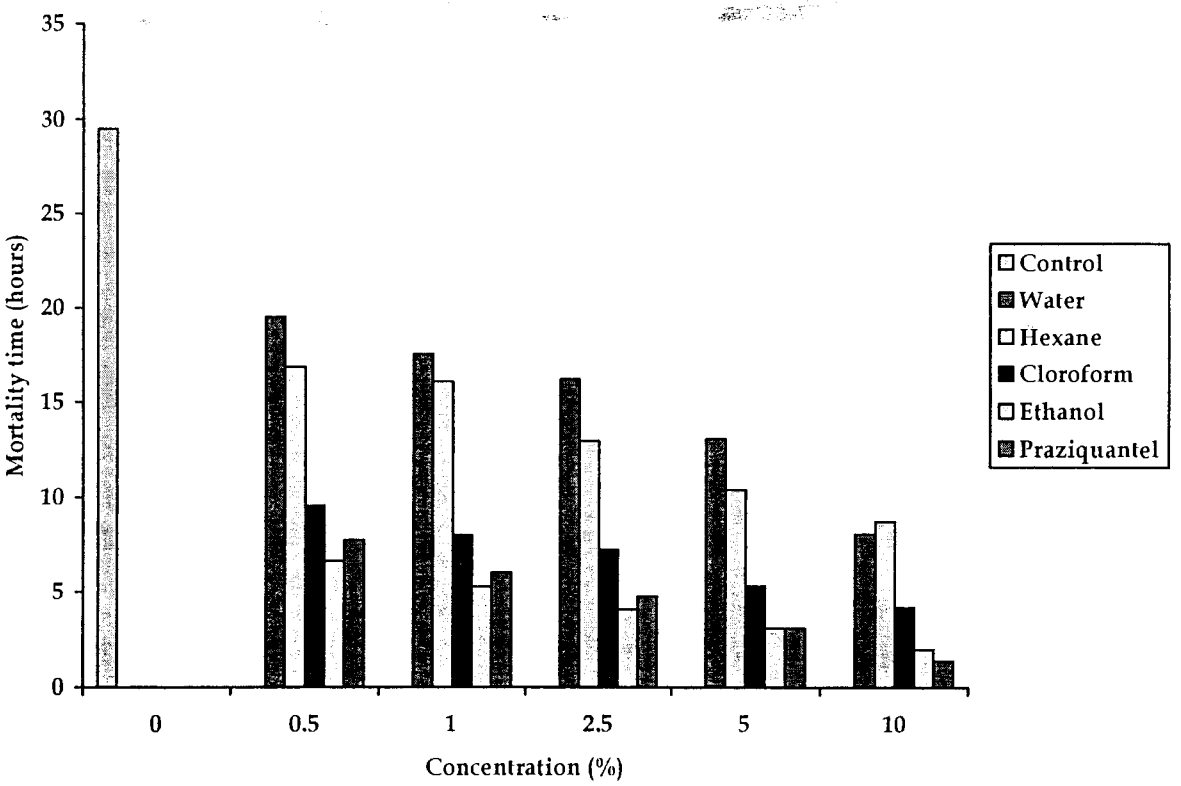

Figure 1. Graphic of mortality time of *H. microstoma* in each treatment group.

The change of stucture of *H. microstoma* after exposure of ethanol extract

Observation using SEM was done at *H. microstoma* 10% of ethanol extract exposured. Selection of ethanol extract in highest concentration was been caused to has highest anticestoda activity (10%) so that it descrip the clearest change compared with *H. microstoma* control group.

The observation using SEM on body surface morphological organisation of tape worm of control group showed illustration of normal body structure of cestoda (Figs. 2A, 2C, 3A, 3C and 3E). Anterior *H. microstoma* composed of scolex and four suckers radicalized around upper site of scolex. Middle site among all suckers has refractical rostelum. Rostelum was not appeared and into rostelum sac showed death *H. microstoma* in relax (Fig. 2A). *H. microstoma* strobila was seen neat and fine surface (Figs. 3A and 3C). *H. microstoma* whole covered regularly tegument and fine in scolex as well as strobila (Figs. 2A and 3A). Tegument surface composed of plait of *microtriches* as net (Figure 3E).

Incubated *H. microstoma* in *Coleus blumei* leaves ethanol extract showed the damage of *H. microstoma* whole, general tophograph (Figure 3B). Scolex experienced high change of it surface in erosion and exhaust of tegument to become vacuole (Figure 2B). Rostelum surrounded hooks appeared stuck out and sucker was damaged.

Proglotid of *H. microstoma* was seen to wrinkle and shorted (Figure 3B), while strobila experienced extensive degenerative (Fig. 3D). Erosion of surface and lesio in vacuole at several parts of strobila (Fig. 3D). Soft plait of *microtriches* experienced permanent damage and there was no that plait anymore and irregularly shapes (Fig. 3F).

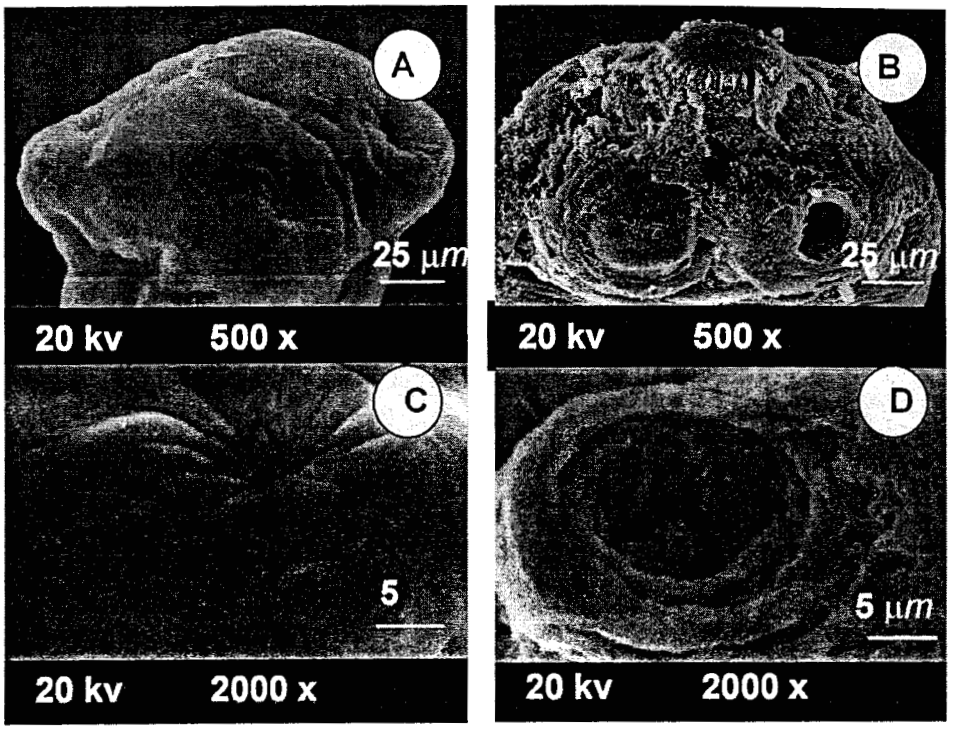

Figure 2. Scolex of incubated *H. microstoma* in control medium (NaCl physiologis and 10% of *Coleus blumei* leaves ethanol extract, A and C, control; B and D, treatment group (SEM).

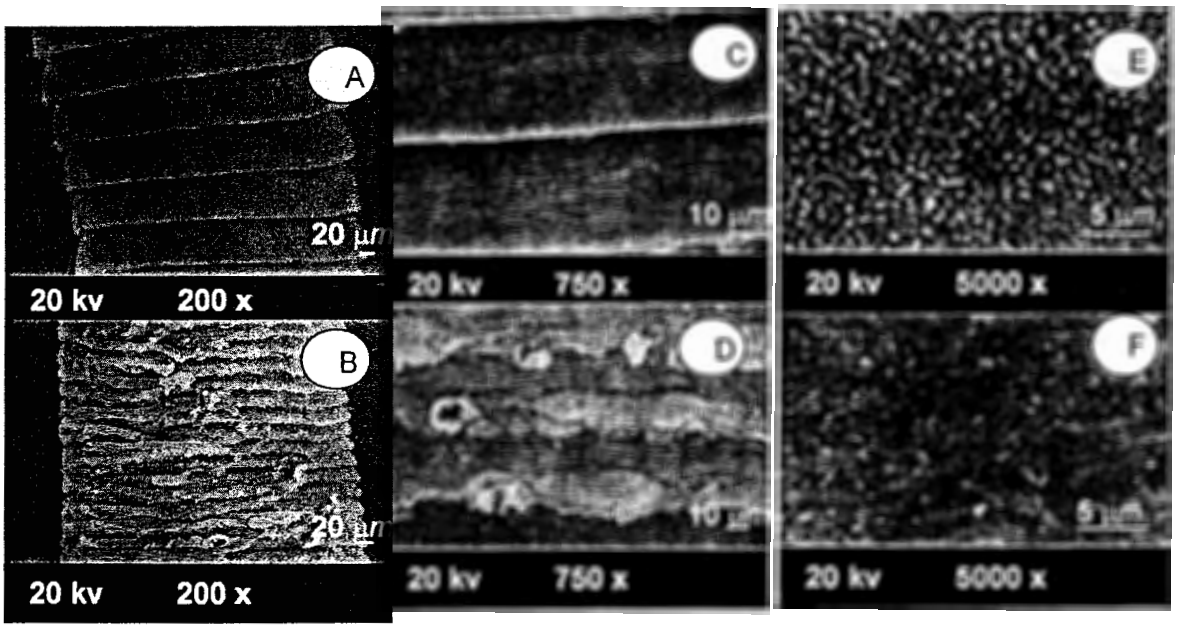

Figure 3. Strobila of *H. Microstoma.* A, C and E, control group; B, D and F : treatment group (SEM)

Parasite tegument is one of the main target of several synthetic and/or natural anthelmintic products (Geary *et al.*, 1992; Tandon *et al.*, 1997; Roy *et al.*, 2008). The change of morphology and structure by anthelmintic agent on tegument of the other species of worm had reported by several investigators (McKinstry *et al.*, 2003; Meaney *et al.*, 2004; Xiao *et al.*, 2003; Roy *et al.*, 2008). Albendazole and the other of the same group already known into parasite tegument through simple diffuse and then it caused disturbance on tegument and muscular layer (Mottier *et al.*, 2003; Markoski *et al.*, 2006). *Hymenolepis nana* experienced vacuolization on tegument in part of tape worm neck that was initially seen 5 minutes post incubation in praziquantel (Becker *et al.*, 1981). The presence of vacuolization caused the disturbance of *syncytia* layer in apical area of tegument.

Ten percents of ethanol extract incubated *H.microstoma* was very clear to show body general tophograph damage and disorganized tegument appearance and *microtriches*. Scolex experienced high change of it surface in erosion and exhaust of tegument to become vacuole in several parts of strobila. Soft plait of *microtriches* experienced damage and disappeared plait anymore and irregular shape. Appeared degenerative effect in this study likes anthelmintic effect of the other anticestoda. Roy and Tandon (1996) and Roy *et al.* (2008) reported the same damage in *Raillietina echinobotrida* after exposure of each extracts of *Flemingia vestia* and *Millettia pachycarpa*. The agent that has role in damage process of the worm body can not exactly yet, but prediction of one or several secondary metabolized agents group of *Coleus blumei* leaves extract such as flavonoid, tanin or aponin have role in that process.

Beside the direct effect on worm body surface, *Coleus blumei* leaves extract predicted to has worm neuromuscular system activity. The strong prediction was proved by the death of worm in contraction condition with shorted strobila. Several synthetic drugs have neuromuscular activity such as *macrocyclic lactones*, it has paralytic activity on worm in host. Paralysis was found at adult *H. diminuta* after exposure of praziquantel and this effect is *reversible* (Andrews and Thomas, 1979).

Even gross extract of *Coleus blumei* leaves showed anticestoda activity, but the right mechanism and structure group that has role to kill *H. microstoma* was not clearly yet.

Conclusions

Coleus blumei leaves extract had anthelmintic activity on *H. microstoma* model. Anthelmintic activity of four extracts of *Coleus blumei* leaves had variation, which highest to lowest of anthelmintic activities, ethanol extract, chloroform, hexane and water, consecutively. Observation using SEM of ethanol extract with 10% concentrate caused damage morphology of *Hymenolepis microstoma*.

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