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

Yusuf Ridwan 1,2, Fadjar Satrija 1, Latifah K. Darusman 2 and Ekowati Handaryani 3

1 Helminthology Laboratory, Department of IPHK, Faculty of Veterinary Medicine-IPB,
2 Study Centre of Biopharmaca LPPM-IPB, 3 Pathology Laboratory, Department of KRP, Faculty of Veterinary Medicine-IPB, e-mail: yusufridwan67@yahoo.com

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 chloroform 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 chloroform, hexane or aqueous 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% (Sasmitha, 1980; Inbandiah, 1995; Retnani et al., 2007). Cestodosis can cause economical loss through decrease of growth, production (meat, milk, egg and wool), reproduction performance, uselessful 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 policity become infection source on human (Over et al., 1992). There are four species of zoonotic 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 latum* (*fish or broad tapeworm*), *Hymenolepis nana* (*duarf 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
Traditional medication has important role as source
of material of antiparasite included effective anti-
estode 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 antestode (He et al.,
1992; Lamttur, 2000; Widdhiasmoro, 2000; Tangpu et
al., 2004; Temjenmongla and Yadav, 2005; Yadav and
Tangpu, 2006; Roy et al., 2008), and no effective
antestode anymore (He et al., 1992; Kusliawan,
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 diluents to get active ingredient
that has anthelmintic activity is still needed explora-
tion. This study was done to know the kind of Coleus
blumei leaves that has anthelmintic 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 Bogorienis 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 anthel-
mintic 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
Prepared H. Microstoma was put in micro-
titration mug which each already contained water
extract solution, ethanol, chloroform 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 concen-
tration 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 antestode 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 prepared for observing SEM.
Preparation of worm for SEM observation that
initiated with to clean specimen in buffer CaCO3
during 2 hours. Then it sonicated by ultrasonic
sonicator for 2 minutes. Then, fixation used 2 % of
glutaraldehyde for overnight. After fixation serial
dipping was done by 2 % of tannic acid for 2-6
minutes, caccodylate buffer for 4 times and each time
for 15 minutes, 1% of osmium oxalate for 2-4 hours and
2 times of distilled water, consecutively.

Dehydration process was done by series alcohol
and t-buthanol, then to be continued using free: 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; Lantiu, 2000; Widdiasmoro, 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 chloroform with anticestoda standard, praziquantel. Needed time of both (ethanol extract and chloroform) 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 caused 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 (Elloff, 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, chloroform and/or hexane. Aktivitas anticestoda activity of Coleus blumei leaves in ethanol and chloroform 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 chloroform). This circumstance also can be seen in increase extract concentration to quicken mortality and representation of the presence of concentration dependent activity.

The highest anthelmintic activity in ethanol extract and chloroform showed that chemical agent that has high anthelmintic activity in highest concentration is semipolar organic agent that capable more dissolved in ethanol and chloroform. 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 several 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 faces and the number of worm particularly nematoda (Athanasia 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**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± standard deviation (X ± SD) of <em>H. microstoma</em> mortality time (hours)</th>
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<tbody>
<tr>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>Control</td>
<td>29.50 ± 0.97</td>
</tr>
<tr>
<td>Praziquantel</td>
<td>7.72 ± 2.53&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>6.61 ± 0.89&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chloroform</td>
<td>9.56 ± 2.42&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hexane</td>
<td>16.83 ± 2.18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Water</td>
<td>19.50 ± 1.87&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes: Different superscript in the same column showed significantly differences (p<0.05)
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![Figure 1. Graphic of mortality time of *H. microstoma* in each treatment group.](image)

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

Observation using SEM was done at *H. microstoma* 10% of ethanol extract exposed. 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 topograph (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 targets 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 syncythaia layer in apical area of tegument.

Ten percents of ethanol extract incubated H. microstoma was very clear to show body general topograph 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 Raillietna echinobrutida after exposure of each extracts of Flemingia vestina and Miletta 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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References


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