Protective Effect of Neem (*Azadirachta Indica*) Leaf Extract on Liver of *Trypanosoma evansi* Infected Rats (*Rattus norvegicus)*

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Abstract: Animal trypanosomiasis is still a devastating disease of both domestic and wild animals around the world. Control of surra is greatly depending on chemotherapy. The aim of this research was to observe histological changes in the liver of *T.evansi* infected rats treated with different concentrations of neem (*Azadirachta indica*) leaf extract. Samples used were livers collected from 24 male Wistar rats randomly divided into 6 treatment groups with 4 replications each. Negative control (K_0) was rats given distilled water only; positive control (K1) was rats infected with *T.evansi* $5x10^4$; K2, K3, K4, and K5 were rats infected with *T. evansi* $5x10^4$ and administered with neem leaf extract 50, 100, 400, and 800 mg/kg body weight (BW) per oral for 3 consecutive days, respectively. On day 4, all rats were sacrificed by ether euthanation for liver collection. Livers obtained were histopathologically processed using standard hematoxylin-eosin staining and microscopically observed. Histological changes in the liver of rats in all treatment groups were as the following. Normal hepatocytes were K_0 63.65, K_1 27.25, K_2 15.15, K_3 12.90, K_4 20.30, and K_5 24.85.Haemorrhagic hepatocytes were K_0 0.00, K_1 15.60, K_2 10.60, K_3 17.75, K_4 6.30 and K_5 9.05. Hyperemic hepatocytes were K_0 0.00, K_1 2.35, K_2 3.70, K_3 3.25, K_4 2.25 and K_5 2.25. Infiltration of inflamation cells were K_0 0.00, K_1 2.75, K_2 3.90, K_3 14.65, K_4 2.55, and K5 2.70. Hepatocyte degeneration was K_0 0.00, K_1 6.30, K_2 6.95, K_3 10.15, K_4 4.15, and and K_5 1.00. Necrotic hepatocytes were K_0 1.55, K_1 32.20, K_2 43.80, K_3 45.00, K_4 31.25, and $K_5$34.20. Neem leaf extract at the dose of 800 mg/kg BW was the best in preventing liver damages caused by *T. evansi* infection in rats.

1. INTRODUCTION

Surra is caused by *T. evansi* and still becomes a problem for animal health (Luckins, 1996). Bad impacts caused by *T. evansi* in infected animals include reduced weight, low reproduction, immunosupression and mortality. According to Damayanti *et al*. (1994), in buffalo *T. evansi* attacks many organs such as brain, kidney, spleen, pulmo, and liver. *T. evansi* infection causes necrosis of hepatocytes in the *Kiernan* triangle, *sentro-perilobulary* lipid degeneration, enlarged sinusoid, and infiltration of polymorphonuclear cells (PMN) around centralis vein (Lazuardi, 2008).

Control of trypanosomiasisis dependent upon synthetic medicines such as suramin, diminazene, isomedium, quinapyramine, and cymelarsan. Several *T. evansi* strains resistant to antitrypanosomal drugs are reported in Vietnam (Stevenson *et al*., 2000). Most *T. evansi* isolates stored at the Tissue Culture Collection of Veterinary Research Central Agency of Bogor are resistant to isometamedium and diminazen aceturate (Sukanto *et al.*, 1988). Therefore, it is necessary to search for new medicine to anticipate the resistance of *T. evansi* isolates to the currently available antitrypanosoma. The use of plants extract containing phytochemicals have a variety of beneficial biologic effects could provide alternatives.

One of plants extensively investigated its medicinal benefits is neem (*Azadirachta indica*). This plant has been known by people as a medicinal plant that has broad spectrum biological activities such as antipyretic, analgesic, antifungal, mosquito repellant, antiinflamasion, antiparasite, antiinsect, and larvasidal as well as anticancer, antoeczema, and antimalaria (Biswas *et al*., 2002; Wahyuningsih *et al*., 2002).

According to Syarmalina and Laksmitawati (2005), phytochemical contents of neem leaves

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include *azadirachtin, paraisin, alkaloid,* and volatile oil containing sulfides. Neem leaves also contain 4 natural compounds that have pesticidal properties namely *azadichtin, salanin, meliatriol,* and *nimbin* (Subiyakto, 2009).

In the previous article we have showed that neem leaves extract 800 mg/bw inhibit the growth of *T. evansi* upto 80.5% in rats (*Rattus norvegicus*) (Fahrimal *et al.* 2017). Effect of neem leaf extract administration on the liver of *T. evansi* infected rats is presented this article.

2. MATERIALS AND METHODS

Trypanosoma evansi isolates used were the collection of the Parasitological Laboratory of Veterinary Faculty of Syiah Kuala University. Experimental animals used were 24 rats randomly assigned into 6 treatment groups. Negative control (K0) and positive control (K1) were consecutively rats given aquadest and intraperioneally infected with *T. evansi* $5x10^4$. Group K2, K3, K4, and K5 were rats infected with *T. evansi* $5x10^4$ and administrated with neem leaf extract of 50, 100, 400, and 800 mg/kgBW per oral for 3 days, respectively. On day 4 all rats were sacrified by ether euthanation for liver collection. Livers were put in 10% NBF solution (pH 6.5-7.5) and subjected for standard histopathological preparation using haematoxylin and eosin staining. The preparates were microscopically observed using a binocular microscope (*Olympus CX21, Japan*) with 400 and 1000 magnification and documented using a photo microscope (Olympus BX41, Japan). Data obtained were analyzed by ANOVA and Duncan test.

3. RESULTS AND DISCUSSION

Microscopic observation indicated that liver of *T. evansi* infected rats administrated with neem leaf extract ranged from 50-800 mg/kgBW showed haemorrhage, hyperemia, inflammation cells infiltration, degeneration, and necrosis (Figure 1). Results of statistical analysis of the observed histological parameters using Duncan test are presented in Table1.

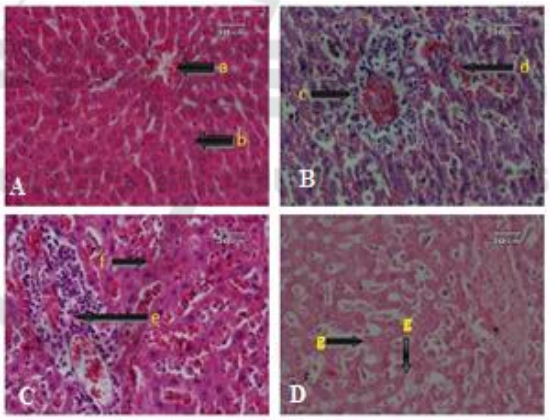

Figure 1. Histopatological profile of livers of control (A) and *T. evansi* infected (B-D) rats treated with with neem leaf extracts. a. Centralis vein, b. Normal hepatocytes c. Hyperemia, d. Haemorrhage, e. leukocyte infiltration, f. degeneration, and g. necrosis (HE, 400x).

Table 1. The changes in the livers of *T. evansi* infected rats treated with neem leaf extracts

Treatment	Parasitemia inhibition (%)*	Normal Cell	Haemorrhage	Hyperemia	Leukocyte Infiltration	Degeneration	Necrosis
K0	0	63.65 ± 9.59^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.55 ± 0.82^{a}
K1	0	27.25 ± 7.32^{b}	9.30 ± 7.81^{bc}	2.35 ± 1.57^{b}	2.75 ± 2.17^{b}	6.30 ± 1.64^{b}	32.30 ± 10.47^{b}
K2	14.64	15.15 ± 4.87^{bc}	10.60 ± 3.82^{b}	3.70 ± 1.55^{b}	3.90 ± 0.99^{b}	6.95 ± 0.25^{b}	43.80 ± 12.93^{b}
K3	23.78	12.90 ± 4.26^{c}	17.75 ± 2.36^{c}	$3,15 \pm 0.57^{b}$	4.75 ± 0.47^{b}	10.15 ± 1.60^{c}	45.00 ± 10.35^{b}
K4	58.68	21.25 ± 2.45^{b}	6.30 ± 5.41^{b}	$2,25 \pm 0.68^{b}$	2.55 ± 0.41^{b}	1.07 ± 0.50^{d}	31.25 ± 10.89^{b}
K5	80.50	20.00 ± 1.36^{b}	9.05 ± 3.10^{b}	$2,55 \pm 1.28^{b}$	2.70 ± 0.99^{b}	1.00 ± 0.71^{d}	$32,55 \pm 3.59^{b}$

* Fahrimal *et al.* (2017)

Note: Different notation a, b, c, and d shows significant difference ($p<0.05$) between treatment groups

K0: negative control (rats given aquadest)
K1: positive control (rats infected by *T. evansi*)
K2: *T. evansi* infected rats given neem leaf extract 50 mg/kgBW
K3: *T. evansi* infected rats given neem leaf extract 100 mg/kgBW
K4: *T. evansi* infected rats given neem leaf extract 400 mg/kgBW
K5: *T. evansi* infected rats given neem leaf extract 800 mg/kgBW

Data in Table 1 shows that in addition to had no hemorrhage, hyperemia and leukocyte infiltration, the rats in the K0 (negative control) had the highest numbers of normal hepatocytes and the lowest necrosis compared to *T. evansi* infected rats, either untreated (positive control K1) or treated with different doses of neem leaves extracts (K2, K3, K4 and K5). Infection of *T. evansi* (K1) significantly reduced the numbers of normal hepatocytes and resulted in moderate hemorrhage, hyperemia, leukocyte infiltration, lipid degeneration and necrosis. The administration of neem leaves extract ranged from 50-100 mg/kgBW could not protect liver of rats from negative effects of *T. evansi* infection, as shown by increased degree of hemoraghe, hyperemia, leukocyte infiltration, lipid degeneration and necrosis. Neem leaves extracts 400 and 800 mg/kg BMW, on the other hands, had protective effects on the liver of *T. evansi* infected rats, as indicated by higher numbers of normal hepatocytes, reduced hemoraghe, hyperemia, leukocyte infiltration and necrosis. These findings are in agreement with those reported by Astuti *et al*. (2012) that *T. evansi* infection causes hepatocytes necrosis, lipid and hydrophic degeneration, pycnosis, light kariolysis, and hyperemia in mice. Other studies in *T. evansi* infected goats also found various damages in the livers such as hepatocytes necrosis (Lazuardi, 2008; Shehu*et al*., 2006), sentro-perilobulary lipid degeneration and PMN infiltration around centralis vein (Lazuardi, 2008).

Reduced normal hepatocytes in *T. evansi* infected rats are predicted caused by the deleterious effects of the high prevalence of the parasite on the tissues. Destruction at hepatocyte structure might increase migration of phagocyted inflammation cells and macrophages travel in blood vessels to damage tissues. Inflamation cells and macrophages produce free radicals that could result in cellular damages. During inflamation occurs proliferation of *Kupffer* cells and leukocytes increase, causing the increase of macrophages (Contran*et al*., 1994). Studies in buffalo showed that *T. evansi* infection is not only caused congestion, intralesional trypanosomes in blood vessel and extramedullary hematopoiesis in the liver but also non specific lesions – edema, congestion and hemosiderosis – in the lungs (Verdillo *et al*., 2012).

Among factors contribute to this bad impact of *T.evansi* in animals are its capability to produces hemolysins, toxic compounds that might lyse erythrocytes (Mbaya *et al*, 2012). Here, erythrocytes, platelets and reticulocytes adhere to the surface of trypanosome surfaces via sialic acid receptors leading to damages to erythrocyte cell (Shehu *et al*., 2006). This is because several areas of discontinuity occur along the surface of erythrocyte membranes where they adhere to the trypanosomes (Mbaya *et al*, 2012).

Haemorrhagioccurred in liver of *T. evansi* infected rats was also reported by Bal *et al*. (2012). This condition was assumed caused by the high prevalence of *T. evansi*in the tissue, resulted in the change of membrane permeability of blood vessels. According to Widodo*et al*. (2012) torn or damage blood vessels due to high permeability of cell wall facilitated erythrocytes leakage out from blood vessels, a clinical condition known as haemorrhage. Mechanical damage to vascular endothelium has been reported when tissue-invading trypanosomes such as the *T. brucei* group penetrate tissues via the interstices (Anosa and Kaneko, 1983).

High infiltration of inflammation cells in the liver might be related to formation of new antigens by *T. evansi* to manipulate antibody of the host. Variable antigenic type (VAT) is the agent encodes variations of antigenic glycoproteins on the surface of *T. evansi* (Wang *et al*., 2010). Every time *T. evansi* grows and develops inside host, it synthesizes new variant of VAT protein. Immune system of the host will adjust this change by creating new, apropriate antibody. This mechanism causes decreased immunity of the host, making it become vulnerable to secondary infections that in turn result in infiltration of inflammation cells (Wang *et al*., 2010).

Degeneration is a sign of the initiation of cell damages from toxins and cells might lose their normal structure that lead to cell death (Assiam *et al*., 2014). Parenchimatous degeneration or albumin degeneration is the failure of oxidation causes accumulation of water inside the cells. As consequences transportation of proteins produced by ribosomes might be disturbed and cause swollen cells, cytoplasm turbidity, and granulated cytoplasm from protein sedimentation (Mitchell *et al*., 2008). Hydrofic degeneration is irreversible degeneration related to the accumulation of lipid and glycogens in the vacuoles containing water (Kasno, 2000). Lipid degeneration might occure in the condition of ischaemia, anaemia, and toxin as well as overconsumption of lipid and protein (Dannuri, 2009). Lipid degeneration is characterized by high proportion of lipid in cytoplasm that leads to the shift of nuclei to the egde of cell and enlarged sinusoid, and cell necrosis (Oktavianti *et al*., 2005).

Number of degenerated cells might reduce if cell become necrosis from toxic effect of high doses of neem extract given. Amalina (2009) explains that higher concentration of chemicals generally causes higher toxicity responses. Necrosis is the death of cells or tissues in the living organisms characterized by smaller, more solid nuclei, folded chromatin and reticular fibrous and eusinophilic/kariolysis cells (Kasno, 2000) (Figure 1).

Administration of neem leaf extracts in *T. evansi* infected rat seems efective in K5 (dose 800 mg/kgBW) where all observed parameters were better although smaller than those in negative control (K0). Increased numbers of normal hepatocyte might be caused by bioactive compounds that are able to kill or at least inhibit the growth of *T. evansi* or to reduce vulnerable effect of toxins produced by the parasite. Choudhary *et al*. (2014) argued that phytochemical analysis indicated that neem leaf extract contains *glycosides, tannin, flavonoids*, and *saponin* that might function as hepatoprotectant. Sonyafitri (2006) added that *azadirachtin* ($C_{35}H_{44}O_{16}$) is the most active compound in neem leaf extract. This limonoid (triterpenoid) inhibits the growth and development of *T. evansi* (Nzelibe *et al.*, 2013).

In addition to azadirachtin, neem leaf extract contain alkaloid, terpenoid, quinolide, and phenolic compounds might act as antiprotozoa (Karira *et al*., 2004). Flavonoid dan terpenoid are are chemicals inhibit the growth and development of *T. evansi* (Eliawardani, 2015). Choudhary *et al*. (2014) have proven that secondary metabolics contained in neem leaf extract could reduce and repair alcoholic induced tissue damages. Result of K5 is also supported by results obtained by Kale *et al*. (2003) that neem leaf extract is able to repair tissue damages caused by medicines. This is because the extract contains chemicals function as hepatoprotective agent (Innih *et al*., 2014).

Beside hepatoprotective, neem leaf extract is also hepatotoxic when used in high doses (Kadiri *et al*., 1999). Hepatotoxisity is damage of liver caused by drugs use. According to Robbins *et al*. (2007) the occurrence of toxin accumulation causes the damage of liver and disrupt membrane permeability, osmotic homeostasis, enzyme and cofactor binding, which in turn disturb cellular work and function. Katsayal *et al.* (2008) added that neem leaf extract, if it was given in a very high dose (up to 2000 mg/kgBW) for 4 weeks, resulted in a numbers of toxicity effects in liver. These include infiltration of inflammation cells, increased Kupffer cells number, hepatocyte apoptosis and necrosis, and narrower blood vessels. Astuti *et al*. (2012) also reported that liver tissue of mice administrated with mindileaf extract 800 mg/kgBW and infected with *T. evansi* showed severe necrosis. Amalina (2009) suggested that higher concentrations of chemical result in stronger toxic effects. This is that causes the damage of liver

as indicated by lession affected the change of cellular function and structure.

4. CONCLUSION

Neem (*Azadirachta indica*) leaf extract 800 mg/kgBW resulted in the best inhibition on the damage of the liver of *Trypanosoma evansi* infected male rats (*Rattus norvegicus*) and inhibition of parasitemia than 50 and 100 mg/kgBW.

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