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THERAPEUTIC EFFICACY OF *ARTEMISIA ANNUA* ETHANOL EXTRACT VERSUS ALBENDAZOLE ON MIGRATING & ENCYSTED *TRICHINELLA SPIRALIS* LARVAE IN MICE

By
MUSLIMAH N. ALSULAMI[1] AND YASSER M MOHAMED[2*]
Department of Biology, College of Science, University of Jeddah, Jeddah, Saudi Arabia[1] and Department of Medical Parasitology, Faculty of Medicine, Assiut University, Assiut, Egypt[2] (Correspondence: yasser_mokh@aun.edu.eg)

Abstract

Trichinellosis the gastro-intestinal (GI) and muscle (parenteral or systemic) are the 2 important phases of the parasite cycle. Infection with *Trichinella spiralis* is dangerous, and severe infections can be lethal affecting several organs including the liver and kidneys in addition to the final habitat which is the muscles. The several side effects limit the benzimidazole derivatives use. This study compared the antiparasitic action of ethanol extract of *Artemisia annua* against migrating and encysted *T. spiralis* larvae in in experimentally infected mice to the common helminthic used drug Albendazole®. Life cycle was developed in BALB/c mice by oral infection with 300muscle larvae. Mice were divided into four groups. Treatments with Albendazole and *Artemisia annua* ethanol extract were administered at the 15th day post infection (PI). On the 30th PI, they were sacrificed to evaluate the treatment, histopathologically of diaphragm, muscle, liver and kidneys to improvement. The *Artemisia annua* ethanol extract was as effective as Albendazole against the *T. spiralis* larvae.

Keywords: *Trichinella spiralis,* Larvae*, Artemisia Annua,* Albendazole*,* Histopathology.

Introduction

Trichinellosis is a foodborne zoonotic parasitic disease worldwide (Murrell and Pozio 2011). Infected mammalians, birds and reptiles were reported (Pozio 2005), with zoonotic trichinosis in both developed and developing countries (Pozio *et al*, 2001). Humans acquired infection by eating undercooked or raw pig meat and products containing *T. spiralis* larvae (Neghina *et al*, 2011a). *Trichinella spiralis* is the species most adapted to domestic and wild swine but can also include synanthropic rats in its life cycle, *Trichinella* exhibited a wide and global distribution (Dick and Pozio, 2001) Also, *Trichinella* can be found in pig, bear, walrus, fox, rat, horse, and lion (Boireau *et al*, 2000).

In Egypt, reports of *T. spiralis* infection in fresh and processed pork were reported (Siam *et al*, 1979). Also, *T. spiralis* was detected in 13.3% (1.025) rodents collected from and around abattoirs in Alexandria (Loutfy *et al,* 1999). By trichinoscope and muscle digestion technique pigs in Cairo Slaughter-Houses over the years (1995-2000) showed an over rate of 1.691% (Morsy *et al,* 2000). Combined albendazole-mefloquine low dose regimen gave highest effect on reducing parasite burden and restored normal histological architecture (Diab and Fahmy, 2021).

Gastrointestinal and muscle (parenteral or systemic) are the 2parasitic phases. For a few days to weeks, both phases of a parasite cycle can coexist. Larvae are discharged in the stomach after consuming infected meat, moult, and grow into adults in small intestinal enterocytes influence by gastric juice. After mating, newly born larvae enter circulation and distribute via tissues and organs, only those penetrate striated muscles grown into muscle larvae, and migrating larvae land in its' main site skeletal muscle cells (Bruschi and Gómez-Morales 2014).

The commonest symptoms are gastrointestinal ones (adults caused) such as nausea, vomiting, diarrhoea, fever, periorbital oedema, and myalgia, but in severe cases, other consequences by larvae migration were encephalitis (Gottstein *et al*, 2009), visual disturbance, and ocular pain or blindness (Alashry and Morsy, 2021). The less common symptoms include macular or urticarial rash, headache, cough, dyspnoea, and dysphagia, as well as occasionally the hepatomegaly

(Neghina and Neghina, 2011).

Trichinella spiralis infection in humans & in the different animal hosts induced an energetically costly liver organomegaly (Meagher and Dudek 2002). Also, it causes CNS manifestations, cardiac inflammation and/or pneumonia (Watt *et al,* 2000). Direct larval damage may be entering sinusoids and cause inflammatory mesenchymal responses (Kuntz, 2006), causing in an excess of liver granulomas (Neghina *et al*, 2010). Kidneys, acute glomerulonephritis indicating renal failure caused by dystrophic lesions and tubuloglomerular changes (Zanc, 2001)

Albendazole and mebendazole are the two commonest anthelmintic drugs used for trichinellosis (Gottstein *et al*, 2009). But, they showed a high level of resistance and a low activity level against encapsulated larvae (Caner *et al*, 2008). Besides, some of these medications were carcinogenic and others to be teratogenic, so they are not recommended for use in pregnant women or children less than 3-years old (Shalaby *et al*, 2010). Thus, safe and effective trichinellosis drugs, particularly from natural cheap compounds were indicated (Basyoni and El-Sabaa 2013).

Abd-Elrahman *et al*. (2020) in vitro found that myrrh crude (*Commiphora molmol* or *C. myrrha*) extracts and volatile oil cured *T. spiralis* larvae with albendazole as a reference drug. Also, Abd-Elrahman *et al*. (2021) showed that AgNPs have marked anti-*T. spiralis* larvae activity, and added that the myrrh extract was used as the green reducing and capping agent for the biosynthesis of AgNPs with less hazardous effects on environment. The developed AgNPs showed good stability with visible changes even after six months. Moreover, larvicidal effectiveness of chemically and green synthesized AgNPs against the *Trichinella* larvae was more or less the same without a significant difference, but, biosynthesis approach was more favourable due to its safety to humans, animals and environment friend agent.

Because the *Artemisia* plants contain a number of bioactive natural compounds (Bora and Sharma 2011) with a good safety profile in treating many human problems (Sofowora *et al*, 2013). *A. annua* proved to be effective against trypanosomiasis and schistosomiasis (Mishina *et al*, 2007), toxoplasmosis and leishmaniasis (de Oliveira *et al*, 2009), and coccidiosis (Jiao *et al*, 2018). Moreover, *A. annua* showed anti-giardiasis activity in vitro (Golami *et al*, 2016).

This study aimed to evaluate the efficacy of *Artemisia annua* ethanol extract against migrating and encysted *Trichinella spiralis* larvae in mice versus Albendazole®.

Material and Methods

The life cycle of *Trichinella spiralis* was maintained by infecting BALB/c mice on a regular basis at Assiut University's Animal House. Each mouse was infected orally with 300muscle larvae. After 30 days post infection (pi), muscle larvae were collected from infected mice by incubating minced skinned mice in artificial digestive fluid in a conical flask at 37°C overnight. To remove tissues, larvae were thieved, washed in PBS, and recovered from conical flask bottom for micrroscopic examinations (Gamble, 1996).

Artemisia annua ethanolic extract: *A. annua* extract was prepared by crushed 250g in clean grinding mortar to fine powder and steeped for 24hrs in methanol (Mishina *et al*, 2007). Extracted material was filtered using Whatman filter paper (No. 3). To have a dried residue, all methanol extracts were collected, evaporated in a rotary evaporator at 40^{o}C under decreased pressure, and dried (16g) was kept at 4°C until the therapeutic dosages were prepared.

Drug doses: A total of 40 clean laboratory bred male albino mice were divided into 4 groups of 10 mice each. GA: Non-infected control (normal control), GB: Infected untreated (infected control), GC: Infected and treated with albendazole® for 3 consecutive days with an dose oral of 50mg/kg started from the 15th day PI (Shalaby *et al*, 2010), and GD: Infected and treated with *A. annua* ethanolic extract with a dose of 500mg/kg, sta-

rted from 15th day post-infection PI for 3 successive days (Caner *et al*, 2008). On the 30th PI, they were sacrificed to evaluate treatment efficacy on the migratory and encysted *Trichinella* larvae (Attia *et al*, 2015).

Histopathological study: Parts of each of diaphragm, muscle, liver and kidney were sectioned to evaluate pathogenicity caused *T. spiralis* larval stages. Small pieces from each part were processed for formalin-fixed, paraffin-embedded tissue blocks, and cut by a microtome (usually 4-5µm) sections. The serial sections of each were deparaffinised in xylene, rehydrated in descending ethanol for one minute each change, washed in distilled water and then tap water for 3-5 minutes. Slides were stained with H & E for 5-7 minutes, and then dehydrated in ascending series of ethanol (50%, 70%, 80%, & 100%) 1 minute for each change, cleaned in xylene, mounted on clean slide in DPX and covered with suitable cover (Morsy *et al,* 1998). Pathogenic changes were evaluated under light microscopy

Ethical statement: The study protocol was approved by the Ethics Committee Board, Faculty of Medicine, Assiut University. The experimental animals used were dealt with according the rules of Helsinki 2000.

Results

Diaphragm of infected control mice showed a large number of *T. spiralis* larvae entrenched inside diaphragm muscle causing necrosis with inflammatory cell infiltrate dissecting muscle of diaphragm, composed of lymphocytes and plasma cells. Sections of Albendazole treated mice showed the diaphragmatic muscle bundles uniform without parasitic larvae. There was mild interstitial edema among some muscle bundles with few chronic inflammatory cells and few necrotic myocytes. Normal histological data were in diaphragm sections of *Artemisia* ethanol extract treated mice, muscle bundles were uniform without larvae, but neither oedema in between muscle bundles nor chronic inflammatory cell infiltration.

Many encysted larvae were in muscle bundles of infected untreated mice sections, significant interstitial oedema with few chronic inflammatory cells infiltrate in between muscle bundles. But, sections of Albendazole treated of infected mice showed uniform muscle bundles without larvae. Also, mild interstitial oedema, few chronic inflammatory cells infiltration and few necrotic myocytes, much more improvement was in muscle sections of *Artemisia* ethanol extract treated mice, with uniform muscle bundles without larvae, interstitial oedema or chronic inflammatory cell infiltration.

The infected untreated mice liver sections showed lobular inflammation scattered foci and modest portal tract enlargement with inflammatory cells, hydropic degenration of hepatocytes and steatosis. Albendazole treated mice showed improved liver architecture and sections showed uniform liver with mild hydropic degenration of hepatocytes. *A. annua* treated mice showed same findings with mild portal tract inflammation and mild hydropic degenration of hepatocytes.

Acute tubular damage was in kidney of infected control mice. Mice treated with either albendazole and/or *A. annua* showed marked histopathologic improvement, with normal glomeruli and minimal degree of acute tubular injury in both treated mice groups.

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

Discussion

Generally, trichinosis (=trichinellosis**)**, is a zoonotic disease caused by nematode; *Trichinella* spp. During initial infection, intestinal invasion can result diarrhoea, abdominal pain, and vomiting, but larvae migrate to muscle, about a week post infection, cause face swelling, eyes inflammation, fever, muscle pains, and rash, but complications may include heart muscle inflammation, CNS involvement, and lungs inflammation. Minor infection may be asymptomatic (CDC, 2015).

Early administration of anthelmintics, such as mebendazole or albendazole, decreases a likelihood of larval encystation, especially if given within 3 days of infection (John and William, 2006). In man, mebendazole (200-

400mg three times a day for 3 days) or albendazole (400mg twice a day for 8-14 days) was given to treat trichinosis (CDC, 2016). Besides, medicinal plants are a rich source in bioactive compounds to product new safe drugs (Tonuci *et al*, 2012).

The present results showed that *A. annua* ethanol extract proved as effective as albendazole in preventing larvae from migrating and encystation. The phytotherapy or herbalism medications are commonly used in all medical settings, making it essential for primary care providers to learn about the products being used and resources they can access for continuing education (Falzon and Balabanova, 2017). Meanwhile, herbs played a major part in Egyptian medicine. The plant medicines mentioned in the Ebers Papyrus for instance include opium, cannabis, myrrh, frankincense, fennel, cassia, senna, thyme, henna, juniper, aloe, linseed and castor oil, though some of translations were less than certain. Cloves of garlic have been found in Egyptian burial sites, including the tomb of Tutankhamen and in the sacred underground temple of the bulls at Saqqara. Many herbs were steeped in wine, which was then drunk as an oral medicine (Patrick *et al,* 2009). Besides, Abdel-Azim *et al.* (2011) added that Egypt is characterized by abundant production of medicinal and aromatic plants that are exported worldwide. Cheong *et al.* (2020) reported that artemisinins isolated from the *A. annua* regulated the expression of pro-inflammatory cytokines, nuclear factor-kappa B (NF-κB), matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), promote cell cycle arrest, drive reactive oxygen species (ROS) production and induce Bak or Bax-dependent or independent apoptosis. They successfully used *A. annua* as anti-malarial herbal treatment.

In the present study, treatments were given for three days on the 15th day post-infection. Histopathologically testing was used to assess the effectiveness. *T. spiralis* larvae generated considerable interstitial edema in non-treated mice, with a persistent inflammatory cell infiltration dividing muscle bundles. Treatment with albendazole or *A. annua* ethanol extract improved oedema and muscular irritation. It is widely recognized that invading muscles with *T. spiralis* larvae results in mononuclear cell infiltration, ending in myositis (Beiting *et al*, 2004). Damage to skeletal muscle cells is induced not only by the parasite, but also by the presence of inflammatory cells, which are responsible for high levels of free radical generation (Bruschi and Lucchi 2001).

In the present study, as compared to albendazole-treated and infected-untreated mice, the inflammatory response to *A. annua* ethanol extract treated infected mice was significantly valuable. Undoubtedly, this was due to the anti-inflammatory activities of *A. annua* ethanol extract (Speroni *et al*, 2005).

In the present study, the liver of infected non-treated mice showed dispersed foci of lobular inflammation and modest portal tract enlargement with inflammatory cells, hydropic degeneration of hepatocytes and steatos is. Guattery *et al*. (1956) found homogeneous fatty deterioration on microscopic inspection due to the most severe form of phosphorus overdose. At necropsy, three others have discovered fatty degeneration. Frothingham (1906) reported migrating larvae outside an artery in an area of parenchymal necrosis and larvae in a sinusoid without obvious surrounding response during an autopsy.

In the present study, treatment of the *Trichinella* infected mice with Albendazole and *A. annua* ethanol extract improved their liver architecture.

In the present study, kidney of non-treated animals showed acute tubular injury, but in mice treated with either albendazole or *A. annua* ethanol extract, renal sections showed marked improvement. No doubt, renal trichinellosis caused tubular necrosis, glomerular congestion and hypercellularity, mesangial proliferative glomerulonephritis, interstitial edema, and renal infarctions (Sitprija *et al*, 1980; Sioris *et al*, 1980).

In human trichinellosis, the patients experience mild proteinria and abnormal urinary sediments, which were cleared up after proper treatment (Askanazy, 2005).

Nevertheless, dietary laws of Judaism and Islam prohibit eating pork, and in the 19th century, when the association between trichinosis and undercooked pork was first established, this association was suggested to be the reason for the prohibition, reminiscent of the earlier opinion of medieval Jewish philosopher Maimonides that food forbidden by Jewish law was unwholesome. This theory was controversial, and eventually fell out of favour (Harris, 1985).

Conclusion

The outcome results showed that *Artemisia annua* ethanol extract (*Shih 'anwa*) was as effective as Albendazole® against the migrating *Trichinella spiralis* larvae, and consequently, protected the infected hosts from trichinellosis' kidney, liver, ocular and other complications.

The best way to prevent trichinellosis is to cook pig-meat to safe temperatures. A food thermometer should be used to measure the internal temperature of cooked meat. Do not sample meat while cooking.

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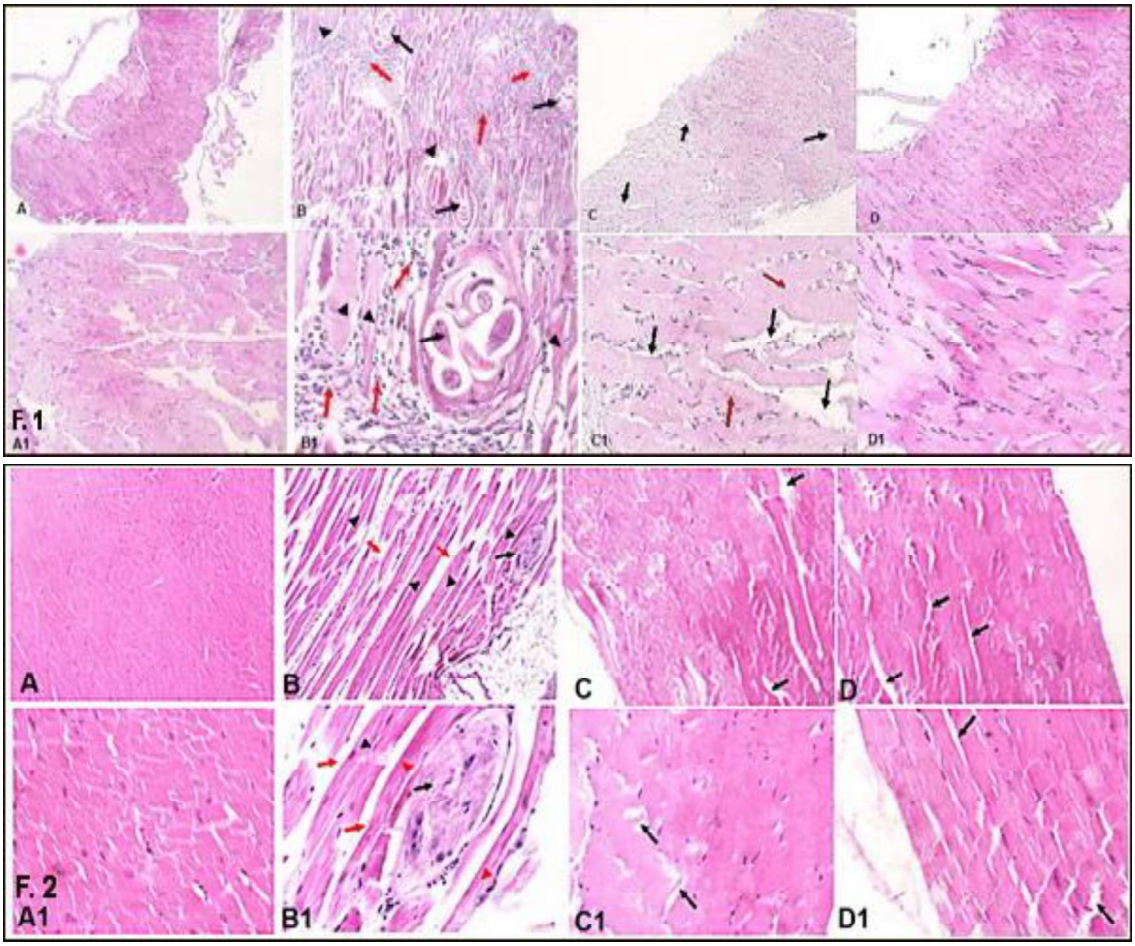
Explanation of figures

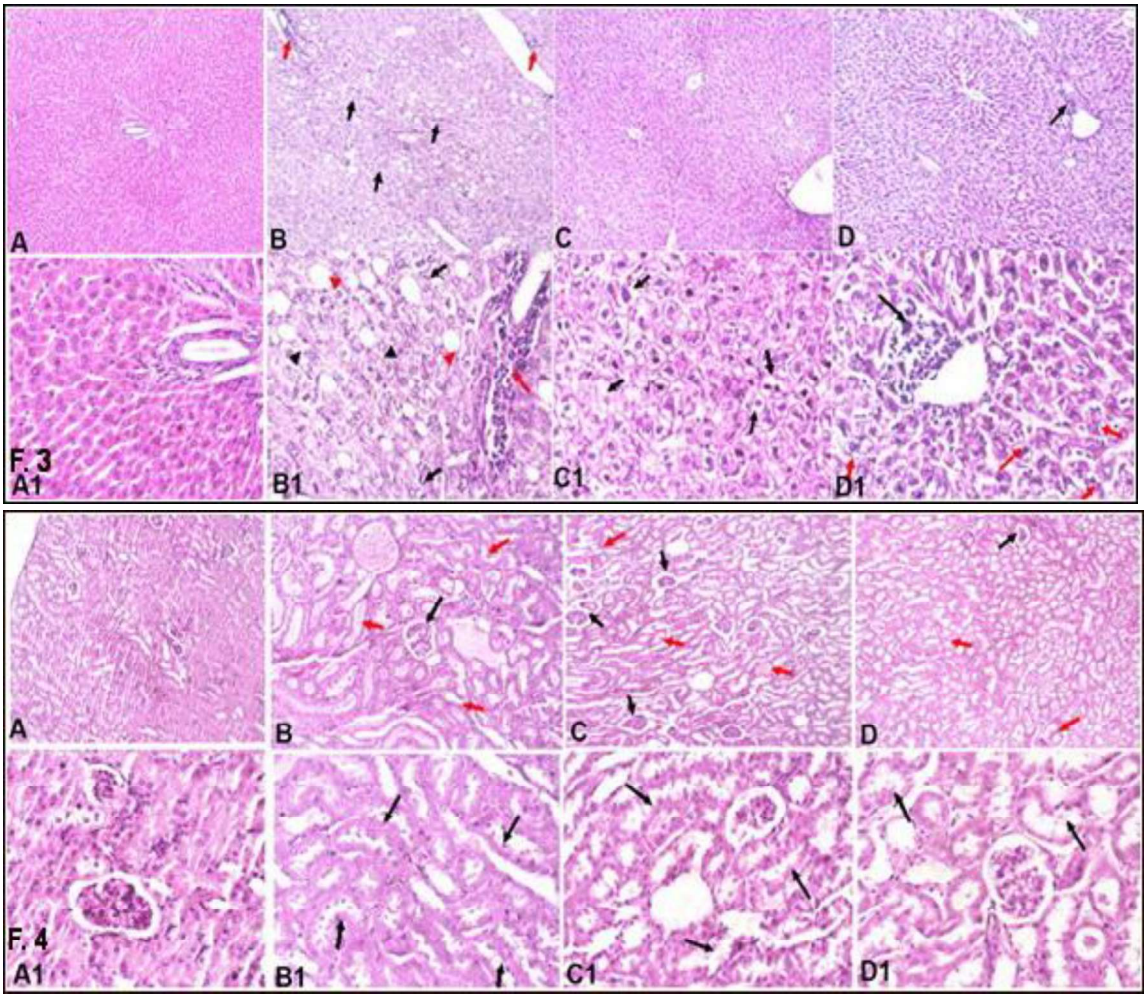
Fig. 1: A: Diaphragm of control mice showed uniform normal muscle bundles without larvae. B: Diaphragm of infected non treated mice showed several larvae (Black arrows) embedded within diaphragm muscle with inflammatory cell infiltrate in diaphragm muscle composed of lymphocytes and plasma cells (Red arrows). C: Diaphragm of Albendazole treated mice showed uniform muscle bundles without larvae. Mild edema (Black arrows) and few myocytes showed necrosis (Red arrows). D: Diaphragm of *Artemisia* treated mice showed uniform normal muscle bundles with no evidence of parasitic larvae (H & E; 10x & 40x).

Fig.2: A: Skeletal muscles of control mice showed uniform skeletal muscle bundles. B: Skeletal muscles of infected non treated mice showed larvae (Black arrow) embedded within muscle bundles (black Arrows) with significant interstitial edema and chronic inflammatory cells infiltrate separating muscle bundles (Red arrows). C: Skeletal muscles of Albendazole treated infected mice showed uniform muscle tissue with mild interstetial edema (Black arrows). D: Skeletal muscles of *Artemisia* treated mice showed uniform muscle bundles without edema in between (H&E: 10x & 40x).

Fig. 3: A: Liver of normal mice showed uniform hepatocytes, with uniform portal tract. B: Liver of infected non treated mice showed scattered foci of lobular inflamamtion (Black arrows) and mild portal tract expansion with inflammatory cells (Red arrows), hydropic degenration of hepatocytes (Black arrowheads) and steatosis (Red arrowheads). C: Liver of Albendazole treated infected mice showed mild hydropic degenration of hepatocytes (Black arrows). D: Liver of *Artemisia* treated infected mice showed mild portal tract inflammation (Black arrow) and mild hydropic degenration of hepatocytes (Red arrows) (H&E: 10x & 40x).

Fig. 4: A: kidneys of normal mice showed uniform renal tissue, normal glomeruli and tubules. B: kidneys of infected, but non-treated mice showed normal glomeruli (Black arrow) and evidence of acute tubular injury (Red arrows). C: kidneys of Albendazole treated infected mice showed renal tissue normal glomeruli (Black arrow), some tubules evidence of acute tubular injury (Red arrows). D: kidneys of *Artemisia* treated infected mice showed renal tissue normal glomeruli (Black arrow), few tubules evidence of acute tubular injury (Red arrows) (H&E: 10x & 40x).

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