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Original Article - Veterinary Medicine

The suppressive properties of sheep bile against the *Plasmodium chabaudi* parasite in mice

[*Propriedades supressoras da bile de ovelha contra o parasita Plasmodium chabaudi em camundongos*]

M. *Murshed*, H.M.A. *Aljawdah*, M.M. *Mares*, S. *Al-Quraishy*

Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

ABSTRACT

Plasmodium species are responsible for the transmission of malaria, which is still one of the most dangerous diseases to humans in the world. This study aimed to evaluate the suppressive effect of SB on parasitemia in mice infected with *Plasmodium chabaudi* infection. A total of 30 disease-free mice were randomly assigned to six groups. The first control non-infected group received only distilled water daily for 7 days by oral route. After being infected with 10^6 of *P. chabaudi* in the other five groups, the mice were gavaged with 0.2mL/mice of a solution containing either 25%, 50%, or 100% of SB, respectively. The fifth group orally received 10mg/ kg chloroquine phosphate (CQ). The sixth set of mice served as the infected group. Following the administration of treatments during a suppression test that lasted for five days, a daily examination of blood smears stained with Giemsa was performed. Sheep bile was able to decrease parasitemia nearly to the used reference drug, chloroquine. In addition, bile significantly decreased the diarrhea rate of infection in mice, the survival rate of mice, the parasitemia percentage, and the suppression ratio. The parasite's caused histological change was enhanced by the SB. After treatment, there was also a rise in the amounts of the liver enzymes alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase. Findings indicate that bile has antimalarial activity and can control parasitemia percentage and parasite suppression. We need further investigations to determine the mechanisms of action of the bile in vivo.

Keywords: malaria, suppression, parasitemia, sheep bile, chloroquine

RESUMO

As espécies de Plasmodium são responsáveis pela transmissão da malária, que ainda é uma das doenças mais perigosas para os seres humanos no mundo. Este estudo teve como objetivo avaliar o efeito supressor do SB sobre a parasitemia em camundongos infectados com Plasmodium chabaudi. Um total de 30 camundongos livres da doença foram divididos aleatoriamente em seis grupos. O primeiro grupo de controle não infectado recebeu apenas água destilada diariamente por 7 dias por via oral. Depois de serem infectados com 106 de P. chabaudi nos outros cinco grupos, os camundongos foram submetidos à gavagem com 0,2mL/camundongo de uma solução contendo 25%, 50% ou 100% de SB, respectivamente. O quinto grupo recebeu oralmente 10mg/kg de fosfato de cloroquina (CQ). O sexto conjunto de camundongos serviu como o grupo infectado. Após a administração dos tratamentos durante um teste de supressão que durou cinco dias, foi realizado um exame diário de esfregaços de sangue corados com Giemsa. A bile de carneiro foi capaz de reduzir a parasitemia de forma quase semelhante ao medicamento de referência usado, a cloroquina. Além disso, a bile diminuiu significativamente a taxa de diarreia da infecção em camundongos, a taxa de sobrevivência dos camundongos, a porcentagem de parasitemia e a taxa de supressão. A alteração histológica causada pelo parasita foi aprimorada pela SB. Após o tratamento, houve também um aumento nas quantidades das enzimas hepáticas fosfatase alcalina, aspartato aminotransferase e alanina aminotransferase. Os resultados indicam que a bile tem atividade antimalárica e pode controlar a porcentagem de parasitemia e a supressão do parasita. São necessárias mais investigações para determinar os mecanismos de ação da bile in vivo.

Palavras-chave: malária, supressão, parasitemia, bile de carneiro, cloroquina

Corresponding author: mutee3@gmail.com; mmurshed@ksu.edu.sa
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INTRODUCTION

Malaria remains a serious threat to public health on a global level (Snow *et al*., 2005), it is one of the most common insect-borne diseases and it is a vital bloody human parasite (Huang *et al*., 2020). It is life-threatening and caused by Anopheles mosquitoes (Ribeiro, 2017). The newest WHO malaria report estimates 241 million malaria infections and 627,000 deaths worldwide in the year 2020. This means 14 million more patients and 69,000 more fatalities in 2020 than in 2019. Two-thirds of these deaths (47,000) were caused by pandemic malaria prevention, diagnostic, and treatment interruptions (World…, 2021). Symptoms of the disease are in the form of fever, chills, weakness, headache, vomiting, diarrhea, anemia, pulmonary and renal dysfunction, and neurologic changes (Ali, 2018). Five main types of Plasmodium parasites cause malaria in humans: *p falciparum, P. vivax, P. ovale, P. malariae,* and *P. Knowlesi*, the most important of these types is P. falciparum, which causes deadly acute malaria (Leslie *et al*., 2014).

Despite ongoing efforts to combat malaria with several medications, such as chloroquine, halofantrine, pyrimethamine, mefloquine, quinine, and artemisinin (Rajendran, 2013), there are many problems, including continued developed resistance to antimalarial medications, lack of effective vaccines, the resistance of mosquitoes that carry insecticides, and social and economic problems, which make the treatment of malaria with chemical drugs somewhat ineffective and has many side problems (Douradinha and Doolan, 2011). The pathogenicity and mortality burden of malaria could be reduced by improving malaria diagnosis, strengthening prevention, using correct therapies, and adopting strategies aimed at preventing drug resistance (Autino *et al*., 2012). Understanding the mechanisms of *Plasmodium* invasion will guide the development of novel vaccines to interrupt the invasion process and prevent disease and transmission of malaria (Garrido-Cardenas *et al*., 2019).

It should be noted that natural products play an effective role in treatments and have been the main source of drugs for many centuries (Zirihi *et al*., 2005). The treatment of malaria mainly in malaria-endemic areas is based on the use of traditional herbal medicines (Castellanos *et al*., 2009), and a worldwide focus on medicinal products in the search for effective anti-malarial drugs (Schuster, 2001). Alternately, natural remedies derived from plants, such as extracts of herbs, are being investigated as potential therapeutic options. Some people in Indonesia believe that consuming intact goat gallbladder is an effective way to treat and prevent malaria (Arwati *et al*., 2020).

The gallbladder is a pear-shaped organ that is responsible for storing bile. Hepatocytes are responsible for the production of bile, which is a digestive fluid. In addition to water and electrolytes, bile is made up of organic components such as bile salts, cholesterol, phospholipids, and bilirubin, as well as ingested substances like proteins. Bile also has bilirubin. When it comes to the digestion and absorption of fats and vitamins that are fat-soluble from the small intestine, bile acids are extremely significant (Boyer, 2019; Jones *et al*., 2019). Traditional Chinese medicine (TCM) has made use of animal bile for the treatment of chronic as well as acute infectious and non-infectious disorders, including malaria, for many centuries. The production of bile is necessary for proper liver function; it also helps break up gallstones and prevents the spread of bacteria and viruses (Wang and Carey, 2014). Research has shown that bile has qualities that make it anti-inflammatory, antipyretic, and antioxidant. However, there is no documented history of the use of goat bile (GB) to treat and cure malaria. Bear bile, on the other hand, is employed in the treatment of liver disease (Li *et al*., 2016). In traditional Chinese medicine, the use of goat bile is relatively common. According to Chinese traditional Medicine, goat bile is used therapeutically in China due effective benefit in treating optic atrophy, acute hemorrhagic conjunctivitis, and different infectious skin illnesses (Sitohang *et al*., 2018).

The acute and subacute toxicity studies performed on BALB/c mice using GB revealed that the mice experienced mild diarrhea (Arwati *et al*., 2020). As a result, the purpose of this research was to determine whether SB has a suppressive effect on parasitemia in mice that have been infected with the *Plasmodium chabaudi* parasite.

MATERIAL AND METHOD

Gallbladders obtained from sheep (*Ovis aries*) were sourced from a local animal slaughterhouse in Riyadh, Saudi Arabia. Gallbladders were removed from four healthy male sheep to study them. Following the spraying of 70% alcohol over the gallbladders, the bile was extracted using a syringe, moved to a clean tube, and pooled there before being diluted with distilled water to create 100% (SB100), 50% (SB50), and 25% (SB25) solutions. Before and throughout the trials, samples were kept at a temperature of 4 ° C.

For passage, cryopreserved parasites of the passage *P. chabaudi* strain were successfully transplanted three times into donor mice. The mice were given an intraperitoneal injection (Wunderlich *et al*., 2005) of a phosphate buffer solution that was 100 microliters in volume containing 10^5 *P. chabaudi* parasitized erythrocytes. To determine the dose that was to be supplied, the Neubauer chamber was utilized. and the parasitemia levels found in this blood were 28%.

Male C57BL/6 mice were used, *P. chabaudi* animal model of malaria that were gavaged diet and water *ad libitum*. Mice of the male gender, aged 10-12 weeks, weighing 19-23 gm, and behaving normally in terms of physical characteristics were active. During the experiment, sick or lifeless mice were not included. The mice in each group (5 mice) were classified according to the same set of inclusion and exclusion criteria. After 1 week of acclimatization, 30 mice were injected with 1×10^5 *P. chabaudi*-infected erythrocytes in 0.2 mL of blood suspension. Mice were then divided randomly into six groups before treatment. The first control non-infected group received only distilled water daily for 7 days by oral route. After being infected with 10^5 of *P. chabaudi*, the mice were gavaged with 0.2 mL/mice of a solution containing either 25%, 50%, or 100% of SB, respectively. the fifth group orally received 10mg/kg chloroquine phosphate (CQ) (Sigma-Aldrich, St. Louis, MO), (daily for 4 days). The sixth set of mice served as the infected group, and they were given sterile water to drink. Following the administration of treatments during a suppression test that lasted for five days, a daily examination of blood smears stained with Giemsa was performed.

Counting the number of red blood corpuscles that were infected with *P. chabaudi* parasites was used to calculate the quantitative relative content of blood parasites, which is also known as parasitemia. The amount of parasite that was present in the blood was determined by performing a blood test after a blood sample was obtained from the tail of the experimental mice. During the process of fixation, methanol has been utilized. Following the drying process, the Giemsa stain was applied. After being cleaned, the slides are inspected using a light microscope. According to Wunderlich *et al*. (2005), the calculation for the parasitemia percentage was carried out. Percentage parasitemia and percentage suppression were calculated as per the formula:

Parasitemia (%) = Number of infected erythrocytes Total number of erythrocytes*100
Chemo suppression% = (Mean Parasitemia of control) - (Mean Parasitemia in treated) (Mean Parasitemia of control)*100

Mice were weighed individually within all experimental classes. Owing to infection and care, body weight change was expressed as a percentage change in the wail relative to the control group.

Liver sections were prepared fresh for histopathological examinations, fixed in 10% neutral buffered formalin, and then embedded in paraffin. Hematoxylin and eosin were used to stain the sections after they had been cut and processed. The liver histology scoring was carried out in compliance with the guidelines provided by Ishak *et al*. (1995).

Alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured in blood plasma using commercial kits (Biomerieux, Marcy l'EtoilFrance) following the manufacturer's instructions.

Values were expressed by using the mean as well as the standard error of the mean. To determine significance a one-way analysis of variance (ANOVA) was performed, and Duncan's test was employed to make statistical comparisons across

the groups. The *P* values were all calculated using two tails, and a value of P less than 0.05 is considered significant for all statistical analyses.

RESULTS

Animals treated with SB developed a minor case of diarrhea. In the current investigation, there was diarrhea in some of the *P. chabaudi*-infected mice that were treated with SB, but not all of them. The greatest number of mice experienced diarrhea when they were given the highest dose of SB (SB100), and the infected group. The survival rate of infected mice treated with SB is shown in Figure 2. Survival estimates of *P. chabaudi*-infected mice treated with sheep bile (SB) compared with controls. All mice in SB100 survived and gave a 100% survival rate. One mouse in SB50 died on day 5 post-treatment and gave an 80% survival rate. One mouse of SB25 died on day 2, and another mouse died on day 4, resulting in a 60% survival rate, while in the infected group, two mice died on day 4 and all mice died on day 7p.i.

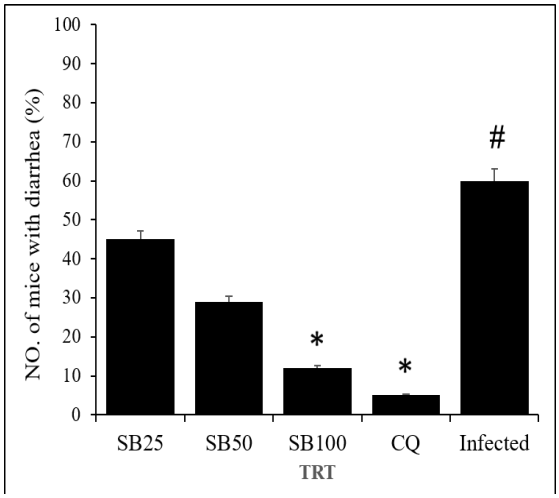

Figure 1. The number of mice with diarrhea, and the positive effect of sheep bile in mice infected with *Plasmodium chabaudi* parasite. * and # are significant compared infected group, at $P < 0.01$.

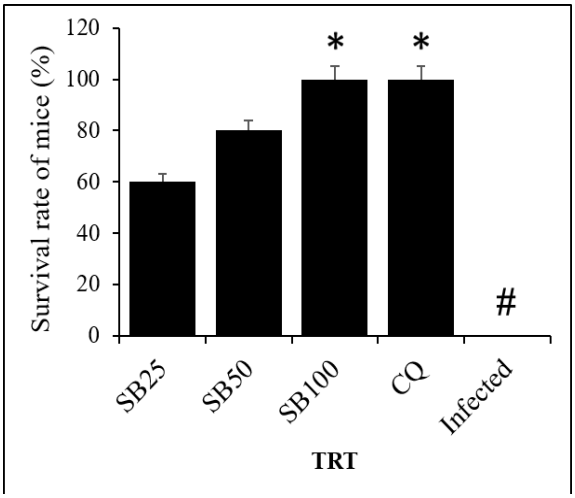

Figure 2. The Survival rate of mice (%), and the positive effect of sheep bile in mice infected with *Plasmodium chabaudi* parasite. * and # are significant compared infected group, at $P < 0.01$.

To control parasitemia in a dose-dependent manner, oral administration of 25%, 50%, and 100% of SB reduced it by 45.12%, 81.99%, and 94.97%, respectively (Fig. 3). The level of parasitemia was lower in the animals who were given SB (Fig. 4). After the treatment was completed, the parasitemia was monitored for another two days. The normal pattern of development of daily parasitemia in infected mice was seen, with a subsequent dramatic increase to 36.33 % on day 6 after treatment. The levels of parasitemia in mice treated with SB25 were comparable. In mice treated with SB50, parasitemia didn't occur until day 3 after treatment and reached 4.48% at that point (p=0.005). Parasites arrived on day 2 after treatment and reached 18.99% of the population at that point. Mice treated with SB100 exhibited suppression in a manner analogous to mice treated with CQ. The prevalence of parasitemia was 1.5% (p = 0.030). On day 5, parasitemia was completely suppressed in animals that had been treated with CQ; however, by day 6, a few erythrocytes had become infected, and by day 7, parasitemia had reached 0.46%. The CQ therapy resulted in a 99.57% reduction in infection (Fig. 5).

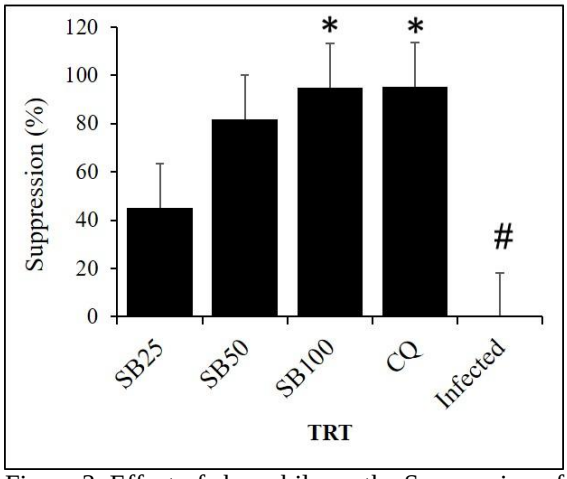

Figure 3. Effect of sheep bile on the Suppression of parasitemia of mice infected with *Plasmodium chabaudi.* * and # are significant compared infected group, at $P < 0.01$.

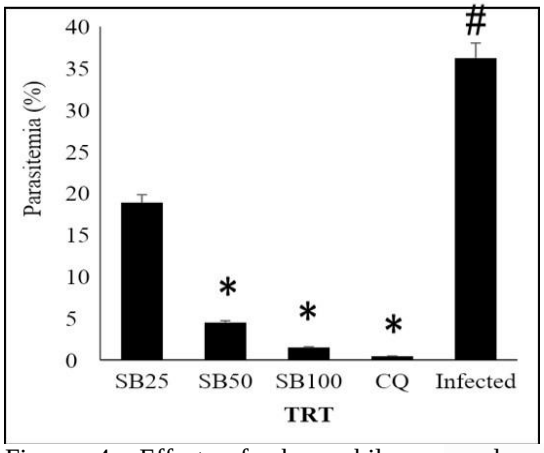

Figure 4. Effect of sheep bile on reduced parasitemia of mice infected with *P. chabaudi*. (*) significance at $p < 0.01$ against the infected group on day 7 parasitemia. * and # are significant compared infected group, at $P < 0.01$.

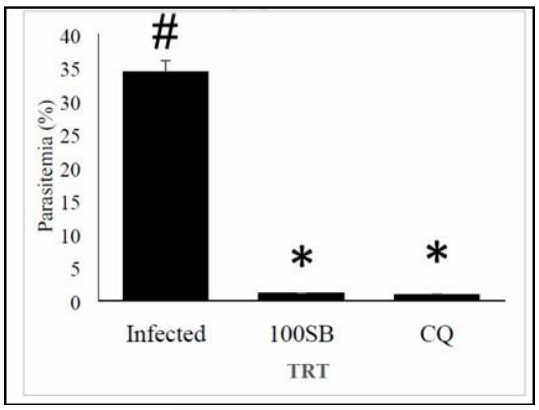

Figure 5. SB reduced parasitemia of mice infected with *P. chabaudi*. (*, #) significance at $p < 0.01$ against the infected group on day 7 parasitemia.

Beginning on day 4 following infection with *P. chabaudi* infected with erythrocytes, SB treatment resulted in a significant drop in the level of parasitemia. On day 7 post-infection, there was a drop in parasitemia of 41.9% in comparison to that in the mice that had been infected (Figure 6). When compared to the infected group, the level of parasitemia suppression that was achieved by administering SB100 to the infected mice was much higher. In addition, 10 milligrams per kilogram of chloroquine (CQ) was administered to the mice.

Plasmodium chabaudi infection resulted in a substantial (P≤0.05) decrease in the weight of mice. However, the rate of weight loss in the infected group was significantly (P≤ 0.05) decreased to 21% when compared to the non-infected group, in which the weight of mice increased by 14%. When compared to the infected group, the weight increased by 27 and 30 % in the treated groups receiving 100 SB dosage and CQ, respectively (Figure).

No alteration was observed in the hepatic architecture in the histological analysis of liver specimens from infected animals (Fig. 9). The liver experienced a significant histological alteration because of the P. chabaudi infection. Hepatic infiltration, apoptotic bodies, dilated sinusoids, malaria pigments, and hyperplasia of Kupfer cells were the manifestations of this. Significantly, the liver's histological abnormalities might be reduced by SB treatment.

As can be seen in Figures 10, 11, and 12, the liver of the infected group (+SB) exhibited a significant malfunction (p<0.01) in comparison to the liver of the control group. A notable dysfunction was seen. During the infection, the levels of AST, ALT, and ALP in the blood plasma were elevated. The administration of SB to infected mice resulted in a reduction in the activity of AST and ALT, while simultaneously causing an increase in the activity of ALP.

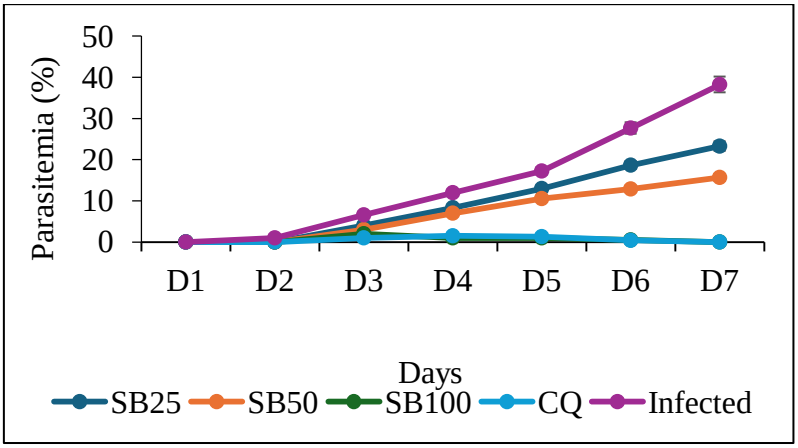

Figure 6. Changes in parasitemia after treatment of *P. chabaudi*-infected mice with SB: SB25, SB50, and SB100 in a 5-day suppressive compared with that of CQ and infected. Values are means ± SD. *Significant against the infected group.

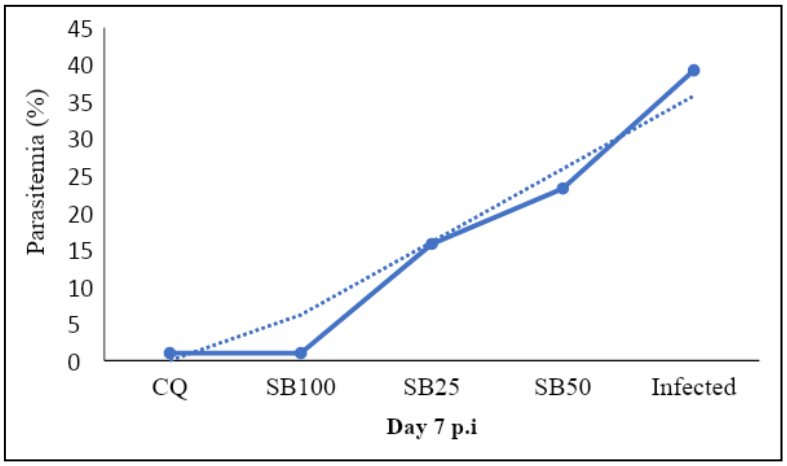

Figure 7. Mean parasitemia % of mice infected with *plasmodium. chabaudi* on day 7p.i for several concentrations.

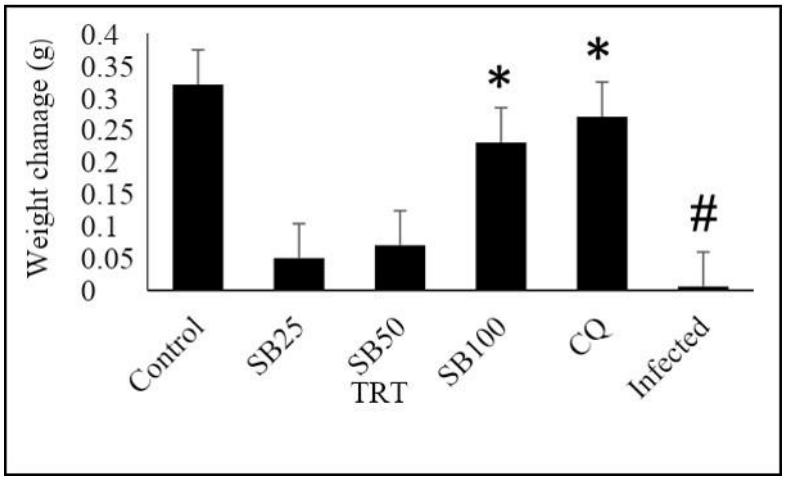

Figure 8. Weight changes of mice due to infection with *P. chabaudi* on day 7 p.i. * and # are significant compared infected group, at $P < 0.01$.

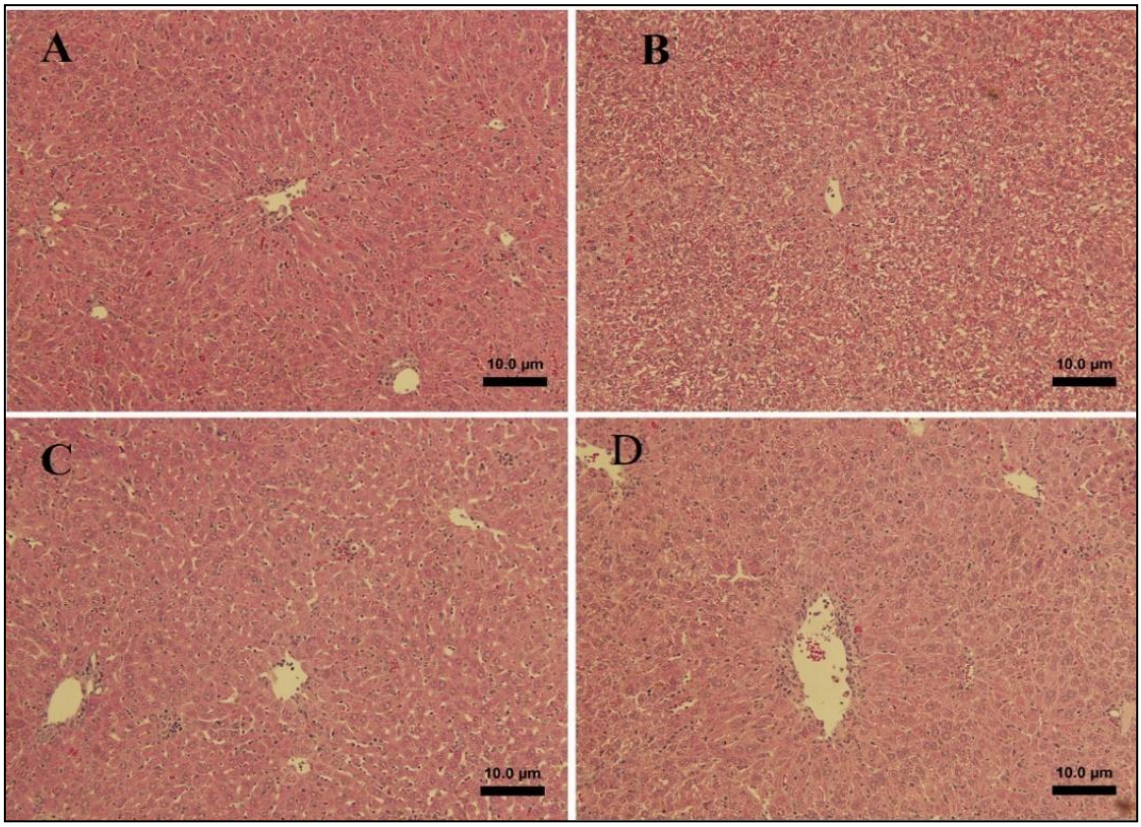

Figure 9. Liver histopathology of mice. a, non-infected control liver sections with hepatic parenchyma radiating from the central vein (cv). b, *P. chabaudi*–infected mice liver exhibiting inflammatory changes, dilated sinusoids with increased cells, apoptotic bodies, and malaria pigments. c, the infected-treated mouse liver 100% SB exhibiting improves tissue structure. d, Infected CQ-treated mice liver exhibiting reduced tissue damage. Sections were stained with hematoxylin and eosin. Bar = 20µm.

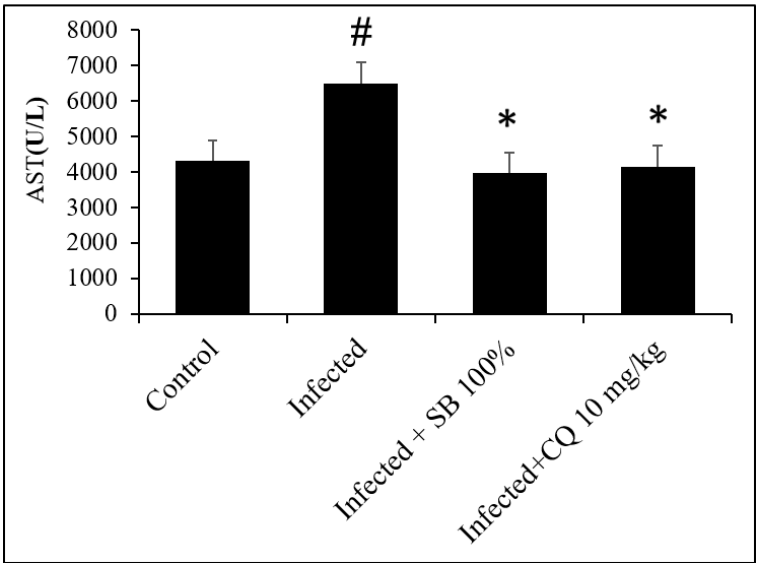

Figure 10. The influence of SB on the liver enzyme ALP, in mice that were infected with *P. chabaudis*. * and # are significant compared infected group, at $P < 0.01$.

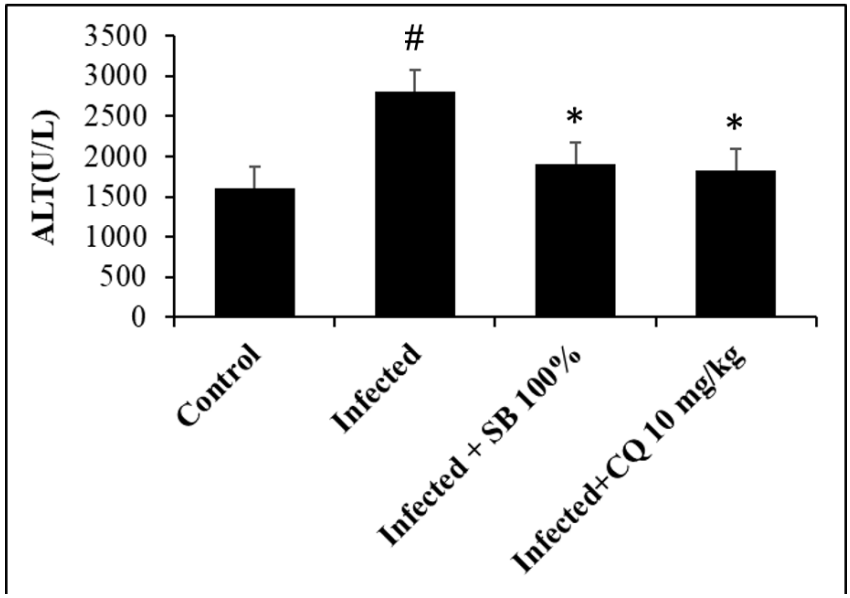

Figure 11. The influence of SB on the liver enzyme AST in mice that were infected with *P. chabaudis.* * and # are significant compared infected group, at $P < 0.01$.

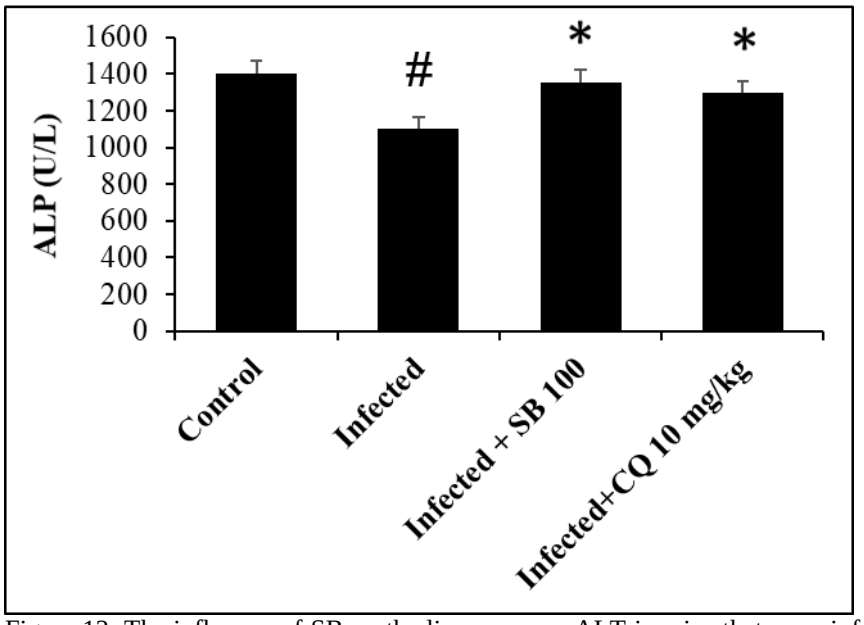

Figure 12. The influence of SB on the liver enzyme ALT in mice that were infected with *P. chabaudi*, * and # are significant compared infected group, at $P < 0.01$.

DISCUSSION

There is evidence that animal bile possesses antimalarial properties (Farrugia and Arasaradnam 2020). Within 2 days of receiving the SB therapy, mice that were not infected had a moderate case of diarrhea (Arwati *et al*., 2020). This response was also observed in mice infected with *P. chabaudi* that had been treated with GB (Farrugia and Arasaradnam 2020). The traditional signs and symptoms of bile acid malabsorption include watery stool, increased bowel frequency, urgency, nocturnal defecation, excessive flatulence, stomach pain, and stool

incontinence (Wardani *et al*., 2020). When there is an excess of bile acid in the human colon, this can also induce bile acid malabsorption. Malabsorption of bile acids and diarrhea ranging from mild to severe were also side effects of orally administering SB to mice. Other symptoms, such as the regularity of bowel movements, were not included in the documentation. There is not a single verifiable case of diarrhea in humans that was caused by the eating of complete sheep gallbladders for the treatment of malaria or to boost stamina.

The fact that the antimalarial treatment was able to virtually completely suppress the parasitemia that was seen in CQ mice demonstrates that the drug is an appropriate choice for use as a positive control (Wardani *et al*., 2020). The levels of parasitemia in mice that had been treated with SB100 were slightly greater than the levels in these control animals, but they were much lower than the levels in mice that had not been treated. This level of effectiveness had previously been documented (Wardani *et al*., 2020). A considerable reduction in parasitemia (94,97%) was seen after treatment with SB100, indicating that its efficacy was comparable to that of CQ. It appears that there is a dose-response relationship at play here because SB was less effective in lower concentrations. The ability of Plasmodium-infected mice to survive was also proportional to the dose of SB administered. Greater amounts of GB related to longer periods of survival. The survival rate of infected mice treated with GB100 was 100%, which was the same as the survival rate reported in animals treated with CQ. However, administration of SB100 was associated with a greater number of cases of diarrhea. Only one of the mice received GB50 and experienced diarrhea as a result. There is a possibility that a dose that is equivalent to SB50 will result in a considerable reduction in parasitemia (81.99%). The indigenous people of Indonesia have traditionally used GB as a treatment for malaria. In this treatment, the entire gallbladder is taken directly, without any preparation (100% GB). The antimalarial activity of SB is consistent with this traditional use of GB.

There is a need for additional research on the mechanism of action that SB employs to inhibit the multiplication of parasites in erythrocytes. This activity may reflect the complexity of bile components, such as the amphipathic characteristics of bile acids, which are linked to both the advantages and the toxicity of the bile. Hydrophilic bile acids such as ursodeoxycholic acid (UDCA) and tauroursodeoxycholic acid assist in the healing of damage and protect against the toxicity of hydrophobic bile acids such as deoxycholic acid (Arwati *et al*., 2020). The high pH of bile may produce an alkaline environment in the parasite's normally acidic feeding vacuole. CQ kills malaria parasites by alkalinizing the vacuole, which is the mechanism of action (Homewood *et al*., 1972), and the susceptibility of malaria parasites to CQ in humans is pH dependent (Yayon *et al*., 1985). The CQ molecule has a weak basic property, and this characteristic controls the accumulation of CQ in vacuoles (Homewood *et al*., 1972; Yayon *et al*., 1985). The malaria parasite feeds on the hemoglobin found in erythrocytes and digests its meal in vacuoles that have an acidic pH. Alterations in pH prevent the breakdown of hemoglobin by cysteinase, which ultimately results in the death of the parasite.

The liver functions as an effector against Plasmodium infection, causing alterations in liver histology that ultimately lead to liver failure. Wunderlich *et al*. (2005) documented the same findings by infecting mice with erythrocytes infected with *P. chabaudi.* In general, sheep bile can lower the activity of enzymes involved in liver function. The study found that administering SB to animals infected with *P. chabaudi* resulted in a reduction of blood plasma ALT, AST, and ALP levels. This indicates that the consequences of liver damage caused by malaria can be ameliorated by bile. The possible therapeutic impact of SB on liver enzyme activity that has been reported may be due to the removal of parasites, which may be obtained from their bioactive components. (Arwati *et al*., 2020; Aini *et al*., 2020).

CONCLUSIONS

The results of this study provide proof that sheep bile is relatively safe and extremely potent in *P. chabaudi*-infected mice, which validates the use of SB in traditional medicine to treat malaria and symptoms connected to the disease. To feed the pipeline for the discovery of antimalarial drugs, however, further research into bile should be conducted to isolate and characterize its bioactive component.

ACKNOWLEDGMENTS

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