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Antischistosomal Effects of Selected Methanolic Plant Extracts in Swiss Albino Mice Infected with *Schistosoma mansoni*

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Authors' contributions

This work was conducted in collaboration between all authors. Authors JMM and SNN designed the study. Authors JMM and JKM was provided the study materials and laboratory reagents. Authors JMM, SNN and MAO participated in data analysis, data interpretation. Author JMM wrote the protocol, carried out the study and wrote the first manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: *Schistosoma mansoni* is a parasite of medical importance because it is the causative agent of intestinal schistosomiasis. The present study was designed to assess the *in vivo* antischistosomal effects of methanolic extracts of three food plants: Apple (*Malus domestica*), Lemon (*Citrus limon*)

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and Onion (*Allium cepa*) on Swiss Albino mice infected with *Schistosoma mansoni.*
Study Design: Swiss albino mice were infected and randomized into groups of five for plant extract treated groups (high and low dosages), negative control were given a vehicle administered via intraperitoneal route twice daily for five days 7 weeks post-infection after which percentage worm reduction and glutathione levels were evaluated.
Place and Duration of Study: The study was conducted at the Centre of Biotechnology Research and Development (Animal Facility), Kenya Medical Research Institute from July, 2013 to May, 2014.
Methodology: Swiss albino mice infected with 90 cercariae each were administered with high and low dosages of plant extracts twice daily for five days (7 weeks post-infection) after which worm densities and hepatic oxidative stress were assessed.
Results: In general, upon administration of increased concentration of the plant extracts, there was significant (P<0.05) reduction in worm densities with a concomitant increase in GSH activity. *Malus domestica* showed significant (P<0.05) antischistosomal activity at concentrations 300 mg/kg and 200 mg/kg giving 85.93% and 72.22% worm reduction respectively, *Allium cepa* at concentrations 500 mg/kg and 300 mg/kg gave 72.59% and 58.52% respectively, with least worm reduction resulting from treatment with *Citrus limon* at dosages of 200 mg/kg and 100 mg/kg (42.96% and 29.63% respectively).
Conclusion: The data demonstrated that, treatment of mice with plant extracts ameliorated oxidative stress induced by Schistosomiasis as indicated by significant (P<0.05) improvement of GSH level compared to schistosome-infected control group. This is expected to have serious ramifications on the management of this tropical disease which hitherto is solely dependent on the use of drugs.

Keywords: Schistosomiasis; glutathione; antioxidants; neglected tropical disease; medicinal plant.

ABBREVIATIONS

GSH-Glutathione; KEMRI-Kenya Medical Research Institute; PZQ-Praziquantel; smTGR-Schistosoma mansoni Thioredoxin/Glutathione Reductase; DTNB-5,5'-Dithiobis-2-nitrobenzoic acid; EDTA-Ethylene Diamine Tetraacetic Acid; TCA-Trichloroacetic Acid; ROS- Reactive Oxygen Species.

1. INTRODUCTION

Intestinal schistosomiasis is a common helminthic infection caused by blood dwelling trematodes, *Schistosoma mansoni*. The disease still remains a major problem among people living in low income countries and especially in conditions of poor sanitation since its mode of transmission tends to limit to people who are in contact with contaminated waters in endemic countries [1]. For this reason, it is endemic in the tropical and subtropical countries and ranks second to Malaria in terms of socio-economic and public health burden [2]. Schistosomiasis affects almost 210 million people worldwide [3] with an estimated 12,000 [4] to 200,000 deaths reported annually and is responsible for causing severe morbidity, anemia, significant growth retardation, educational and nutritional effects not only among children but even to adults living in endemic areas [5].

Schistosomiasis causes a progressive reduction in the levels of protective endogenous antioxiodants such as glutathione (GSH) [6] and increases generation of free radicals [7]. This case of parasite-oxidant/ host-antioxidant system imbalance creates an oxidative stress [8]. Oxidative free radicals inflict host tissue damage, impair mitochondrial function and elevate lipid peroxidation [9]. The oxidative processes that occur during *S. mansoni* infection suppress the host enzymatic detoxification activities, thus playing a role in pathogenesis of schistosomiasis [10].

The parasite, *S. mansoni,* is intravascular and manages to live in seemingly hostile environments in close contact with host humoral and cellular cytotoxic factors [11]. In order for them to thrive in such environments, schistosomes employ strategies for evading host immune responses. For example, they constantly produce antioxidant enzymes that continually degrade host-derived Reactive Oxygen Species. A principal component of this defense system which is exploited by adult schistosomes for survival has been recently identified as

S. mansoni Thioredoxin/Glutathione Reductase (smTGR) [12]. This is a parasite-specific enzyme that combines the functions of two human counterparts: Glutathione Reductase and Thioredoxin Reductase [13-14]. As such, this enzyme presents an attractive new target for anti-schistosomiasis drug development [15].

Currently, the main strategy for managing the parasite in humans relies on the administration of the drug, praziquantel (PZQ) [16]. This is the drug of choice in the treatment of schistosomiasis mainly because it is active against most of the known schistosome species with little or no adverse side effects [17]. Cioli and Pica-Mattoccia [18] hypothesized that the drug selectively dysregulate Ca^{2+} homeostasis targeting the interface between α_1 and β subunits in the voltage-gated Ca^{2+} channels of the parasites leading to spastic muscular paralysis. Additionally, PZQ causes damage to schistosome tegument leading to changes in antigen presentation and activation of host immune response against the parasite. Finally, the drug inhibits adenosine and uridine uptake in schistosomes causing mortality through disruption of *de novo* synthesis of purine nucleotides [19].

While the use of PZQ appears promising for the management of schistosomiasis, there are significant limitations associated with its use. The major one being that there are drug-resistant strains of the parasite that have been reported in endemic areas such as Kenya and Egypt a scenario which is compromising the efficacy of PZQ in managing schistosomiasis [17,20-21].

For that reason, it is important to prospect for novel therapeutic substances that may replace PZQ or be used in combination, in order to mitigate the evolution of resistance. Praziquantel is unable to attenuate the oxidative stress [12] in the tissue directly, but rather it can modulate or decrease the activity of host's antioxidant systems. We focused our attention to the parasite antioxidant pathway, since the parasite is subjected to a high oxidative stress mainly because of host's immune response. Hence interfering with this pathway may help in the search of new therapies [15]. The plant kingdom has continued to play an important role in the discovery of novel and useful phytomolecules that are being tapped in modern medicine. Thus the plants used in this study were selected on the basis of their known properties as follows: apples (*Malus domestica*) have high antioxidant activity [22] which is mostly attributed to phenolic compounds such as flavonoids and phenolic acids [23]. Methanolic extracts of lemon (*Citrus limon*) peels have bioactive compounds including antioxidants such as ascorbic acid, flavonoids, phenolic compounds and pectins [24]. Finally, onions (*Allium cepa*) contain phenolics and flavonoids that also possess antioxidant properties [25]. Since PZQ treatment has been reported to reduce hepatic protein content including GSH [26], it was excluded in this study and we assessed the therapeutic potential of these antioxidant plant extracts alone to evaluate their protective role against *S. mansoni*-induced hepatic oxidative stress in mice.

2. MATERIALS AND METHODS

2.1 Methanolic Plant Extracts Preparation

Fresh plant materials, Apple fruits (red), green Lemon peels and onion bulbs were obtained from a local retail grocery. They were washed in distilled water, hand cut into small pieces (about 2 cm) using a laboratory blender and pulverized in a mortar and pestle. The paste of each fruit was separately air-dried at room temperature (23±2ºC) in darkness until constant weight. 120 g of each powder was then macerated in analytical grade Methanol (300 ml) for 72 hours with intermittent shaking then filtered (Whatmann, 125 mm). The excess methanol was removed under vacuum in a rotary evaporator (Laborota 4000, Germany) at 35-40ºC then air dried yielding a dry weight of 9.30 g (*Malus domestica*), 8.30 g (*Allium cepa*) and 7.56 g (*Citrus limon*). Various concentrations of the plant extracts were prepared and used for bioassays.

2.2 Mice, Parasite and Experimental Infection

Thirty five male Swiss albino mice weighing 20±2 g were used in the experiment. The animals were obtained from Kenya Medical Research Institute (KEMRI) animal breeding facility, Nairobi-Kenya. The animals were randomly divided into seven groups of five mice each and moved into the experimental room for acclimatization six weeks before the start of the experiments. The mice were housed in 15 cm × 21 cm × 29 cm transparent plastic cages. They were maintained under 12 hours of light and 12 hours darkness during the study and fed with pellets (Mice pellets UNGA® feeds) and water *ad libitum*.

Kenya laboratory maintained (KEN-lab) strain of *S. mansoni* parasite was used in the study. The isolate had been routinely maintained in the laboratory at KEMRI by passage in *Biomphalaria pfeifferi* snails and inbred laboratory mice. For mouse infections, cercariae were obtained from infected snails, counted and applied percutaneously by the ring method [27] at a rate of 90 *S. mansoni* cercariae per mouse anesthetized intraperitoneally with Sodium pentobarbital 80 mg/kg (Rompun; Bayer Plc., Newbury, UK).

2.3 Extract Administration

At the seventh week, the plant extracts were reconstituted in a vehicle (10% (v/v) Tween-20 + 10% (v/v) analytical ethanol + distilled water) {In order to avoid neural damage associated with methanol} from which various (high and low dosages) plant concentrations (Apple (300 mg/kg, 200 mg/kg), Onion (500 mg/kg, 300 mg/kg) and Lemon (200 mg/kg and 100 mg/kg) were administered (0.1 ml) to the mice via intraperitoneal route twice daily (0800 hrs and 1500 hrs) for five consecutive days. For the control group, animals were given each 0.1 ml of the vehicle daily.

2.4 Parasitological Procedure

After the final dose (at the 8th week), the animals were anesthetized using Sodium pentobarbital 80 mg/kg, euthanized and *S. mansoni* worms were recovered by portal perfusion [27], with perfusion buffer [phosphate buffered saline, 0.02 µl/ml heparin (monoparin; CP Pharmaceuticals Ltd., Wrexham, UK)]. The worms were washed free of erythrocytes and counted using a dissecting microscope. The adult worms were then counted as described by [28] and percentage worm reduction was calculated following the formulae described below and used by [17].

% Worm Burden Reduction =

[(Mean no. of worms from Control group) - (Mean no. of worms from Treated group) x 100%]/ [Mean no. of worms from Control group]

2.5 Glutathione Assay

Hepatic reduced Glutathione (GSH) levels were determined as described by [29]. About 0.2 g liver tissues were homogenized in 4 ml of 0.02 M EDTA from which 2.5 ml aliquots were obtained then mixed with 2.0 ml of distilled water and 0.2 ml of 50% Trichloroacetic acid (TCA). All tubes were shaken intermittently for 10-15 mins and centrifuged at 3000 x g for 15 mins. 2.0 ml of tissue supernatant was collected into a fresh tube and into it was added 2.0 ml of 0.4 M Tris buffer (pH 8.9) and 0.01 M 5,5'–dithiobis-2-nitrobenzoic acid (DTNB) then shaken slowly. Within 5 mins of addition of DTNB, absorbance was read at 412 nm and compared to a reagent blank (with no homogenate). A standard curve was prepared by addition of known amounts of GSH with corresponding absorbance measurements then expressed as µg/mg of tissue.

2.6 Statistical Analysis

All statistical data in the present study were analyzed by comparing values for different treated groups with the values obtained from control group. Results are expressed as Mean ± Standard Error Mean (S.E.M). The significance difference among the values was performed using One-way analysis of variance (ANOVA) followed by Dunnet's post hoc analysis. All the analyses were carried out at 5% level of significance using SPSS version 17.0.

3. RESULTS

3.1 Worm Burden Reduction

To determine the extent of reduction efficacy effected by the three tested plant extracts, the number of perfused worms were counted and expressed into % as described by formula used by [17]. Upon administration of *M. domestica* extract at 7 weeks post-infection (p.i) at doses of 300 mg/kg and 200 mg/kg (Table 1), percentage worm reduction in *S. mansoni* infected mice was observed as 85.93% and 72.22% respectively. Administration of *A. cepa extract* at dosage of 500 mg/kg and 300 mg/kg resulted in worm reduction of 72.59% and 58.52% respectively whereas; administration of *C. limon* (200 mg/kg and 100 mg/kg) reduced the total worm burden to 42.96% and 29.63% respectively. The results obtained revealed that the most potent methanolic extracts were that of *M. domestica* (Apple) and *A. cepa* (Onion) which had significant (P<0.05) schistosomicidal activity while that of *C. limon* (Lemon peel) showed the lowest schistosomicidal activity. Therefore, *M. domestica* and *A. cepa* extracts were selected for the present study.

As shown in Table 1, upon treatment 7 weeks post-infection (p.i) of *S. mansoni* infected mice using *M. domestica, A. cepa and C. limon extracts;* all the treatment groups showed significance difference (P<0.05) for both mean worm burden as well as mean GSH levels relative to control group (treated with vehicle).

3.2 Glutathione Assay

When *S. mansoni* infected mice were treated with different concentrations of *M. domestica* and *A. cepa* at 7 weeks post infection, their effect on hepatic GSH levels was tested. As shown in Table 1 and Fig. 1, GSH content was significantly (P<0.05) increased in hepatic tissues of *S. mansoni* infected mice following exposure to methanolic extracts of *M. domestica* and *A. cepa* as compared to control group whereby hepatic antioxidant capacity was significantly (P<0.05) suppressed. In comparison with control group which had GSH content of 11.38 µg/mg, *M. domestica* (300 mg/kg and 200 mg/kg) treated mice had hepatic GSH levels of 53.12 µg/mg and 45.62 µg/mg respectively while *A. cepa* treated mice at doses of 500 mg/kg and 300 mg/kg had GSH levels of 34.28 µg/mg and 30.58 µg/mg respectively (Table 1 and Fig. 1). The result in Table 1 revealed that the exposure of the infected mice to the plant extracts had a positive effect on the hepatic GSH levels.

As the worm burdens were significantly reduced in the treated mice, the hepatic GSH levels were significantly (P<0.05) elevated a relationship clearly shown by Fig. 1.

Table 1. Effect of different concentrations of plant extracts on worm density and GSH activities

Group	Plant extract	Concentration (mg/kg)	Worm density (Mean±SEM)	% Worm reduction	GSH activity (µg/mg)	N= no. of mice used (35)
I	Control	Vehicle	54.0±1.14	0	11.38±0.08	5
II	Lemon	100	38.0±1.30**	29.63	12.94±0.04**	5
III	Apple	300	7.6±0.93**	85.93	53.12±0.20**	5
IV	Onion	300	22.4±0.93**	58.52	30.58±0.36**	5
V	Apple	200	15.0±1.90**	72.22	45.62±0.17**	5
VI	Lemon	200	30.8±1.50**	42.96	19.44±0.04**	5
VII	Onion	500	14.8±1.16**	72.59	34.28±0.12**	5

*Values expressed as Mean ± Standard Error Mean (S.E.M) for five mice in each group. ** Statistically significant (P<0.05) difference as compared to infected control group. Statistical analysis was carried out by one way analysis of variance at significance level of P<0.05*

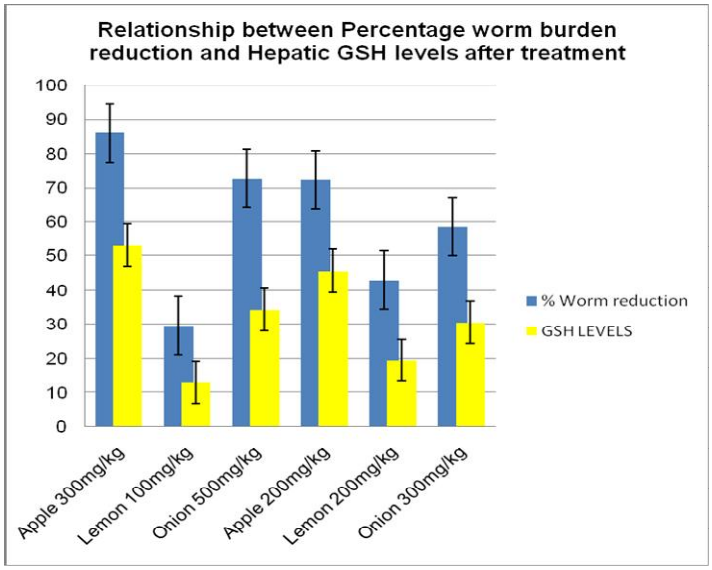

Fig. 1. Relationship between percentage worm reduction and hepatic GSH levels (µg/mg) after treatment

4. DISCUSSION

The current study was done to evaluate *in vivo* antischistosomial effects of the extracts (*M. domestica*, *A. cepa* and *C. limon*) for controlling Schistosomiasis. Medicinal plants have been widely used traditionally for treating or managing schistosomiasis in Eastern and Southern Africa [30-32] as they contain curative and bioactive compounds. The *M. domestica* and *A. cepa* presently proved to have antihelmintic activity. The current study demonstrated that *M. domestica* and *A. cepa* possess schistosomicidal activity against *S. mansoni* adult worms (both female and male worms) as they significantly (P<0.05) reduced the infection compared to control group. Similarly, the study indicated that the different concentrations of plant extracts had a significant (P<0.05) improvement on host antioxidant system depicted by raised levels of enzyme Glutathione reductase compared to that of control group.

Schistosomiasis infection is accompanied by an imbalance between oxidative stress and host-antioxidant enzyme activity which is largely believed to play a role in its pathogenesis such that there is generation of free radicals and disturbances in cellular antioxidant systems [33]. At the same time, the infected host responds to the parasites by producing Reactive Oxygen Species (ROS) that make the parasites susceptible. For this reason, the antioxidant enzymes represent a target for immune elimination of adult worms from the human host [11]. The important role of antioxidants in mediating liver injury/damage in schistosomiasis resulting from elevated production of ROS has been demonstrated by [34]. While there is elevated parasite ROS production in the course of infection; ROS scavenging activity by host antioxidants diminishes concomitantly hence the setting-in of oxidative stress. This result in elevated peroxidation of biomolecules such as lipids, proteins and DNA [33] as well as disturbed antioxidant status [35].

In order to protect themselves from oxidative-mediated killing mechanism of the host, the parasites have developed antioxidant enzyme systems [33]. In *S. mansoni,* Superoxide Dismutase, Glutathione Reductase, Glutathione Peroxidase, Catalase and Glutathione S-transferase are thought to be the main antioxidant enzymes involved in detoxification processes [36] that protect the parasite against damage as a result of Reactive Oxygen Species. However, as the infection progresses, there is decreased generation of Reactive Oxygen-derived free radicals by the host, which may be attributed to the activity of *S. mansoni* Thioredoxin/Glutathione Reductase (smTGR). There is a strong correlation between the *in vivo* sensitivity and action of smTGR and other antioxidants for detoxification of antischistosomals. *S. mansoni* Thioredoxin/ Glutathione Reductase is a key flavoenzyme expressed by schistosomes that bridges two detoxification pathways crucial for the parasite survival in the host's organism [37].

Reactive oxygen species have been implicated in numerous pathophysiological events such as aging, cancer, atherosclerosis and diabetes. The central role in mediating the pathology in many diseases has stimulated interest in the possible role of natural antioxidants in preventing the development of these diseases [38]. Natural antioxidants are involved in metabolic processes hence are believed to play a substantial protective role against oxidant-mediated damage and harmful effects of host factors/substances produced as a result of immune/defense response [39]. *S. mansoni* induces a significant (P<0.05) reduction in host hepatic GSH levels which likely suggests that the parasite liberates its own free radicals. The decrease in glutathione levels has been attributed to inhibition of Glutathione Reductase enzyme that keeps glutathione in its reduced form (GSH) [40-41]. Cunha et al. [42]; Ahmed and Allam, [43] reported that impairment of hepatic GSH content of Schistosoma infected mice decreased antioxidant capacity leading to generation of lipid peroxides that play a central role in the pathology associated with schistosomiasis. This reduction in host's defense mechanism to free radicals can be explained on the basis of *S. mansoni* eggs trapped in the liver which elicit a chain of oxidative processes that may be, at least in part, responsible for the pathology and progression of fibrosis associated with schistosomal infection [33]. Our data showed a significant (P<0.05) improvement of hepatic GSH as a result of treatment compared to control group, indicating that leakage of reduced GSH from liver into the blood circulation [44] as a result of liberation of free radicals and oxidative processes collaborated with *S. mansoni* infection.

In the current study, administration of the plant products appears to augment hepatic GSH activity reducing its depletion. It is possible to make a preliminary assertion that upon uptake of

these plant products the mice are able to better cope with infection. Thus, the plant products ameliorate infection enabling the mice overcome adverse effects of pathogenesis as their hepatic tissue is protected against oxidative stress. The findings of this study corroborate those of [10,45] that in some cases inoculation of Melatonin (an antioxidant) and green tea (*Camellia sinensis*) extracts [46] to *S. mansoni* infected mice resulted in highly significant (P<0.05) increment in GSH levels of liver compared to control subjects. Similarly, Farrag et al. [47] showed that antioxidant compounds possibly modulate host immunity to eliminate the parasite and protecting the mice from schistosomiasis to a significant level. It has been postulated that, the mechanisms whereby consumption of certain plants and plant extracts can affect parasite viability/survival, mobility and fecundity *in vivo* could very likely be associated with: (i) an enhanced immune response of the host towards the parasite [33] or (ii) the phytochemical constituents of the plant extracts. You-Qin et al. [48] showed that flavonoids from *Malus hupehensis* inhibited hepatic fibrosis induced by *S. japonicum.* In this regard, Onions and Apples purposely contain sulfhydryl groups, flavonoids and phenolic compounds with capacity to bind a variety of electrophilic radicals and metabolites with potential to damage cells. On the other hand, the antischistosomal effect of either Apple or Onion extracts may also be attributed to their immunomodulatory effect of host immune response as reported by [49].

The significant (P<0.05) improvement in the studied parameters after treatment of the infected mice with extracts of Apples and Onions resulted from the reduction in worm burden accompanied by significant (P<0.05) increase in GSH level compared to the *S. mansoni* infected control group. The conspicuous worm burden reduction indicates their antischistosomal activity and curative effect on *S. mansoni* infection [33]. Administration of these extracts in the present study resulted in worm reduction whereby the results are in harmony with other investigations which used extracts including garlic and onion for the treatment of *S. mansoni* infection [49-50]. Muchirah et al. [51] reported that PZQ at 1350 mg/kg dose has 65.92% worm reduction effectiveness in Swiss albino mice. Interestingly, the current results showed that *M. domestica* at doses of 300 mg/kg and 200 mg/kg caused significant reduction in worm count by 85.93% and 72.22% respectively which is within the range of PZQ efficacy of 70%-90% worm reduction at recommended dosage. *A. cepa* at dose of 500 mg/kg caused 72.59% worm reduction which is comparable with PZQ efficacy. The results from this study are supported by previous other studies reported on plants with antioxidant activities. Bin Dajem et al. [46] reported that treatment of *S. mansoni* infected BALB/c mice with *Camellia sinesis* extract was effective in ameliorating female *S. mansoni*-induced hepatic lipid peroxidation. You-Qin et al. [48] showed that total flavonoids of *Malus* hupehensis had an inhibitory effect on hepatic fibrosis induced by *S. japonicum in mice*. El Shenawy et al. [7] observed that aqueous extracts of garlic and *Nigella sativa* had anti-schistosomal activities based on their antioxidant properties. Further, Mantawy et al. [33] reported that *Allium cepa* and *Allium sativum* had were potent in treatment of *S. mansoni* infection in mice. Rizk et al. [52] observed that essential oil from *Melaleuca armillaris* leaves had significant effect on *in vivo* antioxidant levels in *S. mansoni* infected mice. Ali [53] reported that *Citrus reticulata* had amelioration effect on hepatic antioxidant parameters in *S.* mansoni infected mice. Aly and Mantawy, [54] showed that ginger (*Zingbar officinale*) was effective against *S. mansoni* infection. The smTGR is a key drug target [16] as inhibiting the enzyme with specific drugs such as Artesunate and mefloquine kills the parasite both *in vitro* and *in vivo*, and substantially, reduces worm burden in infected mice [16,55].

5. CONCLUSION

The use of *M. domestica and A. cepa* as antischistosomal drugs may affect the oxidative imbalance of the adult worms against the oxidative killing by the host effector cells and this may help in the elimination of the worms. The extracts eliminated the products of oxidative reactions and assisted in the immune-mediated destruction of worms. Treatment of *S. mansoni* infected mice with the plant extracts for five consecutive days resulted in remarkable worm reduction accompanied with restored levels of glutathione, which was depleted due to the liver damage by the infection. The present study revealed that for the first time Apples, with their potent free radical scavenging and antioxidant properties, seem to have protecting effect on hepatic tissue against oxidative damage arising from *S. mansoni* infection by stimulating the host hepatic cells to produce sufficient amounts of GSH to counteract the free radicals produced by the parasite. Hence the old adage: “an apple a

day keeps the parasite away" stands. This study could provide scientific basis on the plants being used as antischistosome therapeutic agent or if possible used together with PZQ, the extracts could provide synergistic effects in the control of resistant strains of *S. mansoni* parasite.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The mice used in this study were maintained according to International accepted procedures for animal care and management as recommended by Kenya Medical Research Institute Animal Care and Use Committee (ACUC). All the mice in this experiment were maintained on mice pellets and water *ad libitum*. The mice were humanely handled at all times following anaesthetic and euthanasia procedures during infection and perfusion. After perfusion, the mice were disposed in the incinerator in biohazard plastic bags at the end of study.

All authors declare that "Principle of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments were examined and approved by the Kenya Medical Research Institute (KEMRI) Animal Use and Care committee under protocol number 1191.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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