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Anthelmintic activity of methanolic extract of *Ajuga integrifolia* against *Strongyloides stercoralis*

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ABSTRACT

Strongyloides stercoralis is a persistent pathogenic roundworm commonly known as "hidden killer". Its ability to replicate in human host, cyclic autoinfection that can last for years and asymptomatic behavior make theranostics difficult. *Ajuga integrifolia* is a shrub that is traditionally claimed to possess a very potent effect against persistent gastrointestinal ailments. The objective of this study was to analytically investigate the anthelmintic activity of methanolic extract of aerial parts of *A. integrifolia* on rhabditiform larvae of *S. stercoralis*. The larvae were isolated from fresh stool samples obtained from school children diagnosed to be positive for the parasite. The anti-*S. stercoralis* effect of the extract (in phosphate-buffered saline (PBS): sterile, pH = 7.2, 1.7 mM) against rhabditiform larvae was evaluated by incubating the suspension of the parasites in PBS with different concentrations of the extract for 24 h at 37 ^{0}C in a 5 % CO_2 humidified incubator to determine % survival. A concentration dependent killing effect with an IC_{50} value of 50 µg/mL was recorded. The sensitivity and immediacy of action proved a potential commercial value of the plant for treatment against human strongyloidiasis.

Keywords: *Strongyloides stercoralis*, *Ajuga integrifolia*, crude extract, anthelmintic activity

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INTRODUCTION

Strongyloides stercoralis is a fulminantly lethal parasite *causing* an estimated infection of 30-100 million people worldwide[1,2], with a distribution throughout tropical and subtropical areas. *S.stercoralis* is known to have two life cycles that are described as parasitic generation in human host, and the free-living generation in soil[1,3]. In the parasitic generation, when the filariform larvae come into contact with human skin, they penetrate the small cutaneous blood vessels and are carried through the right heart to the lungs[4]. Then, sexually mature parasitic females settle in the tissues of epithelial mucosa to lay eggs that hatch soon and are discharged in the stools each day[5,6]. When all or some larvae metamorphose into infective filariform larvae autoinfection may be onset by invading the mucosa of the ileum or colon, travel to lungs and then return to the intestine to mature in the mucosa[7,8]. Disseminated strongyloidiasis had been reported in both of two recipients of kidney allografts from a single cadaver donor[9]. One scientific study has reported that almost all deaths due to helminthes in the United States result from *S. stercoralis* hyperinfection mortality rates because the occurrence of hyperinfection can be as high as 87% [10].

The peculiar ability of *S. stercoralis*, unlike other *helminths*, to replicate in the human host permits cycles of autoinfection leading to chronic disease that can last for years. The limited diagnostic yield of stool microscopy for chronic strongyloidiasis, its asymptomatic nature, and complexity of culture techniques are additional challenges in the theranostics of the disease. The parasite is able to persist for years in a human host and then may disseminate and cause a fatal infection, particularly in immunocompromised patients. Therefore, effective and sensitive anthelmintic is essential for timely treatment of the infection.

Ivermectin is a currently recommended commercial drug of choice for treatment against strongyloidiasis, but its use is limited due to its price and accessibility. In addition, it has been reported that a single course of treatment with ivermectin cannot always be relied upon to eradicate the infection in patients. Reported cure rates of ivermectin vary between 67% and 100% [11]. Plants have shown to be successful sources of therapeutic agents and attract the attention of researchers and pharmaceutical companies owing to their accessibility, affordability and ability to address the cause of many diseases and yield superior clinical results. A wide variety of plants have been tested against helminthes of various types[12, 13]. However, very limited plants have been screened against *S. stercoralis*[14].

A. integrifolia, Lamiaceae, is locally known as ‘Annamura’ (Wolaita), has a very bitter taste, moderately to densely hairy grayish green leaves with pale blue/violet flowers[15]. In traditional

health system of Ethiopia, the aqueous and sometimes alcohol infusion of the fresh or dried leaves of the plant is used for the treatment of various diseases including diabetes, malaria, toothache, skin disease, hypertension, persistent gastrointestinal disturbances, pneumonia, liver problem, swelling of legs, retained placenta[16], and Epilepsy[17] and others[18]. Particularly, its potent activity against persistent gastro intestinal disturbances, according to traditional users, led the researchers to envisage its effect on what is commonly known as "hidden killer"-strongyloidiasis. The key objective of this study was, therefore, to investigate the therapeutic effect of methanolic extract of aerial parts of *Ajuga integrifolia* against rhabditiform larvae of *S. stercoralis* isolated from fresh stools samples collected from school children. The ultimate goal is to contribute in the long-run to improved health care of the low income part of the community in Ethiopia.

MATERIALS AND METHOD

The sites of random stool samples were selected to be the elementary schools (from student children) at Dilla District, because *S. stercoralis* was ascertained to be endemic in this region[19,20].

Sample size

A total sample size of 300 student children were randomly examined for *S. stercoralis*. Out of the student children positive for *S. stercoralis* those with relatively heavy worm load of the parasite were used as the sources of stool samples.

Diagnostic Examination

The method applied for the diagnostic examination of the fresh stool samples was Baermann funnel apparatus technique/Water emergence semi-concentration technique. Water emergence semi-concentration technique was used only for detecting the presence of *S. stercoralis* in the student child. Rhabditiform larvae of *S. stercoralis* were isolated from the stool samples using the Baermann funnel apparatus technique. It is only when the rhabditiform larvae of this particular parasite were isolated from the stool samples and maintained in PBS (sterile, pH = 7.2, 1.7 mM) that the *A. integrifolia* extract was applied to (mixed with) the larvae in order to observe the killing effect of the extract.

The isolation of *S. stercoralis* from the fresh stools samples (obtained from student children who were positive for it), using Baermann funnel apparatus technique involved the following steps: Baermann funnel apparatus was constructed and the lower opening of the rubber fitted to the stem of the funnel was closed. Then the PBS solution warmed to 40^0 C was poured into the funnel of the Baermann apparatus. The Cheese cloth that contained the fresh stool sample, tied with its peripheral edges to the rim of the funnel, was partially immersed in the water. This was done

because adults as well as juveniles of *S. stercoralis* would be attracted by the warm temperature of PBS solution (about 37.5 ^{0}C as there was dissipation of heat from the initial 40^{0}C of the added solution to the surrounding materials & equipment) and escape into the warm PBS solution through the pores of the cheese cloth. After 1 hour and 30 minutes, the closed lower end of the rubber tubing was opened, releasing the water found in the funnel of Baermann apparatus into a 500 ml beaker. The stools left behind in the cheese cloth was thrown into the tube of toilet after being treated with a disinfectant (iodine solution) and washed away by a current of water. The PBS solution released and collected in the 500 ml beaker was centrifuged at a speed of 1000 rpm for 2 minutes using a manual centrifuge loaded with 4 centrifuge tubes and anchored to the edge of a table. From each centrifuge tube the supernatant was poured off into a waste collecting bucket to be thrown into the tube of toilet drainage line by treating with the disinfectant. Using a dropper, about 2 ml of the PBS solution was added to the sediment of one of the 4 sediment containing centrifuge tubes and shaked well by closing its mouth with its own fittingly tight lid. The action of shaking was to change the sediment into a transferable suspension. The same suspension was transferred to each of the remaining 3 centrifuge tubes one by one where in each case the centrifuge was shaked well and the sediment was changed into suspension. Next, the sediment collected in the form of suspension from 4 centrifuge tubes was poured into a test tube labeled for pooling the rhabditiform larvae of S. stercoralis isolated from fresh stools samples. The above steps were repeated for the fresh stool sample of each of the remaining 9 student children and the isolated larvae from each are added to the labeled pooling test tube.

Anti- Strongyloides stercoralis bioassay procedure

The PBS (sterile, pH = 7.2, 1.7 mM) was used as drug-free control media. The pH of the three solutions were adjusted to physiological value of 7.2 prior to application to the rhabditiform larvae of *S.stercoralis*. Using a micropipette, a 500 µl suspension of the parasite in PBS where the actively moving rhabditiform larvae of *S. stercoralis* was maintained was taken from the pooling test tube and placed towards one side on a clean and sterile slide whereas another 500 µl suspension of the parasite in PBS was placed on the same slide towards the other side. Added to one of these two drops was a drop of *A. integrifolia* crude extract that was mixed well with a tip of needle to observe its effect on the rhabditiform larvae of *S. stercoralis* and a drop of control (without the extract) was added to the other side. Then, the preparations were carefully observed under suitable objective lenses of a compound light microscope.

The anti-*S. stercoralis* effect of the different concentrations of the extract against rhabditiform larvae of *S. stercoralis*, was further studied as follows. Active rhabditiform larvae obtained from fresh stool samples were transferred to a test tube at a concentration of 50 larvae /ml. 1 ml of the various concentrations (10^6 µg/ml, 10^5 µg/ml, 10^4 µg/ml, 10^3µg/ml, 10^2µg/ml, 10µg/ml, and 0.1 µg/ml) of the extract was added to each of seven test tubes containing 1 ml solution of parasites suspended in the PBS. The eighth test tube contained only 1 ml PBS. The triplicate of eight test tubes was then incubated for 24 h at 37 ^{0}C in a 5 % CO_2 humidified incubator. The viability of the rhabditiform larvae of *S. stercoralis* was microscopically observed as motility or lack of it to determine % survival.

Plant materials

The aerial parts *Ajuga integrifolia* were collected from southern Ethiopia-Boditti (340 km south of the capital, Addis Ababa) in August 2014. The botanical identification of the plant was done at the National Herbarium at Addis Ababa University where a voucher specimen was kept under the cipher ALEM.P2. The plant was selected based on information obtained from informants through semi structured interview. The homogeneity level among the collected information was tabulated by the Informants' Consensus Factor, FIC[21]. The percentage of informants claiming the use of *A. integrifolia* for persistent stomach complaint was calculated as fidelity level (FL): FL (%) = (Np / N) × 100 where, Np = number of informants that claim a use of a plant species to treat a particular disease; N = number of informants that use the plants as a medicine to treat any given disease[22].

Preparation of plant extracts

Prior to extraction, the fresh samples were dried under shade and ground to fine powders using a blender. Then, 300 g of the dried, ground sample was soaked in methanol (1.5 L) for 3 days at room temperature with occasional stirring. Next, the solvent-containing extract was decanted and filtered. From the filtrate the excess solvent was evaporated under reduced pressure using a rotary evaporator to give crude methanol extract. The extract was dissolved in the sterile PBS (sterile, pH = 7.2, 1.7 mM) solution to give the stock solution of 1 g/ml from which a serial dilutions of 1g/ml, 0.5 g/ml, 0.1 g/ml, 0.05 g/ml, and 0.01 g/ml were prepared in sterile PBS (pH = 7.2, 1.7 mM). Then, these different concentrations were tested for the efficacy (bioactivity) of the crude extract against the rhabditiform larvae of *S. stercoralis* isolated from fresh stool samples.

Procedures for qualitative phytochemical screening

Qualitative phytochemical screening was carried out to assess the presence or absence of the class of secondary metabolites such as steroids, alkaloids, phenolic compounds, saponins, tannins,

flavonoids, coumarins, and anthraquinones. Screening was carried out using standard methods[23,24].

Ethical considerations

The study protocol was prepared in accordance with the internationally accepted principles for human subjects and care and reviewed and approved by Institutional Research Ethical Review Board of Dilla University. All human related data collections were conducted as per the guidelines approved by the Board.

RESULTS AND DISCUSSION

Physical examination of killing effects

In order to examine the immediate effects of the extract on the rhabditiform larvae of *S. stercoralis,* a 100 µl of the 1 g/ml extract (in PBS) was applied to a 100 µl suspension of the parasite in PBS. The experiment was performed on a sterile slide to microscopically observe the effect of the extract on the parasite motility and morphology. The parasite in the drug free PBS was observed to be freely swimming in its suspension for several hours. After addition of the extract, however, the parasite remained localized and displayed a dramatic reaction losing its normal swimming, (a) to (d), to an irritated coiling-uncoiling responses, (e) to (g), to eventual complete loss of motility and death, (h), (Figure. 1.). This preliminary observation stimulated a more quantitative investigation of the killing effect of the plant extract.

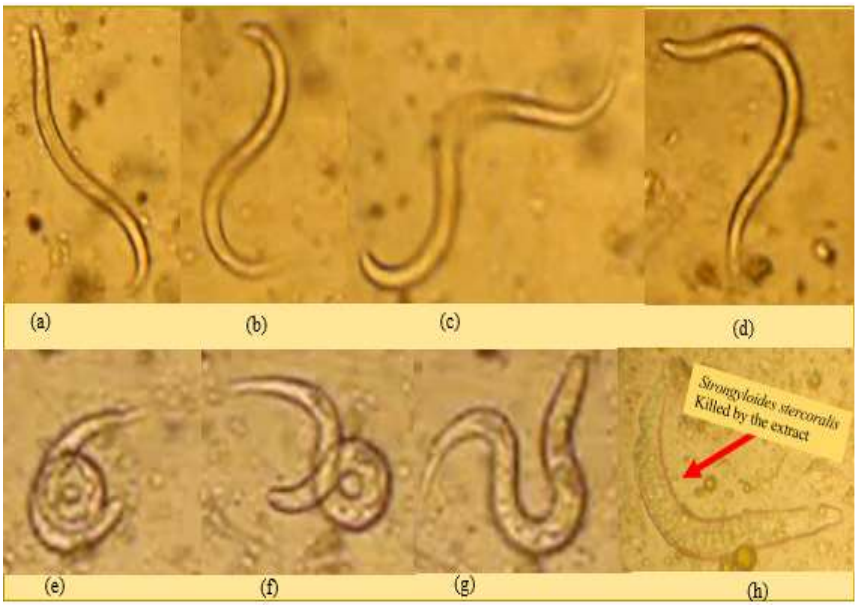

Figure. 1. Freely motile (swimming) rhabditiform larvae of *Strongyloides stercoralis* in PBS (sterile, pH = 7.2, 1.7 mM) with no extract, (a) to (d), and the different irritated responses (coiling-uncoiling) of the parasite, (e) to (g), when subjected to 1 g/ml of methanolic extract of Ajuga integrifolia to eventual death with complete loss of motility, (h).

Determination of the anthelmintic activity

The sensitivity of the plant extract was assayed by directly subjecting the parasite to serial dilutions of it. Our result showed that all concentrations in the range of 0.01 to 1 g/ml exhibited killing effect. However, the time of killing varied with concentration. Very dilute (< 1 mg/ml) or concentrated (> 10 g/ml) solutions of the extract showed delayed killing effect. The effect of dilution suggested the importance of setting a lethal dose. The delayed response of the relatively concentrated solution of the extract could be attributed to the reduced diffusion of the bioactive constituents leading to ineffective interaction with the parasite. The concentrations between 0.01 to 1 g/ml exhibited the killing effect between 84 to 10 minutes, Fig.2. The action of the extract is more dramatic and complete whereas no death or any physical effect was observed with that of the drug free control (PBS; sterile, pH=7.2, 1.7 mM). The parasite remained motile and active during the test time (3 hours).

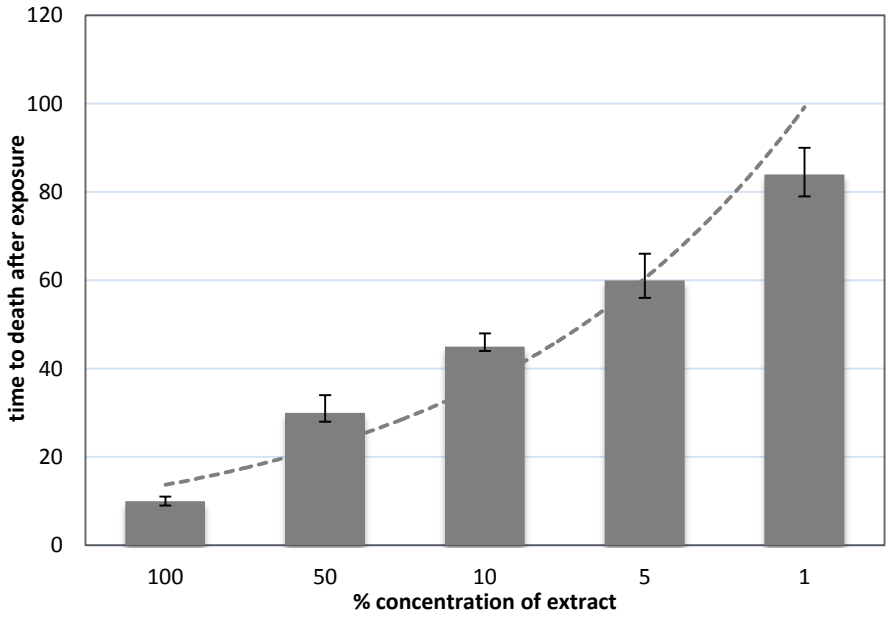

Figure 2. **The plot of % concentrations (100 % = 1 g/ml) of methanolic extract of *Ajuga integrifolia* against post-exposure time till death of the rhabditiform larvae of *Strongyloides stercoralis*.**

Efficacy of the extract

The anti-*Strongyloides stercoralis* effect of the different concentrations of the extract against rhabditiform larvae of *S. stercoralis*, was further studied by determining % viability after exposure to the varied concentrations of the extract. The extract concentrations ≥ 0.01 g/ml (=10^4 µg/ml) showed a 0 % survival of the parasites after 24 h incubation. Concentrations between 0.01 g/ml and 0.1 µg/ml exhibited survival rates that varied according to the concentration. In of the extract

was added to each of seven test tubes containing 1 ml solution of parasites suspended in the PBS. The eighth test tube contained only 1 ml PBS, whereas the ninth test tube contained 1 ml of 1 g/ml ivermectin (in PBS) plus 1 ml of the parasite suspension (50 larvae/ml) respectively. The triplicate of nine test tubes was then incubated for 24 h at 37 ^{0}C in a 5 % CO_2 humidified incubator. The viability of the rhabditiform larvae of *S. stercoralis* was microscopically observed as motility or lack of it to determine % survival.

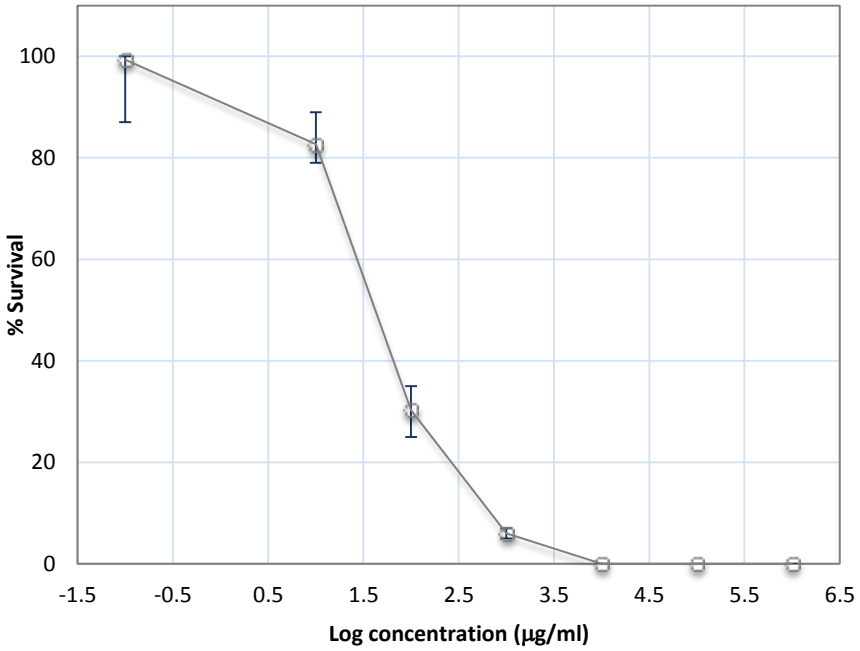

Figure. 3: Percentage survival of the rhabditiform larvae of *Strongyloides stercoralis* after a 24 h incubation with the varied concentration of the methanolic extract of *Ajuga integrifolia*.

Phytochemical screening

Table 1. Phytochemical constituents of *Ajuga integrifolia*

Plant tested	Phenols/ Tannins	Flavonoids	Saponins	Glycosides	Steroids	Terpenoids	Alkaloids
Ajuga integrifolia	+	+	+	+	+	+	+

+ = positive result (or it means present)

Here above in the table, it can be seen that phenols/tannins, flavonoids, alkaloids, glycosides steroids, terpenoids, and saponins were present in the methanol-crude extract of the plant tested.

The effect of *Ajuga integrifolia* crude extract was spectacularly observed to be very strongly deadly on the larvae of *Strongyloides stercoralis* isolated from fresh stools samples. The larvae of *S. stercoralis* that were susceptibly killed by the deadly effect of the methanol-crude extract of the plant studied belonged to the stage of rhabditiform larvae. These larvae are the only source for

autoinfection that is responsible for the most of life time strongyloidiasis with human hosts in the parasitic generation. As far as all the rhabditiform larvae produced are killed off in the intestine and people regularly put shoes on, preventive measures against strongyloidiasis becomes 100% efficient. This is so because the lifespan of individual adult worms of *S. stercoralis* that produce rhabditiform larvae is only 3 or 4 months time. This meant that the parasitic generation of *S. stercoralis* would be prevented completely equivalent to getting the parasite eliminated/eradicated. That is why it is stated that efficient preventive measures against parasitosis and devising effective treatment against a parasite depend on understanding the biology & life cycle of the parasite (in other words, understanding the nature of the disease). It is only the free-living generation in soil that could give rise to the infective filariform larvae. The other possible source of filariform larvae being the reservoir hosts such as dogs.

Live observation of the killing effect of methanolic extracts of *Ajuga integrifolia* on rhabditiform larvae of *S. stercoralis* isolated from fresh stools samples is nearly/almost equivalent to killing these larvae inside the body of the patient (i.e., in the intestine of the patient). This is true because the stools were fresh as if they were inside the intestine and the larvae did not adapt or not changed in their metabolic/physiological characteristics in their internal biochemical environment. In other words, the susceptibility of rhabditiform larvae of *S. stercoralis* to the killing effect of this methanol-extract would be nearly the same inside the body of a human host.

Moreover, the dramatic killing effect of the crude extract on the larvae of the parasite was observed *on vitro* (on slide under suitable objective lenses of a compound light microscope) and not *in vitro*. *On vitro* method is superior to that of *in vitro* for spectacular seeing and showing in reality. *On vitro* observation for accurately recording the deadly effect of the plant extract studied was far better than *in vitro* (in test tube) follow-up. Dying movements (movies) and morphological changes as well as the time of death of the larvae after exposure can be recorded accurately using a digital camera by way of *on vitro* method under a compound light microscope what cannot be done by the *in vitro* method.

Antiparasitic activities of a plant are linked to the class of secondary metabolites. The presence of the secondary metabolite is an important factor to determine the plant's medicinal values[25]. Therefore, phytochemical screening was essential and carried out to verify whether the test plant contained the aforementioned class of compounds or not. The set of phytochemical characteristics of the medicinal plant tested was summarized in Table 1.

CONCLUSION

The methanolic extract of *Ajuga integrifolia* exhibited a spectacular killing effect on rhabditiform larvae *of Strongyloides stercoralis*- a fulminantly lethal parasite. The sensitivity and immediacy of action proved a potential commercial value of the plant for treatment against human strongyloidiasis. We are currently conducting clinical validation to produce an effective anthelminthics against strongyloidiasis and make available to the community.

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