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## ***In vitro* Anti-schistosomal Activity of "*Plectranthus tenuiflorus*" on Miracidium, Cercaria and Schistosomula Stages of *Schistosoma mansoni***

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### **ABSTRACT**

Human schistosomiasis is the second most prevalent tropical disease after malaria and has considerably public health and socioeconomic burden in developing world. The only drug available for treating schistosomiasis is praziquantel, however it lacks efficacy against schistosomulae and there are already reports of resistance to its use in treatment, making it necessary to explore and upsurge for new compounds to combat schistosomiasis. This study was designed to evaluate anti-Schistosoma activity of the methanolic extract of *Plectranthus tenuiflorus* (Vatke) Agnew; an herb on different stages of *Schistosoma mansoni*. Parasite viability and morphological changes were assessed after incubation with different dilutions of the plant extract using inverted as well as scanning electron microscopy. *Plectranthus tenuiflorus* (Vatke) Agnew extract showed moderate anti-cercaria and anti-schistosomulae activity with calculated IC<sub>50</sub> 12.29 and 17.39, respectively compared to lesser anti-miracidium activity with IC<sub>50</sub> 24.37 mg/100 mL. This is the first study to assess anti-schistosoma potency of *Plectranthus tenuiflorus* (Vatke) Agnew extract and may suggest a promising medicinal constituent of *Plectranthus tenuiflorus* (Vatke) Agnew extract importantly against cercaria infective stage and the significantly ambulant invasive *Schistosomulae* inside human body which impose further studies to evaluate different extracts and fractions of this plant.

**Key words:** *Plectranthus tenuiflorus* (Vatke) Agnew, *Schistosoma mansoni*, miracidium, cercaria, schistosomula

### **INTRODUCTION**

Schistosomiasis is the one of the most important communicable disease caused by a blood born fluke of the genus *schistosoma*. It is the second most prevalent tropical disease in Africa after malaria. It is of relatively low mortality; however, it is of high burden in terms of chronic pathogenicity and disability (WHO, 1998).

Praziquantel is the only drug available for the treatment and control of schistosomiasis with good safety and efficacy profile against all schistosome species parasitizing humans (Doenhoff *et al.*, 2008; Black *et al.*, 2009) however, praziquantel lack the efficacy against schistosomulae, the young developing stages of the parasite. This may be explained the low cure rates and rapid re-infection rates in areas of heavy transmission of schistosomiasis, where patients

may be concurrently infected with juvenile and adult parasites (N'Goran *et al.*, 2003). In addition tolerance or resistance to praziquantel may already exist or might be developing with the increasing use of praziquantel (Cioli and Pica, 2003; Keiser and Utzinger, 2007).

Medicinal plants are being used for hundreds of years as therapeutics worldwide. Being natural products are safer than their synthetic equivalent (Elujoba *et al.*, 2005). The family Lamiaceae contains several genera with a rich diversity of ethnobotanical uses. It has an important role as a source of medicinal plants and as an aromatic of commercial importance. *Plectranthus* is a large genus found in Tropical Africa, Asia and Australia and containing about 300 species, 62 of them were reported to be used as medicines, ornamentals, foods, flavors and fodder. Genus *Plecteranthus* (*P.*) which belongs to family Lamiaceae is represented in Saudi Arabia by six species: *P. arabicus*, *P. cylindraceys*, *P. tenuiflorus*, *P. barbatus*, *P. lanuginosus* and *P. asirensis*. *Plecteranthus tenuiflorus* (*P. tenuiflorus*); "Shaar" in Arabic, is one of the medicinal plants in the genus *Plecteranthus* (Colenett, 1998). Ryding and Paton (2001) reported that *Plecteranthus aegyptiacus* is the correct name of *P. tenuiflorus*. *P. tenuiflorus* essential oil as its crude extract showed an anti-phytoviral against different plant viruses like Tobacco Necrosis Virus (TNV) (Othman and Shoman, 2004; Lukhoba *et al.*, 2006).

Thus, our study was designed in attempt to seek out for new natural anti-schistosomal chemotherapy, one plant of the family Lamiaceae, *Plectranthus tenuiflorus* (*P. tenuiflorus*) (Vatke) Agnew was selected to evaluate its methanol leaves extract activity against miracidium, cercaria and schistosomula stages of *Schistosoma mansoni* *in vitro*.

## MATERIALS AND METHODS

**Plant material:** Plant was collected in March 2007 and April 2008 from different region in south and east Saudi Arabia around Jeddah and al Taeif, done by author while a scientific mission stay in Saudi Arabia.

**Preparation of extract:** Fresh herb of *P. tenuiflorus* was cut into small pieces, dried in shade for fifteen days and finally in oven below 60. The dried plant material (1 kg) was ground into fine powder and exhaustively extracted with methanol. The extract was concentrated under vacuum at 40. The dark green viscous residue (65 g) was kept away from light in a refrigerator at 4°C different concentrations of this extract was suspended in 100 mL of methanol and shaken automatically for 30 min then centrifuged. The clear supernatant solution was used to carry out the different experiments.

**Preparation of parasite stages:** Preparation of Egyptian strain of *Schistosoma mansoni* stages miracidium, cercaria and syringe shed schistosomulae was performed freshly just before testing the plant extract by Schistosome Biological Supply Center SBSC team, Theodor Bilharz Research Institute, Imbaba. In brief, collection of eggs from murine intestines was performed according to the method of Liang and Kitikoon (1980), collected viable eggs were pipetted into a small Petri dish (1.5×6 cm) and the dish was placed under ceiling illumination for hatching of miracidia. The entire procedure takes approximately 15 min and miracidia usually appear within 5 min. *Biomphalaria glabrata* snails used in this study were bred and infected with *Schistosoma mansoni* in SBSC lab. The snails were induced to shed cercariae under electric light for nearly 10-20 min, infectious cercaria were examined and counted per one mL. The entire procedure takes less than one hour.

This procedure dramatically reduces the mortality among snails due to prolonged shedding time (Liang *et al.*, 1987). Some of the recently shed infectious cercariae were subjected to sheering stress through a syringe with a 0.8 mm needle. The cercariae were passed through the needle 20 times or more, until they had lost their tail and were transformed into schistosomulae. This is a rough method compared to transformation of cercariae to schistosomulae by passing through a mouse skin (Clegg and Smithers, 1972). Nevertheless, under careful microscopic examination *Schistosomulae* were counted and were assured that only healthy schistosomulae were used in the experiment, only 50 cercariae were added in each well. In control wells only 500  $\mu$ L of cell culture medium was added to the same number of each parasite stage.

***In vitro* evaluation of anti-Schistosomal activities of medicinal plants:** *In vitro* experiments were carried out in flat bottomed microliter plates with 24 wells. The 400 mg/100 mL was attempted as the basic concentration in the first well, knowing that this may not include all plant constituents equally. Four lower concentrations of plant extract 300, 250, 50 and 25 mg/100 mL were used; dilution was done using cell culture medium (100 mL, RPMI 1640 supplemented with glutamin, antibiotics and 5% fetal calf serum) and served as incubation media for all *Schistosoma mansoni* stages in our experiments. To facilitate counting and testing each stage separately, each dilution was prepared then divided into three identical aliquots for the three tested stages. Each concentration was tested in triplicate and at least two experiments were performed on separate occasions. The parasite stages in each well were carefully examined and counted. Since Schistosomulae is a stage that only occurs *in vivo* and survives shortly when produced by this sheering method (Molgaard *et al.*, 2001), as well as the cercariae are known to lose infectivity rapidly, examination of plant effect was carried out after ½, 2, 4 and 6 h of incubation at 37°C and not prolonged to 24 or 48 h. Parasite viability was determined by direct microscopy at 40x magnification using inverted microscope (Nikon). For determination of the extract effect; parasites in each well was counted in duplicate, with the live parasite identified as freely swimming with no structural changes; while dead parasite was recognized by complete loss of motility and was laying at the bottom of the well (Holtfreter *et al.*, 2011).

**Scanning electron microscopy for parasite stages' morphological alteration:** Four hours after incubation with plant extract in different concentrations, parasite stages were processed for electron microscopic examination by the help of the staff in Electron Microscopy Department, Theodor Bilharz Research Institute, Imbaba. In brief; specimen were fixed with 2.5% (v/v) glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4) for 30 min at 22-24°C (room temperature). After rinsing three times with PBS, specimen post fixed with 1% (v/v) Osmium tetroxide in phosphate buffered saline for 30 min at 4°C, afterward was washed thrice with double distilled water at room temperature, dehydrated in ascending ethanol concentration and finally examined and image captured in a high resolution SEM (Philips FEI Inspect) (Robards and Wilson, 1993).

**Statistical analysis:** The rank sum Mann-Whitney U test was used to compare two nonparametric unpaired groups. Also the dose-response curve was applied to measure an agonist/inhibitor's potency for a particular active component. So Graphprism5 software was used to produce nonlinear fitting curve (log inhibitor vs. normalized response-variable slope curve, with

calculation of IC<sub>50</sub> value). The IC<sub>50</sub> simply defines the concentration of inhibitor required to provoke a half response between the baseline and maximum responses.

## RESULTS

**Variable inhibitory effect of plant extract on *S. mansoni* stages:** This study concentrated on evaluating anti-helminthic effect of methanolic extract of *P. tenuiflorus* (Vatke) Agnew on different stages of *Schistosoma mansoni*. Detection of motile viable parasites observed by inverted microscopic examination, methanolic plant extract exhibited statistically significant decrease in viability of *S. mansoni* stages (Fig. 1a). When this plant extract's inhibitory effect was stratified against different time intervals, it confirmed the previous finding, furthermore indicated that the strongest inhibitory effect was on *schistosomulae* rather than on cercariae or miracidium shown in (Fig. 1b). Furthermore; calculated *P. tenuiflorus* (Vatke) Agnew methanol extract concentration that gives a half efficient response IC<sub>50</sub> against miracidium, cercaria and schistosomula was 24.37, 17.39 and 12.29 mg/100 mL, respectively and demonstrated dose-response downhill curve (Fig. 2) with attached table, ensuring that *P. tenuiflorus* (Vatke) Agnew extract had lesser effect on

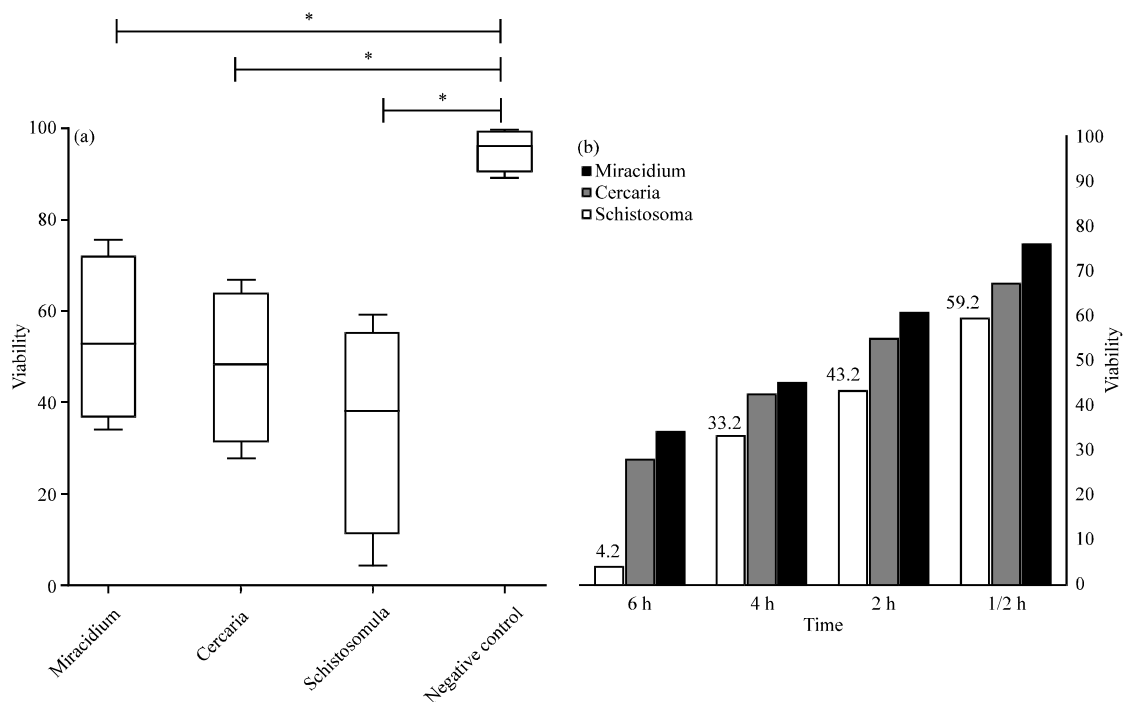
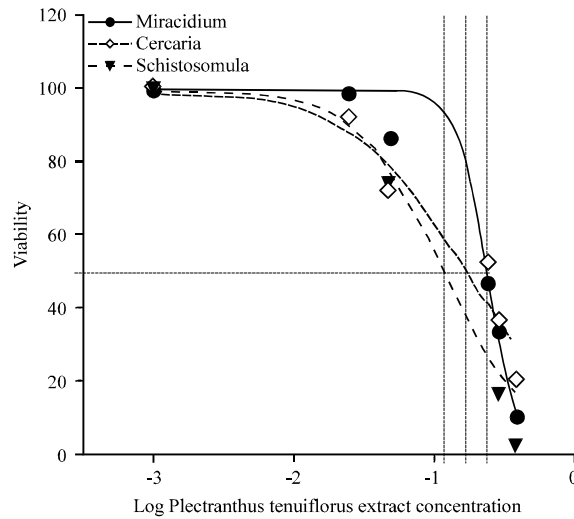


Fig. 1 (a-b): The inhibitory effect of methanol extract of *P. tenuiflorus* (Vatke) Agnew on tested *S. mansoni* stages. (a) show statistically significant inhibitory effect of methanol extract of *P. tenuiflorus* (Vatke) Agnew on all *S. mansoni* stages when compared to negative control, line in bars denote the Median with asterisks for standard deviation and (b) showing effect of the plant extract after different time intervals, with the lowest viability detected when *S. mansoni* schistosomula stages were incubated to the methanolic plant extract. Mann-Whitney U test used to compare two groups. p value (\*) is significant when between 0.01-0.05



	<i>Miracidium</i>	<i>Cercaria</i>	<i>Schistosomula</i>
50% efficient inhibitory extract concentration (IC <sub>50</sub> ). (mg/100 mL)	24.37	17.39	12.29

Fig. 2: The calculated (IC<sub>50</sub>) of *P. tenuiflorus* (Vatke) Agnew extract on *S. mansoni* stages viability. Lines indicate the fitted dose-response curve assuming a non-linear effect of Log concentration of plant extract, solid line for effect on miracidium, dashed line for effect on cercaria and dotted line for effect on Schistosomula. Values of calculated (IC<sub>50</sub>), half inhibitory efficient concentration on different *Schistosoma* stages are shown in small attached table, equation applied  $Y = 100 / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope}))}$ . X = Log conc, Y = response, Hill slope = Slope factor

*miracidium* stage, on the other hand it has more inhibitory effect on cercaria and schistosomula stages.

**Tegument morphological alterations detected by scanning electron microscopy:** Typical signs of injury were *S. mansoni* stages tegument changes which often consisted of vesiculations, thickening, wrinkling or perforations. As the tegument has a vital function, the parasite stages would often be weakened or die as a result of these changes. Obvious changes were detected in cercaria and schistosomula stages when compared to control specimen. *Schistosoma mansoni* cercaria tegument showed major changes represented in crumpling and wrinkling which led to obvious overall shortening of the cercaria Fig. 3a and b compared to Fig. 3e and f, furthermore clearly perforation were observed at the tail tegument Fig. 3c compared to Fig. 3g, with vesiculations/blebs at the forked distal part of the tail Fig. 3d compared to Fig. 3h.

Furthermore; *Schistosoma mansoni* schistosomula tegument exhibited the same overall shortening of the *schistosomula* Fig. 4b, when incubated in *P. tenuiflorus* (Vatke) Agnew extract compared to negative control Fig. 4a, in addition to observable swelling with higher concentration of extract Fig. 4c, this swelling obscured partially wrinkling or crumpling that observed in lower concentration.

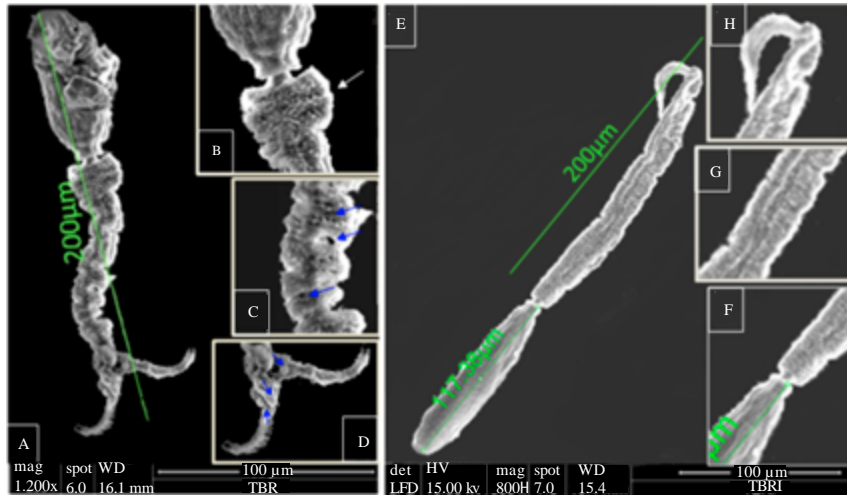


Fig. 3 (A-H): Scanning Electron Microscope (SEM) showing *Schistosoma mansoni* cercaria. (A) Showing the observed morphological changes due to 4 hour incubation in plant extract 250 mg/100 mL. Both body or tail tegument showed visibly tegument shrinkage and thickening compared to negative control (E), with remarkable wrinkling at tail just below the body seen in (B) and compared with (F), Tail teguments showed multiple holes/perforations (arrows) seen clearly in (C) compared to (G). Unmistaken vesicles/blebs (arrows) were observed at the distal forked part of the tail in (D), compared to (H)

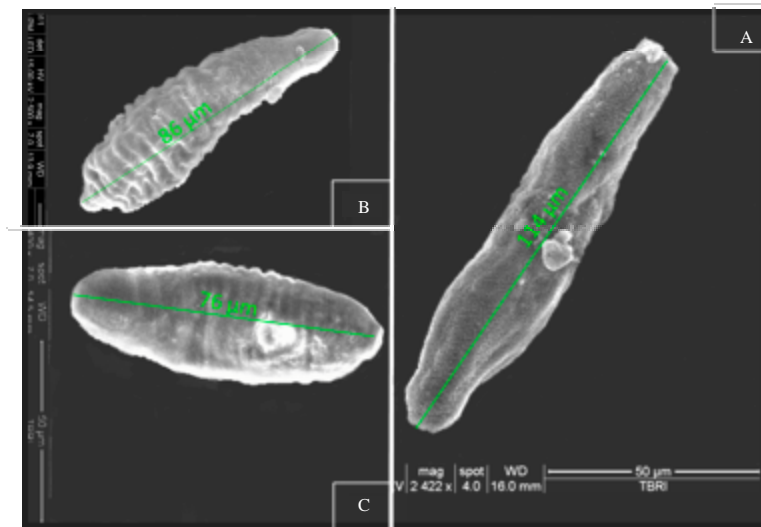


Fig. 4 (A-C): Scanning Electron Microscope (SEM) showing effect of *P. tenuiflorus* (Vatke) Agnew extract on *Schistosoma mansoni* schistosomula after 4 h incubation. (A) show negative control, unaffected stage is 114  $\mu$  in length. (B) Show the tegument wrinkling or crumpling due to 250 mg/100 mL plant extract that led to overall shortening compared to negative control. In (C) further morphological alteration when incubated in higher plant extract 400 mg/100 mL, observed as further shortening with tegument swelling that masked wrinkling or crumpling shown in (B)

## DISCUSSION

Schistosomiasis is a severe snail-borne disease affects more than 200 million people worldwide (WHO, 1998). Among different species of the genus *Schistosoma* known to infect humans *Schistosoma mansoni* is the major transmitted one and endemic in 54 countries and territories in South America, Africa and the Caribbean and the eastern Mediterranean regions (WHO, 1998). Schistosomiasis remains a major health problem despite the efforts to control the disease with significant disabling and financial burdens (Chitsulo *et al.*, 2000). One of the main requirements for treating schistosomiasis is the development of an efficient chemotherapeutic against schistosomulae.

Plants represent an important source of therapeutics from which 25% of the currently used pharmaceuticals has been derived (Farnsworth and Bingel, 1977). Medicinal plants represent natural therapeutic alternatives against pathogenic microorganisms, being safer than their synthetic counterparts.

In a search for natural anti-schistosomal products, the anti-schistosomal activity of ethanol extracts of leaves of one of folk and traditional medicinal plant; *P. tenuiflorus* (Vatke) Agnew against *Schistosoma mansoni* miracidia, cercaria and schistosomula was investigated *in vitro*. *P. tenuiflorus* is one of the Lamiaceae family, it's essential oil has antimicrobial activity against microorganisms including human pathogenic bacteria, yeast and fungi (Al-Garni and Kabli, 2005). It is commonly used as herb for non-specific treatment of ear ache and inflammation of middle ear (Hedberg, 1979) sore throat and laryngitis (Mossa *et al.*, 1987; Rahman *et al.*, 2004), respiratory system infections (Abulfatih, 1987) and digestive and genitourinary disorders (Pakia and Cooke, 2003). *P. tenuiflorus* was able to promote wound healing in rat model through stimulation of fibroblast proliferation both in rat model (*in vivo*) and in culture (*in vitro*) (Khorshid *et al.*, 2010). Due to its anti-inflammatory and anti-microbial activity it might be a candidate to test its anti-Schistosomal activity. To our knowledge *P. tenuiflorus* extracts and essential oil has been not tested for their anti-parasitic activity before and this might be the first study to assess anti-Schistosoma potency of this plant extract.

In our present study we demonstrated significant and reproducible anti-schistosomicidal activity of *P. tenuiflorus* methanol leaf extracts against miracidium, cercaria and schistosomula (Fig. 1-4). Interestingly, the *in vitro* effects of *P. tenuiflorus* methanol leaf extracts against *Schistosoma mansoni* miracidium, cercaria and schistosomula were due to both the effect on muscular function (loss of motor activity) and the extract induced tegumental changes (Fig. 3, 4). Both *Schistosoma mansoni* cercariae and schistosomula were most susceptible to *P. tenuiflorus* methanol leaf extracts with an  $IC^{50}$  of 12.29 and 17.39 mg/100 mL, respectively (Fig. 2), while miracidia being least susceptible with  $IC^{50}$  of 24.37 mg/100 mL (Fig. 2). The observed morphological alterations to cercarial tegument in this study were comparable to SEM findings after *in vitro* praziquantel exposure, as disruption of tegument, blebs formation and vacuolization (Oliveira *et al.*, 2006; Lofty *et al.*, 2009). These finding were proposed to occur at the base of the of the syncytial layer, increase in size, protrude above the surface and result in final bursting of the blebs (Brindley and Sher, 1987), in a process suggested to be accompanied by increased exposure of *Schistosoma* antigens at the parasite surface rendering it more susceptible to host immune system (Harnett and Kusel, 1986).

To date the majority of the phytochemical studies of the plant have concentrated on the isolation of diterpenoids and although these compounds have been shown to have potent antimicrobial activity few have been tested in bioassays that are directly related to the traditional



uses of the species the compounds were isolated from. Natural product chemists have mostly reported on novel diterpenoids in species of *Plectranthus*. Beside diterpenoids other monoterpenes and sesquiterpenes including  $\beta$ -caryophyllene, p-cymene and thymol were isolated from *Plectranthus aegyptiacus* in considerable constituents and many have antimicrobial activity (Smith *et al.*, 1996), where the plant extract lack alkaloids, steroids, anthraquinones and flavonoides (Alsofyani, 2006).

This study may suggest a promising efficacy of *P. tenuiflorus* (Vatke) Agnew methanol extract against *cercaria* which is the infective stage, as well as against the significantly ambulant invasive *Schistosomulae* stage inside human body. Thus, there is a scientific case for further studies to be undertaken on the activity and distribution of different extracts and to identify active compounds of this plant, along with weighing up their biosafety.

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