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▶ **To cite this version:**

Jean-Jacques M Bagalwa, Laurence Voutquenne-Nazabadioko, Charlotte Sayagh, Augustin S Bashwira. Evaluation of the biological activity of the molluscicidal fraction of Solanum sisymbriifolium against non target organisms. Fitoterapia, 2010, 81 (7), pp.767-771. 10.1016/j.fitote.2010.04.003 . hal-01996822

HAL Id: hal-01996822
https://hal.univ-reims.fr/hal-01996822

Submitted on 8 Nov 2021

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Evaluation of the biological activity of the molluscicidal fraction of *Solanum sisymbriifolium* against non target organisms.

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Abstract

The evaluation of the biocidal activity of the fruit of *Solanum sisymbriifolium* involving non target organisms such as aquatic insects, fish and snails lead to the isolation of the steroidal alkaloids, solamargine and β-solamarine, from the active fractions. The fractions A3 and C, with biological activity against fish, snail and aquatic insect and larvae, are able to affect the good functioning of ecosystem found on alimentary chain. The fraction B seems to be less toxic to fish and aquatic insect and larvae. The fraction B could thus be used as molluscicide in the future.

Key Words: Biocidal activity; Environmental degradation; *Solanum sisymbriifolium*; Steroidal alkaloids; Solamargine; Democratic Republic of Congo.

1. Introduction

Shistosomiasis is a parasitic disease that affects over 250 million people in tropical and subtropical areas of the world. The most efficient method of preventing the spread of the disease is destruction of the host snails by use of synthetic molluscicides or plant molluscicides. During our screening of Congolese plants for their possible use as molluscicidal agents, we noted that the ethanolic and aqueous extract of the fruit of *Solanum sisymbriifolium* (Solanaceae) were lethal *in vitro* against snail intermediates of *Schistosoma mansoni* found in the Eastern Democratic Republic of Congo. The saponin fraction showed a powerful molluscicidal activity at 1 mg/l and was planned for use in snail control in Democratic Republic of Congo where schistosomiasis is widespread [1-4]. This new drug from *Solanum sisymbrilifolium* must be evaluated for biocidal activity before use to avoid noxious effects on human beings, animals or plants and also to prevent contamination of the environment [5, 6]. No toxicity was reported for people who drank the infusion prepared from the ground dried fruit to treat ascites, mycosis, ringworms, whooping cough, cough, mumps, malaria, madness, edemas, myalgia and also to stop lactation [7].

Solanum sisymbriifolium is a large schrub native of South America, with yellow prickles and, when ripe, globulose shiny red berries enveloped by a dense prickly accrescent calyx [8]. It is distributed throughout the greater part of the Bushi area, South Kivu province, Democratic Republic of Congo. It is known as a medicinal plant that is used by the native healers both in veterinary and human medicine [7, 9]. *Solanum* species are well known to synthesize steroidal alkaloids and spirostane derivatives and some of which have shown molluscicidal activity [10]. Studies of some Brazilian *Solanum* species have shown that aerial parts of *S. sisymbriifolium* have significant molluscicidal activity [11]. Previous chemical studies on *S. sisymbriifolium* have reported the presence of alkaloids [12], and a spirostane saponin, named nuatigenosido in roots

[13, 14, 15], a steroidal alkaloid, solasodine, in leaves and berries [16, 17], as well as neolignan and sterols in berries [17]. In our study, the methanolic extract of the fruit of *S. sisymbriilofium* was fractionated and the biological activity of each fraction was tested against non target organisms such as aquatic insects, fish and snails. Two known compounds, solamargine (**1**), a chacotriose solasodine, and β-solamarine (**2**), a chacotriose tomatidenol, isolated from the active fraction B are reported for the first time in *S. sisymbriifolium.* This type of investigation would constitute a means to revaluate the African traditional ethnomedical systems on the one hand and Congolese resource on the other hand.

2. Material and Methods

2.1. General

NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. ESIMS and HRESIMS were recorded on a ESI-Q-TOF Micromass spectrometer. Column chromatography was performed with Kieselgel 60 (63 - 200 µm, Merck) silica gel or LiChroprep RP-18 (40 - 63 μm, Merck) silica gel. TLC analysis was run on 60 F_{254} precoated silica gel plates (Merck) and spots were visualized by heating after spraying with 50% H_2SO_4. Snails, aquatic insects (larvae and adults) and fish were identified by reference to the collection of the Laboratory of Malacology and Hydrobiology (Department of Biology, CRSN at Lwiro) where voucher specimens are preserved.

2.2. Plant material

The fruit of *Solanum sisymbriifolium* Lam. (Solanaceae) were collected at Lwiro (2° 14,228' S and 28° 48,441' E) in March 2004. Plant identification was done by comparison with authentic samples from the Herbarium of the Laboratory of Botany of the CRSN at Lwiro (Kivu Province, DR Congo) that contains 12000 plant specimens of the studied area.

2.3. Extraction and isolation of the steroidal alkaloids

The dried and powdered fruit (300 g) was extracted with 96 % methanol. The MeOH extract was concentrated and precipitated with acetone. Then, the crude saponin precipitate was dialyzed to give a saponin rich extract (2.69 g). This extract (2.5 g) was fractionated by VLC on reversed phase C_{18} (MeOH-H_2O, 60:40, 70:30, 80:20 and 100:0, each 300 ml) to give fraction A (1.52 g), B (680 mg), C (277 mg) and D (58 mg), respectively. Fraction A was repurified by RP-18 VLC (MeOH-H_2O, 40:60, 60:40, 80:20 and 100:0, each 200 ml) to give fraction A1 (1.08 g), A2 (64 mg), A3 (116 mg) and A4 (212 mg). A part of fraction B (100 mg) was chromatographed on silicagel CC (4g) eluted with a gradient of $CHCl_3$-MeOH-H_2O (70:30:1 to 70:30:2) to give **1** (20 mg). A part of fraction C (70 mg) was purified by silicagel CC using a gradient of $CHCl_3$-MeOH (9:1 to 7:3) followed by preparative TLC on silicagel with $CHCl_3$-MeOH-H_2O (70:30:5) as eluant, to give solamargine (**1**) (13 mg) and β-solamarine (**2**) (5.8 mg).

*2.3.1. Solamargine, (25R)-3β-{O-α-L-rhamnopyranosyl-(1–>2)-[O-α-L-rhamnopyranosyl-(1–>4)]-β- D-glucopyranosyloxy}-22αN-spirosol-5-ene (**1**)*

White powder, $C_{45}H_{73}NO_{15}$ ESIMS$^+$ m/z (rel. int.): 869 $[M+2H]^+$ (100), 868 $[M+H]^+$ (50), 413 $[C_{27}H_{43}NO_2]^+$ (2). ^{1}H NMR spectral data (500 MHz, CD_3OD): δ 0.88 (3H, s, H-18), 1.06 (1H, d,

J=7.2, H-21), 1.09 (3H, s, H-19), 0.93 (3H, d, *J*=6.3, H-27), 1.20 (1H, ddd, *J*=13.9, 8.3, 5.6, H-14), 1.25 (1H, m, H-12a), 1.27 (3H, d, *J*=6.2, H-6''), 1.29 (3H, d, *J*=6.2, H-6'''), 1.37 (1H, td, *J*=12.2, 6.0, H-15a), 1.48 (1H, td, *J*=13.0, 3.8, H-24a), 1.85 (1H, dm, *J*=13.2, H-12b), 1.86 (1H, dd, *J*=8.1, 7.4, H-17), 2.05 (1H, m, H-15b), 2.33 (1H, td, *J*=12.1, 1.6, H-4a), 2.33 (1H, ddm, *J*=13.2, 2.5, H-4b), 2.67 (1H, t, *J*=11.6, H-26a), 2.79 (1H, brd, *J*=10.2, H-26b), 3.36 (1H, m, H-5'), 3.42 (1H, t, *J*= 9.4, H-4''), 3.43 (1H, t, *J*= 8.4, H-2'), 3.44 (1H, t, *J*= 9.4, H-4'''), 3.55 (1H, t, *J*= 9.2, H-4'), 3.62 (1H, t, *J*= 9, H-3'), 3.65 (1H, dd, *J*= 9.3, 3.6, H-3'''), 3.68 (1H, dd, *J*= 12.1, 3.5, H-6a'), 3.69 (1H, dd, *J*= 9.2, 3.6, H-3''), 3.83 (1H, dd, *J*= 12.1, 1.7, H-6b'), 3.87 (1H, dd, *J*= 3.6, 1.3, H-2'''), 3.96 (1H, dd, *J*= 3.6, 1.3, H-2''), 3.97 (1H, m, H-5'''), 4.15 (1H, dq, *J*= 9.6, 6.2, H-5''), 4.44 (1H, q, *J*=7.3, H-16), 4.53 (1H, d, *J*=7.8, H-1'), 4.86 (1H, brs, H-1'''), 5.23 (1H, brs, H-1''), 5.41 (1H, brd, *J*=4.9, H-6). ^{13}CNMR spectral data (125MHz, CD_3OD): δ 15.2 (C-21), 16.8 (C-18), 17.8 (C-6''), 18.0 (C-6'''), 19.4 (C-27), 19.8 (C-19), 21.9 (C-11), 30.4 (C-24), 30.7 (C-2), 31.0 (C-25), 32.8 (C-8), 33.0 (C-15), 33.2 (C-7), 34.4 (C-23), 38.0 (C-10), 38.6 (C-1), 39.5 (C-4), 40.8 (C-12), 41.8 (C-13), 42.8 (C-20), 47.8 (C-26), 51.7 (C-9), 57.7 (C-14), 61.9 (C-6'), 63.7 (C-17), 69.8 (C-5''), 70.7 (C-5'''), 72.2 (2C, C-2'',C-3'''), 72.4 (C-3''), 72.7 (C-2'''), 73.7 (C-4'''), 73.9 (C-4''), 76.5 (C-5'), 78.0 (C-3'), 79.2 (C-2'), 79.3 (C-3), 80.0 (C-4'), 81.7 (C-16), 99.7 (C-22), 100.4 (C-1'), 103.0 (C-1'''), 102.3 (C-1''), 122.5 (C-6), 141.9 (C-5). HRESIMS (positive ion mode) *m/z* 868.5054 $[M+H]^+$ (calcd. for $C_{45}H_{74}NO_{15}$, 868.5058).

*2.3.2. β-solamarine, (25S)-3β-{O-α-L-rhamnopyranosyl-(1–>2)-[O-α-L-rhamnopyranosyl-(1–>4)]-β- D-glucopyranosyloxy}-22βN-spirosol-5-ene (**2**)*

White powder, $C_{45}H_{73}NO_{15}$ $ESIMS^+$ m/z (rel. int.): 869 $[M+2H]^+$ (100), 868 $[M+H]^+$ (55), 722 $[M+H-C_6H_{10}O_4]^+$ (25), 413 $[C_{27}H_{43}NO_2]^+$ (5). ^{1}H NMR spectral data (500 MHz, CD_3OD): δ 0.87 (3H, d, *J*=6.3, H-27), 0.90 (3H, s, H-18), 1.02 (1H, d, *J*=6.7, H-21), 1.05 (3H, s, H-19), 1.15 (1H,

m, H-14), 1.23 (1H, m, H-12a), 1.23 (3H, d, *J*=6.3, H-6’’), 1.25 (3H, d, *J*=6.2, H-6’’’), 1.31 (1H, m, H-15a), 1.35 (1H, m, H-24a), 1.78 (1H, dm, *J*=12.9, H-12b), 1.74 (1H, dd, *J*=8.4, 7.8 H-17), 2.00 (1H, m, H-15b), 2.29 (1H, tm, *J*=12.3, H-4a), 2.44 (1H, ddm, *J*=12.7, 2.6, H-4b), 2.74 (1H, t, *J*=11.5, H-26a), 2.82 (1H, brd, *J*=11.5, H-26b), 3.31 (1H, m, H-5’), 3.38 (1H, t, *J*= 9.4, H-4’’’), 3.39 (1H, dd, *J*= 8.9, 7.8 H-2’), 3.40 (1H, t, *J*= 9.3, H-4’’), 3.51 (1H, t, *J*= 9.2, H-4’), 3.58 (1H, t, *J*= 8.9, H-3’), 3.61 (1H, dd, *J*= 9.5, 3.0, H-3’’’), 3.64 (1H, dd, *J*= 12.0, 4.2, H-6a’), 3.65 (1H, dd, *J*= 9.2, 3.4, H-3’’), 3.78 (1H, dd, *J*= 12.0, 1.2, H-6b’), 3.82 (1H, dd, *J*= 3.0, 1.3, H-2’’’), 3.91 (1H, dd, *J*= 3.4, 1.3, H-2’’), 3.92 (1H, m, H-5’’’), 4.12 (1H, dq, *J*= 9.5, 6.2, H-5’’), 4.25 (1H, q, *J*=7.2, H-16), 4.49 (1H, d, *J*=7.8, H-1’), 4.83 (1H, brs, H-1’’’), 5.19 (1H, brs, H-1’’), 5.37 (1H, brd, *J*=4.7, H-6). ^{13}CNMR spectral data (125MHz, CD_3OD): δ 15.6 (C-21), 17.2 (C-18), 17.9 (C-6’’), 18.0 (C-6’’’), 19.4 (C-27), 19.8 (C-19), 22.0 (C-11), 27.0 (C-23), 28.7 (C-24), 30.7 (C-2), 31.0 (C-25), 32.7 (C-8), 33.3 (C-15), 33.2 (C-7), 38.0 (C-10), 38.5 (C-1), 39.5 (C-4), 41.0 (C-12), 41.8 (C-13), 43.1 (C-20), 50.3 (C-26), 51.6 (C-9), 57.2 (C-14), 61.9 (C-6’), 63.3 (C-17), 69.8 (C-5’’), 70.6 (C-5’’’), 72.2 (2C, C-2’’,C-3’’’), 72.3 (C-3’’), 72.4 (C-2’’’), 73.7 (C-4’’’), 73.9 (C-4’’), 76.6 (C-5’), 78.0 (C-3’), 79.2 (C-2’), 79.3 (C-3), 80.0 (C-4’), 80.8 (C-16), 99.1 (C-22), 100.4 (C-1’), 102.3 (C-1’’), 103.0 (C-1’’’), 122.6 (C-6), 141.9 (C-5). HRESIMS (positive ion mode) *m/z* 868.5054 $[M+H]^+$ (calcd. for $C_{45}H_{74}NO_{15}$, 868.5058).

2.4. Extraction and preparation of the serial dilutions to determine the lethal concentration (LC)

The fruit of *Solanum sisymbriifolium* were sun-dried, pulverized and 3 kg of the powder were extracted by maceration overnight with 70 % EtOH and intermittent vigorous shaking. The extract was filtered and concentrated to dryness. Five grams of the EtOH extract were dissolved in distilled water to make serial dilutions: 5, 0.5, 0.05, 0.005 g/ml to determine the lethal

concentration [18]. Fractions A1 (65.38 mg), A3 (0.62 mg), B (1.48 mg) and C (1.48 mg) of the MeOH extract were dissolved in distilled water (1 ml) to make the serial dilutions 10^{-1}, 10^{-2}, and 10^{-3}, supplemented in some cases by intermediate half dilutions, to determine the LC_{100}, LC_{50} and LC_0.

2.5. Study of the biocidal activity and experimental design

2.5.1. Piscicidal activity

Adults and subadult fish of *Haplochromis sp*, *Oreochromis nilotica* and *Oreochromis macrochir* (Cichlidae) were caught in the ponds at Lwiro (DR Congo) and kept in an aquarium in the Laboratory of Hydrobiology and fed on vegetation before the test.

Six individual fish were sorted by groups of adults and subadults in separate 5 l containers filled with 4 l of tap water and kept under oxygenation. Serial dilutions of plant drugs were added to each container and the mortality rate was noted after 30 minutes and 24 hours. Untreated containers with fish were used as control. Each concentration was done in triplicate.

2.5.2. Insecticidal activity

Larvae of Anophelidae (*A. gambia*, *A. funestus*), Aeschnidae (*Aeschna sp*), Coenagrionidae, Hydrobidae, Noctonectidae, Gyrinidae, Libellidae, Chironomidae, Baetidae, and Pleidae were collected in the pond at Lwiro using the techniques described by Basabose [19]. The larvae and adult insects were transferred to the Laboratory of Entomology before the test.

Ten aquatic insects (larvae and adults) of each species were put into a 500 ml container and filled with 200 ml of tap water. The plant extract was then added and the mortality rate was monitored every 30 min per each dose [20, 21]. Untreated batches with insects were used as control. Each concentration was done in triplicate.

2.5.3. Molluscicidal activity

Adults of *Biomphalaria pfeifferi* (Planorbidae*),* and *Lymnae natalensis* (Planorbidae) were collected in streams located at Lwiro [2] using a plankton net and kept in the Laboratory of Malacology. Molluscicidal activity was evaluated based on WHO specifications [18]. Uniform *Biomphalaria pfeifferi* and *Lymnae natalensis* snail were tested for the production of cercariae and the snails that produce the cercariae were eliminated from the test of biocidal activity.

Snails (*Biomphalaria pfeifferi* and *Lymnae natalensis*) were incubated at 25 °C for 24 h with extract dilutions using the method previously described [1]. Dead snails were counted and removed and the survivors were maintained in fresh deionized water for a further 24 h. A new count of dead snails was then made. Each concentration was done in triplicate.

3. Results and Discussion

The mortality rate of aquatic animal such as snails, insects and fish due to the crude EtOH extract of *Solanum sisymbriifolium* is presented in Table 1. The results clearly show that the extract of *S. sisymbriifolium* is more toxic to fish than to snail intermediates (*Biomphalaria pfeifferi* and *Lymnae natalensis*). These results are similar to those previously found in the literature [21, 22, 23]. WHO [18] has suggested that the extract of molluscicidal plants have higher toxicities to fish than to snails. The LC_{50} of fish are 10 or 100 times lower than other aquatic animals. This extract has no effect on Pleidae and Aschnidae.

The TLC analysis of the MeOH extract of *S. sisymbriifolium* showed the presence of a major compound with at least four minor compounds. In order to isolate the major compound, the

extract was fractionated by VLC. Fractions A1, A3, B and C were also tested on snails, aquatic insects (larvae and adults) and fish (Table 2 and 3). The TLC chromatographic profile showed that A3 and B contained the major compound of the plant, identified after purification of fraction B and comparison of NMR data to the literature as solamargine (**1**), a common steroid glycoalkaloid of the *Solanum* species [24, 25]. Fraction C contained solamargine (**1**) and a second compound, identified after purification as β-solamarine (**2**) [26, 27]. β-solamarine naturally occurs in a number of *Solanum* species along with solasonine and solamargine [28]. Fraction A1 contained polar compounds such as sugars, and tannins. This fraction is only toxic to fish and to Coenagrionidae (Table 2), and does not contain very much of the active principle.

The toxicity of the fraction A3 isolated from *S. sisymbriifolium* caused 50 % mortality among fish, snails and some aquatic insects and larvae (Anophelidae, Libellidae, Coenagrionidae) at a concentration lower than the toxicity of the Ethiopian Endod-44 (5 mg/ml) [29] (Table 3). Aquatic adult insects (Aeschnidae, Baetidae, Chironomidae, Gyrinidae, Noctonectidae, Pleidae) are the most resistant to the fraction A3, with LC_{50} estimated to 0.38 mg/ml. Compared to the extract of *Maesa lanceolata* [23], the toxicity of this one is high.

The LC_{50} of fraction B (0.73 mg/ml) for fish is tenfold less active after 24 h than that observed for snails and other aquatic insect and larvae (0.08 mg/ml) in general. Thus, the fraction B is less toxic to fish than to aquatic insects and larvae and is a good candidate as a molluscicidal agent [21, 30].

The toxicity of fraction C is 10 fold higher for some aquatic insects and larvae (Anophelidae, Noctonectidae, Chironomidae, Coenagrionidae) than for fish and snails. This fraction is the least active on snails. Fraction C contains solamargine (**1**), β-solamarine (**2**), and another non identified minor compound which are probably responsible for the difference in activity between

fractions B and C. Solamargine and β-solamarine are frequently associated in *Solanum* spp. and the possibility exists of synergism within and between different glycoalkaloid types under natural conditions [28]. Solamargine and β-solamarine are both known to possess molluscicidal activity [27]. Studies on various glycoalkaloids have shown that toxicity was due to acetyl cholinesterase inhibition and cell membrane disruption but these effects do not appear to be pronounced for solamargine [28, 31, 32].

In conclusion, the fractions A3 and C showed biological activity against fish, snail and aquatic insect and larvae but the fraction B seemed to be less toxic to fish and aquatic insect and larvae. The high toxicity of these fractions to biota can affect the good functioning of ecosystem as argue other works [23, 30, 33]. Thus, the fraction B could be used as a molluscicide in the future for snail control programs with careful monitoring to avoid any environmentally damaging effects, especially when indigenous populations use it for fishing during the dry season.

Acknowledgements

The authors are grateful to the laboratory staff of the Department of Biology of Lwiro (DRCongo) and Pharmacognosy of UMR 6229 CNRS (France) for respectively collecting material and fractionation of material.

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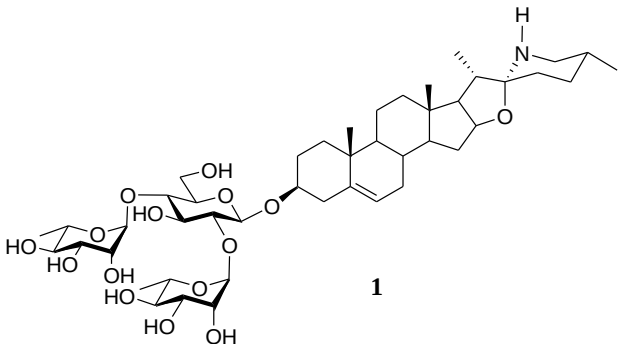
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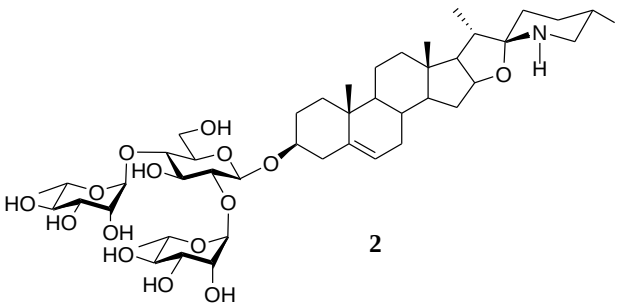
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Table 1. Biocidal activity (g/ml) of crude extract of *Solanum sisymbriifolium*

Species	LC_{100}	LC_{50}	LC_{00}
Fish			
Oreochromis nilotica	0.5	0.05	0.005
Oreochromis macrochir	0.5	0.05	0.005
Haplochromis sp	0.5	0.05	0.005
Insects and larvae			
Anophelidae	5	0.75	0.005
Noctonectidae	5	0.75	0.005
Gyrinidae	10	0.45	0.005
Libellidae	5	0.75	0.005
Pleidae	na	na	na
Aeschinidae	na	na	na
Chironomidae	5	0.75	0.005
Baetidae	5	0.75	0.005
Coenagrionidae	100	50	25
Snails			
Biomphalaria pfeifferi	50	5	0.5
Lymnae natalensis	50	5	0.5

na : not active

Table 2. Biocidal activity (mg/ml) of fraction A1 of *S. sisymbriifolium* after 24 h.

Species	LC_{100}	LC_{50}	LC_{00}
Fish			
Oreochromis nilotica	6.5	3.25	0.065
Oreochromis macrochir	6.5	3.25	0.065
Haplochromis sp	6.5	3.25	0.065
Insects and larvae			
Coenagrionidae	na	0.5	0.05

Table 3. Biocidal activities (mg/ml) of fractions A3, B and C of *S. sisymbriifolium* after 24 h.

	A3			B			C		
Species	LC_{100}	LC_{50}	LC_{00}	LC_{100}	LC_{50}	LC_{00}	LC_{100}	LC_{50}	LC_{00}
Fish									
Oreochromis nilotica	0.062	0.031	0.0062 ±0.002	1.48	0.73 ±0.01	0.0148 ±0.005	1.48	0.74 ±0.01	0.0148 ±0.007
Oreochromis macrochir	0.062	0.031	0.0062 ±0.002	1.48	0.73 ±0.01	0.0148 ±0.005	1.48	0.74 ±0.01	0.0148 ±0.007
Haplochromis sp	0.062	0.031	0.0062 ±0.002	1.48	0.73 ±0.01	0.0148 ±0.000	1.48	0.74 ±0.01	0.0148 ±0.007
Insects and larvae									
Anophelidae	0.062	0.031	0.0062 ±0.002	0.148	0.08	0.0148 ±0.003	0.148	0. 08	0.0148 ±0.008
Noctonectidae	0.62	0.31	0.062 ±0.02	0.148	0.08	0.0148 ±0.003	0.148	0. 08	0.0148 ±0.008
Gyrinidae	0.62	0.31	0.062 ±0.00	0.148	0.08	0.0148 ±0.003	na	na	na
Libellidae	0.62	0.062	0.0062 ±0.002	0.148	0.08	0.0148 ±0.003	1.48	0.8	0.148 ±0.078
Pleidae	0.62	0.31	0.062 ±0.02	0.148	0.08	0.0148 ±0.003	1.48	0.8	0.148 ±0.078
Aeschinidae	0.62	0.31	0.062 ±0.02	0.148	0.08	0.0148 ±0.003	na	na	na
Chironomidae	0.62	0.31	0.062 ±0.022	0.148	0.08	0.0148 ±0.003	0.148	0.08	0.0148 ±0.008
Baetidae	0.62	0.31	0.062 ±0.022	0.148	0.08	0.0148 ±0.003	na	na	na
Coenagrionidae	0.62	0.062	0.0062 ±0.002	0.148	0.0148	0.00148 ±0.003	0.148	0.08	0.0148 ±0.008
Snails									
Biomphalaria pfeifferi	0.62	0.31	0.062 ±0.022	0.148	0.08	0.0148 ±0.003	1.48	0.8	0.148 ±0.078
Lymnae natalensis	0.62	0.31	0.062 ±0.022	0.148	0.08	0.0148 ±0.003	1.48	0.8	0.148 ±0.078

na : not active