Molluscicidal activity of *Hammada scoparia* (Pomel) Iljin leaf extracts and the principal alkaloids isolated from them against *Galba truncatula*

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The molluscicidal activity of Hammada scoparia *leaf extracts and the principal alkaloids isolated from them (carnegine and* N-*methylisosalsoline) were tested against the mollusc gastropod,* Galba truncatula*, the intermediate host of* Fasciola hepatica *in Tunisia. The results indicated that the molluscicidal activity was correlated with the presence of alkaloids. A significant molluscicidal value, according to the World Health Organization, was found with the methanol extract (LC_{50} = 28.93 ppm). Further fractionation of the methanolic extract led to the isolation of two principal alkaloids: carnegine and* N-*methylisosalsoline. These alkaloids are isoquinolines that have not previously been characterised for their molluscicidal activity. The* N-*methylisosalsoline possesses the highest molluscicidal activity (LC_{50} = 0.47 µM against* G. truncatula*).*

Key words: molluscicidal activity - *Galba truncatula* - *Fasciola hepatica* - *Hammada scoparia* - major alkaloids - *N*-methylisosalsoline

Fasciolosis, caused by *Fasciola hepatica,* a parasitic trematode, is of considerable medical and veterinarian importance. This old disease can spread widely because of the large colonisation capacity of the parasite, as well as of the freshwater lymnaeid snail vector species (Mas-Coma et al. 2001). *Galba truncatula* (Lymnaeidae family) has been identified as the principal intermediate host of *F. hepatica* in Southern Tunisia (Ayadi et al. 1997, Hammami & Ayadi 1999, Hammami et al. 2007). One of the major preventive steps against fasciolosis is the control of the vector snail population. Use of molluscicides to eradicate the snail vector is considered the method of choice. Plants containing alkaloids appear to be among the most promising for controlling schistosomiasis and fasciolosis (Melendez & Capriles 2002, El-Ansary et al. 2003, Ahmed & Rifaat 2004, Silva et al. 2006).

Since *Hammada scoparia* (Chenopodiaceae family) has been described as a plant rich in alkaloids (Carling & Sandberg 1970, Benkrief et al. 1990, Jarraya et al. 1993, 2008, Jarraya & Damak 2001), we designed the present study to assess the molluscicidal activity of *H. scoparia* leaf extracts and the principal alkaloids isolated from them against the mollusc gastropod, *G. truncatula*.

H. scoparia (Pomel) Iljin = (*Haloxylon scoparium* (Pomel) Bge. = *Haloxylon articulatum ssp scorparium* (Pomel) Batt. = *Arthrophytum scoparium* (Pomel) Iljin) belongs to Chenopodiaceae family and is locally known as *rimth* in Sfax, Tunisia. Samples of this plant were collected in June 2006 in Sfax, Tunisia and botanically identified by Pr. Mohamed Chaeib, Botanist at the faculty of Science Sfax. Voucher specimens (LCSN101) have been deposited at the Laboratoire de Chimie des Substances Naturelles, Faculty of Science, University of Sfax, Tunisia.

Extracts of air-dried leaves of *H. scoparia* were prepared by two methods. For the first method, 300 g of air-dried leaves were extracted with 10% ethanol in water for 48 h at RT. The ethanol was removed and the remaining aqueous phase was lyophilised to produce the EtOH-H_2O extract (40.52 g).

For the second method, 200 g of air-dried leaves of *H. scoparia* were extracted (for 24 h each time) in a Soxhlet apparatus with hexane, dichloromethane and methanol, in succession. Each extract was concentrated to produce the hexane extract (HE, 2.10 g), the dichloromethane extract (DE, 4.55 g) and the methanol extract (ME, 30.15 g), respectively. Each extract was weighed and kept in a refrigerator at 4°C until use. A sample of each extract was tested for the presence of alkaloids using Mayer's and Dragendorf's reagents.

An acid-base treatment of the methanol extract was performed as follows: 20 g of the methanol extract were dissolved in hydrochloric acid (0.05 M) and then extracted three times with chloroform. After removing and evaporating the chloroform we obtained extract A (1.66 g). The remaining aqueous layer was alkalinised, adjusted to pH = 9 by the addition of ammonia solution 28%, then extracted three times with chloroform. After removing and evaporating the chloroform, we obtained extract B (5 g), containing total alkaloids. Finally, the remaining aqueous layer was extracted three times with butanol. After removing and evaporating the butanol we obtained extract C (3.35 g) containing polar products.

Extract B (5 g) was loaded on a silica gel column (Merck, 230-400 mesh) and eluted with a gradient of chloroform/methanol (100:0 → 0:100). Eleven fractions were collected. Two fractions contained the pure

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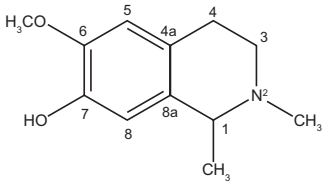
alkaloids: carnegine 1 (1050 mg) and *N*-methylisosalsoline 2 (545 mg), previously isolated from *H. scoparia* (Carling & Sandberg 1970, Benkrief et al. 1990, Jarraya & Damak 2001, Jarraya et al. 2008). The configuration of the chiral centre of C_1 of *N*-methylisosalsoline is Rectus (Figure).



1: R = CH_3 carnegine; 2: R = H *N*-methylisosalsoline.

Adult *G. truncatula* (3-5 mm in length) were collected locally from a drainage canal located in Tozeur's traditional oasis, in February 2007. They were transferred to laboratory aquaria and acclimatised for a minimum of four days in holding tanks containing aerated, dechlorinated tap water and washed sand. They were then exposed to aqueous solutions of the extracts. The toxicity of these preparations was also tested on *G. truncatula* control organisms.

Evaluation of the molluscicidal activity of the *H. scoparia* extracts and $CuCl_2$ (used as positive control) against snails was investigated as recommended by the WHO (1965). A series of aqueous solutions to be used in the bioassays was prepared from each of the four extracts, hexane, dichloromethane, methanol and EtOH-H_2O, respectively. Each series consisted of 20 solutions containing amounts ranging from 5-100 mg of extract material in 5 mg increments. The same method was adopted for the preparation of each concentration of the extracts A, B and C obtained from the methanol extract. For the pure products, the weights ranged from 0.1-1 mg of *N*-methylisosalsoline or carnegine in increments of 0.1 mg. For $CuCl_2$, the weights ranged from 1-20 mg in 1 mg increments. We added sufficient dechlorinated water to each amount of extract material to give a final volume of 1000 mL. Each solution was divided into equal volumes of 500 mL. For each test, we used a control of dechlorinated water without extract, having the same volume as the test solution.

The snails were exposed in groups of 10 (5 replicates) for 48 h (exposure period) to 500 mL of each concentration of one of the materials to be tested: extracts, fractions and products or $CuCl_2$ (the positive control used in each case), as shown in Tables I and II. *G. truncatula* negative control organisms were immersed in dechlorinated water. After exposure, snails were rinsed thoroughly in dechlorinated water and left for 48 h in dechlorinated water (recovery period) before mortality was evaluated. Mortality was recorded after 48 h and dead animals were removed immediately to avoid contamination of live animals. Snail mortality was established by the contraction of the body into the shell. No response to a needle probe was taken as evidence of death.

The concentrations that killed 50% (LC_{50}) or 90% (LC_{90}) of the exposed snails and the confidence interval (95% CI) were determined by the R language analysis, which is an integrated suite of software facilities for data manipulation, calculation and graphical display (Ihaka & Gentleman 1996, Venables et al. 2004).

The molluscicidal activity of *H. scoparia* leaf extracts and $CuCl_2$ against *G. truncatula* (Table I) was highest for the methanol extract (LC_{50} = 28.93 ppm, LC_{90} = 69.96 ppm), next highest for the dichloromethane extract (LC_{50} = 33.90 ppm, LC_{90} = 74.67 ppm) and lowest for the ethanol-water extract (LC_{50} = 46.87 ppm, LC_{90} = 72.10 ppm). Cupric chloride at 11.34 ppm induced the death of 90% of molluscs after six days of treatment (Table I). Chemical tests performed on the extracts for alkaloids were positive, particularly for the methanol extract, followed by the ethanol-H_2O extract. The hexane extract was inactive against *G. truncatula and* did not contain alkaloids.

The molluscicidal activities of extracts A, B and C, obtained after acid-base treatment of the methanol extract, the fractions obtained by column chromatography and the purified alkaloids are reported in Table II. Molluscicidal activity was not observed for extracts A and C and was concentrated in extract B (total alkaloids). Fractions 1-5, obtained from extract B were inactive. These fractions contained carnegine and other minor alkaloids. The molluscicidal activity reappeared in fraction 6, in which *N*-methylisosalsoline started to be eluted from the column and increased in fraction 7, in which pure *N*-methylisosalsoline was eluted (LC_{50} = 0.47 µM LC_{90} = 0.86 µM after 48 h). Then, the molluscicidal activity decreased gradually in fractions 8 and 9 and disappeared in the last fractions. *G. truncatula* control organisms were not affected by dechlorinated water after 48 h exposure.

The prevention of fasciolosis is difficult, despite the efficiency of the measures taken. Chemical molluscicides such as sulphate, copper chloride and the Bayluscides are known to have a strong lethal effect on invasive mollusc species (Rondelaud 1986, Claudi & Mackie 1994, Kennedy et al. 2006). It has been realised, however, that these molluscicides are toxic to non-target animals and have a long-term detrimental effect on aquatic environments (Andrews et al. 1983, Singh et al. 1996). Several plant products with molluscicidal activity against the genus *Lymnaea* have been reported. An aqueous solution of the latex of *Euphorbia splendens* var. *hisloppi* with molluscicidal activity against *Lymnaea columella* has been reported in laboratory (LC_{90} = 0.55 mg/L) and in field conditions (Vasconcellos & Amorim 2003a, b).

H. scoparia is a highly branched, halophytic shrub distributed in Southeast of Tunisia, Spain and parts of Turkey, Iran, Syria and Iraq (Irano-Turanian Region) (Maire 1962, Jafri & Rateeb 1978, Pottier-Alapetite 1981, Greuter et al. 1984). An aqueous extract of this plant has been reported to possess larvicidal (Sathiamoorphy et al. 1997), anti-cancer and anti-plasmodial activities (Sathiamoorphy et al. 1999). The molluscicidal activity of *H. scoparia* has not previously been reported.

During our study, of the four *H. scoparia* leaf extracts, three showed significant molluscicidal activity. However,

only two extracts, the dichloromethane extract and the methanol extract, gave LC_{50} values (33.90 ppm and 28.93 ppm, respectively) against *G. truncatula*, where these fell well below the upper threshold of 40 ppm (WHO 1993). The analyses of *H. scoparia* extracts showed that the methanol one was the richest in alkaloids and the most active against *G. truncatula* (LC_{50} = 28.93 ppm), so alkaloid content may correlate with molluscicidal activity. The predominant alkaloids isolated, carnegine 1 and *N*-methylisosalsoline 2, are isoquinolines that have not previously been characterised for their molluscicidal activity. The results obtained indicated that the *N*-methylisosalsoline 2 (LC_{50} = 0.47 µM) possessed the highest molluscicidal activity against the snails, after 48 h, compared with

TABLE I

Molluscicidal activities of *Hammada scoparia* leaf extracts against *Galba truncatula*

	After 24 h action		After 48 h action		
Extract	LC_{50} (ppm) (95% CI)	LC_{90} (ppm) (95% CI)	LC_{50} (ppm) (95% CI)	LC_{90} (ppm) (95% CI)	Alkaloids
EtOH-H_2O (1-9, v-v)	77.65 (73.46; 81.83)	96.87 (83.52; 110.23)	46.87 (42.38; 51.22)	72.10 (64.44; 85.74)	++
Hexane	82.75 (71.76; 137.72)	122.21 (92.90; 383.26)	82.75 (71.76; 137.72)	122.21 (92.89; 383.26)	-
CH_2Cl_2	33.99 (23.17; 41.47)	89.64 (66.45; 274.71)	33.90 (11.38; 45.57)	74.67 (59.42; 125.87)	++
MeOH	31.68 (23.89; 39.48)	75.96 (64.49; 87.43)	28.93 (21.36; 36.50)	69.96 (59.52; 80.41)	+++
Dechlorinated water	0	0	0	0	-

the $CuCl_2$ showed molluscicidal activity against the mollusca gastropoda *G. truncatula* only after 144 h with: LC_{50} = 6.20 (5.42; 6.94) ppm and LC_{90} = 11.34 ppm. CI: confidence interval; LC_{50}: 50% lethal concentration; LC_{90}: 90% lethal concentration; ++: presence; +++: richness; -: not detected.

TABLE II

Molluscicidal activities of *Hammada scoparia* leaf extracts after acido-basic treatment of the methanolic extract, its fractions and their major isolated alkaloids against *Galba truncatula*

		After 24 h action		After 48 h action	
Extracts	Fraction of extract B	LC_{50} (ppm) (95% CI)	LC_{90} (ppm) (95% CI)	LC_{50} (ppm) (95% CI)	LC_{90} (ppm) (95% CI)
A and C		0	0	0	0
B		37.53 (32.29; 43.01)	50.40 (43.76; 74.80)	29.51 (22.47; 34.96)	49.25 (41.30; 67.65)
	Fraction 1-2	0	0	0	0
	Fraction 3 (carnegine)	0	0	0	0
	Fraction 4-5	0	0	0	0
	Fraction 6	0.38 (0.31; 1.01)	0.66 (0.45; 17.33)	0.22 (0.07; 0.71)	0.52 (0.05; 5.19)
	Fraction 7 (*N*-methyl isosalsoline)	0.27 (0.21; 0.35)	0.47 (0.36; 1.13)	0.098 (0.084; 0.122)	0.18 (0.14; 0.31)
	Fraction 8	0.43 (0.15; 1.28)	1.51 (0.20; 11.37)	0.29 (0.16; 0.51)	1.00 (0.08; 12.29)
	Fraction 9	0.75 (0.19; 2.88)	2.49 (0.15; 41.36)	0.31 (0.17; 0.55)	1.49 (0.22; 10.24)
	Fraction 10-11	0	0	0	0
Dechlorinated water		0	0	0	0

CI: confidence interval; LC_{50}: 50% lethal concentration; LC_{90}: 90% lethal concentration.

other products tested, while carnegine was found to be inactive. It is likely that the phenolic function of the *N*-methylisosalsoline contributes to molluscicidal activity.

In our study, the *N*-methylisosalsoline was more active than the cupric chloride, which showed activity only after 144 h (LC_{90} = 11.34 ppm). A comparison with synthetic molluscicides, such as niclosamide (LC_{90} = 0.45 ppm) and nicotinanilide (LC_{90} = 1.39 ppm), used against *Lymnaea luteola* (Sukumaran et al. 2004), showed that N-methylisosalsoline had comparable molluscicidal activity after 24 h. According to the World Health Organization's guidelines on screening for plant molluscicides (WHO 1983), isolation of the molluscicidal compound *N*-methylisosalsoline from *H. scoparia* may add to the arsenal of methods to control snails that transmit fasciolosis in tropical and third world countries, where fasciolosis is a common disease.

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