MOLLUSCICIDAL EFFECT OF *EOMECON CHIONANTHA* ALKALOIDS AGAINST *ONCOMELANIA HUPENSIS* SNAILS

Fei Peng, Ming Liu, Qiongyao Huang, Nianmeng Liu, Huazhong Yang, Hui Sun, Qi Hu, Fang Feng and Chonghe Jiang

Medical College of Hunan Normal University, Changsha, Hunan, China

Abstract. The molluscicidal effects of *Eomecon chionantha* alkaloids (ECA) against *Oncomelania hupensis* snails were determined by immersion method. The molluscicidal effect was positively related to ECA concentration, immersion time and temperature of the immersion solution. The mortality of the snails reached 100% by 72 hours in ECA at a concentration of 2.5 mg/l at 25ºC. The alanine aminotransferase (ALT) level of liver cells treated with ECA was higher than controls at 24 and 36 hours (57.7 and 60.3 U/l *versus* 39.2 and 49.2 U/l, respectively) but the level decreased at 48-72 hours after treatment. The decrease points to the toxic effect of ECA against liver cells. After ECA treatment, the liver cells were edematous with swollen or disintegrating nuclei; they were enlarged and had vacuolated rER; they had dilated and vesiculated mitochondria with broken crests further indicating a hepatotoxic effect of ECA in *O. hupensis* snails. ECA has a molluscicidal effect that may be of practical use in the field to control *O. hupensis* snails.

Keywords: *Oncomelania hupensis* snail; alanine aminotransferase, *Eomecon chionantha* alkaloids, liver, plant molluscicide, ultrastructure

INTRODUCTION

Schistosomiasis is a common tropical disease causing health problems for people in large parts of the world (Steinmann *et al*, 2006; Dai *et al*, 2008). The World Health Organization (WHO) estimates about 3 billion people reside in schistosomiasis endemic countries and more than 200 million are infected; a third of these are children under 15 years of age (Barbosa, 1995; Steinmann *et al*, 2006; Yang *et al*, 2008). In China, *Schistosoma japonicum* is prevalent and *Oncomelania hupensis* snail is the only intermediate host (Zhou *et al*, 2005). Currently, the snail-infested area covers more than 3.5 billion m^2, mainly in the lake regions along the Yangtze River (Zhang *et al*, 2008; Wu *et al*, 2008). Control of *O. hupensis* snails is one of the most important strategies for interrupting and eliminating schistosomiasis transmission (Xu *et al*, 2004); molluscicidal methods have been the focus of research for half a decade (Zhou *et al*, 2005, 2007).

There are several methods of killing *O. hupensis* snails, including environmental, physical, biological and pharmacological control measures (Tchounwou *et al*, 1991; Souza, 1995). To date, artificial chemical agents have routinely been used, since their molluscicidal effects are more durable and easily limited to distribution

Correspondence: Qiongyao Huang, Medical College of Hunan Normal University, Changsha 410013, Hunan, China.
Tel: +86 731 88912429; Fax: +86 731 88912417
E-mail: huangqy08@yahoo.com

areas of the snail. The drawbacks are potential toxic effects to humans, livestock, fish and overall environmental pollution (Andrews *et al*, 1982; Yang *et al*, 2008).

Native plant molluscicides have become popular snail control research topics, due to easy access, low cost, rapid biodegradation and comparably less environmental damage (Webbe and Lambert, 1983; Marston *et al*, 1993; Li *et al*, 2002; Singh and Singh, 2004; Yang *et al*, 2008). *Eomecon chionantha* alkaloids (ECA), extracted from *Eomecon chionantha* Hance, an herbaceous perennial indigenous to China, have shown encouraging molluscicidal activity and seems less hazardous to non-target organisms (Yang *et al*, 2003b). The present studies were designed to determine the optimal molluscicidal effects of ECA in terms of ECA concentration, immersion period and temperature of immersion solution. To further explore the involved working mechanisms, alanine aminotransferase (ALT) levels evaluating the snail liver were determined along with the ultrastructure of the liver cells.

MATERIALS AND METHODS

ECA extraction

Eomecon chionantha Hance was collected from the Renxing hills of Taojiang County, Hunan Province, China, in November. The rootstalks of the grasses were dried and ground into powder. The powder (400 g) was soaked in 70% alcohol and stored in a one-liter percolator over night, then percolated at 5-8 ml/minute until no alkaloid reaction remained. The alcohol was evaporated under reduced pressure and the pH of the concentrated extract adjusted to pH 2-3 with HCl to better extract the active ingredient more efficiently under acidic conditions. The extract was processed by adding NaCl until a yellow colored precipitate was obtained. The precipitate was filtrated to gain a first precipitate (A); the filtrate was further concentrated and a second precipitate (B) was obtained. A mixture of precipitates A and B was dissolved in distilled water at 45-50ºC after which the above procedures were repeated three times to reach a pure yellow colored precipitate, *ie*, ECA hydrochloride.

Molluscicidal test

O. hupensis snails were collected from Dongting Lake 2-3 days before the experiment. Adult non-infected snails, 6-10 mm long with 6-9 shell striae, were selected for the experiment.

The ECA was prepared at concentrations of 10, 5, 2.5, 1.25, 0.63 and 0.31 mg/l of water (de-chlorinated water was used throughout the experiment). Fifty snails were put into a nylon net bag (10 cm in diameter), and each bag was immersed in a tank with 2,500 ml of different concentrations of ECA solution or water for the experiment. Eight bags with snails were used to test each ECA concentration; four bags were immersed in ECA solution and four in water as controls. One bag was removed from each solution at 24, 48, 72 and 96 hours, washed two times with water and stored out of water for 72 hours. For each concentration, the experiment was performed at 20ºC, 25ºC and 30ºC. The number of dead snails was determined by direct observation of snail movements in water; snails with a closed operculum and/or lack of movement were further tested using the knocking method of Webbe (1961), by inspecting movement of the soft body after crushing the shell. Snails without movement were presumed dead.

Liver alanine aminotransferase (ALT)

Four groups of 40 snails were assigned for liver ALT measurement. Three

Table 1
Molluscicidal effect of ECA at different concentrations, immersion times and temperatures.

ECA (mg/l)	Mortality of snails (%, *n*=50/group)											
	24 hours			48 hours			72 hours			96 hours		
	20°C	25°C	30°C	20°C	25°C	30°C	20°C	25°C	30°C	20°C	25°C	30°C
10.0	12	32	46	38	90	96	60	100	100	82	100	100
5.0	10	18	38	26	80	94	32	100	100	64	100	100
2.5	8	12	16	14	48	86	16	100	100	40	100	100
1.25	4	12	16	10	32	76	14	26	100	40	68	100
0.63	2	8	10	8	30	48	12	16	92	16	36	98
0.31	2	6	6	4	8	24	8	20	42	8	26	100
Control	0	2	0	0	4	0	0	0	6	2	2	18

ECA, *Eomecon chionantha* alkaloids

groups were immersed in ECA solution at concentrations of 5, 2.5 and 1.25 mg/l based on the lowest effective molluscicidal concentrations for 24 - 72 hours, along with one control group in water without ECA. Ten snails from each group were exposed for 24, 36, 48 and 72 hours, at a temperature of 25ºC. The snails were sacrificed immediately after these exposure times and their livers carefully dissected out under a dissecting microscope. A liver homogenate was prepared in 0.1 ml precooled phosphate buffer (0.2 mol/l, pH 7.1) in an ice bath. After centrifugation at 0-4ºC for 5 minutes at 8,000*g*, the upper phase was collected. ALT activity was analysed by Guilbault´s spectrophotometric technique (Guilbault, 1976) at 340 nm, using a DBDA full automatic biochemical analyser (DADE, USA). ALT activity was expressed in international units (U/l).

Liver ultrastructure

Fifty test snails were immersed in ECA (5 mg/l, based on the highest effective molluscicidal concentration) and 50 control snails were immersed in water at 25ºC for 48 hours. After immersion, the live snails were sacrificed, their livers were dissected out and rapidly fixed in 2.5% glutaraldehyde at 4ºC. The samples were rinsed in 0.1 M PBS followed by 1% osmium tetroxide fixation, then dehydrated stepwise with 50, 70, 90 and 100% acetone. The specimens were then immersed in a mixture of acetone and epoxy resin (1:1) followed by embedding in pure epoxy resin. The sample was cut into serial sections of 60 nm. The sections were double stained with acetic acid uranium and lead nitrate and studied with a Hitachi H2600 Transmission Electron Microscope.

Statistical analysis

Data were analyzed with statistical software SPSS (version 11.0 for Windows, SPSS, Chicago, IL). All data are presented as mean±SD. ANOVA was used for multigroup comparisons followed by a least significant difference (LSD) post hoc test; $p < 0.05$ was considered statistically significant.

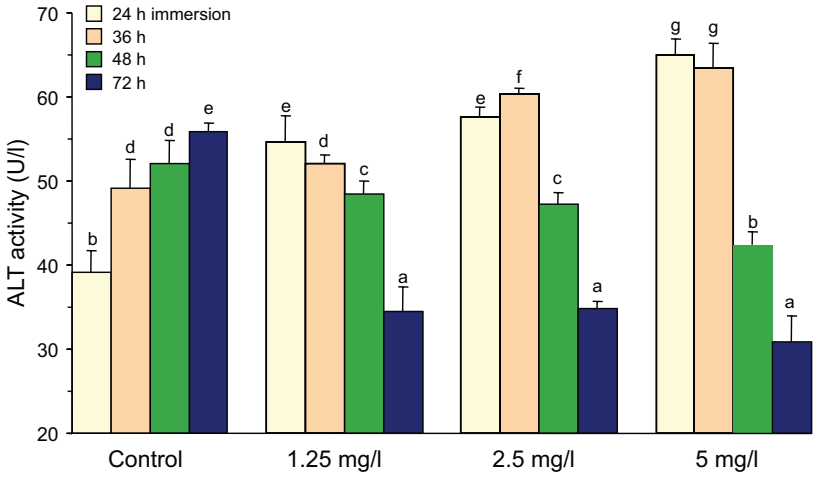

Fig 1–Changes in alanine aminotransferase (ALT) activity in *Oncomelania hupensis* snails with different ECA concentrations at various immersion times. Temperature of immersion solution was 25ºC. The ALT levels in the control animals gradually increased with immersion time, while a bimodal effect was seen after ECA exposure. At short exposure times (24-36 hours) the ALT increased more than the controls, but then it decreased after longer exposure (48-72 hours). Bars without a common superscript letter differ significantly (Mean ± SD, n = 10/group, $p < 0.05$).

RESULTS

Molluscicidal results

A potent molluscicidal effect of ECA against *O. hupensis* was demonstrated in this study (Table 1) with the effect increasing with increasing ECA concentrations, exposure times and temperatures of immersion solution. Immersed in 1.25 mg/l of ECA solution, at a temperature of 30ºC, the mortality rate of the *O. hupensis* snails was 100% at 72 hours. The effect was less when the temperature of the ECA solution was lowered to 25 or 20ºC. At the latter temperature the mortality rate were only 26% and 14% at the same concentrations and exposure times. Even at the highest ECA concentration (10 mg/l), the mortality rate only reached 60% at 20ºC, and increased to 82% at the longest exposure time (96 hours). However, the mortality rate was clearly different from the snails ($p < 0.05$, chi-square test) at the same concentration of ECA (of 1.25 mg/l) and the same temperature (25ºC or 20ºC) but for a shorter time of 24 hours. The required concentration could be decreased to the lowest tested (0.31 mg/l) at 72 hours or longer immersion times. At 30ºC, the differences were significant for all test groups, regardless of ECA concentrations and immersion periods. Using multiple stepwise linear regression, the obtained regression equation that describes the relation found between snail mortality and ECA concentration (C), solution temperature (T) and exposure time (Et) was Y = 5.7xC + 4.2xT + 0.7xEt –120. From these results, an ECA concentration of 2.5 mg/l seems reasonable for field molluscicidal trails.

Changes in liver enzyme activity

Remarkable changes in snail liver alanine aminotransferase (ALT) activity were exhibited after ECA treatment (Fig 1). The ALT activity gradually increased with immersion time in the control group while there was a bimodal effect in the ECA exposure groups. At shorter exposure times (24 and 36 hours) ALT activity was significantly higher to the ECA exposed

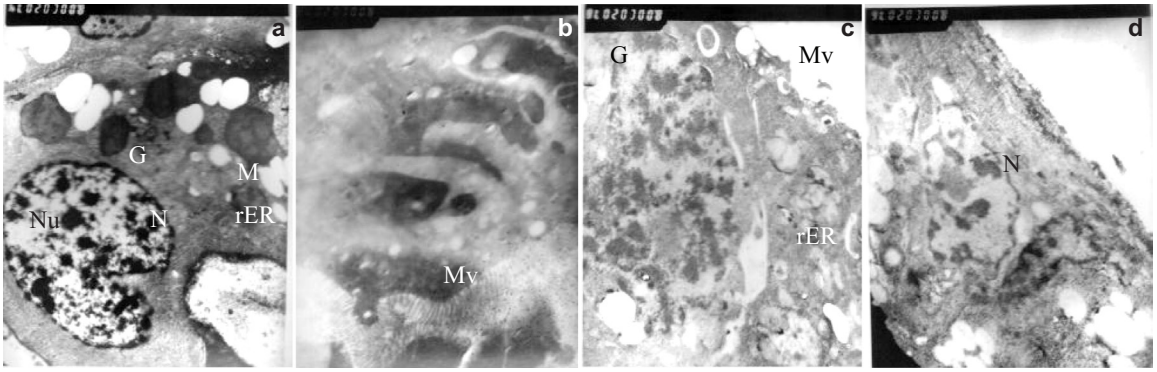

Fig 2–Normal ultrastructure of liver cells in control *Oncomelania hupensis* snails (a, b, c, d, x 800). G: secretary granules (glycogen in **c**); M, mitochondria; N, nucleus; Nu, nucleolus; Mv, microvilli; rER, rough endoplasmic reticulum.

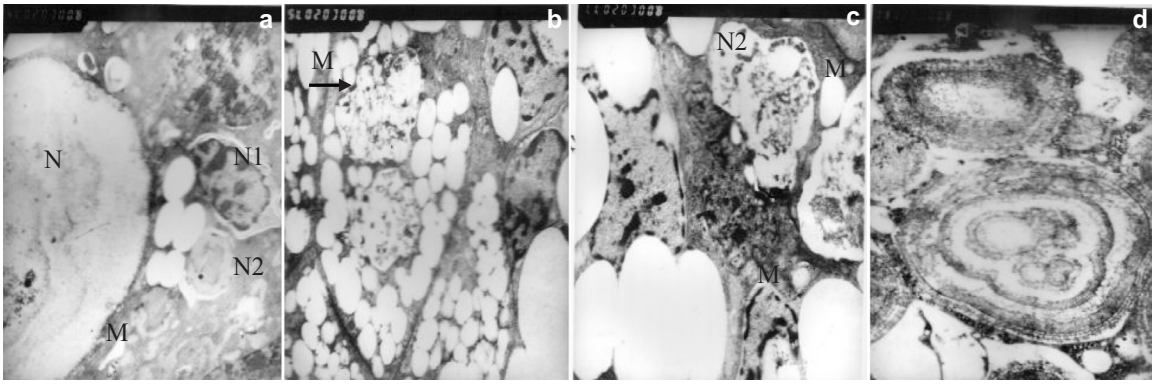

Fig 3–Ultrastructure of liver cells of *Oncomelania hupensis* snails at 48 hours of ECA immersion (a, b, c, d, x 800). M, mitochondria; N, nucleus. Note degenerated liver cells with incomplete and vacuolated mitochondria cristae (b arrow, c), distended bubble-like endoplasmic reticulum; nuclei necrosis (b, c N2), concentric structures between hepatic lobules (d).

group than in controls, reaching 54.7 - 65.0 U/l after 24 hours and 52.0 - 63.5 U/l after 36 hours, compared to 39.2 and 49.2 U/l in the controls ($p < 0.01$). The higher the ECA concentration, the larger the increase in ALT level over both exposure periods ($p < 0.05$). However, with longer exposure periods (48 and 72 hours), the ALT activity decreased significantly compared to earlier values and the controls, decreasing to 42.4 - 48.4 U/l and 30.8 - 34.5 U/l after immersion times of 48 and 72 hours, while the control groups increased to 52.1and 55.9 U/l, respectively. The early increase and late fall in ALT activity after ECA immersion of the snails was the typical finding in this study.

Changes in liver ultrastructure

Control group. The typical ultrastructural appearance of normal *O. hupensis* snail liver cells is shown in Fig 2. The nucleus (N) is oval-shaped with a clear membrane and a large nucleolus (Nu). Chromatin is abundant and granular, scattered in small clumps (Fig 2a). The cell

is rich in high electron dense cytoplasm, containing plentiful secretory granules (G) of different shapes and sizes with variable electron density (Fig 2a, c, d). There are well-developed organelles and abundant rough endoplasmic reticulum (rER), mainly around the nucleus. The rER is equipped with a large number of ribosomes (Fig 2a, c). Mitochondria (M) with vigorous secretory function are distributed throughout the cytoplasm. Microvilli (MV) are densely and orderly arranged along the free surface of the cells (Fig 2b, c).

Test group. After being immersed in 5 mg/l ECA at 25ºC for 48 hours, the liver cells of *O. hupensis* snails were swollen, and in many cells the matrix of the cytoplasm is sparse. Some degenerated cells with ruptured membranes were also observed (Fig 3). The nucleus was swollen and vesiculated with heterochromatin dispersed in the sparse nucleoplasm and without an obvious nucleolus. Some nuclei were shrunken or broken with extremely low electron density, leakage of nuclear material or further breakdown of the heterochromatin (Fig 3a, c). In serious cases, the nuclei were lysed and fragmentized, leaving very few coagulated chromosome plaques (Fig 3c). The organelles were damaged with different levels of vacuolations; secretory granules had broken membranes and less residual content.

The rough endoplasmic reticulum (rER) was distributed in scattered fragments; some of the rER was fractured and organized as tubelets or vacuoles (Fig 3c). Shed ribosomes were also observed. Fewer, apparently degenerated, mitochondria were found, their crests were blurred or missing, some were lysed or enlarged and vesiculated (Fig 3c) and some were broken with fractured inner crests (Fig 3b). Microvilli were fewer than normal and irregularly distributed. The gaps between cells were increased with irregular margins and filled with the residue of damaged cells. Concentric circular structures were observed between the hepatic lobules (Fig 3d).

DISCUSSION

A reliable molluscicidal effect was seen with ECA in the present study as well as in a previous study (Liu *et al*, 2001). The molluscicidal effect of ECA was positively related to ECA concentration, immersion time and temperature of the immersion solution. At a water temperature of 25ºC, the maximal lethal effect was obtained after an immersion time of 72 hours and at an ECA concentration of 2.5 mg/l. The results provide practical variables for field mulluscicide trials, *eg* season (water temperature) for drug application, optimal final concentration of ECA and required time to snail-elimination.

Eomecon chionantha Hance, an herbaceous perennial plant, grows in wet soil on hills and in ditches in eastern, southern and southwestern China, especially along the Yangtze River. Grass is abundant in these areas and easy to collect (Liu *et al*, 2001). The ECA extraction process is simpler and more efficient using alcohol as an extractor compared to water. When combined with the molluscicide niclosamide, ECA counteracts the stimulatory effect of niclosamide on snails, resulting in a synergic molluscicidal effect of the two substances. With this combination, substance usage can be limited (Liu *et al*, 2006). Recently, a main component monomer of *Eomecon chionantha* alkaloids, Sanguinarine, was tested and found to have the same molluscicidal effect (data not shown). Therefore, *Eomecon chionantha* Hance extracts may have the potential

to be developed into a commercially available molluscicide that could play a substantial role in the control of the schistosomias vector *O. hupensis* snails.

Alanine aminotransferase (ALT) is the most important transaminase in *O. hupensis* snails (Wang and Song, 1990). In addition to ammonia transformation, ALT is closely related to the action of the Krebs cycle and linked to protein synthesis and glucose metabolism. The enzyme is found mainly in the liver. ALT changes are a sensitive indicator of liver cell injury (Kolodziejczyk *et al*, 2005). Compared to controls, ALT levels in snails exposed to ECA were significantly higher with short exposure times and increased with ECA concentration. With longer exposure times ALT activity decreased. We assumed the early increase was due to enzyme induction in the liver cells, while the later decrease was the result of toxic damage with disordered function or necrosis of the liver cells. Therefore, measurement of ALT levels may reflect the level of liver damage and point to a possible ECA molluscicidal mechanism.

An increase in ALT activity was also present in the control groups of snails, albeit at a lower level than in the test groups. *O. hupensis* snails are amphibious. Long immersion in water could result in hypoxia, manifested as a moderate increase in the ALT level. Immersion of field in water is an important means for snail control, although eight months are required to reach the aims of eradication of *O. hupensis* snails (Xu *et al*, 2002). In comparison, the present immersion times were short (up to 96 hours), presumably far from the lethal times (although some dead snails were encountered after 96 hours in water at 30ºC, a temperature not used for ALT measurement).

The liver is an important detoxifying organ for *O. hupensis* snails and is one of the most vulnerable organs to toxins. This study showed remarkable alterations in liver cells after ECA treatment, including edema and degeneration accompanied by swelling and dissolving nuclei, enlarged and vacuolated rER and dilated and vesiculated mitochondria with broken crests, giving further support for the hepatotoxic effects of ECA against *O. hupensis* snails. Such hepatotoxic effects could dramatically lower the detoxifying abilities of the liver, and resulting in profound enzymatic and metabolic changes that may eventually poison the snails to death.

Mitochondria dysfunction could affect the process of oxidative phosphorylation, and as a consequence, disrupt the cell's energy supply. Located on the inner membrane of mitochondria and mitochondrial crests, ATPase (Mg^{2+}-ATPase) is the key enzyme for energy production for cell activity from hydrolysis of ATP. Reduced ATPase production could hinder the generation of oxidative phosphorylation coupling and lead to an energy deficit in the cell (Kolodziejczyk *et al*, 2005). Thus ECA induced mitochondria damage, as demonstrated by the present study, could be a cause of snail death.

ACKNOWLEDGEMENTS

This investigation was supported by the Scientific Research Fund of the Hunan Provincial Education Department (No. 03A058), Hunan Provincial Health Department (No. 9686) and Hunan Provincial Scientific and Technical Department, China (No. 2006SK1001). The authors thank Professor Sivert Lindström, Department of Clinical and Experimental Medicine, University of Linköping, Sweden for has constructive advice regarding the paper.

REFERENCES

Andrews P, Thyssen J, Lorke D. The biology and toxicology of molluscicides, Bayluscide. *Pharmacol Ther* 1982; 19: 245-95.

Barbosa FS. Determination and control of schistosomiasis. *Mem Inst Oswaldo Cruz* 1995; 90: 155-9.

Dai JR, Wang W, Liang YS, Li HJ, Guan XH, Zhu YC. A novel molluscicidal formulation of niclosamide. *Parasitol Res* 2008; 103: 405-12.

Guilbault G. Handbook of enzymatic methods of analysis. Clinical and biological analysis. New York: Marcel Dekker, 1976.

Kolodziejczyk L, Siemieniuk E, Skrzydlewska E. Antioxidant potential of rat liver in experimental infection with *Fasciola hepatica*. *Parasitol Res* 2005; 96: 367-72.

Li J, Qian W, Huang F, Jiang Z, Zhang K. Advance of plant molluscicide studies. *Chin J Schisto Control* 2002; 14: 67-9.

Liu N, Huang Q, Peng F, *et al*. Study on synergistic molluscicidal effect of *Eomecon chionantha* alkaloids combined with niclosamide. *Chin J Schisto Control* 2006; 1: 143-5.

Liu N, Peng F, Huang Q, *et al*. Primary study on molluscicidal effects of *Eomecon chionantha* alkaloids. *Chin J Schisto Control* 2001; 13: 303-4.

Marston A, Maillard M, Hostettmann K. Search for antifungal, molluscicidal and larvicidal compounds from African medicinal plants. *J Ethnopharmacol* 1993; 38: 215-23.

Singh A, Singh DK. Effect of herbal molluscicides and their combinations on the reproduction of the snail *Lymnaea acuminata*. *Arch Environ Contam Toxicol* 2004; 46: 470-7.

Souza CP. Molluscicide control of snail vectors of schistosomiasis. *Mem Inst Oswaldo Cruz* 1995; 90: 165-8.

Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J. Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. *Lancet Infect Dis* 2006; 6: 411-25.

Tchounwou PB, Englande Jr AJ, Malek EA. Toxicity evaluation of Bayluscide and malathion to three developmental stages of freshwater snails. *Arch Environ Contam Toxicol* 1991; 21: 351-8.

Wang GF, Song GM. Preliminary studies on transaminase of *Oncomelania* snail. *Chin J Parasitol Parasit Dis* 1990; 8: 110-1.

Webbe G. Laboratory and field trials with a new molluscicide, Bayer 73, in Tanganyika. *Bull World Health Organ* 1961; 25: 525-32.

Webbe G, Lambert JD. Plants that kill snails and prospects for disease control. *Nature* 1983; 302: 754.

Wu XH, Zhang SQ, Xu XJ, *et al*. Effect of floods on the transmission of schistosomiasis in the Yangtze River valley, People's Republic of China. *Parasitol Int* 2008; 57: 271-6.

Xu X, Liu J, Wei F, *et al*. Study of a new technique on the prevention of *Oncomelania hupensis* snail dispersal in the irrigation schemes in middle reaches of Yangtze River. *Chin J Epidemio* 2002; 23: 94-8.

Xu XJ, Wei FH, Cai SX, *et al*. Study on the risk factors of schistosomiasis transmission and control strategy in the Three Gorges Reservoir Areas. *Chin J Epidemiol* 2004; 25: 559-63.

Yang H, Huang Q, Peng F, Liu N, Feng F, Sun H. Observations on acute toxicity of *Eomecon chionantha* Hance alkaloids to fish. *Chin J Schisto Control* 2003; 15: 9-10.

Yang XM, Chen SX, Xia L, Chen J. Molluscicidal activity against *Oncomelania hupensis* of Ginkgo biloba. *Fitoterapia* 2008; 79: 250-4.

Zhang Z, Ong S, Peng W, *et al*. A model for the prediction of *Oncomelania hupensis* in the lake and marshland regions. *Chin Parasitol Int* 2008; 57: 121-31.

Zhou XN, Guo JG, Wu XH, *et al*. Epidemiology of schistosomiasis in the People's Republic of China, 2004. *Emerg Infect Dis* 2007; 13: 1470-6.

Zhou XN, Wang LY, Chen MG, *et al*. The public health significance and control of schistosomiasis in China–then and now. *Acta Trop* 2005; 96: 97-105.