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Toxicity of a molluscicide candidate PPU07 against *Oncomelania hupensis* (Gredler, 1881) and local fish in field evaluation

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HIGHLIGHTS

- PPU07 was a newly-synthesized compound.
- Two dosing forms of PPU07, namely hydrochloride and 25% sulfate WP formulation were obtained.
- 25% PPU07 sulfate WP exhibited similar molluscicidal effects with the only WHO recommended molluscicide – niclosamide.
- In contrast to niclosamide, no obvious toxicity was observed for local fish at the molluscicidal concentrations of PPU07.

GRAPHICAL ABSTRACT

PPU07 mollusicicidal activities

25% PPU07 sulfate MP	Adjusted mortality (%)		
2.0 g/m² (spray)	96.45 (D1*)	90.17 (D3*)	95.28 (D7*)
2.0 g/m³ (immersion)	97.68 (D1*)	94.44 (D2*)	96.52 (D3*)

* D, day

PPU07 toxicity for local fish

	Concn (g/m³)	No. of dead fish (n = 20)[a] 24h	48h	72h	Mortality (%)
25% PPU07 sulfate WP	5	0	0	0	0
50% Niclosamide WP	0.5	20	20	20	100

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ABSTRACT

Schistosomiasis japonica caused by *Schistosoma japonicum* infection is recognized as a considerable economic and public health concern in Asia. *Oncomelania hupensis* is the sole intermediate host of *S. japonicum*. The only molluscicide recommended by World Health Organization (WHO) since 1960s is relative toxic to other aquatic species. In this article, we evaluated the novel molluscicide PPU07 in field trials on their efficiency against *O. hupensis* and toxicity for local fish. 25% PPU07 sulfate WP exhibited similar molluscicidal effect at 2.0 g/m^2 and 2.0 g/m^3 in the spraying and immersion trials with the WHO recommended molluscicide niclosamide (1 g/m^2 and 1 g/m^3). The mortality rates reached 95% and 96%, respectively. Moreover, little toxicity was observed for local fish and other aquatic organisms at the effective molluscicidal concentrations. In all, 25% PPU07 sulfate WP is a promising molluscicide for snail control, particularly in semi-commercial or commercial aquaculture ponds.

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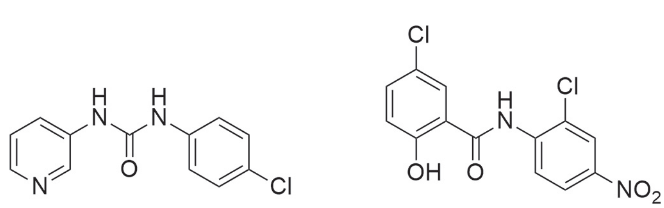
1. Introduction

Schistosomiasis is the second most prevalent endemic disease after malaria in tropical and subtropical regions (Chitsulo et al., 2000; Sady et al., 2013; Thetiot-Laurent et al., 2013). The occurence of schistomiasis has distinct regional character. *Schistosoma japonica* (Katsorada, 1904) is widespread in Asia, while *schistosomiasis mansoni* is popular in Africa, South America, the Caribbean and the Middle East (Berriman et al., 2009; Inobaya et al., 2014; Thetiot-Laurent et al., 2013). More than 58 million people are infected with *S. japonica* worldwide (World Health Organization, 2018). *S. japonica* was previously under control in China. However, it was in high risk of re-emerging especially along the Yangtze River and mountains or hills in southwest China according to recent monitoring data (Guo et al., 2016; Lim, 2014). 413,000 people in 7 provinces (Hubei, Hunan, Jiangxi, Anhui, Jiangsu, Sichuan and Yunnan) are suffering from the disease (Yang et al., 2014). *Oncomelania hupensis* (Gredler, 1881) is the only intermediate host of *S. japonicum*. Among the efforts to control schistosomiasis, snail control is considered to be the most effective methods to interrupt the transmission of *S. japonicum* and chemical molluscicides are widely used for snail control (Yang et al., 2010). Niclosamide (5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide) is the only commercially available molluscicide recommended by World Health Organization (WHO) since 1960s. However, it is toxic to fish and other aquatic animals, which limits its application in aquaculture areas. The development of potent molluscicides with low environmental toxicity is in urgent need.

Great progress has been made in extracting molluscicidal substances from natural products (Alsherbiny et al., 2018; Araujo et al., 2018; Faria et al., 2018; Oliveira-Filho et al., 2010; Rocha et al., 2015; Tallarico and Rapado, 2014). However, the effective extracts vary from the weather and region, which limited their use in field evaluation. And most of the synthetic molluscicides were lack of selectivity and caused damage to the environment (Lima et al., 2002; Upadhyay and Singh, 2011). Recently, we designed and synthesized a series of pyridylphenylureas and evaluated their molluscicidal activities against *Biomphalaria straminea*, the intermediate host of *Schistosoma mansoni* in laboratory (Wang et al., 2018). Most of the derivatives were less active against *B. straminea,* while 1-(4-chloro phenyl)-3-(pyridin-3-yl) urea (named PPU07, Fig. 1) and its 4-bromo phenyl substituted analogue exhibited strong molluscicidal effects (LC_{50} = 0.50 mg/L and 0.51 mg/L) against *B. straminea* snails and good selectivity to local fish, including cyprinoid carp (*Cyprinus carpio*), tilapia (*Oreochromis aureus*), grass carp (*Ctenopharyngodon idella*). The 4-bromo phenyl analogue was relatively less economical, and therefore less suitable in developing countries. Later, PPU07 were evaluated against *O. hupensis* snails via spraying and immersion methods in laboratory, and the 72 h LC_{50} was 0.37 ± 0.05 g/m^2 and 0.25 ± 0.08 mg/L respectively. At 1 g/m^2 or 1 mg/L, PPU07 completely killed the *O. hupensis* snails after 72 h. At 5 mg/L, PPU07 did not cause mortality to any of the tested zebrafish (*Danio rerio*). Herein, we tested the molluscicidal activity of PPU07 with different dosage form in the marshland infested with *O. hupensis* snails. The environmental safety of PPU07 for local fish was also studied in field conditions.



G1714 Niclosamide

Fig. 1. Chemical structures of PPU07 and niclosamide.

2. Material and methods

2.1. Drugs candidates

The niclosamide wettable powder (WP) (active pharmaceutical ingredient, API = 50%) was purchased from Senliang Pharmaceuticals (Suzhou, Jiangsu Province, China). The PPU07 was synthesized as we have reported before (Wang et al., 2018). The hydrochloride and sulfate were obtained by salification of PPU07 with 99% and 98% yield, respectively. The PPU07 sulfate WP (API = 25%) was manufactured by National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention. The 25% PPU07 sulfate WP was obtained from the mixture of kaolin (23 μm, 1240 g), white carbon black (140 g), dispwet wp-410 (120 g), and PPU07 (API, 500 g) via fine-grounding in a ball mill and sieving with a 45-μm mesh sieve.

2.2. Study sites and molluscicidal treatment

The PPU07 hydrochloride field trials were carried out in marshland of Midu County, Dali City, Yunnan Province, China (see Fig. 2). The 25% PPU07 sulfate MP field trials were performed in marshland of Jiangling County, Jinzhou City, Hubei Province, China (see Fig. 3). The researchers followed the same procedures in the two places. A baseline survey of snail density was detected before the field trials (Wang et al., 2000).

For the spraying methods, plots with more than 5 *O. hupensis* snails per square frame (covers 0.11 m^2) in the same ecological environment were selected as study plots. A negative control plot was located separately. Meanwhile, 2 or 3 plots were prepared for PPU07 and one for niclosamide as positive control. Each plot covered an area of 100 m^2 (10 m × 10 m) and was surrounded by low soil dykes interval (5 m width) to avoid the migration of snails. The soil dykes were also treated with corresponding molluscicidal compounds. No weeded higher than 5 cm was reserved before the

Fig. 2. Study plots (**a**) and ditches (**b**) of PPU07 hydrochloride field trials in Midu County, Yunnan Province, China. Molluscicide application in the immersion trials (**c**) and in the spraying trials (**d**).

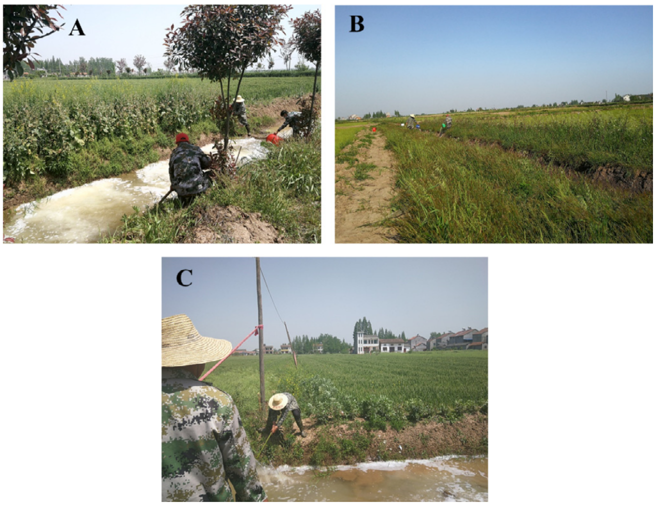


Fig. 3. Molluscicide application of 25% PPU07 sulfate MP in the immersion trials (**a**), the spraying trials (**b**), and the fish toxicity (**c**) in Jiangling County, Hubei Province, China.

molluscicide treatment. The PPU07 hydrochloride or 25% PPU07 sulfate WP were sprayed on the test plots to reach corresponding concentration with mist spraying machines according to the Schistosomiasis prevention handbook by Wang et al., in 2000. In each plot, more than 50 snails from a total of ten square frames were collected by random systematic sampling on day 1 (D1), day 3 (D3), and day 7 (D7) post-treatment. They were washed with dechlorinated water, and recovered for 24 h. The presence of discoloration, the absence of muscle contraction, hemorrhage, and the deterioration of the body tissues indicated the death of the snails. Mortality and adjusted mortality of the snails were calculated as follows (Xia et al., 2014):

$$\text{Mortality} = \frac{Number_{dead\ snails}}{Number_{total\ snails}} \times 100\%$$

$$Mortality_{adjust} = \frac{Mortality_{mulluscicides} - Mortality_{\ control}}{1 - Mortality_{control}} \times 100\%$$

For the immersion method, six ditches (snail density > 5/0.1 m^2) in similar ecological environment were selected to test PPU07 at 4 different concentrations as well as positive control (niclosamide) and negative control. Dykes as interval (50 cm) were built to divide a ditch into several units, and a volume of 3 m^3 water was impounded into each unit. For the PPU07 groups and niclosamide group, the compounds was added to the units and mixed stably to reach corresponding concentrations. The snail-containing soil on the wall of the ditch was shoveled into the ditch water. The snails were collected by a triangular net (0.15 m^2, 20 meshes/inch) after D1, day 2 (D2) and D3 post-treatment. The mortality of the snails was determined as we described above for the spraying methods.

2.3. Fish toxicity evaluation

The fish toxicity test was performed in ditches with the same ecological environment. The ditches were divided into several units by building dykes and a volume of 1.2 m^3 water was impounded into each unit. 25% PPU07 sulfate WP or 50% niclosamide WP was added to corresponding concentrations in the ditches and then local fish (~50–100 g/fish, 20 fish/m^3), including crucian carp (*Carassius carassius*) and grass carp, were immersed with the ditch water in fish cages (0.6 m × 0.5 m × 0.44 m). The fish mortality was investigated on 24 h, 48 h, 72 h after drug treatment.

3. Results

3.1. Molluscicidal effect of PPU07 hydrochloride against O. hupensis in field trials

PPU07 hydrochloride was synthesized to improve the water solubility. Its molluscicidal activities were evaluated via both spraying and immersion methods at the terrain of the snail habitat. For the spraying method, PPU07 hydrochloride was tested at 2 g/m^2 and 4 g/m^2 in comparison with 1 g/m^2 niclosamide (Table 1). Niclosamide was fast-acting with adjusted mortality rates of 90.69% after 1 day treatment and the high rates maintained during our experiments (82.76% at D3 and 85.11% at D7). A time and dose-dependent molluscicidal effect was observed for PPU07 hydrochloride. It was weaker than niclosamide at 2 g/m^2. However, it exerted similar molluscicidal effect with niclosamide at 4 g/m^2 after 3 days (88.23% at D3 and 82.76% at D7).

For the immersion method, PPU07 hydrochloride was tested at 0.5, 1, 2 and 4 g/m^3 in comparison with 1 g/m^3 niclosamide (Table 2). At the WHO recommended molluscicidal effective concentration, niclosamide almost completely killed all the snails within 24 h and the rates maintained at D2 and D3. PPU07 hydrochloride induced time and dose-dependent mortality rates. On D3, the adjusted mortality rates of 1 g/m^3 PPU07 hydrochloride is 98%. And 4 g/m^3 PPU07 hydrochloride completely kills the snails since D2.

3.2. Molluscicidal effect of 25% PPU07 sulfate MP against O. hupensis in field trials

Although PPU07 hydrochloride exhibited potent molluscicidal effects, the field trials results were not as good as expected. Besides, hydrochloride is hard to degrade in the natural environment. Therefore, 25% PPU07 sulfate MP was prepared and tested in field trials according to spraying and immersion methods at the snail habitat (Jiangling County, China) (Wang et al., 2000).

For the spraying method, 25% PPU07 sulfate MP was tested at 2, 4 and 8 g/m^2 in comparison with 1 g/m^2 niclosamide (Table 3). Niclosamide caused fast and sustained molluscicidal effects

Table 1
Molluscicidal effect of PPU07 hydrochloride against *O. hupensis* in field trials by the spraying method.

Molluscicides	Concn (g/m^2)[a]	D1		D3		D7	
		Mortality (%, n/N)[b]	Adjusted mortality (%)	Mortality (%, n/N)[b]	Adjusted mortality (%)	Mortality (%, n/N)[b]	Adjusted mortality (%)
PPU07 hydrochloride	2	21.21 (7/33)	15.37	59.38 (19/32)	58.18	63.33 (19/30)	60.80
	4	63.64 (21/33)	60.95	88.57 (31/35)	88.23	83.87 (26/31)	82.76
50% Niclosamide WP	1	90.91 (30/33)	90.24	83.33 (30/36)	82.76	85.71 (30/35)	85.11
Control	–	6.90 (2/29)	–	2.86 (1/35)	–	6.45 (2/31)	–

[a] Concentration of API.
[b] N, total number of snails in 10 frames; n, number of living snails.

Table 2
Molluscicidal effect of PPU07 hydrochloride against *O. hupensis* in field trials by the immersion method.

Molluscicides	Concn (g/m^3)[a]	D1		D2		D3	
		Mortality (%, n/N)[b]	Adjusted mortality (%)	Mortality (%, n/N)[b]	Adjusted mortality (%)	Mortality (%, n/N)[b]	Adjusted mortality (%)
PPU07 hydrochloride	0.5	41.46 (20/48)	41.46	50.00 (25/50)	46.74	57.14 (28/49)	56.25
	1	44.23 (23/52)	44.23	80.00 (40/50)	78.70	98.04 (50/51)	98.00
	2	34.69 (17/49)	34.69	87.50 (42/48)	86.69	96.08 (49/51)	96.00
	4	63.46 (33/52)	63.46	100 (50/50)	100	100 (50/50)	100
50% Niclosamide WP	1	98.00 (49/50)	98.00	100 (50/50)	100	100 (50/50)	100
Control	–	0.00 (0/50)	–	6.12 (3/49)	–	2.04 (1/49)	–

[a] Concentration of API.
[b] N, total number of snails in 10 frames; n, number of living snails.

(98.05%, 90.48%, and 97.60% on D1, D3 and D7, respectively). Similarly, 25% PPU07 sulfate MP induced more than 90% mortality at all the tested concentrations within 1 day, and the effects lasted till the end of the trials (7 days).

For the immersion method, 25% PPU07 sulfate MP was tested at 0.5, 1, 2 and 4 g/m^3 in comparison with 1 g/m^3 niclosamide (Table 4). Niclosamide killed 100% snails since the first day and the morality effect lasted in the trials. 2 g/m^3 and 4 g/m^3 25% PPU07 sulfate MP exhibited similar molluscicidal effects like the positive control drug. The adjusted mortality rates of 2 g/m^3 25% PPU07 sulfate MP were 97.68%, 94.44% and 96.52% on D1, D2 and D3, respectively.

In the process of the field trials, local loaches, eels, shrimps, tadpoles and frogs, were placed in each test unit (or plot) to test the toxicity of 25% PPU07 sulfate MP on aquatic organisms comparing with niclosamide. No lethal toxicity was observed when exposed with 25% PPU07 sulfate MP. Moreover, 25% PPU07 sulfate MP at the tested concentrations didn't induced obvious toxic symptoms, like balance disorder, bradykinesia, convulsion, and discoloration. The motor activity and feeding of the aquatic organisms were as normal during the trials. However, a lot of tadpoles and frogs died within 2 h after the application of niclosamide.

3.3. *Fish toxicity of 25% PPU07 sulfate MP on local fish*

The toxicity of 25% PPU07 sulfate MP was tested on local fish in comparison with niclosamide. The latter killed all the test fish within 1 h at 0.5 g/m^3 which was half of the WHO recommended molluscicidal effective concentration (1 g/m^3). 25% PPU07 sulfate MP exhibited much less toxicity than niclosamide and were selective against *O. hupensis* snails. At 2.5 or 5 g/m^3, no lethal toxicity was observed. 7.5 g/m^3 25% PPU07 sulfate MP caused 10% death of the fish after 24 h exposure, and 50% death of the fishes after 48 h and 72 h (see Table 5).

Table 5
Fish toxicity of 25% PPU07 sulfate MP on local fish.

Molluscicides	Concn (g/m^3)	No. of dead fish ($n = 20$)[a]			Mortality (%)
		24 h	48 h	72 h	
25% PPU07 sulfate MP	2.5	0	0	0	0
	5	0	0	0	0
	7.5	2	10	10	50
50% Niclosamide WP	0.5	20	20	20	100

[a] n, total number of test fish at each concentration.

4. Discussion

Pyridine is a heterocyclic pharmacophore with low environmental toxicity and wildly used in the fourth-generation pesticides (Di et al., 2007; Maharajan et al., 2018; Vasetska et al., 2015). More

Table 3
Molluscicidal effect of 25% PPU07 sulfate MP against *O. hupensis* in field trials by the spraying method.

Molluscicides	Concn (g/m^2)[a]	D1		D3		D7	
		Mortality (%, n/N)[b]	Adjusted mortality (%)	Mortality (%, n/N)[b]	Adjusted mortality (%)	Mortality (%, n/N)[b]	Adjusted mortality (%)
25% PPU07 sulfate MP	2.0	96.50 (193/200)	96.45	90.56 (163/180)	90.17	95.33 (204/217)	95.28
	4.0	99.05 (208/210)	99.04	96.17 (201/209)	96.01	96.08 (196/204)	96.03
	8.0	100 (218/218)	100	99.52 (207/208)	99.50	97.51 (196/201)	97.48
50% Niclosamide WP	1.0	98.08 (255/260)	98.05	90.86 (169/186)	90.48	97.63 (206/211)	97.60
Control	–	1.48 (3/203)	–	4.00 (4/100)	–	1.05 (2/191)	–

[a] Concentration of API.
[b] N, total number of snails in 10 frames; n, number of living snails.

Table 4
Molluscicidal effect of 25% PPU07 sulfate MP against *O. hupensis* in field trials by the immersion method.

Molluscicides	Concn (g/m^3)[a]	D1		D2		D3	
		Mortality (%, n/N)[b]	Adjusted mortality (%)	Mortality (%, n/N)[b]	Adjusted mortality (%)	Mortality (%, n/N)[b]	Adjusted mortality (%)
25% PPU07 sulfate MP	0.5	4.44 (4/90)	0	22.00 (54/90)	22.00	16.67 (15/90)	12.80
	1	54.44 (49/90)	52.32	76.67 (69/90)	76.67	73.33 (66/90)	72.09
	2	97.78 (88/90)	97.68	94.44 (85/90)	94.44	96.67 (87/90)	96.52
	4	94.44 (85/90)	94.18	95.56 (86/90)	95.56	100 (90/90)	100
50% Niclosamide WP	1	100 (90/90)	100	100 (90/90)	100	100 (90/90)	100
Control	–	4.44 (4/90)	–	0 (0/90)	–	4.44 (4/90)	–

[a] Concentration of API.
[b] N, total number of snails in 10 frames; n, number of living snails.

than 70 pyridine derived pesticides are commercially used, including highly effective inserticides, bactericides, and herbicides (Al-Salahi et al., 2010; El-Zemity and Radwan, 1999; Liu et al., 2015; Xu et al., 2018). For example, niclotinanilide is a broad-spectrum pyridine molluscicide, with no lethal toxicity to fish, tadpoles and frogs in field applications (Chen et al., 1991; Sukumaran et al., 2004). The application of niclotinanilide is limited due to its poor water solubility and the induction of the migration of the snails. We developed a series of pyridylphenylureas as novel molluscicides and studied their structural activity relationship in laboratory against *B. straminea*. PPU07 was one of the most effective molluscicidal agents. We then tested lethal effect of PPU07 against *O. hupensis* to expand the scope of application. Based on the efficiency and safety of PPU07 in laboratory, more soluble formulations, including hydrochloride and sulfate MP, were manufactured and evaluated of their molluscicidal effect in field trials.

PPU07 hydrochloride exhibited a time and dose-dependent lethal effect against *O. hupensis* in the field trials via spraying and immersion methods. At the concentration of 4 g/m^2 in spraying trials and 4 g/m^3 in immersion trials, PPU07 hydrochloride induced around 60% snail deaths after 1 day exposure, while 1 g/m^2 or 1 g/m^3 niclosamide killed more than 90% snails. However, extended administration time would reduce the difference between PPU07 hydrochloride and niclosamide. For example, at D3 and D7, 4 g/m^2 PPU07 hydrochloride had similar mollusicidal effect with 1 g/m^2 in spraying trials. The other formulation of PPU07, namely 25% PPU07 sulfate MP, exhibited more potent mollusicidal effect than its hydrochloride. 2 g/m^2 and 2 g/m^3 25% PPU07 sulfate MP exhibited similar activities (>95% lethallty rate) with 1 g/m^2 and 1 g/m^3 niclosamide in both spraying and immersion methods. Except for the comparable molluscicidal effect, 25% PPU07 sulfate MP caused little toxicity to local fish. At the effective molluscicidal concentrations, 25% PPU07 sulfate MP was lethal to snails but not to fish, local loaches, eels, shrimps, tadpoles, frogs and aquatic plants. Therefore, 25% PPU07 sulfate MP could be applied in snail control program, and have advantages in semi-commercial and commercial aquaculture ponds, such as the fish ponds, while niclosamide is forbidden in these areas due to its lethal toxicity.

The environment impacts and cost affects the application of molluscicides for commercial production and marketing. Niclosamide is prepared by amine of acyl chloride in industry, the process of which is complex, associated with a large number of hydrogen chloride gases, apparatus corrosion, pollution of the environment and low-yield. PPU07 was synthesized from amine with aryl isocyanate, no by-products was formed and there was no concern of corrosion for the reaction apparatus as well as secondary pollution. This suggests that the reaction process is atom economy and green chemistry in nature. In addition, the cost of 25% PPU07 sulfate MP was 5000 CNY/ton cheaper than niclosamide. The former was 45,000 CNY/ton, while the later was 50,000 CNY/ton. The unit cost was also compared. To achieve efficient molluscicidal effect, the unit cost of niclosamide was 0.05 CNY/m^2, and 0.05 CNY/m^3. And the unit cost of 25% PPU07 sulfate MP was 0.09 CNY/m^2 and 0.09 CNY/m^3. Considering of the low toxicity to aquatic animals and plants, the cost of 25% PPU07 sulfate MP would be quite competitive in the market.

More field trials will be needed before the launch of PPU07 in agricultural production. It should be verified the efficiency of PPU07 in other snail habitat environments and against other *O. hupensis* subspecies accordingly. Meanwhile, niclosamide induced acute toxicity to snails and local fish within a few hours, while PPU07 needed more time to kill the target snails. Therefore, the influence of environmental factors and climatic conditions should be evaluated. For example, the terrain, water depth, temperature, precipitation might affect the efficacy of PPU07. Moreover, the environmental fate of PPU07 should also be studied, including but not limited to its risk to more non-target organisms and plants as well as the possibility of bioaccumulation in the organisms.

Based on our research, PPU07 is a promising molluscicide for *O. hupensis*. The 25% PPU07 sulfate MP formulation exhibited comparable molluscicidal effect with the only WHO recommended molluscicide niclosamide dosing via both spraying and immersion methods. 25% PPU07 sulfate MP showed little toxicity to aquatic animals at the therapeutic dose in contrast to the acute toxicity of niclosamide. In all, PPU07 could be an alternative molluscicides of niclosamide in semi-commercial or commercial aquaculture ponds.

Conflicts of interest

The authors declare that they have no conflict of interest.

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