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THE MOLLUSCICIDAL EFFECT OF THE STEM EXTRACTS OF *Tinospora crispa* IN CONTROLLING THE GOLDEN APPLE SNAIL *Pomacea canaliculata*

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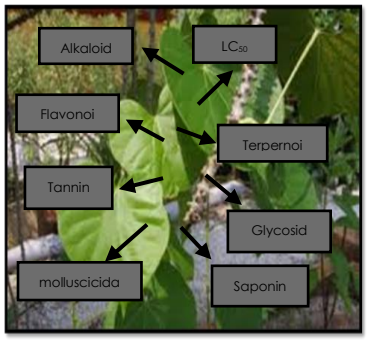
Graphical abstract



Abstract

This study investigated the molluscicidal effect of the stem extracts of *Tinospora crispa* in controlling the golden apple snail *Pomacea canaliculata*. Extracts were prepared in four solvents, i.e., hexane, chloroform, methanol, and distilled water at three concentrations (1,000, 5,000, and 10,000 ppm) per solvent. The phytochemical contents of the extracts were qualitatively identified, and the lethal concentration (LC_{50}) of the extracts for mollusicicidal potential was determined using the probit analysis. The effect of *T. crispa* extracts on the snail was monitored for three days and the snail mortality was recorded every 24 h. The stem extract prepared in methanol at 10,000 ppm showed the highest molluscicidal effect with a mortality of 80% at 72 h. Phytochemicals identified in the stem extracts included alkaloids, flavonoids, saponin, tannin, and terpenoids. Based on the probit analysis, stem extracts of *T. crispa* prepared in methanol showed the lowest LC_{50} value of 3,428 ppm for mollusicicidal potential and followed by extracts prepared in chloroform, hexane, and distilled water at 5,888, 14,771, and 14,993 ppm, respectively.

Keywords: Molluscicidal activity, mortality, phytochemicals, *Pomacea canaliculata, Tinospora crispa*

Abstrak

Kajian ini menilai kesan moluskisida ekstrak batang Tinospora crispa dalam mengawal siput gondang emas (Pomacea canaliculata). Ekstrak disedia dengan empat jenis pelarut, iaitu heksana, kloroform, metanol dan air suling dalam tiga kepekatan untuk setiap pelarut (1,000, 5,000 dan 10,000 ppm). Kandungan fitokimia ekstrak ditentukan secara kualitatif. Kepekatan LC50 untuk potensil moluskisida ekstrak dianggar berdasarkan kaedah analisa probit. Kesan ekstrak terhadap siput gondang emas dipantau selama tiga hari dan kematian direkodkan setiap 24 jam. Ekstrak batang disedia dengan metanol pada kepekatan 10,000 pm mempamerkan kesan kematian tertinggi, iaitu 80% pada jam 72. Fitokimia yang dikenalpasti dalam batang ekstrak termasuk alkaloid, flavonoid, saponin, tanin dan terpenoid. Berdasarkan analisa probit, ekstrak batang T. crispa yang disedia daripada metanol menunjukkan nilai LC50 yang paling rendah untuk moluskisida, iaitu 3,428 ppm dan diikuti oleh ekstrak kloroform, hexan, dan air suling pada 5,888, 14,771, and 14,993 ppm masing-masing.

Kata kunci: Aktiviti moluskisida, kematian, fitokimia, Pomacea canaliculata, Tinospora crispa

1.0 INTRODUCTION

The cultivation of paddy in Malaysia suffers from the attack of a major pest, i.e., the golden apple snail, *Pomacea canaliculata*. The snail was brought from Argentina to Taiwan in the 1980s as food for locals and exportation. However, the demand for the snail was low and thus cause its farming activity was discontinued and the snail manged to flee to the paddy fields [1]. Eventually, the pest spread to Malaysia, Indonesia, Thailand, the Philippines, and other Asian countries, where it would destroy 7 to 24 rice seedlings per day [2]. It usually attacks the young paddy stems and leaves. In particular, individuals of 40 mm and above are the most damaging ones; they could completely damage the paddy seedlings at the growing stage and 20% of the transplanted seedlings [3].

Various methods such as handpicking, biological control (using ducks), and the use of chemicals had been employed to control the infestation of *P. canaliculata* [4]. In particular, chemicals such as niclosamide and metaldehyde are widely used due to their rapid actions and high efficacy. However, the extensive use of chemicals and pesticides would pollute the waterways and the ecosystem [5].

Consequently, the use of natural plant molluscicides is on the rise as they are considered more ecologically friendly [6]. In this respect, the stem extracts of patawali *Tinospora crispa* (Family: Menispermacea) had been reported to be effective in controlling *P. canaliculata* [7, 8, 9, 10, 11, 12]. The present study investigated the molluscicidal effect of the stem extracts of *T. crispa* in controlling the golden apple snail. The phytochemical contents of the extracts were qualitatively identified, and the lethal concentration (LC_{50}) of the extracts for mollusicicidal potential was determined. The study would contribute towards sustainable development by reducing the dependence on chemicals and pesticides in the pest management of paddy cultivation.

2.0 METHODOLOGY

2.1 Preparation of the Stem Extract of *Tinospora crispa*

Stem extracts of *T. crispa* were prepared in four solvents, i.e., hexane, chloroform, methanol, and the distilled water. Extractions in organic solvents generally followed the method of Aiza *et al*. [13], in which 200 g dried powder of *T. crispa* stem was soaked in 500 ml of each hexane, chloroform, and methanol for three weeks. The maceration of each sample (three extracts) was then filtered with Whatman No. 1 filter paper then evaporated using a rotary evaporator to yield crude extracts. They were stored at 4 ℃ until analysis. Meanwhile, the aqueous extraction of *T. crispa* stem was generally based on the method of Safanah *et al*. [14], in which the dried powder of *T. crispa* stem (200 g) was impregnated with water for 24 h, filtered, centrifuged (6,000 rpm, room temperature, 10 min), and dried in an oven (20 ℃) for 2 h. The crude aqueous extract was stored at 4 ℃ until analysis.

2.2 Sampling of *P. canaliculata*

A total of 200 adult snails (18 – 25 mm) were collected from the paddy field in Pekan, Pahang, Malaysia. They were acclimatized to the laboratory environment (25℃) for three days and fed with papaya leaves [15, 16], after which snails were randomly selected for the molluscicidal study.

2.3 Molluscicidal Study

The molluscicidal study via immersion generally followed the method of Zhang & Zou [17]. Suspensions comprising the extract of *T. crispa* stem and solvent were prepared at three concentrations, i.e., 1,000, 5,000, and 10,000 ppm for each solvent with a total of 12 samples or experiments (three concentrations x four extracting solvents). For each experiment, ten adult snails (18 – 25 mm) were placed into a plastic tank (20 cm X 10 cm X 20 cm) containing 1L paddy-field water for 30 minutes, and then 100 ml suspension (extract of *T. crispa* stem with different concentrations of solvents) was added into the corresponding tank.

Also, a negative control was prepared for each solvent; each of these four tanks was similarly filled with 1L paddy-field water and 100 ml control content to treat the same number of snails. Specifically, the 100-ml control content for the aqueous extract of *T. crispa* stem consisted of distilled water, whereas a mixture of dimethyl sulfoxide (DMSO) and methanol at a ratio of 1:1 served as the control content for the *T. crispa* stem extracted from hexane and chloroform, and a 50% methanol was used in the control for the *T. crispa* stem extracted from methanol.

The snail mortality of each tank (16 tanks in total) was recorded every 24 h (3 times). The bodies came out of the shell with the secretion of mucus and a change in the shell colour from light yellowish to discolored is the sign of the snails' death [5, 18]. Dead snails were removed from the tanks to prevent contamination. Meanwhile, the lethal concentration (LC_{50}), was evaluated using the probit analysis and the lethal concentration was calculated using the method of Noorshilawati *et al*. [19].

2.4 Qualitative Phytochemical Testing for the Stem Extract of *T. crispa*

Phytochemicals such as tannins, saponins, flavonoids, alkaloids, terpenoids and glycosides were qualitatively screened using modified methods from Bhandary *et al*., Farhat *et al*., Rauf et al., and

Wadood *et al*. [20, 21, 22, 23]. Tannins were detected by boiling 0.2 g extract in 10 ml of distilled water and filtered. The filtrate then added with ferric chloride solution (a few drops). The formation of a dark green solution would indicate the presence of tannins. For the determination of saponins, for each extract, 0.2 g extract was added into a test tube containing 2 ml distilled water and then vigorously shaken. The test tube was allowed to react for 20 min (room temperature). The formation of foam would indicate the presence of saponins.

Also, the flavonoid was tested by adding 10 ml distilled water to each crude extract, followed by 5 ml diluted ammonia solution and 1 mL concentrated H_2SO_4. The presence of flavonoids would be indicated by the appearance of a yellow coloration. Alkaloids were tested by adding Mayer's reagent (a few drops) into the filtrate derived from the filtration of 0.2 g extract (each of all the four solvents) diluted in 10 ml of 2% H_2SO_4. Reddish-brown precipitation would indicate the presence of alkaloids.

On the other hand, terpenoid was identified by adding 0.2 g extract to 1 ml chloroform followed by the gentle addition of 1.5 ml concentrated H_2S0_4 to form a layer. The formation of a reddish-brown colouration on the surface would show the presence of terpenoids., Finally, the content of glycosides in extracts was examined by hydrolysing 0.2 g crude extracts with hydrochloric acid (HCl) followed by neutralisation with sodium hydroxide (NaOH) and the addition of a few drops of Fehling's A and B solutions with gentle boiling. The formation of reddish-brown precipitation would indicate the presence of glycoside compounds.

2.5 Statistical Analysis

Differences in the relative mortality among various extracts were analysed by the analysis of variance (ANOVA) at the significance level (alpha) of 0.05 using the software Statistical Package for the Social Sciences (version 23, IBM). Mean comparisons were performed using Duncan New Multiple Range Test (DNMRT) at $p < 0.05$. The Pearson correlation analysis was used to examine the relationship among the variables.

3.0 RESULTS AND DISCUSSION

Table 1 shows the relative mortality of the snails treated with *T. crispa* extracted from hexane, in which 10% of snails died after 24 h for each treatment. For the control test, no snails died after 24 h through the end of the molluscicidal study. After 72 h, no significant differences recorded between *P. canaliculata* mortality for the experiments of 1,000 ppm, 5,000 ppm, and 10,000 ppm. However, all the concentration showed significant differences when compared with the negative control. All treatments showed a molluscicidal effect on the snails. However, because the mortality for all treatments was less than 50% even at 72 h, the molluscicidal effect was considered weak.

Table 1 The relative mortality of *P. canaliculata* exposed to *T. crispa* extracted from hexane

Concentration	Relatively mortality (%)		
	24 h	48 h	72 h
1,000 ppm	10^{b}	10^{b}	30^{a}
5,000 ppm	10^{b}	20^{ab}	30^{a}
10,000 ppm	10^{b}	30^{a}	40^{a}
Negative control	10^{b}	10^{b}	10^{b}

*Within the same column, different letters in superscripts show significant differences at $P < 0.05$.

Table 2 shows the relative mortality of the snails treated with *T. crispa* extracted from chloroform. For the first 24 h, only 10% mortality was recorded for 1,000 ppm and 10,000 ppm, and no snails died in 5,000 ppm and the negative control (Table 2). The highest mortality of snails was recorded in 10,000 ppm *T. crispa* stem extract at 72 h, i.e., 70% and this mortality differed substantially from that of the negative control (10%). The molluscicidal effect of *T. crispa* stem extracts might be caused by the presence of saponin, tannin, and alkaloid, which were soluble in chloroform and hence extracted. In particular, saponin would disrupt the cell membrane and thus highly toxic to cold-blooded animals [24, 25].

Table 2 The relative mortality of *P. canaliculata* exposed to *T. crispa* extracted from chloroform

Concentration	Relative mortality (%)		
	24 h	48 h	72 h
1,000 ppm	10^{b}	20^{ab}	30^{ab}
5.000 ppm	0^{b}	10^{b}	40^{ab}
10,000 ppm	10^{b}	30^{ab}	70^{a}
Negative control	0^{b}	10^{b}	10^{b}

*Within the same column, different letters in superscripts show significant differences at $P < 0.05$.

Table 3 shows the relative mortality of the snails treated with *T. crispa* extracted from methanol. All treatments showed molluscicidal activity. In particular, in 10,000 ppm the *T. crispa* extracted from methanol showed the most potent molluscicidal effect at 72 h, i.e., 80% mortality, among other solvent extracts. This result agreed with that of other studies [26, 27, 28]. Phytochemicals extracted with methanol generally contain chemical compounds that possess pesticide effects. Due to the presence of saponin and flavonoids, methanol extracts would be toxic to snails [26]. Besides, *T. crispa* extracted from methanol was once used as an insecticidal agent against the diamondback moth by reducing its feeding activity [27]. For 10,000 ppm, at 24 h after treatment, 20% of the snails died, but the mortality drastically increased to 80% at 72 h. A similar finding was also reported by Comia *et al*. [28], and they found that the presence of saponins, tannins, and

anthraquinones contributed to the molluscicidal activity.

Table 3 The relative mortality of *P. canaliculata* exposed to *T. crispa* extracted from methanol

Concentration	Relative mortality (%)		
	24 h	48 h	72 h
1,000 ppm	20^{b}	20^{b}	40^{ab}
5,000 ppm	10^{b}	20^{b}	50^{ab}
10,000 ppm	20^{b}	40^{ab}	80^{a}
Negative control	10^{b}	10^{b}	20^{b}

*Within the same column, different letters in superscripts show significant differences at $P < 0.05$.

Table 4 shows the relative mortality of the snails treated with *T. crispa* aqueous extract, in which the negative control recorded the lowest mortality (10%) at 72 h, and the highest mortality (50%) was attained at 10,000 ppm. This result was consistent with the findings of Somsak *et al*. [29] and Zulkhairi *et al*. [12], and they found that a higher concentration of *T. crispa* aqueous extract (500 mg/kg) decreased the parasitemia in infected mice. This study also showed that extensive exposure to *T. crispa* treatment in high concentration (5,000 and 10,000 ppm) increased the mortality of the snails, and this result agreed with the finding of Abdullahi *et al*. [30], Li & Zou [16] and Musri [24], which concluded that the molluscicidal activity of phytochemical would depend on the treatment's concentration and the duration of the treatment.

Table 4 The relative mortality of *P. canaliculata* exposed to aqueous extracts of *T. crispa* stem

Concentration	Relative mortality (%)		
	24 h	48 h	72 h
1,000 ppm	10^{b}	20^{ab}	20^{ab}
5,000 ppm	20^{ab}	20^{ab}	30^{ab}
10,000 ppm	10^{b}	30^{ab}	50^{a}
Negative control	0^{b}	10^{b}	10^{b}

*Within the same column, different letters in superscripts show significant differences at $P < 0.05$

The varying outcome of molluscicidal activity appeared to be related to different quantities and types of compounds in various extracts. In general, polar solvent (e.g., methanol) would extract a great number, as well as a higher concentration, of flavonoids and phenolic compounds [31] than nonpolar solvents (e.g., chloroform and hexane). In this respect, chloroform, due to its relatively higher polarity, performed better than hexane in terms of exerting effectiveness of molluscicidal activity when used to prepare extracts of *T. crispa* stem (Tables 2 & 3). Overall, negative controls in all the solvents showed 10 to 20% mortality of *P. canaliculata* at 72 h, and the deaths were likely due to environmental stress that resulted from warm and stagnant water [4, 32]. Together, *T. crispa* appeared to be a good molluscicide [33].

Table 5 shows the phytochemicals identified in various extracts of *T. crispa* stem via qualitative tests. For methanol extracts, five phytochemicals were identified in this study, i.e., alkaloid, flavonoids, saponins, tannins, and terpenoids. These active compounds would likely play an important role in the pest management of snails in the cultivation of paddy as they could fatally disrupt the nervous system of snails [34]. Incidentally, Shakirah *et al*. [35] also detected tannin, alkaloid, flavonoid, and polyphenol substances from the *T. crispa* stem extracted from methanol, while Noor & Ashcroft [36] found saponin and alkaloid in the *T. crispa* stem extracted from methanol.

Table 5 Phytochemicals identified in the extracts of *T. crispa* stem via qualitative tests

Compound	Hexane	Chloroform	Methanol	Aqueous
Tannins	-	+	+	-
Saponin	-	+	+	+
Flavonoid	+	-	+	+
Alkaloid	+	+	+	-
Terpenoid	-	-	+	-
Glycoside	-	-	-	-

** + present - absent

For chloroform extract, three phytochemicals were identified in this study, i.e., alkaloid, saponins, and tannins. This finding was in general agreement with that of Shakirah *et al*. [35]., who also detected alkaloid and triterpenoid types of saponins in the *T. crispa* stem extracted from chloroform. For hexane extracts, only two compounds were detected, i.e., flavonoids and alkaloids. Fewer compounds in the *T. crispa* stem were extracted from hexane might have contributed to their weak molluscicidal activities (Table 1). However, this finding differed somewhat from that of Shakirah et al. [35], who also detected polyphenol in the *T. crispa* stem extracted besides alkaloid and flavonoid substances. The reason for such a difference remained unclear. Also, only two compounds were detected in the aqueous extract of *T. crispa* stem, namely, flavonoids and saponin.

Different active compounds may interact with each other to enhance the molluscicidal activity [37]. Musman [5] reported that extracts containing both flavonoid and saponins exerted a high mortality effect on *P. canaliculata* as compared to extracts containing only flavonoids. Prabhakaran *et al*. [38] found that the combinations of neem, tobacco and Nerium (1: 1: 1) and tobacco, piper, and nerium (1: 1: 1) and were effective in controlling *Pomacea maculata* with LC_{90}, 191.52 mg/L and 180.35 mg/L. Keshav *et al*. [39] in their study found that the toxicity of the mixture of *Azadirachta indica* oils, *Embelia ribens and Cedrus deodara* against *Lymnaea*

acuminata and *Indoplanorbis exustus* is 10 times more active than the toxicity of only two, *Azadirachta indica* oils and *Cedrus deodara*. Meanwhile, they found *Embelia ribens* itself did not shows any sign of toxicity. The combination of custard apple seed powder (*Annona squamosa*), neem oil (*Azadirachta indica*) and cedar oil (*Cedrus deodara*) have high molluscicidal activity in contolling *Lymnaea acuminata* than the individual plants [40]. Molusicicides from the combination of *Allium sativum* and *Cedrus deodara* oil and the combination of *Cedrus deodara* and *Azadirachta indica* oil can disturb the life cycle of African snail (*Achatina fulica*) [41].

Table 6 shows the LC_{50} values of the *T. crispa* stem extracts calculated from the probit analysis. Extracts from methanol had the most potent mollusicicidal effect with an LC_{50} value of 3,428 ppm. Thus, the suggested concentrations for *T. crispa* stem extracted from hexane, chloroform, and aqueous extracts to control 50% of the *P. canaliculata* population were 14,771 ppm, 5,888 ppm, and 14,993 ppm, respectively (Table 6).

Table 6 The lethal concentration (LC_{50}) of *T. crispa* stem extracts for mollusicicidal potential, which was estimated based on the probit analysis

Extract	Suggested concentration
Hexane	14,771 ppm
Chloroform	5,888 ppm
Methanol	3,428 ppm
Aqueous	14,993 ppm

4.0 CONCLUSION

Extracts of *T. crispa* stem were found to have mollusicicidal potential. Based on the probit analysis, stem extracts of *T. crispa* prepared in methanol showed the lowest LC_{50} value of 3,428 ppm for mollusicicidal potential followed by extracts prepared in chloroform, hexane, and distilled water at 5,888, 14,771, and 14,993 ppm, respectively. Further studies would be essential to screen new biopesticide with high efficiency and low toxicity for the pest management of paddy cultivation.

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