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Effects of *Nicotina tobacum* (Linnaeus) on the survival and behavioral response of the freshwater leeches, *Hirudinaria* sp.

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Abstract: We investigated the efficacy of *Nicotina tobacum* as an anti-parasitic chemical to freshwater leeches of the genus *Hirudinea*, by assessing their behavioral and survival responses to different concentrations and ages of *N. tobacum* (i.e. 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 g L^{-1}) at 25±1°C. We observed longer escape time from freshly prepared tobacco solutions compared to 1- day 2- day and 3-day-old solutions, suggesting a gradual degradation of nicotine toxicity with time. The percentage survival decreased significantly (P<0.05) with increasing concentrations of *N. tobacum* and exposure time. Concentrations of 15.0, 17.5 and 20.0 g L^{-1} recorded highest hazard ratios and low survival rates of leeches. The lethal concentration (LC_{50}) and time (LT_{50}) at which 50 % of leeches died were 15.0 gL^{-1} and 73 min respectively. This finding can be applied to control leeches, which are potential human disease vectors in aquatic ecosystems. Further investigations should target effects of nicotine on cultured animals and water quality parameters.

Key words: *Hirudinea* sp., *Nicotina tobacum*, leeches, lethal concentration, lethal time

Introduction

Leeches are hermaphroditic invertebrates (phylum: annelid, genus: *Hirudinea*), often found in terrestrial and aquatic habitats (Sasaki and Tani, 1997; Sket and Trontelj, 2008). Leeches reproduce by egg deposition in their cocoons secreted by the clitellum through direct ontogeny without larval stages (Sawyer, 1986; Sket and Trontelj, 2008). In aquatic ecosystems, the parasitic leeches affect growth and survival of fishes (Sakiti *et al*., 1999; Iyaji and Eyo, 2008). For example, the genus *Hirudinea* have been reported in a wider range of African fishes e.g. Clariidae, Synodontidae, mugilidae, Mormyridae and Cichlidae (Mann, 1962; Oosthuizen, 1989; FAO, 1996), where it causes haemorrhage, fibrin deposition and oedema (Volonterio *et al*., 2004). The leeches also erode mucus membrane and damages the fish skin (Roubal, 1986; Volonterio *et al*., 2004), hence become a predisposing factor to opportunistic bacterial and fungal infections (Kabata, 1985).

In humans, leeches can cause aenemia and haemorrhage (Kazemi and Bajoghli, 2002; Yaghmaee, 2002) thanks to their anti-coagulant histamine chemical (Bahmani *et al*., 2012). They also cause ophthalmorrhagia (Davari, 2008), pain, itching, hypersensitivity, and anaphylactic reactions (Ahmadiz-adeh, 2001). To control such incidences, chemical drugs have been commonly used (Bahmani *et al*., 2012). However, these chemicals have longer residual effects on the environment and may affect non-target organisms. Therefore, studies are needed to investigate environmentally safe chemicals (Sasaki and Tani, 1997). *Nicotina tobacum* is an agricultural product processed from the leaves of plants in the genus *Nicotina*. Studies have documented that nicotine; a highly toxic substance found in tobacco leaves, can kill and repel different pests and predators (Bahmani *et al*., 2012) including molluscs (Spector, 1956; FAO, 1970; Aleem, 1988; FAO, 1997;

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Tangkoonboribun, 2009; Ogello *et al*., 2012). *N. tobacum* is easily available and highly biodegradable and can also double as an organic fertilizer in fish ponds (Aleem, 1988; Ogello *et al*., 2012). Despite investigating the anti-parasitic effects of nicotine drug on leeches (Bahmani *et al*., 2012), there is still limited information in literature regarding the lethal concentration and time under the influence of nicotine. This study investigated tobacco as a natural anti-leeches drug by determining the lethal concentration (LC_{50}) and time (LT_{50}) of nicotine solution at which 50 % of the leeches would die.

Materials and methods

Source of leeches

This study was conducted in the laboratory of Kenya Marine and Fisheries Research Institute (KMFRI), Kegati Aquaculture Research Station, Kenya located at 00° 42"S; 034°47"E. We obtained the leeches (*Hirudinaria* sp), from muddy pond bottom in the station. We collected leeches with body length of 8 - 10 cm in 500 mL glass bottles containing pond water and acclimatized them in a basin, with aeration at 25.0±1°C for 12 hours. Next, we identified the leeches up to genus level according to Davis (1971) and Boris and Trontelj (2008).

Experimental design

We obtained dry tobacco leaves from nearby tobacco farmers and made fine powder out of it. The tobacco powder was used to make solutions of different concentrations: i.e. 0.00 (control), 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 gL^{-1} in triplicates using fresh pond water (Ogello *et al*., 2012). Some solutions were left to stay for 24 h, 48 h, and 72 h before use. Freshly prepared tobacco solution was also used. To start the experiment, 30 *Hirudinea* sp. were selected at random and placed in each 1-L beaker containing 400 mL of freshly prepared, 1-day (24 h), 2-day (48 h) and 3-day (72 h) old tobacco solution. The behavior of the *Hirudinea* sp. was monitored along with the time taken to crawl out of the solution in the beaker in all the treatments. For survival studies, only the freshly prepared tobacco solutions were used. In this study, we placed 30 *Hirudinea* sp. in a sealed 500 mL conical flasks (three replicate flasks per treatment, including control) and we determined survival at 2-day intervals for 12 days (Ogello *et al*., 2012) using similar volumes of tobacco concentrations as described previously at an average temperature of 25±1°C.

Data analysis

The data were analyzed using R statistical software (version 3.2.1 of the R Foundation for Statistical Computing Platform © 2015). The Bartlett test of homogeneity of variances was used to test for the normality of the data. Two-way ANOVA was used to identify significant effects of concentration and age of *N. nicotina* on the escape time and survival of the leeches. Tukey's HSD Post Hoc Test was performed to determine where the differences were situated. The survival analysis was performed using SAS 9.1 (SAS Institute, Cary, NC, USA). Cox regression model (PROC PHREG in SAS) was used to estimate survival. The survival time of each member was assumed to follow its own hazard function hi (t) expressed as: $h_i(t) = h_o(t) \exp z_i\beta$; Where $h_o(t)$ is an arbitrary and unspecified hazard function, z_i is the vector of measured explanatory variables for the i-th individual and β is the vector of unknown regression parameters associated with the explanatory variables. For the data presented herein β will contain two parameters, one relating to initial percentage survival and the other to tobacco waste concentration. The model assumes that for a given initial proportion, the hazards at different concentrations of tobacco waste are proportional to each other. The analysis included all *Hirudinea* sp. till time of death in the beaker. The survival rates among beakers was compared using multiple comparisons with a Bonferroni-corrected alpha (significance considered at a value of $P < 0.008$) to minimize the possibility of Type I statistical error. The chi-square test was used in the determination of variations in variables at a value of $P < 0.05$.

Results

Behaviorally, the Leeches reacted strongly to the tobacco waste solutions by showing vigorous coiling movements and producing a foam-like substance as they attempted to move out of the beaker. There was no significant difference (P>0.05) in mean escape time at 2.5, 5.0 and 7.5 gL^{-1} of all solutions of tobacco leaves (Tab. 1). However, the mean escape time significantly increased (P<0.01) with increasing concentrations of tobacco in all the solutions tested

Tab. 1: Leeches mean escape time ± SE (min) in different concentrations and age of *N. tobacum* solution.

	Mean *Hirudinea* sp. escape time (min)				ANOVA (n = 30)	
Concentration (gL^{-1})	**Freshly prepared**	**1 day old**	**2 days old**	**3 days old**	***F***	***P***
2.5	21.00±4.56	19.00±5.11	15.67±3.14	15.00±3.66	33.652	0.051
5.0	26.00±5.21	25.67±4.25	19.00±6.22	19.00±6.14	45.352	0.069
7.5	38.33±6.33	29.00±6.99	23.67±5.14	20.00±5.44	24.697	0.054
10.0	61.00±6.97	38.33±9.87	31.00±9.77	21.33±3.14	245.361	0.001
12.5	198.00±10.23	71.33±7.22	61.00±6.11	31.00±6.44	101.147	0.002
15.0	251.67±9.99	188.00±14.22	139.00±10.11	63.33±7.29	56.699	0.000
17.5	263.67±14.11	249.33±10.47	203.33±9.97	149.67±9.41	154.247	0.000

(Tab. 1). No leech was able to move out of the beaker at 20 gL^{-1} of tobacco solution (Fig. 1). The leeches took significantly longer ($P<0.05$) time to move out of freshly prepared tobacco solution as compared to 1-day, 2-day and 3-day old solutions (Tab. 1). The leeches did not move out of the beaker in the control experiment (0.0 gL^{-1}) therefore, no time results were recorded for the control experiment. The experiment, recorded least escape time for all the leeches in the 3 days old solutions (Tab. 1).

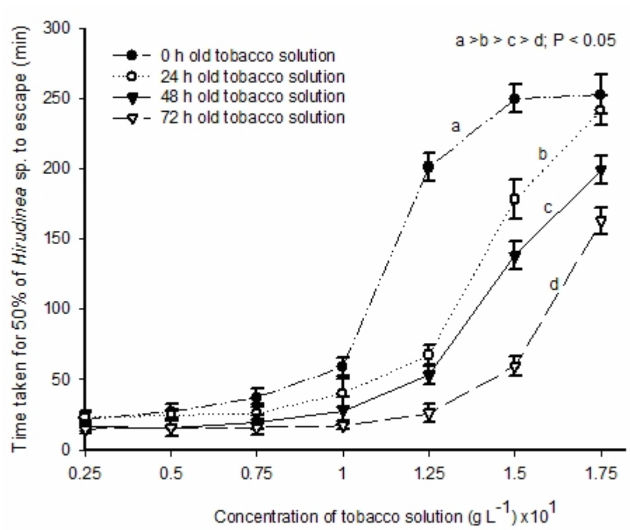

Fig. 1: Time-behavioral response curves of the leeches, *Hirudinea sp*. exposed to various concentrations of freshly prepared (0 h), One day old (24 h), 2-day old (48 h) and 3-day old (72 h) tobacco waste solutions during the study period. The vertical bars represent mean escape time for 50% of leeches ± Standard Error, *n* = 30.

Percentage survival of the leeches at different concentrations of tobacco solutions is summarized in Figure 2. The survival decreased significantly ($P<0.05$) with increasing concentrations of tobacco and time of exposure. All the leeches (100%) survived in the control unit (0.0 gL^{-1}). The highest leech survival was observed in 2.5 gL^{-1} of tobacco solution, where 100% survived after 144 h of exposure to tobacco solution. However, this declined to about 30% after 288 h (Fig. 2). Survival was poorest at 20 gL^{-1}, where only 20% of the leeches survived after 48 h. The experiment recorded LT_{50} of 258, 240, 192, 162, 120, and 72 h for concentrations of 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 gL^{-1} respectively (Fig. 2). The most lethal doses were 17.5 gL^{-1} and 20.0 gL^{-1} as they recorded less than 50% survival after only 48 h (Fig. 2). The mean survival time was 144.5±2.9 h (range 48 - 288 h). Both longer and shorter leeches experienced significantly similar survival probabilities ($P>0.05$). The parameter estimates and standard errors for the Cox proportional hazards model are given in Table 2. The presence of both initial proportions of leeches and tobacco waste concentration were found to be insignificant (change in - 4 log likelihood from model without covariates = 127.11 on 2 d.f., $P=0.000$). No interaction between initial proportions and tobacco waste concentration or quadratic effects could be detected. The model for the i-th individual is h_i (t) = exp* (4.17 x initial proportion + 1.33 x tobacco concentration) h_o (t).

Tab. 2: Estimated values of the coefficients of the covariates on fitting a proportional hazards model

Covariate	Estimate of β	SE of β	Wald X^2	*P*
Proportions	4.17	0.54	14.69	0.000
Concentration of tobacco solution	1.33	0.61	55.87	0.000

The hazard of death increased with increasing concentration of tobacco waste and time of exposure. For every 48 h increment, the hazard ratio increased by a factor of exp (0.48 x 4.17) = 2.00 (95% confidence interval 2.11 to 4.28). Similarly, for every

2.5 gL^{-1} increase in nicotine concentration, the hazard ratio increased by a factor of exp (2.5 x 1.36) = 3.4 (95% confident interval 0.30 to 0.35). To clarify these results, a plot of the survivor function estimates for the eight doses of tobacco waste concentration is shown in Figure 3. The leeches survival rates for treatment with 20.0 gL^{-1} and 17.5 gL^{-1} were the lowest and while survival rates in the other six treatments differed significantly (Bonferroni-corrected probability, $P<0.005$).

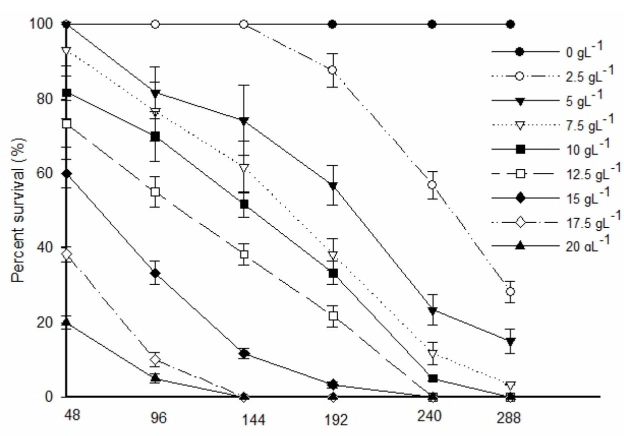

Fig. 2: Percent time-survival curves of the *Hirudnea sp.* exposed to various concentrations of freshly prepared tobacco waste solutions during the study period. The vertical bars represent mean survival % ± Standard Error, *n* = 30.

On the other hand, cumulative survival probability decreased significantly ($P<0.05$) with increase in time of exposure of the leeches to the different concentrations of tobacco solutions (Fig. 3). When compared with 20.0 gL^{-1}, the hazard ratios for treatments 5.0 gL^{-1}, 7.5 gL^{-1}, 10.0 gL^{-1}, 12.5 gL^{-1} and 15.0 gL^{-1} were 0.18, 0.21, 0.24, 0.33 and 0.37 respectively. The hazard probabilities for the leeches increased with increased time of exposure to the different concentrations of tobacco solution (Fig. 4).

Discussion

This study investigated the anti-parasitic effects of *N. tobacum* against freshwater leeches of the genus *Hirudinea* by observing behavioral and survival changes in different concentrations and ages of tobacco solutions. The leeches spent less time to move out of low concentrated nicotine solutions (2.5, 5.0 and 7.5 gL^{-1}) but took significantly ($P<0.05$) longer time to escape from highly concentrated tobacco solutions probably due to increased nicotine irritation. This observation mirrors those of Narahashi *et al*. (2000), who reported that nicotine interacts with nicotinic acetylcholine receptors to cause muscle weakness. The escape time was longer in freshly prepared solutions compared to 1-day, 2-day and 3-day-old solutions, suggesting degradation of nicotine toxicity with time. Similar observations were reported for freshwater gastropods (Aleem, 1988; Ogello *et al*., 2012). The foam-like gel substance released by the leeches was perhaps a mechanism by the leeches to neutralize the effects of the irritating nicotine.

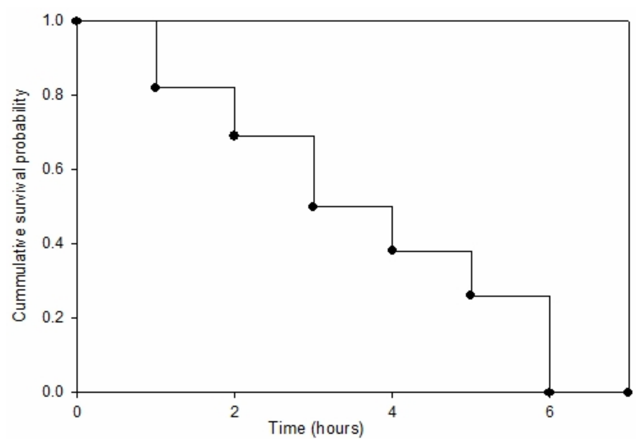

Fig. 3: Overall cumulative survival probabilities of the *Hirudnea sp*. exposed to different concentrations of nicotine solutions. For the x-axis: 1 = 48 h, 2 = 96 h, 3 = 144 h, 4 = 192 h, 5 = 246 h and 6 = 288 h.

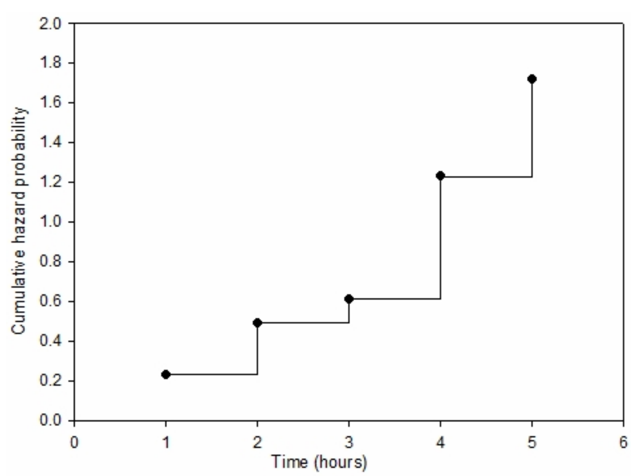

Fig. 4: Overall cumulative hazard probabilities of the leeches when exposed to different concentrations of nicotine solutions. For the x-axis: 1 = 48 h, 2 = 96 h, 3 = 144 h, 4 = 192 h, 5 = 246 h and 6 = 288 h.

The percentage survival rates of *Hirudinea sp*. significantly (P<0.05) decreased with increasing concentrations of tobacco solution and the time of exposure to the solutions. The death rate was faster in the concentrations of 15.0 gL^{-1}, 17.5 gL^{-1} and 20.0 gL^{-1} as they contained the lethal nicotine ratios. This study showed that lethal concentrations (LC_{50}) could be found at or slightly above the threshold of 15 gL^{-1} and the lethal time (LT_{50}) of 73 hours. However, the finding of this study contrasts those of Bahmani *et al*. (2012), obtained 130 gL^{-1} as the LC_{50} of nicotine for freshwater leeches. Further investigations are necessary to provide more insights on these variations.

The survival and hazard function were used to model the times to death for each of the eight groups of tobacco concentrations (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 gL^{-1}). The survival function is the probability that an individual leech survives longer than a certain time while the hazard function defines the probability that an individual leech experiences death in the next small time interval, given that the individual is alive at the beginning of this time interval. When compared with 20.0 gL^{-1}, the hazard ratios for treatments of 5.0 gL^{-1} to 15.0 gL^{-1} were between 18 % and 37.0 %, which can be interpreted as the ratio of the estimated hazard for leeches to the estimated hazard at concentration of 20.0 gL^{-1} (Allison, 1995). In other words, the likelihood of mortality for the leeches at other concentrations lies between 18.0 - 37.0% of the likelihood of mortality for the leeches at 20.0 gL^{-1}. Further, the probability of death increased with increased time of exposure of the leeches to increased concentration levels (Fig. 3) probably due to the toxic influence of the solution. These findings mirror the studies of Konar (1970), who reported reduced respiratory activity of snails at elevated nicotine concentrations, hence increasing the death probabilities at certain hazard ratios. According to Fry (1971), all toxicants have threshold toxicity, above which any animal dies while below it, the animal thrives in a tolerance zone.

The LC_{50} (15 gL^{-1}) and LT_{50} (73 hours) of this study can be applied in leech infested ponds as it indicates that appreciable amounts of nicotine toxicity shall have degraded by the end of 73 hours. Musa et al. (2013) recommended that 1.56 $g\,L^{-1}$ of tobacco leaf dust is optimal for pond preparation for *Clarias gariepinus* fingerling if applied three days before stocking. Tobacco is biodegradable and can as well be used as organic fertilizers in fish ponds (Aleem, 1988). It is therefore prudent to suggest that fish stocking in earthen ponds should be done approximately 4 days to 1 week after nicotine treatment. However, further investigations should be done in practical conditions for more conclusive remarks. As at now, there is no chemical drug that eliminates leech populations without side effects on the environmental biology of other aquatic animals such as fish. Many authors (Aleem, 1988; Sasaki and Tani, 1997; Adamu, 2009; Tangkoonboribun, 2009; Bahmani *et al*., 2012) have reported that *N. tobacum* is a cheap, effective and environmentally sound control method to aquatic animal pests.

In conclusion, this study revealed that *N. tobacum* plant has anti-pesticide activities and can be effective in controlling leech populations. The concentrations of 15, 17.5 and 20.0 gL^{-1} had high hazard ratios and low survivals rates and could be the most effective for application in leech infested ecosystems. This study would provide possible insights on environmentally safe ways of leeches reduction and/or elimination in aquaculture ponds. Further studies are recommended to investigate whether *N. tobacum* influence water quality parameters.

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