Asian Fisheries Science **19**(2006):363-375
ISSN: 0116-6514
https://doi.org/10.33997/j.afs.2006.19.4.004

Asian Fisheries Society, Manila, Philippines

Ichthyotoxicity and Rotenone Stability of *Derris trifoliata* (Leguminosae) Acetone Formulations

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Abstract

The stability of the acetone extract of *Derris trifoliata* (as a more accessible piscicide for fishpond management) was compared with those of *Derris elliptica* (Leguminosae) for a duration of seven months with respect to toxicity and rotenone content to *Oreochromis niloticus* (Cichlidae). Extract formulation of *D. elliptica* was found to retain its fish toxicity throughout the duration of the experiment without exposure to sunlight while that of *D. trifoliata* was found to decrease after only about a month. The toxicity of *D. trifoliata* and *D. elliptica* formulations were maintained longer, when their extracts were refrigerated at around 9 – 10°C.

Chromatographic analysis (HPLC) revealed that although fish toxicity is maintained, rotenone concentration continued to decrease in *D. elliptica* extracts much faster at conditions where the extracts were exposed to sunlight than if kept in a refrigerator or simply kept inside a room at ambient conditions. However, for the *D. trifoliata* extract, not much difference was found if it was kept exposed or not to sunlight, as the rotenone concentration in both conditions continued to drop. Placing the extract at 9 – 10°C in a refrigerator prevented this rapid degradation of rotenone. A better way of preventing the toxicity to decrease and the rotenone to degrade was by partitioning the acetone extract between chloroform and water, taking the chloroform extract, evaporating, collecting and reformulating it when needed. This new formulation showed better rotenone stability and consistent toxicity throughout the seven months of experimental observation.

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Introduction

In our previous report, we have formulated acetone extracts of *Derris trifoliata*, in comparison with those of *Derris elliptica* and one Derris "uwak" for use in fishpond management (Sumera and Conato 2006). The use of *D. trifoliata* extract over those of *D. elliptica* was prompted by the plant's availability near fishponds using brackish water. The rotenone content and the LC_{50} of each of the formulations were determined and subsequently applied with success to several fishponds. Cost analysis showed that fish farmers can economize using the plant extract instead of buying environmentally hazardous chemicals such as sodium cyanide or banned insecticides. For fisheries management purpose, liquid preparations of rotenone have been reportedly preferred because their emulsions are more easily dispersed in water than powders and have a greater capacity to penetrate stratified water bodies (Almquist 1959). Derris formulations contain rotenone and other rotenoids known to degrade in the presence of water, air, light, and low or high pH of the solution (Cheng et al. 1972; Marking and Bills 1976). Solutions of rotenone in organic solvent, when exposed to light and air, become successively yellow, orange and finally deep red due to oxidation. It is therefore important to determine the stability of these formulations before they are distributed for use. The objective of this research was to determine whether the *Derris trifoliata* (collected from estuarine locations near fishponds) formulations in acetone which we demonstrated to be efficient in cleaning fishponds from weed fish are stable with time especially if the formulation is to be kept before use. The other objectives were to compare these same properties with those of *Derris elliptica* and one Derris "uwak" collected from secondary forest usually near mountain sites and to know the conditions and proper treatment of the formulations to keep them from rotenone degradation and subsequent loss of their toxicity.

Materials and Methods

Sample Collection and Identification

The *Derris trifoliata* plant (or sila-sila) was obtained from the mangrove swamps of San Roque, Paombong, Bulacan. The roots of the mature plant (0.9–1.1 cm in diameter) were cut into about 15 cm pieces prior to

their transport to the University of the Philippines, Natural Science Research Laboratory where the bark were stripped for further extraction. Specimens of the plant were identified by Mr. Leonardo Co of the Dr. Jose Vera Santos Herbarium, Institute of Biology, University of the Philippines at Diliman.

The tubli plants, *Derris elliptica*, and *Derris* specimen "uwak" were collected at the foot of the hills of Bato, Catanduanes with the help of villagers who use the plant for fishing during spring tides on nearby shores. The diameter of the roots collected was 2–3.5 cm for *D. elliptica* and 3–4 cm for *Derris* "uwak". The roots were cut into 15-cm segments before transport to UP NSRI laboratory where they were further chopped to smaller pieces just before extraction. Plant specimens were identified by Dr. Edwino Fernando of the Makiling Center for Mountain Ecosystem, UP, Los Baños, Laguna.

Formulation Studies

Several kilograms of Derris root or root bark (~ 15 kg) were extracted with CP grade acetone (~20L) by soaking about 2 kg of new plant part (root or root bark) after every 24 hours. The extraction was stopped only when the bioactivity of the extract reached its maximum or when the activity was comparable to a reference insecticide used as fish poison. The extracts' ichthyocidal activities were monitored by comparing activity with reference insecticide (Telothion-40) commonly and illegally used by fishpond owners. The volume of extract needed for fishpond application was determined from this comparison with Telothion-40, from now on referred to as reference fish poison.

For the bioassay, a given volume of the extract was poured and immediately stirred into a glass tank containing 10 L of tap water and five tilapia fingerlings (*O. niloticus*). One milliliter of the reference fish poison was used. The time when all the five fingerlings died was noted.

Below are the different formulated extracts used previously in fishpond management and whose stabilities were studied (Table 1). Formulations I, II, and III were the direct acetone extracts of the plant parts indicated while formulation IV is the acetone formulation of the $CHCl_3$ extract after the direct acetone extract has been concentrated and partitioned.

Table. 1. Formulated Extracts from Derris Plants

Formulation (Extract)	Solvent	Source	Plant part used
I	Acetone	*D. trifoliata*	dried rootbark
II	Acetone	*D. elliptica*	root
III	Acetone	*Derris* "uwak"	root
IV	Acetone	*D. trifoliata*	rootbark

Formulation's Stability: Decrease in Toxicity and Rotenone Degradation

Formulations of each derris species, *D. trifoliata*, *D. elliptica*, and derris "uwak" were kept in four different locations and conditions. The rotenone contents and toxicity towards *O. niloticus* (fingerlings) of these formulations were then monitored every month to see if there were any decrease in activity or decomposition and degradation of the rotenone in the formulation. The first location A, where the formulations were placed was inside a locker (or cabinet). The second location B was in an open shelf of the research laboratory room subject to the rays of the fluorescent lamp when the room is in use. The third location C was at the top of the institute building provided with shade by a roof but no walls and open to the rays of the rising and setting sun. The fourth location D was inside a refrigerator at constant temperature of 9–10^{o}C. Monthly determinations of the rotenone content of the different formulations in the different locations and conditions and the corresponding bioactivity of the formulations to *O. niloticus* were made.

Each trial in the stability experiment involved duplicate determinations of toxicity and single point determination of rotenone concentration in the rotenone degradation studies. Separate experiments were made to determine the standard deviation involved in the determination of rotenone. This experiment involved one time rotenone determination in triplicate for one high and one low concentration of rotenone in formulations of *D. elliptica* and *D. trifoliata*.

HPLC Analysis of Rotenone

A volume of the acetone formulation was evaporated and then extracted with chloroform. The chloroform extract was then evaporated and redissolved in 25 ml acetonitrile for High Pressure Liquid Chromatography (HPLC) analysis. The rotenone concentration in this acetonitrile solution was determined by comparison with calibrated concentration of rotenone standard using HPLC. The standard procedure used was from the Manual of Analytical Methods of National Institute for Occupational Safety and Health (NIOSH 1994).

Standard rotenone (Sigma) solutions were prepared in 10 mL acetonitrile; 0.01 $mg \bullet mL^{-1}$, 0.03 $mg \bullet mL^{-1}$, 0.05 $mg \bullet mL^{-1}$, 0.07 $mg \bullet mL^{-1}$ and 0.095 $mg \bullet mL^{-1}$. The HPLC column used was μ-Bondapak C18, with UV at 290 nm detector and mobile phase 60% methanol: 40% water with a flow rate of 1.0 $mL \bullet min^{-1}$.

The rotenone peak of the Derris extracts was identified by the standard addition technique and verified by mass spectrometric method. Here, HPLC fractions from the extract corresponding to the retention time of rotenone were collected and subjected to mass spectroscopy (Finnigan LC-MS). The MS spectrum of this fraction was then compared to the MS spectra of standard rotenone.

Bioassay against O. niloticus

The extracts' ichthyocidal activities were monitored by comparing activity with known insecticide (Telothion-40, Shell Chemical Company) used also as fish poison by fishpond owners.

For the bioassay, a given volume (mL) of the extract was poured and immediately stirred into a glass tank containing 10L of tap water and five tilapia fingerlings (*O. niloticus*). The time when all the five fingerlings died was noted. The insecticide Telothion (1 mL) was used as the reference fish poison. A blank test accompanied each of the experiments.

Fingerlings (3-4 cm; 0.4-1.0 g) of the Nile tilapia (*O. niloticus*) were obtained from a nursery of a commercial tilapia fingerling distributor, Mr Arnold Billanes of Bgy. Pulo, Malolos City, Bulacan. The fish were acclimated in laboratory holding tanks before the bioassay experiments.

Results and Discussion

Stability of Toxicity of the Formulations

Monthly analysis of the toxicity of the Formulations II (*D. elliptica*) and III (derris "uwak") towards *O. niloticus* did not show large variation from their initial toxicity (1 mL extract in 10 L water) except very slightly for both formulations under conditions A and C which showed slight decrease in toxicity starting from the month of March (Table 2). This lack of or small decrease in toxicity also was observed in Formulation IV, a reformulated *D. trifoliata* extract under condition A. However, large decrease in toxicity was observed in Formulation I (*D. trifoliata* extract) starting from February where 1 mL equivalent of reference fish poison fell to 4 mL for the formulations. This decrease in toxicity is evident in conditions A, B, and C except in condition D which showed its decrease in toxicity arrested to constancy at 4 mL of formulation (compared to 1 mL of reference fish poison). This showed that decrease in toxicity of *D. trifoliata* extract could be arrested by refrigerating the formulation at 9 – 10^{o}C.

Toxicity can be related to the degradation in rotenone occurring in the formulation as shown in figures 1, 2, 3, and 5 which plot the concentration of rotenone against the age of formulations in days.

Stability of Rotenone in the Formulation

While the bioassay in table 2 showed only slight decrease in toxicity, on the contrary, here in figures 1, 2, 3 and 5, clearly reveal that rotenone degradation occurred in all formulations. The standard deviation for the HPLC determination of rotenone concentration in the formulation was first determined to get approximate standard deviation of single concentration determination throughout the duration of the experiment and is shown in table 3.

The degradation of rotenone from *D. elliptica* was maximum under condition C (roof top, open to sun rays). The formulation is shown to rapidly loose its rotenone content immediately after the formulation was placed under condition C and reached its lowest after 4 months. However, table 2 shows that this last condition of the formulation has still a toxicity equivalent to 1 ml of reference fish poison.

Table 2. Stability of formulations: monthly analysis of toxicity of formulations against *O. niloticus* (under formulation conditions, A = inside cabinet, B = inside room, C = on roof top, D = inside refrigerator at 9 – 10°C)

Formulation	January ml(min)	February ml(min)	March ml(min)	April ml(min)	July ml(min)
II (*D. elliptica*))					
A	1(14,14)[a]	1(10,10)[a]	1(15,14)[a]	1(13,16)[a]	1(37,42)[a]
B		1(11,14)[a]	1(12,12)[a]	1(14,14)[a]	1(16,15)[a]
C		1(11,13)[a]	1(46,43)[a]	1(16,21)[a]	1(38,39)[a]
D		1(14,14)[a]	1(10,12)[a]	1(10,10)[a]	1(18,17)[a]
III (Derris "uwak")					
A	1(13,15)[a]	1(12,10)[a]	1(15,14)[a]	1(12,16)[a]	1(20,21)[a]
B		1(11,11)[a]	1(11,13)[a]	1(14,14)[a]	1(16,15)[a]
C		1(15,14)[a]	1(20,20)[a]	1(18,20)[a]	1(35,34)[a]
D		1(14,10)[a]	1(12,13)[a]	1(12,12)[a]	1(16,15)[a]
I (*D. trifoliata*)					
A	1(40),2(20)[b]	4(22,19)[b]	8(34,48)[b]	10(31,35)[b]	20(22,21)[a]
B		4(21,40)[b]	10(27,29)[b]	10(32,33)[b]	20(24,21)[a]
C		4(23,24)[b]	10(31,33)[b]	10(25,25)[b]	20(25,24)[b]
D		4(10,11)[b]	4(25,20)[b]	4(17,17)[b]	4(20,20)[b]
IV (*D. trifoliata*)	1(18),2(11)[b]	1(15,16)[a]	1(18,18)[a]	1(19,16)[b]	1(17,15)[a]
[Reference Fish Poison]	1(27,25)[a]	1(20,21)[a]	1(21,25)[a]	1(21,14)[a]	1(25,24)[a]
	1(42,38)[b]	1(28,28)[b]	1(29,33)[b]	1(17,20)[b]	1(19,20)[b]

[a] First day toxicity determination using corresponding reference poison 1( )[a]
[b] Second day toxicity determination using corresponding reference poison 1( )[b]

Note that the dotted line in the graphs is an extrapolation of the decrease in rotenone concentration (conditions A and B at room temperature) starting from the date of extraction to the start of rotenone analysis in January.

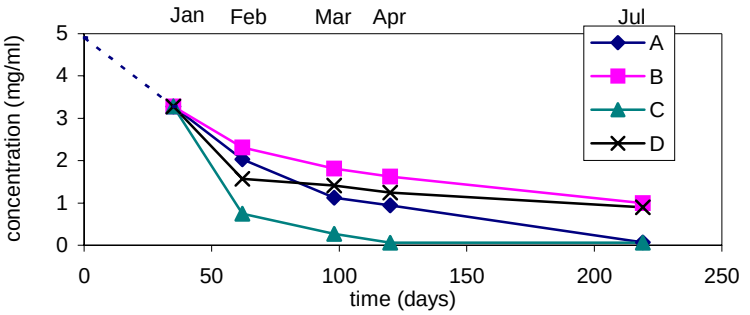

Figure 1. Stability of rotenone in formulation II (*D. elliptica*); conditions of formulation: A = inside cabinet, B = inside room, C = on roof top, D = inside refrigerator

Table 3. Standard deviation for the HPLC determination of rotenone concentration in the formulation

Formulation II (*D. elliptica*)	Rotenone concentration (mg/mL)
Trial 1	0.9435333
Trial 2	0.9553000
Trial 3	0.9793000
	Ave.= 0.9593777 S.D. = 0.0149
Formulation I (*D. trifoliata*)	
Trial 1	0.0084383
Trial 2	0.0096000
Trial 3	0.0098166
	Ave. = 0.0092849 S.D. = 0.000605

Slower degradation of rotenone is observed in conditions A and even much slower in B (inside and outside the cabinet in the laboratory room). The figure shows that these two formulations can last more than four to five months with comparable or even still greater toxicity than the reference fish poison. The line that trace the rotenone concentration of the formulation under D (refrigerator) condition shows a break in the graph of the degradation of rotenone, an indication of a slow down of degradation of rotenone in February. This steeper decrease in rotenone in the concentrations of D could be due to precipitation in the formulation under very low temperature decreasing the solubility of rotenone and its availability in the solution. After such precipitation, the concentration showed slightly better stability than formulations A and B. This big drop in concentration of rotenone in the early period can also be observed in Formulation III D. "uwak".

In figure 2, where the rotenone concentration of the D. "uwak" formulation is monitored, we see the same trend of degradation where condition C shows maximum degradation of rotenone with ageing, although the rotenone concentration after four months is still much higher than in C of figure 1. Again the toxicity trend in table 1 for Formulation III (Derris called "uwak") condition C shows only very slight decrease in toxicity, shown in the months of March onward.

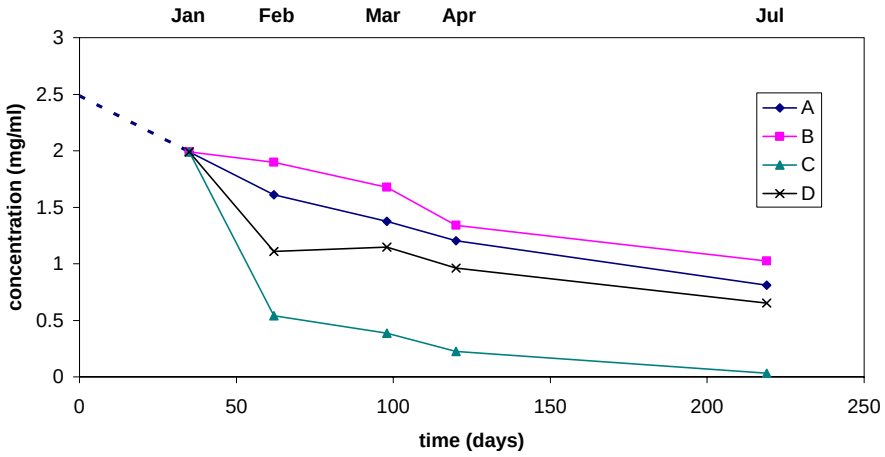

Figure 2. Stability of Rotenone in Formulation III (D. "uwak"). Conditions of formulation: A = inside cabinet, B = inside room, C = on roof top, D = inside refrigerator

Here, formulations in A and B show a moderate and gradual decrease in rotenone concentrations while as before in figure 1, formulation in D shows a sudden drop in concentration but remained stable afterwards. This sudden drop as has been explained before may be due to precipitation and equilibration once the formulation is placed under a temperature of 9 – 10^{o}C. Also, the plot of A and B in both figures 1 and 2 show a lower trend in rotenone concentration in A than in B signifying that there is no advantage in keeping the formulation inside the cabinet or in complete absence of light (nor even inside a refrigerator for Formulation III) as long as the extract is kept away from direct sunlight. Note that the dotted line in the graph is an extrapolation of the decrease in rotenone (conditions A and B) starting from the date of extraction to the start of rotenone analysis in January.

In figure 3, rotenone degradation in *D. trifoliata* formulation, did not differ much if the formulations were kept from sunlight or not (A, B, and C).The rotenone degradation occurred anyway except only if the formulation is kept in a refrigerator at 9 – 10^{o}C. In condition D, we see no big drop in concentration in rotenone as in Formulation II and III. This is probably due to the absence in precipitation occurring in the formulation as was observed. The concentration of rotenone in these formulations for *D. trifoliata* is relatively dilute compared to the Formulations II and III. This decreasing trend in rotenone degradation in Formulation I parallels the loss

in toxicity as shown in table 1 and much more coherently than the previous correlation of toxicity and rotenone degradation in Formulations II and III. Thus it appears that the more concentrated the formulation in rotenone (Formulations II and III are more concentrated in rotenone) the lesser its effect since the toxicity is probably not directly proportional at all times to rotenone concentration.

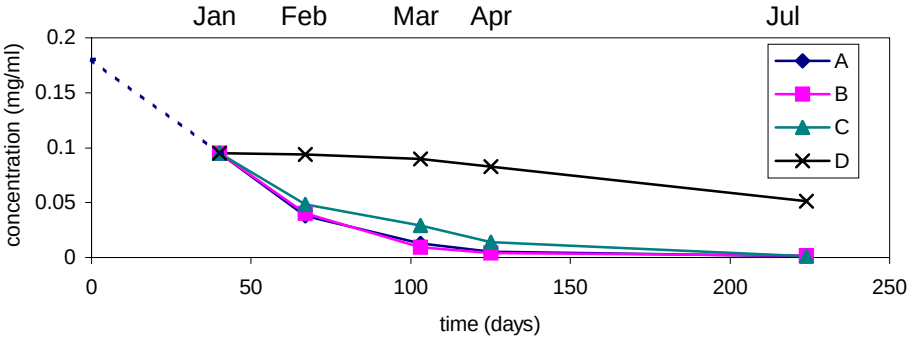

Figure 3. Stability of Formulation I (*D. trifoliata*). Conditions of formulation: A = inside cabinet, B = inside room, C = on roof top, D = inside refrigerator

A plot of toxicity versus concentration of rotenone from data collected in this research is shown in figure 4. This is a plot of toxicity expressed as toxicity of the reference fish poison (min) over toxicity of formulation (min). Values of bioactivity equal to one means equivalent toxicity with reference; values lesser than one means lower toxicity than the reference, while larger than one means greater toxicity than the reference. All the data represented by these points were taken from the stability of formulation studies. As shown in the graph (at lower rotenone concentration) only a portion of the curve shows direct proportionality between toxicity and concentration. Above the toxicity of the reference fish poison, the response of the curve to higher concentration of rotenone is shown to plateau, where the bioactivity becomes relatively constant at higher concentration of rotenone.

This only means that formulations containing larger than 0.20 – 0.50 mg/ml concentrations of rotenone (at the intersection of the curve with the toxicity of 1 ml of reference fish poison) should be reformulated and diluted to provide a more efficient use of the Derris extract.

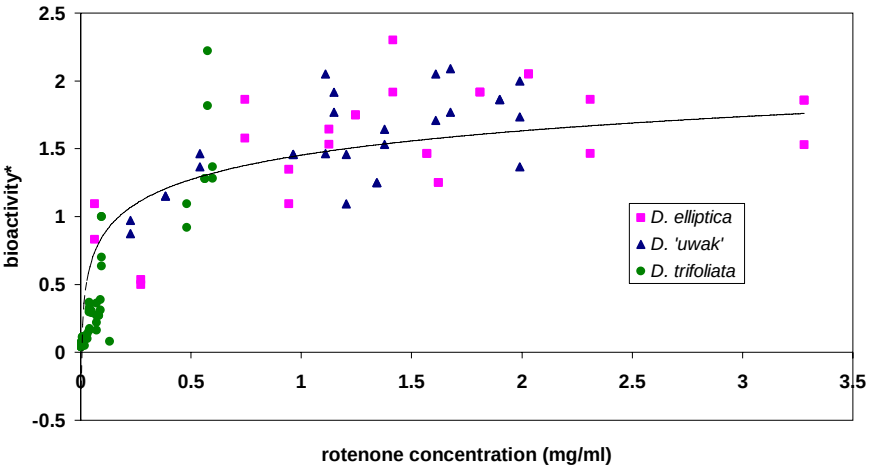

Figure 4. Toxicity vs Rotenone Concentration of different Derris species on *O. niloticus*
*Bioactivity = ave. time of death in reference (min) / ave. time of death in formulation (min)

Furthermore, a promising result on stability was provided by Formulation IV, a formulation made from *D. trifoliata* where only the water insoluble and chloroform extract of the formulation (the aqueous layer discarded) was reconstituted with acetone. The formulation which was produced almost five months before its analysis and kept inside the cabinet (condition A) provided an almost constant toxicity (Table 1) at 1ml equivalent to the reference poison. This formulation also provided a graph indicating better stability of the rotenone concentration in the course of the stability study (4 months) without showing large drop in rotenone concentration. Furthermore this formulation was not kept at low temperature as in condition D, but still maintained a relatively constant concentration in rotenone. This could only mean that if rotenone is separated from the water soluble fraction of the formulation or extract, its degradation can be held off or arrested even without refrigeration. This means then that there may be harmful substances in the water soluble fraction capable (if not removed) of degrading rotenone in the acetone formulation.

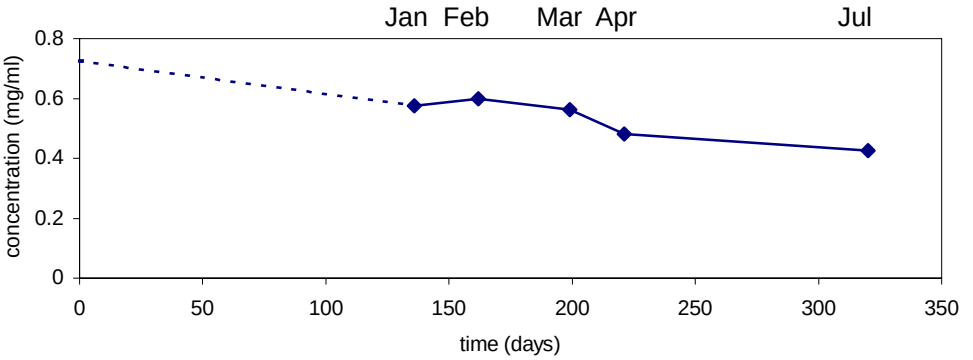

Figure 5. Stability of Rotenone in Formulation IV

Thus, rapid degradation in rotenone and decrease in toxicity can be arrested if the rotenone fraction ($CHCl_3$ extract) is separated from the aqueous fraction as in the case of Formulation IV (*D. trifoliata* reconstituted extract). Once the rotenone fraction is separated, the formulation need not even be kept in the refrigerator but simply away from sunlight. Note that the dotted line in the graph is an extrapolation of the decrease in rotenone concentration (condition A) starting from the date of extraction/partition to the start of rotenone analysis.

Conclusion

The ichthyotoxicity of *D. trifoliata* formulation decreases quickly immediately after extraction, and the preparation must immediately be cooled in a refrigerator at 9 – 10^{o}C to arrest the decrease in toxicity to fish. However, the decrease in toxicity in the formulations of *D. elliptica* and *Derris* "uwak" was not noticeable during the 4 to 7 months of observation except when the formulation was exposed to sunlight. Degradation of the formulations was better observed if the rotenone concentration is monitored. As observed from the monitoring of the rotenone concentration, degradation of rotenone can be minimized in *D. trifoliata* formulation only if the formulation is kept inside a refrigerator at 9 – 10^{o}C as was also observed when the toxicity was monitored. Degradation in rotenone is also large in the formulations with *D. elliptica* and *Derris* "uwak", when the formulation was exposed to sunlight but could be arrested if placed in

refrigerator at 9 – 10°C. Degradation in rotenone and decrease in toxicity can also be prevented if the rotenone fraction ($CHCl_3$ extract) is separated from the aqueous fraction as in the case of Formulation IV (*D. trifoliata* extract). Once separated, the formulation need not be even kept in the refrigerator but simply away from sunlight. The concentration of rotenone in the formulation should be above 0.2 – 0.5 $mg \cdot ml^{-1}$ to have the same toxicity as the reference fish poison. Over this concentration the formulation should be diluted to save on cost since the activity tend to plateau with increasing rotenone concentration.

Acknowledgment

We would like to acknowledge the generous funding from the Department of Agriculture-Bureau of Research and the Natural Sciences Research Institute of the University of the Philippines. This funding has been realized with the help of Dr. Irene Villaseñor, research representative of the Institute of Chemistry, University of the Philippines at Diliman.

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