Two Isoquinuclidine Alkaloids of a Tropical Yam, *Dioscorea hispida* (Dioscoreaceae) as Antifeedant and Toxin Against Lepidopteran Insects.

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ABSTRACT Toxic compounds in the rhizome of a tropical toxic yam, *Dioscorea hispida* Schlussel were extracted and evaluated for biological activity against larvae of the diamondback moth (DBM), *Plutella xylostella* (Linnaeus) and rice armyworm, *Psudoletia saparata* (Walker) (RAW). Two alkaloids, dioscorine and dioscorine N-oxide showed antifeedant activity, retarding of development and insecticidal activity against the larvae of DBM. Individually, the alkaloids significantly inhibited larval feeding at 50 µg/ml and in combination based on a natural ratio (dioscorine and dioscorine N-oxide, 75:25) also deterred feeding of insects at 25 µg/ml. They also significantly retarded larval molting and reduced larval weight when DBM larvae were reared on alkaloid-laden radish seedlings. LC_{50} values of dioscorine and dioscorine N-oxide to DBM larvae were 25.6 and 31.9 µg/ml, respectively. Typical symptom in RAW larvae injected with the alkaloids suggested that these chemicals could act as depressants in the nervous system.

KEY WORDS : *Dioscorea hispida*, alkaloids, diamondback moth, rice armyworm, antifeedant, toxicity

INTRODUCTION

Yam (*Dioscorea* spp.) comprises a major carbohydrate source in tropical countries. Many wild species of Dioscoreaceae are, however, highly toxic due to their content of isoquinuclidine alkaloids. These alkaloids induce dizziness, nausea, vomiting, and later sleepiness in humans (Leyva and Gutierrez, 1937; Pinder, 1952; Leete, 1977; Science Education Center, 1980; Leete and Michelson, 1989; Huxtable, 1992; Goh *et al*., 1994). Several authors have suggested that some tropical Dioscoreaceae species could be potential source of natural control agents against several insect pests (Heal and Rogers, 1950; Wada *et al*., 1970; Grainage *et al*., 1985). In the Philippines, a homogenate of *Dioscorea hispida* Schlussel rhizome has been used by farmers to control insect pests in agricultural fields (Ecological Farming Program, 1990).

In earlier studies it was demonstrated that alkaloidal fractions as well as ether soluble non-alkaloidal fractions in the methanol extracts of *D. hispida* rhizome inhibit feeding, delay development and cause larval and pupal death in the diamondback moth (DBM), *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) (Banaag *et al*., 1998,1997). The present work now focuses on the isolation and identification of two active alkaloids and their antifeedant activity, effects on growth and development, and toxicity against larvae of diamondback moth (DBM). Mode of action of the alkaloids isolated is also discussed based on comparative intoxication test between alkaloids and synthetic insecticides in the larvae of rice armyworm (RAW), *Psudoletia saparata* (Walker) (Lepidoptera: Noctuidae).

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MATERIALS AND METHODS

Insect

DBM larvae were provided by the Nihon Bayer Agrochem K.K. Research Center (Yuki, Ibaraki, Japan). DBM larvae were reared on radish seedlings in a plastic container (7.5 cm x 13 cm) at 25 ± 2°C under a 16L:8D photoregime. RAW larvae were obtained from National Institute of Sericulture and Entomology (Tsukuba, Ibaraki, Japan) and reared on the Silkmate artificial diet (Nihon Nosan Kogyo Co.) under the same conditions as for DBM.

Plant

Rhizomes of *D. hispida* were collected at Catarman, Camiguin, Philippines in March 1994, April 1995 and March 1996, and some of them were planted in Agricultural and Forest Research Center, University of Tsukuba for harvesting leaves and stems.

Extract Preparation and Isolation of Alkaloids

Procedures for preparation and isolation of alkaloids were the same as already reported by Banaag *et al*. (1997) (Fig. 1). All fractions were assayed after each step. Active fractions were further fractionated and assayed repeatedly. Since two alkaloid fractions (fractions 4 and 5) were shown to be antifeedants against DBM larvae (Banaag *et al*., 1997), they were further re-fractionated into following six fractions by an open column chromatography (Wakogel C-100, 23.5 cm in height x 1.5 cm diameter). Elution was accomplished with

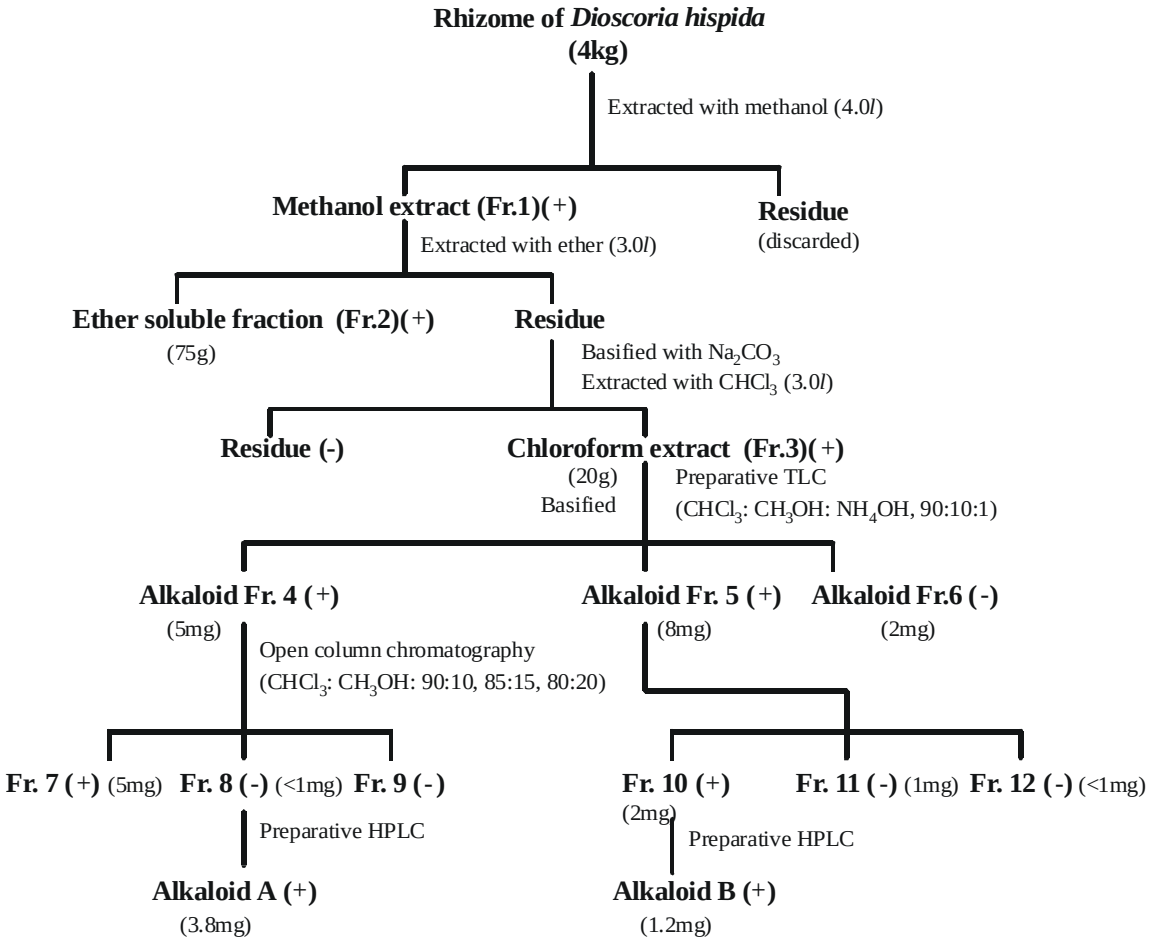

Fig. 1. Fractionation scheme for the methanol extract of *D. hispida* rhizome and its antifeedant activity. (+): active, (-): not active

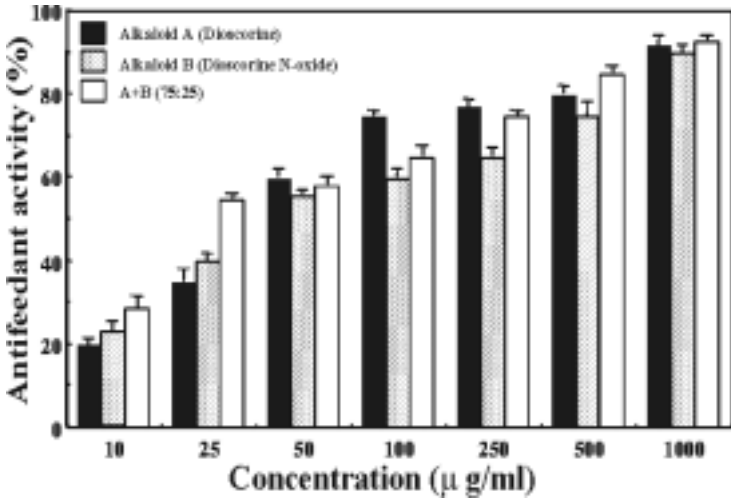

Fig. 2. Antifeedant activity of alkaloid A , B and their mixture (75 : 25) at different concentrations against DBM larvae. *, **: significantly different at P<0.05 and P<0.01, respectively, by paired t-test. ns: not significantly different.

500 ml each of $CHCl_3$-MeOH-NH_3 mixture at 90:10:1, 80:20:1 and 60:40:1 ratios. After these procedures, Frs. 7, 8, 9 (from Fr. 4) and Frs. 10, 11, 12 (from Fr. 5) were obtained. Antifeedant activity of all six fractions was then examined by the leaf disk test assay against DBM larvae (Banaag *et al*., 1997).

Fractions 7 and 10 showing significantly high antifeedant activity to DBM larvae were further purified by a preparative high-performance liquid chromatography (HPLC) (Shimadzu LC-10A system). Chromatographic conditions followed were: column; HRC-ODS (46mm x 150 mm, Shimadzu); solvent system EtOH-H_2O (90:10); flow rate 2ml/ min. The chromatogram was monitored with UV at 254nm (SPD-10Avp, Shimadzu). Alkaloids purified were designated as alkaloid A and B, respectively. Contents and profiles of these two alkaloids in rhizomes, stems and leaves of *D. hispida* were also determined by analytical ODS-HPLC.

Chemical Analysis

Isolated fractions were monitored by thin layer chromatography (TLC) on silica gel plate (60 F_{254}, Merck) using $CHCl_3$-MeOH-NH_3 (90:15:1) and ODS silica gel plate (KC_{18} F, Whatman) with EtOH-H_2O (70:30) as solvents. Detection was under UV_{254} and spraying with Dragendorff's reagent (Stahl, 1969). Mass spectra of the alkaloids were obtained with a Hitachi M-80 B High Resolution Mass Spectrometer (in direct inlet mode). ^1H NMR (270 MHz) and ^{13}C NMR (67.8 MHz) spectra were recorded in $CDCl_3$ with TMS on JEOL EX-270 spectrometer. IR spectrum was obtained as a KBr-pellet with IRA-1 (JASCO Co.).

The two alkaloids (A and B) were subjected to structural transformation to confirm N-oxidation in a quinuclidine ring by oxidation or reduction reactions. Alkaloid A was oxidized with 30 per cent H_2O_2 and PtO in MeOH, and after filtration, reacted product was concentrated and dissolved in $CHCl_3$. Alkaloid B was reduced with 2N H_2SO_4 and zinc dust in MeOH, and after removing particulate the product was finally passed through ODS Sep-pack Plus (Waters). These converted alkaloids and original alkaloids were chromatographed on TLC and their Rf values were compared with each other.

Bioassays for Antifeedant Activity

Antifeednt activity of alkaloid A and B were determined by using a leaf disk "two-choice" test, as described earlier (Banaag *et al*., 1997). The leaf disks were treated with individual alkaloids and also with a mixture of the alkaloids in a natural ratio of 75: 25.

The toxicity of the alkaloids A and B was evaluated by the leaf disks assay in no-choice system.

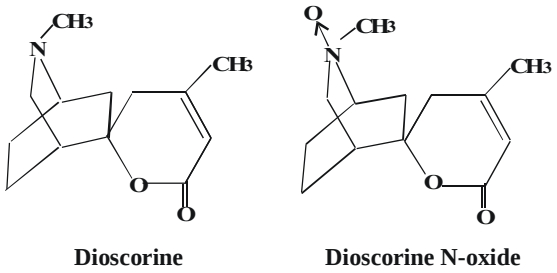

Fig. 3. Structures of dioscorine and dioscorine N-oxide. (from Goh et al., 1994)

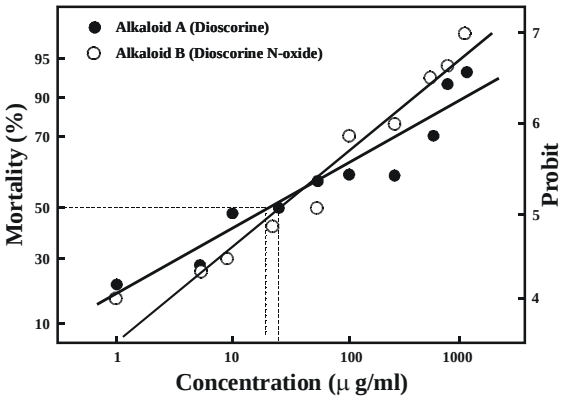

Fig. 4. Toxicity of alkaloid A and B of *D. hispida* rhizome against DBM from 2nd instar larvae until pupae.

The cabbage leaf disks were treated with the alkaloids by dipping at concentrations of 1, 5, 10, 25, 50, 100, 250 500, 750, and 1000 μg/ml. Ten starved third instar DBM larvae were allowed to feed continuously on the alkaloid-laden leaf disk at 25± 2 °C under a 16L:8D photoregime. All tests were replicated 3 times. Leaf disks were changed daily, and pupae were transferred into a plastic cup until adult emergence. Mortality was corrected by Abbott's formula (Abbott, 1925) and then subjected to probit linear regression analysis to determine the LC_{50} (effective concentration to kill 50% of larvae) values.

Symptomology

The 3rd instar larvae of RAW (ca. 0.22 mg in weight) were used to observe characteristic intoxication symptoms and to assess the mode of action of the alkaloids. Alkaloid A and B (>95% in purity), nicotine (95%, Nakarai Tesque Inc.), cartap (98.5%, Takeda Chem. Ind. Ltd.) or dichlorvos (98.5%, Nippon Soda Co.) was dissolved in 3μl of sterilized distilled water containing 1 per cent Tween 60 at 3μg for the two alkaloids and at 0.3μg for the insecticides. Test solutions were injected into insects through the prolegs by a micro-syringe (Hamilton). The control larvae were treated with solvent only. The treated larvae were placed in 9cm-petri dishes with no provision of food under the same rearing conditions mentioned above. Intoxication symptoms were observed consecutively during the first 0.5h and at 1, 12 and 24h after the treatment. Symptoms were categorized and recorded as (i) normal, (ii) depressive effects, characterized by immobility and stretching of the body, (iii) hyperactive effects, characterized by struggle and curved body by muscular contraction, and (iv) death.

RESULTS

Antifeedant Activity of Fractions.

As shown in Fig. 1, antifeedant activity in the crude MeOH extracts (Fr.1) of *D. hispida* rhizome was separated into ether soluble fraction (Fr.2) and chloroform soluble fraction (Fr.3). Fr. 2 contained a toxic unsaturated fatty acid (Banaag *et al*., 1998) and the main components in Fr. 3 seemed to be alkaloids (Banaag *et al*., 1997). A preparative TLC further fractionated alkaloidal Fr. 3 into Fr. 4 to Fr.6, and remarkable antifeedant activity was found in Fr. 4 and Fr. 5. Of six fractions obtained from Fr.4 and Fr.5 by silica gel column chromatography with different solvent systems, Fr.7 and Fr.11 showed potent antifeedant activity when tested individually, but the other four fractions Fr.8, 9, 11 and 12 were not active at any concentration tested. From Fr. 7 and Fr. 10, two active components, which were positive to Dragendorff's reagent on TLC were obtained as alkaloid A (3.8mg) and alkaloid B (1.2mg), respectively (Fig.1). HPLC analysis of a crude alkaloid fraction gave a natural ratio of 75: 25 between alkaloid A and B. Both alkaloids purified showed antifeedant activity at concentrations of 50μg/ml and their binary mixture showed antifeedant activity even at 25μg/ml (Fig. 2).

Table 1. Mortality and intoxication symptoms of the 3rd instar rice armyworm larvae at different times after injection with dioscorine (alkaloid A), dioscorine N-oxide (alkaloid B), cartap, dichlorvos and nicotine.

	Normal (%)					Inhibitory symptom (%)					Hyperactive symptom (%)					Mortality (%)				
Time (h)	D	DN	CAR	DIC	NIC	D	DN	CAR	DIC	NIC	D	DN	CAR	DIC	NIC	D	DN	CAR	DIC	NIC
0.5	2(2)	4(1)	2(1)	0	0	98(1)	96(2)	88(3)	0	0	0	0	0	87(4)	80(5)	0	0	12(2)	3(1)	20(2)
1	2(1)	2(1)	0	0	0	90(3)	93(3)	85(3)	0	0	0	0	0	85(4)	72(4)	8(2)	5(1)	13(2)	15(4)	28(2)
6	0	2(1)	0	0	0	90(5)	90(4)	72(2)	0	0	0	0	0	80(5)	68(2)	10(2)	8(2)	18(4)	20(5)	32(4)
12	0	0	0	0	0	86(3)	88(4)	77(2)	0	0	0	0	0	70(5)	57(6)	14(2)	12(2)	23(3)	30(2)	43(5)
24	0	0	0	0	0	79(6)	87(4)	73(2)	0	0	0	0	0	65(7)	48(7)	21(3)	13(2)	27(3)	35(3)	47(7)

D, DN, CAR, DIC, NIC: dioscorine, dioscorine N-oxide, cartap, dichlorvos and nicotine, respectively.
Injected dose: 3mg/larva in D and DN, 0.3mg/larva in CAR, DIC, NIC.
Each experiment was replicated 3 times with 10 larvae.
Number in parenthesis indicates SD.
No mortality of larvae was observed in control.

Structural Elucidation of Alkaloid A and B

High-resolution mass spectrometry of alkaloid A gave an M^+ ion at *m/z* 221 and the following fragment ions and intensities (%); *m/z* 148(9), 110(6), 96(20), 82(32), 70(100,base ion), 55(20), and 42(30)(Fig. 3a). While Alkaloid B gave an M^+ ion at *m/z* 237(4) and the following fragment ions; *m/z* 152(4), 137(8), 114(21), 96(17), 70(29), 60 (100, base ion), 53(17) and 42(34)(Fig. 3b). IR spectrum (KBr) showed absorption at 2924 cm^{-1}(C-H), 1710 cm^{-1} (C=O), and 1230 cm^{-1} (C-O), respectively, in both alkaloids.

^{13}C NMR spectra of alkaloid A gave 13 carbons as follows; δ (ppm): 52.4 (CH, C1), 53.8 (CH_2, C3), 35.1 (CH, C4), 81.3 (C, C5), 40.9 (CH_2, C6), 20.0 (CH_2, C7), 19.4 (CH_2, C8), 39.5 (CH_2, C9), 155.7 (C, C10), 116.5 (CH, C11), 165.1 (C, C12), 23.3 (CH_3, C13), 42.6 (NMe).

^1H NMR spectra of alkaloid A yielded 19 protons; δ (ppm): 2.62 (1H, br. s), 2.45 (1H, dd, J = 10.89, 1.98 Hz), 3.00 (1H, ddd, J = 13.85, 2.97 Hz), 1.95 (1H, d, 9.9 Hz), 2.62 (2H, br. s), 1.55 (1H, m), 1.95 (1H, dd, J = 9.9 Hz), 1.50 (1H, m), 2.15 (1H, ddd, J = 5.28, 2.64 Hz), 1.80 (1H, dd), 2.08 (1H, d), 5.85 (1H, s), 2.00 (3H, d), 2.35 (3H, s).

The ^{13}C NMR assignment of alkaloid B gave 13 carbons as follows; $\delta_{TMS}^{CDCl_3}$ ppm: 68.1 (CH, C1), 72.1 (CH_2, C3), 35.4 (CH, C4), 75.5 (C, C5), 38.1 (CH_2, C6), 20.9 (CH_2, C7), 16.9 (CH_2, C8), 35.7 (CH_2, C9), 156.4 (C, C10), 115.9 (CH_2, C11), 164.1 (C, C12), 23.2 (CH_3, C13), 59.3 (NMe).

^1H NMR of alkaloid B also yielded 19 protons; δ (ppm): 3.40 (1H, m), 3.55 (1H, dd, J = 13.35, 2.31 Hz), 3.70 (1H, d, 12.87 Hz), 2.28 (1H, t), 1.80 (1H, dd, J = 15.02, 2.48 Hz), 3.22 (1H, dd, J = 16.82, 1.65 Hz), 2.05 (2H, t, J = 2.64), 1.40 (1H, m), 2.32 (1H, m), 2.70 (1H, d, J = 17.81 Hz), 3.10 (1H, dd, J = 17.81), 5.85 (1H, s), 2.00 (3H, d) and 3.32 (3H, m). These NMR spectra of alkaloid A and B were in good agreement with isoquinuclidine alkaloids, dioscorine and dioscorine *N* -oxide (Beecham *et al*., 1969; Leete, 1977; Leete and Michelson, 1989; Goh *et al*., 1994).

The speculation that *N* -oxidation creates quaternary nitrogen in a quinuclidine ring was confirmed by reducing alkaloid B to alkaloid A and by oxidizing alkaloid A to alkaloid B. The Rf values of oxidized alkaloid A and reduced alkaloid B were well coincidental with untreated alkaloid B (Rf 0.22) and A (Rf 0.46), respectively, on TLC. Therefore Alkaloid A and alkaloid B were identified as dioscorine and dioscorine *N* -oxide, respectively (Fig. 3) (Goh *et al*., 1994).

Insecticidal Activity and Behavioral Effects of Alkaloids

All DBM larvae were continuously exposed to the alkaloids in the range of 1 μg/ml to 1000μg/ml, and total mortality from larvae to pupae was determined. The alkaloid A and B showed insecticidal activity with an LC_{50} value of 25.6μg/ml and 31.9μg/ml, respectively (Fig. 4).

Table 1 summarizes intoxication symptoms and mortality of RAW larvae injected with alkaloids or

three different synthetic insecticides. All tested chemicals paralyzed the RAW larvae, but the time course of intoxication was different among the chemicals. Alkaloid A and B paralyzed larvae within 0.5 h while dichlorvos, cartap and nicotine elicited paralysis within 10 min. During the first 0.5 h no mortality was observed, but, mortality increased in the alkaloid treated larvae as time progressed. Most of affected larvae showed typical depressive symptom in the form of stretched body. The total mortality for the alkaloids after 24 h was 21 per cent and 13 per cent, respectively. Similar curved bodies characteristic as depressive symptoms were evoked by cartap, and 27 per cent of mortality was recorded after 24 h. Dichlvos and nicotine elicited paralysis after convulsions, followed by contractions as hyperactive symptoms, and finally the death after 24 h was 35 and 47 per cent, respectively.

DISCUSSION

Toxicity of wild yam, *D. hispida* has been well known in various species of mammals including human, and dioscorine and dioscorine *N* -oxide has been reported as toxic alkaloids from this plant. Banaag (1992) tested possible biological activities of a squeeze of *D. hispida* rhizome against *Papilio demoleus* Linnaeus larvae because of its local use as a traditional pest control agent in Philippines. Results of our previous work (Banaag *et al*., 1997) and present work confirm that antifeedant activity and toxicity of *D. hispida* against lepidopteran larvae is due to two isoquinuclidine alkaloids, dioscorine and dioscorine *N* -oxide.

Adams and Bernays (1978) has pointed out that combinations of antifeedants elicit additive or synergistic effects when compared to each single compound. Generally insects under natural conditions may encounter not merely a given concentration of a specific compound but the total phytochemical profile (Bell, 1987). Binary mixture of two alkaloidal fractions corresponding to dioscorine and dioscorine *N* -oxide, showed significant antifeedant activity at 50µg/ml in 1: 1 ratio against DBM larvae (Banaag *et al*., 1997). A mixture of these alkaloids in natural ratio showed a potent activity even at lower concentration (25µg/ml) suggesting an additive or synergistic action of the alkaloids under natural conditions as well. The potency of these alkaloids as antifeedants seems to be comparable to azadirachtin from *Azadirachta indica*, which at 50µg/ml gave 37 to 75 per cent feeding deterrence in *Achaea janata*, (Linnaeus) *Spodoptera litura* (Fabricius) (Ramachandran *et al*., 1989), *S. frugiperada* (Raffa, 1987), *Peridroma saucia* (Hübner) (Isman *et al*., 1990) and *Ostrinia nubilalis* (Hübner) (Arnason *et al*., 1985).

In addition to these alkaloids, *D. hispida* rhizome contained a non-alkaloidal toxic compounds (Banaag *et al*., 1998), and the lowest concentration of the alkaloids for antifeedant activity was 10µg/ml when they were mixed with the toxic non-alkaloidal fractions (Banaag *et al*., unpublished data). These facts strongly indicate that *D. hispida* has evolved a multi-component system in chemical defense against herbivores, a phenomenon described recently for neem allelochemicals (Koul *et al*., 2003, 2004).

The leaves and stems of *D. hispida* also contain dioscorine and dioscorine *N* -oxide equal to 2.4 and 0.5mg/kg dry weight, respectively, and the total content of the alkaloids amounts to 1.5 times higher than that of rhizome. Thus *D. hispida* may be highly protected from herbivores as both terrestrial as well as underground parts are rich in toxic alkaloids, more so the leaves or stems of the yam are superior to rhizomes as source of antifeedants.

Both dioscorine and diosorine *N*-oxide showed no antifeedant effect at 25µg/ml (Fig. 2), but this concentration was close to LC_{50} value for each of the compounds in oral no-choice situation. Death of DBM larvae at lower concentration apparently is due to the forced feeding in no-choice situation. However, at higher concentrations behavioural effects seem to be more prominent and mortality of larvae due to long-term starvation was obvious.

Based on symptoms of human intoxication, Huxtable (1992) concluded that dioscorine has picrotoxin-like effects on the central nervous system. Picrotoxin is known to block the $GABA_A$-receptor-complex (GABAR) (Yoo *et al*., 1993). If dioscorine or dioscorine *N*-oxide could have the same mode of action as picrotoxin, hyperactive symptoms in RAW larvae are justified. In the present work, dioscorine

or dioscorine N-oxide showed depressive actions on the RAW larvae similar to those of cartap (Table 1). Cartap is known to be a non-competitive open channel blocker of the nicotinic acetylcholine receptor (nAChR) (Nagata *et al*., 1997,1998). Thus, dioscorine or dioscorin N-oxide may act on the nAChR rather than GABAR. Nagata *et al.* (1999) confirmed by patch clamp experiments that these alkaloids act as open-channel blockers of nAChR in rat phaeochromocytoma (PC12) cells, and have no effect on GABAR in rat dorsal root ganglion neurons.

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