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LIMONOIDS FROM *CITRUS RETICULATA* AND THEIR MOULT INHIBITING ACTIVITY IN MOSQUITO *CULEX QUINQUEFASCIATUS* LARVAE

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Key Word Index—*Citrus reticulata*; Rutaceae; limonoids; limonin; nomilin; obacunone; *Culex quinquefasciatus*; moult inhibition.

Abstract—Three limonoids, namely limonin, nomilin and obacunone, were isolated from the seeds of *Citrus reticulata* [Blanco Coorg Mandarin]. Their structures were confirmed by spectroscopic studies. With 4th instar larvae of mosquito *Culex quinquefasciatus*, the EC_{50} for inhibition of adult emergence was 6.31, 26.61 and 59.57 ppm for obacunone, nomilin and limonin, respectively. The pattern of mortality at around the EC_{50} levels was indicative of moult inhibiting activity. The efficacy of a limonoid enriched fraction was similar to that of limonin, probably due to the high content (70%) of limonin. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

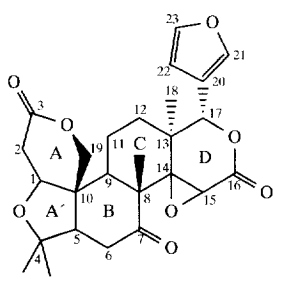
Limonoids are a group of chemically related triterpenoids found in Rutaceae and Meliaceae families of the order Rutales. Out of 38 limonoids reported to occur in citrus and its hybrids [1], limonin is largely responsible for delayed bitterness in citrus juice and processed citrus products [2].

Limonoids have attracted attention due to their insect antifeedant, growth regulating activity [3] and, more recently, their anticarcinogenic and antitumorogenic activity [1]. The Rutaceae limonoids, limonin (**1**), nomilin (**2**) and obacunone (**3**) present in large quantities in the byproducts of the citrus industry [4] have previously been investigated for their insect antifeedant activity [3].

In the present communication we report the isolation and purification of limonin (**1**), nomilin (**2**) and obacunone (**3**) from *Citrus reticulata* [Blanco Coorg Mandarin] and their moult inhibiting activity in the mosquito *Culex quinquefasciatus* Say.

RESULTS AND DISCUSSION

The limonoid compounds after purification were analysed by TLC and HPLC. TLC analyses of the compounds gave mobilities which matched with that of standard limonoids (R_f value of 0.53, 0.25 and 0.17 for compounds **3**, **1** and **2**, respectively). No additional spot(s) were visualised on two plates with either Ehrlich reagent or iodine thus confirming the absence of any additional compounds. The purity of the compounds was further confirmed on HPLC using a C-18 reverse phase column under isocratic conditions [2]. The compounds **1**, **2** and **3** had retention times of 7.14, 11.59 and 19.40 min, respectively, which matched with those of standards. The ^{1}H NMR and ^{13}C NMR spec-



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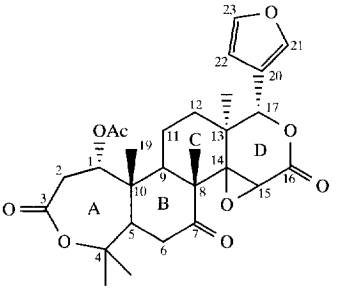

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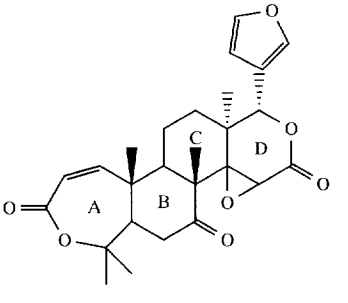

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Table 1. Efficacy of chronic exposure of fourth instar *C. quinquefasciatus* larvae to citrus limonids

Limonoids	Regression equation	EC_{50} (ppm)	SE_M	EC_{95} (ppm)
Limonin (**1**)	1.1599+2.1958	59.57	±0.0307	668.30
Nomilin (**2**)	1.3790+2.5700	26.61	±0.0297	177.80
Obacunone (**3**)	1.5542+1.9233	6.31	±0.0365	44.67
Enriched fraction	1.2939+2.1815	50.12	±0.0286	281.80

trum of compounds **1**, **2** and **3** matched well with standard reported values [5–7] for limonin, nomilin and obacunone, respectively.

The purified limonoids were tested for their effect on *Culex quinquefasciatus* larvae. The results (Table 1) show that obacunone was most active, followed by nomilin and limonin. Significant mortalities at larva-pupa moult (L-P) and emerging-adult (PA_2) (Fig. 1) were indicative of moult inhibiting activity. Although intermediate in activity between limonin (**1**) and obacunone (**3**) (Table 1), nomilin (**2**) exhibited maximum moult inhibition judged by low larval mortalities and the spread of mortalities at L-P and PA_2 stages (Fig. 1(a)). At higher concentrations, all three compounds exhibited toxicity resulting in high mortality of mature larvae (Fig. 1(b)).

Since all the three citrus limonoids exhibited moult inhibiting activity in larvae of *C. quinquefasciatus* but are apparently harmless to animals due to their presence in citrus fruits [4], it was thought worthwhile to test enriched fractions of these limonoids on 1st and 4th instar mosquito larvae. Further, since other components in the mixture could enhance activity, such enriched fractions would be less expensive to produce. The EC_{50} of the enriched fraction to 4th instar larvae was close to that for limonin (**1**) (Table 1) probably due to the high content (70%) of limonin. However, at higher concentrations of the enriched fraction, the more active obacunone (**3**) and nomilin (**2**) were probably responsible for lowering the EC_{95} value (Table 1). With the 1st instar larvae, the enriched fraction, even at the 5 ppm level, did not exhibit significant toxicity. Further, the speed of development of the larvae in the treatments was not significantly different from that of the control.

As an antifeedant against a variety of phytophagous insect pests, nomilin (**2**) and obacunone (**3**) were equally active but they were about ten times more effective than limonin (**1**) [3]. This trend in activity prompted the conclusion that the 7-membered lactone ring (ring A) influenced the antifeedant activity [4, 8]. However, in the present study obacunone (**3**) was about two times and ten times more active than nomilin (**2**) and limonin (**1**), respectively, to 4th instar *C. quinquefasciatus* larvae (Table 1). The least active compound, limonin (**1**), has a 6-membered lactone (A ring) and tetrahydrofuran (A′ ring) moiety. Between obacunone (**3**) and nomilin (**2**), the higher activity of the former was due to the absence of the acetoxy group in ring A. On the other hand, saturation of ring A by introduction of the acetoxy group at C-1 of nomilin and the resultant change in conformation of a large part of the molecule, improved the moult inhibiting activity. Thus, the 7-membered lactone and more specifically C-1 is implicated in the toxic and moult inhibiting activity of these limonoids (Fig. 1(a)). With more studies on various insect pests on the structure–activity relationships of these limonoids and with suitable modification in their structure and application method, these could be exploited for the formulation of eco-friendly pesticides.

EXPERIMENTAL

Materials. Dried seeds of *Citrus reticulata* [Blanco Coorg Mandarin] were procured from a local fruit processing factory in February–March. Mandarin oranges are cultivated in South India on a large scale in Coorg [9]. All the solvents/chemicals used were of AR/HLPC grade. Mp: uncorr. Silica gel TLC plates were developed with cyclohexane–EtOAc (2:3) [10]. The plates were sprayed with Ehrlich's reagent (2% *p*-dimethylamino benzaldehyde in EtOH) and developed in an HCl gas chamber. Typical pink/reddish coloured spots were obtained for limonoids [11]. HPLC analysis of the compounds was carried out on C-18 reverse phase column (150 × 4.6 mm, 5 μm particle size, Shimadzu LC-6A model). The elution was carried out at a flow rate of 1.0 ml min^{-1} under isocratic conditions using MeCN–MeOH–H_2O (10:41:49). The compounds were detected by UV absorption at 210 nm [2] and were quantified using a Shimadzu C-R4A Chromatopak data processor. The standard limonoids were also run under similar conditions for comparison.

Extraction of limonoids. Sun-dried citrus seeds were cleaned, powdered and extracted in a Soxhlet with petrol (60–80° for 6 h) for the removal of oily material. The residue was extracted 3 × by soaking overnight with MeOH (200+200+150 ml per 100 g of residue) with occasional stirring. The MeOH extract was concentrated *in vacuo* to yield a viscous liquid. This was diluted with H_2O and extracted 3 × with CH_2Cl_2. The extract was concentrated and crystallised. Most of the limonin (**1**) crystallized as needle-shaped crystals and was collected by filtration. In earlier reports, limonin (**1**) and nomilin (**2**) were obtained by repeated recrystallization from an Me_2CO extract of seeds using

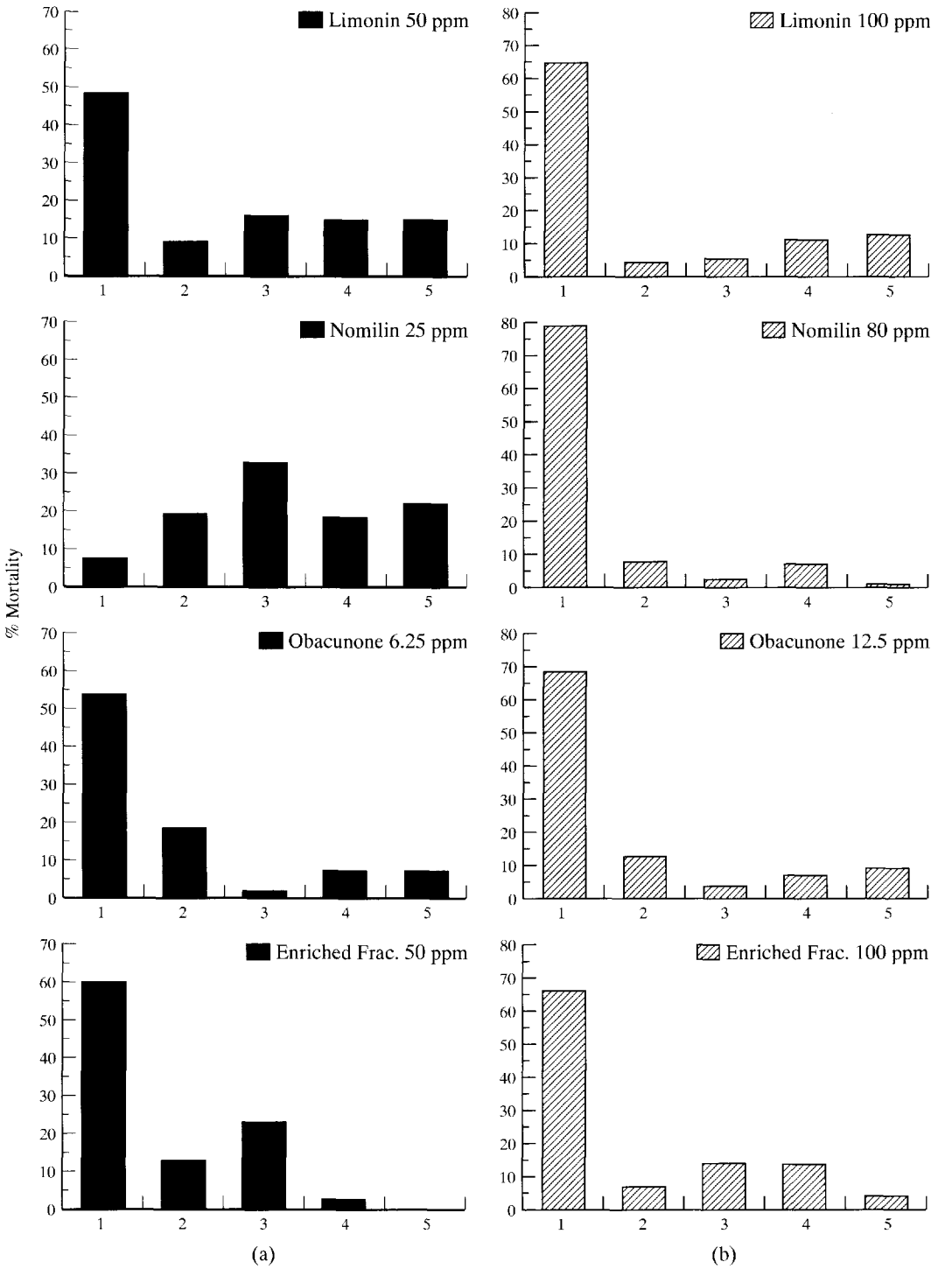

Fig. 1. Histograms showing mortality at various developmental stages of *C. quinquefasciatus* following chronic exposure of fourth instar larvae to citrus limonoids at concentrations affording ~50% (a) and >70% (b) kill. 1, Larva; 2, arva-pupa moult; 3, white pupa; 4, pupa; 5, pharate adult.

CH_2Cl_2 [12]. In the present study, 60% pure limonin (**1**) was obtained by crystallization from an Me_2CO extract using CH_2Cl_2, but pure nomilin (**2**) could not be obtained. Hence, the limonoids were separated by silica gel column chromatography. The remaining fraction was loaded on to a silica column and eluted with cyclohexane–EtOAc mixture with stepwise increases in the concentration of EtOAc. Compound **3** was eluted at 25% EtOAc in cyclohexane while compound **1** and compound **2** were eluted by 30% and 35% of EtOAc in cyclohexane, respectively. Compounds **1**, **2** and **3** were further purified by crys-

tallization with CH_2Cl_2, Me_2CO and MeOH, respectively.

Preparation of enriched fraction. To increase the concentration of nomilin (**2**), and obacunone (**3**) present in the crude extract, limonin (**1**) was crystallized using CH_2Cl_2. After collecting limonin crystals by filtration, the remaining solution was analysed by HPLC (limonin 70%, nomilin 1.6% and obacunone 0.3%) and used for biological work.

The mps of compounds **1**, **2** and **3** were 294–296°, 275–278° and 228–230°; $[\alpha]_D^{20}+118°$ (Me_2CO; *c* 1), $-94.5°$ (Me_2CO; *c* 1) and $-50°$ ($CHCl_3$ *c* 1), respectively. IR spectrum of the compound **1**: ν_{max} cm^{-1} 1765, 1745, 1715, 1510, 880; compound **2**: 1746, 1713, 1508, 880; compound **3**: 1752, 1716, 1507 and 879.

Insect bioassay. The test insect *C. quinquefasciatus* was drawn from a laboratory colony maintained for several years without exposure to insecticides. The larvae were fed finely ground dog biscuits.

For the assay, up to 0.2 ml soln of the test compound in Me_2CO was applied to 50 ml distilled water in a 100 ml beaker. The controls received 0.2 ml Me_2CO alone. Two hours after treatment, 25 larvae of 1st or 4th instar were released in each beaker. The 1st instar larvae (<2 hr old) were drawn into a tube (4 mm i.d.) and gently released over a strip of Whatman no. 1 filter paper. The H_2O was allowed to drain off, the larvae stranded on the paper strip were counted and dipped into the test soln. Dead specimens were observed under a binocular microscope and staged [13, 14]. For experiments with 1st instar larvae, the speed of development was calculated [15].

The experiments were carried out $28\pm1°C$. Each test comprised of 4 replicates with at least 4 concentrations along with the control. The data were subjected to regression analysis. From the regression line, the EC_{50} and EC_{95} values were read representing the effective concentrations for 50% and 95% inhibition of adult emergence.

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