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# BIOLOGICAL ACTIVITIES AND MODE OF ACTION OF LIGNANS FROM THE *PIPERACEAE*

CLAUDE BOURRET-BERNARD

Thesis submitted to the
School of Graduate Studies and Research
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in partial fulfilment of the requirements for the Degree of

Doctor in Philosophy

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**à Philippe**

# ABSTRACT

Tropical American *Piper spp.* have insecticidal activities comparable to those of previously studied African and Asian *species*. Effects of dietary administration of the crude ethanolic extracts of *Piper spp.* to the European corn borer, *Ostrinia nubilalis* Hübner, include a reduction of larval growth, a high death rate of larvae, pupae and adults, and a limited effect on the consumption and digestion of food. Among 16 *Piper* extracts the most active American *species* were, in decreasing order of growth reducing activity: *P. tuberculatum, P. aduncum, P. pseudo-lindenii, P. lanceiaifolium, P. guanacastensis, P. decurrens, P. carilloanum,* and *P. auritum*. Bioassay-guided isolation, using mosquito larvae, led to the isolation of a monolignan, dillapiol, as an active principle of *P. aduncum,* two neolignans as active principles of *P. decurrens,* and an amide, piperlonguminine as an active principle of *P. tuberculatum*.

The new isolation of the neolignans and the amide gives further evidence of the biological significance of these two classes of compounds as chemical defenses of the *Piperaceae*.

The activities of a series of 15 pure monolignans, lignans, and neolignans were investigated with the polyphagous lepidopteran herbivore, the European corn borer, which has a high adaptive capacity to tolerate plant allelochemicals. The lignans (at a dose of 100 μg/g in the diet) were generally not highly toxic to the larvae, except for the monolignan dillapiol. However, delayed toxicity was revealed as mortality increased later in the life cycle, reaching at the adult stage a cumulative mortality of 95%, 68%, 60%, 43% and 40% with dillapiol, epiashantin, podophyllotoxin, epiyangambin and cubebin, respectively. The growth profile of larvae further showed that lignans are slow acting toxins, except for dillapiol and cyclolignans of the podophyllotoxin series. Antifeedant activity of the lignans was generally poor. Cubebin was the most active antifeedant with respect to the ECB with a protective concentration, $PC_{50}$, of 74 μg/g. There was a trend of increasing toxicity of lignans which generally parallelled the evolutionary advancement in biosynthesis.

Because of their role in reversing insecticide resistance, lignans were tested for their activity on multidrug resistant (MDR) cells with overexpression of the membrane P-glycoprotein. MDR cells responded to the group of cyclolignans in a way similar to the response of other drugs

which belong to the multiresistant group, while they showed important collateral sensitivity to the furans and even more to the monolignan dillapiol. An increased uptake of the anticancer drug vinblastine was observed upon co-administration of dillapiol. This activity constitutes a novel mode of action of this lignan, and possibly other insecticide synergists such as piperonyl butoxide, with regards to resistance to drugs and insecticides.

# RÉSUMÉ

Les espèces tropicales américaines de *Piper* ont une activité insecticide comparable à celle des espèces africaines et asiatiques déjà étudiées. Les activités des extraits éthanoliques de *Piper* sur la pyrale du maïs, *Ostrinia nubilalis* Hübner, comprennent une réduction de la croissance larvaire, une grande incidence de mortalité chez les larves, les chrysalides et les adultes, et un effet restreint sur la consommation de nourriture et la digestion. Parmi les 16 extraits de *Piper*, les espèces américaines les plus actives sont, en ordre décroissant: *P. tuberculatum, P. aduncum, P. pseudo-lindenii, P. lanceiaifolium, P. guanacastensis, P. decurrens, P. carilloanum* and *P. auritum*. L'isolation de composés actifs guidée par les tests biologiques a montré l'importance, dans l'activité de la plante, du dillapiol chez *P. aduncum,* de deux néolignans chez *P. decurrens,* et de la piperlonguminine chez *P. tuberculatum*. La présence de deux néolignans décrits pour la première fois chez les *Piperaceae,* et d'une amide décrite pour la première fois chez *P.*

*tuberculatum*, souligne l'importance biologique de ces deux classes de composés dans la défense chimique des *Piperaceae*.

Nous avons étudié les activités d'une série de 15 lignans, néolignans et monolignans purs sur la pyrale du maïs, un Lépidoptère polyphage qui présente de hautes capacités d'adaptation aux composés allélochimiques des plantes. Les lignans, à la dose de 100 $\mu$g/ml dans la nourriture, à part le monolignan dillapiol, ne sont généralement pas hautement toxiques. Cependant, avec le dillapiol, l'épiashantine, la podophyllotoxine, l'épiyangambine et la cubébine, on observe une toxicité reportée sur les stades ultérieurs, comme le montre la mortalité apparaissant chez les chrysalides et culminant chez les adultes respectivement . Le profil de croissance des larves a montré en outre que les lignans sont des toxines à action lente, sauf pour les cyclolignans et le dillapiol. L'activité antiappétante des lignans est généralement faible. La cubébine est le meilleur antiappétant avec un $PC_{50}$ (concentration qui protège à 50%) de 74 $\mu$g/g. Le degré de toxicité d'un lignan correspond de manière générale à sa position dans la séquence de sa biosynthèse au cours de l'évolution.

En ce qui concerne l'activité des lignans sur les cellules multirésistantes aux médicaments avec hyper-expression de la P-glycoprotéine membranaire, on peut distinguer deux groupes. Face au groupe des cyclolignans, la réponse des cellules multirésistantes est semblable à celle

des autres anticancéreux qui font partie du groupe des drogues multirésistantes. Face au groupe des furolignans, et plus encore face au monolignan dillapiol, les cellules multirésistantes révèlent une toxicité collatérale, c'est à dire s'avèrent plus sensibles que leurs cellules parentales. L'accumulation par la cellule multirésistante du composé anticancéreux, la vinblastine, est augmentée en présence de dillapiol. Ceci représente un mode d'action nouveau de ce monolignan, et peut être d'autres synergistes d'insecticides comme le pipéronyl butoxide, pour lutter contre la résistance aux drogues et aux insecticides.

## REMERCIEMENTS

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## 1.0 INTRODUCTION

The biological role of secondary compounds in plants has long remained obscure. It was only with Fraenkel's contribution (1959) on the feeding behaviour of insect herbivores, later followed by Ehrlich and Raven's proposal (1965) on the importance of plant chemistry in the plant-animal coevolution processes, that their defensive role was realized. Alkaloids, amides, non-protein amino acids, flavonoids and terpenoids have attracted most of the attention with regard to insects, because they are either potent deterrents and insecticides, or play an important role as attractants (Harborne, 1989). This has often allowed integration of these compounds in the management of insect pests. Phenolics, found in all plants, are also known to play a role in plant-insect interactions.

Lignans, are a less investigated family of phenolic plant metabolites. They are formed by 2 phenylpropane units joined by the central carbon of the propane chain and are often present in large quantities in plant tissues (Pelter, 1986). Lignans have long been used as folk remedies (Kelly and Harwell, 1954), and some of their derivatives are used in current cancer therapy (MacRae et al., 1989). A review of the biological activities of lignans can be found in MacRae and Towers (1984) and Ayres and Loike (1990). However, little is known of their role in plants in relation to herbivores, except that sesamin-type lignans can synergize the natural insecticide pyrethrin (Tomar *et al.*, 1987), myristicin synergizes the activity of coumarins (Berenbaum and Neal, 1985) with which it may co-occur.

Large numbers of lignan structures are present in the pepper family, *Piperaceae* (Jensen *et al.*, 1993), a family of vines and shrubs growing mainly in the tropics. This family of 2000 species has a long history of medicinal and pesticidal use in Asia (Atal *et al.*, 1975) and Africa (Gbewonyo *et al.*, 1993). However, species from the neotropics have attracted little scientific attention in spite of an equally long utilization in local pharmacopoeia (Schultes and Raffauf, 1990). In particular, the role of lignans in the defense arsenal of American pepper species against insect herbivores is almost unknown.

The capacity of several lignans to inhibit the detoxification enzymes in insects has been related to their synergism with insecticides (Ahmad *et al.*, 1986). Other modes of action of lignans towards mammals are known (Ayres and Loike, 1990), which may suggest new avenues for evaluating the effects of these compounds on insects. This is relevant because selected physiological systems in insects and higher organisms, such as mammals, present similarities. For instance, to detoxify xenobiotics encountered in their plant food, herbivores utilize a pool of enzymes among which the polysubstrate monooxygenases (PSMOs) and glutathione transferase (GSH) appear to perform comparable chemical reactions in insects and mammals (Wilkinson *et al.*, 1984; Hodgson, 1985). Moreover, resistance to insecticides or to xenobiotics is observed in insect populations under insecticide or xenobiotic pressure, as resistance to drugs occurs in tumour cells subjected to repeated chemotherapy. Lignans are useful modifiers of insect resistance. Podophyllotoxin is a potent anti-tumour agent and one of its derivatives, etoposide, is often included in the therapy of recurrent ovarian cancers (Ayres and Loike, 1990). However the effect of other lignans on drug resistance is uninvestigated.

The present thesis is designed to study lignans at several levels. It first investigates their role in the bioactivity of extracts of neotropical *Piperaceae* (Chapter 3). These largely uninvestigated *species* were a good source of lignans for the study. It secondly determines the bioactivities towards an insect herbivore of the isolated pure compounds and other related structures from our collections of pure lignans (Chapter

4). It finally explores a novel alternative mode of action of lignans to their well known synergistic activity to insecticides (Chapter 5).

# 1.1 THE PIPERACEAE

## 1.1.1 Traditional uses of the Piperaceae.

Several ethnomedical, ethnopharmacological and cultural studies of the traditional use of plants have demonstrated the potential of the *Piperaceae* as medicines and against human pests (Dragendorff, 1898; Perrot and Paris, 1971; Atal *et al.*, 1975; Jacobson, 1990; Schultes and Raffauf, 1990). The pepper family, *Piperaceae*, includes over 2000 species in four genera and is a very extensive tropical family of small trees, shrubs and more or less woody pantropical vines with conspicuous stem nodes. The family is economically important as a source of spices and stimulants. These include the black pepper, *Piper nigrum*, collected and now cultivated throughout the tropics. It also includes *P. betle*, a source of aromatic essences in Southeast Asia, the leaves of which are wrapped around the masticatory stimulant betel nut, *Areca catechu*. *P. cubeba*, cubeb, has long been used as a medicine in India and as a fragrance. Kawa, *P. methysticum*, is a source of psychoactive drugs and a somnifacient in the Pacific islands. Because of their fragrance, many species enter into the folk-medicine of the tropics worldwide, including Central and Northern South America, or are used as pest control agents (Table 1.1 at the end of section 1.1).

The pleasant fragrance of several species can be attributed mainly to monolignans such as safrol, eugenol, asarone, apiol, dillapiol, or also menthol (Table 1.2 at the end of section 1.1). This may have contributed to their selection as pharmaceuticals by traditional healers.

The three American genera of *Piperaceae* native to the neotropics are represented in the pharmacopoeia of the area. For example, in Colombia, yerba Maria (Colombia), *Lepianthes peltatum* (formerly called *Poptomorphe peltata*), is claimed to induce abortion three days after the ingestion of a decoction of the leaves (Schultes and Raffauf, 1990). *Lepianthes umbellatum* (formerly *Potomorphe umbellata*), is added to curare in Ecuador to prepare an arrow poison, and the same use is reported from *Piper caudatum*, in the Rio Negro area of Brazil (Table 1.1).

Numerous ailments of the digestive tract are traditionally cured with plants of the *Piperaceae*. Extracts are used to alleviate toothache and prevent decay (*Lepianthes umbellatum* in Ecuador, *Piper daguanum and P. conejoense* in the Amazon), and to treat stomach poisoning (*P. arboreum, P. macerispicum, Peperomia putumayensis*, for instance). A number of *Piperaceae* are used as strong diuretics (*Lepianthes umbellatum, Peperomia macrostachya, Piper puberulibracteum* and several other *Piper spp.* from Table 1.1)(Schultes and Raffauf, 1990). These activities often appear to be related to anaesthetic or psychoactive effects. For instance, the aromatic leaves of *Peperomia emarginella, P. serpens*, or also *Piper nervum* and *P. serpens* relieve the irritant sting of the conga ant common in these tropical areas. The crushed leaves of *Peperomia victoriana*, applied on the forehead relieve headaches, those of *Piper iteritum* are used as a substitute for tobacco in Peru (Table 1.1), and several others, sometimes non-identified *Piper spp.*, prescribed only by a shaman, are used in traditional ceremonies (Schultes and Raffauf, 1990).

In Central America a fish poison has been made from extracts of *P. tuberculatum* and *P. hispidum* (Table 1.1). Although none of the species from the Amazonian investigated by Schultes and Raffauf (1990) were reported to be highly toxic to humans, the bark and the root, alone or in combination with other plants, were used as curare on arrows (*Lepianthes umbellatum, Peperomia serpens, Piper caudatum, P. dumosum, P. hispidum*). Other examples of the activities of the *Piper species* from the American Tropics can be found in Table 1.1.

### 1.1.2 *Piper* species and insect control.

The use of *Piper* species to protect humans, animals and crops from pests has been best recognized in Asia and Africa. For instance, *P. guineense* and *P. nigrum* have long been used as insecticides and molluscicides in several parts of Africa (Su and Horwat, 1981; Ivbijaro and Bolaji, 1990). The Indian species *P. longum, P. betle, P. peepuloides* and *P. cubeba* have demonstrated insecticidal activity against mosquitos and flies (Srivastava, 1970; Miyakado *et al.*, 1983; 1989), and were shown to repel several grain pests. For instance, cubeb oil, from *P. cubeba,* repelled *Blattella germanica* adults and killed 100% of the mosquito larvae exposed to it (Kokate *et al.*, 1980). The leaves of *P. futokatsura* from Taiwan and Japan are known as a feeding deterrent to the larvae of *Spodoptera litura* (Matsui and Munakata, 1975). Jacobson (1990) estimates a "satisfactory insect repellent" activity of 8 species of *Piperaceae* out of the 11 reported in the literature. Examples from the neotropics include the traditional use in Haiti of *P. aduncum* to repel ants (Table 1.3 at the end of section 1.1).

Marquis (1991) in La Selva, Costa Rica, observed a limited number of insects feeding on *Piper species*, and could not correlate it with leaf characteristics, soil, or other environmental factors. Some of the *Piper species* most avoided by weevils and geometrids, *P. aduncum, P. aequale, P.*

*darienense, P. decurrens* and *P. reticulatum*, are traditionally used locally or in surrounding countries against pests or in the pharmacopoeia (Table 1.1). Their individual chemistry appears to be an essential factor to investigate.

### 1.1.3 Phytochemistry of the genus *Piper*.

The activities of the *Piperaceae*, and in particular their insecticidal activity, have often been attributed to their alkaloid and amide constituents, quite frequent in this genus (Table 1.2). In fact, it has been shown that wheat and cow peas can be protected from *Sitophilus oryzae* and *Tribolium confusum*, respectively in several African countries, by coating the grains with 3000 ppm of two amides isolated from the fruits of *P. guineense* (Su and Horwat, 1981). Piperine and several co-occurring amides from *P. nigrum*, the world distributed black pepper, were more toxic than pyrethrin to *Musca domestica* (Miyakado *et al.*, 1983; 1989). A combination of amides from *P. guineense* were toxic to fruit flies, azduki bean weevil, cockroaches and several other insect species (Gbewonyo *et al.*, 1993). Olefinic and alkyl isobutylamide, such as piperine, piperettine, trichostachine, peepuloidin, piplartin, trichonine are most frequently encountered in this genus (Atal *et al.*, 1975; Greger, 1988; Tables 1.2 and 1.3). From their type of insecticidal activity, it was postulated that they may be neurotoxins (Gbewonyo *et al.*, 1993).

However, several of the active species, for example *Peperonia macrostachya*, a febrifuge, stimulant and diuretic, *Piper aduncum*, a styptic, or *P. umbellatum*, used as an antimalarial, antipyretic and hallucinogenic, do not contain alkaloids or amides (Tables 1.1, 1.2, and 1.4, at the end of section 1.1).

Phenylpropanoids are group of potentially active chemicals of common occurrence in the *Piper spp.*. They include monolignols such as apiol, dillapiol, myristicin, eugenol or safrol, and dimers of phenylpropanoids such as the lignans sesamin, cubebin, yangambin or diaeudesmin (Table 1.2 and 1.4). They often constitute a large part of the essential oil of the plant, as for instance in *P. aduncum*, where dillapiol constitutes up to 80% of the oil (Nair and Burke, 1990), or in *P. auritum*, where safrol is present in similar quantities (Nair *et al.*, 1989)(Table 1.4). They do not appear to be highly toxic to insects, with a few exceptions such as safrol, which has long been used as a natural insecticide (Coats, 1994). Several of these compounds are known to synergize natural and synthetic insecticides. Sesamin and sesamolin, for instance, synergize the activity of natural and synthetic pyrethrins (Singh *et al.*, 1976), and their synthetic analogue, piperonyl butoxide, is currently included in most commercial preparations of pyrethrin

(Worthing and Walker, 1993). Dillapiol, closely related to piperonyl butoxide, synergizes several carbamates and organochlorates (Mukerjee *et al.*, 1979; Parmar & Tomar, 1983). In addition, lignans and their monomers can repel, or are antifeedant to, several grain pests (Harmatha and Nawrot, 1988; Pelter, 1986)(Table 1.3). Cubebin and hinokinin are among the most active and are also quite frequent in the *Piperaceae* (Table 1.2). Kobusin and dillapiol can reduce the growth of larvae of insect pests (MacRae and Towers, 1984). In particular, dillapiol was found to inhibit growth of the European corn borer, *Ostrinia nubilalis* (Bernard *et al.*, 1990).

### 1.1.4 *Piper spp.* from Costa Rica used in the present study

Whereas in tropical areas of Africa and Asia, the use of *Piperaceae* to control pests has been extensive, such studies, in the American tropics, appear to be restricted to local areas, and the potential of *Piper spp.* remains largely uninvestigated. The abundance of *Piper spp.* available from tropical forest reserves in Costa Rica allowed us to collect 14 species of *Piper* and further to investigate the activity of neotropical species towards insects (Bernard *et al.*, 1995) as well as analyze some of them phytochemically (Chauret *et al.*, submitted).

Four of the collected species, *P. aduncum, P. aequale, P. auritum* and *P. marginatum*, are used in the traditional medicine of the Amazonian plateau (Table 1.1). *P. tuberculatum* and *P. aduncum* are also reported to be insecticidal (Siever *et al.*, 1949; Schultes and Raffauf, 1990).

The chemical constituents of the *Piperaceae* collected (Table 1.4) are representative of the general chemical profile of *Piper spp.* from other parts of the world (Table 1.2), with a high incidence of lignans and amides. Monolignans, in particular, may constitute a large part of the essential oil from the leaves, as, for instance, up to 90% dillapiol in leaf samples of *P. aduncum* from Panama (Gupta *et al.*, 1983), or 85% safrol in *P. auritum* from El Salvador (Castro and Poveda, 1983). No chemical investigation has been reported from nine of the *Piper spp.* collected. However, their fragrance was characteristic of *Piper spp.* with monolignols or menthol, a possible indicator of their chemical constituents. *P. betle* and *P. guineense*, used against insects in India and Africa, respectively, both contain large quantities of lignans and amides (Table 1.4). They were assayed with the insects of the present study for comparative purposes.

# TABLES ABOUT THE *PIPERACEAE*

TABLE 1.1: ETHNOBOTANICAL USE OF PIPER spp. IN THE NEOTROPICS

| PIPER SPP. | LOCATION | TRADITIONAL USE | REFERENCE # |
|---|---|---|---|
| P. aduncum | N. Amazonia | dried leaf: excellent styptic | 1, 2 |
| P. aequale | N. Amazonia | leaf: liver complaints, carminative | 2 |
| P. allenii | Panama | pains from snake bite | 3 |
| P. amazonica | N. Amazonia | leaf: febrifuge, purgative, as poultice on abdomen in children | 2 |
| P. amalago | Mexico, Jamaica | root, leaf, bark: anti-inflammatory, gastrointestinal, chest pains, fever, oedema | 4 |
| P. angustifolium | Panama | leaf: astringent, helps healing, against vaginal trichomonas, stomach ailments | 5 |
| P. arboreum | Fr. Guiana, | leaf tea: stimulant, for stomach poisoning | 2 |
| P. augustum | N. Amazon | whole plant: prevents tooth decay, carminative | 2 |

| | | | |
|---|---|---|---|
| *P. auritum* | El Salvador, Panama | leaf, root:<br>feeding attractant for fish, emmenagogue, to remove ticks | 3 |
| *P. callosum* | Brazil, Bolivia | leaf tea:<br>tooth, stomach akes | 6 |
| *P. carpunya* | Peru | leaf tea:<br>carminative, against pneumonia | 7 |
| *P. caudatum* | Brazil | fruits:<br>carminative, also in preparation of curare | 2 |
| *P. conejoense* | N. Amazonia | whole plant:<br>blacken teeth to prevent deacay | 2 |
| *P. crassinervium* | Nov. Gen. | leaf:<br>alleviates pain from stings of the conga ant | 2 |
| *P. cubeba* | West Indies | fruit:<br>bronchitis, urinary antiseptic | 8 |
| *P. dactylostigma* | Brazil, Amazonia | leaf, stem:<br>chewed to numb tongue | 3 |
| *P. daguanum* | Amazonia | root:<br>toothhache | 2 |
| *P. divortans* | N. Amazonia | leaf:<br>colics | 2 |
| *P. dilatatum* | Argentina | leaf:<br>abortifacient | 9 |
| *P. dumosum* | Amazon | ingredient in arrow poison, stimulant, abundant in fallows | 2 |

| | | | |
|---|---|---|---|
| *P. el-metanum* | N. Amazonia | leaf:<br>toothache | 2 |
| *P. elongatum* | Peru | leaf decoction:<br>cicatrizes wounds, ulcers | 7 |
| *P. erythroxyloides* | Colombia,<br>Venezuela | whole aerials:<br>aphrodisiac, analgesic | 3 |
| *P. fadyenii* | Jamaica | insecticidal | 10 |
| *P. falculispicum* | N. Amazonia | leaf poultice:<br>sting of ants and other insects | 2 |
| *P. futuri* | N. Amazonia | leaf tea:<br>sick stomach | 2 |
| *P. geniculatum* | Mexico | emmenagogue | 9 |
| *P. guianense* | Guiana | leaf infusion:<br>small amounts as appetite stimulant for infants | 2, 3 |
| *P. hispidum* | Brazil,<br>Ecuador, Peru,<br>Mexico | leaf, fruit:<br>to remove head lice, against tooth decay, to blacken teeth, for tonsilitis pains, as diuretic.<br>also mixed with other plants to prepare curare, to treat malaria:<br>mixed with leaves of *Phyllanthus*: fish poison | 2, 3 |
| *P. hostmannianum* | N. Amazonia | leaf poultice:<br>warts | 2 |

| | | | |
|---|---|---|---|
| P. interitum | Peru | dry leaf and root:<br>aphrodisiac, used as substitute of tobacco snuff | 2, 3 |
| P. jamaicense | Jamaica | leaf decoction:<br>stomach pains | 8 |
| P. lenticellosum | Colombia | stimulant | 11 |
| P. macerispicum | N. Amazonia | leaf decoction:<br>abdominal cramps, stomach pains | 2 |
| P. marginatum | Brazil, Costa Rica, Puerto Rico | leaf, stem:<br>anti-cercarial, -bacterial, prevents tooth decay, | 12 |
| P. metanum | N. Amazonia | leaf:<br>rheumatic pains | 2 |
| P. nigrum | Guatemala, entire Tropics | fruit, essential oil:<br>insecticide, ascariscide, antiviral, -bacterial, antistrychnine poisoning, barbiturate potentiation... | 13 |
| P. nigridonum | Jamaica | twig decoction:<br>fever, cold, stomachache | 8 |
| P. obliquum | Peru, Chili, Ecuador | Leaf:<br>odontological purposes | 2 |
| P. ombumbratum | S-W. Amazonia | leaf:<br>toothache | 2 |
| P. papillicaule | N. Amazonia | leaf:<br>antirheumatic | 2 |
| P. parthenium | Brazil | emmenagogue, various uterine troubles | 6 |

|  |  |  |  |
|---|---|---|---|
| P. peltatum | Haiti | stomachache, | 14 |
| P. phytolaccaefolium | Amazonia | sap:<br>a few drops to treat blindness;<br>stem:<br>chewed to traaet sore throat, to blacken teeth, to prevent dental caries | 2 |
| P. puberulibracteum | N. Amazonia | leaf decoction:<br>diuretic | 2 |
| P. rotundistipulum | Amazonia | dried leaf:<br>insect repellent in clothing, general insecticide, fish poison | 2, 3 |
| P. sacchamatesense | N. Amazonia | leaf poultice:<br>against burns | 2 |
| P. sanctum | Mexico | diarrhea, abortifacient | 9 |
| P. schultesii | Colombia | hot tea of leaf and stem:<br>coughs, tuberculosis, strong diuretic | 2 |
| P. serpens | Amazonia | leaf and stem poultice:<br>stigs of conga ants | 2 |
| P.soledadense | Peru | leaf and stem:<br>sores of the mouth | 2 |
| P. tingens | Peru | entire plant:<br>against tooth decay | 3 |
| P. trigonum | N. Amazonia | leaf tea:<br>diuretic, prostate | 2 |

| | | | |
|---|---|---|---|
| *P. tuberculatum* | Brazil | root, leaf, bark:<br>moluscicidal, insecticide, minor fish poison | 2, 15 |
| *P. umbellatum* | Mexico, Brazil | leaf, root:<br>malaria, diarrhea, liver diseases, laxative, insecticidal, antiepileptic | 16 |
| *P. spp. unidentified* | N-W. Amazonia | witchcraft for mental blindness and to obtain clear thinking, psycho-stimulant, to strengthen men... | 2 |

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# :REFERENCES FOR TABLE 1.1:
1: Stimson, 1971; 2: Schultes and Raffauf, 1990; 3: Schultes, 1975; 4: Dominguez *et al*., 1986; 5: Gupta *et al*., 1979; 6: Van den Berg, 1984; 7: Ramirez *et al*., 1988; 8: Ayensu, 1981; 9: Martinez, 1984; Martinez-Crovetto, 1981; 10: Nair *et al*., 1986; 11: De Smet and Rivier, 1985; 12: Frischkorn *et al*., 1978; 13: Miyakado *et al*., 1980; 1983; Su and Horvat, 1981; Kiuchi *et al*., 1988; 14: Weniger *et al*., 1986; 15: Siever *et al*., 1949; 16: Chatrol, 1964; Lewis and Elvin-Lewis, 1984.

TABLE 1.2: EXAMPLES OF CHEMICAL CONSTITUENTS FOUND IN THE PIPERACEAE

| PIPER species | PLANT PART | LIGNANS | AMIDES | REF |
|---|---|---|---|---|
| P. album | fruit | - | piperine | 1 |
| P. amalago<br>Mexico<br>Jamaica | root<br>leaf | - | piperine-like | 2 |
| P. aborescens | | | - | 3 |
| P. arboricola<br>India | whole plant | 3,4-dimethoxy phenylpropionic acid | piperine | 4 |
| P. argyrophyllum<br>India | whole | - | piperine, dienamide | 5 |
| P. aurantiacum<br>India | seed | - | auranamide, aurantamide | 6 |
| P. attenuatum | | galbelgin | piperolactam D | 7 |
| P. banksii<br>Australia | stem | dillapiol, elemicin | n-isobutyl dienamide | 8 |
| P. brachystachyum<br>India | leaf | apiol, asarinin, fargesin, pluviatilol, sesamin | n-isobutyl brachystamide A & B, sylvetin, guineensin, pipercide retrofractamide A | 9 |
| P. boehmerifolium | whole | - | piperolactam D | 10 |

|  |  |  |  |  |
|---|---|---|---|---|
| P. callosum<br>Bolivia<br>Brazil | root<br>leaf | safrol | n-isobutyl dienamides, pipercallosinine pipercallosidin, piperovatine | 11 |
| P. capense |  | schmiditin, isodihydrofutokinol | - | 12 |
| P. cavalcantei<br>Brazil | essential oil | eugenol, safrol | - | 13 |
| P. clarkii |  | clarkinol | - | 14 |
| P. clusii<br>India | whole | azaronaldehyde, benzodioxoles, clusin, dihydrocubebin, cubebin, hinokinin, deoxypodorhizon, yatein, sesamin | piperine | 15 |
| P. cubeba<br>India<br>Thailand<br>Malaysia<br>Zaire | fruit | cubebin & related cpds, ashantin, hinokinin, ariensin, butyrolactone, clusin, yatein, isoyatein, monosalin, sesamin, thujaplicatin | piperine, piperidine | 16 |
| P. demeraranum<br>Trinitad | earial parts | - | amides non specified | 17 |
| P. fadyenii<br>Jamaica | aerial parts | methylenedioxy-lignans | fadyenolide | 18 |
| P. farnechoni |  | - | piperine | 19 |

| | | | | |
|---|---|---|---|---|
| P. futokatsura<br>Japan<br>Taiwan<br>China | leaf<br>stem | asarone, futoenone,<br>futoquinol & derivatives,<br>futoxine, kadsurenone,<br>kadsurin a & b, piperenone | futoamide | 20 |
| P. guayranum<br>Venezuela<br>Trinitad | aerial parts | - | tembamide acetate,<br>alatamide | 21 |
| P. hancei<br>China | whole plant | bicyclolignans,<br>burchellin, denudatin,<br>hancinone, kadsurenone | iso-butyl dienamides,<br>futoamide,<br>piperlonguminine,<br>isobutylpyrazine | 22 |
| P. hookeri | | safrol | - | 23 |
| P. interuptum | | eupomantene | - | 24 |
| P. jaborandi | | - | jaborandine | 19 |
| P. lacunosum | leaf | cubebin | - | 25 |
| P. lenticellosum<br>Colombia | leaf | elemicin, methyl-eugenol,<br>isosafrol, sarisan | - | 26 |
| P. lowong | | sesamine | - | 27 |
| P. longum<br>India<br>Thailand<br>China<br>Japan | fruit<br>root<br>seed | asarinin,<br>trimethoxycinnamate,<br>eudesmin, diaeudesmin,<br>fargesin, pluviatol,<br>pluviatilol, sesamin | n-isobutyl dienamide,<br>eicosa-trienamide,<br>piperine, pipercide,<br>piperlongine,<br>piperlongumine,<br>piperlonguminine,<br>piplartine, sylvatine,<br>piperadione, guineensine | 28<br>29 |

| | | | | |
|---|---|---|---|---|
| P. methysticum<br>Hawai<br>Fiji<br>India | leaf<br>root<br>fruit | - | pipermethystine, cepharadione | 30 |
| P. nepalense<br>India | wood | trimethoxy-allyl benzenes | dienamides, piperine, piperlonguminine | 31 |
| P. nigrum<br>India<br>Japan<br>Africa<br>C-America<br>Indonesia | fruit<br>essential oil | caffeic & coumaric acid derivatives, cubebin, eugenol, methyl-, safrol, myristicin, dillapiol | n-isobutyl dienamides, feruperine, guineensine, pellitorine, piperamide, piperanine, pipercide, piperettine, piperine, piperidine, piperoleine, pipernonaline, piperundecalidine | 32 |
| P. novae-hollandiae<br>Australia | wood | dillapiol, isodillapiol | chavicine, n-isobutyl-dienamides, fagaramide, piperidine, piperine & derivatives, piperlonguminine | 33 |
| P. officinarum | | - | chavicine, piperine, (MDP)-isobutyl-trienamide | 34 |
| P. ovatum | | - | piperovatine | 19 |
| P. peepuloides<br>India | fruit<br>leaf | sesamin, diasesamin, diaeudesmin | n-isobutyl- dienamide, peepuloidin, pipatalin, piperidine, pyrrolidine, piperine & derivatives | 35<br>36 |
| P. polysyphorum | | wallichine, grandisin, polysyphorin, virolongin A | - | 37 |

|  |  |  |  |  |
|---|---|---|---|---|
| P. retrofractum<br>Thailand<br>India<br>S-Korea | fruit<br>stem | sesamin | n-isobutyl- trienamide, filfiline, piperine, retrofractamine A & C, piplartine, piperidine, pipereicosalidine, piperoctadecalidine | 38 |
| P. ribesioides<br>Thailand | aerial part | cubebin, hinokinin, eupomatenoid-7 | n-isobutyl dienamides | 39 |
| P. sanctum<br>Mexico | root<br>stem | cinnamylidenbutenolide, methysticin | piperolide, epoxypiperolide, cepharadione A & B | 40 |
| P. sarmentosum<br>Thailand<br>China | fruit<br>leafs | asarone, asaronaldehyde | pellitorine, sarmentine, sarmentosine, piperine | 41 |
| P. schmitii | whole | isodihydro-futokinol A & B, futokinol, malichin G, zuionin A, calopiptin, kadsurin, schmiditin | - | 42 |
| P. sumatranum |  | asarinin, monolignol | - | 43 |
| P. sylvaticum<br>India | seed<br>root | eudesmin, sesamin, sylvatesmin, sylvone | n-isobutyl dienamide, piperine, piperlonguminine, sylvamide, sylvatine | 44 |
| P. thomsonii |  | galbelgin | - | 45 |
| P. trichostachyon<br>India | stem<br>fruit | cubebin, dihydrocubebin, hinokinin, trichostin & derivatives | cyclopiperstachin cyclostachin a & b, piperstachin, tricholeine, trichonine | 46 |
| P. umbellatum<br>Brazil<br>Mexico | leaf | dillapiol, cinnamic acid derivatives | - | 47 |

|  |  |  |  |
|---|---|---|---|
| P. wallichii | galgravin, kadsurenon,<br>denudatin B, wallichinine,<br>hancinon | - | 48 |

#: REFERENCES FOR TABLE 1.2: 1 : Glasl et al., 1976; 2: Durand et al., 1962; Dominguez et al., 1986; 3: Duh et al., 1990; 4: Ho et al., 1981; 5: Banerji and Nandi, 1988; 6: Banerji and Das, 1975; 7: Desai et al., 1990; Sumathykutty and Rao, 1991; 8: Loder and Nearn, 1972; 9: Singh and Atal, 1969; Koul et al., 1988b; Dutta and Banerjee, 1976; Banerji and Das, 1989; 10: Desai et al., 1990; 11: Pring, 1982; Gottlieb et al., 1981; Maia et al., 1987; 12: Green et al., 1991; Green and Wiemer, 1991; 13: Alencar et al., 1974; 14: Jensen et al., 1993; 15: Koul et al., 1983; 1984; 16: Hadorn and Jungkunz, 1951; Prabhu and Mulchandani, 1985; Badheka et al., 1986; 1987; 17: Maxwell and Rampersad, 1989b; 18: Nair et al., 1986; 19: Glasby, 1991; 20: Takahashi et al., 1969; Takahashi and Ogiso, 1970; Matsui and Munakata, 1976; Shen et al., 1985; 21: Maxwell and Rampersad, 1989a; 22: Han et al., 1986; Qiu et al., 1986; Li et al., 1987; 23: Singh et al., 1969; 24: Thebpatiphat et al., 1988; 25: Vanegas et al., 1984; 26: Diaz and Dorado, 1986; 27: Peinemann, 1896; 28: Atal et al., 1966; 29: Chatterjee and Dutta, 1967; Dutta et al., 1975; Dutta and Banerjee, 1976; Tabuneng et al., 1983; Desai et al., 1988; 30: Smith, 1979; 1983; Jaggy and Achenbach, 1992; 31: Gupta et al., 1972; 32: Russel and Jenning, 1969; Traxler, 1971; Raina et al., 1976; Natakani and Inatani, 1981; Schultz and Herrman, 1980; Lin et al., 1981Semler and Gross, 1988; 33: Loder et al., 1969; Falkiner et al., 1972; 34: Gupta et al. 1976a; 1976b; 1977; 35: Atal et al., 1967; 36: Dhar and Raina, 1973; Gupta et al., 1978; Shah et al., 1986; 37: Ma et al., 1991; 38: Mishra and Tewari, 1964; Banerji et al., 1985; 39: Kiijoa et al., 1988; 1989; 40:Hansel and Pelter, 1971; Pelter and Hansel, 1972; Hansel et al., 1973; 41: Likhitwitayawuid et al., 1987; 1988; 42: Koul et al., 1988b; Joshi et al., 1990; 43: Tyagi et al., 1993; 44: Banerji and Ghost, 1973; Banerji and Dhara, 1974; Banerji et al., 1974; Banerji and Pal, 1982; Banerji et al., 1984; 45: Malhotra et al., 1990; 46: Singh et al., 1971; Joshi et al., 1975; Singh et al., 1976a; Koul et al., 1988a; 47: Chatrol, 1964; 48: Han et al., 1986.

## TABLE 1.3: THE USE OF PIPERACEAE AGAINST INSECTS

| TYPE OF ACTIVITY | PIPER SPECIES | PLANT PART / MODE of UTILISATION | INSECT SPECIES AFFECTED | REF # |
|---|---|---|---|---|
| repellent | *Piper. aduncum | leaves | ants | 1 |
| avoided | *P. auritum | leaves | leaf cutting ant | 2 |
| insecticidal | | juice of crushed leaves | remove ticks | 5 |
| weak insecticide | P. capense | dry leaves powder | Rhipicephalus appendiculatus | 3 |
| repellent | P. cubeba | oil | Blatella germanica | 1 |
| repellent | | oil | mosquito larvae | 1 |
| insecticide | P. fadyenii | oil | | 16 |
| deterrent | P. futokadsura | leaves benzene extract | Spodoptera litura larvae | 1, 17 |
| not insecticide | P. geniculatum | | | 4 |
| repellent | P. guineense | alkaloids fruit used to coat grains | Sitophilus oryzae | 1 |
| repellent | | — | Tribolium confusum | 1 |

| | | | | |
|---|---|---|---|---|
| knockdown | | 4 alkaloids from the roots | Schistocerca gregaria | 5 |
| insecticidal | P. hispidum | dry powdered fruit | dog louse | 6 |
| insecticidal | | crushed in water | head lice | 6 |
| repellent | P. longum | fruit oil | Blatella germanica | 1, 14 |
| repellent | P. nigrum | fruit oil | Blatella germanica | 1, 15 |
| repellent | | dry fruit powder | Acanthoscelides obtectus | 1 |
| repellent | | EtOH extract in topical application | Sitophilus oryzae | 1 |
| repellent | | " | Callobrochus maculatus | 1 |
| repellent | | containers of grains coated with pepper seeds | Callosobruchus chinensis | 1 |
| repellent | | ground sprayed on grain | Heliothis zea | 1 |
| inhibition of oviposition | | mixture of pepper seeds and yeast | Tribolium castaneum | 1, 15 |
| insecticidal | | EtOH extract topical | Anthonomus grandis adults | 1, 15 |
| repellent | | MeOH extract in diet | Bombyx mori larvae | 1 |
| repellent | | piperine fraction from fruit extract | Musca domestica | 1 |

| | | | | |
|---|---|---|---|---|
| not insecticidal | | acetone extract | Epilachna varivestis | 7 |
| insecticidal | | fruit | Acanthoscelides obtectus | 8 |
| larvicidal | | acetone extract | Culex quinquefasciatus | 7, 15 |
| strong aromatic perfume, repellent | P. oleosum | oil, for storage goods, used like Eucalyptus | insects | 9 |
| knock down effect | P. peepuloides | petroleum extract spray | Aedes aegypti | 10 |
| knock down | | petroleum extract spray | Musca domestica nebula | 10 |
| knock down | | petroleum extract | Musca domestica vicina | 10 |
| larvicidal | | petroleum extract | Aedes eagypti | 10 |
| insecticidal | P. propinquum (perhaps Ottonia spp.) | used in place of pyrethrum | spp. not mentioned | 9 |
| moderate repellent | P. retrofractum | seed oil | Blatella germanica adults | 1 |
| repellent for clothing | *P. rotundistipulum | powder of dry leaves | insects non specified | 11 |
| insecticidal | *P. tuberculatum | dry whole plant | insects non specified | 4 |
| weak repellent | *P. umbellatum | essential oil | insects non specified | 12 |

| | | | | |
|---|---|---|---|---|
| short lived repellent | _Lepianthes umbellatum (= Potomorphe umbellata)_ | essential oil | insects | 12 |
| antimalaria | *_Peperomia nigropunctata_ | - | - | 12 |
| antimalaria | *_Peperomia trifolia_ | - | - | 13 |

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* : _Piperaceae spp._ from the Neotropics.

# : REFERENCE FOR TABLE 1.3 : 1: Jacobson, 1990; 2: Maia _et al_., 1987; 3: Van Puyvelde _et al_., 1985; 4: Siever, 1949; 5: Schultes, 1975, 6:Atschul, 1973; 7: Hartzell, 1947; 8: Lathrop and Keirstead, 1946; 9: Dragendorff, 1898; 10: Srivastava, 1970; 11: Schultes and Raffauf, 1990; 12: Chatrol, 1964; 13: Ayensu, 1981; 14: Kokate _et al_., 1980; 15: Miyakado _et al_., 1980; 1983; 1989; Su and Horvat, 1981; 16: Nair _et al_., 1986; 17: Oatsui and Munakata, 1975; Matsui _et al_., 1976.

TABLE 1.4 : CHEMICAL CONSTITUENTS REPORTED FROM THE PIPERACEAE COLLECTED IN COSTA RICA FOR THE PRESENT STUDY (Chapter 3).

| PIPER SPP. | [e]LIGNANS | REF # | [e]AMIDES | REF # | [e]OTHERS | REF # |
|---|---|---|---|---|---|---|
| P. aduncum | | | | | | |
| Brazil: | dillapiol | | none detected | 2 | | |
| var. aduncum | leaf dist., 74% | 1 | | | | |
| var. cordulatum | leaf dist., 84% | 1 | | | | |
| Fiji: | dillapiol<br>leaf dist., 58%<br>after storage<br>up to 90% | 2 | | | monoterpenes,<br>leaf 4% | 2<br>4 |
| | pseudo-dillapiol,<br>myristicin,<br>neo-lignans<br>fruit, 1% | 3 | | | | |
| Panama: | leaf dist., 90% | 4 | | | flavones 1% | 3 |
| Colombia: | whole | 5 | | | | |
| Peru: | leaf,<br>benzodioxole,5-methoxy lignan | 6 | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Papua-New Guinea: | leaf, benzoic acid deriv. | 7 | | | chromenes | 7 |
| *P. auritum* | leaf, | | | | benzoic ac. and derivatives | 10 |
| El-Salvador: | safrol, 85% | 8 | aporphine-type alkaloid | 8 | | |
| Costa Rica: | dillapiol, 2%<br>elimicin,<br>eugenol,<br>myristicin | 9 | | 9 | | |
| Panama: | | | | | mono-, sesquiterpenes | 9 |
| *P. hispidum* | | | | | | |
| Peru: | leaf, dillapiol & derivatives | 3 | | | | |
| Mexico: | | | | | | |
| Jamaica: | | | | | fruit, benzoid acid | 3 |
| Brazil: | | | | | flavonoids, 0.01% | 11 |

| | | | | | | |
|---|---|---|---|---|---|---|
| P. marginatum | | | | | | |
| Brazil: | leaf, | | piperine | 12 | | |
| | anethol, 40% | 12 | | | | |
| | dillapiol,0.7% | 13 | | | | |
| | elemicin,1.3% | | | | | |
| | stem, | | | | | |
| | estragole,.09% | 13 | | | | |
| | eugenol,1% | 12 | | | | |
| | kakuol,.05% | 14 | | | | |
| | myristicin,0.2% | 13 | | | | |
| | safrol,0.5% | 14 | | | | |
| Costa Rica: | trans-anethol | 15 | | | | |
| | methylenedioxy | | | | | |
| | lignans | | | | | |
| P. tuberculatum | | | | | | |
| Brazil: | root bark, | 16 | piplartine,1% | 16 | | |
| | trimethoxy | | - dimers,.02% | | | |
| | cinnamic ac. | | | | | |
| | leaf, | 17 | | | | |
| | methylenedioxy | | | | | |
| | cinnamic ac. | | | | | |
| P. betle | | | | | | |
| India: | fruit, seed, | | fruit, | | | |
| Taiwan: | dillapiol and | 18 | guineensine, | 18 | mono-, | 18 |
| Philippines: | derivatives, | | pipercide, | 19 | sesquiterpenes | |
| | eugenol, | | brachistine, | | 5-8 %, | |
| | safrol, 0.1% | | brachyamide, | | sitosterol | |

|  |  |  |  |  |
|---|---|---|---|---|
| Fiji: | leaf, |  |  |  |
|  | chavicol,0.6% | 19 | sylvetine | 19 |
|  | chavibetol,16% |  |  |  |
|  | estragole,7% |  |  |  |
|  | catechols,0.6% |  |  |  |
| P. guineense |  |  |  |  |
| Guinea: | fruit, stem, | 20 | guineensine, | 20 |
| Ghana: | root, |  | dihydropiperlong |  |
| Congo: | safrol, |  | uminine,0.02% |  |
| = ashanti pepper | yangambin, 0.2% |  | piperine,0.5% | 21 |
|  | epiyangambin, |  | piperidine,0.02% |  |
|  | myristicin, |  | sylvatine,0.01% |  |
|  | dihydrocubebin, |  | trichostachine, |  |
|  | 0.02% |  | 0.1% |  |
|  |  |  | wisanine | 22 |
| Cameroon: | sesamin, | 21 | wiswanidine |  |
|  | eudesmin |  | ..... |  |
|  | ashantin |  |  |  |
| P. aequale |  |  |  |  |
| P. carrilloanum |  |  |  |  |
| P. decurrens |  |  |  |  |
| P. guanacastense | no chemistry |  |  |  |
| P. lanceiaifolium | reported |  |  |  |
| P. pseudo-lindenii |  |  |  |  |
| P. obliquum |  |  |  |  |
| P. schiedianum |  |  |  |  |
| P. spp. |  |  |  |  |

* : Percent of constituent in essential oil.

# : REFERENCES FOR TABLE 1.4 : 1: Gottlieb *et al*., 1981; 2: Smith and Kassim, 1979; 3: Burke and Nair, 1986; 4: Gupta *et al*., 1983; 5: Diaz *et al*., 1984; 6: Burgos-Macedo and Gibaja-Oviedo, 1987; 7: Orjala *et al*., 1989; 1993 8: Castro and Poveda, 1983; 9: Gupta *et al*., 1985; 10: Ampofo *et al*., 1987; 11: Vieira *et al*., 1980; 12: Foungbe *et al*., 1976; 13: Ramos *et al*., 1986; 14: Diaz and Gottlieb, 1979; 15: Hussain *et al*., 1988; 16: Braz-Filho *et al*., 1981; 17: Simmonds and Stevens, 1956; 18: Amonkar *et al*., 1986; Dutt, 1956; Nigam and Purohit, 1962; Sharma *et al*., 1983; 19: Ueda and Toyosaku, 1951; 20: Dwuma-Badu *et al*., 1975; 1976; Hadorn and Jungkunz, 1951; 21: Sondengam and Kimbu, 1976; Sondengam *et al*., 1977.

## 1.2. THE LIGNANS

Lignans and related phenlypropanoid monomers of plant origin have long been a source of biologically active products for pest control and traditional medicine (Casida, 1970; Mukerjee *et al.*, 1979; MacRae and Towers, 1984; MacRae *et al.*, 1989). More recently, they have been used as lead compounds for the synthesis of potent synergists of insecticides (piperonyl butoxide), or for antiviral and antineoplastic agents (etoposide). More than 400 structures of lignans have been reported up to 1988 (Ayres and Loike, 1990), with approximately 30 new ones since this date, distributed throughout the plant kingdom (Cole and Wiedhopf, 1978; MacRae and Towers, 1984). They are found in several plant parts, with the highest concentrations in resins and heartwood, or also sometimes in leaves. The tropical family of the black pepper, the *Piperaceae*, synthesizes a large diversity of structures (see section 1.1 and Jensen *et al.*, 1993).

While the chemistry of lignans, the identification of new structures, the chemical synthesis of interesting bioactive ones and more recently their biosynthesis, have given rise to a large number of publications (Hearon and MacGregor, 1955; Gottlieb, 1974; Rao, 1978; Pelter, 1986; Whiting, 1987; Massenet *et al.*, 1989; Ayres and Loike, 1990; Davin and Lewis, 1992), the question of their natural role has not been thoroughly investigated. However, the fungistatic properties of a number of neolignans (MacRae and Towers, 1984), the antifeedant activity of some lignans to grain insect pests (Harmatha and Nawrot, 1988), and their occurrence in plants as synergists of other plant toxins (Berenbaum and Neal, 1985), suggest that they play a role in the chemical defense of plants. Other biochemical and physiological activities may be related to natural plant defense. These include their role in the regulation of plant growth (Van Sumere *et al.*, 1972), or their antitumour, antiviral and antimitotic activities (MacRae *et al.*, 1989).

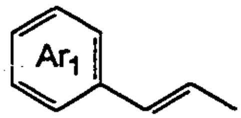
The present chapter surveys the recent findings on the biosynthetic pathway of lignans as well as the present theories on the evolution of these structures, both relevant to the discussion of the present work. It also reviews the current knowledge of the function and the biological activities of lignans, with a particular focus on insect studies.

### 1.2.1 Biosynthesis in plants

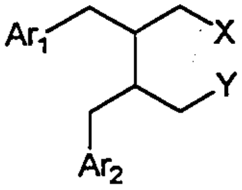
Although lignans and neolignans represent an important branchpoint pathway in phenylpropanoid metabolism, this class of compounds has been largely overlooked in terms of their specific biosynthesis, when compared to other phenylpropane of this biosynthetic pathway. Initially the term "lignan" was restricted to dimers of phenylpropanoids, i.e. $C_6C_3$ compounds linked via an 8,8′ carbon-carbon bond (Rao, 1978; Pelter, 1986). The word "neolignan" was used to designate other types of bond formation (Gottlieb, 1978) represented in Figure 1.1. This distinction is being questioned as lignan and neolignan biosynthesis require the same monolignol precursors and enzymes (Davin and Lewis,1992), and as higher oligomers as well as glucoside forms are being reported. Most lignans are found in optically active forms, a fact that is not well understood considering that the biosynthetically related lignins are not optically active. For the purpose of the present discussion, a classification separating lignans into monolignans (monomers of lignans) and lignans (dimers) including diarylbutanes, diarylbutyrolactones, furans, furofurans (furolignans), tetralins (cyclolignans) and neolignans (Fig 1.1), as described by Ayres and Loike (1990), will be adopted.

It is generally agreed that the development and elaboration of the phenylpropanoid pathway was essential for the transition

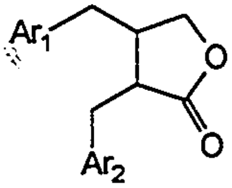
Figure 1.1 CLASSIFICATION OF LIGNAN STRUCTURES (after Ayres and Loike, 1993)



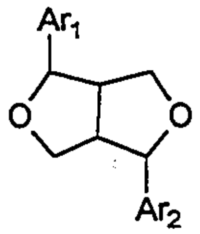
Monolignan



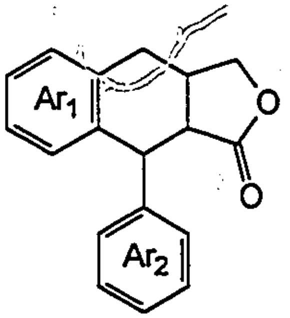
Diarylbutane



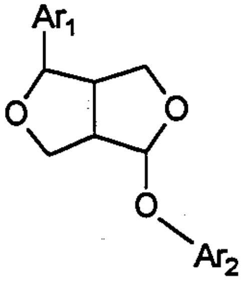
Oxodiarylbutane



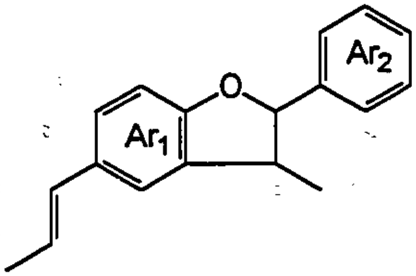
Furofuran



Cyclolignan



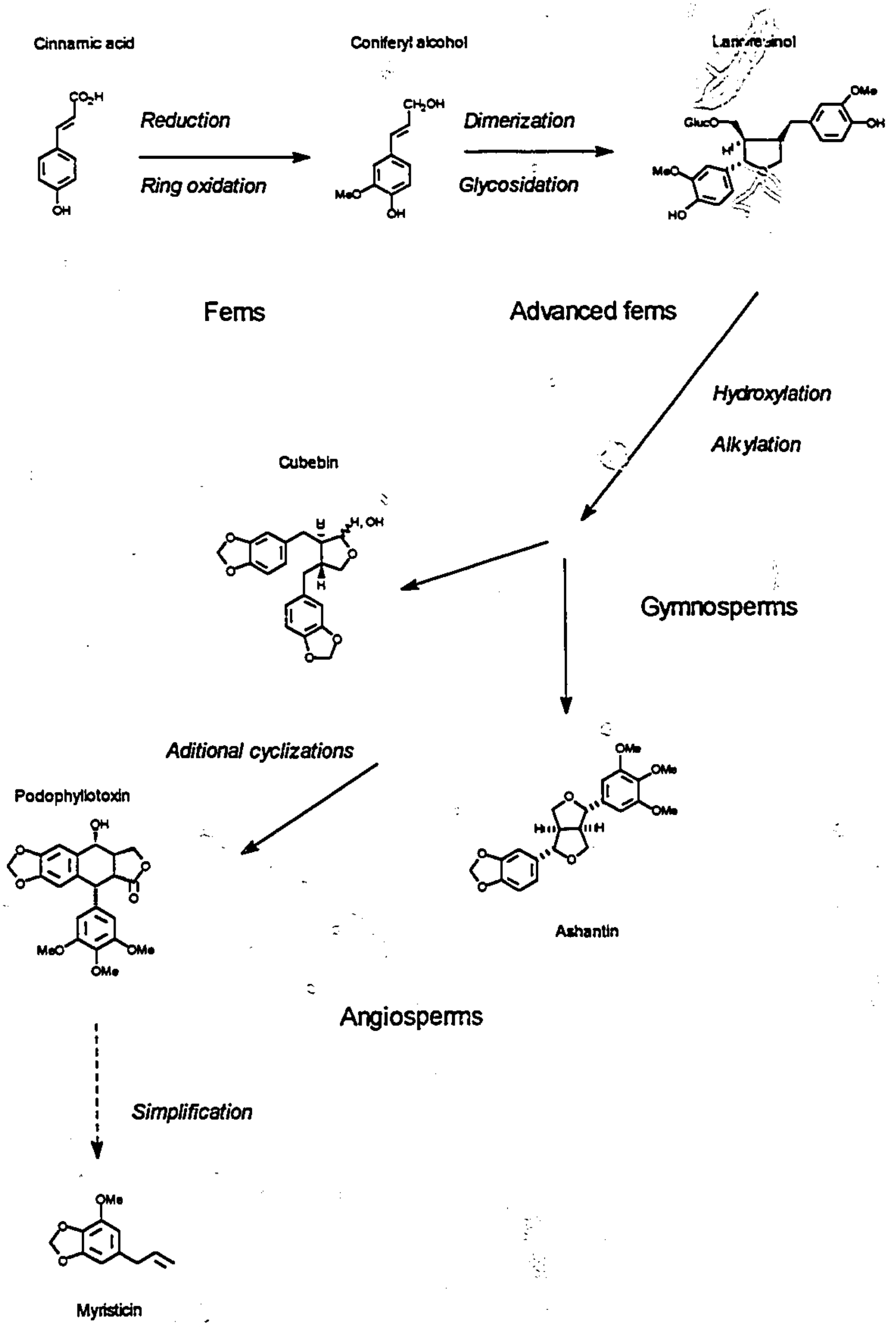
Epoxylignan



Neolignan

of aquatic plants to the terrestrial environment (Lewis and Davin, 1994). Furthermore, based on available evidence, aquatic algae, fungi, and also mosses or liverworts do not contain lignans. Lignans first appeared in the ferns (*Pteridophyta*), possibly as dimers of caffeic acids. Gradual evolution of the ferns was putatively accompanied by site-specific methylation and reduction of hydroxycinnamic acids to give the monolignols (Fig 1.2). Reduction occurred in later ferns, with dimers of the monolignol coniferyl alcohol. With the subsequent emergence of the gymnosperms, novel transformations are seen: first hydroxylation and methylation of the aromatic rings, as in most furolignans, followed by the formation of methylenedioxy bridges, and aryltetrahydronaphtalene skeletons to give plicatic acid and podophyllotoxin type lignans (Lewis and Davin, 1994). Further elaboration of the pathway and cyclization led to allylphenols derivatives such as gomisin, in *Schizandra chinensis*. According to Lewis and Davin (1994), these allylphenol derivatives represented a peak of structural complexity elaborated by the *Magnoliflorae,* which was followed by a shortening of the pathway as evolution progressed, leading to the monolignans. A series of lignan structures discussed further in this work are given in Appendix I.

Figure 1.2 EVOLUTION OF LIGNAN STRUCTURE (after Lewis and Davin, 1994)


Cinnamic acid
Reduction
Ring oxidation
Coniferyl alcohol
Dimerization
Glycosidation
Lariciresinol
Ferns
Advanced ferns
Hydroxylation
Alkylation
Cubebin
Gymnosperms
Aditional cyclizations
Podophyllotoxin
Ashantin
Angiosperms
Simplification
Myristicin

### 1.2.2 Antimitotic, antitumour activity

There has been a growing appreciation of the importance of lignans, neolignans and monolignans as biologically active compounds over the last decade. A total of at least 33 lignans are known to have antitumour activity (MacRae and Towers, 1984; MacRae *et al.*,1989; Ayres and Loike, 1990). The better investigated ones are podophyllotoxin, peltatins, and several of their derivatives (methyl, methyl-ether, and glucoside acid). These compounds act like colchicine in binding to tubulin, although podophyllin and colchicine occupy adjacent sites rather than the same one (Brewer *et al.*, 1979). Structural features such as a five-membered lactone ring, a 3,4,5-trimethoxyphenyl group, a methylenedioxy group, as well as the stereochemistry of the molecule, appear to be important for this type of activity (MacRae and Towers, 1984). The synthetic deoxypodophyllotoxin glucoside derivatives, etoposide and teniposide, have an optimized antitumour activity and are used in the clinical treatment of human cancers (Jardine, 1980). They are used clinically under the brand name of VP-16 and VM-26, respectively. They do not inhibit microtubule assembly, as do their aglycone counterparts, but rather induce breaks in single and double stranded DNA, possibly because of their interaction with topoisomerase II (Ayres and Loike, 1990). Together with lignans of the podophyllotoxin group, they inhibit nucleoside transport into mammalian cells, somehow interfering with their protein carrier (Loike and Horwitz, 1976), through a mechanism that is yet unknown. The synthesis of several derivatives of podophyllotoxin (Glinski and Durst, 1983) and etoposide, led to the 2-chloro compounds with significant activity against leukaemia P388 cells, and lower toxicity than podophyllotoxin (Glinski *et al.*, 1987).

### 1.2.3 Antiviral activity

It has been known for many years that the resin of the May apple, *Podophyllum spp.*, is effective in the treatment of papillomas (Bennet and Grist, 1985) and venereal warts (Kelly and Hartwell, 1954). The use of May apple extracts has only recently been placed on a sound experimental foundation. It revealed that the effect on herpes simplex type II, influenza A and vaccinia viruses of the crude extract of *Podophyllum peltatum* correlated with the activity of its lignans, i.e. podophyllum, alpha- and beta-peltatin, deoxypodophyllotoxin and picropodophyllotoxin. MacRae *et al.* (1989), also evaluated 18 lignans for their antiviral activity on murine cytomegalovirus and sindbis virus of mouse 3T3-L1 cells. Podophyllotoxin and alpha-peltatin were the most active ones, inhibiting virus replication at an early stage of its adsorption on cells rather than having a direct effect (virucidal) upon the virus.

### 1.2.4 Biochemical effects

These include effects on nucleic acids, inhibition of DNA, RNA and protein synthesis, activities which may have a direct bearing upon their antitumour and antiviral effects. After the report that podophyllotoxin reduced cellular respiration in rat tumours (Waravdekar *et al.*, 1955), several lignans were found to inhibit enzymes involved in respiration, including cytochrome oxidase, cytochrome c and succinic dehydrogenase (MacRae and Towers, 1984). The differential inhibition of cAMP phosphodiesterase by matairesinol, arctigenin, revealed that a guaiacyl ring (3′-methoxy, 4′-hydroxyphenyl), such as in matairesinol, was a better structural feature for this activity, than the veratryl ring (3′-,4′-dimethoxyphenyl) as in arctigenin (Nikaido *et al.*, 1981).

### 1.2.5 Activities towards insects

The three major reviews on the bioactivities of lignans (MacRae and Towers, 1984; Pelter, 1986; Ayres and Loike, 1990) provide little coverage of their effects on insects. There has been little interest in the subject in spite of the early demonstration (Wachs, 1947) of the synergistic properties of sesamin with pyrethrins, and the extensive success of its synthetic derivative, piperonyl butoxide. Most of the research on botanical insecticides was aimed at the discovery of highly toxic compounds, such as alkaloids or cardenolides.

The mode of action of the sesamin-type synergists and their synthetic derivatives is fairly well understood. They interfere with the rate of detoxification of the insecticide (Wilkinson and Hicks, 1969), by preferentially binding to the active sites of the main detoxification enzymes in insects, the cytochrome P-450 dependent polysubstrate nomooxygenases (PSMOs)(Ahmad *et al.*, 1986). This activity was, in part, related to the presence of a methylenedioxyphenyl (MDP) ring (Casida, 1970). There is a high incidence of this structural features in lignans with synergistic activity. Monomers of lignans such as apiol, dillapiol, safrol, all contain an MDP structure, and are potent synergists of several classes of insecticides, including pyrethrins, carbamates and organophosphates (Lichtenstein *et al.*, 1974; Mukerjee *et al.*, 1979).

However, the inhibition of the detoxification enzymes cannot explain other bioactivities of lignans such as growth reduction, antifeedant and hormone-like activities in insects (Table 1.3 and 1.5). Three lignans out of the 8 examined by Harmatha and Nawrot (1988) for their antifeedant activity against grain pests, cubebin, hinokinin and epiashantin, were more potent than azadirachtin on adult *Sitophilus granarius* beetles, and these 3 lignans together with yatein and the monolignan latifolon, had good antifeedant activity to larvae of

TABLE 1.5 : ACTIVITIES OF LIGNANS AND ANALOGUES ON INSECTS

| 'STRUCTURAL CLASS | [a]TYPE OF ACTIVITY | INSECT SPECIES | REF # |
|---|---|---|---|
| **DIARYLBUTANES:** | | | |
| N.D.G.A. | phototoxic, antifeedant | avoided by several <u>spp.</u> | 1 |
| **OXODIARYLBUTANES** | | | |
| cubebin | weak antifeedant | <u>Peridroma saucia</u> (LEP.)<br><u>Sitophilus granarius</u> (COL.)<br><u>Tribolium confusum</u> (COL.)<br><u>Trogoderma granarium</u> (COL.) | 2, 5 |
| dihydrocubebin | antifeedant | grain pests | 5 |
| hinokinin | synergist | three grain pests | 5 |
| yatein | antifeedant | grain pests | 5 |
| **FUROFURANS (dihydrofurans)** | | | |
| sesamin | synergist<br>weak JH<br>growth inhibitor | <u>Bombyx mori</u> (LEP.)<br><u>Ostrinia nubilalis</u> (LEP.) | 3 |
| asarinin | synergist | | 4 |

|  |  |  |  |
|---|---|---|---|
| savinin | synergist |  | 4 |
| eudesmin | antifeedant | *Spodoptera litura* (LEP.) | 6 |
| epieudesmin | antifeedant | id | 6 |
| epiashantin | antifeedant | id | 4 |
| justicin A & B | synergist |  | 1 |
| pinoresinol | not antifeedant | grain pests | 3 |
| matairesinol | synergist antifungal |  | 3 |
| **CYCLOLIGNANS** |  |  |  |
| β-peltatin | toxic | *P. saucia* (LEP.)<br>*O. nubilalis* (LEP.) | 3, unpub |
| podophyllotoxin | toxic<br>antifeedant | *O. nubilalis* (LEP.) | unpub |
| **NEOLIGNANS** |  |  |  |
| β-phenylpropanoid | antifeedant<br>growth inhibitor | *B. mori* (LEP.) | 6 |
| pseudolignan | antifeedant | id | 8 |
| sesamolin | synergist<br>antifeedant<br>weak JH activity growth inhibitor | *B. mori* (LEP.) | 4 |
| piperenone | antifeedant | *S. litura* (LEP.) | 6 |

| | | | |
|---|---|---|---|
| **MONOLIGNANS** | | | |
| dillapiol | synergist<br>toxic<br>growth inhibitor | *Cylas formicarius* (COL.)<br>*Periplaneta americana* (BLA.)<br>*Musca domestica* (DIP.)<br>*Tribolium castaneum* (COL.)<br>*Tricopulsia ni* (LEP.)<br>*O. nubilalis* (LEP.) | 6, 8, 9 |
| latifolon | antifeedant | grain pests | 7, 8 |
| myristicin | synergist<br>antifeedant<br>growth inhibitor | *Heliothis zea* (LEP.) | 6, 8, 10, 12 |
| isoasarone | antifeedant | *S.litura* (LEP.) | 6 |
| safrol, isosafrol | synergist<br>antifeedant | *H. zea* (LEP.) | 4, 12 |
| piperenone | antifeedant | *S.litura* (LEP.) | 6 |

REFERENCES FOR TABLE 1.5: 1: Downum, 1986; 2: Nawrot *et al*., 1991; 3: McRae & Towers, 1984; 4: Casida, 1970; 5: Harmatha and Nawrot, 1984; 1988; 6: Koul, 1982; Schoonhoven, 1982; 7: Nawrot *et al*., 1991; 8: Vigneron, 1978; 9: Tomar *et al*., 1979; Parmar and Tomar, 1983; Bernard *et al*., 1991; 10: Berenbaum and Neal, 1985; 11: Pelter, 1986; 12: Neal, 1989.

COL: Coleoptera; LEP: Lepidoptera; BLA: Blattodea; DIP: Diptera.

#: classification follows Ayres and Loike, 1990.

$^{e}$: considered antifeedant if activity is ≥ 50% that of azadirachtin.

*Tribolium confusum* and *Trogoderma granarius* (Nawrot and Harmatha, 1994). The mechanism of action involved in these activities is unknown.

The diversity of biological properties of lignans, as well as the diversity of the organisms that they affect, strongly suggest that their mechanism of action is not unique, and that as yet non described biochemical target sites reached by lignans remain to be uncovered.

# 1.3 P-glycoprotein

## 1.3.1 Multi-resistance to insecticides and drugs:

The development of multi-resistance, or cross-resistance to insecticides is a major challenge to pest management and research (Brattsten *et al.*, 1986). It affects over 500 species of pest insects and mites (Georghiou, 1990), and it is estimated that 50% of all new insecticide applications for agricultural pests in the United States are made as a result of insects having become resistant to existing insecticides (Moberg, 1990). Multi-resistance occurs when a population of insects can no longer be controlled either with the usual insecticides, or with several other biochemically unrelated insecticides to which the insects may never have been exposed. Resistant insects often exhibit an enhanced detoxification of the insecticide, through the polysubstrate monooxygenases (PSMOs), or elsewhere on the detoxification pathway (Hodgson, 1985), resulting in levels of the insecticide which are too low to affect their biological targets. This was considered to be the major mechanism involved in multi-resistance to insecticides (Raffa and Priester, 1985). Lignans and their derivatives, dillapiol, myristicin, piperonyl butoxide, sesamex, are inhibitors of enzymes of the detoxification pathway, in particular of the PSMOs (Bernard *et al.*, 1989; Bernard and Philogène, 1993), and restore the sensitivity of insects to insecticides.

Multi-resistance to drugs in mammals, fish, and bacteria shares several common patterns with insect multi-resistance to insecticides. Tumours responding to a first chemotherapy, or cells killed by a specific drug, fail to respond to a second treatment with the same drug or with several chemically unrelated drugs. They display the multidrug resistant (MDR) phenotype. Because of its negative impact on the success of cancer chemotherapy, the phenomenon has been extensively studied, mostly in mammals (Bradley *et al.*, 1988; Endicott and Ling, 1989; Gottesman and Pastan, 1993). Drugs involved in multidrug resistance include a variety of cytotoxic agents of plant origin, such as vincristine, directly extracted from *Vinca major (Catharanthus major)*, etoposide (VP-16), a derivative from the lignan podophyllotoxin found in the may apple, *Podophyllum peltatum,* antibiotics of fungal origin and their semi-synthetic derivatives, and a number of other molecules such as taxol, extracted from the pacific yew tree, *Taxus breviofolia* (Table

1.6). These drugs are known to be transported by a membrane protein, the P-glycoprotein, responsible for multiresistance (Gottesman and Pastan, 1993).

### 1.3.2 The membrane transporter, P-glycoprotein:

Pharmacological and biochemical studies have demonstrated that drug-resistant cells can maintain a low intracellular level of drug, compared to their parental sensitive cell line, and that the level of both intracellular drug and resistance are related to the level of activity of a class of integral transport proteins, the multidrug resistance (MDR) transporters, called P-glycoprotein (P-gp, Permeability-glycoprotein)(Ling and Thompson, 1973). This membrane-bound protein can actively pump a variety of drugs out of normal cells and, to a much higher degree, out of MDR cells (Gottesman *et al.*, 1994).

Mammalian P-gps belong to the superfamily of energy-dependent membrane transporters, the ABC (ATP binding cassette) transporter which contains highly conserved sequences among animal, bacteria and even plant species (reviews in Juranka *et al.*, 1989; Higgins, 1992), where they are active transporters of nutrients, peptides, polysaccharides and toxins (Hayes and Wolf, 1990; Gottesman *et al.*, 1994). For instance, the protein required for the transport of haemolysin in *Escherichia coli*, the HlyB protein, shows extensive sequence homology with the

TABLE 1.6 : COMPOUNDS THAT INTERACT WITH P-GLYCOPROTEIN AND MODIFY DRUG RESISTANCE (*).

**ANTICANCER DRUGS AND CYTOTOXIC AGENTS:**

*Vinca* alkaloids: vinblastine
Antibiotics: actinomycin D
Others: mitomycin C, taxol, topotecan
Antimicrotubule: colchicine, podophyllotoxin, taxol, vinblastine
Epipodophyllotoxin and derivatives: etoposide
Protein synthesis inhibitors: emetine, puromycin
Toxic peptides: gramicidin
Plant cardiac glycoside: digitoxin (1)

**AGENTS THAT REVERSE DRUG RESISTANCE:**

Calcium channel blockers: verapamil, azidopine
Anti-arrhythmics: quinidine, aminodarone
Hypertensives: reserpine
Antibiotics: hydrophobic cephalosporins
Antihistamines: terfenadine
Immunosuppressants: cyclosporine A
Steroid hormones: progesterone
Diterpenes: forskolin
Detergents: Tween-80
One plant lignan: phyllanthin (2)
Plant amides: biamides (3)
Other plant alkaloids: thaliblastine (4), roemerine
Several other hydrophobic, amphipathic drugs

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(*): Adapted from Gottesman and Pastan, 1993; (1): Lannoy *et al.*, 1992; (2): Somanabandhu *et al.*, 1993; (3): Saifah *et al.*, 1993; (4): Chen *et al.*, 1993.

nucleotide binding domain of mammalian P-glycoprotein (Hyde *et al.*, 1990). Pigment precursors in *Drosophila melanogaster* are imported into the pigment producing cells of the eye by a protein transporter of this superfamily (Dreesen *et al.*, 1988). The a-peptide, a pheromonal mating factor in common yeast, *Saccharomyces cerevisiae*, is transported by the protein STE6 which shares 57% sequence homology with the mammalian P-glycoprotein (McGrath and Varshavsky, 1989). A mutation in the STE6 gene altered the STE6 protein and provoked defective mating. Insertion of the murine mdr3 cDNA in the yeast restored the transport of a-peptide and successful mating capacities (McGrath and Varshavsky, 1989).

The wide distribution of P-glycoprotein-like efflux pumps in animals, bacteria, and insects, suggests the hypothesis that some herbivorous insects adapted to feeding on plants containing highly toxic allelochemicals, may have been selected for a P-glycoprotein-like defense mechanism. For instance, nicotine, a major allelochemical of tobacco plants, is innocuous to the tobacco hornworn, *Manduca sexta*, although it is highly toxic to most insects and mammals. A blood-brain barrier in these species, partially impermeable to nicotine, delays the passage of the toxin to the nicotine-sensitive nerve cord (Morris and Harrison, 1984). Recent studies using P-glycoprotein antibodies have shown that these insects also possess a P-glycoprotein-like protein in the blood-brain barrier as well as in the epithelial wall of their Malpighian tubule (Murray *et al.*, 1994). The contribution of both defense mechanisms, the blood-brain barrier and the P-glycoprotein-like pump, accounted for the low, non toxic levels of nicotine in the sensitive sites of the tobacco hornworm. This first evidence of the presence of a P-gp pump in insects will be supplemented by the current research of the same group of investigators to characterize the gene(s) coding for the protein in insects.

### 1.3.3 Reversal of resistance

The possible involvement of a P-gp-like pump in the malaria parasite, *Plasmodium falciparum,* was indirectly suggested by the reversal of resistance to chloroquine with known inhibitors of the pump. Verapamil, a calcium channel blocker and inhibitor of the P-glycoprotein pump in MDR mammalian cells (Gottesman *et al.,* 1994), as well as several analogues, had a remarkable capacity to reverse chloroquine-resistance *in vitro*, an activity unrelated to the calcium channel (Ye and Van Dyke, 1994).

Several agents that reverse drug resistance in mammalian cells have been reported (Table 1.6). They are not toxic in themselves but restore the sensitivity of MDR cells to a drug by competing for the transport system (the P-glycoprotein) responsible for resistance (Gottesman *et al.*, 1994). Recently, the lignan phyllantin has been shown to inhibit binding of the anticancer drug vinblastine to P-glycoprotein membrane vesicles derived from the multidrug resistant, P-glycoprotein rich, KB-V1 cells (Somanabandhu *et al.*, 1993).

Different cell lines have been developed to help to define the link between the multiresistance phenotype and P-glycoprotein. The Chinese hamster ovarian cells, CHO-AUXB1, gave rise through stepwise selection with colchicine to the two multiresistant cell lines, $CH^RC5$ and $CH^RB30$ (Ling *et al.*, 1983). In contrast, the multidrug resistant cell line G185 was obtained through the transfection and overexpression in the Swiss mouse fibroblast culture, NIH/3T3, of a human gene coding for P-glycoprotein (Gottesman *et al.*, 1994). In cells selected for resistance by continuous exposure to MDR drugs, the extent of P-glycoprotein expression was shown to be directly proportional to the degree of resistance (Kartner *et al.*, 1983: Pearce *et al.*, 1990). In other words, for a given drug or agent from the MDR group such as colchicine, vinblastine, vincristine, actinomycin D, etoposide and several others, the lethal concentration is higher for resistant cell lines (resistance level >1 for C5 and B30, Table 1.7), than for its parental sensitive line (AuxB1). This is also true for cells with a transfected MDR gene such as G185 (Table 1.7).

TABLE 1.7 : EFFECT OF DRUGS AND OTHER AGENTS ON TWO SERIES OF CELL LINES.

| | CELL LINES ′ (Resistance level) | | | | | |
|---|---|---|---|---|---|---|
| | Swiss mouse fibroblast | | Chinese hamster ovary cell | | | |
| @ | NIH sens. | G185 MDR | AUXB1 sens. | C5 MDR | B30 MDR | I10 rev. |
| **MDR group of drugs:** | | | | | | |
| colchicine | 1 | 25 | 1 | 96 | 680 | 28 |
| actinomycin D | 1 | 30-100 | 1 | | >4 | |
| vincristine | 1 | 100 | 1 | | 75 | |
| vinblastine | 1 | 10 | 1 | | 50 | |
| VP-16 (etoposide) | 1 | 10 | 1 | | 10 | |
| **MDR inhibitor:** | | | | | | |
| verapamil | 1 | 1 | 1 | | 1 | |
| **Drugs showing collateral sensitivity:** | | | | | | |
| Tween 80 | | | 1 | 1.3 | 0.9 | 1.2 |
| Triton X100 | | | 1 | 0.6 | 0.4 | 1.1 |
| phenolic DBP | | | 1 | 0.2 | 0.06 | 1.1 |
| NP-40 | | | 1 | 0.5 | 0.09 | 1.2 |

′ Resistance level: $LD_{50}$resistant cell line/ $LD_{50}$sensitive parental cell line.

@ Loe and Sharom, 1993; Preliminary work to Chapter 5.

### 1.3.4 Changes associated with the MDR phenotype

MDR cells resulting from stepwise selection with different agents express different patterns of cross-resistance (Juranka *et al.*, 1989). To explain this phenomenon, these authors suggested that the amplification of the gene for P-glycoprotein may be accompanied by the coamplification of stretches of flanking DNA. In fact, in a Chinese hamster ovary MDR cell line, CH$^R$C5, at least six classes of genes were coamplified and overexpressed (Bradley *et al.*, 1988).

A few drugs or agents have been shown to be more toxic to MDR cells than to their parental cell line, a phenomenon called collateral sensitivity. For instance MDR cells showed collateral sensitivity to a series of membrane-active detergents such as NP-40, Triton X100, the phenolic DBP, but not to Tween 80 (Loe and Sharom, 1993)(Table 1.7). The phenomenon was eliminated in revertant, drug-sensitive cells CH$^R$I10, which were selected from CH$^R$C5 on the basis of the collateral sensitivity of the MDR line to 1-dehydrotestosterone. This indicated that both hypersensitivity and resistance to the detergents were an intrinsic part of the MDR phenotype in these cell series.

Properties of lignans such as the restoration of sensitivity to insecticides in resistant populations, or also the inhibition of tumours (see section 1.1 and Table 1.5), could potentially be related to their effects on a P-gp-like pump, or a system similar to it. Consequently, the study of the activities of lignans on multidrug resistant, P-glycoprotein rich, cell systems, may add to our understanding of the mode of action of these compounds.

# CHAPTER 2 CENTRAL HYPOTHESIS AND OBJECTIVES

## 2.1 HYPOTHESIS

Members of the *Piperaceae* are rich in bioactive lignans. As with their Asian and African counterparts, the American peppers have a long history of use in local pharmacopoeia. Their chemistry has been little investigated.

> HYPOTHESIS 1 : Piperaceae from the neotropics share a similar chemical profile with species from other parts of the world, which explains their insecticidal activity.

Lignans occur in a large number of plant families. They display a wide array of biological activities against animals, fungi and bacteria. Their known ability to synergize insecticides does not explain satisfactorily these other properties.

> HYPOTHESIS 2 : Lignans have activities towards insects that may be related to a role in plant-animal, in particular plant-insect interactions, i.e. as phytochemical defenses against insect herbivores.

The major role played by lignans in the reversal of insect cross-resistance to insecticides, and the occurrence of a phenomenon of similar consequences in mammals and other animals, multidrug resistance, suggests that lignans could interact with the toxicity of xenobiotics in a new way.

> HYPOTHESIS 3: Lignans have a modulating effect on the passage of toxins across membranes with multidrug resistance characteristics.

## 1.2 OBJECTIVES

The general objective of the present work was to increase our understanding of the defensive role of plant lignans, in particular *Piperaceae* lignans, towards herbivorous insects.

Three specific objectives are presented in the present work:

OBJECTIVE 1. To study the insecticidal activities of *Piperaceae* extracts collected in the neotropics on the European corn borer and to isolate the phytochemical constituents responsible for insecticidal activity by bioassay guided fractionation (Chapter 3).

OBJECTIVE 2. To investigate the antifeedant and growth reducing activities of a series of lignans of different structural groups with an insect herbivore, the European corn borer (Chapter 4).

OBJECTIVE 3. To assay the modulating activity of lignans towards vinblastine accumulation in cultured multiresistant cells (Chapter 5).

# CHAPTER 3

# INSECTICIDAL DEFENSES OF PIPERACEAE FROM THE NEOTROPICS

## 3.1 INTRODUCTION

The role of plants from the pepper family (*Piperaceae*) in traditional pharmacopoeia is well established in tropical areas where these vines and shrubs are particularly abundant (Atal *et al.*, 1975; Schultes and Raffauf, 1990). Throughout the world, *Piper* plants have traditionally also been used against pests. For example, the Amazonian species, *Piper rotundistipulum,* is used locally as an insecticide and a fish poison (Schultes and Raffauf, 1990). *Piper. guineense* and *P. nigrum* are used as insecticides and molluscicides in several parts of Africa (Su and Horwat, 1981; Ivbijaro and Bolaji, 1990). The Indian species, *P. longum, P. betle, P. peepuloides,* and *P. cubeba,* have demonstrated insecticidal activity against mosquitos and flies (Srivastava, 1970; Miyakado *et al.*, 1983), and were shown to repel grain pests (Kokate *et al.*, 1980). The leaves of *P. futokatsura* from Taiwan and Japan are known as a feeding deterrent to the larvae of *Spodoptera litura* (Matsui and Munakata, 1975). Also, the leaves of *P. umbellatum, P. hispidum, P. auritum,* and plants reported only as *Piper spp.*, which are native to Central America and the Northwest Amazonian basin, are used by indigenous peoples to prevent malaria and to remove head lice (Schultes, 1975; 1980).

Our knowledge about the compounds responsible for all of these insecticidal activities is limited. However, it is known that amides are frequently found in the *Piperaceae* (Greger, 1988), in particular olefinic or alkyl isobutylamides such as piperine, piperettine, trichostachine, peepuloidin, piplartin and trichonine (Atal *et al.*, 1975). They are toxic to fruit flies, azduki bean weevils, cockroaches and several other insect species (Gbewonyo *et al.*, 1993; Miyakado *et al.*, 1983; 1989; Su and Horwat, 1981). They act as neurotoxins (Gbewonyo *et al*, 1993).

Phenylpropanoids are another group of potentially active chemicals of common occurrence in *Piper spp*. They include monolignans such as apiol, dillapiol, myristicin, eugenol and safrol, and lignans such as sesamin, cubebin, yangambin or diaeudesmin. They often constitute a large part of the essential oil of the plant, as for instance 85% safrol in the oil of the leaves of *P. auritum* (Castro and Poveda, 1983).

However, the toxicity of these substances, with a few exceptions such as safrol which has long been used as a natural insecticide (Coats, 1994), has not been established.

On the other hand, several of these compounds are known to synergize natural and synthetic insecticides. Sesamin and sesamolin, for instance, synergize the activity of natural and synthetic pyrethrins (Singh *et al.*, 1976), and their synthetic analogue, piperonyl butoxide, is currently included in most commercial preparations of pyrethrum (Worthing and Walker, 1993). Dillapiol, related to piperonyl butoxide, synergizes not only pyrethrins but also several carbamates and organochlorates (Handa and Dewan, 1974; Mukerjee *et al.*, 1979; Parmar and Tomar, 1983). In addition, lignans and their monomers are known to repel or act as antifeedants to several grain pests (Harmatha and Nawrot, 1988; Nawrot and Harmatha, 1994; Pelter, 1986; Tomar *et al.*, 1979). Kobusin and dillapiol have been shown to reduce the growth of larvae of insect pests (MacRae and Towers, 1984), including the larvae of a major pest of maize in North America, the European corn borer *Ostrinia nubilalis* (Bernard *et al.*, 1990).

These observations, together with our interest in the bioactivities of lignans in insects (Bernard *et al.*, 1989; 1990), prompted us to investigate the activities of a group of *Piper spp.* from the neotropics (Costa Rica) largely unknown from the point of view of their chemical ecology, against our model insect, the European corn borer. One African (*P. guineense*) and one Asian species (*P. betle*) were included for comparison with previous studies. We also report here the structure of a neolignan isolated for the first time from this family and the occurrence of dihydropiperlonguminine as the component responsible for the insecticidal activity of another newly investigated *Piper* species.

## 3.2 MATERIAL AND METHODS

### 3.2.1 Instrumentation

Melting points were determined on a Kofler hot stage and are uncorrected. $^1$H NMR spectra were obtained on a Bruker 500 MHz spectrometer, and chemical shifts (d) are reported in parts per million downfield from TMS. Data in parentheses: number of protons, multiplicity (s=singlet, d=doublet, q=quartet, quint=quintet, m=multiplet, non=nonet), coupling constant, and assignment.

Mass spectrometry was carried out by a VG analytical 7070E spectrometer. UV visible and IR absorption spectra were both recorded on Perkin-Elmer spectrophotometers.

All data were collected and interpreted under the supervision of Dr T Durst, Department of Chemistry, University of Ottawa, with the collaboration of Dr HG Krishnamurthy.

### 3.2.2 Plant material

Plants collected in Costa Rica by C. Bernard, L. Poveda and C. Hasbun in February 1992 included: *Piper aduncum* L., *P. auritum* Kunth, *P. aequale* Vahl, *P. hispidum* Sw. and *P. reticulatum* L. (E.A.R.T.H. station in Guapiles); *P. decurrens* C.DC, *P. marginatum* Jacq. and *P. pseudo-lindenii* C.DC. (C.A.T.I.E. in Cartago), and *P. tuberculatum* Jacq. in the city of Colon. *Piper lanceiaifolium* Kunth was collected by J.T. Arnason, L. Poveda and P. Sanchez in July 1993 near Colon, *P. obliquum* Ruiz & Pav. near Wilson Botanical Garden, *P. guanacastensis* C.DC. near Mirador, Guanacaste, *P. carrilloanum* C.DC. and an unidentified *Piper sp.* on the Peninsula de Osa in October 1993 by J.T. Arnason, K. Downum, L. Poveda, N. Moreno, and P. Sanchez. The fruits of *P. guineense* Schum & Thom came from Nigeria and were provided by F. Ewete, while the leaves of *P. betle* L. were collected in 1991 in Java, Indonesia by J.T. Arnason and Y. Razali. Identification of species was carried out by L. Poveda and P. Sanchez, and voucher specimens are retained at the Universidad Nacional de Heredia and the University of Ottawa.

Fresh leaves or fruits were immediately immersed on site in 95% ethanol (EtOH), and were stored in this state 2 weeks or more, until processed. This first EtOH extract was set aside, leaves were blended in a similar volume of EtOH and macerated for another 2 days. Both EtOH extracts were combined, filtered and concentrated to dryness on a rotary evaporator under reduced pressure at 30°C. Water was further removed by freeze drying the material for 12 hours which resulted in a powder or a gum, i.e. "crude extract", depending on the plant species. Dry weight of plant material was calculated from the plant solids remaining on the filter paper and the dry crude extract.

### 3.2.3 Insect bioassays

Larvae of *Ostrinia nubilalis* Hübner (*Lepidoptera: Pyralideae*) from a laboratory colony were maintained according to procedures described previously (Arnason *et al.*, 1985). The freeze dried crude EtOH extract of each *Piper spp.* (leaves, or fruits) was incorporated into a meridic diet at 30°C at a final concentration of 4% and 0.4% (w/w). Twenty naive (not exposed to the allelochemical) second instar (6 day old larvae weighing 8-12 mg) larvae per *Piper* diet were individually distributed in 12 mL glass vials closed with a cotton plug, and returned to the rearing conditions of the colony culture. The weight (Wt) of each larva, the diet placed, the diet remaining and the frass in each vial were determined at five-day intervals. The relative growth rate (RGR = Wt gain/ mean Wt during the period of study), and nutritional indices were calculated as described in Reese (1979) (Table 3.1):

TABLE 1 : EFFECT OF PIPERACEAE EXTRACTS (0.4%) ON INSECT GROWTH AND FOOD MANAGEMENT

| PIPER SPP | @ | RGR | CI | ECI | AD | ECD |
|---|---|---|---|---|---|---|
| *P. aduncum (leaves)* | | 3.63 * | 591.11 * | 5.23 * | 148.62 * | 3.43 * |
| *P. aequale (leaves)* | | 78.93 | 127.33 | 85.12 | 100.00 | 100.00 |
| *P. aequale (twigs)* | | 59.26 * | 162.91 * | 58.45 * | 102.61 * | 56.02 * |
| *P. auritum (leaves)* | | 68.58 | 164.48 | 55.42 | 109.63 | 49.83 * |
| *P. auritum (twigs)* | | 56.17 | 161.69 | 63.37 | 101.24 | 62.66 |
| *P. betle (fruits)* | | 55.56 * | 171.29 | 61.70 | 119.62 | 59.82 * |
| *P. decurrens (leaves)* | | 68.31 | 155.73 | 78.55 | 119.80 | 64.87 * |
| *P. guanacastense (leaves)* | | 75.42 | 86.62 | 90.50 | 98.87 | 90.69 |
| *P. guineense (fruits)* | | 0.18 * | 336.32 * | 0.35 * | 104.11 * | 0.33 * |
| *P. hispidum (leaves)* | | 73.36 | 123.63 | 73.00 | 106.38 | 71.74 |
| *P. marginatum (leaves)* | | 101.86 | 100.45 | 119.38 | 91.37 | 214.30 * |
| *P. pseudo-lindenii (leaves)* | | 40.83 * | 256.15 * | 36.81 * | 113.62 | 32.48 * |
| *P. reticulatum (leaves)* | | 30.65 * | 274.84 * | 29.46 * | 116.32 * | 25.72 * |
| *P. tuberculatum (leaves)* | | 0.26 * | 831.24 * | 0.28 * | 121.85 * | 0.23 * |

@ Data expressed as % of control.
Data followed by an * are significantly different from their control in Tukey's multiple range test (P=0.05). The mean RGR, CI, ECI, AD and ECD (stdev) values of control groups were: 1.7(0.4), 2.7(0.6), 5.3(1.2), 0.7(0.1) and 0.07(0.03).

*Consumption index (CI) = Wt of food ingested / mean Wt of larvae over the same time*

*Approximate digestibility (AD) = [(Wt of food ingested - fecal Wt) / Wt of food ingested] X 100*

*Efficiency of Conversion of Ingested food (ECI) = [Wt gained / Wt of food ingested] X 100*

*Efficiency of Conversion of Digested food (ECD) = [Wt gained / (Wt of food ingested - Wt of feces)] X 100*

All statistical analysis were performed using Tukey's general linear models procedure (Tukey, 1949) with SAS for PC.

A second insect species was used to monitor the isolation of chemicals from the plants: the rock hole breeding mosquito, *Aedes atropalpus* L. (*Diptera: Culicidae*). The small amount of material needed for the bioassays with larvae of these species when compared to the ECB, as well as the rapidity of the toxic response, made it a system of choice to follow the activity of the successive fractions of the plant extracts. Rearing conditions were previously reported (Watt *et al.*, 1981). Ten second instar larvae were pipetted into each of the glass vials containing the crude plant extract at concentrations of 10, 50 and 100 ppm. After 24 hours at room temperature, the surviving larvae were counted. The experiment was repeated 3 times. The pattern of toxicity of the 16 *Piper* extracts to mosquito larvae generally paralleled that obtained with the ECB (Table 3.2), confirming the suitability of mosquito larvae as assay guides for the isolation compounds with activity on the test herbivore.

TABLE 3.2 : INSECTICIDAL ACTIVITY OF *PIPER SPP*. CRUDE EXTRACTS TO MOSQUITO LARVAE.

| | Concentration of extract | | |
|---|---|---|---|
| *PIPER SPP* | 10µg/ml | 50µg/ml | 100µg/ml |
| P. *aduncum* | 7 (5) [•] | 2 (2) | 0 |
| P. *aequale* | 9 (1) | 8 (1) | 3 (2) |
| P. *auritum* | 8 (1) | 1 (1) | 0 |
| P. *betle* | 10 (0) | 10 (0) | 9 (1) |
| P. *carrilloanum* | 10 (0) | 9 (1) | 9 (1) |
| P. *decurrens* | 7 (1) | 5 (2) | 0 |
| P. *guanacastense* | 8 (1) | 7 (1) | 7 (1) |
| P. *guineense* | 0 | 0 | 0 |
| P. *hispidum* | 8 (0) | 6 (1) | 2 (1) |
| P. *marginatum* | 9 (1) | 8 (1) | 2 (1) |
| P. *obliquum* | 9 (1) | 7 (2) | 2 (1) |
| P. *pseudo-lindenii* | 9 (1) | 7 (1) | 6 (1) |
| P. *reticulatum* | 8 (1) | 7 (2) | 4 (3) |
| P. *tuberculatum* | 1 (1) | 0 | 0 |
| P. *non identified* | 9 (1) | 8 (2) | 8 (2) |

---

[•] Number of surviving larvae: mean of 3 trials of 10 larvae (standard deviation), after 24h.

For the assays on fractions of the crude extracts, a concentration of 100 ppm was arbitrarily selected. The activity of the fraction was expressed as % of the toxicity of the corresponding crude extract at the same concentration (Table 3.3).

### 3.2.4 Phytochemical isolation and identification

#### *3.2.4.1 Piper aduncum*

Fifteen g of dry crude leaf extract were dissolved in a minimal volume of acetone, and adsorbed onto 30 g of silica gel 70-230 mesh. The solvent was evaporated under reduced pressure. The resulting powder (silica-crude extract) was deposited in a Buchner funnel. Five hundred mL of the following solvents were added successively: hexanes (HEX), methylene chloride ($CH_2Cl_2$), ethyl acetate (EtOAc) and methanol (MeOH). Each fraction was then evaporated to dryness, weighed (0.2g, 1.02g, 0.01g, and 0.2g, respectively) and redissolved in the water of the mosquito bioassay to achieve the selected concentration. Most of the activity was found in the first two fractions (Table 3.3). These two were combined and a pale yellow oil, dillapiol (Figure 3.1a), the major component (11 mg), was isolated following the method described by Orjala *et al*. (1989). The identification of

TABLE 3.3 : DISTRIBUTION OF THE INSECTICIDAL ACTIVITY TO MOSQUITO LARVAE OF FRACTIONS OF THREE *PIPER SPP*.

| PIPER SPP. [a] | HEX | $CH_2Cl_2$ | EtOAc | MeOH |
|---|---|---|---|---|
| *P. aduncum* | 26 (3) | 72 (7) | 2 (1) | 0 |
| *P. decurrens* | 88 (3) | 8 (2) | 3 (1) | 1(1) |
| *P. tuberculatum* | 54 (6) | 44 (7) | 2 (1) | 0 |

---

[a] : Percent mortality (standard error) at 100 ppm.

the purified material was made by comparison with the spectral data of a sample of pure dillapiol (Soerensen and Soerensen, 1969).

### 3.2.4.2 *Piper decurrens*

The chemistry of this species had not been previously reported. Sixty g of the crude dried extract were adsorbed on silica gel and washed successively with four solvents (HEX, $CH_2Cl_2$, EtOH and MeOH), as described above. This resulted in the distribution of the toxicity shown in Table 3.2. The HEX and $CH_2Cl_2$ fractions were combined, and passed through a column of silica gel (70-230 mesh; 200 g), with HEX and EtOAc as eluents (100:0 to 0:100). From the resulting fractions, a fine white solid (92 mg) was isolated by crystallization from cold HEX. The physical and spectroscopic data allowed to identified this solid as the neolignan, conocarpan (Figure 3.1b). IR (KBr pellet, $cm^{-1}$) $3410^{(br)}$, $3242^{(br)}$, 2963, 1607, 1607, 1520, 1476, 1239, 925, 830; $^1H$ Nmr ($CDCl_3$, ppm) $\delta$ 1.40 (3H, d, J=6.8 Hz, $CH-C\underline{H}_3$), 1.87 (3H, dd, J=6.5 Hz, 1.5 Hz, $CH=CH-C\underline{H}_3$), 3.40 (1H, apparent quint, J=7.3 Hz, $C\underline{H}-CH_3$),5.09 (1H, d, J=8.8 Hz, $O-C\underline{H}$), 6.45 (1H, dq, J=15.7 Hz, 6.5 Hz, $CH=C\underline{H}-CH_3$) 6.37 (1H, dd, J=15.7 Hz, 1.5 Hz, $C\underline{H}=CH-CH_3$), 6.77 (1H, D, J=8.2 Hz, Ar-H ortho to OR), 6.83 (2H, d, J=8.5 Hz, Ar-H ortho to OH), 7.13 (1H, d, J=8.2, Ar-H ortho to CH=CH), 7.30 (2H, d, J= 8.5 Hz, Ar-H meto to OH); MS (EI, m/z) 267(28), 266(100), 251(17), 233(10), 159(12), 133(12), 131(11), 121(12), 119(18), 107(17); Anal. calculated for $C_{18}H_{18}O_2$: C, 81.17; H, 6.81.

### 3.2.4.3 *Piper tuberculatum*

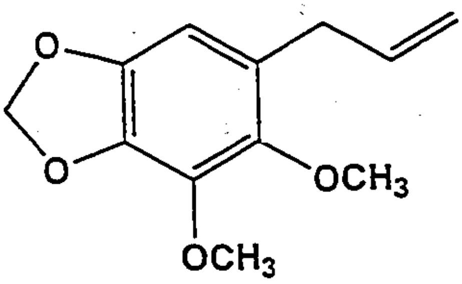
Seventy g of crude EtOH extract from fresh leaves were treated as for the two previous *species*. The bioactivity of the four basic fractions is shown in Table 3.2. The HEX and $CH_2Cl_2$ fractions were combined and chromatographed on a large silica gel column (70-230 mesh, 500g), eluting successively with pure HEX, mixtures of Hex-EtOAc of increasing polarities, pure EtOAc and finally with EtOAC-MeOH (1:1, v:v). The largest fraction contained one major compound, white silky needles, recrystallized from cold benzene-hexanes mixtures. The physical (mp = 116-118 °C) and spectroscopic data [IR (KBr pellet, $cm^{-1}$) 3300, 2941, 1667, 1624, 1550, 1490, 1444, 1249, 1041, 928, 812; $^1$H NMR (acetone-d6, ppm) $\delta$ 0.89 [6H, d, J=6.9 Hz, $CH(C\underline{H}_3)_2$], 1.77 [1H, non, J=6.9 Hz, $C\underline{H}(CH_3)2$], 2.44 (2H, dq, J=7.5 Hz, 1.5 Hz, $C\underline{H}_2$-CH=CH), 2.69 (2H, t, j=7.5 Hz, Ar-$CH_2$), 3.16 (2H, t, J=6.9 Hz, NH-$C\underline{H}_2$), 5.95 (2H, s, O-$C\underline{H}_2$-O), 5.96 (1H, dt, J=15.2 Hz, 1.5 Hz, $CH_2$CH=CH), 6.69 (1H, dd, J=7.9 Hz, 1.7 Hz, Ar-H meta and para to O-$CH_2$O), 6.75 (1H, dt, J=15.2 Hz, 7.5 Hz, $CH_2$-C$\underline{H}$=CH), 6.75 (1H, D, J=7.9 Hz, Ar-H ortho to O-$C\underline{H}_2$O and meta to R), 6.76 (1H, d, J=1.7 Hz, Ar-H ortho to O-$CH_2$-O and R) ; MS (m/z M+ 275, $C_{16}H_{21}O_3N$)] of this compound matched those given in the literature for dihydropiperlonguminine (Figure 3.1c).

FIGURE 3.1 KNOWN COMPOUNDS ISOLATED IN THE PRESENT STUDY:

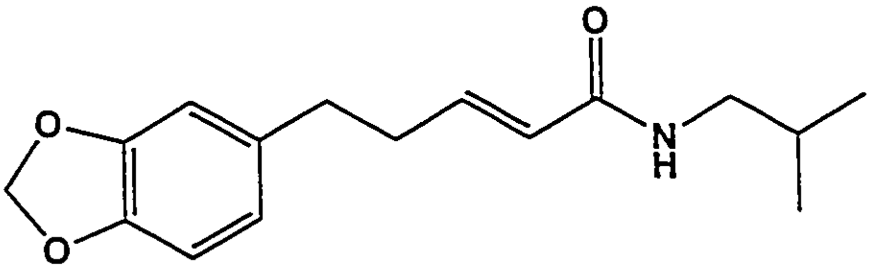
a, dillapiol

b, conocarpan

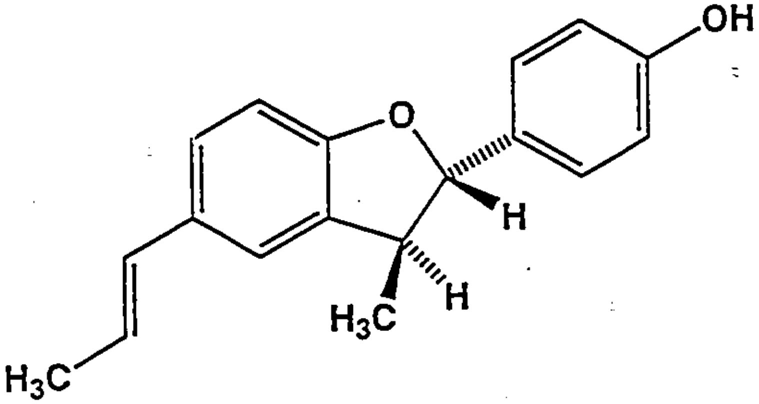
c, dihydropiperlonguminine.



.a. Dillapiol



c. dihydropiperlonguminine



b. Conocarpan

## 3.3 RESULTS AND DISCUSSION

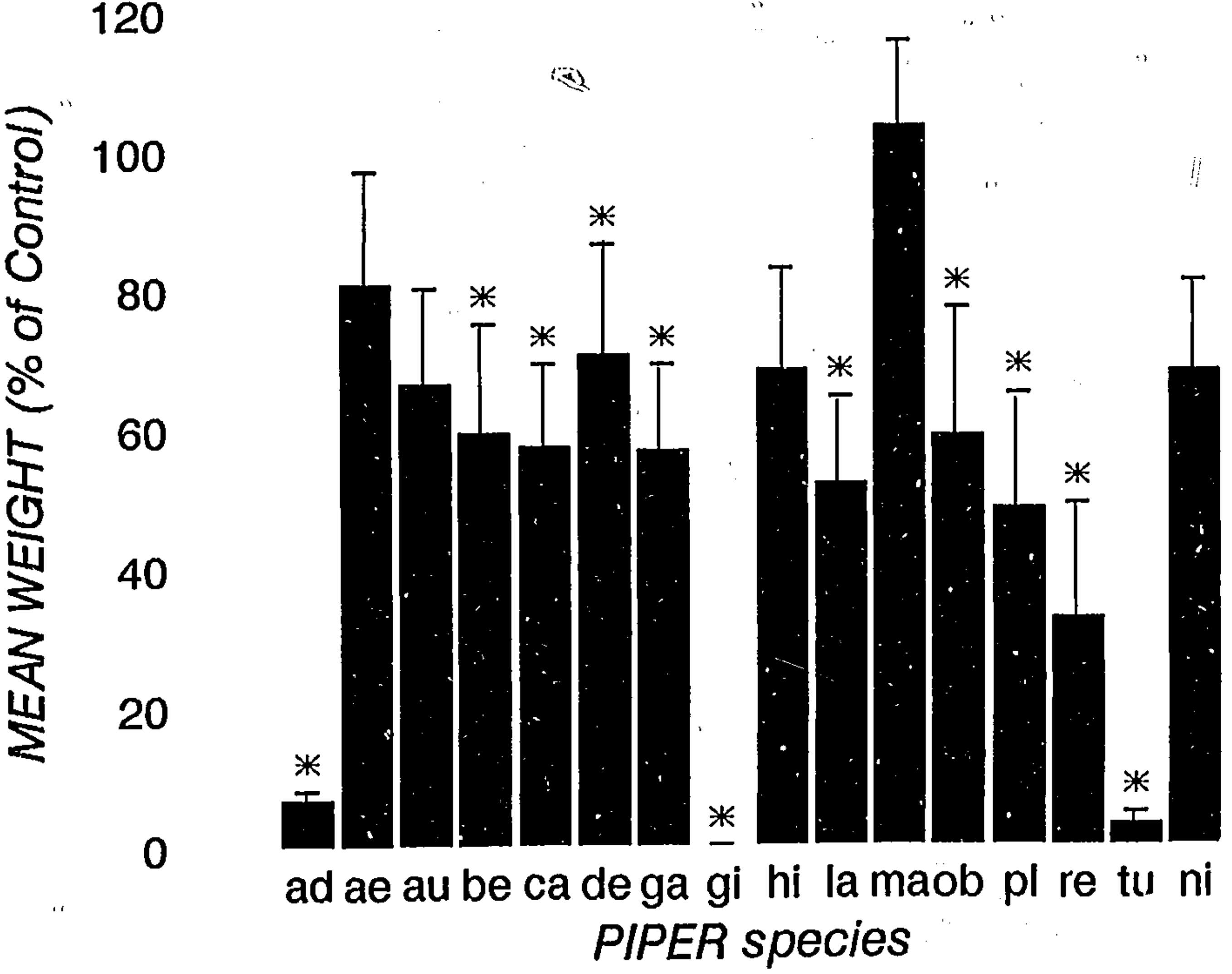
### 3.3.1 Activity of crude extracts towards the European corn borer larvae

The present study establishes the growth reducing activity on the European corn borer (ECB) of the majority of the leaf extracts of the *Piper* species collected (Figure 3.1 and 3.2). *Piper aduncum* (ad), *P. tuberculatum* (tu) and *P. guineense* (gi) had the highest activity in the diet (Figure 3.2). The mortality data (not shown) generally parallelled the growth reducing data and all larvae in the *P. aduncum*, *P. tuberculatum* and *P. guineense* groups died by day 12 of treatment. In addition, seven other species, *P. betle* (be), *P. decurrens* (de), *P. guanacastensis* (ga), *P. lanceiaifolium* (la), *P. obliquum* (ob), *P. pseudo-lindenii* (pl), *P. reticulatum* (re) and *P. carrilloanum (ca)* significantly reduced the growth rate of the larvae (Table 3.1), with larval weights after a period of 10 days on these diets ranging from 27% to 68% of controls. For

FIGURE 3.2 : GROWTH REDUCING ACTIVITY OF *PIPER* EXTRACTS (0.4% in the meridic diet) TO EUROPEAN CORN BORER LARVAE.

*ad:* P. *aduncum*
*ae:* P. *aequale*
*au:* P. *auritum*
*be:* P. *betle*
*ca:* P. *carrilloanum*
*de:* P. *decurrens*
*ga:* P. *guanacastensis*
*gi:* P. *guineense*
*hi:* P. *hispidum*
*la:* P. *lanceiaifolium*
*ma:* P. *marginatum*
*ob:* P. *obliquum*
*pl:* P. *pseudo-lindenii*
*re:* P. *reticulatum*
*tu:* P. *tuberculatum*
*ni:* P. not identified.

*Piper* species with an asterisk are significantly different from their control, P = 0.05 at Tukey's general linear models procedure. Twenty second instar larvae, at day 6, were fed treated diets and weights determined on day 20.


MEAN WEIGHT (% of Control)
0
20
40
60
80
100
120
ad
ae
au
be
ca
de
ga
gi
hi
la
ma
ob
pl
re
tu
ni
PIPER species
*

the larvae that survived the treatment, the percentage pupating was decreased in all cases except for *P. marginatum*.

Nutritional indices (Table 3.1) showed a limited effect of the extracts on the food consumption (CI) of the larvae, indicating little feeding deterrence. In fact, the three most insecticidal species induced a 3 to 8 times higher consumption of food. The digestion of the insect was also little affected by the allelochemicals of the plant food but the ECD was lowered, indicating a toxicity of the digested food components beyond the gut-haemolymph barrier.

### 3.3.2 Phytochemical isolation of pure compounds

Dillapiol was the main active component of *P. aduncum* and caused 92% mortality of mosquito larvae (Table 3.4).

Dihydropiperlonguminine was isolated from the active fraction of *P. tuberculatum*. This isobutylamide has been isolated from other *Piperaceae* (Tabuneng *et al.*,1983), along with longuminine and other derivatives. Dihydropiperlonguminine is toxic to mosquito larvae (Table 3.4).

Conocarpan was isolated from the active fraction of *P. decurrens extract.* Conocarpan could account for part of the insecticidal activity of this plant (Table 3.4). Conocarpan has been previously isolated from the wood of *Conocarpus erectus* (*Combretaceae*) (Hayashi and Thomson, 1975), but this is its

TABLE 3.4 : ACTIVITY OF THE NEWLY ISOLATED PURE COMPOUNDS TO MOSQUITO LARVAE.

| SPECIES | % MORTALITY |
|---|---|
| *P. aduncum:* | |
| dillapiol | 92 (4) |
| *P. decurrens:* | |
| conocarpan | 47 (5) |
| *P. tuberculatum:* | |
| dihydropiperlonguminine | 47 (2) |

[*]: Percent mortality (standard error from 3 assays of 10 larvae). Concentrations of bioassays were 0.1 ppm for *P. aduncum* and *P. decurrens,* and 0.01 ppm for *P. tuberculatum*.

first isolation from the *Piperaceae*. Large quantities of sitosterol (118 mg) were also purified from this fraction, as well as a number of less abundant neolignans structurally close to conocarpan, which account for the remaining activity. These are presently being analyzed and will be described elsewhere (Chauret *et al.*, 1995, submitted).

### 3.3.3 Lignans and amides of the *Piperaceae*

More than 15 species of *Piper* have been reported in the literature to have insecticidal activity. According to Marquis (1991), the highly insecticidal activity of most of the *Piper spp.* observed in the field in relation to two phytophagous insects, could not be correlated with the leaf characteristics, the soil, or other environment factors, "leaving the individual chemistry as a possible factor to investigate".

The two species of *Piper* with the highest toxicity to the ECB, *P. tuberculatum* and *P. guineense* contain large amounts of amides (Table 1.4). The isobutylamide, dihydropiperlonguminine, is a major component of *P. tuberculatum* leaves, as described in the present study (Table 3.4). In the only other chemical investigation reported on this species, the pyridine alkaloid piplartine and its dimer were isolated from the root bark (Filho *et al.*, 1981).

*P. guineense* is by contrast well studied (Table 1.4). A series of isobutylamides, including pipercide, guineensinamide, guineensine, pellitorine and kalecide have been isolated from the roots of *P. guineense*, and shown to be responsible for the insecticidal activity of this species (Gbewonyo *et al.*, 1993). The first three amides were the most active against adults of *Musca domestica*. Each of these contains a methylenedioxyphenyl (MDP) structural moiety.

The isobutylamide newly isolated from *P. tuberculatum*, dihydropiperlonguminine (Figure 3.1c) also has an MDP ring, and is the main component of the active fraction of the leaf extract. Greger (1988) pointed out that these types of amides, including olefinic and alkyl isobutylamides, are common to a restricted number of related plant families, namely the *Piperaceae*, the *Asteraceae* and the *Rutaceae*. All three families are abundant in the tropics, particularly in the humid tropics in the case of the *Piperaceae*, where herbivory is a potent selective force. The isobutylamides found in the *Piper* family have low molecular weights, contain a single nitrogen, and are thus not 'expensive' to biosynthesize. The pungent, numbing sensation caused by some of the psychoactive species, such as *P. methysticum*, may be a reason for their avoidance by herbivores, as observed by Marquis (1991), in particular with *P. auritum*. The *Piperaceae* are mostly vines or small shrubs in the tropics; short lifecycle species would be expected to protect themselves with simply synthesized, low cost, molecules. The isobutylamides could be described as belonging to this category of defense compounds.

The most active insecticidal species in our bioassays was *P. aduncum*. Surprisingly, no amides have been reported from this species (Table 1.4). It does, however, contain several lignans, including dillapiol, first found in the Indian dill, *Anethum sowa* (Fabaceae), where it constitutes up to 35% of the essential oil of the seeds of Indian cultivars (Tomar *et al.*, 1979), and also in the leaves of *P. aduncum* from Panama where it is the only major component (Gupta and Arias, 1983). Dillapiol can synergize several natural insecticides, including pyrethrum (Mukerjee *et al.*, 1979), azadirachtin (Bertrand, 1992) and tenulin (Bernard *et al.*, 1990), at the concentration of 100 ppm in the diet of the European corn borer, a concentration well below the level in the leaves of *P. aduncum* (approximately 60% of the oil). It was also previously found to be toxic to the ECB (Bernard *et al.*, 1989). It is the main insecticidal component of this *Piper spp.* Monolignans and lignans with the methylenedioxyphenyl (MDP) moiety are characteristic chemicals of the *Piperaceae*. Lignan toxicity differs from that of classical neurotoxic insecticides, as it is chronic rather than acute. Several lignans inhibit a vital biochemical defense of herbivores, the polysubstrate monooxygenase (PSMO) detoxification enzymes (Ahmad, 1986; Bernard *et al.*,1989; Berenbaum and Neal, 1985). Thus, several potentially toxic components in the food of the insect, that are usually gradually eliminated, could accumulate to a toxic level. These specific lignans, myristicin, dillapiol, epiyangambin, ashantin and eugenol, are distributed in most of the *Piper* spp. and often occur in large quantities in the plant.

The elucidation of the structure of conocarpan, a neolignan, as the main but not highly insecticidal component of *P. decurrens*, further suggests the importance of lignans in the *Piperaceae*. Only lignans have been isolated from the extracts having a growth reducing rather than an acute toxic effect on insect larvae, i.e. *P. marginatum, P. decurrens* and *P. hispidum* of the present study which afforded the lowest larval mortality. This leads to the possibility that *Piper* lignans not associated with amides, excluding monolignols, may have a long term effect and low toxicity, as further suggested by the low incidence of pupation in the groups fed on these three species. This possibility warrants further investigation.

In any case, isobutylamides and lignans, combined in the same plant, constitute an inexpensive, nevertheless effective, means of defense elaborated by a limited number of highly successful plants from the neotropics.

The present results support previous studies on the chemical components of the *Piperaceae,* and stresses the importance of neolignans and amides as the insecticidal components of this family. It establishes that tropical American *Piper spp.* have a chemical and insecticidal profile similar to African and African species.

# CHAPTER 4

# BIOLOGICAL ACTIVITIES OF PURE LIGNANS ON THE EUROPEAN CORN BORER

## 4.1: INTRODUCTION

Lignans, neolignans and their monomers can be found in large quantities in plants. For instance, the cyclolignan podophyllotoxin may constitute up to 2% of the resin of several *Podophyllum spp.* (*Berberidaceae*) (Hartwell and Schrecker, 1958), the monolignan dillapiol up to 35% of the seed oil of *Anethum sowa* (Apiaceae) (Singh *et al.*, 1976), and the dry leaves of *Larrea divaricata* (Lauraceae) may contain up to 12% nordihydroguaiaretic acid (Downum, 1986). The pepper family (*Piperaceae*), in particular, contains a large number of monolignans (see Tables 1.2 and 1.4), together with at least 79 different lignans (Jensen *et al.*, 1993) and neolignans (Chapter 3).

Lignans and their monomers, have a variety of biological activities towards animals, plants or microorganisms (Ayres and Loike, 1990). Some of them are at the origin of the present therapeutical use of potent antiviral and antineoplastic agents, other ones are at the origin of the widely used insecticide synergist, piperonyl butoxide, and a few are synergists to plant toxins (Neal, 1989). Feeding deterrence to insect grain pests (Table 1.5) has also been documented (Harmatha and Nawrot, 1984; 1988; Nawrot *et al.*, 1991). The level of activity of some lignans compares to that of azadirachtin, a potent antifeedant to over 80 species of insects. Dillapiol, a monolignan, is toxic to larvae of *Aedes spp.*, with an $LD_{50}$ of 11 ppm (Lichtenstein and Casida, 1963). A few other lignans reduce the growth, and delay or impair the development of Lepidopteran larvae (Casida 1970, MacRae and Towers, 1984; Bernard *et al.*, 1990). Also, whole plant extracts with lignans as major constituents, such as in some pepper species (Table 1.3 and 1.4), have shown significant insecticidal activity (Atal *et al.*, 1975; Bernard *et al.*, 1995).

Although lignans represent an important branchpoint in the biosynthetic metabolism of phenylpropanoids (Davin and Lewis, 1992), this class of compounds has been largely overlooked in term of their function, both within plants and in plant-animal interactions, when compared to other chemicals with which they share the same initial biosynthetic pathway. The antimicrobial, fungistatic, insecticidal and allelopathic properties of several lignans strongly suggest that these compounds have a role to play in the plant-host defense system. Hence, we investigated the activity of a series of 15 pure lignans on a model insect, the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae), a highly polyphagous insect and a major pest of corn, sweet pepper and several other crops.

## 4.2 MATERIAL AND METHODS

### 4.2.1 Source of pure compounds

Dr J Lam, Department of Chemistry, University of Aarhus, Denmark, provided the series of furofuran lignans, sesamolin, ashantin, epiashantin, yangambin, epiyangambin, diasesartemin and episesartemin. Dr K Downum, Department of Biological Sciences, Florida International University, Miami, provided the only reported phototoxic lignan, nordihydroguaiaretic acid. Dr J Harmatha, Czeck Academy of Sciences, Prague, Czech Republic, provided the oxodiarylbutane lignans, cubebin, dihydrocubebin, hinokinin and yatein. Dr T Durst, Department of Chemistry, University of Ottawa, provided the cyclolignans, α-peltatin, ß-peltatin, podophyllotoxin and podophyllin, and assisted with the spectroscopic identification of the two lignans from *Piper decurrens* and *P. aduncum*.

### 4.2.2 Insect cultures

Insects were reared on meridic diets as described by Guthrie (1971) for *Ostrinia nubilalis* Hübner (*Lepidoptera: Pyralidae*), and maintained according to procedures described previously by our laboratory (Arnason *et al.*, 1985). Specific growth conditions were 27°C, 80% relative humidity and a photoperiod of 18h-6h light-dark under fluorescent Vitalite R (100 W/m2).

### 4.2.3 Feeding deterrence tests: a no-choice leaf disk bioassay

The effects of 14 lignans on the feeding behaviour of young *O. nubilalis* larvae was evaluated using an apparatus derived from that described by Ascher and Glotter (1981). Pieces of fresh corn leaves (the 6th leaf of 6 weeks old 'Silver Queen' corn plants), excluding the central nervure, were enclosed in a plastic Petri dish with, between lid and bottom, an inverted lid with a hole exposing a disk of leaf of 130 $mm^2$. Ten µL of the lignans solutions at 30, 100, 300, 700, 1000 µg/mL, with additional concentrations of 3000 and 10000 µg/Ml for podophyllotoxin and dillapiol, were sprayed over the leaf surface. Two separate replicates of the bioassays were performed, each of 20 insects per concentration, totalling 40 insects for a given lignan concentration. After evaporation of the solvent, one second instar larva was enclosed in each Petri dish. Experiments were conducted in a Conviron ET growth chamber under the conditions described above. After 24 h, all larvae were removed and leaf pieces from the same group set aside in methanol. Evaluation of the area eaten per $mm^2$ by the insect was performed under a binocular stereomicroscope with samples resting on a millimetre graph paper lighted from below through a lucite glass plate. Any $mm^2$ showing evidence of insect feeding damage was scored as consumed, and data expressed as % of controls. Larvae feeding on NDGA were exposed to a regime of visible light without and with near UV (4 $W/m^2$, 1 hour), provided by a bank of four 20W black light blue tubes (Westinghouse).

### 4.2.4 Test diets

Appropriate amounts of the test compounds in 95% EtOH or a mixture of $EtOH:H_2O$ (1:1), were added to liquid diet (30 to 35°C) in 50 Ml beakers, and incorporated in the diet by vigourous hand mixing . Controls contained the same amount of EtOH, which did not exceed 1% of the diet weight. Fresh diet was prepared every 5 days and refrigerated until used.

### 4.2.5 Growth studies

Larvae of the second instar (5-6 days old, 1 mg ± 0.2) were reared individually in 12 mL clear glass scintillation vials with one cube (approximately 1.5 g) of the appropriate treated diet, with control larvae reared in the same conditions. Groups included 20 larvae. Larval and diet weights were measured at days 1, 5 and 10. The initial dry weight of the diet was calculated from the dry weight/fresh weight ratio (= 0.177), as were both the initial, middle and final weights of the larvae (dry/fresh ratio = 0.121, 0.211 and 0.241, respectively). Frass were dried before weighing. Larvae were then moved (at day 15) to a regular diet (without lignan), and allowed to continue their life cycle. Weights of pupae and adults were recorded after oven drying.

Insect mortality was evaluated during the larval, the pupal and the imaginal stages. Cumulative mortality for a given lignan was expressed as % of the mortality of controls run at the same time. Pupae not responding to a slight pressure on the ventral part and weak moribund emerging adults (these insects die within one or two days), were included in mortality data.

### 4.2.6 Nutritional analysis

Collection of data and the Calculation of the nutritional indices was performed using the formulae derived from Reese (1979) as described in Chapter 3.

### 4.2.7 Topical application

To investigate the toxicity and the effects of 4 lignans of the podophyllotoxin series on larval feeding behaviour, 1 µl of the test solution (50 µg/ml in acetone) was applied to the back of second instar larvae (day 6 after hatching). The application was repeated two days later, resulting in a cumulative dose of lignan of 100µg /larva. Larvae were maintained throughout the experiment on normal meridic diet. Weights were measured and nutritional indices calculated as described above.

### 4.2.8 Data analysis

For the antifeedant test using corn leaves treated with pure lignans, results from assay I and assay II were analyzed separately as they were performed one year apart, but data sets were both treated identically. Each response in $mm^2$ of leaf consumed was expressed as % of its control, and a regression calculated (SAS for PC) from the log concentration of the lignan on the leaf disk, as it was found to be the best fitting model (Probit calculations did not improve the validity of the model). This gave rise to two values of $PC_{50}$ (concentration needed to inhibit 50% larval feeding) which were reported on a bar graph. Actual values can be calculated as follows: 10 µL of the 100 µg/µL concentration (for example) resulted in 1 µg / 130 $mm^2$ of leaf, as the solvent was totally evaporated.

Data sets from all treatment groups of insects fed on treated meridic diet and topical applications of lignans were also expressed as % of their controls. The actual data were log transformed before their expression in % of controls and tested for homoscedasticity before performing an ANOVA using Tukey's multiple range test (Tukey, 1949; SAS for PC).

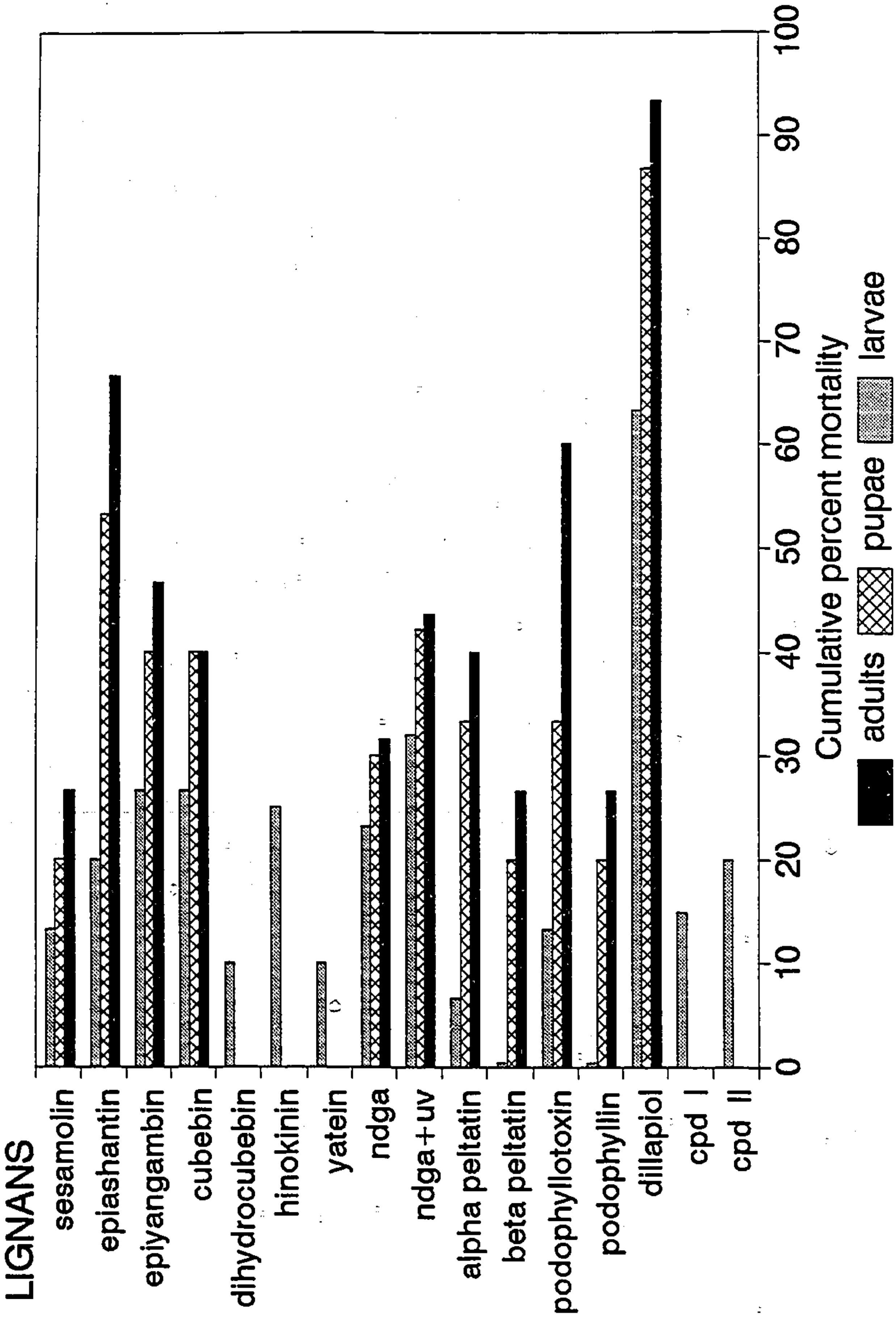
## 4.3 RESULTS and DISCUSSION

### 4.3.1 Acute toxicity

Lignans, monolignans and neolignans (Figure 4.1), were classified in groups of similar basic structures, with the furofurans (epiashantin and yangambin, and an oxydated furofuran, sesamolin), followed by the oxodiarylbutanes (nordihydroguaiaretic acid=NDGA, cubebin, dihydrocubebin, hinokinin and yatein), the cyclo- (α-peltatin, ß-peltatin, podophyllotoxin), the mono- (dillapiol) and neo-lignans (cpd I and cpd II=conocarpan). Podophyllin, a complex mixture of several lignans including podophyllotoxin, α-peltatin, ß-peltatin, several of their glucoside derivatives, and flavones, was included in the Figures and Tables for comparison of its activity with the pure cyclolignans. For most lignans, elevated mortality was not restricted to feeding larvae, but rather, toxicity continued beyond the feeding period on lignan-treated diets.

Dillapiol-treated diet consumed by larvae for 10 days, at

**Figure 4.1** Cumulative percent mortality for *Ostrinia nubilalis* reared ten days on meridic diets treated with lignans at 100 $\mu g \cdot g^{-1}$ diet.


LIGNANS
sesamolin
epiashantin
epiyangambin
cubebin
dihydrocubebin
hinokinin
yatein
ndga
ndga+uv
alpha peltatin
beta peltatin
podophyllotoxin
podophyllin
dillapiol
cpd I
cpd II
0
10
20
30
40
50
60
70
80
90
100
Cumulative percent mortality
adults
pupae
larvae

100 $\mu g/g$, was not only lethal to 63% of the larvae, but also caused a delayed toxicity that contributed to increase the cumulative mortality (to 86.7% and 93.3%) in pupae and adults, respectively (Figure 4.1). As a result, only 6.7% of the insects on a dillapiol diet eventually completed their life cycle. A lower toxicity of dillapiol to the mosquito larvae, *Aedes aegypti*, and to the fruit fly, *Drosophila melanogaster,* has been reported (Lichtenstein *et al.*, 1974), but both assays were performed over a shorter time period. Similarly, assays on the synergistic activity of dillapiol with pyrethrins (Handa and Dewan, 1974), with parathion and carbaryl (Parmar and Tomar, 1983), on the red flour beetle, *Tribolium castaneum* were evaluated within 72 hours. These studies probably revealed only part of the potential toxicity of dillapiol.

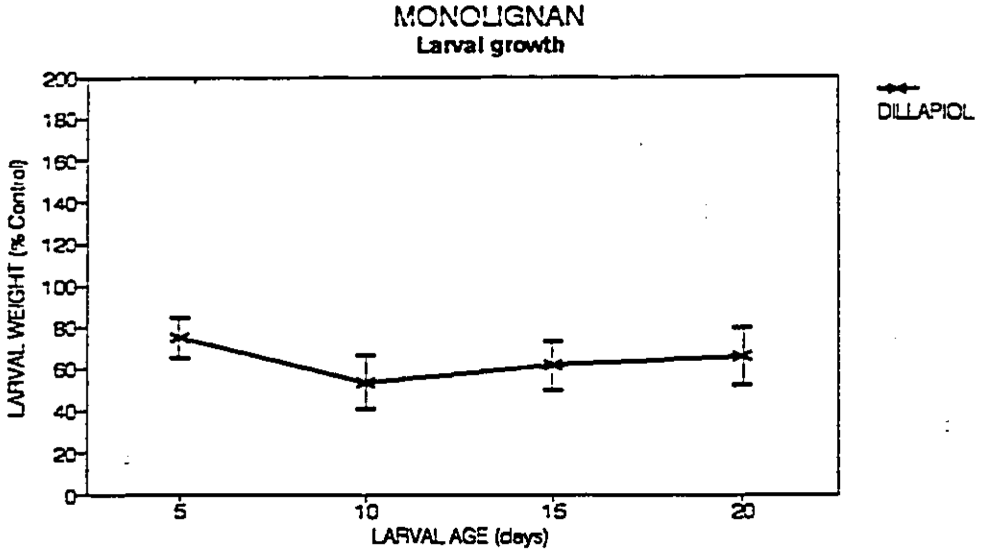
Delayed toxicity was also pronounced in the group of cyclolignans, in the group of furofuran lignans (Figure 4.1). Delayed toxicity is consistent with a hormonal or systemic rather than a neurotoxic mode of action of lignans. Also, most of the activities of lignans on simple organisms, cells or viruses, the inhibition of nucleoside transport, of microtubule assembly, of DNA or viral replication, as with podophyllotoxin-type lignans (Ayres and Loike, 1990; Jardine, 1980; MacRae and Towers, 1984; MacRae *et al.*, 1989), would not be expected to cause an immediate death of the larvae, as would be the case with a knock-down, neurotoxic type of insecticide. Rather, a progressive sickness or physiological disequilibrium leading to premature death might be expected. Inhibition by NDGA of enzymes affecting respiration, mitochondrial transport and specifically NADH-oxidase (Pardini *et al.*, 1970), may have some bearing upon the delayed lethality of this lignan (Figure 4.1). In fact, damage caused to tumours by the three cyclolignans, α-peltatin, β-peltatin and podophyllotoxin, was closely correlated with the inhibition of respiratory enzymes by these lignans (MacRae and Towers, 1984).

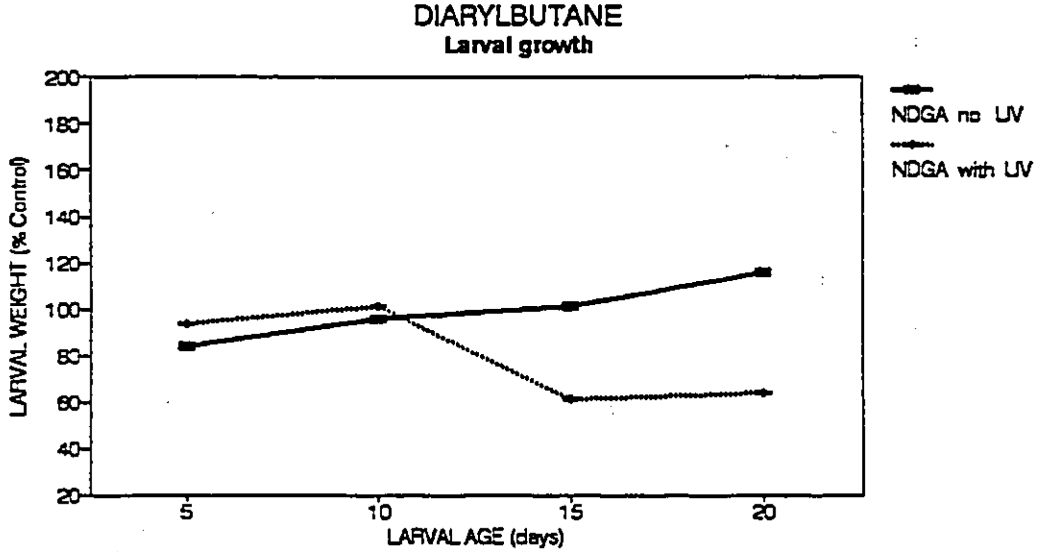
Inhibition of the activity of detoxification enzymes by lignans may also affect their level of toxicity. Sesamolin and cubebin have been shown to inhibit 50% of the ECB polysubstrate monooxygenases (PSMOs) activity *in vitro* with $10^{-4}$M or more (Bernard *et al.*, 1989). What can be considered as a low inhibitory effect *in vitro*, corresponded for these lignans, in the present study, to a low toxicity *in vivo* on the ECB (Figure 4.1). In contrast, the same degree of enzymic inhibition is obtained with $10^{-7}$M dillapiol, in the same study. This monolignan is highly toxic to the ECB.

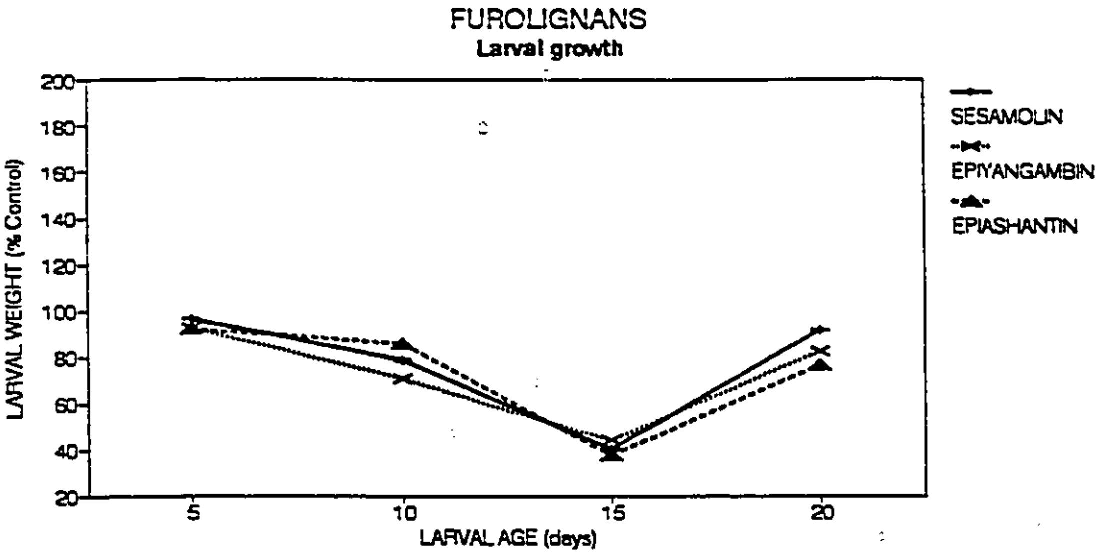
### 4.3.2 Growth reduction

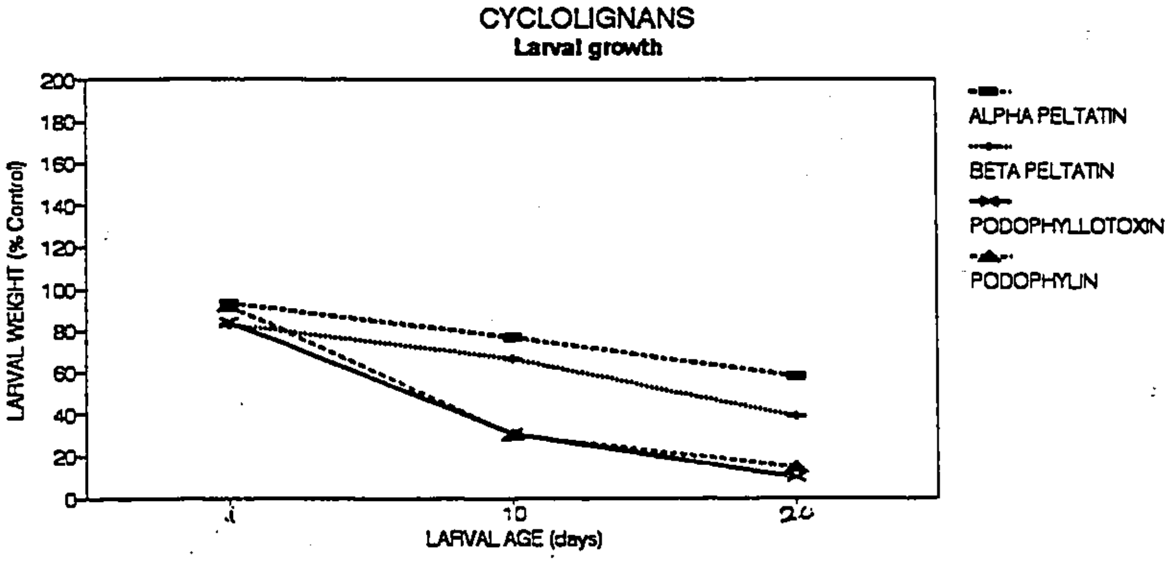
A closer look at the reduction of growth of the surviving larvae fed diets with 100 ppm lignans generally further supports the conclusions from mortality data. The majority of the lignans studied are slow-acting toxins (Figure 4.2).

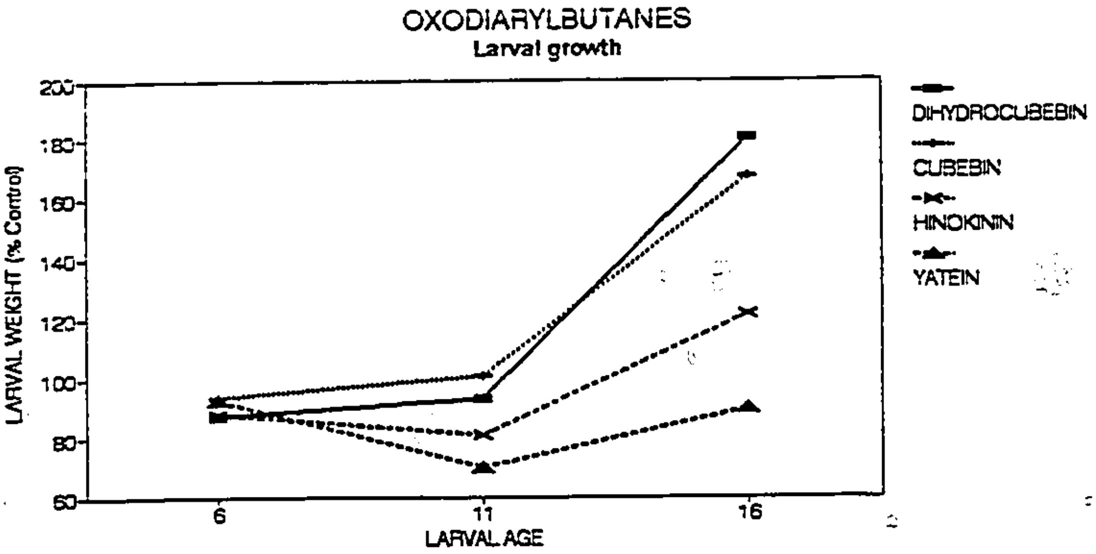
**Figure 4.2** Growth of *Ostrinia nubilalis* during a feeding period of 10 days (from day 5 to day 10 of their larval life) on meridic diets laden with lignans at 100 µg/g fresh diet. Standard deviation was comparable from one group to the next, and are presented for dillapiol and omitted for clarity elsewhere.

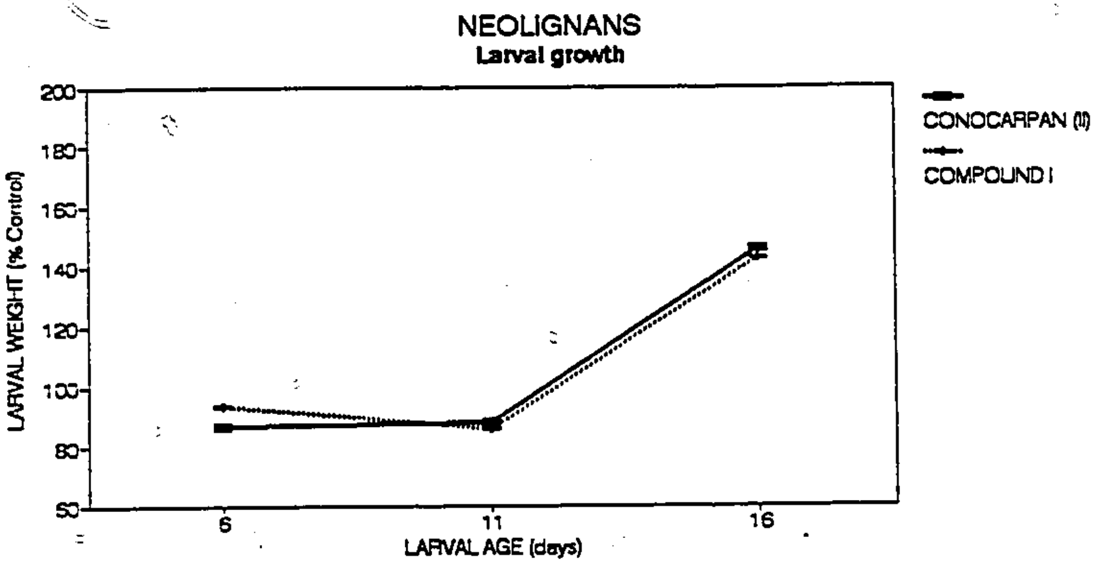

MONOLIGNAN
Larval growth
DILLAPIOL
LARVAL WEIGHT (% Control)
200
180
160
140
120
100
80
60
40
20
0
5
10
15
20
LARVAL AGE (days)


DIARYLBUTANE
Larval growth
NDGA no UV
NDGA with UV
LARVAL WEIGHT (% Control)
200
180
160
140
120
100
80
60
40
20
5
10
15
20
LARVAL AGE (days)


FUROLIGNANS
Larval growth
LARVAL WEIGHT (% Control)
200
180
160
140
120
100
80
60
40
20
5
10
15
20
LARVAL AGE (days)
SESAMOLIN
EPIYANGAMBIN
EPIASHANTIN


CYCLOLIGNANS
Larval growth
LARVAL WEIGHT (% Control)
200
180
160
140
120
100
80
60
40
20
0
5
10
20
LARVAL AGE (days)
ALPHA PELTATIN
BETA PELTATIN
PODOPHYLLOTOXIN
PODOPHYLIN


OXODIARYLBUTANES
Larval growth
LARVAL WEIGHT (% Control)
200
180
160
140
120
100
80
60
6
11
16
LARVAL AGE
DIHYDROCUBEBIN
CUBEBIN
HINOKININ
YATEIN


NEOLIGNANS
Larval growth
LARVAL WEIGHT (% Control)
200
180
160
140
120
100
80
50
6
11
16
LARVAL AGE (days)
CONOCARPAN (II)
COMPOUND I

However, larvae may or may not recover, i.e. recuperate the weight of their controls, depending on the group of lignans considered. Larvae feeding on lignans of the oxodiarylbutane group or neolignans recover after 5 days of treatment, while those feeding on lignans of the furofuran group and on sesamolin recover only after treatment has ceased (Figure 4.2). Moreover, in the cubebin or dihydrocubebin group, larvae become overweight, a reversal of toxicity which appeared to be related to a behavioral response, an overfeeding that will be closer analyzed later in this chapter (section 4.3.4). Growth reduction continuing after the end of the treatment is observed with the cyclolignans as well as with the monolignan, dillapiol (Figure 4.2). The different responses obtained could be an indication of differences in modes of action according the structural group of particular lignans (see Chapter 5).

NDGA is the only phototoxic lignan reported to date (Downum, 1986). As with several polyacetylenes and thiophenes, including phenylheptatriyne at the same concentration of $100\mu g.g^{-1}$ on the ECB (Champagne *et al.*, 1986), no or minimal toxic effect on the ECB was observed under normal light throughout the larval stage, but a 40% growth reduction occurred when short UV radiations was applied even for a short time (UV control were included in the experiment).

### 4.3.3 Antifeedant activity

In order to investigate the specific part played by feeding behaviour in toxicity and growth reduction, we examined the antifeedant properties of lignans in a no-choice leaf disk bioassay.

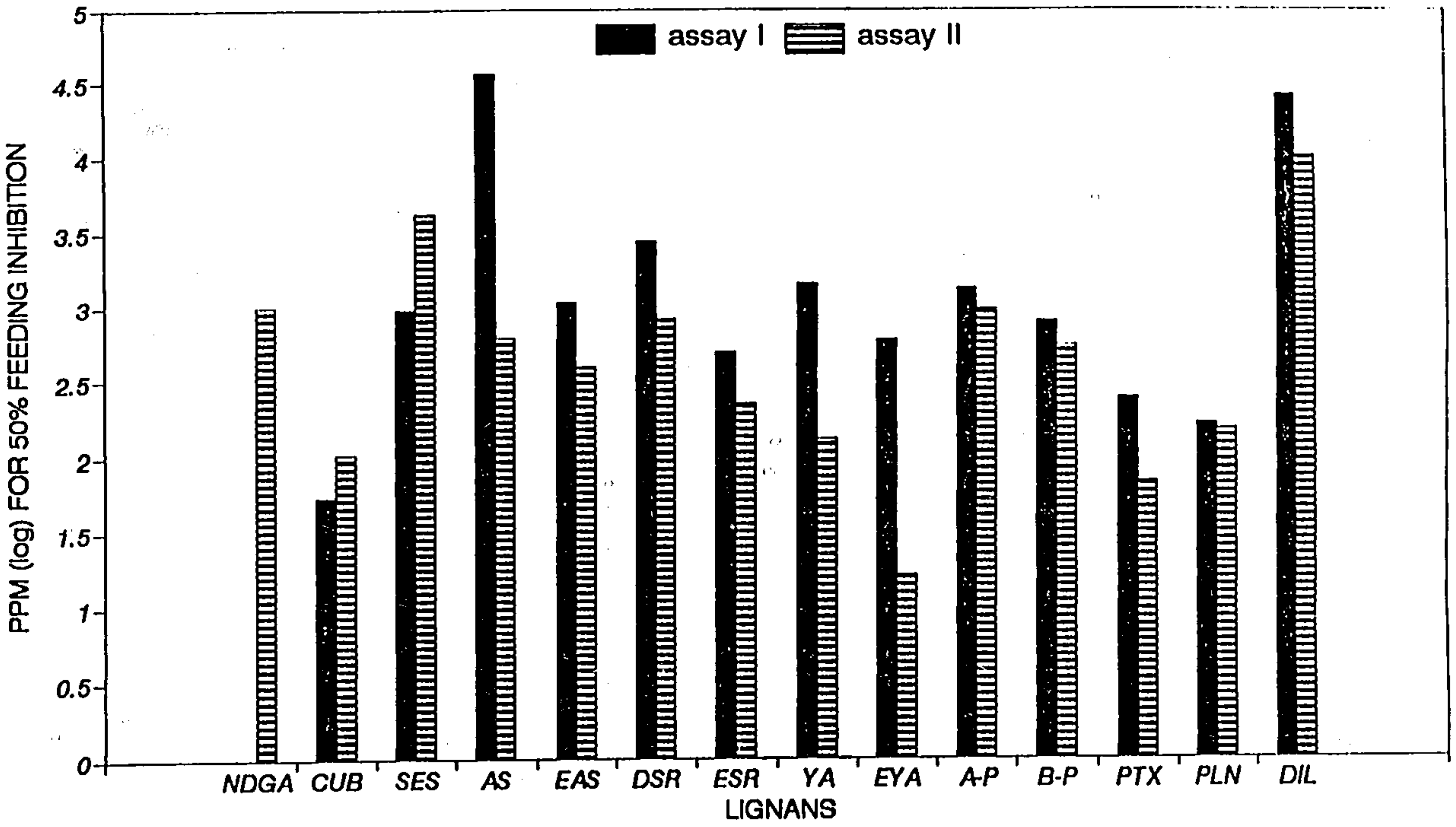
Feeding deterrence is an important factor of toxicity and may largely account for the activity of several allelochemicals such as terpenes, quinones, phenols or coumarins (Koul, 1982). Cubebin, previously found to be a good antifeedant to three grain pests (Harmatha and Nawrot, 1988), was also the best antifeedant in our study ($PC_{50}$ = 74 ppm, Figure 4.3). However, it did not result in a particularly high toxicity to young larvae, and older larvae became overweight (Figures 4.1 and 4.2). Such apparent discrepancy in insect physiological and behavioral responses has been previously reported with insects, particularly the ECB, fed on meridic agar-based diets, in which the agar prevented detection of allelochemicals (Canney and Gardner, 1988).

Our results show that lignans, as a group (Figure 4.3 classified according to their structural group), are moderate antifeedants to the ECB. Dillapiol clearly does not exert its toxicity through deterrence. A dose of 25000 ppm of dillapiol, calculated from the regression curve, would have to be reached to inhibit 50% of feeding in the ECB. In fact, dillapiol remained undetected or ignored over a large range of

Figure 4.3 Feeding response of second instar *Ostrinia nubilalis* larvae to natural lignans, in a no-choice feeding bioassay.

NDGA : nor-dihydroguaiaretic acid
CUB : cubebin
SES : sesamolin
AS : ashantin
EAS : epiashantin
DSR : diasesartemin
ESR : episesartemin
YA : yangambin
EYA : epiyangambin
A-P : α-peltatin
B-P : ß-peltatin
PTX : podophyllotoxin
PLN : podophyllin
DIL : dillapiol.

Assay I and Assay II each include 20 second instar larvae.


assay I
assay II
PPM (log) FOR 50% FEEDING INHIBITION
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
NDGA
CUB
SES
AS
EAS
DSR
ESR
YA
EYA
A-P
B-P
PTX
PLN
DIL
LIGNANS

concentrations (30 ppm to 10000 ppm, data not shown). Large amounts of this compound are found in the leaves of *Piper aduncum* (Table 1.5) which were not avoided by some insect species in the fields (personal observations). In contrast, another monolignan, isoasarone, structurally close to dillapiol, was considered to be a good antifeedant for the fall armyworm, *Spodoptera litura,* at the concentration of 10000 ppm (Matsui and Munakata, 1976). For the group of lignans investigated here, concentrations found to induce 50% inhibition of feeding ($PC_{50}$) ranged from 10 ppm to 3000 ppm (Figure 4.3), a result which further confirms the low participation of feeding inhibition in the toxicity of lignans on the ECB.

Isomeric differences in lignan structures have been reported to greatly affect antifeedant activity (Matsui et al., 1976). A total rejection of food by *S. litura* was obtained with 10000 ppm in the case of eudesmin and 500 ppm with epieudesmin. A similar trend was observed in our three sets of isomers, the epi- series having more antifeedant potential than its counterpart. This suggests a possible role of the stereoisometry of the molecule in antifeedant activity.

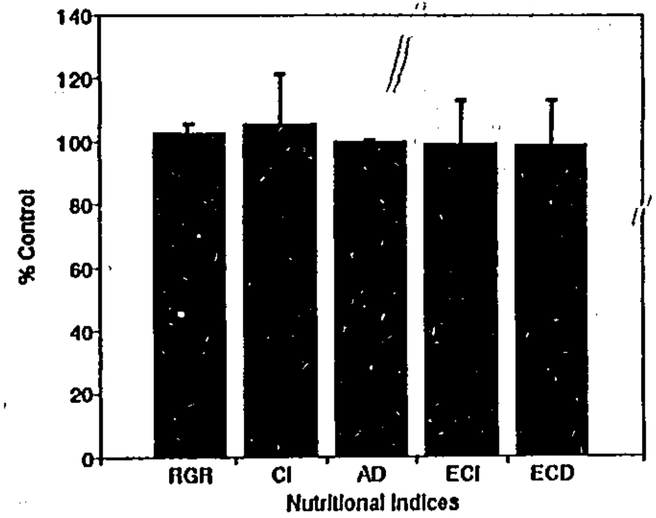
Feeding deterrence does not appear to be a major mode of action of lignans, as determined from our results with the European corn borer. This is not surprising considering that the ECB normally attacks about every wild and cultivated plant, including species with high levels of lignans (Caffrey and Worthley, 1927; Bourret-Bernard, 1988).

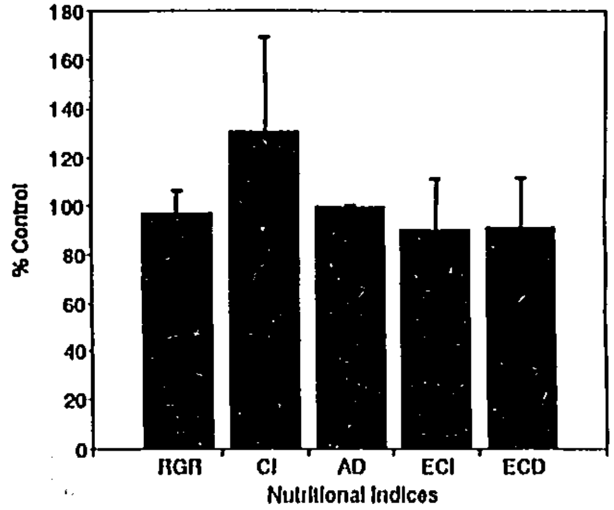
### 4.3.4 Nutrient utilization

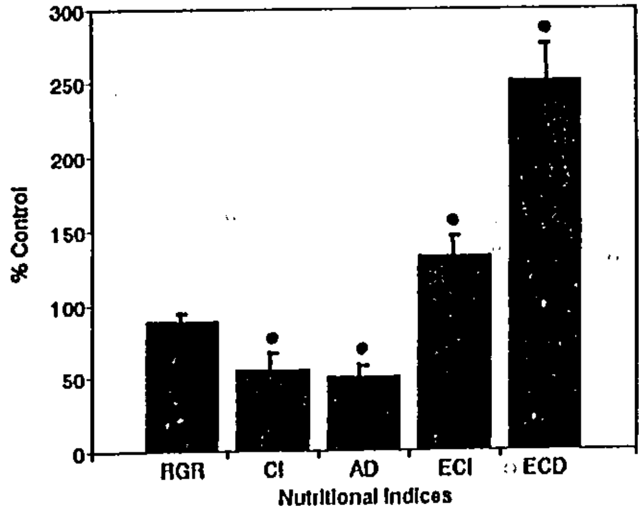
Digestibility (AD) was significantly modified from controls by only one lignan, hinokinin (Figure 4.4), indicating that lignans have, in general, little effect on the accessibility of diet components to the ECB. In light of the known pharmacokinetics of some these low molecular weight compounds, crossing the gut barrier should not represent a problem. Safrol, sesamin, and myristicin are substrates of the P-450 dependent monooxygenases, abundant in the smooth endoplasmic reticulum of the gut wall of most *Lepidoptera* (Ahmad *et al.*, 1986). They may be delivered from there to other part of the body.

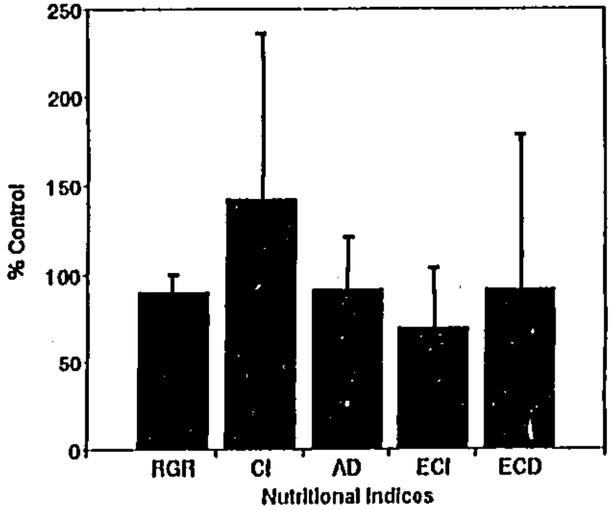
The high food intake (CI) of larvae in the presence of podophyllotoxin, cubebin, epiyangambin, an overall CI increase of 20% to 50% from controls for the group of lignans examined (Figure 4.4), could represent a physiological response to, perhaps, a feeling of starvation induced by a less suitable food, due to the presence of these lignans. A simple dilution of the normal diet may induce up to 200% increase of food consumption in the velvet bean caterpillars, *Anticarsia gemmatalis* (Slansky and Wheeler, 1989). Whether the energetic content of the digested food is insufficient or when a toxin restricts its availability to, or its utilization by metabolic

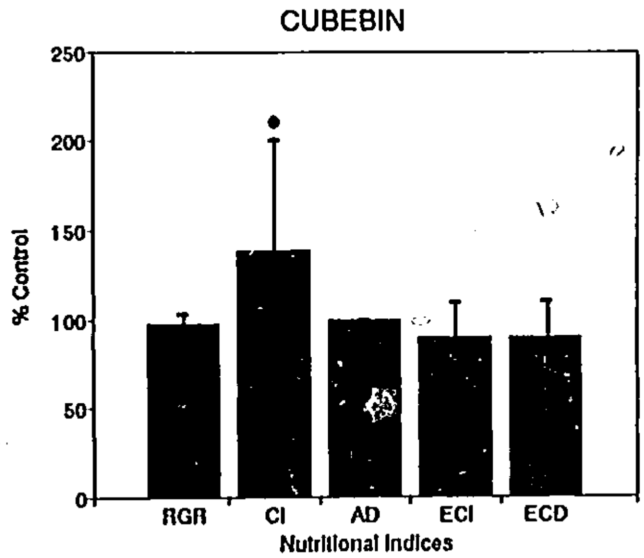
**Figure 4.4** Relative growth rate (RGR) and nutritional indices (CI, AD, ECI, ECD) of *Ostrinia nubilalis* larvae reared, from day 5 to day 10 after hatching, on diets treated with lignans at 100 $\mu g.g^{-1}$ in fresh diet (see section 3.2.3 for definitions). Bars topped with a • are significantly different from their control in Tukey's multiple range test (P=0.05). The mean RGR, CI, AD, ECI and ECD (standard deviation) values of control groups were: 1.7 (0.4), 2.7 (0.6), 0.7 (0.1), 5.3 (1.2) and 0.07 (0.03), respectively.

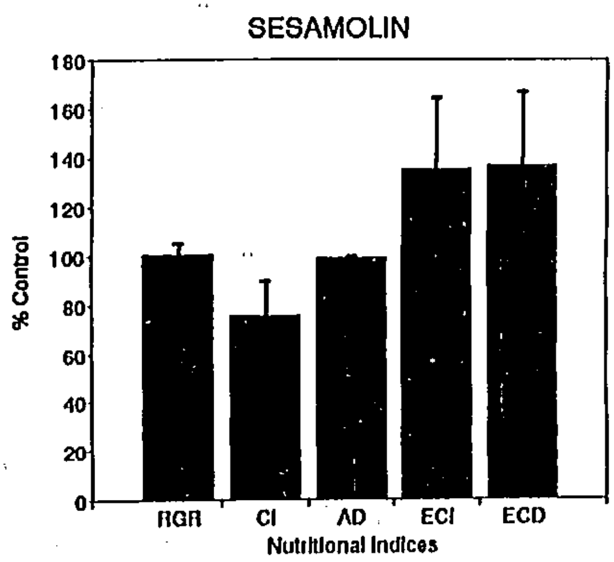

NORDIHYDROGUAIARETIC ACID (no UV)

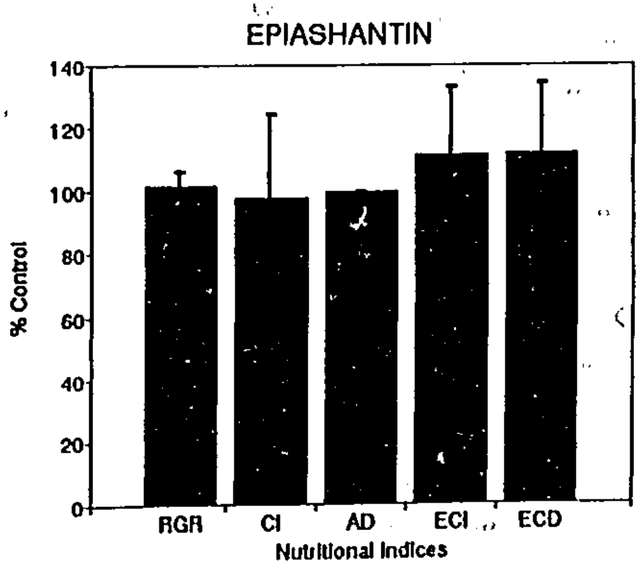

NORDIHYDROGUAIARETIC ACID (with UV)

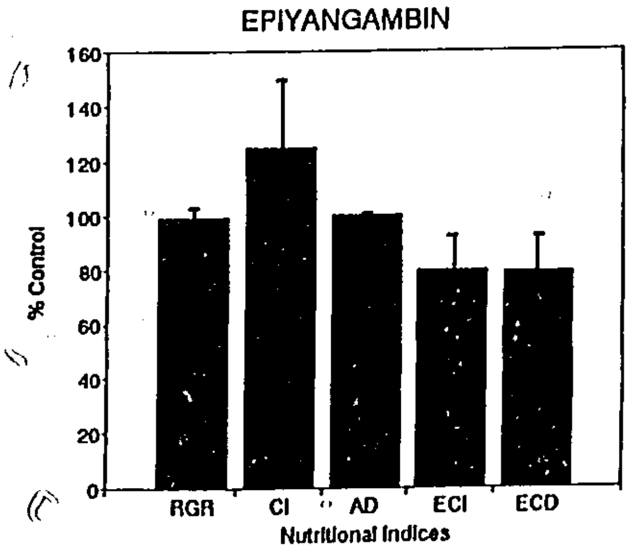

HINOKININ

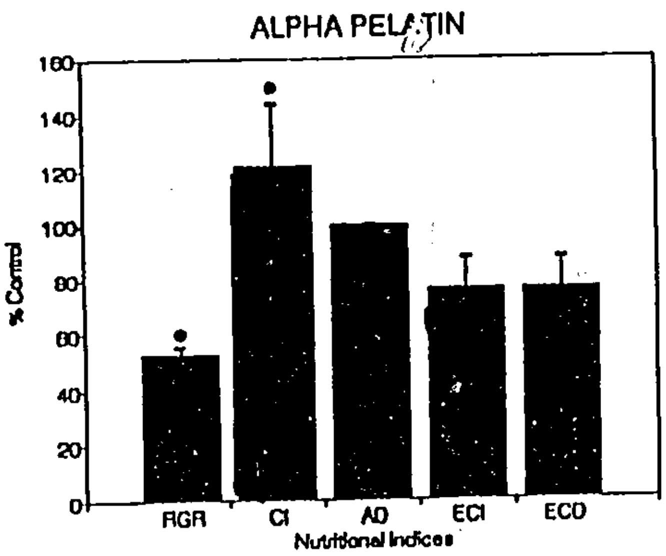

YATEIN


CUBEBIN

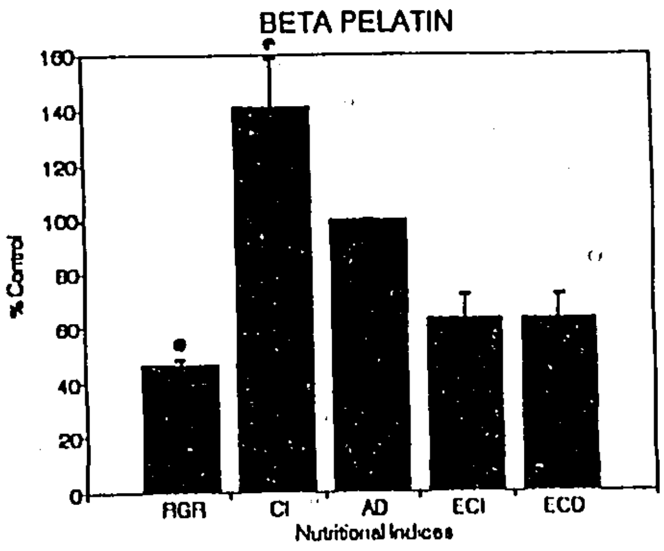

SESAMOLIN


EPIASHANTIN

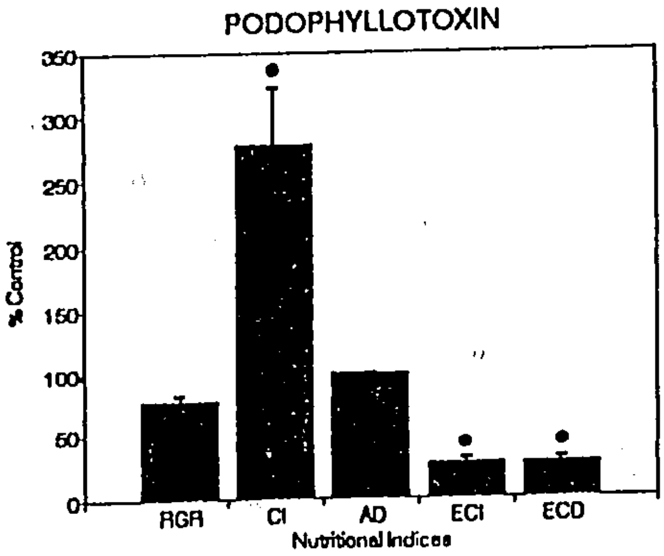

EPIYANGAMBIN



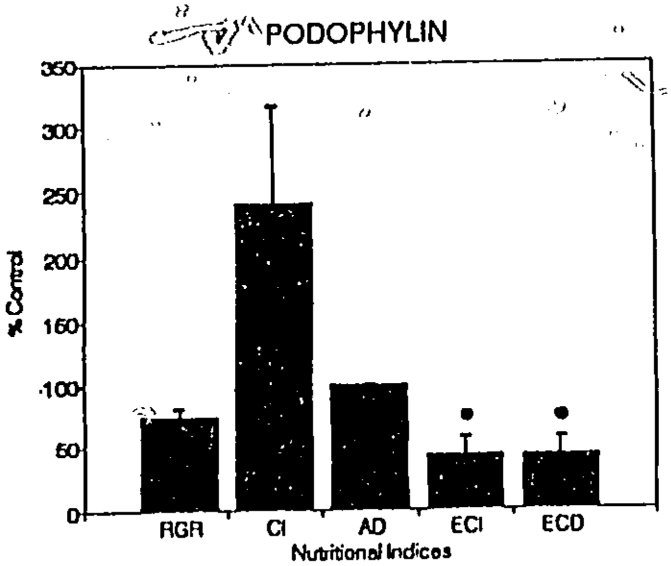
ALPHA PELATIN



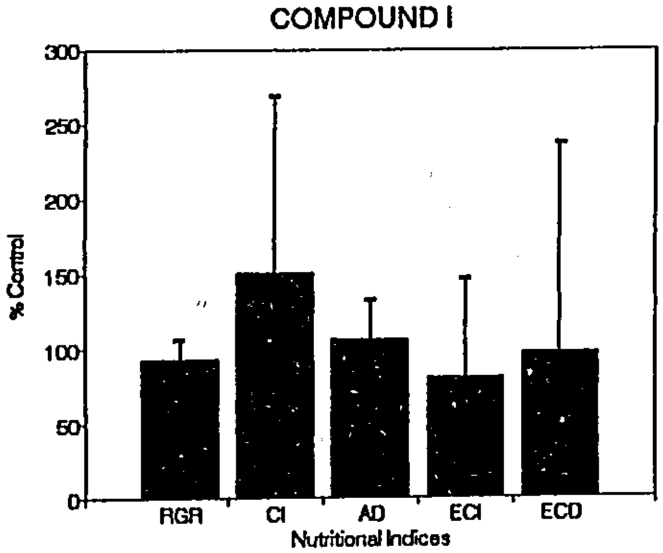
BETA PELATIN

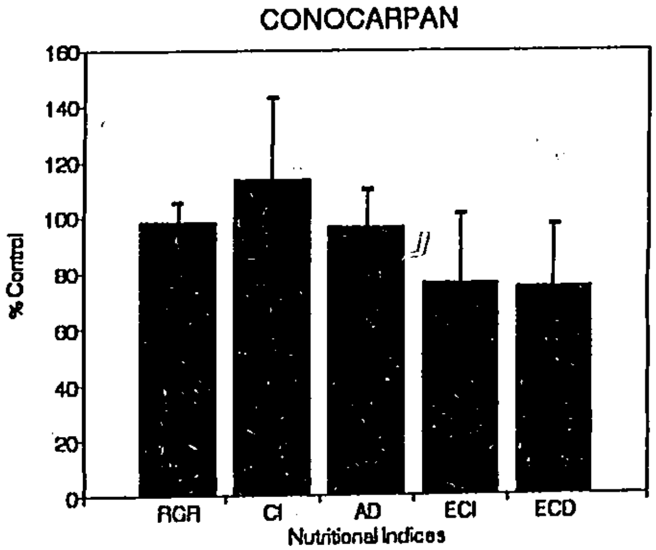


PODOPHYLLOTOXIN



PODOPHYLIN


COMPOUND I


CONOCARPAN

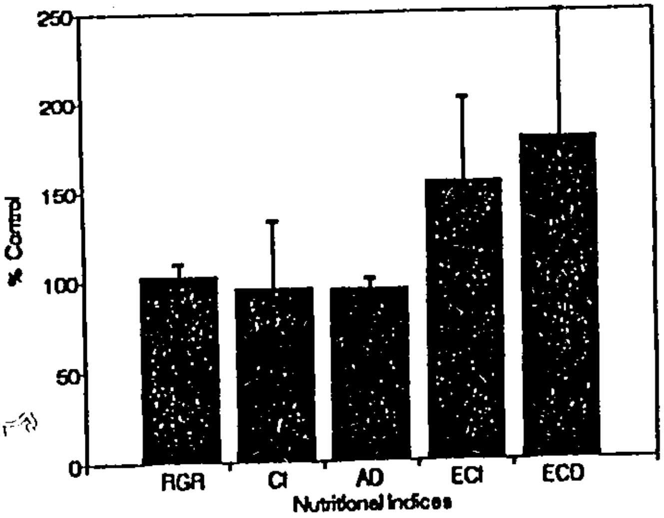
processes, the behavioral answer appears identical: an increased food intake. Such a behaviour partially restores in several cases a normal growth rate.

In contrast, the conversion of assimilated diet to biomass (ECD) was strongly reduced, particularly in the groups treated with podophyllotoxin and related cyclolignans, and to a lesser extent, with several other lignans (Figure 4.4). Cyclolignans also reduced the larval growth to some extent, with an approximate reduction of 70% to 50% (Figure 4.4), in spite of a compensatory increase of food intake. The previously demonstrated high toxicity of cyclolignans (Figures 4.1 and 4.2), could be related to disruption of the capacity of insects to transform food into biomass (decreased ECI and ECD).
High reductions of ECI and ECD are known to accompany the ingestion of a number of highly toxic allelochemicals with different specific modes of action (Beck and Reese, 1976).

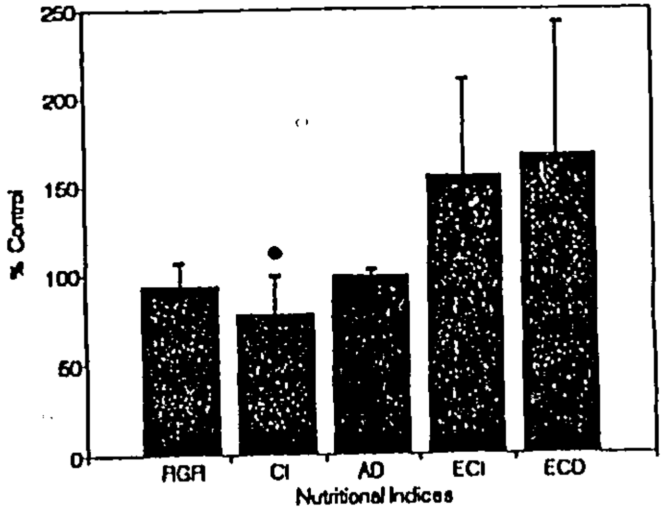
Dorsal topical application of cyclolignans of the podophyllotoxin series on the larvae, while the larvae were feeding on normal diet, revealed a decrease in feeding in this group of lignans (Figure 4.5), rather than a compensatory overfeeding, with up to 50% less food intake for podophyllin. This decreased feeding could be the insect response to a feeling of 'sickness' linked to metabolic disorders due to the lignans. The mechanism leading to the elevation of the ECI and ECD under these experimental conditions cannot be explained at this point. However, further study of this phenomenon may reveal another

**Figure 4.5** Relative growth rate (RGR) and nutritional indices (CI, AD, ECI, ECD) of *Ostrinia nubilalis* second instar larvae fed on normal meridic diet. One µL of a 50 µg.mL$^{-1}$ of the lignan in acetone was applied dorsally on day 6 and on day 8 after hatching. Conditions were otherwise those of Figure 4.4.

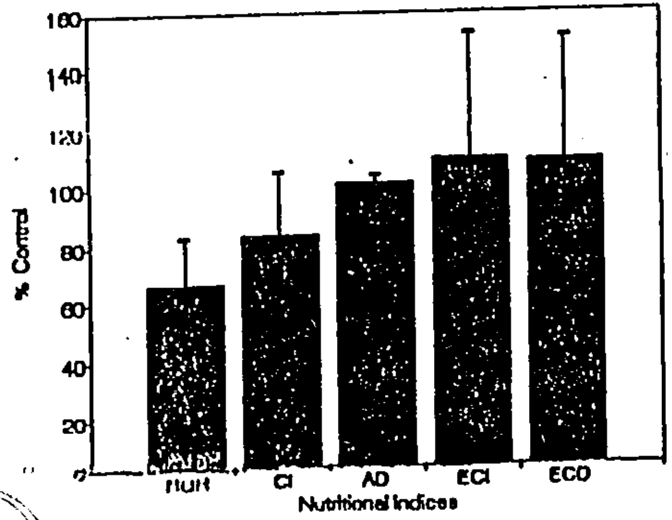
ALPHA - PELTATIN (topical)



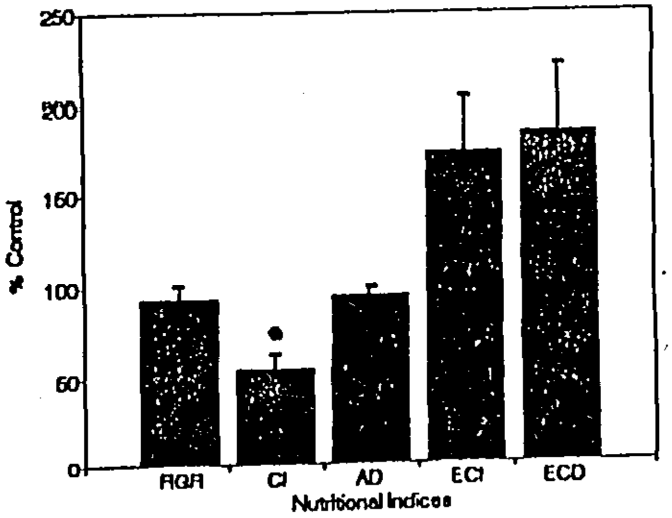
BETA - PELTATIN (topical)



PODOPHYLLOTOXIN (topical)



PODOPHYLIN (topical)



example of the high capacity of the ECB to adjust to its chemical environment.

### 4.3.5 Structure-activity relationships and evolutionary considerations on lignan toxicity

The cyclolignans were probably elaborated late in the evolution of flowering plants, when compared to lignans with a lower degree of oxidation such as NDGA (Davin and Lewis, 1992). Lewis and Davin (1994) propose an evolutionary scenario for lignans, by which a two phenylpropane structure gradually evolved from an open propane chain to a furan ring, with a later addition of a lactone or hydroxyl moiety. Also, the degree of oxidation of the phenolic rings gradually increased, with the replacement of one to three of the hydrogens of the phenol rings with one or more methoxy groups and/or the formation of methylenedioxy (MDP) group. A further step was putatively the cyclization of the propane chain to the phenol, such as seen in podophyllotoxin type lignans (Figure 1.2).

The level of toxicity of the lignans of our study appears to generally parallel their position in this evolutionary sequence. The simpler lignan, NDGA, has a low toxicity, except when photoactivated. Among the furofuran series, the advantage, for the toxic effect on animals, i.e. for the defense of the plant, of a methylenedioxy ring over two or three methoxy groups is not clear. The advantage of an MDP has been associated with the inhibition of the detoxification enzymes in insects (Casida, 1970; Ahmad et al.,1986). The best inhibitor in the present study, dillapiol, possesses this group. More specific features, such as the position of the methoxy group, can also confer advantages to the plant. It has been shown that the presence of a 3-oxomethylene is more essential to the insecticidal activity than a 4-oxomethylene (Yamauchi *et al.*, 1992). However, the lignans of plant origin studied here are MDP or 3-oxomethylene lignans (Appendix I).

Further understanding of the different ways by which plant lignans affect herbivores, particularly insects, and the mechanism of action of these allelochemicals, is needed before an explanation of their diversity in plants can be made. To do this will necessitate more ecologically and physiologically relevant research.

# CHAPTER 5

# EFFECT OF LIGNANS ON MULTIDRUG RESISTANCE PHENOTYPES

## 5.1 Introduction

Multi-resistance to insecticides most probably arises from a combination of several mechanisms (Gottesman and Pastan, 1993). One of them is the induction of cytochrome P-450 mediated polysubstrate monooxygenases (PSMOs), which results in an increased biotransformation of the insecticide and the reduced exposure of cellular targets in insects to toxic levels of the insecticide. Resistant insects become resistant not only to the insecticide that triggered the enzyme induction, but also to a number of chemically unrelated insecticides. Insecticide synergists which can prevent or inhibit specific detoxication enzymes are useful tools in the management of such cases (Raffa and Priester, 1985).

Another, more recently uncovered, mechanism of resistance hinders cancer chemotherapy. Often, tumours which successfully respond to a first treatment, have become insensitive to further therapy, even to drugs to which they have never been exposed. These cancer cells have developed a multidrug-resistant (MDR) phenotype. One of the mechanisms by which the phenotype arises is via increased expression of one or more genes of a highly conserved multidrug resistance (MDR) gene family (Gottesman and Pastan, 1993). These genes code for a transmembrane protein, called P-glycoprotein (for Permeability-glycoprotein: P-gp), which, in resistant cells, appears to pump out of the intracellular space a number of chemically unrelated xenobiotics, including plant allelochemicals and their derivatives commonly used as chemotherapeutic agents (Gottesman *et al.*, 1994). By removing these drugs from cells, the P-gp maintains the exposure and binding of the drugs to their intracellular target at a non-toxic level.

In humans, the presence of P-glycoprotein has been demonstrated in several excretory sites, on the luminal face of the intestine and the colon, on the luminal border of proximal tubules, on the biliary face of hepatocytes as well as in the endothelium of cortex and testis capillaries (review in Gottesman and Pastan, 1993). The high expression of the MDR1 gene in germinal cells during embryogenesis is also thought to ensure the protection of the embryo against the toxicity of xenobiotics (Oudard and Poupon, 1993).

Lignans and their derivatives interfere with the toxicity of xenobiotics. They are insecticide synergists that inhibit the activity of the PSMOs in an insect microsomal preparation (Bernard *et al.*, 1989). In addition, there is a possibility that other, perhaps complementary, mechanisms of resistance, such as the P-gp, may be affected by xenobiotics. Gant *et al.* (1991) demonstrated that two inducers of the cytochrome P4501A enzymes, 3-methylchoranthrene and 2-acetylaminofluorene, were also efficient inducers of the multidrug-resistant (MDR1) gene coding for P-gp. Also, in the nicotine-resistant tobacco hornworm, *Manduca sexta,* physiological resistance to several alkaloids resulted from the combined activity of detoxifying enzymes as well as that of a P-glycoprotein-like pump at the excretory malpighian tubule and possibly at the blood-brain barrier (Morris, 1983; Murray *et al.*, 1994). There is also some evidence that a multidrug resistance gene, closely related to the mammalian MDR1 gene, is involved in the resistance of the malaria parasite, *Plasmodium falciparum*, to chloroquine (Foote *et al.*, 1989). Verapamil, a P-gp inhibitor, could reverse chloroquine resistance *in vitro* (Ye and Van Dyke, 1994). Also, the description of a MDR-like gene in the fruit fly, *Drosophila melanogaster* (Schinkel and Borst, 1991), further suggests that a P-glycoprotein-like mechanism plays a role in insect resistance to xenobiotics.

One of the partial structural requirements for a modifier of P-glycoprotein activity appears to be the presence of two planar aromatic rings (Pearce *et al.*, 1989), as in lignans. In addition, binding to P-glycoprotein, a prerequisite for a direct action on the P-glycoprotein pump, has been demonstrated with one lignan, phyllanthin (Somanabandhu *et al*., 1993).

The present study was designed to investigate the activity of a group of lignans on the MDR phenotype in a cultured cell model which included cell lines selected for multidrug-resistance as well as cells transfected with MDR1 gene that confers the P-glycoprotein phenotype.

## 5.2 Material and Methods

### 5.2.1 Cell lines

Cultured NIH/3T3 (later called NIH) cells, established from NIH Swiss mouse fibroblast cultures were used, and their multidrug resistant derivatives transfected with a human MDR1 gene, NIH-MDR-G185 (G185), provided by the National Cancer Institute, Bethesda, MD, USA. The Chinese hamster ovarian cell lines, the parental, AUXB1 and its two MDR lines successively selected with colchicine, $CH^RC5$ (C5) and $CH^RB30$ (B30), were kindly supplied by Dr V. Ling, Ontario Cancer Institute, Toronto, Canada. All three multidrug resistant cell lines have exhibited stable level of cross resistance to anthracyclines, etoposide (VP-16) and Vinca alkaloids for several years, with highest level of expression of P-glycoprotein in B30 (Ling *et al.*, 1983).

Cells were grown in plastic flasks (Falcon) in a humidified atmosphere at 37°C, 5% $CO_2$. The mouse cells, NIH and G185, were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco BRL) containing glutamine, and the hamster cells, AUXB1, C5 and B30, in alpha minimum essential medium (αMEM, Gibco BRL), containing nucleosides and glycine. All culture media were supplemented with 10% fetal calf serumalbumin (Wittaker Bioproducts), penicillin (50 units/mL) and streptomycin (50 μg/mL) (Gibco BRL). Resistance levels were maintained with a weekly addition of colchicine to the cultures, 0.06 μg/mL in G185, 15 μg/mL in C5 and 30 μg/mL in B30.

### 5.2.2 Compounds

The following pure lignans or neolignans were either extracted or derivatized in our laboratory: α-peltatin, β-peltatin, podophyllotoxin and podophyllin (Dr T Durst, Department of Chemistry, University of Ottawa), dillapiol and its 5 derivatives (S Delorme). Other pure lignans, sesamolin, ashantin, epiashantin, diasesartemin, episesartemin, yangambin and epiyangambin, were kindly supplied by Dr J Lam (Department of Chemistry, University of Aarhus, Denmark), and cubebin, dihydrocubebin, hinokinin and yatein by Dr J Harmatha (Czeck Academy of Sciences, Prague, Czech Republic). The nordihydroguaiaretic acid was kindly supplied by Dr K Downum (Department of Biological Sciences, Florida International

University, Miami, USA). Other pure compounds resulted from work previously reported in this study (see Chapter 3). Compound I and conocarpan are neolignans from *Piper decurrens (Piperaceae)*, piperine and piperlonguminine are isobutylamides from *Piper tuberculatum, (Piperaceae)*, and VK1 is a neolignan from *Virola kochnii (Meliaceae)*. Piperonyl butoxide (PBO) was purchased from Sigma. Stock solutions of the lignans were kept at 4°C in 50% EtOH (in dimethylsulfoxide (DMSO) for the highest concentration of dillapiol and PBO presented in Table 5.1).

### 5.2.3 Determination of resistance levels

A screening of the dose toxic to the four cell lines (NIH, G185, AUXB1 and B30) was achieved with lignan final concentrations in the assay from 0.25 ng/mL to 25000 ng/mL. Adherent cells in the culture flask were detached with a solution of trypsin (0.5% in PBS with 0.2% EDTA), centrifuged at 1000 rpm for 15 minutes and resuspended in medium at a final concentration of 500 cells/mL. One mL of the cell suspension was distributed in each well of a 24-well plates (Falcon), already containing one mL of the lignan, or other allelochemical, at the appropriate concentration (at least 6 concentrations). Control wells contained cells and the solvent (0.1% EtOH or DMSO) used to solubilize the drug. Cells were incubated for 8 days at 37°C, 0.5% $CO_2$. The media were discarded and cell colonies stained with 0.25% methylene blue (in 50% EtOH) for 15 min. The concentration beyond which cell growth ceases was used for the assessment of the toxicity of the compounds.

### 5.2.4 Drug accumulation and transport: Uptake of $^3$H-vinblastine.

Drug accumulation studies were carried out at room temperature following a method modified from Lemontt *et al.* (1988). In short, trypsinized cells were washed and resuspended in PBS-glucose (10 mM), at a concentration of 1.5 X $10^6$ cells/mL. At specific intervals (1, 15, 30, 60 and 90 min) after mixing the 1 mL cell suspension with 2 $\mu$l $^3$H-vinblastine/mL (specific activity, 11 Ci/mM), with or without the potential resistance modifying compounds, 300 $\mu$L of the mixture were layered onto 250 $\mu$L of a silicone oil and mineral oil mixture (4:1, v:v; Aldrich silicone oil, d = 1.05 : Fisher light mineral oil), and immediately centrifuged 10 sec at 14 000 rpm in a countertop Eppendorf centrifuge. The aqueous phase was removed, the upper part of the tube rinsed with 500 $\mu$L PBS to eliminate risks of contamination of the pellet with the unincorporated radioactivity, the oil removed and the pellet digested overnight in 500 $\mu$L NaOH 0.1N. Five mL of scintillation liquid were added to the pellet and counting of tritium activity was done on a Beckman LS1701 scintillation counter.

The validity of the system was established by using verapamil (15 μM), a specific P-gp inhibitor, as positive control in each experiment.

## 5.3 Results and discussion

### 5.3.1 Resistance level of cells treated with lignans and other compounds

In this study, we discovered that lignans generally produce one of two types of response in the MDR cell lines (Table 5.1). Lignans of the cyclolignan series behaved like classical substrates of P-glycoprotein (P-gp), as does the derivative of podophyllotoxin, the anticancer drug etoposide (VP16). In contrast, lignans of the furan series, the monolignan and neolignans, were more toxic to the P-gp rich MDR cells than to their parental P-gp poor cells, a phenomenon called collateral sensitivity which has been described with a few agents of various origin (see Table 1.7).

Sensitivity to lignans and other modulating compounds varied with the cell line studied. The murine fibroblast MDR cells, G185, were generally more resistant to lignan toxicity,

Table 5.1 : TOXICITY of plant-derived LIGNANS to SENSITIVE (NIH and AUXB1) and MULTI DRUG RESISTANT (G185 and B30) cell lines.

| @ LIGNANS | * MIC (ng/ml) | | | |
|---|---|---|---|---|
| | NIH | G185 | AUXB1 | B30 |
| **FURANS** | | | | |
| ashantin | >2500 | >2500 | >2500 | 25 |
| epiashantin | >2500 | >2500 | >2500 | 25 |
| diasesartemin | >2500 | >2500 | >2500 | 25 |
| episesartemin | >2500 | >2500 | >2500 | 25 |
| diayangambin | >2500 | >2500 | >2500 | 25 |
| epiyangambin | >2500 | >2500 | >2500 | 25 |
| sesamolin | >2500 | >2500 | >2500 | >2500 |
| **OXODIARYLBUTANES** | | | | |
| cubebin | | | 25000 | 2500 |
| dihydrocubebin | - | - | 12500 | - |
| hinokinin | - | - | 12500 | - |
| yatein | - | - | 200 | 200 |
| **CYCLOLIGNANS** | | | | |
| podophyllotoxin | 250 | 250 | 2.5 | 250 |
| alpha-peltatin | 2.5 | 2.5 | 2.5 | 25 |
| beta-peltatin | - | 2.5 | 0.25 | 25 |
| podophyllin | 2.5 | 2.5 | 2.5 | 250 |
| etoposide: (der. of podophyllotoxin) | 25 | 250 | 250 | 2500 |
| **MONOLIGNANS** | | | | |
| dillapiol | >500 | >500 | >500 | 0.5 |
| piperonyl butoxide (der. of dillapiol) | 50 | 5 | 50 | 0.5 |
| **NEOLIGNANS** | | | | |
| from *Piper decurrens*: | | | | |
| HG1 | >2500 | >2500 | >2500 | 250 |
| conocarpan | >2500 | >2500 | >2500 | 250 |
| from *Virola kochnii*: | | | | |
| VK1 | >2500 | >2500 | >2500 | >2500 |

* MIC: Minimum Inhibitory Concentration for cell growth (no growth over a period of 8 days).

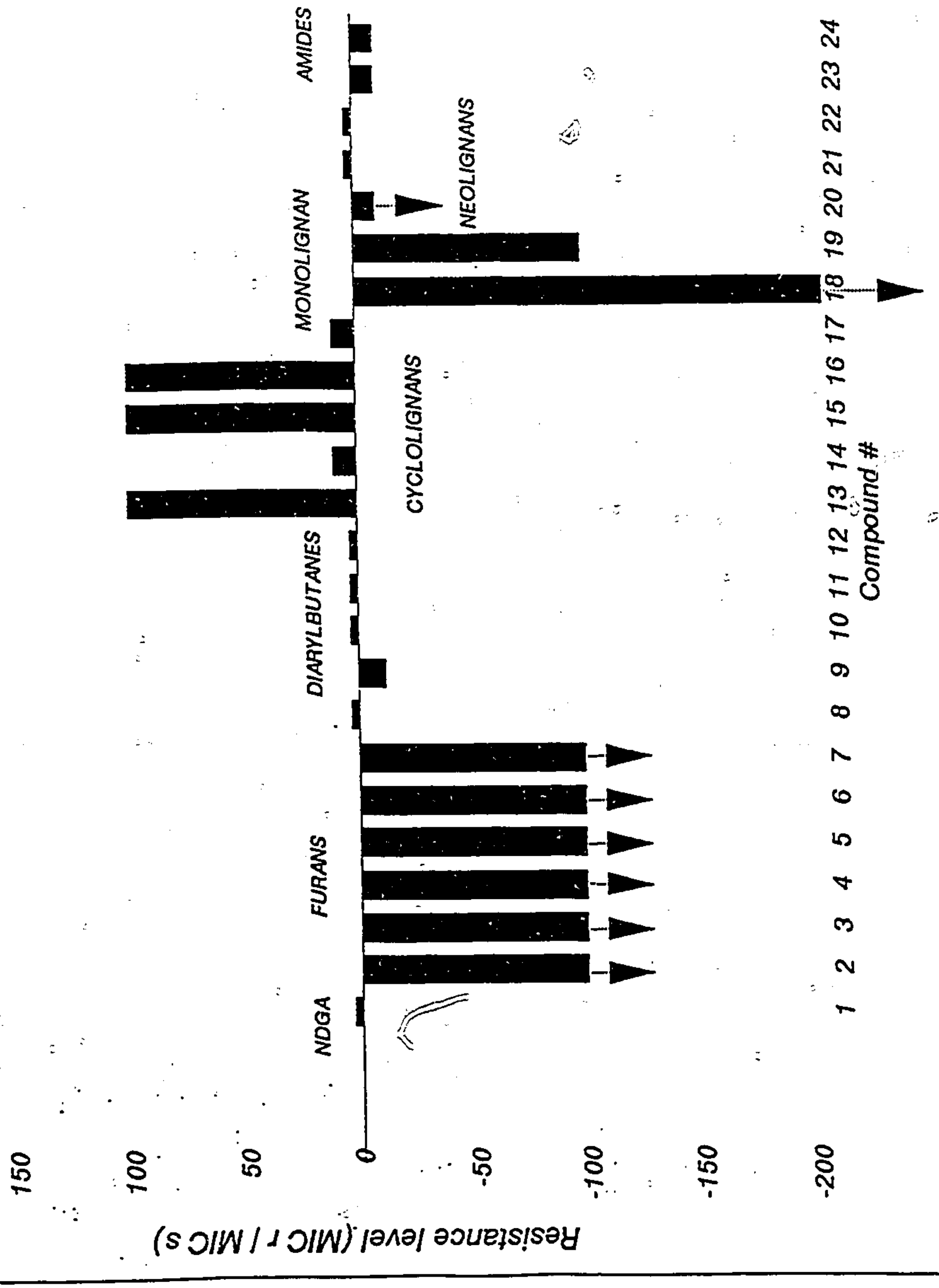
@ : Concentrations of lignans for the assays were: 0.05, 0.5, 5, 50 and 500 ng/ml for the monolignans; up to 50000 ng/ml for oxydiarylbutanes; 0.25, 2.5, 25, 25, and 2500 for all others.

Figure 5.1 Level of resistance of hamster multidrug resistant cells to a series of lignans and other compounds. An arrow indicates that the actual level of resistance lies beyond the concentration range assayed.

MIC: minimum level of the compound needed for the inhibition of cell growth, in drug sensitive (MIC s) and multidrug resistant (MIC r) cells.

COMPOUNDS #:

1: nordihydroguaiaretic acid
2: ashantin
3: epiashantin
4: diasesartemin
5: episesartemin
6: yangambin
7: epiyangambin
8: sesamolin
9: cubebin
10: dihydrocubebin
11: hinokinin
12: yatein
13: podophyllotoxin
14: α-peltatin
15: β-peltatin
16: podophylin
17: etoposide
18: dillapiol from *Piper aduncum*
19: piperonyl butoxide
20: conocarpan from *Piper decurrens*
21: neolignan from *Piper decurrens* (hg2)
22: neolignan from *Virola kochnii*
23: piperlonguminine from *Piper tuberculatum*
24: piperine.


Resistance level (MIC r / MIC s)
150
100
50
0
-50
-100
-150
-200
Compound #
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
NDGA
FURANS
DIARYLBUTANES
CYCLOLIGNANS
MONOLIGNAN
NEOLIGNANS
AMIDES

particularly to lignans of the furan series, than the hamster MDR cells, B30 (Table 5.1). The range of lignan concentrations assayed was limited by their solubility in the culture media. Consequently, the minimum inhibitory concentrations (MIC) to induce mortality in the murine cell lines, NIH and G185, as well as in the sensitive hamster line, AUXB1, could not always be precisely evaluated with the furano-lignans, the neolignans or dillapiol. The MIC was above 2500 ng/mL and 500ng/mL for the furans and dillapiol, respectively.

Comparing the cytotoxic effects of a series of lignans in sensitive and multiresistant cell lines revealed striking differences in cell specificity. These are presented for the first time here. Multidrug resistant sublines appeared several fold more sensitive to a number of lignans, than their parental cells (Table 5.1). They display collateral sensitivity. This was particularly true with the hamster cell lines (Figure 5.1). Furano-lignans, monolignans, and, to a lesser extent, cubebin as well as one of the neolignans, had a resistance ratio below one (MICr, multidrug resistant / MICs, sensitive).

Collateral sensitivity of MDR cells, i.e. an MDR phenotype leading to hypersensitivity to drugs, has been reported with a few chemicals of various origin (Loe and Sharom, 1993) (see also Table 1.7). However this phenomenon is poorly understood. Another lignan, phyllanthin, an oxodiarylbutane, is also more toxic to the multiresistant cell line KB-V1 ($LD_{50}$ = 9$\mu$g/mL), than to its drug-sensitive parental line KB-3 ($LD_{50}$ > 20$\mu$g/mL)

(Somanabandhu *et al.*, 1993). Collateral sensitivity is reported in MDR hamster ovary carcinoma ($CH^RC5$ and $CH^RB30$), and in MDR cells of other origin (P388), with membrane active agents, including detergents, anaesthetics and steroids (Bech-Hansen et *al.*, 1976; Riordan and Ling, 1985). Schuurhuis *et al.* (1990) report the same phenomenon with several MDR modifiers, including calmodulin inhibitors (trifluoperazine), calcium channel blockers (verapamil, bepridil) and a few other non-related compounds. Changes in membrane permeability and composition were evoked as a possible mechanism for collateral sensitivity, as in doxorubicin-resistant P388 cells (Ramu *et al.*, 1983). Ultrastructural changes in the membrane layer of MDR cells and modifications of its physical properties have also been described by Arsenault *et al.* ( 1988), and by Kessel (1988).

In a series of MDR cell lines with different degrees of colchicine resistance and different P-glycoprotein expression levels, including the colchicine selected multiresistant hamster cells C5 and B30, Loe and Sharom (1993) showed that collateral sensitivity to a series of detergents is directly linked to the MDR phenotype. Whereas the level of resistance to colchicine ($IC_{50}$ resistant cells / $IC_{50}$ sensitive cells) for C5 and B30 cells, relative to AUXB1 drug-sensitive cells, was 96 and 680, respectively, the ratio was inverted with nonionic phenolic detergents with polyethylene chains of various length, such as Tween 80 (0.4 and 0.9), Triton X-100 (0.6 and 0.4) and a phenolic compound of small molecular weight called DBP (0.2 and

0.06), respectively for C5 and B30 (see Table 1.7). The phenomenon was eliminated in revertant, drug-sensitive, cells CH$^{R}$I10 selected from CH$^{R}$C5. This indicates that both hypersensitivity and resistance to these detergents are an intrinsic part of the MDR phenotype in these cell series (Loe and Sharom, 1993).

Collateral sensitivity to the group of furanolignans was less marked, in murine MDR cells transfected with the human MDR1 gene, G185, than in Chinese hamster ovary cells selected for its MDR phenotype, B30 (Table 5.1). MDR G185 cells were killed by a dose of piperonylbutoxide (PBO) 10 times lower than the parental NIH cells , while the dose for MDR B30 cells was 100 times lower than for AUXB1 (Table 5.1). A similar result was reported by Loe and Sharon (1993). MDR Chinese hamster LR73 cells transfected with the murine MDR1 gene, were less hypersensitive to a series of detergents than MDR hamster cell lines overexpressing endogenous hamster P-gp genes. This was, in spite of an *MDR1* expression level in the transfected cells comparable to that of the CH$^{R}$C5 line, as estimated by P-glycoprotein-specific monoclonal antibody (Loe and Sharom, 1993). This strongly suggests that changes in MDR cell membranes, other than an elevated expression of P-glycoprotein, accompanies the selection for multidrug resistance.

It is known that during the amplification of the P-gp gene, adjacent genes (at least 6 other genes) are also amplified and overexpressed (Bradley *et al.*, 1988). Therefore, it is possible in the case of the MDR cells C5 and B30, that collateral sensitivity to various compounds in MDR cells may be due to coamplification of yet unknown gene(s), beside P-gp-coding genes.

It is interesting to note that the hamster MDR B30 cell line was extremely sensitive to all lignans with a furofuran structure, except sesamolin. The insertion of an oxygen between furan and phenolic rings, as in sesamolin, resulted in a dramatic elimination of the selective collateral sensitivity of B30 to the group of furanolignans (Table 5.1 and Figure 5.1).

The anti-tumour lignan, podophyllotoxin, as well as α-peltatin and ß-peltatin, affected AUXB1 and B30 cells, in a way similar to etoposide (VP-16). Etoposide is a derivative of podophyllotoxin used in human cancer therapy to which tumours eventually become resistant (Oudard and Poupon, 1993), a resistance related to the overexpression of P-gp (Gottesman and Pastan, 1993). Multiresistance appears to be extended to the whole group of natural lignans related to etoposide, as well as to the mixture of lignans, podophyllin (Table 5.1; Figure 5.1).

The common structural feature of the podophyllotoxin group is a cyclization at $C_{2-7'}$, an additional C ring, forcing a rigid conformation of the molecule. Rigidity of etoposide, and other podophyllotoxin derivatives, was evoked to explain the mechanism of action of these molecules, particularly their interaction with tubulin (Ayres and Loike, 1990). Steric configuration was aslo an important structural features for a P-gp substrate in a series of alkaloid derivatives (Pearce *et al.*, 1989; Chevallier-Multon *et al.*, 1990). Further investigations are needed to shed light on the steric requirements for the interaction of cyclolignans with the P-glycoprotein.

The largest effects on the MDR phenotypes were obtained with the natural insecticide synergist, dillapiol, and with its derivative, piperonyl butoxide (PBO). Concentrations of PBO that inhibit cell growth in MDR cells (0.5 and 5 ng/mL with B30 and G185, respectively), are far below the concentrations found in currently commercialized insecticidal mixtures (Worthing and Walker, 1990). Similarly, the very low MIC of dillapiol (0.5 ng/mL with B30), is far below the concentration found in plants, in particular the *Piperaceae* (see section 1.1).

As this could prove to be of significance in the management of resistant tumour cells, and potentially of resistant insect pests, further attention was given to the mechanism of action of dillapiol on multidrug resistant cells.

### 5.3.2 Modification of the uptake of $^3$H-vinblastine in MDR cells by dillapiol and other lignans

A number of mechanisms have been shown to be involved in the cross-resistance to drugs, both in *in vitro* and *in vivo* systems: a decreased uptake of the drugs, an increased energy-dependant efflux, a lower affinity to intra cellular binding sites as well as a slower conversion of the drugs to alkali-labile materials, when compared to drug-sensitive cells (review in Gottesman *et al.*, 1994). Agents that modify resistance patterns of MDR cells may act upon any one, or combinations, of these mechanisms. The net accumulation of $^3$H-vinblastine by snesitive and MDR cells was measured in search for an explanation to the differential toxicity of dillapiol and furanolignans to P-gp-rich and P-gp-poor cells.

Accumulation of $^3$H-vinblastine correlated with the resistance level of the five cell lines studied (Table 5.2). A plateau was reached after 60 minutes in all cell types (Figure 5.2). There was 2.5 times more vinblastine in NIH cells than in the multidrug resistant cells G185, while the hamster drug-sensitive AUXB1 cells had 2.5 more than C5 MDR cells, and 4.2 more than B30 MDR cells. These counts represent the net cellular loading that results from the combination of the uptake of the drug by cells, minus its active efflux by the membrane bound P-gp pump. Counts were stable from one experiment to the next, once the proper protocol was established (see section 5.2.3), and were comparable to results reported from other studies using vinblastine and other drugs recognized and transported by the P-glycoprotein (Table 1.7) (Ling and Thompson, 1973; Lemontt *et al.*, 1988; Loe and Sharom, 1993).

TABLE 5.2 : UPTAKE OF $^{3}$H-VINBLASTINE BY MURINE AND HAMSTER CELL LINES WITH DURG SENSITIVE AND MDR PHENOTYPES.

| •CELL LINES | DPM per $10^{6}$ CELLS |
|---|---|
| Swiss mouse embryo: | |
| NIH/3T3 (NIH) | 55000 ± 5000 |
| NIH-MDR-G185 (G185) | 22000 ± 2000 |
| Chinese hamster ovarian cells: | |
| CHO-AUXB1 (AUXB1) | 72000 ± 9000 |
| CH$^{R}$C5 (C5) | 29000 ± 2700 |
| CH$^{R}$B30 (B30) | 17000 ± 3200 |

• The name usually referred to in the text is in parenthesis.

Figure 5.2 Differential uptake of $^3$H-vinblastine by drug-sensitive and multi-drug resistant hamster and murine cells.

A: hamster cells

AUXB1, parental drug-sensitive

C5, multidrug resistant

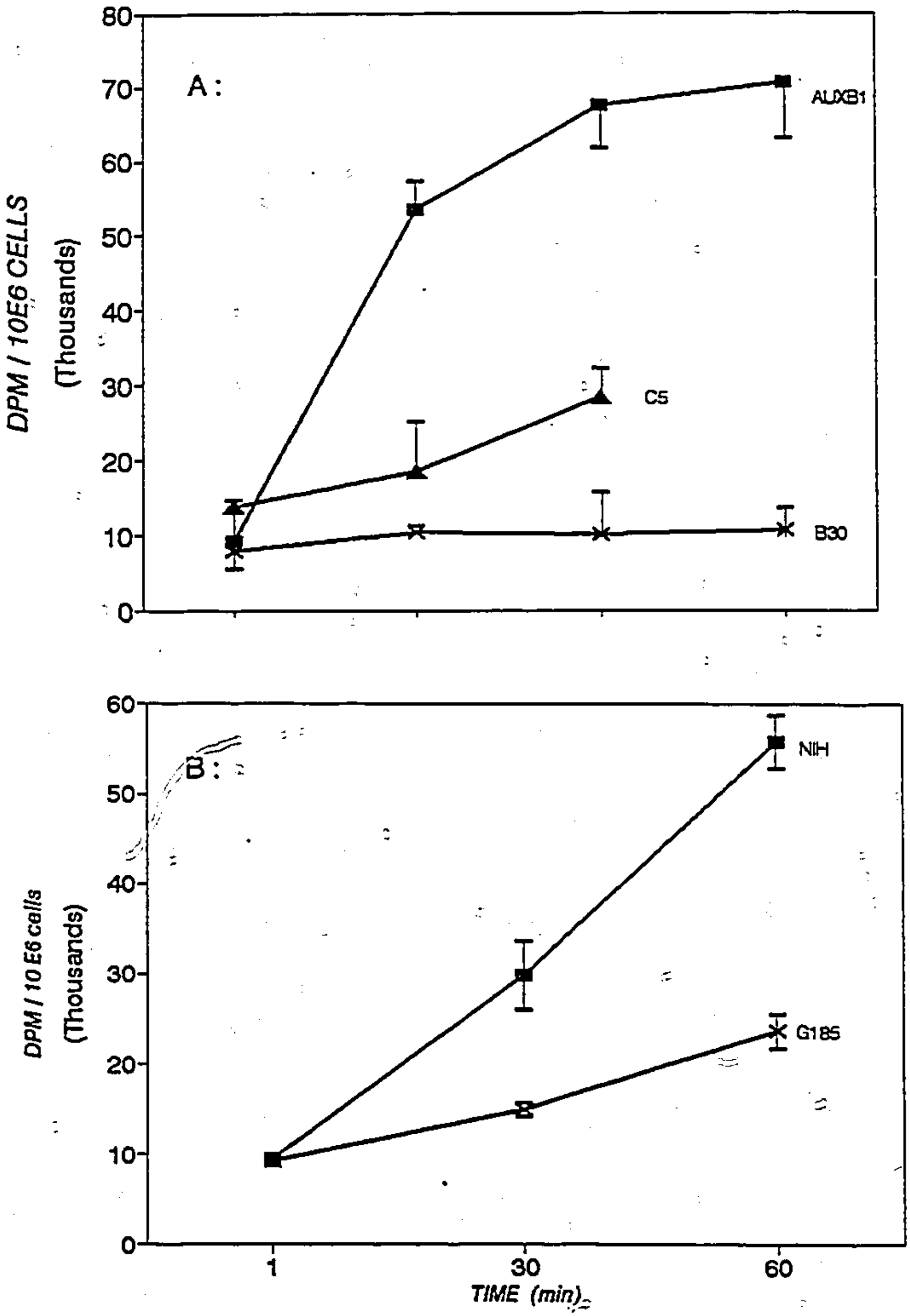
B30, multidrug resistant

B: murine cells

NIH, parental drug-sensitive

G185, multidrug resistant

Data represent the mean of 2 (C5) or 4 experiments (all other cell types).


A:
DPM / 10E6 CELLS
(Thousands)
80
70
60
50
40
30
20
10
0
AUXB1
C5
B30
B:
DPM / 10 E6 cells
(Thousands)
60
50
40
30
20
10
0
NIH
G185
1
30
60
TIME (min)

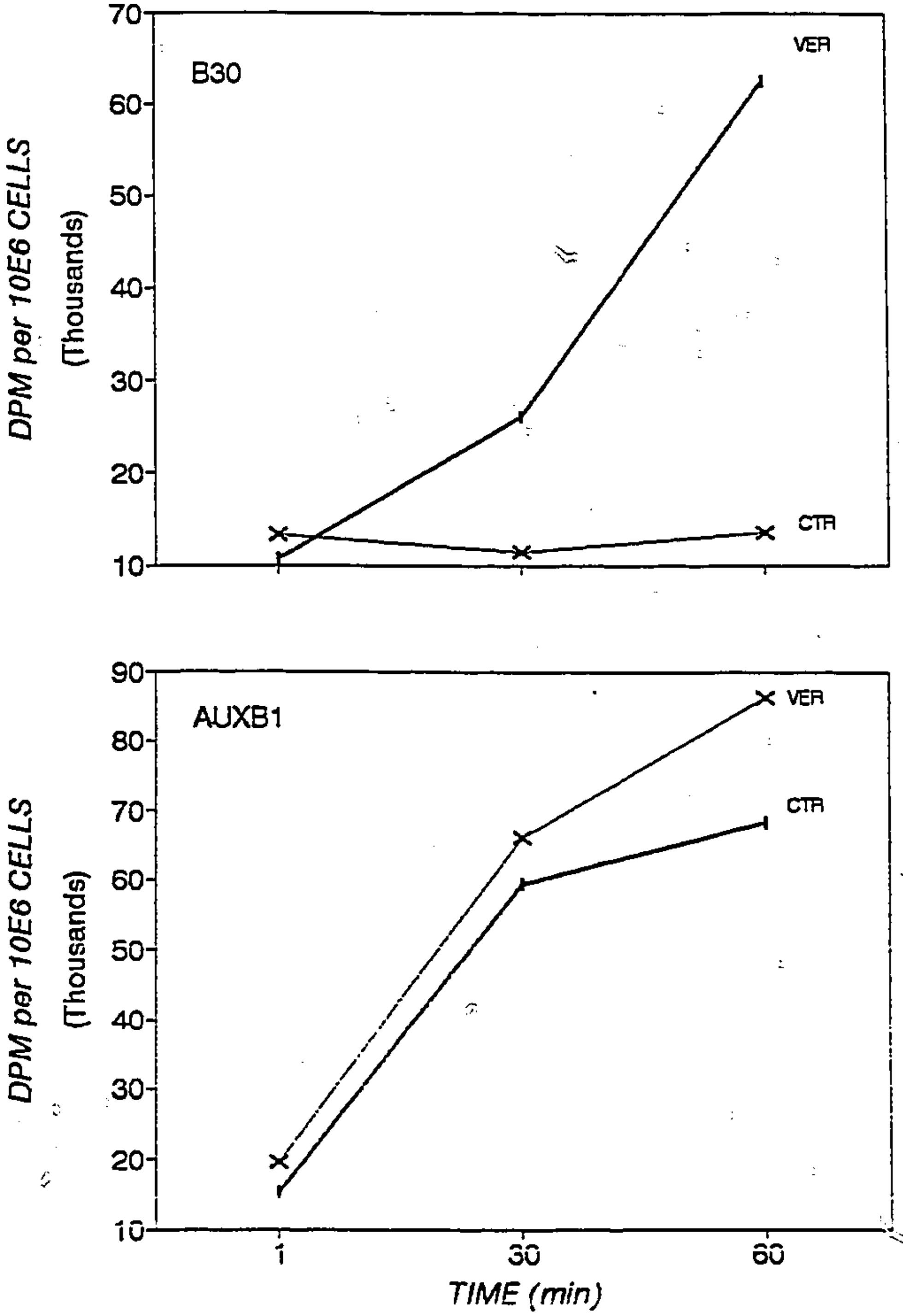
The major role of P-glycoprotein in the resistance of B30 cells (Lemontt *et al.*, 1988; Loe and Sharon, 1993), is demonstrated here by the capacity of verapamil to specifically inhibit P-gp activity and to restore the level of vinblastine accumulated by B30 cells to that of the drug sensitive AUXB1 cells (Figure 5.3). This constituted a positive control run with each experiment.

Dillapiol had a major effect on the net accumulation of $^3$H-vinblastine in the MDR hamster cells (B30), and a limited effect on sensitive AUXB1 cells (Figure 5.4). While the lower doses (9 nM and 18 nM) had little effect on the net uptake of parental AUXB1 cells, these low concentrations increased $^3$H-vinblastine uptake ten times in the resistant cells (e.g. DPM/$10^6$ cells at 45 min in the MDR B30 18000 ± 4000 without, and 173000 ± 9000 with 9 nM dillapiol). Dillapiol was toxic to B30 cells in culture at 0.5 ng/mL, or 2.2 nM. Its capacity to inhibit the efflux of a toxin from the MDR cell, potentially extended to other lignans of the furanofuran group, may help explain the collateral sensitivity described earlier (Figure 5.1 and section 5.3.1).

The MDR selective modulating effects of dillapiol on the $^3$H-vinblastine uptake was more rapid and obtained with much lower concentrations than with verapamil. After 15 minutes, the net accumulation of vinblastine in dillapiol-treated cells was three times that of verapamil-treated cells, in the resistant

**Figure 5.3** Differential uptake of $^3$H-vinblastine by AUXB1 and B30 with the known inhibitor of the P-glycoprotein pump, verapamil.

B30 : multidrug resistant cells
AUXB1 : parental drug sensitive cells
VER : verapamil, 160 µM
CTR : control



B30
VER
CTR
DPM per 10E6 CELLS
(Thousands)
70
60
50
40
30
20
10
AUXB1
VER
CTR
DPM per 10E6 CELLS
(Thousands)
90
80
70
60
50
40
30
20
10
1
30
60
TIME (min)

cells (Figure 5.4).

None of the doses of dillapiol in the vinblastine uptake assays were toxic to AUXB1. Dillapiol is not toxic to AUXB1 until 500ng/mL, or 2.2 μg/mL (Figure 5.1). Because cytotoxicity for non-tumour tissues limits to a narrow range of concentrations the therapeutic use of drugs such as vinblastine, a tool to circumvent cell resistance is highly needed.

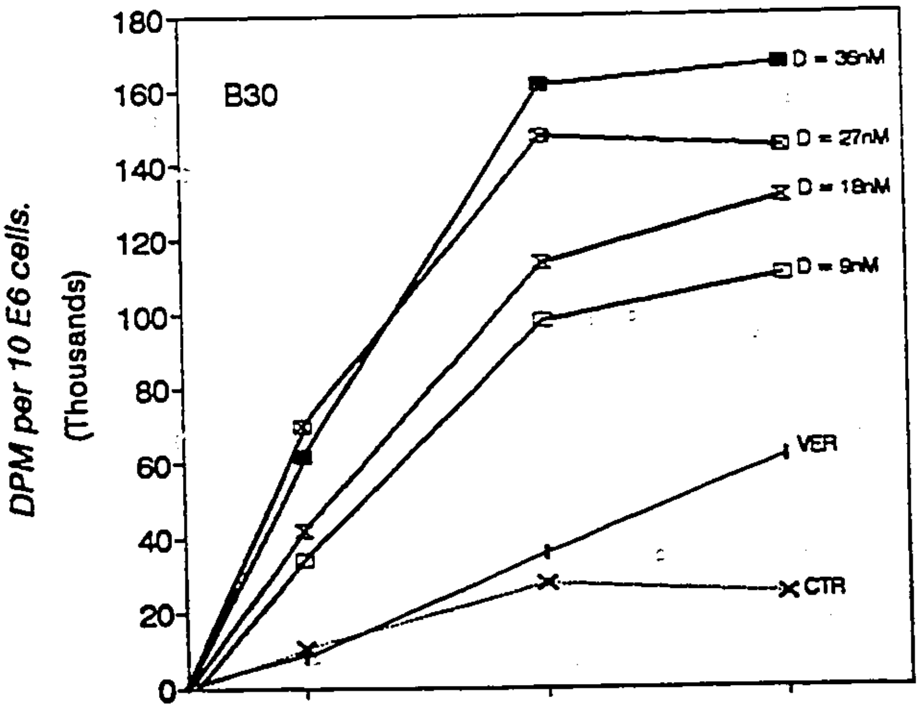
In addition, dillapiol, and phenylpropenes closely related to it, are widespread in edible food and spices, a fact that could indicate a low dietary toxicity. For instance, dillapiol is a major constituent of dill and celery seeds, estragole is used as a flavour in foods and liqueurs and is found in *Piper betle*, which is chewed, with betel nuts, on a daily basis in Asian countries. Myristicin is found in carrots, nutmeg and celery, elemicin is found in carrots, eugenol in nutmeg, oregano cinnamon and cassia, safrole is found in basil and nutmeg (Harborne and Baxter, 1993).

At high doses of dillapiol (135 nM, data not shown), the retention of $^3$H-vinblastine in the MDR B30 cell pellet decreased after 30 minutes, sugging an apparent efflux which we hypothesize is actually due to cell lysis and release of the $^3$H-vinblastine from the cells. This concentration of dillapiol is extremely toxic to MDR B30 cells.

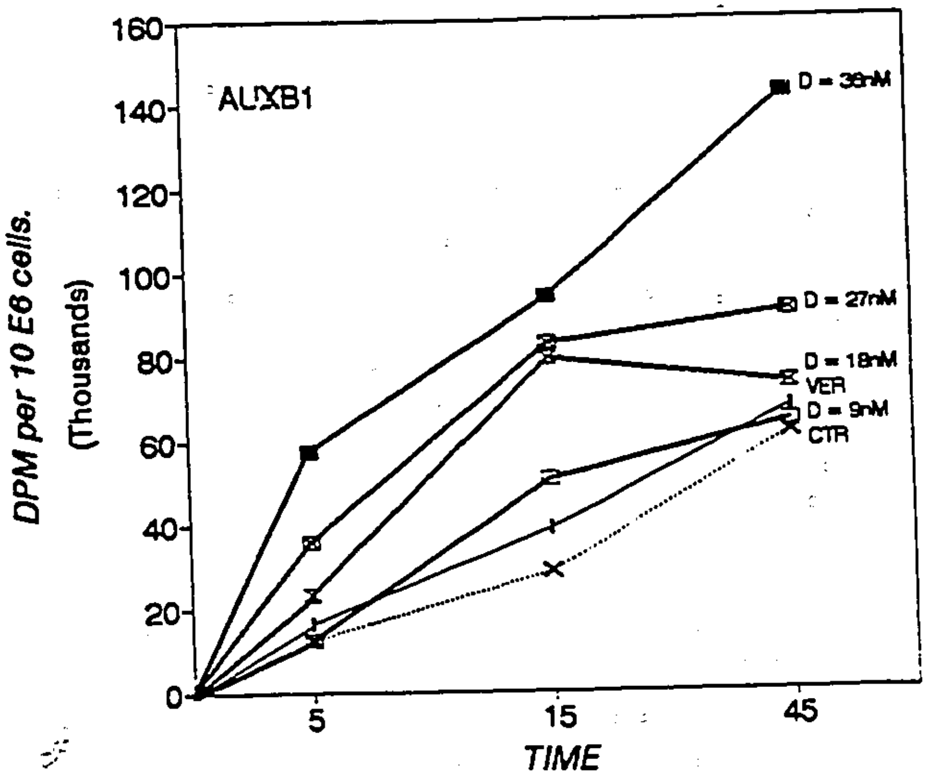
Results of vinblastin retention in cells are consistent with a mode of action of collateral sensitivity involving membrane active effects. They also show that the selective

**Figure 5.4** Differential uptake of $^3$H-vinblastine by AUXB1 and B30 with the known inhibitor of the P-glycoprotein pump, verapamil (VER) and with dillapiol (D) at various concentrations.

B30 : multidrug resistant cells
AUXB1 : parental drug sensitive cells
VER : verapamil, 160 $\mu$M
CTR : control



B30
DPM per 10 E6 cells.
(Thousands)
0
20
40
60
80
100
120
140
160
180
D = 36nM
D = 27nM
D = 18nM
D = 9nM
VER
CTR



AUXB1
DPM per 10 E6 cells.
(Thousands)
0
20
40
60
80
100
120
140
160
D = 36nM
D = 27nM
D = 18nM
VER
D = 9nM
CTR
5
15
45
TIME

effect of dillapiol on MDR cells, rather than on drug-sensitive cells is restricted to a limited range of concentrations of this lignan. It strongly suggests that dillapiol has an effect on the permeability of the membrane of MDR cells to drugs.

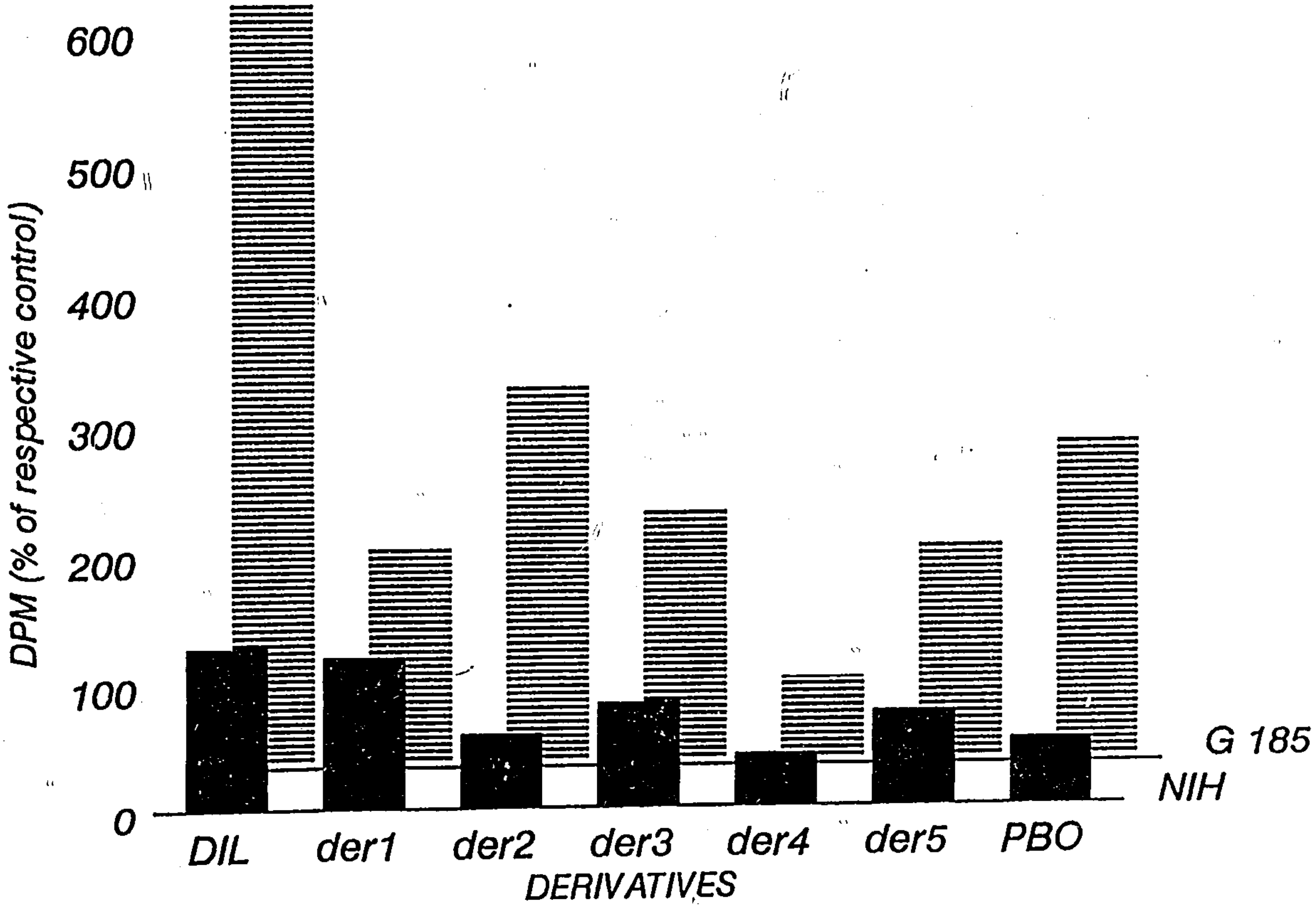
Collateral sensitivity of G185 (MDR) cells to dillapiol could not be shown because of the limited solubility of dillapiol in the rearing media (Table 5.1). However, the increase of vinblastine uptake in G185 cells with dillapiol (65 nM, Figure 5.5), compared to NIH sensitive cells, parallels that seen in B30 and AUXB1. Clearly, a non-toxic concentration to NIH cells (Table 5.1), has a marked effect on vinblastine uptake in MDR G185 cells (6-time increase). A similar response was obtained with PBO and some of the synthetic derivatives (Appendix II) (Figure 5.5). These results further indicate an of action of dillapiol and related compounds closely linked to the P-glycoprotein phenotype.

Dillapiol, PBO and several structurally related natural compounds such as myristicin and sesamex, are known inhibitors of cytoplasmic polysubstrate monooxygenases (PSMOs) (Bernard *et al.*, 1991). Although, an an elevation of the level or activity of the PSMOs possibly limiting the metabolism of vinblasting cannot be ruled out in drug-selected MDR B30 cells, MDR-transfected G185 cells should not be expected to experience altered levels of the intracellular enzymes. This leaves the possibility of an action of dillapiol and its derivatives on the cell membrane.

**Figure 5.5** Differential uptake of $^3$H-vinblastine by NIH and G185 cells after 60 minutes of incubation with dillapiol and selected dillapiol derivatives:

DIL : dillapiol, 65 nM
der1 : derivative 1, 150 nM
der2 : derivative 2, 150 nM
der3 : derivative 3, 150 nM
der4 : derivative 4, 150 nM
der5 : derivative 5, 150 nM
PBO : piperonyl butoxide, 65 nM

Uptake in control was 53000 ± 2000 dpm and 19000 ± 1500 dpm / $10^6$ cells for NIH and G185, respectively.


600
500
400
300
200
100
0
DPM (% of respective control)
DIL
der1
der2
der3
der4
der5
PBO
DERIVATIVES
G 185
NIH

The three most active derivatives of dillapiol on MDR cells, derivatives 1, 2 and 5, possess a highly substituted phenolic ring, with two methoxy groups in position 1 and 2 and a 3,4-methylenedioxy group. Dillapiol also possesses the same substituents in the same steric position (Appendix I and II). Derivative 4 does not have these features and has the lowest activity in our series (Figure 5.5), in spite of the fact that it is a potent synergist of carbaryl (Tomar *et al.*, 1978). The same group reported that compound 2 had little advantage over dillapiol as synergist of pyrethrins (Mukerjee *et al.*, 1979). Structural requirements for lignan interaction with P-gp rich cells or membranes differ from those required for the interaction with PSMO-rich membranes of the endoplasmic reticulum. They have yet to be investigated.

Agents that act preferentially on resistant tumours, with MDR phenotype, rather than on sensitive cells, are certainly greatly needed in cancer chemotherapeutical arsenal. As lignans are already present in the human diet, efforts could concentrate on elaborating a protocol of their use in patients food under cancer chemotherapy, to obtain a modulating effect on cells which have become resistant. Further investigations may establish the use of lignans or their derivatives as specific toxins of MDR phenotypes or potentially as adjuvents of other drugs with recognised efficacy.

The fact that dillapiol and lignans of the furan type are inhibitors of PSMOs, and act preferentially on multiresistant

resistant cells with high expression of P-glycoprotein, both point at a possible role of lignans as antagonists of resistance mechanisms. The coevolution of insects and plants may have favoured the selection of lignans in plants to act on particular mechanisms related to resistance in insects. Future research may reveal the potential of such compounds in the major area of management of insects pests.

# CHAPTER 6

# GENERAL DISCUSSION AND CONCLUSIONS

The present study brings a new understanding of the biology of lignans. It shows their definite role in the chemical defense of plants, in particular in the family of the *Piperaceae*. It uncovers unexpected biological properties of lignans and puts them in a new evolutionary perspective with respect to plant-insect interactions. The selective toxicity of a series of lignans to multidrug resistant cells, and the selective effect of dillapiol on the retention of drugs by these cells, opens new avenues for the understanding of multiresistance to xenobiotics, one of the major challenges to biological sciences of our time.

## 6.1 Lignans in the chemical defense of the *Piperaceae*

Traditional use of extracts of the *Piperaceae* in local medicine and against pests is well documented in tropical areas in the world but little investigated in the American neotropics. We demonstrated that:

1. The *Piperaceae* from the American tropics have insecticidal activity comparable to previously studied Asian and African species.

2. American *Piper spp.* contain large quantities of amides and lignans, particularly monolignans, a chemical profile identical to other *Piper spp.* worldwide.

3. Our bioassay-guided isolation of the compounds toxic to insects, designate isobutylamides and lignans, either together or alone in a plant, as insecticidal allelochemicals in the *Piperaceae*.

Isobutylamides and lignans are a wise choice for the chemical defense of the plant that synthesizes them. The isobutylamides of the *Piperaceae* have a low molecular weight, contain a single nitrogen, and are thus inexpensive to make. They can be considered as a protection well adapted to the defense needs of this family of small shrubs and vines with a short life-cycle. Similarly, lignans are low molecular weight molecules and only necessitate carbon, hydrogen and oxygen molecules which are easily available in a tropical environment where there is carbon fixation in excess of growth requirements.

The insecticidal activity of the isobutylamides of the *Piperaceae* is well documented. These compounds are fast-acting neurotoxins that account for the major insecticidal activities of species such as *Piper guineense*, *P. cubeba* and also *P. tuberculatum*, from the present study.

The addition of lignans to the chemical arsenal of the *Piperaceae* represents a sophisticated and potentially very successful defense strategy. They synergize the toxicity of other allelochemicals, have an intrinsic toxicity or combine both activities. In fact, all of the 12 *Piper spp.*, dicussed in chapter 1 with traditional or recognized insecticidal activities contain one or several lignans. In addition, lignans, particularly monolignans such as dillapiol, safrol, sarisan, or elemicin, may be the only known allelochemicals in the plant, such as in *P. umbellatum, P. sumatranum, P. lenticellosum, P. hookeri* and *P. cavalcantei*.

The association of isobutylamides and lignans in the *Piperaceae* may constitute a major factor in the success and diversification of this tropical Family to tens of thousands *species* worldwide.

## 6.2 Mode of toxicity of lignans

The present investigation which covers a unique collection of pure natural lignans collected or isolated over the past 20 years by our laboratory and other collaborators, allows us to put lignans in a new toxicological perspective.

1. These simple phenylpropanoid molecules of low molecular weight have a non-neurotoxic mode of toxicity. They have a chronic, rather than an acute toxicity.

2. The nutritional indices show that they act on the post-digestive metabolism of the insect rather on the regulation of its food selection.

3. The insecticidal activity of lignans generally increases as more elaborate structures are selected by evolution. It is in increasing order of activity: NDGA, oxodiarylbutanes, furofuran-lignans, cyclolignans. A further step of evolution is thought to be a simplification of the biosynthesis with the monolignans of high insecticidal potential.

4. Lignans act on at least two adaptative mechanisms by which organisms cope with the potentially toxic effects of xenobiotics, i.e. on the activity of PSMOs and on a yet not totally defined functionality of membranes of multidrug resistant cells which over-express P-glycoprotein.

5. Lignans can be clearly separated in two structural groups in regard to their activity on resistant cells. The cyclolignans act like other xenobiotics of the MDR group that are substrates of the P-glycoprotein. The furanolignans and the monolignan display the phenotype of collateral sensitivity.

Adapted insect herbivores have had to develop a series of strategies to overcome the toxicity of allelochemicals in their food. Lignans were previously shown to interfere with the detoxification of natural insecticides by insects, countering a generally highly efficient adaptative feature used by herbivores, they inhibit the PSMOs. Lignans, and particularly monolignans, the most recent compound in the evolutionary sequence of this class of compounds, appear to counter an even more elaborate resistant strategy. They are highly toxic to multiresistant cells overexpressing P-glycoprotein and have little toxicity to normal cells. The demonstration by a co-worker, C Murray, at the time of the present study of the presence of P-glycoprotein in an insect adapted to high levels of nicotine, the tobacco hornworn, puts lignan activity against cells overexpressing P-glycoprotein in a totally new perspective of plant-animal coevolution. The synthesis of lignans by plants may represent a successful strategy to prevent (PSMOs) and eliminate (P-glycoprotein) counterstrategies that allow insects to survive on an food rich in allelochemicals of all sorts.

The action of dillapiol and a series of lignans with a furan structure is specific to multidrug resistant (MDR) cells rather than to drug-sensitive ones. The consequence of the stimulation of drug accumulation by dillapiol is that drugs such as vinblastine of the MDR group are retained in the MDR cell at concentrations high enough to be toxic. There is no need to increase the therapeutic dose towards the limits imposed by unacceptable side effects.

## 6.3 Lignans in an evolutionary perspective

Our results on the relative toxicity to a herbivore of a large group of lignans structures arrive at a good time to support the postulate of Lewis and Davin (1994) on the evolution of lignans.

> A gradient of increasing toxicity from NDGA to dillapiol was found, which follows a recently proposed evolutionary scheme represented in figure 1.2, chapter 1.

The first lignans biosynthesized such as nordihydroguaiaretic acid or cubebin, were not highly toxic and they may have persisted because of collateral systems that enhanced their toxicity to herbivores, such as photoactivation or antifeedant activity, respectively. More elaborate structures, such as the furolignans, acquired the definite selective advantage of countering directly at least two known major adaptative mechanisms that allow organisms to overcome the toxicity of xenobiotics. The furanolignans include several PSMO inhibitors such as sesamin and myristicin. A significant result of the present work is the discovery that this group of furanolignans also bypasses another adaptative mechanism, the cellular resistance brought by P-glycoprotein. In the next step of evolution highly efficient cellular toxins were biosynthesized with the group of cyclolignans, such as podophyllotoxin. Highest efficiency of the biotoxic mechanism was reached with the monolignans, whose biosynthesis is seen by Lewis and Davin (1994) as a simplification in the biosynthetic pathway reached later in evolution. Monolignans, such as dillapiol, combine a toxic defense and the capacity to prevent or circumvent adaptation in the target organism. Their structure is readily available from cinnamic acids, an unavoidable cross point in the biosynthetic pathways of lignins. They are yet another step towards plant defense efficiency.

## 6.4 Future work

Two main wide research avenues have been opened with the present work:

1. One is the largely uninvestigated coevolution of plant lignans and herbivores.

2. The second is the new definition of their potential as agents countering adaptative or resistance mechanisms to xenobiotics toxicity in animal, the mechanisms that cause failure of cancer therapy and insect control.

The fascinating possibilities offered by the combination of lignans and isobutylamides in the *Piperaceae* has to be examined in relation to the evolutionary trend with a variety of generalist and specialist insects of the *Piperaceae*.

The daily use of several species as anaesthetic, psychostimulants, and aphrodisiacs, the presence of high quantities of lignans in edible plants and the daily use of plants with complex associations of lignans and toxins, such as amides of the pepper (*Piperaceae)*, appears to be well tolerated by humans. This constitutes a promising starting point for their development as safe chemotherapies for cancer prophyllaxy and therapy. With over 2 000 *species* in the *Piperaceae*, scientists have really only begun to appreciate the diversity which has obviously been of considerable interest to traditional cultures worldwide.

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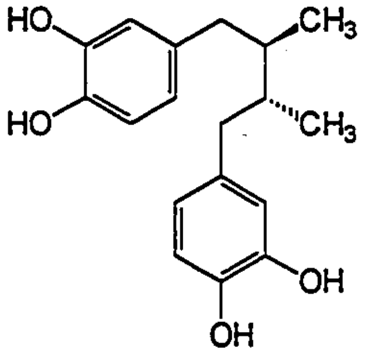
# APPENDIX I

## Plant lignans and neolignans from the study



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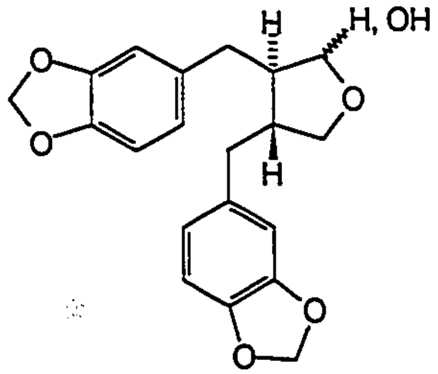

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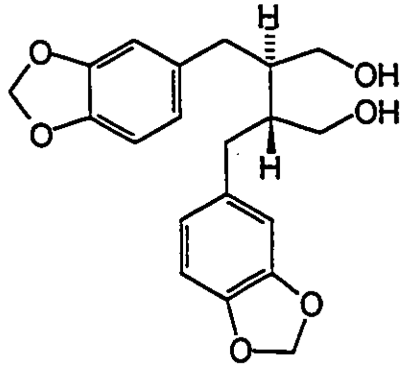

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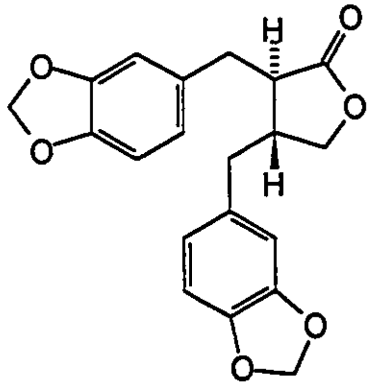

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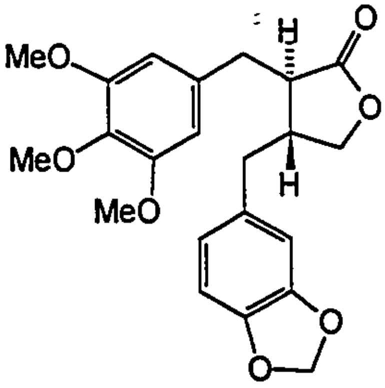

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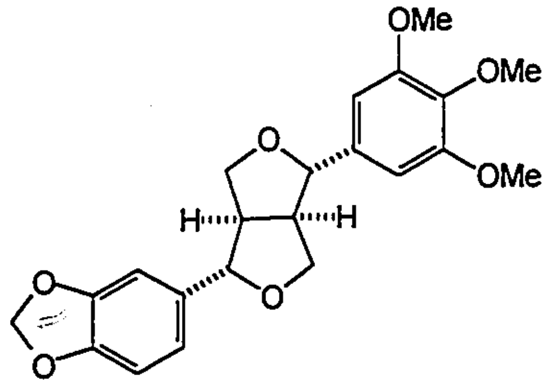

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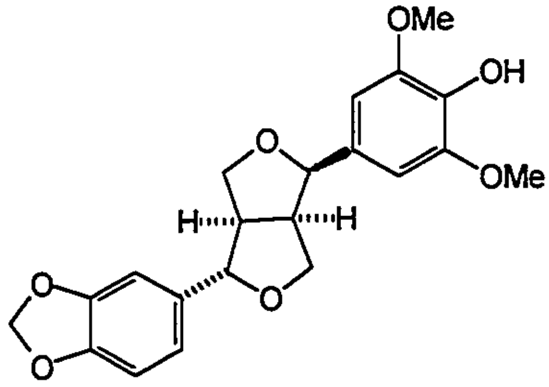

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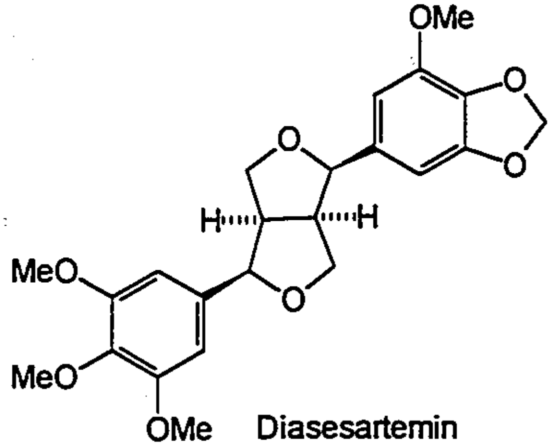

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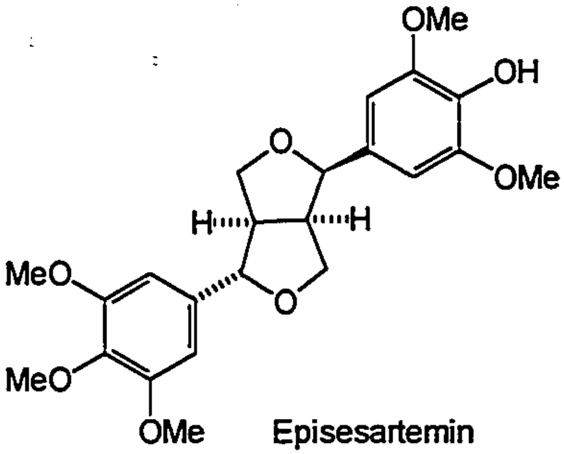

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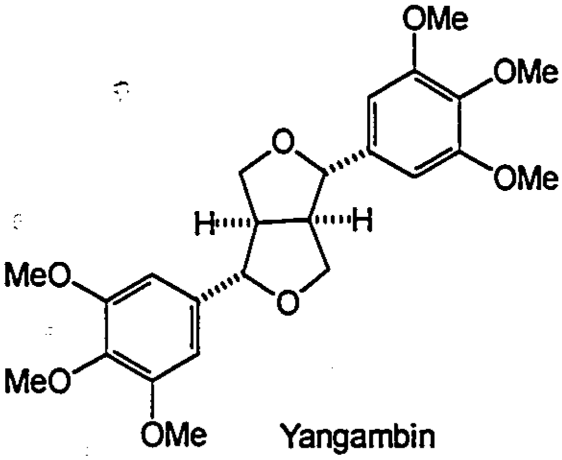

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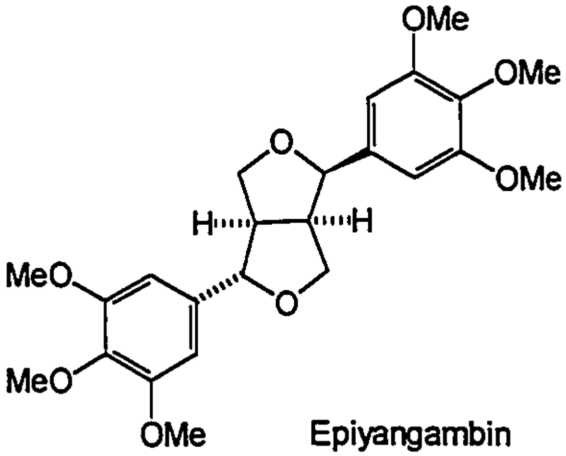

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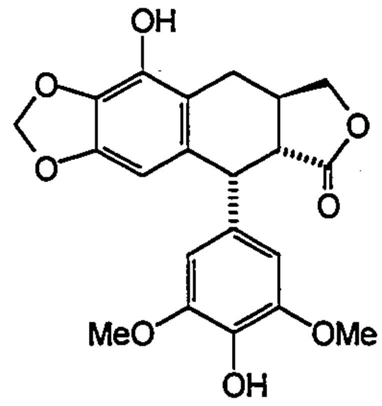
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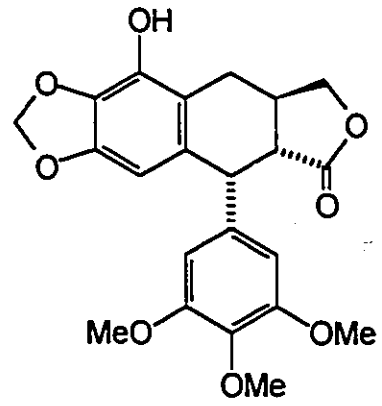
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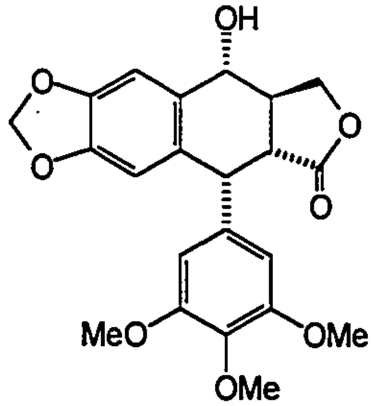
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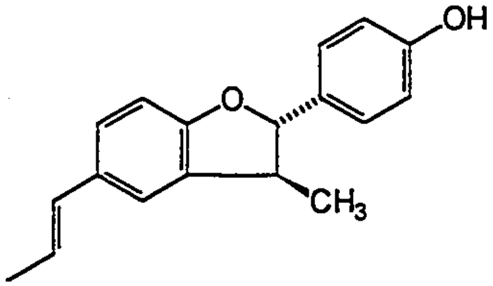

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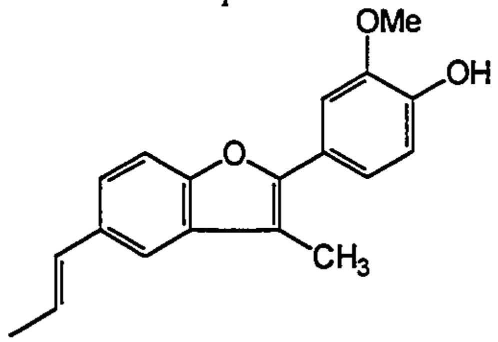

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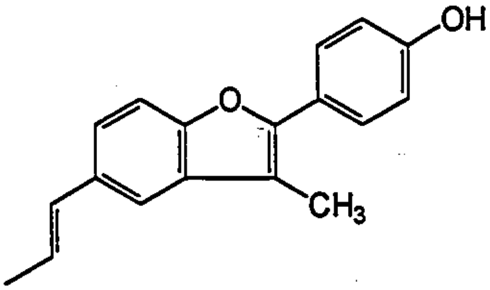

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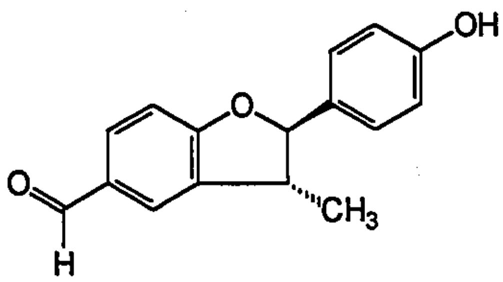

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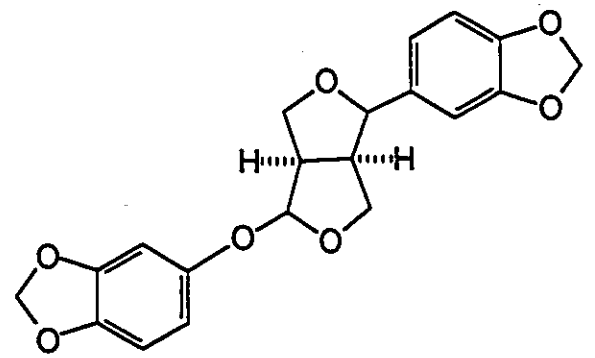

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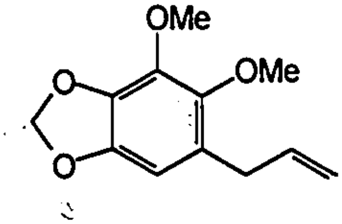

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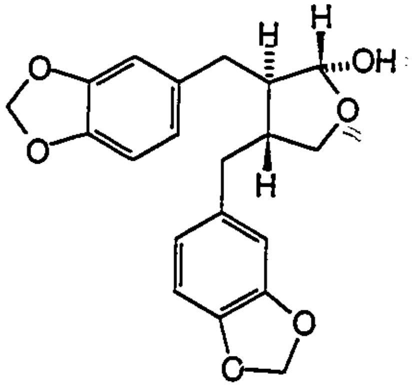
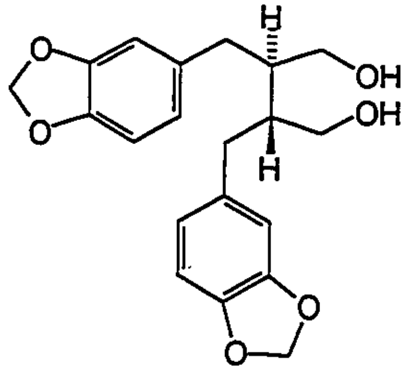


(-) Cubebin



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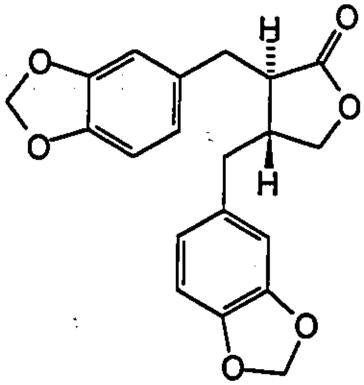

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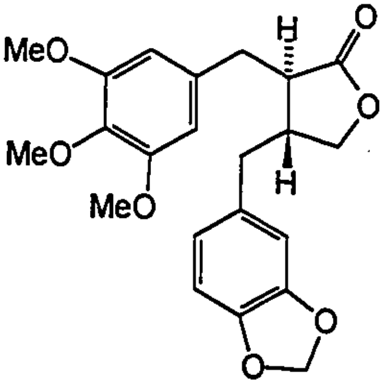
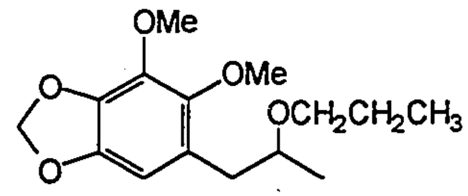


(-) Yatein

# APPENDIX II

# Derivatives of dillapiol



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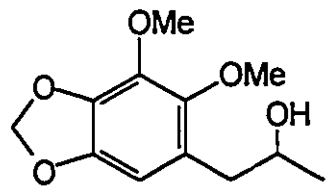

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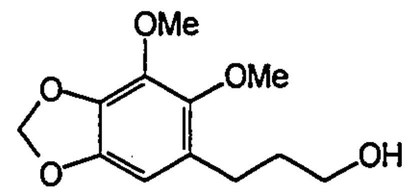

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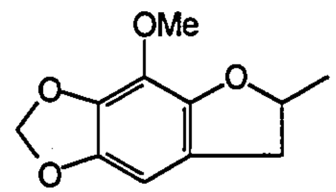

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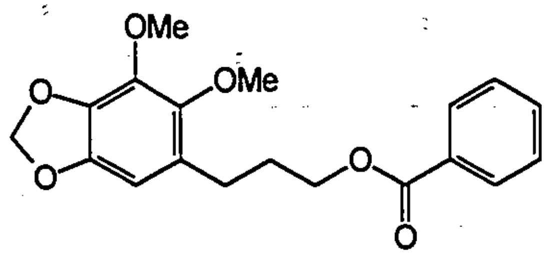

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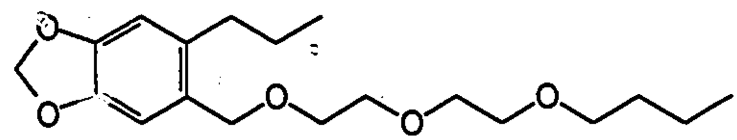

**PBO**